GENETICS

Comparative Flow Cytometric Analysis of the Human Sperm Acrosome Reaction Using CD46 Antibody and Lectins

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Purpose: *Our purpose was to determine the most suitable marker for the human sperm acrosome reaction, based on detection of CD46 antibody binding compared with lectin binding.*
Methods: *Flow cytometric analysis of CD46 antibody versus lectins (PNA, PSA, and Con A) was used to quantify the acrosome reaction of human sperm.*
Results: *Neither PSA nor Con A was able to detect significant changes in the spontaneous and ionophore-induced acrosome reactions compared to CD46 antibody. However, PNA was found to exhibit a binding pattern similar to that observed with CD46 and could be used to quantify measurable changes in acrosomal response to ionophore, albeit of a lower magnitude than the responses detected by CD46.*
Conclusions: *We conclude that PNA binds to the inner acrosomal membrane of acrosome-reacted sperm and is suitable for use as a marker of the acrosome reaction by flow cytometry. Data are presented which clarify the assessment of the acrosome reaction when CD46 and lectins are used.*

KEY WORDS: lectins; CD46; flow cytometry; acrosome.

INTRODUCTION

A variety of methods for assessing and quantifying the human sperm acrosome reaction is currently available, among which are epifluorescent microscopy and flow cytometry. However, there appears to be no common consensus in the literature as to standardized methodologies for any of the techniques. Further, there is also no consensus regarding the validity of interassay comparisons, especially with regard to assays utilizing the binding of lectins for assessment of the acrosome reaction. Lectins are known to bind to various different saccharide moieties on cell membranes, including sperm cell membranes (1). Kallajoki *et al.* (1) examined the binding properties of six different fluorescently conjugated lectins to sperm membranes, by epifluorescent microscopy, and discovered that the binding properties to the various sugar moieties was greatly dependent upon the method of sperm treatment prior to, and during, labeling, i.e., dependent upon whether the sperm was fixed, permeabilized, air-dried, or untreated. If we examine the various epifluorescent microscopic and flow cytometric techniques, we can see that a variety of lectins is in use as well as the various sperm preparation protocols, also in consideration of the use of concomitant sperm viability assessment. For example, if we look first at the literature regarding the use of *Pisum sativum* (PSA; which reportedly binds to an α-mannose moiety on the sperm outer acrosomal membrane), we find several technical differences between authors: some perform prefixation of sperm with ethanol prior to labeling with FITC-PSA and utilize no supravital staining protocol (2–7); others use the same protocol as those above, yet incorporate the supravital stain H33258 into their protocols (8–14). A further protocol, utilizing paraformaldehyde prefixation plus the H33258 supravital stain, is also used (15,16). Thus, there can be seen to be at least different approaches for the first major lectin, PSA.

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In examining the second popular lectin, *Arachis hypogaea* (PNA; reportedly binds to a galactosyl (β-1,3) *N*-acetylgalactosamine residue on the sperm outer acrosomal membrane and matrix), we find the following techniques in use: one uses a biotinylated rather than FITC-conjugated PNA (17)—using ethanol fixation prior to staining but no supravital stain, whereas others (18) use the ethanol fixation plus the supravital stain H33258. Conversely, others (19) utilize a method of no fixation prior to, or after, lectin binding, plus they use H33258 as the supravital stain. Further workers use a methanol fixation plus the hypo osmotic swelling test to assess sperm vitality (20–22). An adaptation of the methodologies is also used but with flow cytometry and ethanol fixation after lectin binding and propidium iodide as the supravital stain (23).

When we examine the case for *Canavalia ensiformis* (Con A; reportedly binding to α-mannose and α-glucose residues on the inner acrosomal membrane), we find that some groups use a formaldehyde prefixation of sperm, with no supravital stain (24–26), while others use the same method but add the H33258 supravital stain (27,28). A further group (17) adheres to the biotinylated lectin protocol and utilize ethanol fixation with no supravital stain.

A further lectin may be considered—*Triticum vulgaris* (WGA; reportedly binds to a *N*-acetylgalactosamine residue on the inner acrosomal membrane and equatorial segment of sperm); one group utilizes this particular lectin using a paraformaldehyde prefixation with no supravital stain (29).

On further examination of the various protocols mentioned above, we can also see that there are variations between fixation times, incubation times, air-drying techniques, fixation after air-drying or before air-drying, etc.

If we consider flow cytometric analysis further, we can see that it is gaining wider acceptance as a technique for assessing the acrosome reaction and viability simultaneously. Tao *et al.* (30) examined the use of two monoclonal antibodies (MH61 and CD46) for acrosome reaction assessment, while others have concentrated on CD46 alone (31,32). Comparing these assays to the more widely used epifluorescent microscopic techniques, the flow cytometric analysis is able to give a far more simple and objective method of analysis, especially with regard to correlation of fertilization with acrosome reactivity potential (16,23,33).

In this study we have attempted to elucidate the binding properties of the three most popular lectins in use today (namely, PSA, PNA, and Con A) by using flow cytometry in parallel with our previously described CD46 assay (32). This is to compare and validate the use of lectins in the assessment of the true sperm acrosome reaction *in vitro*. It should be noted that we have concentrated solely on nonpermeabilized and nonfixed sperm to compare more accurately the true lectin binding profile with the flow cytometric analysis of CD46 binding. It has been stated that all of the lectins examined in their study displayed distinct binding patterns to human sperm (1); however, it was found that staining intact sperm, in suspension, gave results different results from those obtained with staining after air-drying. Further, prefixation with paraformaldehyde increased the number of acrosome-reacted sperm and this was similar for detergent-treated sperm. It was concluded that lectin staining of intact cells in suspension most likely represented the true surface binding pattern for the lectins, as fixation/permeabilization techniques and air-drying may disrupt the sperm plasma membrane and thereby expose acrosomal and intracellular glycoconjugates to lectin binding, thereby giving false-positive surface staining data.

The purpose of this study is therefore to present data which may assist in clarifying the assessment of the acrosome reaction when lectins are used and, also, to recommend a more uniform and consistent assay protocol, which more properly assesses sperm under physiological conditions.

MATERIALS AND METHODS

Reagents and Stock Solutions

Calcium ionophore (A23187) was obtained from Sigma Chemical Co. St. Louis, MO). A stock solution of 5 m*M* was made up in dimethyl sulfoxide (DMSO; Sigma) and stored at −40°C in 0.25-ml aliquots. A working stock was made up by diluting 1:10 in unsupplemented human tubal fluid medium (40) and was equilibrated at 37°C in 5% CO_2 in air for 3 hr before use. The same batch of A23187 was used throughout the series of experiments. Ten microliters of the working stock was added to each 500 μl of sperm suspension, giving a final concentration of 10 μ*M* (32). Percoll (Sigma) gradients were used to separate motile sperm from seminal plasma (32) using HTF-BSA as the diluent (BSA, low endotoxin, 0.3%; Irvine Scientific, Santa Ana, CA).

Anti-human CD46 monoclonal antibody (Immunotech., Westbrook, ME) was made up as a stock solution of 10 μg/ml in PBS (Sigma). A stock solution of fluorescein-conjugated goat anti-mouse Ig (Becton

Dickinson Immunocytometry Systems, San Jose, CA) of 50 μg/ml, and a stock solution of propidium iodide (Sigma), also 50 μg/ml, were both made up in PBS.

FITC-conjugated lectins (PSA, PNA, and Con A; Vector Laboratories, Burlingame, CA) were made up as a stock solution of 0.2 mg/ml in PBS.

Sperm Preparation

Semen specimens from 24 males were collected after 48–72 hr of sexual abstinence and were analyzed by a CASA system (CellSoft 2000) as described previously (34). Sperm morphological analysis by strict criteria was performed according to Kruger *et al.* (35). Criteria for inclusion in the study comprised a count of >50 million/ml, >50% progressive motility, >30% normal forms, and an ARIC score (acrosome response to ionophore challenge) of >10 (33) on at least one previous occasion. Motile sperm were separated from liquefied semen by Percoll buoyant density-gradient centrifugation, as described previously (32). Resulting pellets were resuspended up to 5 ml with HTF-BSA and centrifuged at 300*g* to remove residual Percoll. The final pellet was resuspended up to 1.0 ml with HTF-BSA, and a post-Percoll semen analysis carried out as described above. Each final sperm suspension was diluted to 50 million/ml motile sperm.

Induction of the Acrosome Reaction

Each specimen was treated as described previously (32). Briefly, the prepared specimen was divided into two 0.5-ml aliquots (labeled A and B). To portion A was added 10 μl of HTF-BSA, and to portion B was added 10 μl of calcium ionophore A23187 (final dilution, 10 μ*M*). Each tube was capped lightly and incubated at 37°C/5% CO_2 for 45 min. At the end of this period, each was made up to 6.0 ml with HTF-BSA and centrifuged at 300*g* for 10 min. Supernatants were aspirated and the wash was repeated. Final pellets were made up to 0.5 ml and taken for further processing via CD46 binding or lectin binding.

Incubation with Lectins and CD46

Specimens were treated according to the flowchart in Fig. 1. Briefly, after incubation for acrosome reaction induction, all aliquots were made up to 8.0 ml with PBS and further divided into four 2.0-ml aliquots. The first pair of aliquots (with and without ionophore treatment) was allocated for CD46 incubation, and the

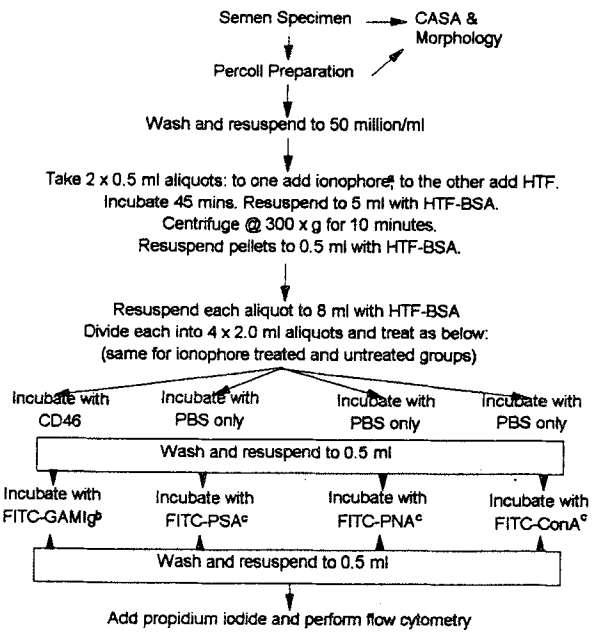

Fig. 1. Flowchart showing the allocation of specimen treatments. [a]Calcium ionophore (A23187). [b]FITC-conjugated goat anti-mouse Ig. [c]FITC-conjugated *Pisum sativum*, *Arachis hypogaea*, and *Canavalia ensiformis*.

remaining three pairs for lectin incubation (PSA, PNA, and Con A, respectively).

For CD46 binding, sperm suspensions were centrifuged at 500*g* for 5 min. Pellets were resuspended in 20 μl reconstituted anti-human CD46 mcAB (final concentration, 10 μg/ml). Tubes were incubated at room temperature (RT) for 30 min, followed by centrifugation, as above, with 2.0 ml phosphate-buffered saline (PBS; Sigma). Supernatants were discarded and 4 μl FITC-conjugated goat anti-mouse Ig was added to each 0.1-ml pellet. Suspensions were incubated for 30 min at RT in the dark, followed by two washes in PBS (as above). Final pellets were resuspended in 1.0 ml PBS and analyzed on the FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). Immediately before analysis, 20 μl propidium iodide (50 μg/ml) was added, to give a final concentration of 1 μg/ml.

For lectin binding, Sperm suspensions were centrifuged at 500*g* for 5 min. Pellets were resuspended in 10 μl reconstituted FITC-PSA/FITC-PNA/FITC-ConA (2 mg/ml stock; final concentration, 20 μg in 10 μl). Tubes were incubated at RT in the dark for 30 min, followed by centrifugation, as above, with 2.0 ml PBS. Final pellets were resuspended in 1.0 ml PBS and analyzed on the FACScan flow cytometer. Immediately before analysis, 20 μl propidium iodide

(50 μg/ml) was added, to give a final concentration of 1 μg/ml

Data were collected for a minimum of 5000 cells in each specimen. A gate was set to exclude cells which fluoresced red (propidium iodide positive)—these were the dead cells in the population. After setting the "live" gate, the number of cells that fluoresced green (CD46 positive) was counted and is expressed as a percentage of the live population.

Data were analyzed using LYSIS II Research Software and stored on disk for subsequent statistical analysis.

RESULTS

Statistical Analysis

All data were analyzed by vector analysis using the Hotelling t^2 test. The test was performed to compare the results of the CD46 marker with the three lectin markers (PSA, PNA, Con A) and, also, the lectin markers with each other. Results were calculated for the spontaneous acrosome reaction (SAR), ionophore-induced acrosome reaction (IAR), and derived acrosome response to ionophore challenge (ARIC), which is calculated by subtracting the SAR from the IAR. Also, viability values were compared between the CD46 and the lectin markers, for the SAR and IAR. A significance level of 1% ($P = 0.01$) was used throughout.

Data

All data are graphically represented in Figs. 2a to 2d and Fig. 3.

In Fig. 2a we show the assessment of the acrosome reaction with CD46. On the basis of the SAR, data were grouped into normal and abnormal responses; six specimens were found to demonstrate a SAR of >10 (which, in our laboratory, is the upper limit of normal for the SAR, as defined previously) (21,33). The bar dividing the graph therefore splits the two groups. The data clearly show that CD46 does not bind to non-AR sperm, whereas there is a significant increase in binding after induction with ionophore (IAR) ($P < 0.0001$).

In Fig. 2b we show the assessment of the acrosome reaction with PSA. It can be seen that both the SAR and the IAR data demonstrate binding with PSA and that these two groups of data are significantly different ($P < 0.0001$).

In Fig. 2c we show the assessment of the acrosome reaction with PNA. The IAR group is significantly higher than the SAR group ($P < 0.0001$). In Fig. 2d we show the assessment of the acrosome reaction with Con A. There is no significant difference between the SAR and the IAR values ($P > 0.037$), demonstrating that Con A is not specific for either the inner or the outer acrosomal membranes.

In Fig. 3 we show sperm viability for each marker, with and without ionophore treatment. The results demonstrate that lectin markers compromise sperm viability, as well as ionophore treatment.

Interpretation of Results

In interpreting the results we have made the assumption that sperm not treated with ionophore is not acrosome reaction induced (SAR) and that sperm treated with ionophore is acrosome reaction induced [IAR; as

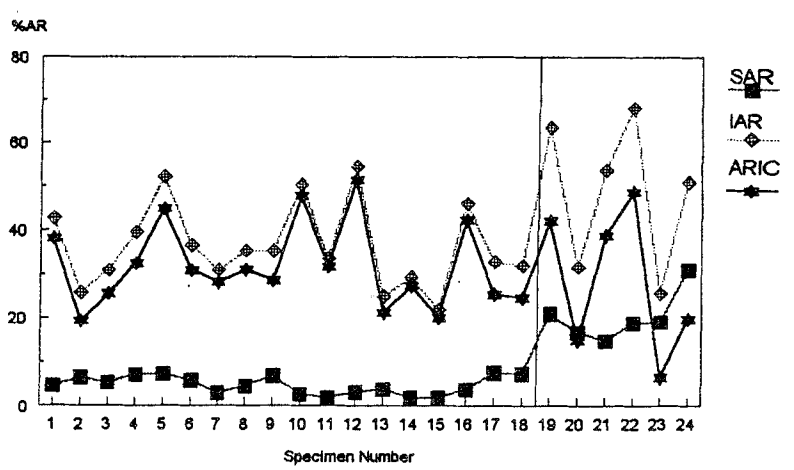

Fig. 2a. Acrosome reaction data using the CD46 marker.

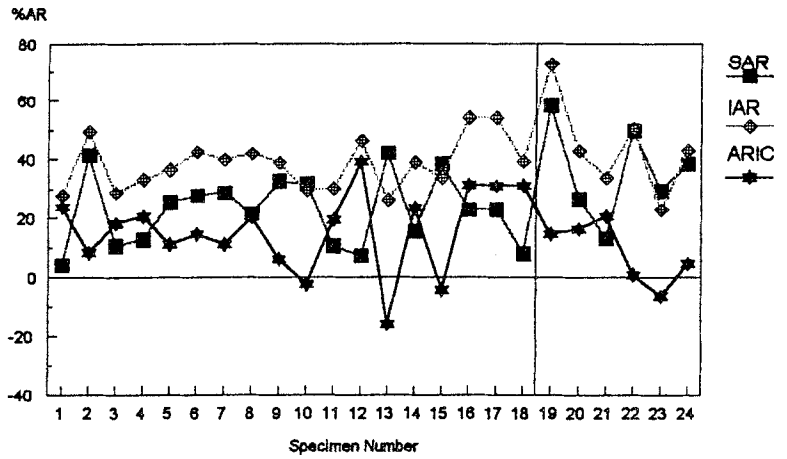

Fig. 2b. Acrosome reaction data using the PSA marker.

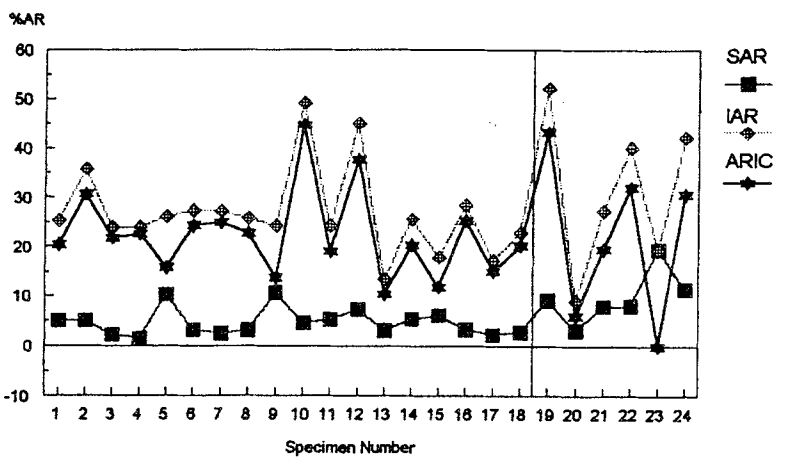

Fig. 2c. Acrosome reaction data using the PNA marker.

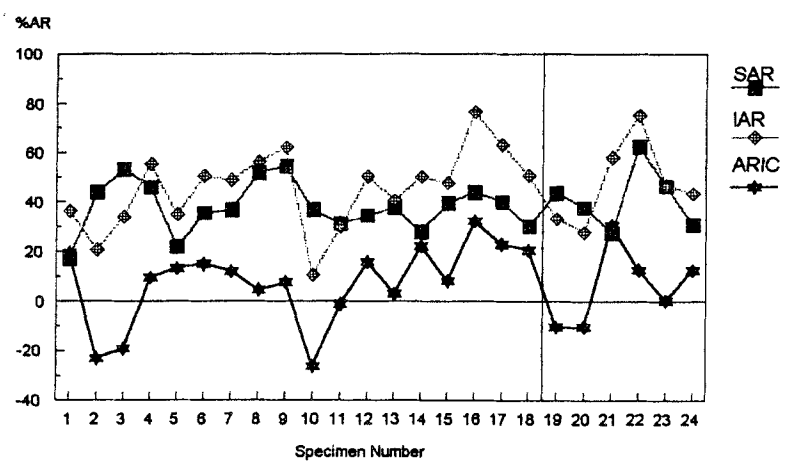

Fig. 2d. Acrosome reaction data using the Con A marker.

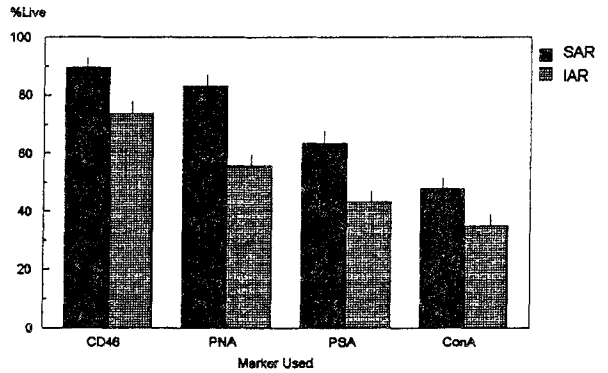

Fig. 3. Sperm viability measurement for each marker. For SAR values: CD46 > PNA > PSA > Con A ($P = 0.0013$, $P < 0.0001$, $P < 0.0001$). For IAR values: CD46 > PNA > PSA > Con A ($P < 0.0001$, $P < 0.0001$, $P < 0.0002$). Values are means ± SE.

reported previously (32)]. It is also assumed that the actual induction of the AR is the same whichever marker is used. This is due to the fact that the same specimen (and same group) was subjected to the same treatment protocol prior to labeling, either with CD46 or with the lectins. The data show that all markers, except for Con A, display significantly higher values after ionophore-induced acrosome reaction ($P < 0.0001$). This indicates that all of the markers, with the exception of Con A are detecting a positive ARIC score. The difference between SAR and IAR observed in the Con A group is not significant ($P > 0.037$), thus indicating that Con A is not suitable for use as an acrosome reaction marker.

When we compare data between groups, the following conclusions can be drawn: if we compare CD46 with PNA (Figs. 2a and 2c), we find that the data for ARIC score and IAR are significantly higher in the CD46 group ($P < 0.0001$), whereas the data for SAR are not significantly different between CD46 and PNA ($P = 0.0452$). This indicates that only acrosome-intact sperm show similar labeling patterns for both CD46 and PNA. However, if we examine the binding patterns after AR (IAR), we can see that both markers demonstrate an increase in labeling, thereby indicating that PNA is, in fact, binding to an intracellular structure in a manner similar to that in which CD46 is binding. If we compare these two groups (IAR) with respect to the acrosome reaction, PNA gives significantly lower labeling than does CD46 ($P < 0.0001$).

When we compare the differences between CD46 and PSA (Figs. 2a and 2b), we see that both the SAR and the ARIC scores are significantly different ($P < 0.0001$ and $P < 0.0001$); the data for IAR are not significantly different between CD46 and PSA ($P = 0.9778$). This indicates that only acrosome-reacted sperm show a similar binding pattern with PSA and CD46. The high SAR binding with PSA could indicate that PSA is not a specific marker for the inner acrosomal membrane of human sperm, as the binding occurs before the AR is induced.

When we compare the differences between PNA and PSA (Figs. 2b and 2c), we see that all data groups are significantly different ($P < 0.0001$). This is to be expected, as PNA recognizes β-D-galactose and PSA recognizes α-D-mannose.

If we split the data into normal and abnormal specimens based on the spontaneous acrosome reaction (see above) and compare the t^2 results for each marker group, we find that only CD46 is capable of distinguishing between abnormal and normal sperm ($P < 0.0001$), whereas, PSA, PNA, and Con A do not distinguish between normal and abnormal spontaneous acrosome reactions ($P = 0.2296$, $P = 0.0843$, and $P = 0.8518$, respectively). However, of the three lectins, only the PNA marker needs further examination to determine its true cutoff value with respect to abnormal specimens if it is the marker of choice in acrosome reaction testing for fertility screening.

In using propidium iodide to discriminate between live and dead sperm, we see that all of the lectins used have negative effects on sperm viability and that these effects are more pronounced after ionophore treatment (Fig. 3). The most damaging lectin appears to be Con A, which gives a significantly lower viability than CD46 both with and without ionophore ($P < 0.0001$, $P < 0.0001$). Conversely, the least harmful marker appears to be CD46, with PNA as a close second. We cannot account for the negative effects of these markers by technical differences, as the same protocol was adhered to throughout. The results are particularly alarming with respect to Con A and PSA. We can postulate that these markers (which both bind to α-D-mannose moieties) may block glycoproteins necessary for normal sperm function, thereby compromising sperm viability. We could also postulate that the binding of PNA and CD46 to the receptor may be beneficial to sperm viability.

DISCUSSION

A major problem in studying the acrosome reaction of human spermatozoa is that acrosomal loss cannot be observed directly. In most previous investigations spermatozoa were permeabilized before labeling with lectins, to expose binding sites. The staining of intact

spermatozoa in suspension differs essentially from that of air-dried specimens, as the acrosomal region is more intensely stained in air-dried specimens (1). To distinguish true acrosome-reacted sperm from membrane-permeabilized, acrosome-intact sperm is very difficult, as both may be labeled. Once the membranes are permeabilized (by air-drying, fixation, or detergent treatments), the opportunity to measure the true acrosome reaction and to make the comparison of lectin labeling before and after the acrosome reaction is lost. Thus, the preservation of membrane integrity is paramount in studies of the acrosome reaction of human sperm. Many methods have been proposed to assess the human sperm acrosomal status, but as yet no single method is widely accepted as an indicator of the true acrosome reaction for clinical purposes.

Tao *et al.* (36) compared flow cytometry and epifluorescent microscopy with various lectins and indicated that there is no significant difference between the two methodologies for detection of the acrosome reaction ($P > 0.010$). However, it has been argued that lectins do not bind specifically to the acrosomal region of the sperm (23,26) and that other binding sites can be easily distinguished by epifluorescence microscopy, whereas flow cytometry identifies the signal from the entire sperm. Therefore, lectins with a high specificity for the acrosomal region are required to resolve different populations of sperm. Purvis *et al.* (23) found a single normal distribution, by flow cytometry, when labeling frozen sperm, which exhibit a degree of loss of membrane integrity. This is not in agreement with our data, which clearly show the presence of a low, or unlabeled, population and a labeled population, as was also demonstrated in mice by Tao *et al.* (36). One single normal distribution is not in agreement with the biology of the acrosome reaction, as not all sperm are able to acrosome react (37). Tao *et al.* (36) also state that PNA is a more reliable acrosome reaction marker compared to PSA, Con A, and SBA. Kallajoki *et al.* (1) further state that PNA represents intracellular rather than surface binding and is, therefore, suitable as an acrosome reaction marker for nonfixed and nonpermeabilized sperm. This conflicts with the findings of other workers (38,39), who state that PNA binds specifically to the outer acrosomal membrane of detergent (Nonidet P40)-permeabilized sperm by stating that the lectin must bind to an intracellular structure, probably to the outer acrosomal membrane. We agree that PNA must bind to an internal structure but argue that this is more likely to be the inner acrosomal membrane. This is also supported by Kallajoki *et al.* (1), who state that for nonpermeabilized, nonfixed sperm, the FITC-PNA indicates intracellular binding. From a biological point of view, both the plasma membrane and the outer acrosomal membranes fuse and vesiculate during the acrosome reaction, which would preclude binding to the outer acrosomal membrane on nonpermeabilized sperm. Also, it is questionable as to how one could permeabilize the plasma membrane without affecting the underlying outer acrosomal membrane.

Others suggest the use of nonfixed and nonpermeabilized sperm, as formaldehyde and ethanol often produce intensely labeled acrosomal regions, and ethanol may solubilize some glycoproteins that are not lost during spontaneous lysis (8).

It may be argued that when comparing fluorescent microscopy assays with flow cytometry, one is examining "patterns" of fluorescence rather than fluorescence intensity, i.e., the flow cytometer is not capable of discriminating sperm which have a fluorescent marker bound to the equatorial segment or over one of the acrosomal membranes (19,38,39). However, when we utilize flow cytometric assessment of the acrosome reaction to predict fertilization in IVF cycles, the problem of identifying specific regions of fluorescent intensity ceases to be an issue (33), i.e., the significance lies in the magnitude of fluorescence (after incubation and binding to an antibody conjugated to a fluorescent marker) expressed by acrosome-reacted and nonreacted sperm and the differences between the two values.

Our findings indicate that the only reliable lectin to use, without compromising sperm viability and membrane integrity, is PNA. We have shown that this particular lectin binds in a similar manner to CD46 antibody, i.e., to the inner acrosomal membrane of acrosome-reacted sperm, and is specific for this membrane (due to the low level of binding seen on non-acrosome-reacted sperm). Thus, PNA is capable of differentiating the acrosome reacted sperm from a given population. Further research is necessary to redefine acceptable limits for scoring an acrosome reaction measured by PNA, since the ionophore-induced acrosome reactions and the ARIC scores are essentially of a lower magnitude than those seen with CD46. It is possible that, in taking CD46 antibody binding as the "gold standard, we are imparting an unfair bias to the data for comparison with the lectins. However, in observing that only PNA gives a specific comparison between non-acrosome-reacted and acrosome-reacted sperm, the differences between the two markers are merely a matter of magnitude. In utilizing PNA in the assay system, one would need to redefine the limits of acceptability as to the actual ARIC score for diagnostic purposes. Stud-

ies are in progress to elucidate the true nature of binding of the various lectins to sperm membranes by electron microscopy and image analysis.

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