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Annexin V Binding and Merocyanine Staining Fail to Detect Human Sperm Capacitation

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ABSTRACT: The signaling pathways that characterize the process of capacitation of human spermatozoa are still largely unknown. Modifications in the lipid architecture of the sperm plasma membrane have been described in spermatozoa from different species, including translocation of phosphatidylserine (PS) from the inner to the outer leaflet and increased phospholipid disorder in the membrane. In human spermatozoa, however, results of PS exposure are controversial. In the present study, we used flow cytometry to investigate both membrane PS exposure by Annexin V (Ann V) binding and lipid disorder by merocyanine 540 (M540) staining, in swim-up–selected live spermatozoa after incubation in conditions leading to capacitation. Our results indicate that neither probe is able to detect capacitation-related membrane modifications. Investigation of the nature of PS exposure and M540-positive live cells was then carried out. We found that M540 stains elements devoid of nuclei are present in seminal plasma. Live PS-exposing cells were mainly represented by damaged spermatozoa as revealed by the occurrence of a negative correlation between PS exposure and normal morphology and motility in unselected samples. The same cells were also positive for M540. These results demonstrate that Ann V and M540 binding in human sperm samples mainly detects cells with early membrane degeneration as well as dead cells, which is in agreement with findings obtained for somatic cells in which the two probes recognize cells with a damaged membrane due to the apoptotic process.

Key words: FACScan, membrane architecture, phosphatidylserine translocation.

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Capacitation is an essential process that a spermatozoon must undergo in the female reproductive tract to become able to fertilize an egg. As a consequence of capacitation, sperm become responsive to stimuli that induce the acrosome reaction and develop a particular pattern of motility known as hyperactivation (Yanagimachi, 1994). Up to now, the molecular mechanisms underlying capacitation have not been completely disclosed. Several molecular modifications involved in the initiation of the process have been identified, including cholesterol efflux from the plasma membrane, increased membrane fluidity, modulation in intracellular ion concentrations, increased protein tyrosine phosphorylation, and hyperpolarization of the plasma membrane (Yanagimachi, 1994; Baldi et al, 2000; Visconti et al, 2002), although the precise relation between such modifications and the development of capacitation is not completely understood.

Capacitation can be achieved in vitro by incubating sperm in defined media. Two essential components of these media are serum albumin and bicarbonate. Serum albumin is believed to facilitate the efflux of cholesterol from the sperm plasma membrane by acting as an acceptor for the lipid (Visconti et al, 2002), whereas entry of the bicarbonate ion into spermatozoa has been shown to be involved in the observed increase in intracellular pH during capacitation (Zeng et al, 1996). In addition, bicarbonate appears to be involved in the activation of adenylyl cyclase and in the consequent increase in intracellular levels of cyclic adenosine monophosphate (cAMP) (Okamura et al, 1985) and tyrosine phosphorylation of proteins (Osheroff et al, 1999) that have been observed during capacitation. In some mammalian species, it has been shown that bicarbonate-induced cAMP increase provokes the loss of the membrane asymmetry due to a transbilayer scrambling of membrane phospholipids, resulting in an increased lipid disorder in the membrane (Gadella and Harrison, 2000; Fletsch et al, 2001; Rathi et al, 2001) and external exposure of phosphatidylserine (PS) (Gadella and Harrison, 2002). The increased disorder in the phospholipid membrane package of capacitated viable sperm has been detected by staining with the lipophilic dye merocyanine 540 (M540) (Gadella and Harrison, 2000; Fletsch et al, 2001; Rathi et al, 2001), whereas the external exposure of PS has been revealed by the Annexin V (Ann V) binding assay in viable sperm (Gadella and Harrison, 2002).

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In human sperm, studies of PS translocation from the inner to the outer leaflet of the membrane of viable cells are conflicting. Our group and other groups reported evidence that PS exposure in live sperm represents an early sign of cellular damage (D'Cruz et al, 1998; Ramos et al, 2001; Muratori et al, 2003), whereas Barroso et al (2000) reported that an increased level of Ann V binding is present in the most motile fraction of the sperm populations, and De Vries et al (2003) showed an increase in this parameter with capacitation, as it occurs in boar sperm (Gadella and Harrison, 2002). More recently, Moustafa et al (2004) demonstrated the occurrence of a strong correlation between PS exposure and sperm DNA damage in semen from infertile patients. It might be postulated that both damaged and capacitated spermatozoa, which are present in human ejaculates due to the great heterogeneity of sperm population in this species, are characterized by PS exposure on the surface, thus generating some confusion in the interpretation of the results. Because M540 staining has been proven able to detect capacitated sperm in some mammalian species (Gadella and Harrison, 2000; Fletsch et al, 2001; Rathi et al, 2001), this probe could be useful in discriminating among capacitated and damaged human sperm, thus clarifying the nature of those with membrane PS translocation. Therefore, in the present study, we investigated whether staining with M540, coupled to flow cytometry detection, is able to reveal capacitated human spermatozoa. In addition, using flow cytometry, we investigated M540 staining and Ann V binding in swim-up–selected viable sperm following incubation in capacitating or noncapacitating medium. Moreover, the amount of live PS-exposing sperm was evaluated in unselected sperm and correlated to morphology, motility, and viability. Our results clearly show that capacitation of human sperm is not associated with either PS membrane exposure or M540 staining, and that PS exposure is higher in sperm populations characterized by abnormal morphology and reduced motility.

Methods

Chemicals

Human tubal fluid (HTF) medium, modified HTF (HTF/HEPES) medium, and human serum albumin (HSA) were purchased from Celbio (Milan, Italy). M540 and YOPRO-1 (Y1) were from Molecular Probes Europe BV (The Netherlands). PureSperm was obtained from Nidacon (Gothenberg, Sweden). All reagents for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Amersham Pharmacia Biotech Italia (Cologno Monzese, Italy). Reagents for protein measurement were from Bio-Rad Laboratories (Hercules, Calif). Peroxidase-conjugated PY20 antibody was obtained from Calbiochem (La Jolla, Calif). Rat anti-AKAP3 FSP95 antibody was kindly provided by Prof John Herr (Charlottesville, Va). Antibody anti-p42ERK was kindly provided by Prof M.J. Dunn (Medical College of Wisconsin, Milwaukee, Wis). Testsimplets slides and the BM enhanced-chemiluminescence system were purchased from Roche Diagnostics (Milan, Italy). Polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), the conjugated anti-rat secondary antibody, and the other chemicals was from Sigma Chemical Company (Milan, Italy).

Sperm Media

Swim-up selection and incubation were carried out in HTF medium supplemented with 10% HSA, from here on indicated as CM (capacitating medium) or in HTF/HEPES medium (supplemented with 1 mg/mL PVA), from here on indicated as NCM (noncapacitating medium). HTF medium and HTF/HEPES medium have the same composition, except for $NaHCO_3$ (respectively, 24 and 4 mM) and HEPES (21 mM) contained only in the latter. NCM (devoid of serum albumin and with low concentrations of $NaHCO_3$) was used to exclude the occurrence of capacitation in the sperm samples (Courmier et al, 1997; Choi and Toyoda, 1998; De Vries et al, 2003).

Semen Sample Collection and Preparation

Semen samples were collected according to World Health Organization (1999) criteria from subjects undergoing routine semen analysis for couple infertility in the andrology laboratory of the University of Florence. Samples with any detectable leukocytes in the semen were excluded from the study. Detection of leukocytes was performed by carefully determining their percentage on the total amount of round cells after smearing semen onto prestained slides (Testsimplets).

Sperm morphology and motility were assessed by optical microscopy, according to World Health Organization criteria. Sperm morphology was scored by determining the percentage of normal and abnormal forms, after smearing semen onto prestained slides (Testsimplets). Sperm motility was scored by determining the percentage of progressive motile, nonprogressive motile, and immotile spermatozoa. For experiments assessing Ann V and M540 binding related to capacitation, only semen samples from normozoospermic (World Health Organization, 1999) subjects were used. For the remaining experiments, semen samples were randomly collected from normozoospermic, teratozoospermic, asthenoteratozoospermic, and oligoasthenoteratozoospermic subjects (World Health Organization, 1999). Table 1 reports the mean values of sperm concentration, motility, morphology, volume, and pH of the semen samples, and the age of the subjects included in the study.

The swim-up–selection technique was used for all the experiments assessing Ann V binding and M540 staining related to capacitation. Swim-up selection was performed by layering 1 mL of CM or NCM on the top of an equal volume of semen fluid. After 1 hour of incubation at 37°C in 5% CO_2 atmosphere, 900 μL of medium was carefully collected.

For experiments in which sperm samples were prepared by gradient separation, semen samples were layered on 50%, 70%, and 95% PureSperm fractions (prepared in HTF/HSA medium) and centrifuged at 500 × *g* for 30 minutes at 26°C. The resulting interfaces between seminal plasma and 50% (fraction 1), 50% and 70% (fraction 2), 70% and 90% (fraction 3), and the 95%

Table 1. *Semen parameters of subjects included in the study**

Parameters	N (n = 35)	AT (n = 25)	T (n = 14)	OAT (n = 18)
Sperm concentration (sperm/mL)	95.7 ± 63.0	62.3 ± 48.1	51.2 ± 21.7	8.3 ± 5.4
Total motility (%)	74.7 ± 10.3	53.9 ± 12.5	74.6 ± 4.7	42.1 ± 16.2
Progressive motility (%)	59.8 ± 9.8	33.2 ± 10.6	59.6 ± 6.3	22.9 ± 13.2
Normal Morphology (%)	38.3 ± 7.3	17.7 ± 7.5	18.1 ± 6.0	10.5 ± 9.0
Ejaculate volume	3.4 ± 1.4	3.1 ± 1.4	3.2 ± 1.4	4.0 ± 1.8
pH	7.4 ± 0.2	7.4 ± 0.2	7.4 ± 0.2	7.4 ± 0.2
Age	33.8 ± 6.3	35.4 ± 7.9	30.8 ± 9.6	34.6 ± 5.4

* Values are mean ± SD. N indicates normozoospermic; AT, asthenoteratozoospermic; T, teratozoospermic; and OAT, oligoasthenozoospermic.

pellet (fraction 4) were collected and transferred to separate test tubes. Then, each fraction was washed with an equal volume of medium.

For experiments performed in unselected sperm, semen samples were washed twice with HTF medium.

Measurement of Intracellular Calcium Concentration

Intracellular calcium concentration ($[Ca^{2+}]_i$) was evaluated with a spectrofluorimetric method as previously described (Baldi et al, 1991), with the exception that, in the present experiments, we used a Perkin-Elmer (Foster City, Calif) LS50B instrument equipped with a fast rotary filter shuttle for alternate 340- and 380-nm excitation (Luconi et al, 1999). Briefly, swim-up–selected spermatozoa obtained as described above in CM or NCM were loaded with 2 μM Fura-2/AM for 45 minutes at 37°C. After centrifugation, the pellet was resuspended in FM medium (125 mM NaCl, 10 mM KCl, 2.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 19 mM Na-lactate, 2.5 mM Na-pyruvate, 2 mM HEPES, 0.3% bovine serum albumin [BSA] pH 7.5) and incubated for an additional 30 minutes. Fluorescence measurements were converted to $[Ca^{2+}]_i$ by determining the maximal fluorescence (F_{max}) with 0.01% digitonin followed by minimal fluorescence (F_{min}) with 10 mM ethyleneglycotetraacetic acid pH 10. $[Ca^{2+}]_i$ was calculated according to the method described by Grynkiewicz et al (1985) using the ratio 340:380 and assuming a dissociation constant of Fura-2 for calcium of 224 nM. $[Ca^{2+}]_i$ was determined both in basal condition and after stimulation with progesterone (10 μM).

SDS-PAGE and Western Blot Analysis

After incubation in CM or NCM, sperm (about 5×10^6 cells) were washed and resuspended in lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 0.25% NP-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride [PMSF]) for total sperm extract. In some experiments, sperm (about 30×10^6 cells) were processed for preparation of sperm tail and head purified fractions (Luconi et al, 2004). Briefly, after washing, spermatozoa were sonicated at 8 bursts (3×10 seconds) in Na_3VO_4-containing phosphate-buffered saline (PBS) on ice. After $500 \times g$ centrifugation for 5 minutes, the supernatant corresponding to tail fractions and the pellet corresponding to head fractions were examined with a microscope and then extracted in lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 0.25% NP-40, 1 mM Na_3VO_4, 1 mM PMSF). After protein measurement (Comassie kit), the sperm extracts containing approximately 20 μg of protein were diluted in an equal volume of 2× Laemmli reducing sample buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 2.5% pyronin, and 200 mM dithiothreitol), incubated at 95°C for 5 minutes and loaded onto 8% polyacrylamide-bisacrylamide gels. After SDS-PAGE, proteins were transferred to nitrocellulose (Sigma, St Louis, Mo). Membranes were blocked for 2 hours, washed in TTBS (Tris-buffered saline containing 0.1% Tween 20 pH 7.4), and incubated overnight with primary antibodies. After blocking at room temperature for 2 hours in 10% BM blocking buffer (Roche, Milan) in TTBS solution, nitrocellulose membranes were washed and then immunostained with peroxidase-conjugated antiphosphotyrosine antibody (PY20-HRP, 1:1000). The antibody-reacted proteins were revealed with an enhanced-chemiluminescence system. For membrane reprobing, the nitrocellulose membranes were washed for 30 minutes at 50°C in stripping buffer (10 mM Tris pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) and reprobed with anti-AKAP3 FSP95 antibody (1:3000) or anti-p42 ERK (1:1000), followed by conjugated secondary antibody.

Staining With M540 in Live and Dead Sperm

Staining with M540 was performed in the same media used for sperm preparation, except for the addition of PVA and PVP (0.5 mg/mL each; Fletsch et al, 2001; Rathi et al, 2001). Unless otherwise stated (see ''Results''), M540 was used at the concentration of 0.27 μM. In most experiments, the cell-impermeable nuclear stain, Y1 (25 nM), was added to discriminate dead and live cells (Fletsch et al, 2001; Rathi et al, 2001). Working aqueous solutions (100×) of M540 and Y1 were prepared from corresponding stock solutions in dimethylsulfoxide. To define the optimal incubation time for staining, spermatozoa were incubated with the dye for increasing times. These experiments demonstrated that 15 minutes of incubation was the shorter staining time required for reaching a plateau of labeling (data not shown). Incubation of spermatozoa (10^6) with the stains was carried on in 500 μL of medium in the dark at 37°C in a 5% CO_2 atmosphere for experiments assessing the state of sperm capacitation.

In each experiment, two sperm suspensions were prepared for instrumental setting and data analysis: 1) by omitting both M540 and Y1 staining (nonspecific fluorescence sample), and 2) by omitting only the M540 staining (sample for compensation, see below). Y1 green fluorescence and M540 red fluorescence were revealed by using, respectively, an FL-1 (515–555 nm wavelength band) and an FL-2 (563–607 nm wavelength band) detector of a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif) equipped with a 15 mW argon-ion laser for excitation. Fluorescence compensation was set by acquiring

sperm labeled with only Y1. For each sample, 10 000 events were recorded within the characteristic flame-shaped region in the forward light scatter/side light scatter (FSC/SSC) dot plot corresponding to sperm population (Muratori et al, 2000, 2003). Percentages of subpopulations were determined after gating out debris, as previously reported (Muratori et al, 2000, 2003).

For observation by fluorescence microscopy, double-stained sperm were smeared on slides and examined using a fluorescence microscope (type 307-148002; Leitz, Wetzlar, Germany) equipped with E4 and N2.1 filters (Leica, Milan, Italy) by an oil immersion 100× magnification objective.

Staining With Methylene Blue and Cresyl Violet Acetate

Ten microliters of semen were smeared onto prestained Testsimplets slides. After 15 minutes, slides were observed under a light microscope with an oil immersion 100× magnification objective.

Binding of Annexin V in Live and Dead Sperm

Sperm (10^6 cells) were suspended in 250 μL of the medium used for cell preparation, supplemented with $CaCl_2$ up to 2.5 mM. Then, Annexin-V-FLUOS (Ann V-F, Annexin V conjugated to fluorescein, supplied at the 200× concentration by Roche Molecular Biochemicals [Milan, Italy]) was added, and samples were incubated for 20 minutes in the dark at 37°C (in a 5% CO_2 atmosphere in experiments to asses the state of sperm capacitation). After centrifugation (250 × *g*, 10 minutes), sperm were resuspended in 500 μL of the above medium, stained with 10 μL of propidium iodide (PI, 30 μg/mL in PBS), and acquired by flow cytometry after a further 5 minutes at 37°C. For each experimental set, two sperm suspensions were prepared for instrumental setting and data analysis: 1) by omitting both Ann V-F and PI staining (nonspecific fluorescence sample), and 2) by omitting only the PI staining (sample for compensation, see below). Ann V-F green fluorescence and PI red fluorescence were revealed by using FL-1 and FL-2 detectors, respectively. Fluorescence compensation was set by acquiring sperm labeled with only Ann V-F. For each sample, 10 000 events were recorded within the characteristic flame-shaped region in the FSC/SSC dot plot corresponding to sperm population (Muratori et al, 2000, 2003). Percentages of subpopulations were determined after gating out debris, as previously reported (Muratori et al, 2000, 2003).

Double Staining With Annexin V-F and M540 in Human Sperm

Swim-up–selected or unselected sperm were first stained with Ann V-F as described above. After the 20-minute incubation, sperm were suspended in HTF/HEPES medium containing 2.5 mM $CaCl_2$, PVP 0.5 mg/mL, and PVA 0.5 mg/mL. Then, M540 0.27 μM was added, and samples were incubated for 15 minutes at 37°C. Data analysis was performed within the region containing the population of interest in the FSC/SSC dot plot (see ''Results'').

Statistical Analysis

Data were analyzed with Microcal Origin software, 6.1 version (MicroCal Software Inc, Northampton, Mass). Unless otherwise stated, results are shown as the mean ± SE. Analysis of variance and the Student's *t* test were used to assess statistically significant differences between the investigated parameters. Correlation tests were performed by linear regression analysis.

Results

Staining With M540 in Human Sperm

In order to define the optimal concentration of M540 to stain human sperm, swim-up–selected and unselected sperm from the same semen samples were labeled with increasing concentrations (0.027, 0.27, 1.35, and 2.7 μM) of the fluorochrome. After gating the characteristic flame-shaped region corresponding to human sperm (Figure 1A), we found that the lowest concentration able to sharply separate less fluorescent ($M540^{dim}$) from brighter ($M540^{br}$) cells was 0.27 μM both in selected and in unselected sperm (Figure 1B, upper panels: selected sperm; lower panels: unselected sperm). At this concentration, the histogram of the $M540^{dim}$ cells overlapped the fluorescence of the unstained sample (nonspecific fluorescence, open histogram in Figure 1B). Higher concentrations of the dye were also able to discriminate between $M540^{dim}$ and $M540^{br}$ cells and yielded the same percentages of $M540^{br}$ sperm (Figure 1B), determining a shift of both populations to higher intensities of fluorescence. Hence, we concluded that the different concentrations of the probe yielded equivalent results.

In order to discriminate between live and dead spermatozoa within the M540-positive sperm population, M540 (0.27 μM) staining was coupled to Y1 (25 nM) labeling (Fletsch et al, 2001; Rathi et al, 2001). Figure 1C shows the dot plots corresponding to selected (upper panel) and unselected (lower panel) sperm. In selected sperm, all the cells stained by M540 were also positive for Y1, and thus were considered dead. On the contrary, a percentage of $M540^+/Y1^-$ events was present in unselected sperm populations.

The presence of staining with M540 in unselected but not in selected live sperm was surprising and suggested that the $M540^+/Y1^-$ population is not selected by swim-up. Because it has been reported that M540 binds to mammalian capacitated sperm (Gadella and Harrison, 2000; Fletsch et al, 2001; Rathi et al, 2001), we reasoned that the lack of M540 staining in swim-up–selected sperm could be due to their being not fully capacitated, as staining was performed immediately after swim-up. To test this hypothesis, we determined the percentage of $M540^+/Y1^-$ events in sperm after a total of 3 hours of incubation (1 hour for swim-up selection plus 2 additional hours) in CM or in NCM. Although it has been shown previously by our group that such a procedure supports sperm capacitation (Baldi et al, 1991, 1993; Luconi et al, 1996, 1998a,b), actual induction of capacitation during incuba-

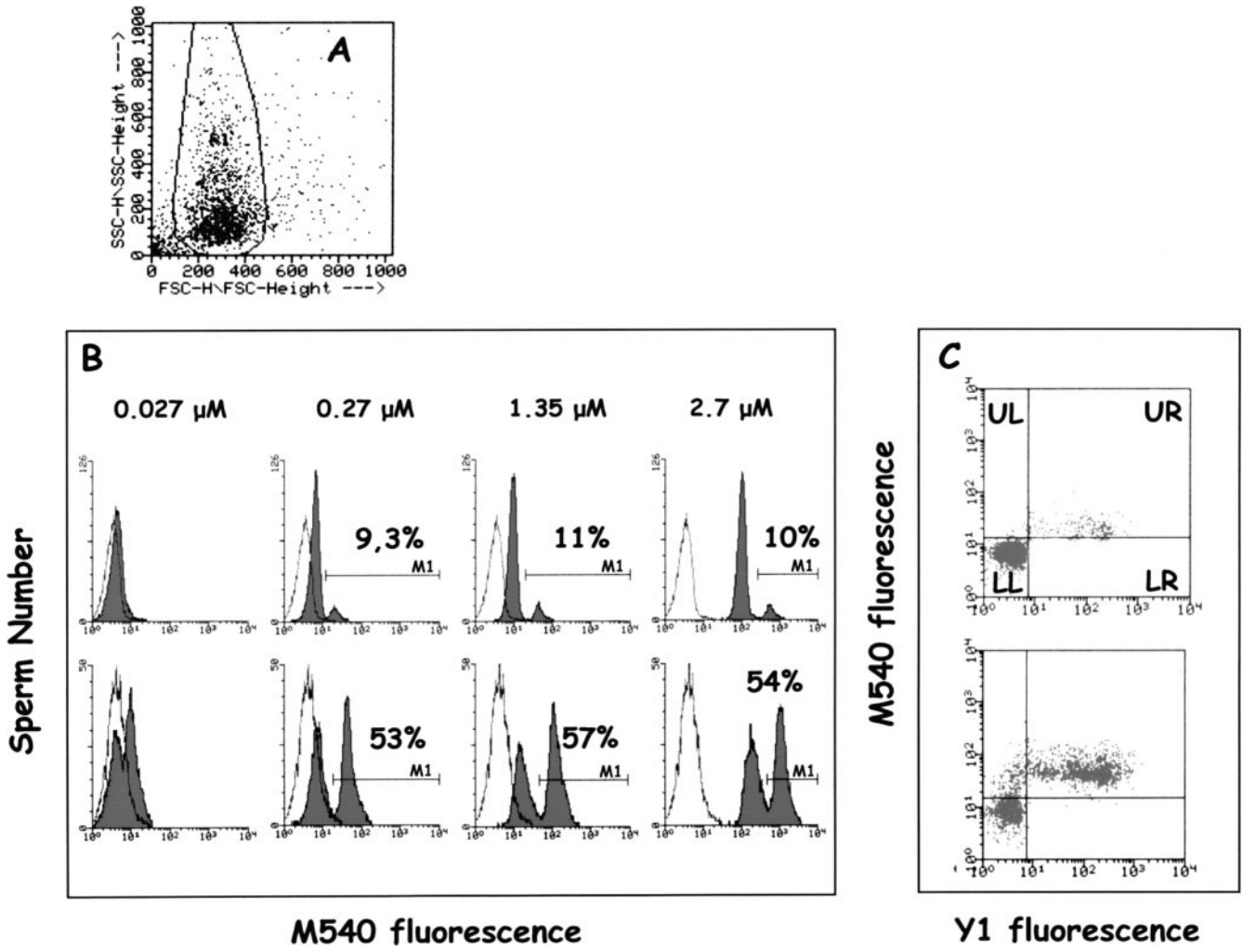

Figure 1. **(A)** FSC/SSC dot plot showing the flame-shaped region (R1) containing sperm. **(B)** M540 fluorescence histograms of human sperm stained with increasing concentrations of the fluorochrome (upper panels, swim-up selected sperm; lower panels, unselected sperm). In each panel, M1 indicates the region including the $M540^{br}$ (brighter) cells. $M540^{dim}$ (less fluorescent) cells are all the events out of such regions. Percentage of $M540^{br}$ cells in the different conditions are indicated in each panel. The histogram corresponding to the nonspecific fluorescence is also shown (open histogram). A typical experiment out of 3 similar is shown. **(C)** Double staining of human sperm with M540 (0.27 µM) and Y1 (upper panel, swim-up–selected sperm; lower panel, unselected sperm). LL indicates lower left quadrant: $M540^-/Y1^-$ (live, M540 unstained population); UL indicates upper left quadrant: $M540^+/Y1^-$ (live, M540 stained population); UR indicates upper right quadrant: $M540^+/Y1^+$ (dead, M540 stained population); and LR indicates lower right quadrant.

tion in CM in the present experiments was verified by two different procedures: 1) by measuring the basal level of $[Ca^{2+}]_i$ and the increase in the same parameter in response to progesterone (Baldi et al, 1991; Garcia and Meizel, 1999); and 2) by determining, by Western blot analysis, tyrosine phosphorylation in total sperm lysates and lysates from tail and head preparations. Indeed, it has been shown that an increase in tyrosine phosphorylation occurs during sperm capacitation (Luconi et al, 1995, 1996), in particular in flagellar proteins, such as the antigen A-kinase anchoring proteins AKAP3 and AKAP4 (Mandal et al, 1999; Bajpai and Doncel 2003; Ficarro et al, 2003). Results of these experiments are reported in Figure 2. As expected, the progesterone-stimulated $[Ca^{2+}]_i$ increase was much higher in swim-up–selected spermatozoa prepared and incubated in CM compared to those prepared and incubated in NCM (Figure 2A) (Baldi et al, 1991; Garcia and Meizel, 1999). Furthermore, results of Western blot analysis of tyrosine phosphorylated proteins performed in total sperm lysates incubated in CM demonstrated an increased phosphorylation compared to sperm incubated in NCM (Figure 2B, left panel). In addition, Western blot analysis of tyrosine phosphorylation in purified tail and head fractions from swim-up–selected sperm incubated in the two different conditions demonstrated that the increase in tyrosine phosphorylated proteins in CM is mainly localized in the tails (Figure 2C, left panel). Stripping and reprobing of the two membranes

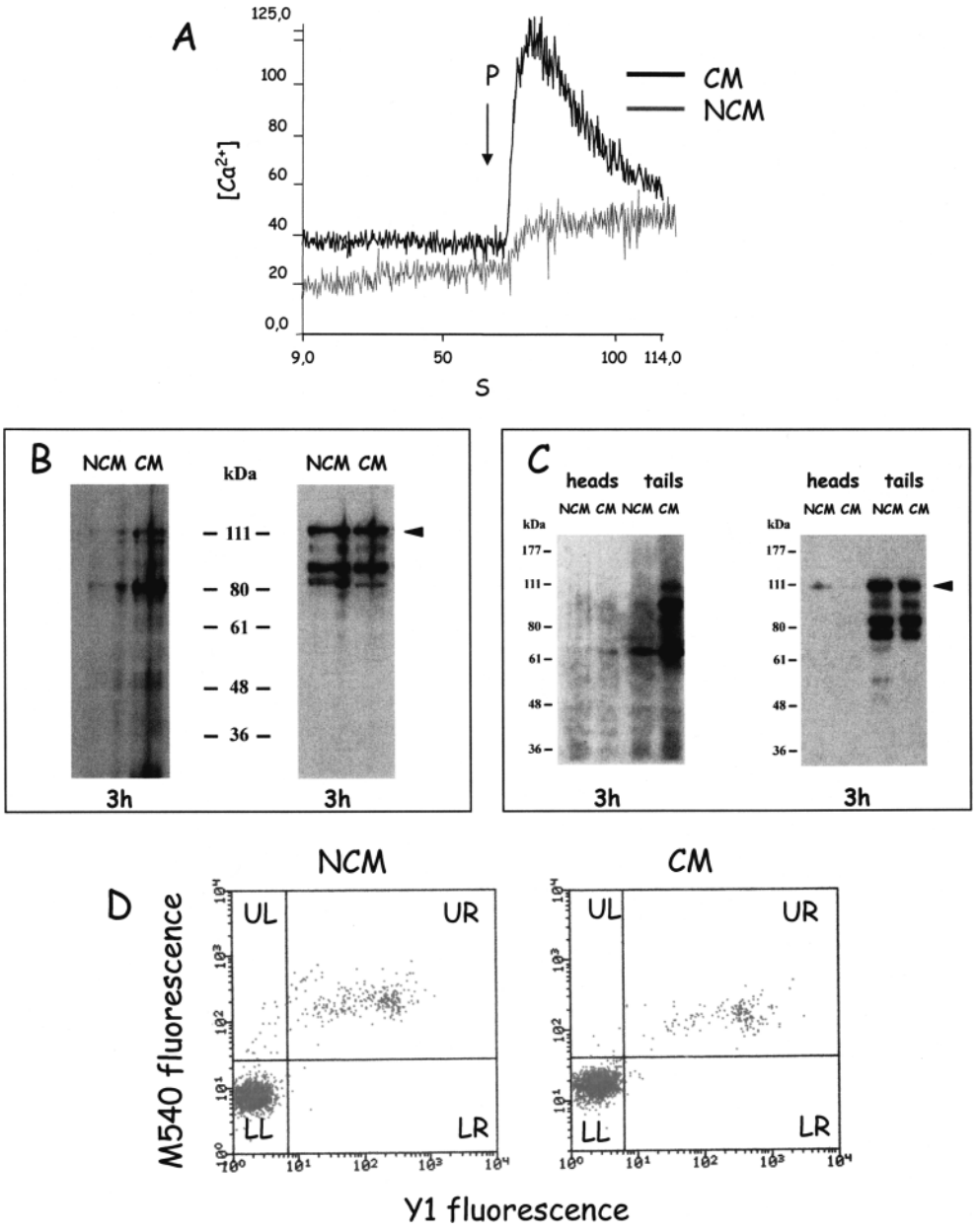

Figure 2. Effect of sperm incubation in capacitating (CM) and noncapacitating (NCM) medium on intracellular calcium and tyrosine phosphorylation of sperm proteins. **(A)** Basal and progesterone-stimulated $[Ca^{2+}]_i$ levels in swim-up–selected sperm incubated for 3 hours (1 hour for swim-up selection followed by a 2-hour incubation) in CM (black trace) or in NCM (gray trace). A typical experiment out of 3 with similar results is shown. The arrow indicates the addition of progesterone (10 μM). **(B)** and **(C)** Western blot analysis of total **(B)**, tail and head extracts **(C)** from swim-up–selected sperm incubated for 3 hours (1 hour for swim-up selection followed by a 2-hour incubation) in CM or NCM. Equal amounts of sperm protein extracts (20 μg) were subjected to 8% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was first blotted with PY20-HRP antibody to reveal tyrosine phosphorylated proteins (left panels) and, after stripping, reprobed with FSP95 antibody to reveal AKAP3 in order to show equal loading of the lanes (right panels). Molecular weight markers (kDa) are indicated in the middle **(panel B)** or on the left **(panel C)** of the blots. Representative of 3 similar experiments. **(D)** Double staining with M540 and Y1 of swim-up spermatozoa incubated for 3 hours in NCM (left panel) or in CM (right panel). A typical experiment out of 9 with similar results is shown.

using an antibody directed against the fibrous sheath AKAP 3 (Mandal et al, 1999) demonstrated equal loading of the lanes in total sperm lysates and in tail preparations (Figure 2B and C, right panels). Because AKAP3 is much less expressed in head fractions (Mandal et al, 1999), the blot in Figure 2C was stripped and reprobed using anti-p42 ERK (Luconi et al, 1998a,b), which demonstrated equal loading of the two lanes (NCM and CM) in head fractions (not shown). In pilot experiments (n = 3) to test M540 staining in $Y1^-$ capacitated and noncapacitated sperm, we used M540 at the concentrations of 0.27 and 2.7 μM. Again, the only difference between the two con-

centrations was a shift toward the higher values of the M540 fluorescence (data not shown). Thus, the remaining experiments of M540 staining were performed at the concentration of 0.27 μM. Figure 2D shows M540/Y1 dot plots corresponding to the capacitated and noncapacitated selected sperm. The percentage of $M540^+/Y1^-$ elements is very low in both samples and does not show any difference in the two experimental conditions (respectively, CM: 1.1% ± 0.2% versus NCM: 1.8% ± 0.4%; n = 9; $P > .05$; Figure 2D). A small percentage of Y1-positive cells (upper right quadrants) is present in both conditions (8.6% ± 1.0%, and 14.0% ± 1.8%, respectively, in CM and NCM media), probably due to the staining procedure, because motility immediately after swim-up was >90% in all the samples. To further characterize $M540^+/Y1^-$ elements, we used unselected samples, in which their concentration is higher. First, we investigated the morphology of these elements by double staining unselected sperm with M540 and Y1 and observing them under a fluorescence microscope. Using appropriate filters for M540 and Y1 fluorescence, we found that $M540^+/Y1^-$ elements, showing only red fluorescence (Figure 3A, indicated by thin arrows), are characterized by a round shape with variable dimensions, including those similar to sperm heads (as expected by their inclusion in the gate established in the FSC/SSC dot plot) and do not show any morphological sign of spermatozoa (Figure 3A). On the contrary, the M540-positive spermatozoa are also positive for YI (Figure 3A and B, indicated by thick arrows), as expected on the basis of the results shown in Figure 1C (lower dot plot). To verify the presence of these elements with similar size as a sperm head in seminal fluid, the latter was smeared on Testsimplets slides and observed via microscopy. Round elements are indeed present in seminal fluid (Figure 3C). These elements apparently lack methylene blue labeling, suggesting an absence of nuclei. It is thus possible that the lack of staining with Y1 in $M540^+$ elements (Figure 3A and B) could be due to the absence of nuclei rather than to cell viability. To confirm this possibility, unselected sperm were stained with M540 and Y1 before and after induction of cell permeabilization by treatment with digitonin 200 μg/mL. As shown in Figure 3D, the percentage of $M540^+/Y1^-$ elements (upper left quadrants) does not change after this treatment (10.6% ± 3.6% vs 11.3% ± 2.6%; n = 4; $P > .05$), whereas all $M540^-/Y1^-$ elements (lower left quadrant, left panel) become $M540^+/Y1^+$ (upper right quadrant, right panel), indicating that the treatment has effectively determined the permeabilization of the cells, allowing staining of their nuclei. We conclude that $M540^+/Y1^-$ elements lack a nucleus. In the remaining part of the paper, we term ''M540 bodies'' the $M540^+/Y1^-$ elements. By using fluorescence microscopy M540 bodies were never detected in swim-up–selected spermatozoa, suggesting that a very low percentage of M540 staining detected in the upper left quadrants of Figure 1C (upper panel) and Figure 2D is due to a nonspecific signal.

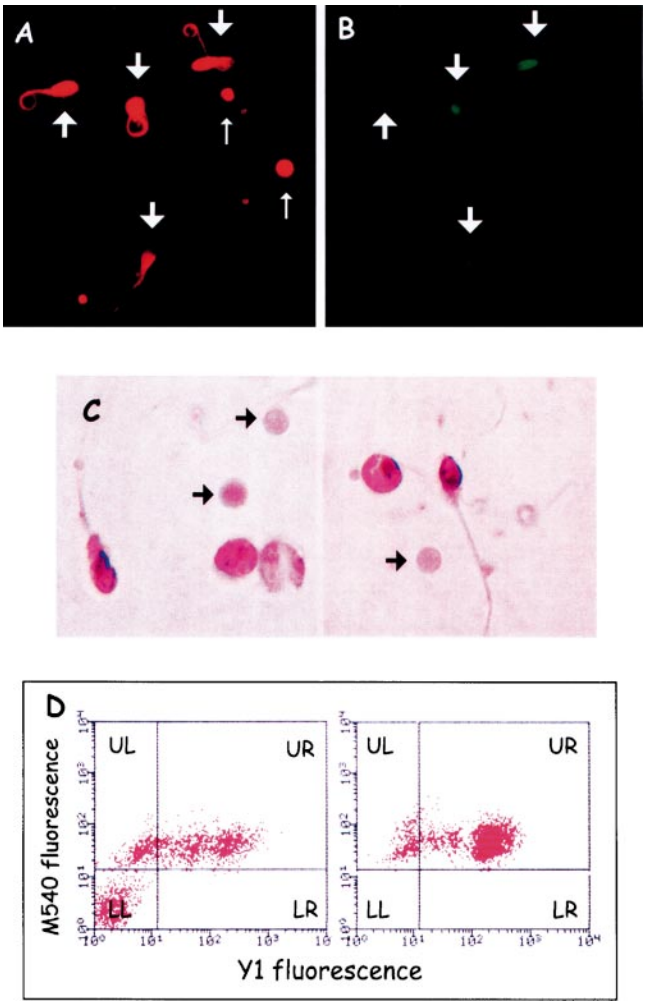

Figure 3. **(A, B)** Micrographs of unselected sperm obtained by fluorescence microscopy after double staining with M540 and Y1. **(A)** shows the red fluorescence (M540 staining) and **(B)** the green fluorescence (Y1 staining) from the same field. Note the round red elements in **(A)** (thin arrows) are not stained with Y1, while sperm (thick arrows) exhibit both colors of fluorescence **(A and B)**. **(C)** Micrographs of two different fields obtained by light microscopy, after smearing unselected sperm on prestained Testsimplets slides. Note the presence of round elements apparently lacking a nucleus (black arrows). **(D)** Double staining with M540 and Y1 of unselected sperm before (left panel) and after (right panel) induction of cell permeabilization by treatment with digitonin 200 μg/mL. Note the passage of lower left quadrant events (viable cells) of the left panel to the upper right quadrant of the right panel (dead cells), while the events of upper left quadrant remain in the same position. Results of 1 out of 4 similar experiments are shown.

To separate M540 bodies from the whole semen, we performed centrifugation on discontinuous density gradient of human semen and stained sperm migrating in the different fractions with M540 and Y1. We found that the percentage of M540 bodies (Figure 4A, upper left quadrants) decreased from fraction 1 to fraction 4. The mean

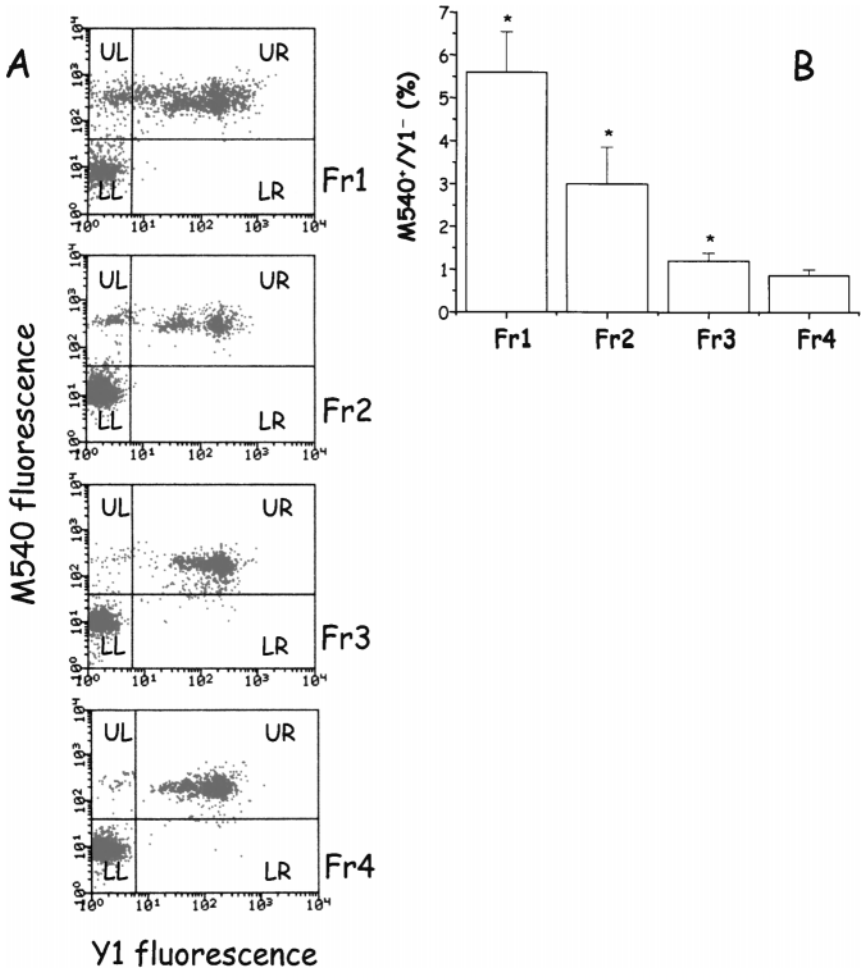

Figure 4. **(A)** Double staining with M540 and Y1 of spermatozoa collected from fractions 1 (Fr1), 2 (Fr2), 3 (Fr3), and 4 (Fr4) (see ''Materials and Methods'') of discontinuous density gradient. LL indicates lower left quadrant; UL, upper left quadrant; UR, upper right quadrant; and LR, lower right quadrant. **(B)** Mean values of percentages of ''M540 bodies'' in fractions 1, 2, 3, and 4. *$P < .05$; n = 7, paired *t* test vs Fr4.

values for percentages of M540 bodies in each fraction, as calculated from 7 experiments, is shown in Figure 4B.

The presence of elements (termed ''apototic bodies'') resembling the bodies resulting from apoptosis of somatic cells, with similar characteristics to M540 bodies observed in the present study, has been described previously in semen from subfertile men (Baccetti et al, 1996; Gandini et al, 2000). They are considered the terminal stages of the apoptotic process in the testis (Gandini et al, 2000) and may be extruded during secretion of spermatozoa. Because germ cell apoptosis appears to be higher in subfertile men (Baccetti et al, 1996; Sakkas et al, 1999a), we tested whether the percentage of M540 bodies was different in pathological conditions of seminal fluid. As shown in Figure 5, the percentage of M540 bodies is much higher in semen samples from oligoasthenoteratozoospermic subjects compared to normozoospermic subjects ($P < .01$). A slight, though significant, increase in M540 bodies is also present in teratozoospermic ($P < .05$) and asthenoteratozoospermic ($P < .05$) subjects compared to normozoospermic subjects. We found, interestingly, a significant, negative correlation between the percentage of M540 bodies in semen and both sperm concentration ($r = -.49$; SD = 44.4; $P < .01$; n = 29) and normal morphology ($r = -.54$; SD = 10.2; $P < .01$; n = 29).

Binding of Annexin V in Viable Human Sperm

As mentioned above, the meaning of PS exposure on the surface of human sperm is not completely understood. To evaluate whether PS exposure (as measured by Ann V binding) is associated with development of capacitation in human sperm, we stained swim-up–selected sperm simultaneously with Ann V-F and PI (the latter stain is used to discriminate between live and dead cells) after 3 hours of incubation (1 hour for swim-up selection plus 2 additional hours) in CM or in NCM. Induction of capacitation in our incubation conditions was verified as described above by determining, by Western blot analysis, tyrosine

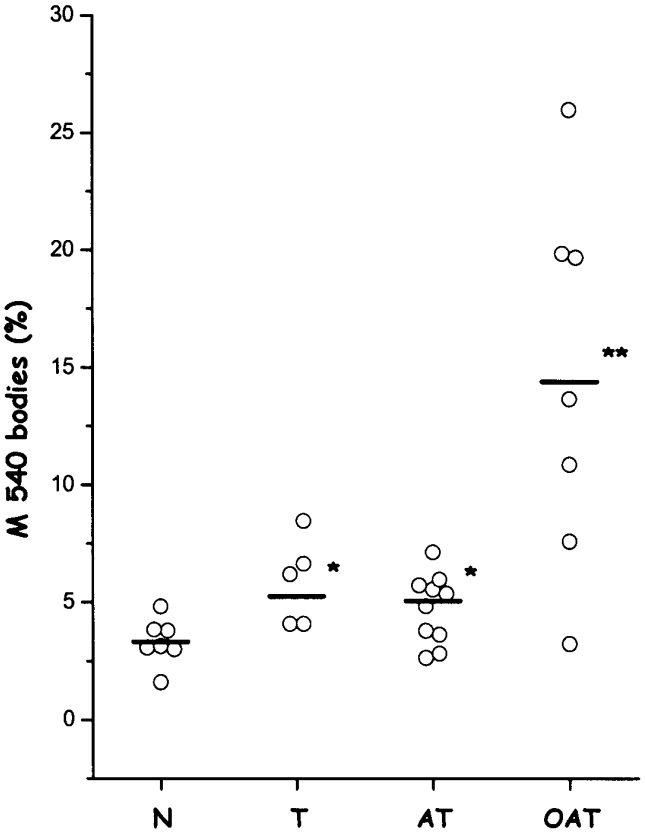

Figure 5. Percentages of ''M540 bodies'' on total sperm population in unselected sperm samples from normozoospermic (N, n = 7), asthenoteratozoospermic (AT, n = 10), teratozoospermic (T, n = 5), and oligoasthenoteratozoospermic (OAT, n = 7) subjects. *$P < .05$, **$P < .01$. Independent *t* test. Mean values of the percentage of ''M540 bodies'' in each group are indicated by the bar symbol.

phosphorylation in the total extracts and in the extracts of tail and head preparations from sperm (see Figure 2B and C). As shown in Figure 6, the percentage of live Ann V^+ sperm (lower right quadrants) does not show any increase in CM with respect to NCM (5.1% ± 1.7% vs 7.5% ± 1.6%; n = 6; $P > .05$). Most of the viable cells are Ann V^- (Figure 6A, lower left quadrants). A small percentage of PI-positive cells (upper right quadrants) is present in both conditions (12.5% ± 2.3% and 18.7% ± 3.7%, respectively in CM and NCM media), probably due to the staining procedure, because motility immediately after swim-up was >90% in all the samples.

To better clarify the nature of Ann V^+ viable sperm, Ann V binding was determined in swim-up–selected and the corresponding unselected sperm. We found that the percentage of Ann V^+/PI^- as well as of Ann V^+/PI^+sperm is much greater in unselected than in corresponding swim-up–selected sperm (Figure 6B). Because unselected samples are characterized by a poorer motility, morphology, and viability with respect to swim-up–selected sperm, we tested whether Ann V binding correlated with these sperm parameters in 15 unselected samples. We found a strong correlation between Ann V binding in live sperm, and both sperm morphology and sperm motility (total and progressive motility, Table 2). No statistically significant correlation was found with viability, although there was a tendency toward a negative relation between the two parameters (Table 2).

Direct Comparison Between M540 and Ann V-F Staining in Human Sperm

To verify whether viable Ann V^+ sperm bind M540, we performed double staining with M540 and Ann V-F in swim-up–selected sperm (not containing M540 bodies) showing live PS-exposing sperm, as ascertained by double staining with Ann V-F and PI (Figure 7A, left dot plot). After staining with M540, (Figure 7A, right dot plot), all the Ann V^+ spermatozoa (viable and not viable) were also stained by M540 (as demonstrated by their location in the upper right quadrant), indicating that Ann V^+/PI^- spermatozoa can bind M540. The finding that a fraction of swim-up–selected live sperm (Ann V^+/PI^-) binds M540 is in apparent contrast with experiments performed in swim-up–selected sperm using Y1 as tool to discriminate viable sperm. Indeed, in these experiments (see Figure 2), no live, Y1-negative swim-up–selected sperm appear to bind M540. In addition, by fluorescence microscopy evaluation, we have not been able to detect any $M540^+/Y1^-$ sperm (not shown, see Figure 3A). To verify whether a difference in the percentage of positive cells occurs by using the two stains, we simultaneously labeled the same samples with PI and Y1. We found that higher percentages of positive sperm were always detected by Y1 (not shown), possibly explaining the discrepancy described above. This finding is not surprising, because it has been reported that Y1 is able to detect somatic apoptotic cells after exclusion of dead cells (Idziorek et al, 1995).

To investigate whether M540 bodies bind Ann V, we used unselected sperm from patients with oligoasthenozoospermia in whom a high percentage of M540 bodies is present (Figure 7B left, upper dot plot). After staining with Ann V-F and M540, the percentage of elements positive only for M540 in the upper left quadrants does not change (Figure 7B, upper dot plots; n = 5; $P > .05$). In addition, the percentage of the Ann V^+ population does not change with respect to that determined by double staining with Ann V-F and PI in the same sample (Figure 7B, right dot plots, upper right plus upper left quadrants; n = 5; $P > .05$). In the insert in Figure 7B, micrographs from these sample obtained after observation by fluorescence microscopy are reported. As shown, M540 bodies (left panels, thin arrows) emit only red fluorescence (M540 labeling), whereas sperm (left and right panels,

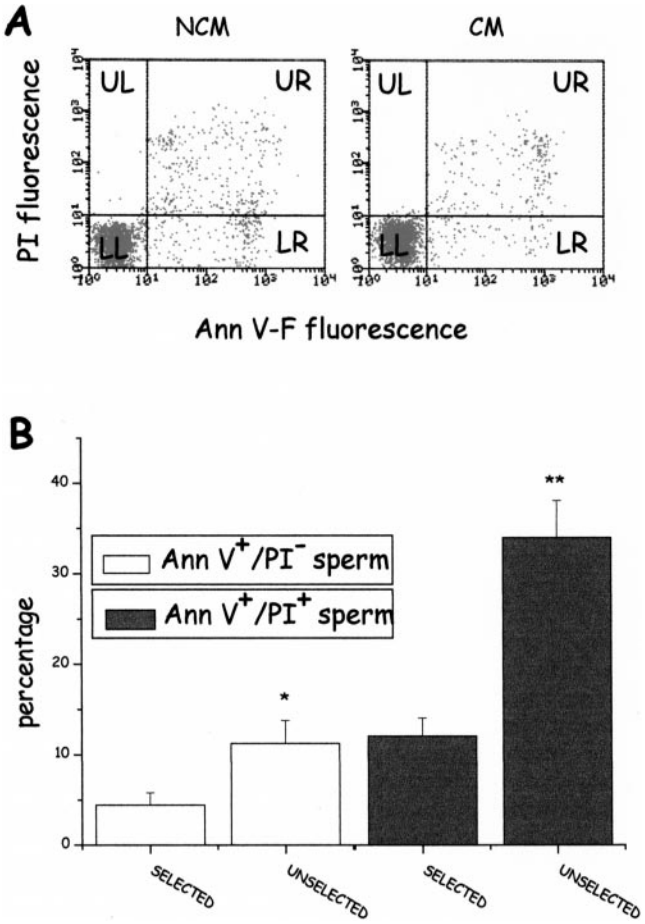

Figure 6. **(A)** Double staining with Ann V-F and PI of swim-up spermatozoa incubated for 3 hours (1 hour for swim-up selection followed by a 2-hour incubation) in NCM (left panel) or in CM (right panel). A typical experiment out of 6 similar is shown. LL indicates lower left quadrant (Ann V^-/PI^-, live sperm); LR, lower right quadrant (Ann V^+/PI^-, live PS exposing sperm); UR, upper right quadrant (Ann V^+/PI^+, dead sperm); and UL, upper left quadrant. **(B)** Mean values of percentages of Ann V^+/PI^- sperm and Ann V^+/PI^+ in swim-up selected sperm and in their corresponding unselected samples. $*P < .05$; $**P < .01$; n = 6, paired *t* test, selected vs unselected.

thick arrows) emit both red and green fluorescence (M540 and Ann V-F labeling).

Discussion

Sperm capacitation is a poorly understood phenomenon. A limit in the progress of knowledge in this field can partially be attributed to the lack of an objective method suitable to measure a large number of cells. Recently, in mammalian sperm, it has been shown that the increased disorder in phospholipid membrane package, which occurs during capacitation, can be detected by M540, a lipophilic molecule sensitive to changes in the membrane architecture (Aussel et al, 1993; Mower et al, 1994). Indeed, in boar (Gadella and Harrison 2000) and stallion (Rathi et al, 2001) sperm, M540 fluorochrome was able to detect such a modification in viable spermatozoa. We reasoned that, if M540 is also able to detect capacitation-related membrane modifications in human spermatozoa, then it could be used as a tool to investigate the nature of spermatozoa exposing PS in the membrane, as conflicting results have been reported regarding the nature of these cells (D'Cruz et al, 1998; Barroso et al, 2000; Ramos et al, 2001; De Vries et al, 2003; Muratori et al, 2003).

In the present study, we show that staining of live

Table 2. *Linear regression analysis of the relationship between the percentage of Ann V binding in live sperm in whole semen**

Parameter	Mean ± SD	Number of Samples	Correlation Coefficient (*r*)	Probability (≤)
Normal morphology	25.5 ± 12.8	15	−.65	.01
Total motility (%)	66.4 ± 13.7	15	−.62	.05
Progressive motility	51.7 ± 18.2	15	−.65	.01
Necrosis (%)	44.6 ± 12.7	15	.42	.09

* Mean ± SD = 10.7 ± 5.5; range, 4%–24.9%.

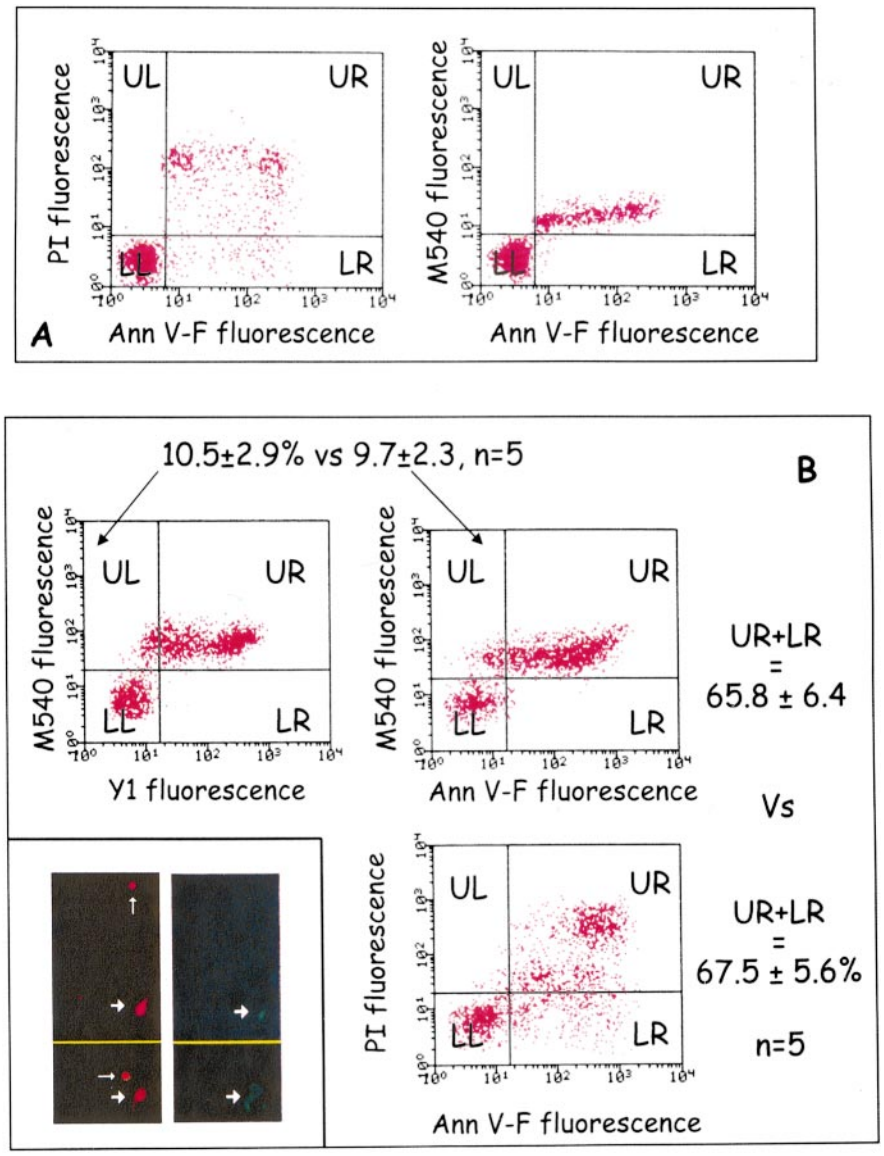

Figure 7. **(A)** Swim-up selected spermatozoa from the same sample, double-stained with Ann V-F and PI (left panel) and with M540 and Ann V-F (right panel). **(B)** Unselected spermatozoa from the same sample, double-stained with M540 and Y1 (left dot plot), M540 and Ann V-F (upper right dot plot) and with Ann V-F and PI (right lower dot plot). Values of percentages of events in upper left quadrants of M540/Y1 and M540/Ann V-F dot plots are indicated on the top. Values of the percentages of events in upper right and lower right quadrants (total Ann V-positive cells) of M540/Ann V and Ann V/PI dot plots are indicated on the right. LL indicates lower left quadrant; UL, upper left quadrant; UR, upper right quadrant; and LR, lower right quadrant. Insert: micrographs of unselected sperm obtained by fluorescence microscopy after double staining with M540 and Ann V-F. Left panels show the red fluorescence (M540 staining) of both sperm (thick arrows) and M540 bodies (thin arrows). Right panels show the green fluorescence (Ann V-F staining) of sperm (thick arrows) from the same field.

sperm with M540 is not a suitable technique for revealing and investigating capacitation in human spermatozoa. By using Y1 to reveal viable spermatozoa, we found virtually no staining with this dye after swim-up selection and subsequent incubation in CM. On the contrary, within the Y1-negative sperm in unselected samples, a variable amount of $M540^{+}$ elements, disappearing after swim-up selection, was observed. Investigation of the morphology of these elements revealed that they are not sperm, and further studies demonstrated that they are: 1) surrounded by a membrane, packaged or organized differently from normal cell membranes (or both), because they are labeled by M540; 2) round shaped, as observed via light microscopy; 3) devoid of a nucleus, as observed after staining in Testsimplets slides and by the failure of the nuclear probe Y1 to label these elements after the loss of membrane integrity induced by treatment with digitonin; and 4) exhibit variable dimensions, including those similar to sperm heads and thus included in the region in the FSC/SSC dot plot characteristic of human spermatozoa

(Muratori et al, 2000, 2003). The heterogeneousness in size as well as in density is also suggested by the finding that these elements migrated differently after centrifugation in density gradient. We termed these elements ''M540 bodies.''

''M540 bodies'' resembled apoptotic bodies observed by electron microscopy in human semen as round and variably dimensioned bodies, often devoid of nuclei (Baccetti et al, 1996; Gandini et al, 2000). Apoptotic bodies are the terminal stages of the apoptotic process in the testis and may be extruded during secretion of spermatozoa. On the other hand, an abortive apoptosis has been hypothesized as a process that stems in the testis, fails to complete, and to delete apoptotic germ cells and their residues, which appear in the human ejaculates of subfertile patients (Sakkas et al, 1999b). In recent years, detection of apoptotic features in human ejaculates from subfertile patients (Sakkas et al, 1999a, 2002), as well as apoptotic-like ultra structures (Baccetti et al, 1996) have been considered signs of abortive apoptosis. Because the fluorochrome M540 is able to detect membrane modifications occurring during apoptosis in several types of somatic cells (Laakko et al, 2002), it is reasonable that it may bind apoptotic bodies in semen, as demonstrated in the present study for ''M540 bodies.'' The finding that the percentage of these bodies is higher in semen from men with oligoasthenozoospermia, which is in agreement with the higher expression of the apoptosis-related FAS receptor in the same group of patients (Sakkas et al, 1999a), further substantiates our hypothesis that M540 bodies may represent apoptotic bodies. It may be argued that if M540 bodies are apoptotic bodies, then they should be also labeled with Ann V-F, due to PS exposure on the surface as a consequence of the apoptotic process. However, double staining of sperm populations with M540 and Ann V-F demonstrated that this is not the case (Figure 7). The failure to expose PS on the surface in these bodies might, however, explain why these elements escape phagocytosis. Indeed, PS exposure is one of the main signals for recognition of somatic apoptotic cells and their removal by phagocytes (Williamson and Schlegel, 2002). A human disorder, in which a defect in lipid scrambling determines failure to expose a sufficient amount of PS, has been described (Bevers et al, 1999; Williamson and Schlegel, 2002).

Although apoptotic bodies are the semen components most resembling M540 bodies, we cannot exclude that the latter could represent different semen elements. In order to definitively assess the actual nature of M540 bodies, further investigations are necessary.

The second part of the present study investigated the occurrence of viable Ann V^+ spermatozoa in capacitated and noncapacitated samples. As for M540 binding, these results are in contrast with observations in sperm of other mammalian species, in which capacitation is accompanied by both external exposure of PS (Gadella and Harrison, 2002) and M540 binding (Gadella and Harrison, 2000; Fletsch et al, 2001; Rathi et al, 2001). We conclude that, at difference with sperm capacitation in some mammalian species, human sperm capacitation is not accompanied by changes in the membrane architecture that can be detected by M540 staining as well as by Ann V binding. On the other hand, species-specific differences in sperm capacitation have been documented (Baldi et al, 2000; Harrison, 2003). While the role of albumin in capacitation-induced changes in sperm membrane as a sink for cholesterol efflux has been well-defined for many species (Visconti et al, 2002), the role of bicarbonate is less clear when comparing spermatozoa from different species. In particular, it is not clear at which levels bicarbonate acts, in the pathway of signaling of capacitation. In pig sperm, it has been reported that, via activation of adenylate cyclase, the ion promotes changes in the membrane, including external exposure of PS and phosphatidylcoline, which in turn, facilitates the efflux of cholesterol by albumin (Flesch et al, 2001). On the contrary, in human and mouse sperm, the action of bicarbonate seems to be located at a downstream level with respect to the action of albumin in promoting activation of protein phosphorylation (Osheroff et al, 1999) as well as functional capacitation (Visconti et al, 1999). Hence, it is possible that the observed changes in the architecture of the sperm membrane (detectable with M540 and including PS translocation) do not occur in human sperm or do not precisely overlap those of other mammalian species. However, we cannot exclude that 3 hours of incubation of human sperm in CM, even if it is sufficient to induce both responsiveness to progesterone and an increase in phosphorylation, is not enough to provoke the membrane changes detectable with Ann V binding and M540 staining, which is at variance with other mammalian species.

It must be mentioned that in a recent report, De Vries et al (2003), using a method similar to ours and a similar time of capacitation, demonstrated that PS exposure accompanies capacitation in human spermatozoa. External exposure of PS during capacitation in human sperm has also been hypothesized by Barroso et al (2000), who found a higher, but not significant, percentage of Ann V binding in the most motile fraction of sperm population. Later on, the same group (Schuffner et al, 2002) showed that a short incubation of Percoll-selected sperm (from both fertile and subfertile patients) in a noncapacitating medium (similar to the NCM of the present study) determined a time-related increase in Ann V binding. Such an increase was much lower in the presence of HSA (0.3%–3%) and was significantly associated with a decline in motility in the same samples (Schuffner et al, 2002). Moreover, Weng et al (2002) found higher PS exposure

in the low-motility fractions of human sperm. In a previous study (Muratori et al, 2003), we show that the basal amount of Ann V^+/PI^- in swim-up–selected sperm is highly predictive of the development of death and DNA fragmentation during in vitro incubation, speculating that these sperm may be considered cells in an early phase of cellular deterioration. The finding that the percentage of Ann V^+/PI^- sperm is lower in selected than in unselected sperm, in which it is negatively correlated with normal morphology (present study and Shen et al, 2002) and motility (present study), together with the demonstration of concomitant binding with a probe able to detect apoptotic somatic cells (Laakko et al, 2002) such as M540, is consistent with our previous hypothesis (Muratori et al, 2003). The hypothesis that PS-exposing human spermatozoa represents damaged or immature cells has been postulated by other authors (Amzar et al, 2002; Shen et al, 2002; Schuffner et al, 2002; Weng et al, 2002; Moustafa et al, 2004). In addition, induction of PS exposure by sperm have been obtained after exposure to several cytotoxic conditions (D'Cruz et al, 1998; Glander and Schaller, 1999; Ramos et al, 2001). At present, we do not have an explanation for the different results obtained by us and those of De Vries et al (2003). It is possible that methodological differences are responsible for the different results, although in partial agreement with our results, Schuffner et al (2002) reported that PS exposure during in vitro incubation of selected human spermatozoa is higher in a medium devoid of albumin, concluding that albumin has a protective role against deterioration of sperm in vitro.

In conclusion, the present study shows that both M540 and Ann V fails to detect capacitation-related membrane changes in human sperm, suggesting that such changes do not precisely overlap those observed in other mammalian species. In addition, our study shows strong evidence that exposure of PS in human sperm is characteristic of damaged cells.

Acknowledgments

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