UTEROGLOBIN AND TRANSGLUTAMINASE MODULATE HUMAN SPERM FUNCTIONS.

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Uteroglobin and Transglutaminase Modulate Human Sperm Functions

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ABSTRACT: During the process of capacitation, spermatozoa undergo significant changes in membrane composition, including removal of decapacitating factors (DFs), which are present in seminal plasma, that lead to increased sensitivity to physiological stimuli of the acrosome reaction. In the present study we investigated the presence, localization, and effects on human spermatozoa of 2 proteins of seminal plasma origin, uteroglobin (UG) and transglutaminase (TG). These 2 proteins interact with one another because TG promotes covalent links of UG to sperm surface proteins. We found that UG is localized around the entire surface of ejaculated human sperm, whereas TG is predominantly localized in the neck. FACScan analysis confirmed the surface localization of both antigens and demonstrated that swim-up selection of spermatozoa was associated with a significant reduction in the contents of the 2 substances when compared with unselected samples. Western blot analysis of UG in total sperm lysates confirmed the lower content of the protein in swim-up–selected sperm. Swim-up–selected sperm were characterized by their ability to undergo a spontaneous, time-dependent increase of capacitation-characteristic chlortetracycline pattern of fluorescence and increase in responsiveness to progesterone. Such changes were not observed in unselected sperm. Exogenous addition of TG, together with recombinant rabbit UG, prevented the spontaneous increase in responsiveness to progesterone (acrosome reaction and intracellular calcium) at 24 hours in swim-up–selected sperm, suggesting the occurrence of a capacitation-inhibiting activity of the 2 substances. In addition, we found that endogenous UG and TG contents, as determined by FACScan analysis, were negatively correlated ($P < .0001$) with sperm motility and that exogenous addition of the 2 substances resulted in a substantial reduction of progressive motility ($P < .01$). Collectively, these data indicate that TG and UG represent 2 DFs, and contribute to understanding the biochemical mechanisms that characterize the process of capacitation.

Key words: Capacitation, spermatozoa, decapacitation factor.
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Capacitation is one of the essential events in the process of fertilization of an oocyte (Yanagimachi, 1994). This process is characterized by several biochemical events, including changes in the concentration of intracellular ions and pH, tyrosine phosphorylation of proteins, and other molecular events that involve surface proteins leading to responsiveness to physiological agonists of the acrosome reaction (Yanagimachi, 1994; Baldi et al, 1996). The biochemical mechanisms generating this process are known only in part and involve spontaneous activation of several signal transduction pathways, including intracellular calcium, cyclic adenosine monophosphate (cAMP), and tyrosine phosphorylation (Baldi et al, 1996). In vitro, this process can be modeled by removing seminal plasma and incubating spermatozoa in defined media with a composition similar to tubal fluids (Yanagimachi, 1994). The absence of capacitation in spermatozoa kept in seminal plasma (Chang, 1957; Shivaji et al, 1990) and the reversion of the process (decapacitation) upon reincubation in seminal fluid (Chang, 1957) has led to the hypothesis that seminal plasma contains ''decapacitating factors'' (DFs; Yanagimachi, 1994; Fraser, 1995). It has been hypothesized that these factors form a coating on the entire surface of the sperm cells and are removed or masked during the process of capacitation (Yanagimachi, 1994; Fraser, 1995). Some of the factors that form such coating material have been totally or partially characterized (eg, Yanagimachi, 1994) and include peripheral proteins and glycoproteins.

Uteroglobin (UG) is a low-molecular-weight, homodimeric secreted protein that was initially detected in rabbit uterus and subsequently in several extrauterine tissues (Miele et al, 1994). Among these, high levels of UG have been found in male reproductive tissues, including prostate (Manyak et al, 1988; Peri et al, 1993) and seminal vesicles (Noske and Gooding, 1978). A number of biological effects have been attributed to UG (Miele et al, 1994). In particular, suppression of sperm and embryo antigenicity (Mukherjee et al, 1982; Mukherjee et al,

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1983) and inhibition of soluble phospholipase A2 (Levin et al, 1986) suggest a role for the protein in reproductive functions and in modulation of inflammatory processes. Recently, the generation of UG-deficient mice by gene targeting in embryonic stem cells demonstrated that UG is essential to maintain normal renal function in the mouse (Zhang et al, 1997).

Transglutaminases (TGs) are multifunctional, ubiquitous enzymes that catalyze the formation of an isopeptide bond by cross linking a γ-glutamine residue and the γ-amino group of a peptide-bound lysine, or the primary group of a polyamine (Greenberg et al, 1991). TGs are expressed in a variety of tissues, including the prostate, where a tissue-specific TG exists (Dubbink et al, 1996), and are involved in post-translational modifications of proteins as well as in the incorporation of polyamines into proteins (Greenberg et al, 1991).

The high UG and TG levels in prostatic secretions (Folk et al, 1980; Peri et al, 1993), the demonstration that UG cross links into high-molecular-weight sperm proteins in the presence of TG (Manjunath et al, 1984), and the occurrence of immunostaining for UG on the entire surface of TG-treated epididymal rabbit spermatozoa (Mukherjee et al, 1983) indicate a possible role for both UG and TG in the modulation of other sperm functions besides suppression of sperm antigenicity (Mukherjee et al, 1983; Peluso et al, 1994). The demonstration of a physiological role of TG in the modification of rat epididymal sperm proteins (Paonessa et al, 1984) further supports this hypothesis.

In the present paper we characterized expression of UG and TG in ejaculated human spermatozoa by immunohistochemistry, Western blot, and FACScan analysis. We found that swim-up selection of human sperm resulted in a significant decrease in UG and TG sperm levels and that these levels were inversely related to sperm motility. Readdition of the substances to a swim-up sperm population prevented development of capacitation and decreased motility. These results strongly indicate that these 2 molecules are involved in the process of capacitation of human sperm.

Materials and Methods

Chemicals

Recombinant rabbit uteroglobin (rec-rUG), goat anti-rabbit UG, and goat anti-human UG antibodies were a kind gift of Prof Anil Mukherjee. Percoll was obtained from Pharmacia LKB (Uppsala, Sweden). Human serum albumin (HSA)-free human tubal fluid (HTF) was from Irvine (Santa Ana, Calif). All the other chemicals, tranglutaminase, and all secondary antibodies were from Sigma Chemical Company (St Louis, Mo). Reagents for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein measurement were from BioRad Labs (Hercules, Calif). BM chemiluminescence substrate was from Boehringer (Mannheim, Germany). Ionomycin, Fura-2/AM, and fluorescein isothiocyanate (FITC)-labeled *Arachis hypogea* (peanut) lectin were obtained from Calbiochem (La Jolla, Calif). Chlortetracycline (CTC), Hoechst bis-benzimide 33258, cystein, polyvinylpyrollidone (PVP-40), and 1,4-diazabycyclo[2,2,2]-octane (DABCO) were from Sigma.

Preparation and Incubation of Spermatozoa

Human semen was obtained from men undergoing semen analysis in our laboratory, with normal semen parameters according to World Health Organization (WHO) guidelines (WHO, 1992). Semen was collected according to WHO-recommended procedures (WHO, 1992) by masturbation after 3 to 4 days of sexual abstinence. Samples with a linear progressive motility of less than 50% at 60 minutes and with leukocytes, immature germ cell concentration, or both greater than 10^6/mL were not included in the study. Spermatozoa were selected by swim-up procedure for 1 hour in 1% HSA-HTF medium at 37°C. Unselected samples represent spermatozoa that did not migrate to the supernatant during swim-up procedure and seminal plasma was removed by centrifugation. Spermatozoa were counted and incubated at 37°C in a 5% CO_2 atmosphere at different concentrations and under different conditions required in each experiment. Sperm motility was evaluated according to WHO guidelines (WHO, 1992).

Collection of Epididymal Spermatozoa

Epididymal spermatozoa were retrieved from 2 azoospermic men with epididymal cysts who were undergoing epididymal sperm aspiration for diagnostic purposes. Spermatozoa were collected in HSA-containing HTF medium. Sperm motility was evaluated within 15 minutes of retrieval and was found to be >80% in both patients.

Immunofluorescence of Human Spermatozoa

Immunofluorescence of spermatozoa was performed as previously described (Luconi et al, 1998). Briefly, sperm samples were washed in PBS and incubated with anti-human UG (1:200) or anti-human TG (1:100) antibodies in 0.1% BSA-PBS for 1 hour under continuous shaking. After washing in PBS, samples were incubated with FITC-conjugated rabbit anti-goat-IgG (1: 300 0.1% BSA-PBS), washed 2 times, and fixed in either 3.7% formaldehyde or cold methanol. Finally, 10 μL of the suspension was placed on slides and allowed to dry. After mounting with glycerol:PBS (1:9), slides were observed using a fluorescence microscope (Leitz, Type 307-148002, Wetzlar, Germany). Negative controls were performed by processing slides exactly as described earlier, but omitting incubation with the primary antibody.

Western Blot Analysis

After the different incubations, samples were processed for SDS-PAGE as previously described (Luconi et al, 1995, 1996). Briefly, sperm samples containing 10^7 cells/mL were added with 1 mM Na_3VO_4, centrifuged at 400 × *g* at 4°C for 10 minutes, washed in HSA-free HTF, and resuspended in 20 μL of lysis

buffer (20 mM Tris pH 7.4, 150 mM NaCl, 0.25% Nonidet P40, 1 mM Na_3VO_4, 1 mM PMSF). After protein measurement, aliquots of sperm extracts that contained the same protein amounts were diluted in equal volumes of reducing 2× SB (Laemmli's sample buffer = 62.5 mM Tris pH 6.8, containing 10% glycerol, 20% SDS, 2.5% pyronin, and 10% β-mercaptoethanol), incubated at 95°C for 5 minutes, and loaded onto 20% or 8% polyacrylamide-bisacrylamide gels. After SDS-PAGE, proteins were transferred to nitrocellulose (Sigma) and stained with Ponceau to verify equal protein loading. In some experiments, equivalent protein loading was verified by staining parallel gels with Coomassie. The nitrocellulose was blocked in 5% BSA for 2 hours in Tris-buffered saline containing 0.1% Tween 20 (TTBS; pH 7.4), washed, and then immunostained with primary antibody (anti-rabbit [1:2000] or anti-human [1:100] UG-antibody followed by peroxidase-conjugated secondary IgG). The reacted proteins were revealed by BM chemiluminescence substrate. For reprobing experiments, the blots were washed for 30 minutes at 50°C in stripping buffer (10 mM Tris pH 6.8, 1% SDS, 100 mM β-mercaptoethanol) in order to remove bound antibodies, then the immunostaining was performed as described earlier. Occurrence of complete stripping was verified by exposing the blot to BM before reprobing.

FACScan Analysis

Spermatozoa were selected by the swim-up procedure as described earlier. Unselected and selected sperm suspensions capacitated for 2 and 24 hours were incubated in the presence or absence (negative controls) of anti-UG (1:200) or anti-TG (1:100) antibodies for 1 hour under continuous shaking to avoid sperm aggregation. At the end of incubation, the presence of agglutinated sperm was carefully verified under a microscope. After several washes, samples were incubated for 30 minutes with FITC-labeled rabbit anti-goat IgG (1:300). Finally, the washed pellets were resuspended in PBS for flow cytometric analysis. FITC fluorescence was revealed by using the FL-1 detector of a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif) and displayed as frequency distribution histograms. For each sample, 10 000 cells were analyzed at a rate of 200 to 300 cells/s. Percentage of labeled spermatozoa was determined by subtracting nonspecific fluorescence of the negative control, which were processed identically but without the addition of the primary antibody.

To assess morphometric characteristics of white blood cells, 100 mL of blood were added to 2 mL of Auto Prep plus Diluent (Becton Dickinson). The sample was then acquired by FACScan, and forward scatter (FSC) and side scatter (SSC) detectors were set in order to display white blood cells and to exclude platelets, red blood cells, and debris (threshold = 180). The same instrument setting, but with a threshold of 30, was used to acquire sperm samples. The percentage of UG-positive cells was determined in the fluorescence histograms as described earlier, before and after gating white blood cells from the FSC/SSC cytogram.

Evaluation of CTC Fluorescence

Spermatozoa were selected by swim-up procedure as described earlier. Unselected and selected sperm suspensions were incubated for 3 minutes in vital dye Hoechst bis-benzamide 33258 solution (DasGupta et al, 1993), then washed by centrifugation at 830 × *g* for 6 minutes through 4 mL of 2% PVP40 solution (3% PVP40-PBS). The resulting pellet was resuspended in 45 μL of HSA-HTF diluted (1:1 v/v) with CTC solution (DasGupta et al, 1993) and thoroughly mixed. Cells were fixed by adding 8 μL of 12.5% (w/v) paraformaldehyde in 0.5 mM Tris-HCl (pH 7.4). Slides were prepared by adding 10 μL of stained, fixed sperm suspension onto a microscope slide to a drop of 0.22 M of 1,4-diazabycyclo[2,2,2]-octane dissolved in glycerol:PBS (9:1) to retard fluorescence fading. Slides that were covered with coverslips and sealed were examined using an epifluorescent microscope. Each cell was first observed under ultraviolet illumination (excitation at 330–380 nm and emission at 420 nm) to determine live/dead status. Cells with bright blue staining of the nucleus were considered to be dead and not counted. At least 100 live cells were then examined under blue-violet illumination (excitation at 400–440 nm and emission at 470 nm) for CTC patterns (DasGupta et al, 1993): F, with uniform fluorescence on the whole head, which is characteristic of uncapacitated, acrosome-intact cells; B, with a fluorescence-free dark band in the postacrosomal region, which is characteristic of capacitated, acrosome-intact cells; AR, with dull or absent fluorescence, which is characteristic of capacitated, acrosome-reacted cells.

Measurement of Intracellular Calcium Concentration

Spermatozoa prepared as described earlier were loaded with 2 μM Fura-2/AM for 45 minutes at 37°C, washed, 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% BSA, pH 7.5). Intracellular calcium concentration ($[Ca^{2+}]_i$) before and after stimulation with progesterone (10 μM) was measured as described previously using a spectrofluorimetric method (Baldi et al, 1991; Krausz et al, 1996), with the difference being that in the present experiments, we used a Perkin-Elmer LS50B instrument equipped with a fast rotary filter shuttle for alternate 340 nm and 380 nm excitation. Fluorescence measurements were converted to $[Ca^{2+}]_i$ by determining maximal fluorescence (F_{max}) with digitonin (0.01% final concentration), followed by minimal fluorescence (F_{min}) with 10 mM EGTA, pH 10. $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz et al (1985) using the ratio 340/380 while assuming a dissociation constant of Fura-2 for calcium of 224 nM.

Evaluation of Sperm Acrosome Reaction

Sperm acrosome reaction was evaluated using the fluorescent probe, FITC-labeled *Arachis hypogea* (peanut) lectin, as previously described (Krausz et al, 1996). Incubation in hyposmotic swelling medium for 1 hour at 37°C was performed to discriminate live from dead spermatozoa. Fluorescence was observed under a fluorescence microscope (Leitz, Type 307–148002, Wetzlar, Germany) and the acrosome reaction was evaluated on a total of 100 spermatozoa/slide. According to the method of Aitken et al (1993), only curly tailed spermatozoa were considered viable and thus scored.

In experiments in which acrosome reaction in response to progesterone was used as an index of capacitation, unselected and swim-up–selected spermatozoa were incubated for 24 hours in the presence or absence of TG (3.5 U/mL), UG (2 mg/μL), and

TG/UG. After washing, samples were stimulated for 1 hour with progesterone (10 μM) or with solvent. Acrosome reaction following progesterone challenge (ARPC) represents the difference between the percentage of acrosome reactions obtained in progesterone-stimulated spermatozoa and the percent of spontaneous acrosome reactions.

Assessment of Sperm Viability

Sperm viability was assessed by incubating spermatozoa in hyposmotic swelling medium for 1 hour at 37°C (WHO, 1992). Briefly, after centrifugation, spermatozoa were fixed in cold methanol and suspension-layered onto slides. Under light microscopy, only curly tailed spermatozoa were considered viable.

Measurement of Proteins

Protein concentrations were evaluated by the BioRad (Hercules, Calif) protein assay reagent exactly as indicated by the manufacturer, with BSA used as standard.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical comparisons were made with Student's *t*-test for paired or independent data. Correlation coefficients were evaluated by Spearman's analysis.

Results

Immunolocalization of UG and TG in Human Spermatozoa

Immunofluorescence analysis of live, nonpermeabilized ejaculated human spermatozoa performed with anti-human UG antibody localizes UG on sperm surfaces both at the acrosomal and tail levels, whereas the neck is less stained (Figure 1A). This pattern does not substantially differ from the UG localization pattern demonstrated in rabbit epididymal spermatozoa after treatment with UG + TG (Mukherjee et al, 1983). On the contrary, immunofluorescence staining with anti-TG antibody (Figure 1B) reveals a rather selective localization of positivity on sperm surfaces overlaying the neck, confirming previous studies (Ablin and Whyard, 1991). No positivity is observed in negative controls (Figure 1C). Western blot analysis of total sperm lysates confirms the presence of UG and TG both in unselected and swim-up–selected spermatozoa and demonstrates that UG and TG contents are clearly higher in unselected spermatozoa (Figure 2). A slight shift in the expected molecular weight of UG is observed when compared with the recombinant rabbit UG used as a positive control (Figure 2). Different apparent molecular weights in SDS-PAGE migration, ranging between 10 and 17 kd for the dimeric forms, have been described for cellular UG by other authors (Mantile et al, 1993). Moreover, it has been shown that in the presence of TG, UG undergoes several biochemical modifications that alter its gel mobility (Porta et al, 1990). It is also possible that such differences may be due to different migratory properties of recombinant rabbit UG in SDS gel with respect to human cellular UG.

For UG content in swim-up and unselected sperm populations, these results are indirectly confirmed by incubation of live spermatozoa with anti-UG antibodies. These experiments were initially performed with the aim of demonstrating a physiological role for UG in sperma-

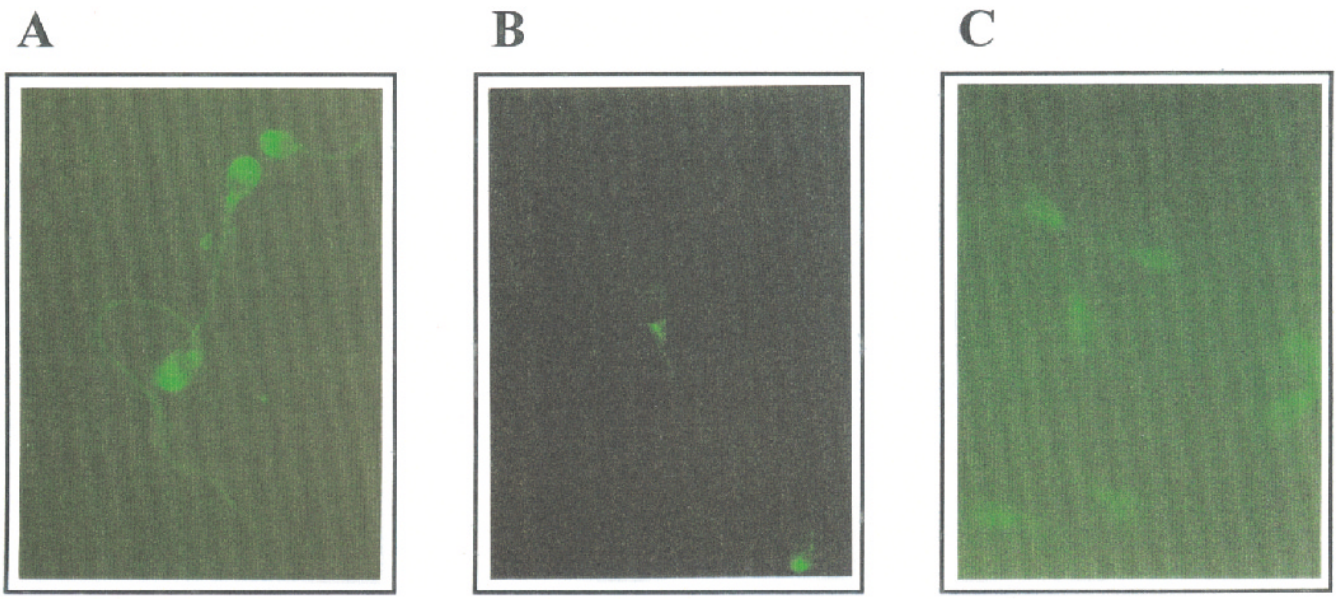

Figure 1. Localization of UG and TG in human spermatozoa. Spermatozoa were fixed in methanol or paraformaldehyde and analyzed by staining with polyclonal goat anti-UG **(A)** and anti-TG **(B)** antibodies followed by FITC-conjugated secondary swine anti-goat antibody. The immunostaining shown in the figure is representative of 3 similar experiments. **(C)** Negative control stained with peroxidase-conjugated secondary antibody alone.

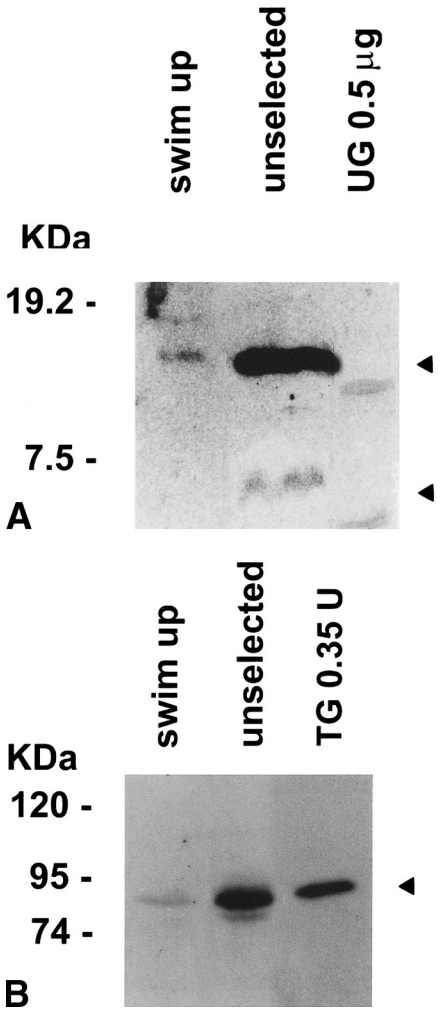

Figure 2. Western blot analysis (20% and 8% SDS-PAGE respectively for panels A and B) of proteins from 2-hour-capacitated unselected and swim-up–selected spermatozoa revealed with anti-UG **(A)** or anti-TG **(B)** antibodies. Purified UG and TG have been run as positive controls. Molecular weight markers are indicated to the left of the blots. Arrowheads indicate position of the bands in the sperm lysates.

tozoa by neutralizing endogenous UG with the antibodies. However, incubation with the antibodies (2 hours) results in the formation of agglutinates in unselected spermatozoa (Figure 3A), while swim-up–selected spermatozoa do not agglutinate, even if incubation with the antibodies is carried out for a longer time period (data not shown). It is possible that the antibodies act as a sort of bridge and interact with UG present on the surface of spermatozoa. It is worth noting that in these agglutinates, spermatozoa are still motile. Agglutination is prevented when incubation with the antibodies is conducted in the presence of excess rec-rUG (75 μM, Figure 3B). Conversely, no agglutination is observed either in swim-up–selected or unselected spermatozoa using nonimmune goat serum (not shown) or anti-TG antibody, which indicates the high specificity of the effect of the UG antibody. Spermatozoa retrieved from epididymal aspiration in 2 men did not stain for UG (data not shown). This finding is in agreement with a previous study performed in the rabbit (Mukherjee et al, 1983), and indicates that UG is attached to the sperm surface following contact with fluids distal to the epididymis.

FACScan Analysis of UG and TG Content in Human Sperm

To confirm surface localization and to quantify the percentage of sperm that were positive for UG, FACScan analysis of swim-up–selected and unselected spermatozoa was performed. UG positivity in swim-up–selected and unselected spermatozoa at 2 different times of capacitation (2 and 24 hours) was evaluated. Incubation at 24 hours was chosen because spermatozoa may remain in the female genital tract, where UG is present, for such a long time. In addition, the longer incubation period was chosen to demonstrate eventual removal of the 2 substances from the sperm surface during capacitation. Results of a typical experiment for swim-up–selected (panel A_1) and unselected (panel A_2) sperm in 3-dimensional view are shown in Figure 4. Traces a, b, and c show, respectively, the negative control (incubation with FITC-conjugated secondary antibody only), and UG positivity at 2 and 24 hours. In Figure 4B, average values are reported for UG positivity at both time points in the 2 sperm populations for the indicated number of experiments. The higher number of UG-positive cells revealed by FACScan analysis in unselected compared with swim-up–selected spermatozoa (Figure 4) confirms results of Western blot analysis (Figure 2A) and is statistically significant at both time points (Figure 4B). To exclude the possibility that the higher signal observed in unselected sperm could be due to the presence of nonsperm cells (for example leukocytes), we acquired by FASCan a population of white blood cells and established the morphometric characteristics of these cells. We then set a gate around the white blood cell population in the cytogram FSC/SSC (Figure 5A) to exclude these cells from unselected sperm samples (Figure 5B). After such an analysis, the percentage of UG-positive cells in unselected samples at both 2-hour (Figure 5C, compare tracings 2 and 3) and 24-hour (not shown) incubations does not differ from that obtained in the total cell population, indicating that the UG signal in unselected cells is totally due to sperm cells.

Surprisingly, a significant increase in the percentage of UG-positive cells between 2 and 24 hours of incubation in capacitating conditions was observed in unselected spermatozoa (Figure 4B). This increase has been consistently found in 9 experiments performed in spermatozoa from 9 different men. A small increase of UG-positive cells is also present in swim-up–selected sperm (Figure $4A_1$); however, on average, such an increase was not sta-

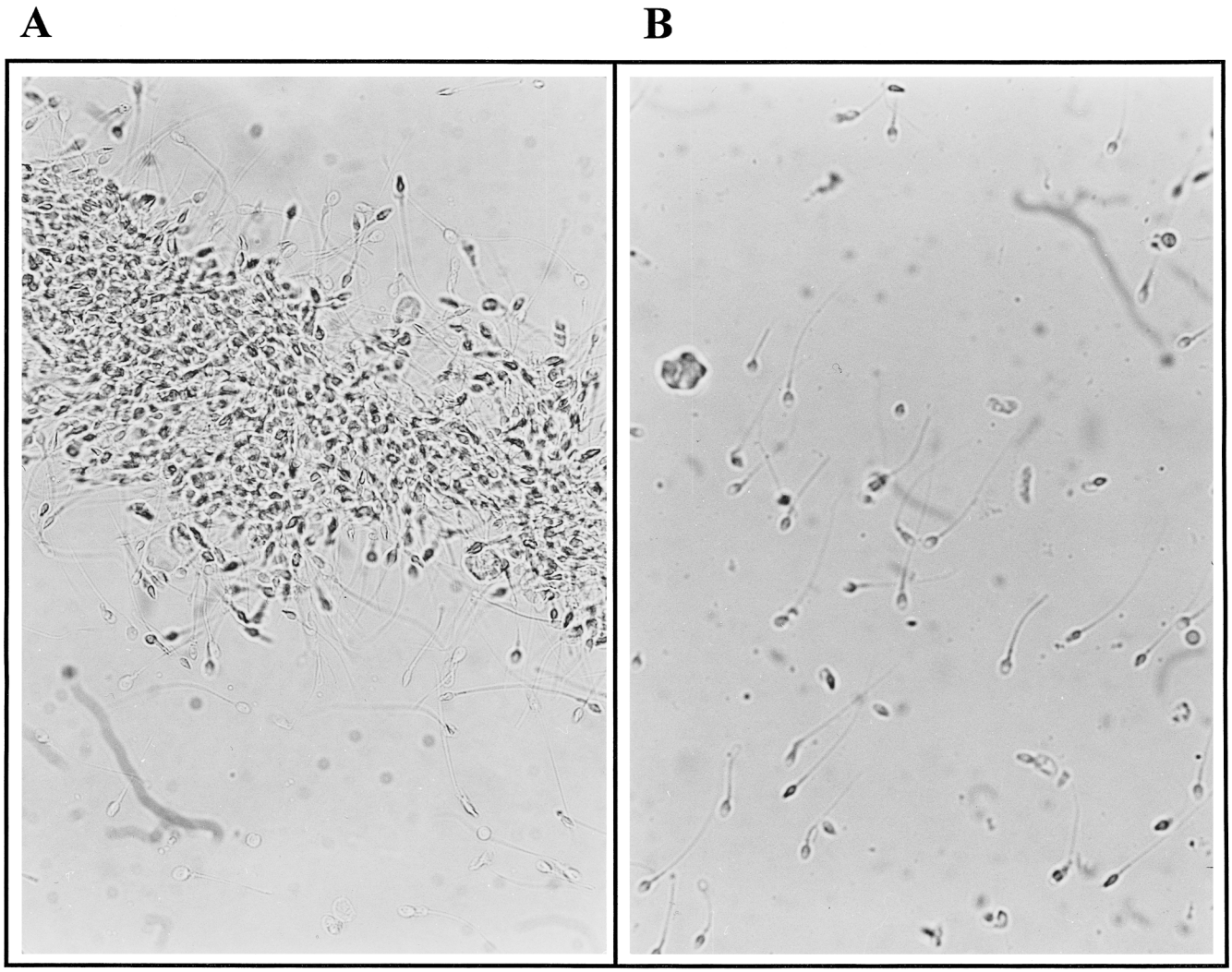

Figure 3. Formation of agglutinates of human spermatozoa upon treatment with anti-UG antibody. Unselected spermatozoa (10^6/mL) were incubated for 2 hours with anti-UG antiserum **(A)**. In **(B)** , anti-UG antiserum has been preabsorbed with excess recombinant UG (75 μM for 2 hours at room temperature). Representative of 6 similar experiments.

tistically significant (Figure 4B). These results are also confirmed by Western blot analysis of UG in sperm lysates at 2 and 24 hours of capacitation (Figure 6). Usually, an increase in protein content at different time points reflects an increase in protein synthesis by the cells; however, sperm cells are transcriptionally inactive, and thus the increase cannot be explained by such phenomena. On the other hand, cells other than those of the germinal line may be present in unselected sperm samples, representing a possible source of UG during the incubation period. To exclude this possibility we performed the experiment in the presence of cycloheximide to eliminate new protein synthesis. However the increase in percentage of UG-positive cells, evaluated by FACScan analysis, is also detected when the protein synthesis inhibitor is present in the medium (mean ± SEM percentage UG-positive sperm in unselected samples at 24 hours: 46.5 ± 8.4 in control; 48.4 ± 8.6 and 53.9 ± 10.5, respectively with 100 μg/mL and 1 mg/mL cycloheximide in 3 different subjects). Because no protein synthesis occurs during the incubation period, we hypothesized that UG may originate from a different source. In particular, we considered the hypothesis that UG might be present in the incubation medium as a contaminant of HSA. To verify this possibility we conducted the experiment in both HSA-free and HSA-containing media in the same subjects. The increase in the percentage of UG-positive sperm at 24 hours is observed in both samples (data not shown), excluding the possibility that UG could be present in the medium as a contaminant of HSA.

We also evaluated sperm TG content by FACScan analysis. For UG, a higher percentage of TG-positive cells was found in unselected sperm compared with swim-up–selected sperm (percentage positive cells: 33.8 ± 3.6 in

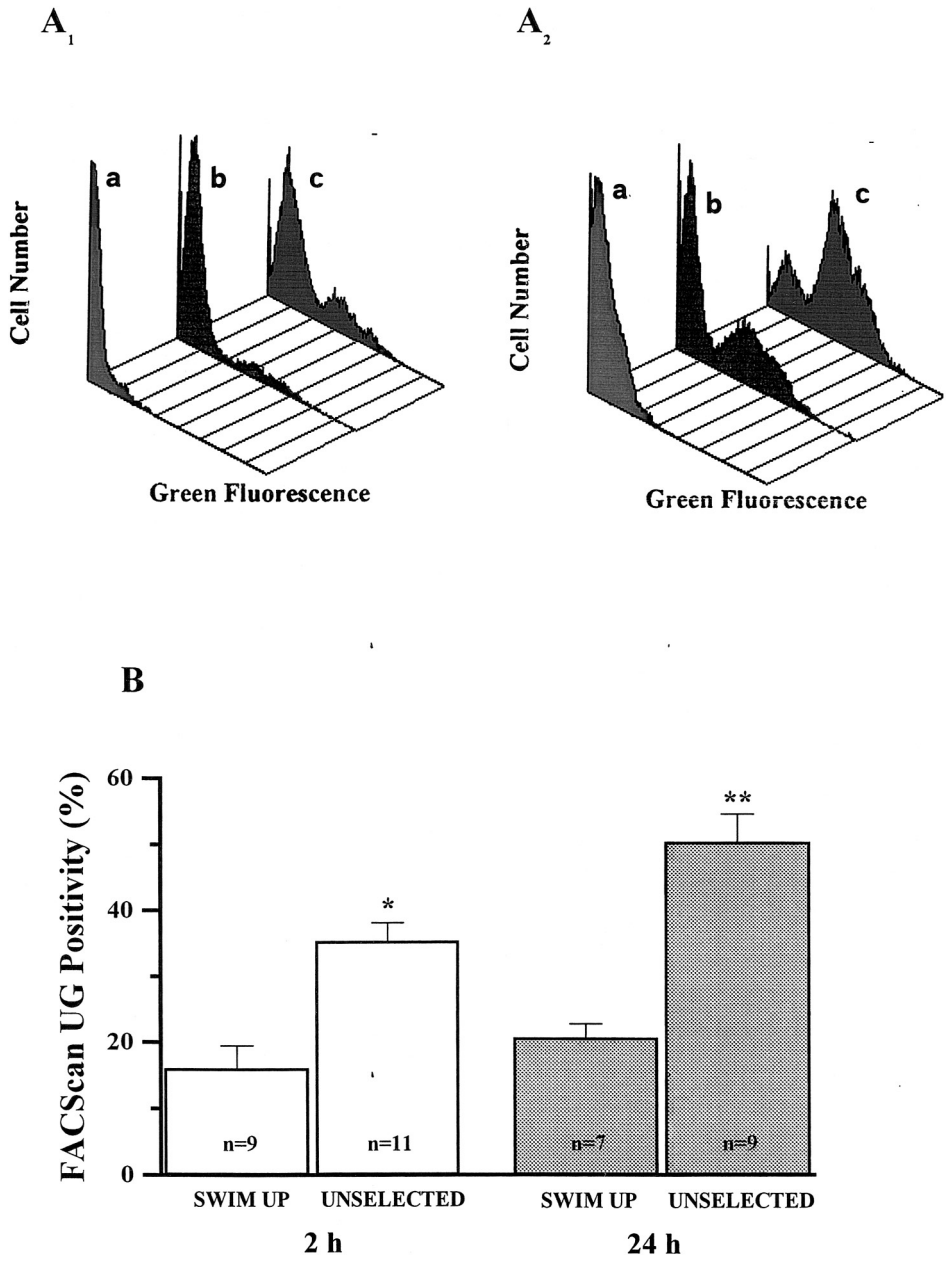

Figure 4. FACScan analysis of surface UG expression in human spermatozoa. **(A_1)** and **(A_2)**: Representative frequency histograms of negative control (incubation with FITC-conjugated secondary antibody but not primary antibody) **(a)**, 2 hours **(b)**, and 24 hours **(c)** capacitated swim-up–selected (panel A_1) and unselected (panel A_2) spermatozoa. **(B)**: Mean ± SEM percentage UG-positive cells in swim-up–selected and unselected spermatozoa for the indicated number of experiments in the different conditions. *$P < .001$ vs swim-up–selected sperm at 2 hours. **$P < .001$ vs swim-up–selected sperm at 24 hours and unselected sperm at 2 hours.

unselected vs 11.9 ± 2.4 in swim-up–selected sperm, n = 5, see Figure 2). However, contrary to results involving UG, TG content assessed both by Western blot analysis (not shown) and FACScan (not shown) is similar at 2 hours and at 24 hours in both sperm populations.

UG and TG Modulate Human Sperm Capacitation

The different endogenous contents of UG and TG in the 2 sperm populations studied might also be related to their different levels of capacitation. Indeed, as shown in Figure 7, basal and progesterone-stimulated $[Ca^{2+}]_i$ (Cross, 1996; Luconi et al, 1996; Visconti et al, 1998) as well as CTC pattern of fluorescence (Fraser, 1995), 2 well-established indices of capacitation, are significantly higher in swim-up–selected versus unselected populations at both 2- and 24-hour incubation times. In particular, the expected increase in CTC pattern of capacitation and basal/progesterone-stimulated $[Ca^{2+}]_i$ after 24

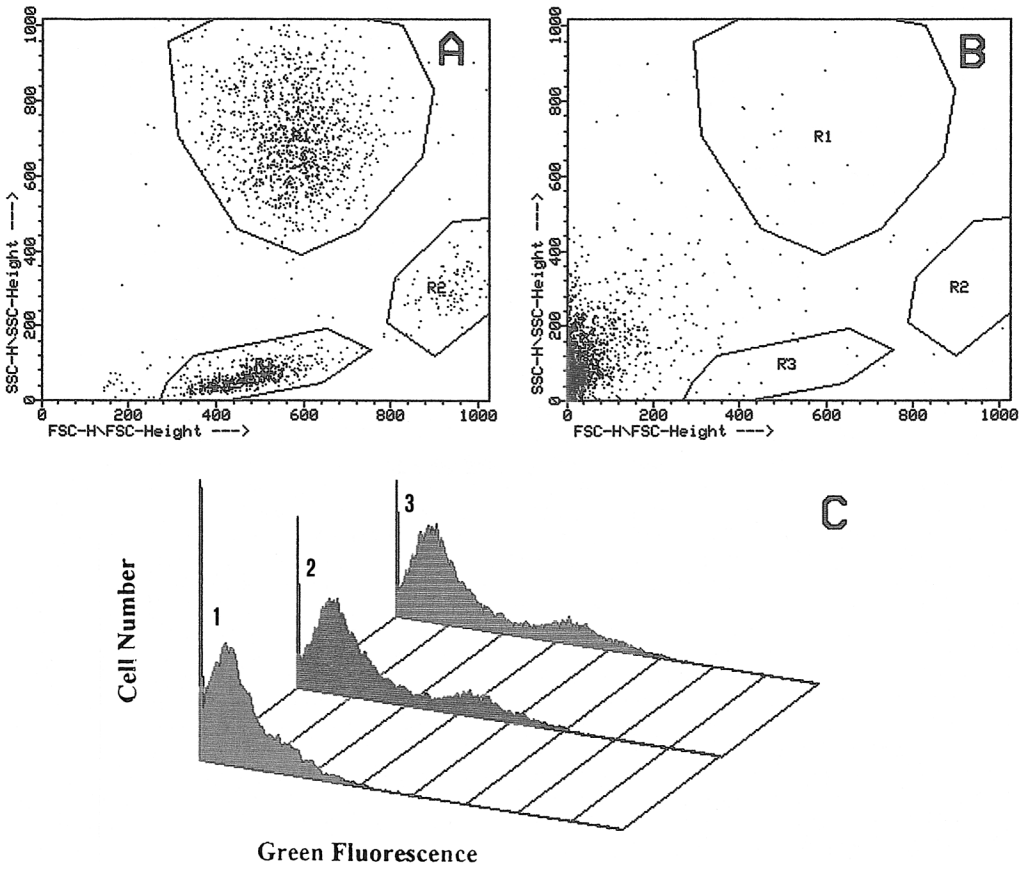

Figure 5. Panel A: Morphometric characteristics of white blood cells in the cytogram FSC/SSC: a gate has been set around the 3 cell populations (R1, R2, and R3). Panel B: Morphometric characteristics of unselected spermatozoa in the cytogram FSC/SSC. R1, R2, and R3 represent the gates for the 3 white blood cell populations as established in panel A. Panel C: FACScan analysis of UG sperm content in unselected spermatozoa in control conditions (histogram 2) and after gating off white blood cells (histogram 3). Histogram 1 represents negative control (incubation only with secondary antibody).

hours incubation in capacitating conditions are present only in the swim-up population (Figure 7). To assess the role of UG and TG in capacitation, we evaluated whether exogenous readdition of the 2 substances to swim-up–selected sperm affected sperm responsiveness to progesterone. Because human UG was not available to us, we used rec-rUG in view of its high degree of homology to human UG (Singh et al, 1990). Swim-up–selected sperm were incubated for 24 hours in the presence or absence of UG or TG alone, or added together. After washing, spermatozoa were incubated for 1 hour with progesterone (10 μM). Rec-rUG alone did not affect acrosome reaction following progesterone challenge (ARPC = difference between the percentage of acrosome-reacted sperm after progesterone stimulation and in basal conditions) of live, capacitated spermatozoa (Figure 8A), whereas in the presence of TG, it dramatically inhibits the ability of swim-up–selected spermatozoa to respond to progesterone (Figure 8A for acrosome reaction and Figure 8B for calcium increase). It is interesting that the presence of the anti-UG antiserum (1:500) reverts the effect of UG + TG on sperm responsiveness to progesterone in swim-up–selected sperm (% ARPC:control = 12 ± 2, UG + TG = 2.5 ± 0.5, UG + TG + anti-UG antiserum = 9.5 ± 3.5, anti-UG antiserum = 14 ± 3, n = 2). However, TG reduces responsiveness to progesterone in this sperm population, also in the absence of UG (Figure 8). It is possible that such an effect by TG in response to progesterone is due to a toxic effect by the molecule on swim-up–selected sperm. Indeed, we observed a decrease of sperm viability after incubation with TG for 24 hours in this population (Table); however, the acrosome reaction is evaluated only in curly tailed, viable spermatozoa, and thus the eventual effect on sperm viability should not interfere with results on acrosome reaction. In addition, while the influence of TG on sperm viability tends to be reverted in the presence of UG (Table), its effects on ARPC appear to be greater in its presence (Figure 8A). Moreover, incubation with TG for 24 hours increases UG levels in swim-up–selected sperm (mean ± SEM % UG-positive spermatozoa evaluated by FACScan analysis: 27.5 ± 5.1 in control vs 38.9 ± 6.2, $P < .05$, n = 5), suggesting that the

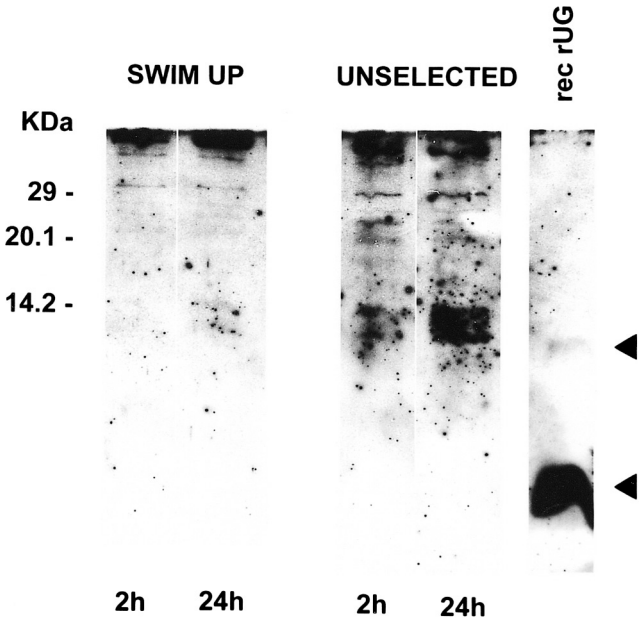

Figure 6. Western blot analysis of UG contents at different capacitation times. Protein lysates from swim-up–selected and unselected spermatozoa at 2 and 24 hours incubation in capacitating conditions were run on 20% SDS-PAGE and stained with anti-UG antiserum. Recombinant rabbit UG has been run as control and the 2 bands corresponding to the monomer and the dimer marked with arrowheads. Molecular weight markers are indicated on the left of the blots.

effects observed with TG alone may be due to increased UG sperm content.

UG and TG Contents are Negatively Correlated With Sperm Motility

The higher percentage of UG- and TG-positive sperm in unselected spermatozoa, characterized by lower motility, suggests that the 2 molecules may be deleterious for motility. In keeping with this hypothesis, we find a significant negative correlation between % UG-positive sperm evaluated by FACScan and total ($R = -.76$, $P < .0001$, n = 35, Figure 9A) as well as progressive motility ($R = -.63$, $P < .0001$, n = 35, Figure 9B). A similar correlation is found between TG content and total ($R = -.77$, $P < .001$, n = 10) and progressive motility ($R = -.73$, $P < .016$, n = 10). To investigate whether UG and TG have a direct effect on sperm motility, unselected and swim-up–selected spermatozoa were incubated for 2 hours with TG and rec-rUG, the latter in the presence or absence of TG. We found that in the absence of TG, UG does not affect motility (Figure 10). In contrast, in the presence of TG, UG decreases motility only in swim-up–selected sperm (Figure 10).

Discussion

It is well known that incubation with seminal plasma has detrimental effects on spermatozoa, impairing some aspects of capacitation such as motility (Tomes et al, 1998) and responsiveness to progesterone (Cross, 1996), and resulting in inhibition of their fertilizing ability. These effects have been attributed to as-yet-unidentified DFs present in seminal plasma. DFs may be of seminal vesicle, prostate, or epididymal origin. They are hypothesized to form a coating on the entire surface of the sperm cell, keeping the cell in a noncapacitated state. During capacitation, these molecules are removed from the sperm surface, allowing exposure of sperm membrane molecules that are involved in induction of the acrosome reaction (Yanagimachi, 1994). Several substances present in sem-

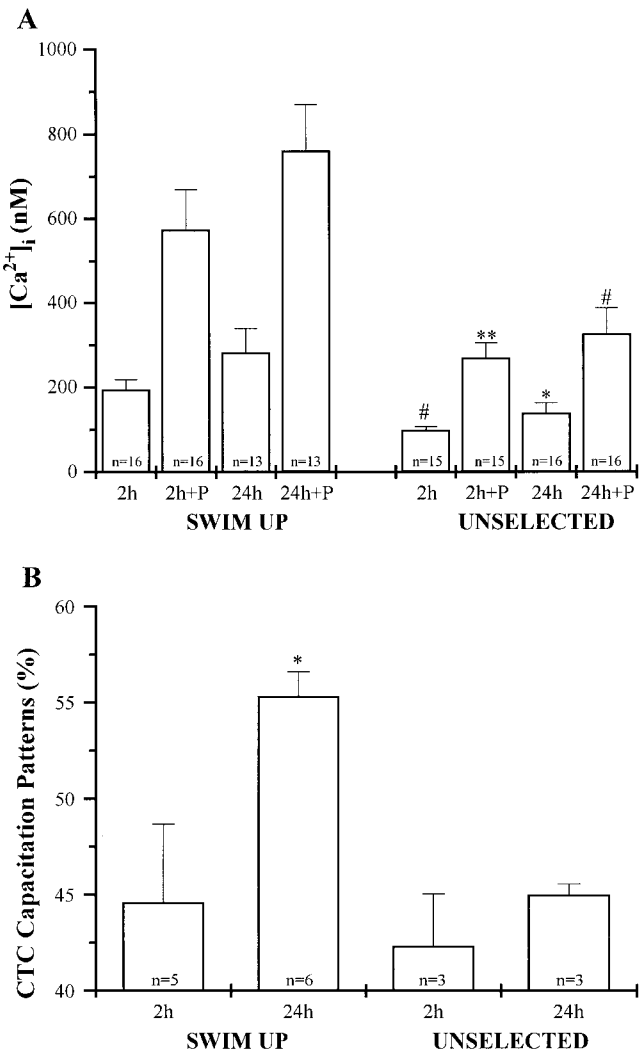

Figure 7. Intracellular calcium concentrations ($[Ca^{2+}]_i$) and chlortetracycline (CTC) capacitation patterns of swim-up–selected and unselected spermatozoa at 2 and 24 hours incubation in capacitating conditions. **(A)** Mean ± SEM basal and progesterone (P)-stimulated $[Ca^{2+}]_i$ for the indicated number of experiments. *$P < .05$; **$P < .01$; #$P < .005$ vs respective swim-up bars. **(B)** Mean ± SEM percentage of swim-up and unselected spermatozoa expressing capacitation patterns at 2 and 24 hours incubation as evaluated by CTC fluorescence analysis for the indicated number of experiments. *$P < .001$ vs unselected at 24 hours and $P < .05$ vs swim-up at 2 hours.

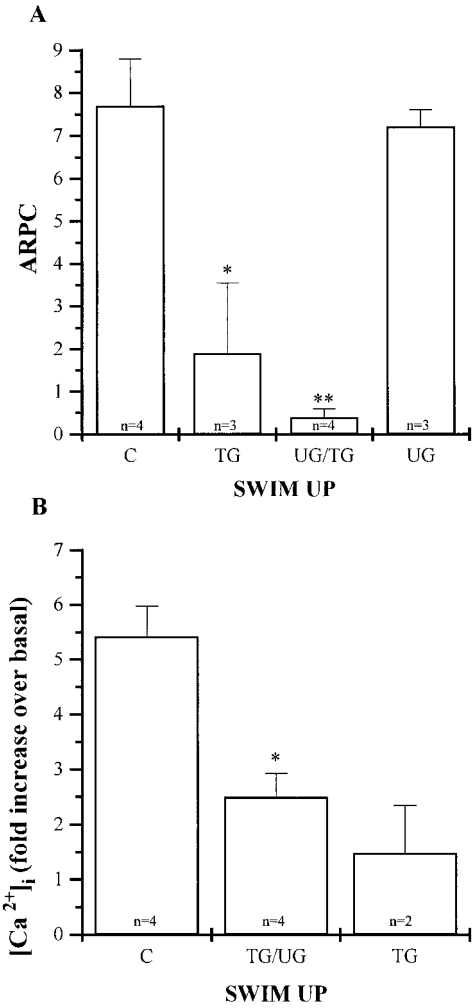

Figure 8. Influence of TG, UG, and the combination of the 2 on the ability of spermatozoa to undergo acrosome reaction (panel A) and intracellular calcium increase (panel B) in response to progesterone. Swim-up–selected spermatozoa were incubated in capacitating medium in the presence or absence of TG (3.5 U/mL), UG (2 μg/mL) and TG + UG for 24 hours and then stimulated for 1 hour with progesterone (10 μM) for evaluation of acrosome reaction or loaded with fura-2/AM for evaluation of $[Ca^{2+}]_i$ increase in response to progesterone. ARPC indicates acrosome reaction following progesterone challenge (% AR in response to progesterone minus spontaneous). *$P < .05$ vs C; **$P < .001$ vs C.

inal plasma from different mammalian species have been indicated as possible DFs (Yanagimachi, 1994), including a high-molecular-weight protein named *acrosome stabilizing factor* (Eng and Oliphant, 1978), spermine (Rubinstein and Breitbart, 1991), free cholesterol (Cross, 1996), and spermadhesins (Dostalova et al, 1994). The mechanism by which DFs are attached to the sperm surface (by specific membrane receptors or by binding to membrane phospholipids or proteins) is not completely understood. The results shown in this report strongly suggest that UG and TG, 2 proteins present in large amounts in prostatic fluid and in seminal vesicles, may represent 2 DFs of seminal plasma. This assumption is based on several ex-

Sperm viability after incubation with transglutaminase for 24 hours

	Sperm			
Protein†	Swim Up	n	Unselected	n
C	80.8 ± 2.7	11	68.3 ± 5.8	3
TG	55.9 ± 5.0*	8	66.3 ± 7.7	3
UG/TG	68.9 ± 5.4**	7	69.0 ± 4.0	2
UG	87.5 ± 1.5	2	60.0 ± 0.8	2

† C indicates control; TG, transglutaminase; and UG, uteroglobin.
* $P < .0005$ vs C.
** $P < .05$ vs C.

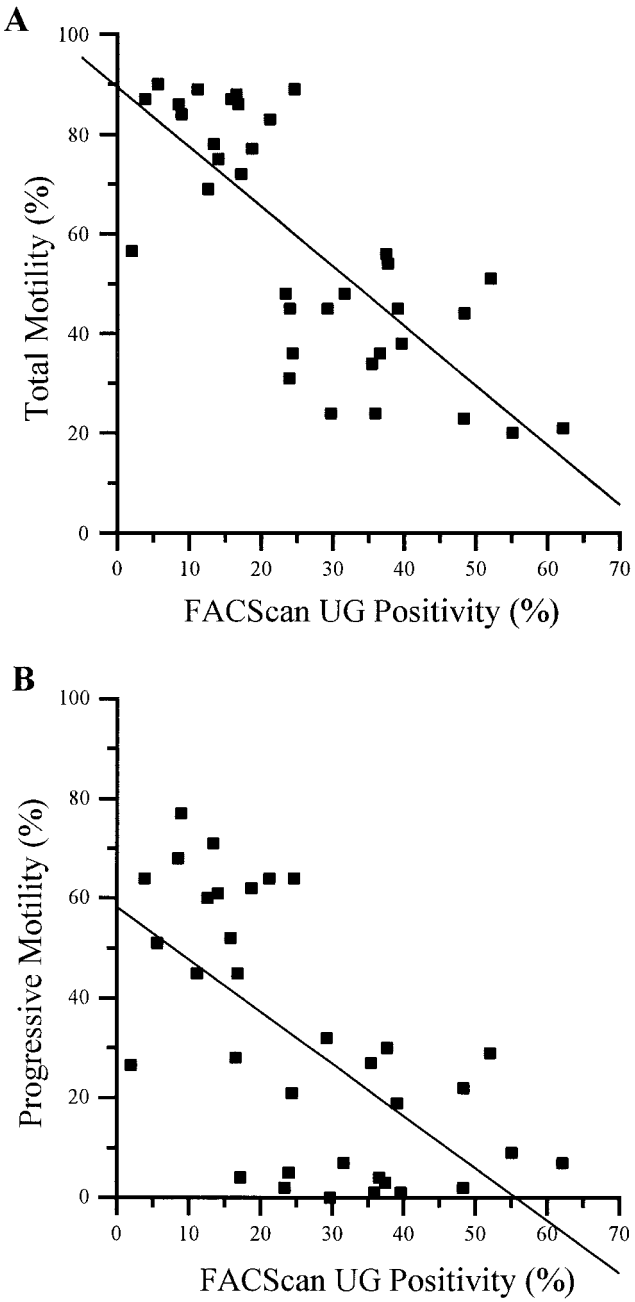

Figure 9. Negative correlation between percentage UG-positive spermatozoa and total **(A)** or progressive **(B)** motility.

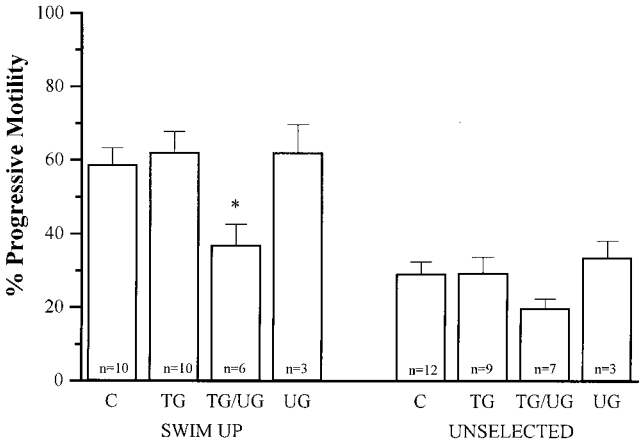

Figure 10. Effect of TG, UG, and the combination of the 2 on human sperm motility. Swim-up–selected and unselected spermatozoa were incubated in capacitating medium in the presence or absence of TG (3.5 U/mL), UG (2 μg/mL) and TG + UG for 2 hours and progressive motility evaluated. *$P < .01$ vs C.

perimental results. First, both proteins are present on the surface of a high proportion of live ejaculated human sperm. In addition, UG was not found on spermatozoa obtained from an epididymal cyst, confirming previous reports that the origin of the molecule is the seminal vesicle, prostate, or both (Noske and Gooding, 1978; Manyak et al, 1988; Peri et al, 1993). Second, levels of UG and TG are found to be significantly lower in swim-up–selected sperm populations, characterized by a higher percentage of capacitated cells. Third, levels of both molecules are significantly inversely correlated with sperm motility. Fourth, exogenous readdition of the 2 agents to swim-up–selected sperm significantly reduces their motility as well as the ability of these cells to capacitate, while no effects are observed in unselected sperm, where high levels of UG and TG are already present in basal conditions.

The highly significant negative correlation found between sperm motility and UG and TG contents, and the effects of the 2 substances on motility in swim-up–selected samples, demonstrate that the presence of these factors on sperm surface represents an obstacle to the development of motility. Thus, we can postulate that during the swim-up procedure, only cells with low or absent levels of UG and TG are allowed to migrate in the upper buffer, while sperm with high levels remain trapped in the bottom phase. Because sperm selection in the female genital tract occurs during transit through the cervical mucus, it can be speculated that only spermatozoa with low or absent levels of the 2 compounds, and thus with higher motility, are allowed to penetrate the mucus and reach the uterus.

From the results of our study it appears that the presence of TG is necessary to obtain the effects of UG on motility and capacitation, because in its absence, the molecule does not have any effect on the 2 parameters. However, if UG is removed from the medium with an antibody, the decapacitating effect of TG + UG is no longer present and the acrosome reaction in response to progesterone occurs as in control samples. It is well known that UG cross links to human sperm proteins in the presence of TG (Manjuath et al, 1984). From our study it appears that TG-induced UG cross linking to the sperm surface is necessary to obtain the effects of UG. On the other hand, TG exerts its effects on the capacitation of swim-up–selected sperm also in the absence of exogenously added UG (Figure 8). It is possible that part of UG present on the sperm surface is not covalently linked to sperm proteins and cross links when TG is exogenously added. In keeping with this hypothesis, we found that at 24 hours in the presence of TG, percentage of UG-positive spermatozoa in swim-up–selected samples increases even if incubation is performed in the absence of added UG. It is also possible that TG, besides cross linking UG, might catalyze acyl transfer to sperm proteins of other substrates. In particular, it is well-established that TG catalyzes incorporation of polyamines into proteins, including spermine and other polyamines present in seminal fluid (Folk et al, 1980) involved in the regulation of sperm motility and capacitation (De Lamirande and Gagnon, 1989; Rubinstein and Breitbart, 1991; Breitbart et al, 1997).

Western blot analysis of UG in total sperm lysates reveals the presence of additional protein bands besides those corresponding to recombinant UG (Figure 6). In particular, the anti-UG antibody reveals additional bands with a molecular weight raging from 20 kd to 30 kd and higher. Whether these proteins represent cross linked multimers of UG cannot be established at present. However, the presence of additional high-molecular-weight protein bands after Western blot analysis has also been reported in other tissue lysates such as human prostatic extracts (Manyak et al, 1988) and rabbit pulmonary alveolar cells (Guy et al, 1992). In particular, in rabbit alveolar cells, some of these bands have the same molecular weight as proteins detected in immunoprecipitation of ^{35}S-labeled proteins with UG antibody in the same cells, leading to the hypothesis that they may represent covalently cross linked multimers of UG (Guy et al, 1992).

It must be noted that DFs are hypothesized to be lost or inactivated as capacitation proceeds (Yanagimachi, 1994; Fraser, 1995), while our experiments clearly show that the percentage of cells expressing UG on their surface increases following overnight incubation. At present we do not have a clear explanation for this surprising result. Apparently, no protein synthesis is involved, and UG is not present in the medium as a contaminant of albumin. It is possible that during the first 2 hours of

capacitation spermatozoa release UG into the medium, and during overnight incubation TG cross links the molecule to the surface. The increase of UG observed at this time may reflect a decapacitated state of spermatozoa, which is particularly evident in unselected sperm (rich in endogenous TG). It has been recently shown that sperm capacitation is transient and that, within a sperm population, there is a ''continuous process of replacement of capacitated spermatozoa'' (Cohen-Dayag et al, 1995), indicating that capacitation is asynchronous (Yanagimachi, 1994). In addition, capacitation seems to be maximal at 50 minutes (corresponding to about 2 hours from the beginning of sperm selection) and decreases thereafter (Cohen-Dayag et al, 1995). Furthermore, no capacitated spermatozoa were found in the unselected population (Cohen-Dayag et al, 1995). Although a comparison between our work and that of Cohen-Dayag et al (1995) is not possible because of the different modes of sperm selection used, the increase in UG observed in our experiments might justify the changes in the status of capacitation detected by these authors in their work.

In conclusion, the present paper demonstrates that UG and TG might represent 2 decapacitating factors of seminal plasma. The equilibrium between UG and TG present on sperm surface and in the female genital tract may play an important role in the process of fertilization by modulating sperm motility and capacitation. In particular, in the rabbit, the lowest levels of uterine UG are found during the proliferative phase and increase following ovulation (Peri et al, 1994), suggesting that UG must be kept at low levels in order to favor sperm transit and the fertilization process.

Acknowledgments

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