

Human zona pellucida recognition associated with removal of sialic acid from human sperm surface

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The ability of human spermatozoa recovered from highly motile sperm fractions to bind wheat germ agglutinin (WGA) after discontinuous Percoll gradient centrifugation was studied. WGA could bind to almost all motile spermatozoa, whereas fewer than 25% of spermatozoa could bind peanut (PNA) and concanavalin A (Con A) agglutinin, two lectins that specifically bind acrosomal membranes. After removal of the plasma membrane with 0.04% Triton X100, WGA, PNA and Con A bound more than 80% of spermatozoa, but binding sites for WGA on the anterior acrosomal region were markedly reduced. The expression of sialic acid on human sperm plasma membrane was demonstrated, since WGA, which specifically recognizes both sialic acid (NeuNAc) and *N*-acetylglucosamine (GlcNAc), bound almost all intact motile spermatozoa, whereas succinylated WGA, which recognizes only GlcNAc, bound less than 10% of intact motile spermatozoa. Moreover, binding of WGA was compared with that of three other lectins (*Sambucus nigra*, SNA; *Maackia amurensis*, MAL and *Limulus polyphemus*, LPA) with specificity for different NeuNAc linkages. Only SNA, which requires the presence of the disaccharide structure NeuNAc $\alpha(2,6)$ Gal/GalNAc, showed a positive correlation with sperm motility as observed with WGA. Moreover, there was a strong inhibition of WGA binding on spermatozoa preincubated with bovine submaxillary mucin containing (2,6)-linked NeuNAc. These results demonstrate the presence of NeuNAc $\alpha(2,6)$ Gal/GalNAc glycoconjugate sequences on the plasma membrane of the motile human spermatozoon. Treatment of spermatozoa with *Arthrobacter ureafaciens* neuraminidase to cleave NeuNAc residues led to a dose-dependent decrease of WGA binding at the sperm surface and to the enhancement of sperm attachment to the zona pellucida. We hypothesize that the release of sialic acid from the sperm plasma membrane could be one of the capacitation events necessary for unmasking certain sperm surface antigens implicated in zona pellucida recognition.

Introduction

Maturation of the spermatozoon (acquisition of motility and fertilizing ability) seems to be acquired through the epididymis (Orgebin-Crist and Fournier-Delpech, 1982; Bellvé and O'Brien, 1983; Primakoff *et al.*, 1987). Many modifications of the sperm surface, such as masking or unmasking surface molecules or addition of new molecules, result from interactions between the spermatozoa and the surrounding epididymal fluid and especially with glycoproteins synthesized and secreted by the epididymal epithelium (Brown *et al.*, 1983; Eddy *et al.*, 1985; Srivastava and Olson, 1991).

Sialic acid (NeuNAc) residues are secreted by the epithelium of the epididymis as terminal sugars of sialoglycoproteins that bind to the sperm surface during epididymal transit (reviewed by Toshimori *et al.*, 1991). Sialic acid residues have significant biological functions such as masking of antigens, i.e. anti-

recognition molecules (reviewed by Schauer, 1985) or as receptor determinants (Herrler *et al.*, 1992; Yoshida *et al.*, 1992; Powell *et al.*, 1993). The presence of sialic acid bound to the surface of epididymal spermatozoa has been demonstrated in several mammals (rats: Toowicharanont and Chulavatnatol, 1983; mice: Toshimori *et al.*, 1991; rams and bulls: Holt, 1980). Sperm surface sialic acid seems to be conserved, at least in rams and bulls, after ejaculation (Holt, 1980). The loss of sperm surface sialic acid allows phagocytosis of mouse spermatozoa by macrophages *in vitro* (Toshimori *et al.*, 1991). Seminal phagocytic cells play a role in the destruction of abnormal human spermatozoa from the ejaculate (Tomlinson *et al.*, 1992) and leucocytes are recruited at the human cervix in a specific response to spermatozoa (Pandya and Cohen, 1985). The presence of sperm surface sialic acid may render certain spermatozoa immune from phagocytosis (Holt, 1980), whereas its absence leads to the destruction of spermatozoa of abnormal morphology by phagocytosis in the ejaculate and during their transit through the female genital tract.

The aim of our work was to study (i) the presence of sialic acid at the sperm surface either on the plasma membrane before

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Table 1. Lectins used in this study and their sugar specificities

Lectins	Sugar specificities	References
PNA Peanut agglutinin <i>Arachis hypogaea</i> (Sigma)	D-Gal β 1,3 D GalNAc > D GalNAc > D Gal	1
Con A Concanavalin A <i>Canavalia ensiformis</i> (Sigma)	Man α -1,2 Man α -1,2 Man > Man α -1,2 Man > α Glc > α GlcNAc	2
Succinylated WGA Wheat germ agglutinin <i>Triticum vulgare</i> (Vector)	GlcNAc β -1,4 GlcNAc β -1,4 GlcNAc > GlcNAc β -1,4 GlcNAc > GlcNAc	2
WGA Wheat germ agglutinin <i>Triticum vulgare</i> (Sigma)	NeuNAc α -2,6 and α -2,3 D Gal/GalNAc and GlcNAc β -1,4 GlcNAc	3–5 2, 4
MAL <i>Maackia amurensis</i> leukoagglutinin (Vector)	Necessitate the trisaccharide structure: NeuNAc α -2,3 Gal β -1,4 GlcNAc/Glc	6–8
SNA Elderberry bark agglutinin <i>Sambucus nigra</i> (Vector)	Necessitate the disaccharide structure: NeuNAc α -2,6 Gal/GalNAc	6, 8, 9
LPA Horseshoe crab agglutinin <i>Limulus polyphemus</i> (Sigma)	NeuNAc α -2,3 GalNAc > NeuNAc α -2,6 GalNAc > NeuNAc GalNAc	10 11

(1) Pereira *et al.*, 1976; (2) Magargee *et al.*, 1988; (3) Furukawa *et al.*, 1986; (4) Monsigny *et al.*, 1980; (5) Wright, 1992; (6) Knibbs *et al.*, 1991; (7) Wang and Cummings, 1988; (8) Heerze and Armstrong, 1990; (9) Shibuya *et al.*, 1987; (10) Zeng and Gabius, 1992; (11) Miller *et al.*, 1982.

and after capacitation or after the loss of the plasma membrane and (ii) the relationships between sialic acid expression and sperm fertilizing ability of spermatozoa.

Materials and Methods

Preparation of spermatozoa

Ejaculated fresh semen was obtained with consent from normal donors and liquefied for 30 min at 37°C. Whole semen, 500 μ l, was diluted with 5 ml PBS plus 0.4% (w/v) BSA (fraction V, Sigma La Verpillère), and then centrifuged at 600 g for 7 min. The sperm pellet was resuspended in 1 ml B2 medium (B2 Menezo, Biomerieux SA, 69280 Marcy-l'Etoile). This sample constituted the entire sperm sample. One millilitre of whole semen was separated on a discontinuous Percoll gradient as described by Lassalle and Testart (1992) except that two concentrations (90 and 45%) of Percoll were used rather than three. After centrifugation (25 min at 600 g), Percoll fractions were carefully separated and washed by dilution with 5 ml PBS-BSA and then centrifuged at 600 g for 7 min. The

pellets were resuspended in 1 ml B2 medium. Before addition of lectin probes, the numbers of motile and immotile spermatozoa were determined on a Malassez cell to estimate the percentage of motile spermatozoa for each sperm suspension.

Labelling of spermatozoa with lectins

The ability of spermatozoa from selected populations to bind particular lectins conjugated to fluorescein isothiocyanate (FITC) or biotin was studied. Lectins were purchased from Sigma or Vector (Vector Laboratories Inc., Burlingame, CA). The common names, taxonomic names and specificities of these lectins are given (Table 1). Wheat germ agglutinin (WGA) and succinylated WGA (sWGA) were used to demonstrate the presence of cell surface receptors containing sialic acid (Monsigny *et al.*, 1980). WGA can recognize both NeuNAc (sialic acid) and GlcNAc (*N*-acetylglucosamine), whereas sWGA recognizes only GlcNAc (Monsigny *et al.*, 1980). Peanut agglutinin (PNA) and concanavalin A (Con A) were chosen for their ability to bind specifically to the outer and the inner acrosomal membrane of human spermatozoa, respectively

(Mortimer *et al.*, 1987; Holden *et al.*, 1990), and were used to assess sperm acrosomal status when compared with WGA. WGA was also compared to *Limulus polyphemus* (LPA), *Maackia amurensis* (MAL) and *Sambucus nigra* (SNA) agglutinins that exhibit specificity for different NeuNAc-linkage from Gal/GalNAc (Table 1).

Each treated sperm suspension was incubated for 30 min at 37°C with lectin at optimal concentrations according to previous reports (Table 1) i.e. 10 µg ml⁻¹ (PNA, Con A) or 5 µg ml⁻¹ (SNA, MAL, LPA, WGA and sWGA). Lectin-treated sperm suspensions were washed twice in PBS-BSA and pellets were resuspended in 100 µl PBS-BSA. Sperm pellets from samples pretreated with biotinylated lectins were incubated for an additional 30 min at 37°C with 10 µl of streptavidin-FITC (0.25 mg ml⁻¹) solution (Sigma) and then washed twice in PBS-BSA.

Changes in lectin binding after incubation of spermatozoa in vitro and release of sperm plasma membrane

Binding of PNA, Con A, WGA and sWGA to the sperm head was also studied after incubation of spermatozoa *in vitro* or treatment that induces total release of the sperm plasma membrane. Incubation of spermatozoa *in vitro* was performed at 4°C for 24 h. Total release of the sperm plasma membrane was induced by treatment with 0.04% Triton X100 for 30 min at 37°C (Langlais *et al.*, 1981; Giroux-Widemann *et al.*, 1991). Ultrastructural studies have found that about one-third of fresh (Barthelemy *et al.*, 1990) and all Triton X100-treated (Langlais *et al.*, 1981; Giroux-Widemann *et al.*, 1991) human spermatozoa have lost their plasma membrane. Sperm samples that had been incubated or treated with Triton X100 were diluted with 5 ml PBS-BSA before centrifugation (600 g for 10 min) and finally the pellet was resuspended in 1 ml B2 medium before addition of lectin probes.

Microscope analysis of lectin binding

Sperm suspensions, 10 µl, were placed between slide and coverslip for observation under a Leitz microscope equipped with epifluorescence. Percentages of spermatozoa displaying fluorescence anywhere on the head surface (i.e. whatever the precise binding location on the sperm head) or on the anterior acrosomal region were estimated from at least 100 spermatozoa per preparation. Binding of PNA, Con A, WGA and sWGA to the anterior acrosomal region was studied for untreated spermatozoa recovered from the 90% Percoll fractions and after treatment with Triton X100. WGA binding on the anterior acrosomal region of untreated spermatozoa was compared with that of capacitated spermatozoa and of those recovered in the 45% Percoll fraction.

Inhibition of sperm WGA binding by glycoproteins or polysaccharides containing terminal NeuNAc Gal/GalNAc sequences

Sugar-binding specificity of lectins is usually determined by measuring the ability of a sugar to inhibit either blood cell agglutination by the lectin, precipitation of the lectin by

polysaccharides or glycoproteins or binding of the lectin to glycosubstances (reviewed by Zeng and Gabius, 1992). WGA-FITC, 5 µg ml⁻¹, in 1 ml B2 medium was incubated alone (control) or with increasing concentrations of either *N*-acetylneuraminic acid (Sigma), bovine submaxillary mucin, BSM (Sigma) or bovine fetuin (Sigma) to compare the effects of glycoproteins containing terminal NeuNAc linked α2,3, α2,6 or both with penultimate Gal or GalNAc. After incubation for 1 h at 37°C, 10⁶ spermatozoa (100 µl) from the highly motile sperm fraction (90% Percoll fraction) were added. These mixtures were incubated for an additional 1 h at 37°C and spermatozoa were then washed twice before observation under a microscope. The percentage of spermatozoa showing fluorescence on the head surface was estimated for each inhibitor tested. Concentrations of fetuin and BSM were expressed on the basis of NeuNAc content. Results were given as total inhibition (absence of WGA bound spermatozoa) or 50% inhibition when percentages of spermatozoa with bound WGA reached 50% of the control value.

Effect of sialic acid release by neuraminidase on sperm-zona pellucida recognition

A preliminary experiment was done to study WGA binding on the sperm head after treatment with increasing concentrations of neuraminidase from *Arthrobacter ureafaciens* (Sigma). Spermatozoa recovered from the 90% Percoll fraction were treated for 1 h with 0, 200, 300 or 500 mU neuraminidase ml⁻¹ at 37°C, and washed in PBS-BSA. Sperm pellets were then resuspended in 1 ml B2. Labelling with WGA-FITC, washing and control of WGA binding were performed as described above.

Human oocytes that were not fertilized by 40–48 h after insemination *in vitro* were stored at 4°C in ammonium sulfate solution for optimal conservation of the zona pellucida (Yoshimatsu *et al.*, 1988). Two hours before insemination, the zonae pellucidae were thoroughly rinsed for 90 min in four successive baths of PBS-BSA and then placed for 30 min in B2 medium at 37°C before insemination. Spermatozoa recovered from the 90% Percoll fraction were either untreated or treated for 1 h with 500 mU neuraminidase ml⁻¹ at 37°C before washing in PBS-BSA. Sperm pellets were labelled by suspension in 200 µl of the FITC stock solution (200 µg FITC ml⁻¹, Sigma) according to Liu *et al.* (1988). The suspension was incubated at 37°C for 15 min and spermatozoa were washed twice by centrifugation (600 g for 7 min), resuspended in B2 medium and diluted to a concentration of 5 × 10⁵ spermatozoa ml⁻¹ with B2 medium. Five hundred microlitres of untreated or neuraminidase-treated sperm suspensions were placed in macrowells of Nunclon delta dishes (Nunc, Roskilde) before addition of zonae pellucidae. After incubation for 2 h (at 37°C under 5% CO₂), zonae pellucidae were thoroughly rinsed in three successive baths of PBS-BSA and then placed between slide and coverslip as described by Lassalle and Testart (1991). Observations were made under a Leitz microscope equipped with epifluorescence and the number of fluorescent spermatozoa on each zona pellucida was determined. These experiments to evaluate the ability of human spermatozoa to bind human zonae pellucidae were approved by the French Comité National d'Ethique.

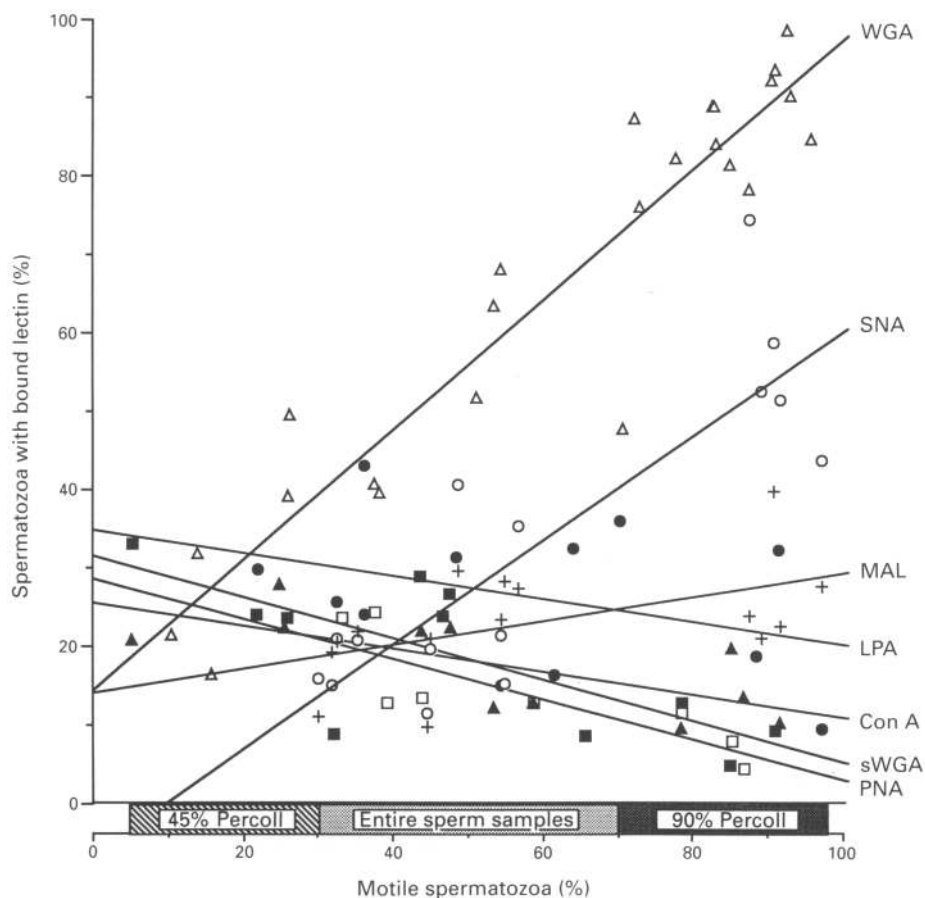


Fig. 1. Lectin binding according to sperm motility. Entire sperm samples and sperm populations recovered in the 45% and 90% Percoll fractions after Percoll density gradient centrifugation were obtained from each ejaculate. Percentages of motile spermatozoa were 34.0–56.9%, 70.4–95.5% and 5.2–30.0% in the entire sperm samples, and in the 90% and 45% Percoll fractions, respectively. Equations of the regression straight line and correlation coefficients were obtained and linearity was tested using *F* tests. PNA: $R^2 = 0.70$, $P < 0.01$; Con A, $R^2 = 0.50$, $P < 0.02$; sWGA, $R^2 = 0.56$, $P < 0.01$; WGA, $R^2 = 0.90$, $P < 0.0001$; SNA, $R^2 = 0.72$, $P < 0.001$; MAL, $R^2 = 0.27$, $P < 0.05$. No significant correlation was found between LPA binding and sperm motility. At least four ejaculates from different normal donors were used for each lectin tested. (□) PNA, peanut agglutinin; (▲) Con A, concanavalin A; (Δ) WGA, wheat germ agglutinin; (■) sWGA, succinylated wheat germ agglutinin; (●) LPA, *Limulus polyphemus* agglutinin; (+) MAL, *Maackia amurensis* leucoagglutinin and (○) SNA, *Sambucus nigra* agglutinin.

Statistical analysis

Data were analysed using Statview 4.0 software provided by a Macintosh II si computer. Means (\pm SEM) were compared using Student's *t* test and one-way analysis of variance (ANOVA). Regression equations and correlation coefficients were obtained and linearity was tested using *F* tests.

Results

Labelling of spermatozoa with lectins according to sperm motility

Percentages of motile spermatozoa were 34.0–56.9%, 70.4–95.5% and 5.2–30.0% in the entire sperm sample, in the 90% and in the 45% Percoll fractions, respectively. For three of the tested lectins, PNA, Con A and sWGA, percent-

ages of spermatozoa that exhibited positive lectin binding on the sperm head decreased (PNA, $R^2 = 0.70$, $P < 0.01$; Con A, $R^2 = 0.50$, $P < 0.02$; sWGA, $R^2 = 0.56$, $P < 0.01$) in relation to the increasing proportion of motile spermatozoa (Fig. 1) and varied between 14 and 25%. On the contrary, WGA bound 76% or 33% of spermatozoa when sperm motility was higher or lower than 50%, respectively, and a positive correlation ($R^2 = 0.90$, $P < 0.0001$) was observed between WGA binding and the number of motile spermatozoa (Fig. 1). Binding of WGA occurred in nearly all the motile spermatozoa, especially those from the 90% Percoll fraction (Fig. 1). A positive correlation was also observed between motility and lectin binding for SNA ($R^2 = 0.72$, $P < 0.001$) and MAL ($R^2 = 0.27$, $P < 0.05$) (Fig. 1). However, when the sperm motility was higher than 50%, SNA and MAL bound 44% and 27% of spermatozoa, respectively (data not

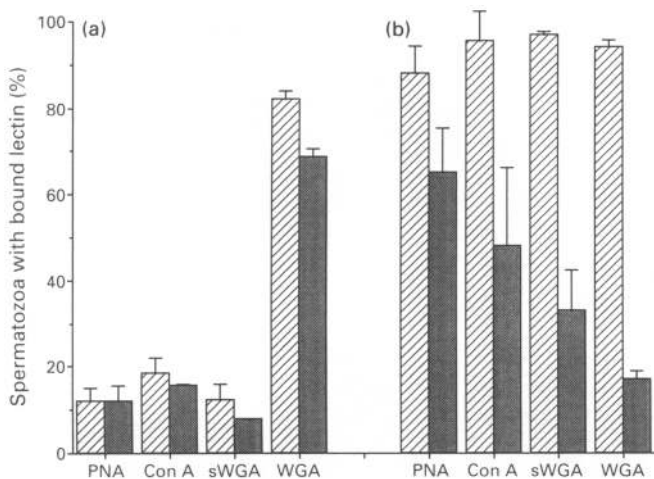


Fig. 2. Binding sites of lectins on the head of spermatozoa (a) untreated and (b) treated with Triton X100. Wheat germ agglutinin (WGA) was compared with peanut agglutinin (PNA), concanavalin A (Con A) and succinylated wheat germ agglutinin (sWGA) lectins. (▨) lectin-binding sites on sperm heads whatever the precise location; (■) lectin-binding sites at the anterior acrosomal region. Data are means \pm SEM from at least three experiments.

shown), compared with 76% using WGA. No significant correlation was found between LPA binding and sperm motility (Fig. 1).

Lectin distribution in relation to incubation of spermatozoa in vitro and release of sperm plasma membrane

Proportions of untreated or Triton X100-treated spermatozoa with binding sites for PNA, Con A, sWGA and WGA on the sperm head or at least on the anterior acrosomal region were compared (Fig. 2). Fewer than 20% of untreated spermatozoa bound PNA, Con A and sWGA, whereas WGA binding occurred in at least 80% of this sperm population (Fig. 2). After sperm demembration with Triton X100, all tested lectins bound more than 80% of spermatozoa (Fig. 2). Whereas proportions of demembrated spermatozoa with lectin binding sites on the anterior acrosomal region tended to increase for PNA (12.0 versus 65.1%, $P < 0.01$), Con A (15.6 versus 48.1%, not significant) and sWGA (7.7 versus 33.0%, not significant), these proportions decreased for WGA (68.9% versus 16.9%, $P < 0.001$) (Figs 2 and 3). WGA bound particularly on the equatorial segment and the post-acrosomal region (with or without binding on the anterior acrosomal region) after Triton X100 treatment but similar proportions of untreated (76.2%) and Triton X100-treated (73.6%) spermatozoa had binding sites for these two sperm regions (data not shown).

Preliminary studies in which incubation was for 24 h at 4°C showed that percentages of acrosome-reacted spermatozoa assessed by PNA-FITC or Con A-FITC, two lectins used to assess sperm acrosome reaction (Mortimer *et al.*, 1987; Holden *et al.*, 1990), were higher ($P < 0.05$) for capacitated (39.3%) than for untreated spermatozoa (15.1%) (data not shown). Proportions of spermatozoa that bound WGA anywhere on the sperm head surface or on the anterior acrosome were lower in the

45% Percoll fraction and after incubation compared with that in spermatozoa from the 90% Percoll fraction (Fig. 3). However, after sperm demembration with Triton X100, only proportions of those spermatozoa that exhibit WGA binding on the anterior acrosomal region were low (Fig. 4).

Inhibition of sperm WGA binding by glycoproteins or polysaccharides containing terminal NeuNAc Gal/GalNAc sequences

Total inhibition of WGA binding on the sperm head was observed for 4 mmol NeuNAc l^{-1} contained in bovine submaxillary mucin (Fig. 5). About 10 mmol NeuNAc l^{-1} contained in fetuin or 150 mmol free NeuNAc l^{-1} were needed for 50% inhibition of WGA binding and total inhibition was not reached with 20 mmol fetuin l^{-1} (Fig. 4). No sign of WGA inhibition was observed with 32 mmol NeuNAc l^{-1} contained in NeuNAc $\alpha(2,3)$ lactose and 50% inhibition was obtained with 200 mmol GlcNAc l^{-1} (data not shown).

Effects of treatment of spermatozoa with neuraminidase on binding of WGA to spermatozoa and sperm-zona pellucida recognition

Neuraminidase from *Arthrobacter ureafaciens* has been used to cleave $\alpha(2,3)$, $\alpha(2,6)$ or $\alpha(2,8)$ -linked NeuNAc residues (Powell *et al.*, 1993). Binding sites for WGA were observed on 73.4, 39.6, 31.4 and 20.2% of spermatozoa treated with 0, 200, 300 and 500 mU *Arthrobacter ureafaciens* neuraminidase ml^{-1} , respectively (Fig. 6). Neuraminidase treatment induced a dose-dependent decrease ($R^2 = 0.87$, $P < 0.01$) in the percentage of spermatozoa that bound WGA. Treatment of spermatozoa with 500 mU *Arthrobacter ureafaciens* neuraminidase ml^{-1} did not affect the sperm motility and the number of motile spermatozoa that were identical to untreated sperm samples. Moreover, no sign of sperm agglutination or adhesion to the plastic culture dish was observed after neuraminidase treatment. In three assays using different sperm donors, the average number of spermatozoa bound per human zona pellucida increased significantly (donor 1, 2.9 versus 18.0, $P < 0.001$; donor 2, 3.4 versus 15.8, $P < 0.001$; donor 3, 16.9 versus 35.4, $P < 0.01$) after pretreatment of spermatozoa with 500 mU *Arthrobacter ureafaciens* neuraminidase l^{-1} (Table 2, Fig. 7).

Discussion

Human spermatozoa are characterized by a high heterogeneity in morphology, motility and nuclear maturity. Percoll density gradient centrifugation is used to separate sperm populations according to sperm density (Bolton and Braude, 1984) and spermatozoa with a dense and homogeneous nucleus were recovered mainly in the highest density fraction (90%) of the Percoll gradient. Spermatozoa of normal and motile spermatozoa are mainly recovered in the 90% Percoll fraction (Le Lannou and Blanchard, 1988) and that fraction contains spermatozoa with high fertilizing ability (Hyne *et al.*, 1983; Berger *et al.*, 1985; Sapienza *et al.*, 1993). Our results show that less than 20% of freshly ejaculated spermatozoa recovered in the 90% Percoll fraction exhibited positive PNA, Con A and

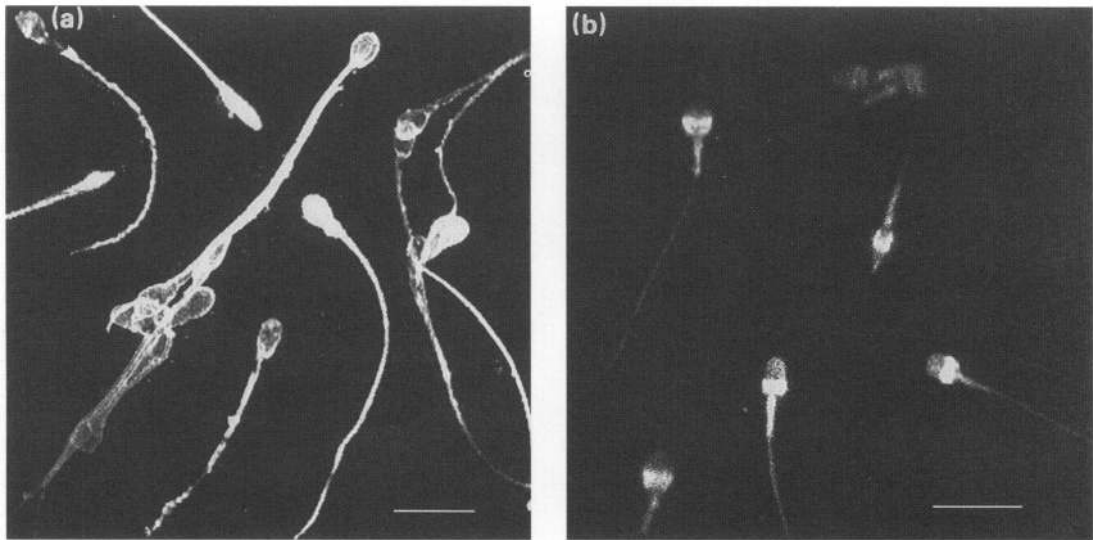


Fig. 3. (a) Untreated spermatozoa from the 90% Percoll fraction exhibited wheat germ agglutinin (WGA)–fluorescein isothiocyanate (FITC) binding on all surfaces including the sperm tail. (b) After release of the plasma membrane with 0.04% Triton X100, WGA–FITC binding was not evident in the anterior acrosomal region and tail. Scale bars represent 10 μm .

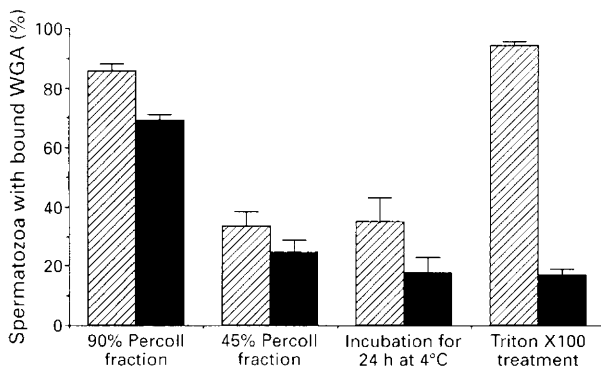


Fig. 4. Wheat germ agglutinin (WGA)–binding sites on the head of spermatozoa recovered in the 90% Percoll fractions after incubation for 24 h at 4°C and after treatment of spermatozoa with Triton X100. Comparison with untreated spermatozoa from 90% and 45% Percoll fractions. (▨) WGA sites on sperm heads whatever the precise binding location; (■) WGA sites at the anterior acrosomal region. Data are means \pm SEM from six experiments.

sWGA binding, whereas WGA could bind to more than 80% of spermatozoa from this fraction. Binding of WGA was positively related to sperm motility, whereas a negative relationship was observed for PNA, Con A and sWGA binding.

PNA and Con A have been used to assess the acrosomal status of human spermatozoa (Mortimer *et al.*, 1987; Holden *et al.*, 1990). Mortimer *et al.* (1987) demonstrated specific PNA labelling of the outer acrosomal membrane, whereas Con A binds predominantly to the inner acrosomal membrane (Holden *et al.*, 1990). In the study reported here, PNA, Con A and WGA (as sWGA) bound more than 80% of spermatozoa after release of the sperm plasma membrane with Triton X100. However, this treatment unmasked PNA- and Con A-binding sites at the anterior acrosomal region, i.e. on acrosomal

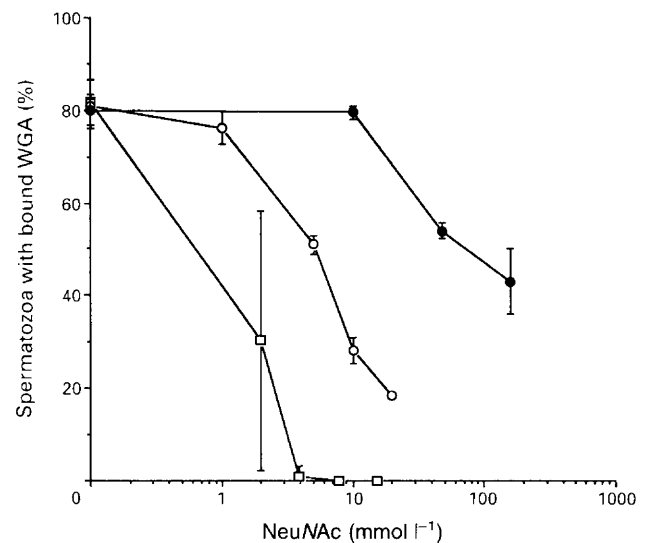


Fig. 5. Competitive inhibition of wheat germ agglutinin (WGA) binding on human sperm head with NeuNAc (●), fetuin (○) and bovine submaxillary mucin (◻). WGA–fluorescein isothiocyanate (FITC) at 5 $\mu\text{g ml}^{-1}$ in 1 ml B2 medium was incubated alone (control) or with increasing concentrations of either *N*-acetylneuraminic acid (NeuNAc), bovine submaxillary mucin (BSM) or bovine fetuin. After incubation for 1 h at 37°C, 10⁶ spermatozoa (100 μl) from the 90% Percoll fraction were added and incubated for a further 1 h at 37°C. Spermatozoa were washed twice before observation under a microscope and the percentage of spermatozoa showing fluorescence on the head surface was estimated for each inhibitor tested. Concentrations of fetuin and BSM were expressed on the basis of NeuNAc content. Data are means \pm SEM from five (NeuNAc), two (fetuin) and six (BSM) experiments.

membranes of spermatozoa, whereas WGA binding sites on the anterior acrosomal region seemed to be released with the plasma membrane. After treatment of spermatozoa with Triton

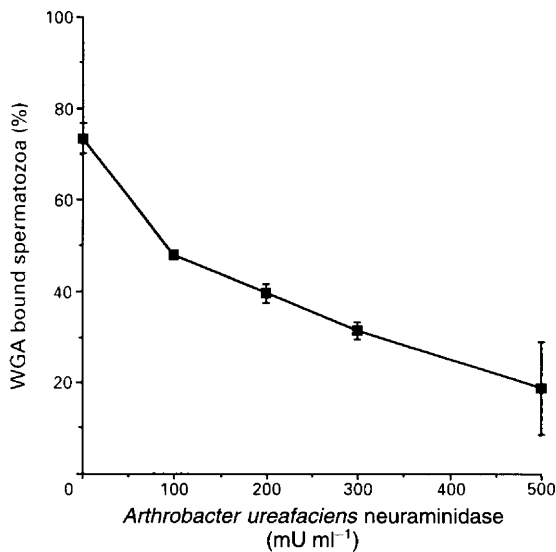


Fig. 6. Inhibition of wheat germ agglutinin (WGA) binding on the surface of human spermatozoa by neuraminidase. Spermatozoa were pretreated with increasing concentrations of neuraminidase from *Arthrobacter ureafaciens* before addition of WGA–fluorescein isothiocyanate (FITC) probes. The percentage of spermatozoa with bound WGA decreased for 200 ($P < 0.001$), 300 ($P < 0.001$) and 500 ($P < 0.01$) mU neuraminidase ml⁻¹ compared with the control. Each point represents the mean \pm SEM of two experiments.

Table 2. Average number of human spermatozoa bound per zona pellucida after sperm treatment with neuraminidase

Donor	Concentrations of <i>Arthrobacter ureafaciens</i> neuraminidase	
	0 mU ml ⁻¹	500 mU ml ⁻¹
1	2.9 \pm 1.2 (14) ^a	18.0 \pm 2.1 (15) ^a
2	3.4 \pm 0.5 (13) ^b	15.8 \pm 2.5 (16) ^b
3	16.9 \pm 3.3 (16) ^c	35.4 \pm 5.3 (16) ^c

Spermatozoa recovered from the 90% Percoll fraction were either untreated or treated for 1 h with 500 mU *Arthrobacter ureafaciens* neuraminidase ml⁻¹ at 37°C before labelling with fluorescein isothiocyanate stock solution. After washing by centrifugation, spermatozoa were resuspended in B2 medium and diluted to 5×10^5 spermatozoa ml⁻¹. Five hundred microlitres of each sperm suspension was placed in macrowells of Nunclon delta dishes before addition of human zonae pellucidae. After 2 h incubation (at 37°C under 5% CO₂), zonae pellucidae were thoroughly rinsed in PBS-BSA then placed between slide and coverslip for observation under a microscope.

The number of human zonae pellucidae used is indicated in parentheses.

^{a,b} $P < 0.001$; ^c $P < 0.01$.

X100, WGA binding sites were observed particularly on the equatorial segment and the post-acrosomal region. This leads us to hypothesize that additional WGA binding sites could be unmasked on the equatorial segment and on posterior acrosomal regions after sperm plasma membrane release. In agreement with the work of Liu *et al.* (1991) on mouse spermatozoa, these results support the contention that WGA-binding sites are at the surface of intact human spermatozoa.

Although the exact mechanism of the capacitation process

remains to be clarified, capacitation is viewed as a reversible phenomenon that results in a net decrease in negative surface charge, an efflux of membrane cholesterol, and an influx of calcium ions between the plasma and outer acrosomal membrane (reviewed by Langlais and Roberts, 1985). Holt (1980) suggested that sialic acid was responsible for the net negative surface charge of the spermatozoon, whereas the loss of the negative surface charge results from the removal of sialic acid (Srivastava and Farooqui, 1980). In the study reported here, we observed a marked decrease in binding to WGA, a lectin which recognizes sialic acid, after incubation for 24 h at 4°C compared with untreated sperm samples. Moreover, incubation of spermatozoa for 24 h at 4°C increased the acrosome reaction and fertilizing ability of human spermatozoa (Carrell *et al.*, 1992). In the study reported here, the percentage of acrosome-reacted spermatozoa increased after incubation for 24 h at 4°C. These results support the contention that there is a decrease of the net negative surface charge and an influx of calcium ions, two events characteristic of the sperm capacitation process. The percentage of spermatozoa with WGA bound anywhere on the sperm head and those with WGA bound on the anterior acrosomal region both decreased after incubation compared with that of untreated and Triton X100-treated spermatozoa. After incubation, WGA-binding sites on the plasma membrane were not evident, although the plasma membrane persisted. Similar results were obtained using sperm populations with low percentages of motile spermatozoa from the 45% Percoll fraction. However, sperm populations recovered from low Percoll density fractions present lower percentages of motile spermatozoa of normal morphology, and of mature spermatozoa compared with those from the 90% Percoll fractions (Le Lannou and Blanchard, 1988).

WGA and sWGA have been used to determine the number of cell surface receptors containing sialic acid (Monsigny *et al.*, 1980). WGA can recognize both NeuNAc and GlcNAc, whereas sWGA recognizes only GlcNAc (Monsigny *et al.*, 1980). The percentages of untreated spermatozoa with bound sWGA, PNA and Con A were similar ($< 20\%$), whereas WGA bound more than 80% of untreated spermatozoa. However, sWGA and WGA bound high percentages of Triton X100-treated spermatozoa without great differences between percentages of spermatozoa with lectins bound on the anterior acrosome. From these results, we cannot exclude the possibility that GlcNAc is the only sugar residue recognized by both sWGA and WGA after demembration with Triton X100. However, we found that NeuNAc was expressed on the plasma membrane of motile spermatozoa, especially at the anterior acrosomal region and that this expression was lost after capacitation or plasma membrane release or on spermatozoa (live or dead) that are immotile.

The sialic acid residue is generally located on the terminal position of carbohydrate chains (Schauer, 1985), usually in $\alpha(2,3)$ or $\alpha(2,6)$ linkage to galactose (Gal) or *N*-acetyl-D-galactosamine (GalNAc) residues of glycoproteins or glycolipids. The secondary and tertiary structures of carbohydrate chains associated with the particular polysaccharide in the terminal position seem to play a very significant role in lectin binding, as shown by the much stronger interaction between lectins and native oligosaccharides than with the corresponding simple sugar. WGA requires the presence of terminal NeuNAc

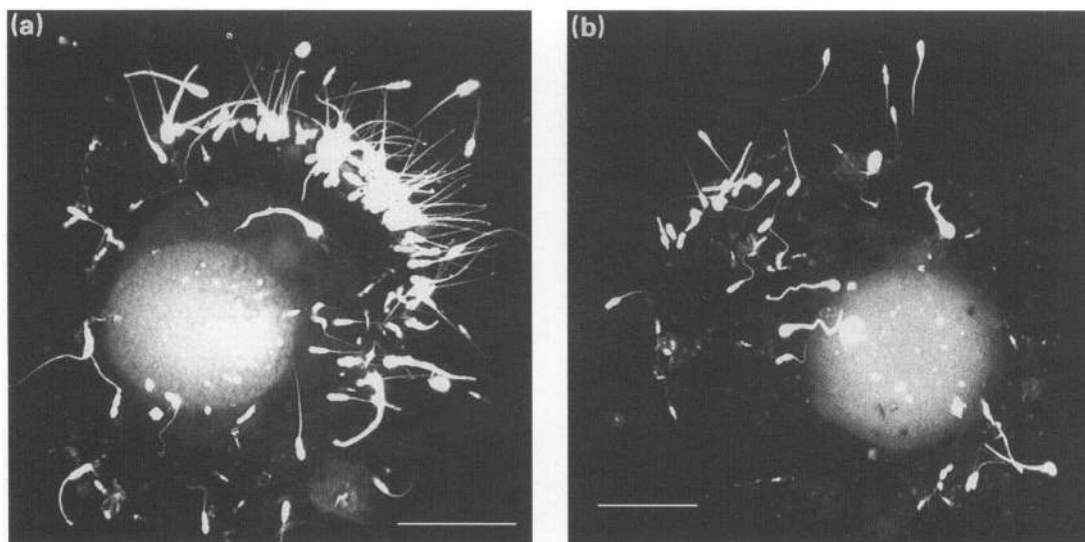


Fig. 7. Effect of pretreatment of spermatozoa with neuraminidase from *Arthrobacter ureafaciens* on human sperm-zona pellucida recognition. The ability of human spermatozoa to recognize human zona pellucida increased after sperm pretreatment with (a) 500 mU *Arthrobacter ureafaciens* neuraminidase mol⁻¹ compared with (b) control. Scale bars represent 50 μm.

in $\alpha(2,6)$ or $\alpha(2,3)$ linkage to the penultimate galactose or *N*-acetylgalactosamine for high-affinity binding (Monsigny *et al.*, 1980; Furukawa *et al.*, 1986) with a preference for $\alpha(2,6)$ -linked NeuNAc (Wright, 1992). WGA was compared with three other lectins (SNA, MAL and LFA) with specificity for different NeuNAc-linkage to Gal/GalNAc to determine what kind of NeuNAc linkage was expressed on the plasma membrane of human spermatozoa. Only SNA binding showed a positive correlation with sperm motility as observed with WGA, but with a lower slope of regression curve indicating differences in specificity between these two lectins. SNA requires the presence of the disaccharide structure NeuNAc $\alpha(2,6)$ Gal/GalNAc, whereas LFA and MAL require NeuNAc $\alpha(2,6)$ or $\alpha(2,3)$ GalNAc (Zeng and Gabius, 1992) or the trisaccharide structure NeuNAc $\alpha(2,3)$ Gal β 1,4 GlcNAc/Glc (Wang and Cummings, 1988; Knibbs *et al.*, 1991), respectively. At comparable NeuNAc concentrations, inhibition of WGA binding on spermatozoa was higher with bovine submaxillary mucin which contained $\alpha(2,6)$ -linked NeuNAc than with fetuin containing a roughly equal proportion of $\alpha(2,3)$ - and $\alpha(2,6)$ -linked NeuNAc (Heerze and Armstrong, 1990), NeuNAc $\alpha(2,3)$ lactose and free NeuNAc. The specificity of WGA for NeuNAc $\alpha(2,6)$ Gal/GalNAc glycoconjugate sequences was confirmed and the presence of such sequences on the plasma membrane of the human spermatozoa was demonstrated.

Capacitation is associated with the decrease in the net surface charge and loss of carbohydrates, including sialic acid, and such a decrease in electronegativity of the sperm surface occurs during treatment of spermatozoa with follicular fluid or neuraminidase (reviewed by Sidhu and Guraya, 1989). In the study reported here, treatment of spermatozoa with neuraminidase led to a dose-dependent decrease in WGA binding on the sperm surface and to the enhancement of the number of spermatozoa that could recognize the surface of the zona pellucida. This result differs from that of Lambert and Le (1984) in mouse (*Mus musculus*) in which treatment of

capacitated spermatozoa with high doses of neuraminidase (25000 mU neuraminidase ml⁻¹ versus 500 mU ml⁻¹ in this study) destroyed their ability to bind and fertilize eggs. However, this treatment had no effect on spermatozoa of *Mus caroli*. These observations are in agreement with the proposal that there are intra- and inter-species differences in molecular mechanisms involved in sperm-zona pellucida recognition.

Our results are consistent with an antigen-masking (anti-recognition) function of sialic acid in several biological events and cells (reviewed in Takahashi, 1992). The release of sialic acid from many blood proteins by treatment with neuraminidase results in rapid clearance of these proteins from the blood circulation as a consequence of uptake by hepatic membrane receptors with specificity for galactose residues (Ashwell and Morell, 1975). Neuraminidase-treated and senescent erythrocytes which have binding sites with high specificity for β -galactose were sequestered by phagocytes (Kiehne and Schauer, 1992). We suggest that sialic acid protects the spermatozoon and sperm surface antigens against the immune system and that its absence from the sperm plasma membrane represents one of the capacitation events necessary for unmasking some sperm surface antigens implicated in zona pellucida recognition.

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