

Sperm Surface Galactosyltransferase Activities during In Vitro Capacitation

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ABSTRACT Studies using genetic and biochemical probes have suggested that mouse sperm surface galactosyltransferases may participate during fertilization by binding *N*-acetylglucosamine (GlcNAc) residues in the egg zona pellucida. In light of these results, we examined sperm surface galactosyltransferase activity during in vitro capacitation to determine whether changes in enzymatic activity correlated with fertilizing ability. Results show that surface galactosyltransferases on uncapacitated sperm are preferentially loaded with poly *N*-acetylglucosamine substrates. As a consequence of capacitation in Ca^{++} -containing medium, these polyglucosaminyl substrates are spontaneously released from the sperm surface, thereby exposing the sperm galactosyltransferase for binding to the zona pellucida. Sperm capacitation can be mimicked, in the absence of Ca^{++} , either by washing sperm in Ca^{++} -free medium, or by pretreating sperm with antiserum that reacts with the galactosyltransferase substrate. In both instances, sperm galactosylation of endogenous polyglucosaminyl substrates is reduced, coincident with increased galactosylation of exogenous GlcNAc, and increased binding to the zona pellucida. Binding of capacitated sperm to the egg can be inhibited by pronase-digested high molecular weight polyglucosaminyl glycosides extracted from epididymal fluids or from undifferentiated F9 embryonal carcinoma cells. Thus, these glycosides function as "decapacitation factors" when added back to in vitro fertilization assays. These glycoside "decapacitation factors" inhibit sperm-egg binding by competing for the sperm surface galactosyltransferase, since (a) they are galactosylated by sperm in the presence of $\text{UDP}[^3\text{H}]\text{galactose}$, and (b) enzymatic removal of terminal GlcNAc residues reduces "decapacitation factor" competition. On the other hand, "conventional" low molecular weight glycosides, isolated from either epididymal fluid or differentiated F9 cells, fail to inhibit capacitated sperm binding to the zona pellucida. These results define a molecular mechanism for one aspect of sperm capacitation, and help explain why removal of "decapacitation factors" is a necessary prerequisite for sperm binding to the zona pellucida.

This paper addresses the molecular basis of one particular aspect of mouse sperm capacitation. Before mammalian sperm are capable of binding to the egg zona pellucida or completing the acrosome reaction, they must be capacitated. According to present knowledge, sperm capacitation is a multifaceted process involving changes in sperm surface glycoconjugates, antigens, lipids, intramembrane particles, fluidity, ion permeability, and sperm intermediary metabolism (see references 4 and 5 for review). One of the first events occurring during capacitation is the release of an epididymal fluid glycoconjugate from the sperm surface, which can inhibit capacitated sperm binding to the zona pellucida when added back to in vitro fertilization assays. These "coating" or "decapacitation factors" can be

released by either elevated ionic strength (6), or by glycosidase digestion, such as β -*N*-acetylhexosaminidase (7). These, and other results, suggest that epididymal glycoconjugates are absorbed to the sperm surface and are released coincident with capacitation. After the release of sperm surface glycosides, capacitation results in an increase in sperm respiration, in sperm forward motility, and enables the sperm to complete the acrosome reaction (4, 5, 8). There is much species variation in the timing of the acrosome reaction, but in mouse, it is thought that only acrosome-intact sperm are capable of binding the zona pellucida (9). After zona binding, the acrosome releases hydrolytic enzymes that allow the sperm to penetrate the egg investments.

Capacitation is thus required for sperm recognition and penetration of the zona pellucida. Much research is directed at identifying the receptor on sperm surfaces that mediates recognition of the zona pellucida. A lectinlike protein has been isolated from sea urchin sperm (10), which may participate in fertilization by binding vitelline layer glycoconjugates (11). Recent work on mouse sperm suggests that sperm surface galactosyltransferases may participate during fertilization by binding GlcNAc residues in the zona pellucida (1-3, 12). This conclusion is supported by several genetic and biochemical observations. For example, mutant *t*-sperm that have a genetic predisposition for increased fertilizing ability have a specific fourfold increase in surface GlcNAc:galactosyltransferase activity, while eight other *t*-sperm enzyme activities are indistinguishable from normal. Sperm bearing recombinant *t*-chromosomes, which do not affect fertilization, have galactosyltransferase activity equal to normal (1, 2). In addition, the presence of either a galactosyltransferase inhibitor, or a specific galactosyltransferase modifier protein, inhibits both sperm surface galactosyltransferase activity and sperm binding to the zona pellucida, but does not inhibit a variety of other sperm enzymes (3).

As part of our analysis of sperm surface galactosyltransferases during mouse fertilization, we examined whether in vitro capacitation had any effect on surface galactosyltransferase activity. We focused our attention on the requisite release of epididymal glycoconjugates that occurs during capacitation, since we thought this could have dramatic consequences on sperm surface galactosyltransferase activity. Specifically, we wanted to know whether mouse sperm capacitation involved the release of galactosyltransferase substrates from the sperm surface, thereby exposing the enzyme for binding to the zona pellucida. Additionally, we examined whether the released galactosyl acceptors could serve as competitive "decapacitation factors" when added back to in vitro fertilization assays. This paper reports experiments aimed at testing these possibilities. The accompanying paper (3) presents our biochemical evidence for sperm surface galactosyltransferase binding to the zona pellucida.

MATERIALS AND METHODS

Gametes

Viable cauda epididymal sperm were collected from either CD1 (Charles River) or inbred normal-tail BTBRTF/Nev males, filtered through 35 μ m mesh nylon cloth, washed by centrifugation and prepared for galactosyltransferase assay as previously described (1). Epididymal fluid contamination of the final sperm pellet was diluted by over 1.25×10^5 -fold (1). Eggs were isolated from superovulated CD1 females in a modified complete medium (CM) (13) minus lactose, plus 5.6 mM fructose. The eggs were freed from the surrounding cumulus cells with 0.1% hyaluronidase (Sigma Chemical Co., St. Louis, MO) (23°C, 10 min), then washed three times in CM and used for in vitro sperm binding assays as described below.

In Vitro Fertilization Assays

Viable sperm were removed from minced cauda epididymides and capacitated in CM for 1 h in a 37°C, 7% CO₂ tissue-culture incubator. The sperm concentration and motility were determined with a hemocytometer. 40- μ l aliquots of capacitated sperm were added to ~30 cumulus-free eggs in 400 μ l of CM, under mineral oil. Within any one set of assays, on a given day, control and experimental incubations were prepared from a common sperm suspension. In this way, sperm concentration and motility were identical in all cultures, thus eliminating inevitable variations between males sacrificed on different days. Sperm binding to the zona pellucida, as assayed below, was proportional to the final sperm concentration from $1-5 \times$ sperm/ml. Assays usually contained 3×10^6 sperm/ml final concentration, of which >50% were motile.

In our initial experiments the cultures were incubated in a reciprocating (30 reciprocations/min) 37°C water bath, while in our more recent assays the cultures were incubated in a stationary 37°C, 7% CO₂ tissue-culture incubator. Results were qualitatively the same under both incubation conditions. After 20 min of incubation, the entire 440- μ l suspension was applied to the top of a discontinuous microgradient composed of 50 μ l of CM, 25 μ l of 1.8% dextran, and 25 μ l of 2.25% dextran containing 2.5% glutaraldehyde (14). The gradient was centrifuged for 90 s at 100 g, sedimenting the eggs with adhering sperm into the glutaraldehyde-containing dextran layer. Unbound sperm partitioned in the CM and adjacent 1.8% dextran layers. The egg pellets were removed and the number of sperm bound/egg was counted using phase-contrast microscopy. The number of eggs examined for each data point is given in the appropriate table.

Galactosyltransferase Assay

Sperm galactosyltransferase activity was assayed as previously described under optimal enzymatic conditions, in which enzyme activity is linear for at least 3 h (1, 2). Incubations contained 0.5×10^6 sperm, 203 μ M UDP[³H]galactose (UDPGal) (197 mCi/mmol) (New England Nuclear, Boston, MA), and 10 mM MnCl₂ in 50 μ l of Medium B (NaCl, 7.5 g/l; KCl, 0.4 g/l; HEPES buffer, 4.76 g/l; pH 7.2). When assaying for exogenous acceptor activity, 30 mM GlcNAc was also added. After the indicated incubation times at 37°C, the assays were terminated with 10 μ l Na EDTA, pH 7.2, of which 50 μ l was subjected to high-voltage borate electrophoresis (3,000 volts, 280 mA, 42 min) to separate the reaction products from unused UDPGal and UDPGal degradation products (1, 2). All incubated assays have had background levels of radioactivity (0°C incubations) subtracted from them.

In some assays, heat-inactivated (60°C, 30 min) aliquots of the epididymal supernatant were added to extensively washed sperm, so that soluble epididymal acceptors could be glycosylated by sperm galactosyltransferases.

Galactosyl Product Characterization

To characterize the galactosyltransferase reaction products, the standard incubation mixture was scaled up sixfold in volume and incubated for 2 h at 37°C. One set of assays was extracted with chloroform:methanol (2:1) (15) to determine the presence of galactosylated glycolipids. Another set of assays was detergent-extracted with 30 mM *n*-octylglucoside (Sigma Chemical Co.) for 1 h on ice. After extensive trituration, the detergent extract was centrifuged to remove the cells (1,200 g, 10 min, 10°C), and the supernatant was extensively dialyzed against Medium B to remove detergent and unused sugar nucleotide. The resulting preparation was heat-inactivated (60°C, 30 min) to destroy any endogenous enzyme activity.

Dialyzed, detergent-extracted, galactosylated products were digested with either 10 mg/ml pronase (Calbiochem-Behring Corp., San Diego, CA) with 10 mM CaCl₂, or 15 mU endo- β -galactosidase (keratanase purified from *Pseudomonas*) (16), (Miles Laboratories Inc., Elkhart, IN) for 48 h at 37°C, after which additional pronase (10 mg/ml) or keratanase (15 mU) was added to the appropriate tubes and incubated for an additional 48 h. The resulting material was chromatographed on Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ) as described below. Endo- β -galactosidase reportedly had no detectable protease, α -fucosidase, α - and β -galactosidase, α - and β -glucosidase, α - and β -*N*-acetylgalactosaminidase, α - and β -*N*-acetylglucosaminidase, α - and β -mannosidase, β -mannosaminidase, β -xylosidase, sulfatase, hyaluronidase, nor chondroitinase contamination. All enzyme units are defined as 1 U liberating 1 μ mol reducing sugar/min.

Column Chromatography

Undigested, pronase-digested, and endo- β -galactosidase-digested labeled glycoconjugates were applied to Sephadex G-50 (fine) columns (1.8 \times 60 cm) and eluted with 0.05 M acetic acid, adjusted to pH 6.0 with NH₄OH. Eighty 2.0-ml fractions were collected, with a flow rate of 30 ml/h, and the radioactivity in each was determined using ACS (Amersham Corp., Arlington Heights, IL) aqueous scintillant. Generally, blue dextran eluted at fractions 23-31, glycopeptides prepared from parietal endoderm (2,000-3,500 mol wt) at 41-50, and UDPGal at 57-63.

Immunoprecipitation

Syngeneic anti-F9 antiserum recognizes a family of complex poly *N*-acetylglucosamine glycoconjugates on embryonal carcinoma (EC) cells, sperm and embryonic cells (17-19). Anti-F9 antiserum and 129/SV normal mouse serum were prepared as previously described (17), such that antiserum reacted specifically with EC cells, and not with lymphocytes nor with yolk sac endoderm. 200 μ l of the dialyzed, detergent-extracted, galactosylated material was incubated with

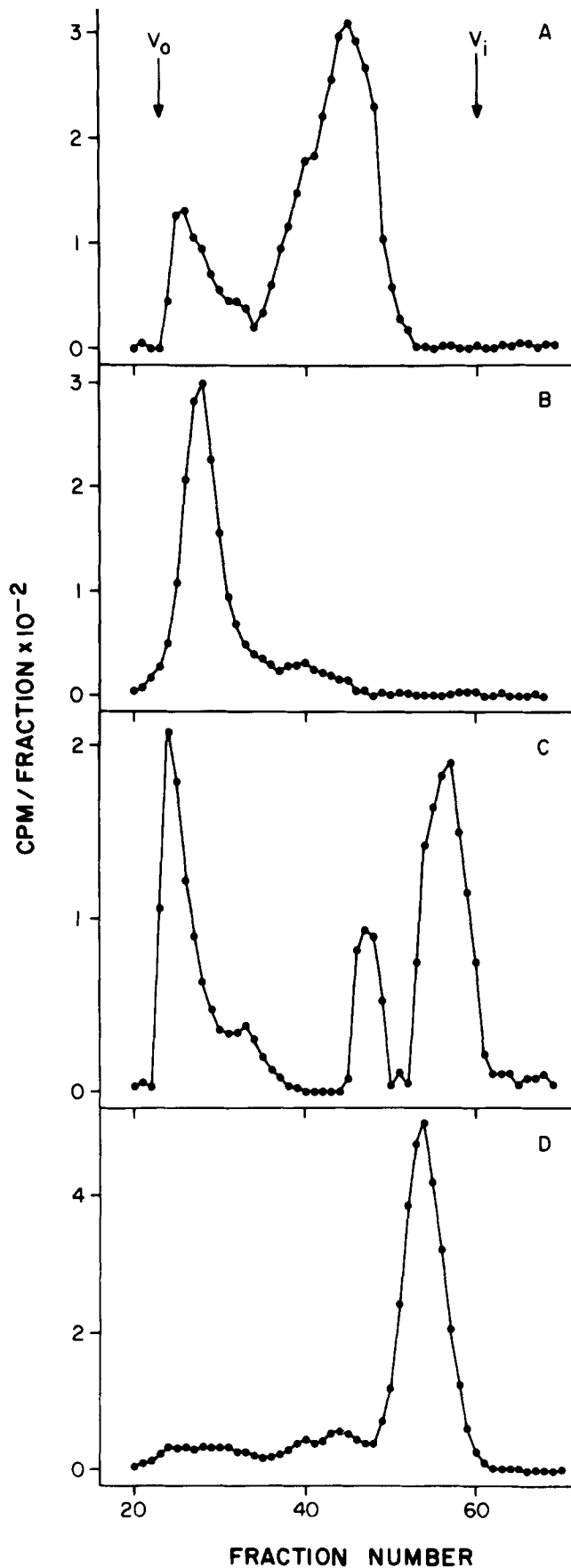


FIGURE 1 Sephadex G-50 elution profiles of detergent-extracted galactosyltransferase reaction products. (A) Pronase-digested glycopeptides from fresh epididymal sperm suspensions. (B) Pronase-

anti-F9 antiserum or normal mouse serum (1/60 final dilution) for 1/2 h at 37°C, followed by 1/2 h at 4°C. 100 μ l of goat IgG antimouse IgG, IgM, and IgA heavy and light chain antiserum (Cappel Laboratories, Cochranville, PA) were then added, and the tubes were incubated for another hour at 37°C, and then overnight at 4°C. The pellets were collected by centrifugation, washed twice with Medium B, and dissolved with 200 μ l 1 N NaOH. This immunoprecipitation was repeated three times for each aliquot of galactosylated glycoconjugate, the dissolved precipitates were pooled, 2 ml H₂O were added, and the precipitates were counted using ACS aqueous scintillant.

Enzymatic Digestion of Intact Sperm

Suspensions of epididymal sperm were centrifuged and resuspended in 0.1 ml of Medium B, to which were added 60 μ l of the protease inhibitor, Aprotinin (Sigma Chemical Co.), and either 15 mU endo- β -galactosidase, 1 U β -N-acetylglucosaminidase, or an equivalent volume (90 μ l) of Medium B. The sperm suspensions were incubated for 90 min with gentle agitation at 37°C, after which they were washed with 10 ml of Medium B and resuspended for galactosyltransferase assay.

Detergent Extraction of F9 and Retinoic Acid-treated F9 Cells

Cell surface glycoconjugates were extracted from F9 EC and retinoic acid-treated EC cells as previously described (19). Pronase-digested F9 cell extracts are highly enriched for poly N-acetylglucosamine glycosides, while retinoic acid-treated cells do not synthesize these glycoconjugates.

RESULTS AND DISCUSSION

Glycoprotein:galactosyltransferases normally transfer galactose from UDPGal to terminal GlcNAc residues, or to free GlcNAc, to produce N-acetylglucosaminyl linkages (i.e., Gal \rightarrow GlcNAc) (20). Results below show that glycoprotein:galactosyltransferases on the surface of uncapacitated sperm are loaded with polyglucosaminyl substrates (i.e., GlcNAc \rightarrow (Gal \rightarrow GlcNAc)_n). By assaying galactosyltransferase activity towards both endogenous and exogenous (i.e., GlcNAc) substrates, we could show that endogenous polyglucosaminyl galactosyltransferase substrates are spontaneously released from the sperm surface during in vitro capacitation. Furthermore, intentional removal of surface galactosyltransferase substrates mimics capacitation. These released substrates serve as decapacitation factors by binding back to the sperm surface, and thus inhibit sperm binding to the zona pellucida.

Characterization of the Sperm Surface Galactosyltransferase Substrates as Polyglucosaminyl Glycosides

In our first experiments, a freshly isolated cauda epididymal sperm suspension, essentially free of somatic cells, was assayed for galactosyltransferase activity towards endogenous acceptors. Previous studies have shown that the galactosyltransferase assay conditions were optimal for UDPGal, GlcNAc, and MnCl₂ concentrations, proportional to sperm concentration, and linear with incubation time (1). The reaction products were isolated and pronase-digested (see Materials and Methods), and the resultant glycopeptides were chromatographed on Sephadex G-50 to determine their relative sizes. Fig. 1A shows

digested glycopeptides from heat-inactivated epididymal fluids galactosylated by washed sperm. (C) 48-h endo- β -galactosidase digestion of galactosylated products used in B. (D) 96-h endo- β -galactosidase digestion of galactosylated products used in B. Galactosyltransferase assays, enzymatic digestion and column chromatography were conducted as described in Materials and Methods. V₀: void volume. V_i: included volume.

that two different size classes of glycopeptides were galactosylated. This elution profile was not affected by continued incubation with additional pronase.

We wanted to determine whether either of the galactosyltransferase substrates in the epididymal fluid was glycosylated by sperm surface galactosyltransferases. To do this, the sperm were removed by centrifugation, and the epididymal supernatant was heat-inactivated to destroy soluble galactosyltransferase. Mild heat-inactivation did not affect the endogenous high and low molecular weight (mol wt) glycoside substrates, since they could be galactosylated by unheated epididymal suspensions. The sperm pellet was extensively washed by centrifugation, resulting in a 1.25×10^6 -fold dilution of epididymal fluid contamination. The final sperm pellet showed negligible levels of endogenous substrates, but retained high levels of activity toward exogenous GlcNAc, in agreement with previous studies (1, 3). The heat-inactivated epididymal supernatant was added back to extensively washed sperm and assayed for sperm galactosyltransferase activity towards epididymal acceptors. The resulting pronase-digested galactosylated products eluted from Sephadex G-50 as seen in Fig. 1*b*. Only the larger glycopeptide, eluting near the void volume, was obtained. Thus, sperm specifically galactosylated the larger molecular weight glycopeptide, while the smaller molecular weight glycopeptide was a substrate specific for soluble, epididymal fluid galactosyltransferases. These results show that the cauda epididymal sperm suspension contained at least two distinct galactosyltransferase activities, only one of which was specifically associated with the sperm surface. Therefore, the following studies used heat-inactivated epididymal fluid supernatants as an acceptor source for sperm surface galactosyltransferases.

The large molecular weight galactosyl acceptors glycosylated by sperm surface galactosyltransferases were partially characterized as follows. First, the reaction products were extracted with chloroform:methanol to determine the presence of glycolipid substrates. Only 8% of the total products were chloroform:methanol soluble (279 cpm out of 3,474 cpm total). Second, sperm surface galactosyltransferase reaction products were digested with purified endo- β -galactosidase (keratanase) to determine whether lactosaminyl residues (i.e., Gal \rightarrow GlcNAc) were present. As shown in Fig. 1*c*, 63% of the galactosyl acceptors were degraded into smaller oligosaccharides after 2 d of digestion, as assessed by Sephadex G-50 chromatography. Endo- β -galactosidase digestion for 4 d degraded over 90% of the galactosylated products (Fig. 1*d*). The intermediate-sized oligosaccharides seen in Fig. 1*c* were chased into low molecular weight di- and trisaccharides (Fig. 1*d*), demonstrating the poly-lactosaminyl nature of the reaction products.

Further insight into the nature of the large mol wt sperm surface galactosyl acceptors was obtained by specific immunoprecipitation with anti-F9 antiserum. Antiserum raised against syngeneic F9 embryonal carcinoma (EC) cells, and absorbed with syngeneic lymphocytes and differentiated EC cells, recognizes a class of poly-*N*-acetyl-lactosamine glycoconjugates on F9 cells and embryonic cells (17-19, 21). Anti-F9 antiserum also reacts with sperm (22), so we determined whether the sperm surface galactosyltransferase acceptor substrates were also recognized by syngeneic anti-F9 antiserum. Anti-F9 antiserum immunoprecipitated 87% (1,043 cpm) of the galactosylated product(s), while under identical conditions (see Materials and Methods), normal mouse serum precipitated only 9.2% (110 cpm) of the product.

Competition between Endogenous Poly-lactosaminyl Substrates and Exogenous GlcNAc for the Sperm Surface Galactosyltransferase

To assess the level of galactosyltransferase activity during capacitation, it was necessary to establish that exogenous (GlcNAc) and endogenous (poly-lactosaminyl) substrates were being galactosylated by the same enzyme. Therefore, the following experiments assayed for competition between endogenous and exogenous substrates for the sperm surface galactosyltransferase. Epididymal sperm suspensions were pretreated with either endo- β -galactosidase, to create terminal GlcNAc residues, or buffer, washed once by centrifugation, and assayed for galactosylation of either endogenous glycosides or exogenous GlcNAc. Fig. 2 shows that endo- β -galactosidase pretreatment elevated activity towards endogenous substrates to the same degree that it inhibited galactosylation of exogenous GlcNAc. On the other hand, β -*N*-acetylglucosaminidase pretreatment, which cleaved terminal GlcNAc residues, produced the reciprocal effect, inhibiting endogenous galactosylation by 50% while simultaneously stimulating galactosylation of exogenous GlcNAc nearly twofold (Fig. 3). Therefore, by selectively exposing or removing endogenous GlcNAc residues on poly-lactosaminyl acceptors, galactosylation of exogenous GlcNAc was either inhibited (Fig. 2) or stimulated (Fig. 3), respectively, showing the competitive nature of the endogenous poly-lactosaminyl acceptors and exogenous GlcNAc.

We examined whether anti-F9 antiserum could also be used to demonstrate competition between endogenous and exogenous substrates, since we knew that anti-F9 antiserum bound the endogenous galactosyltransferase substrate (see above). This was found to be the case, since the presence of anti-F9 antiserum inhibited glycosylation of endogenous acceptors while stimulating galactosylation of exogenous GlcNAc. Normal mouse serum had no significant effect on either endogenous or exogenous acceptor glycosylation (Table I). Galactosyltransferases endogenous to the sera were heat-inactivated before the experiment. For these assays, the sperm suspension was washed once by centrifugation to remove soluble enzyme activity, while maintaining moderate levels of endogenous acceptors.

To assess the specificity of the anti-F9 antiserum, another UDPGal-requiring enzyme, nucleotide pyrophosphatase, was examined. After 90 min of incubation without antiserum, UDPGal accounted for 88.1% of the total soluble radioactivity; Gal-phosphate, 10.2%; and Gal, 1.7%. In the presence of anti-F9 antiserum, UDPGal hydrolysis was indistinguishable from controls; UDPGal 87.4%; Gal-phosphate, 10.8%; and Gal, 1.8%. These results are similar to previous studies, which have also shown that, under identical conditions, anti-F9 antiserum had no effect on sperm surface sialyltransferase, alkaline phosphatase or acid phosphatase activities (1).

Reduced Levels of Endogenous Poly-lactosaminyl Substrates Correlate with Sperm Capacitation In Vitro

Fresh epididymal sperm are uncapacitated in that they are relatively unable to bind or penetrate the zona pellucida. The following experiments examined whether sperm capacitation

in vitro is associated with removal of competing poly lactosaminyl substrates from the sperm surface galactosyltransferase. Sperm capacitation was functionally assayed by determining the number of sperm bound to the zona pellucida as described in Materials and Methods.

Other workers have shown that in vitro mouse sperm capacitation (i.e., increased sperm binding to the zona) is Ca^{++} -

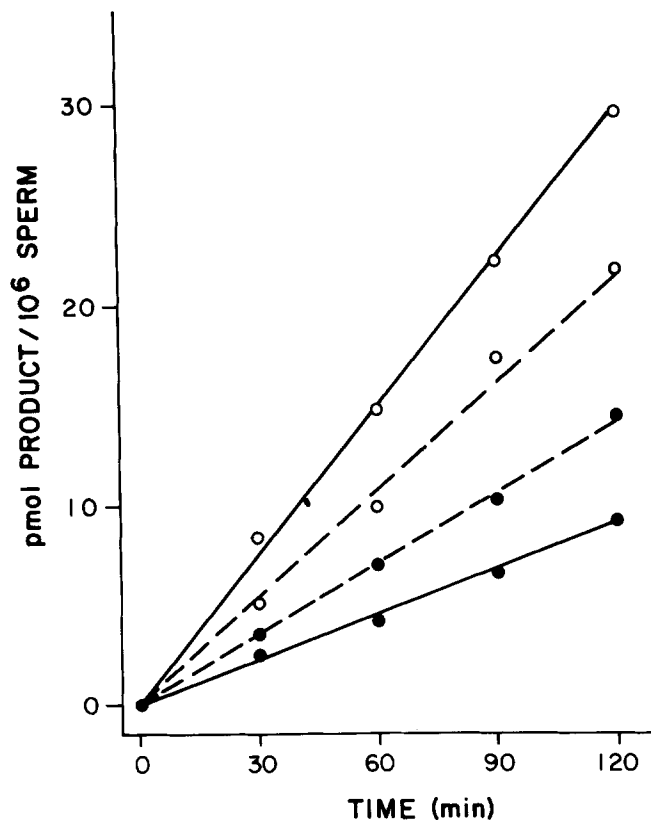


FIGURE 2 Sperm surface galactosyltransferase activity towards endogenous, cell-bound acceptors after either endo- β -galactosidase (●—●) or buffer (○—○) pretreatment. The effect of endo- β -galactosidase (○—○) or buffer (○—○) pretreatment on galactosyltransferase activity towards exogenous GlcNAc is also shown. Sperm preparation, enzymatic digestion, and galactosyltransferase assay were as described in Materials and Methods.

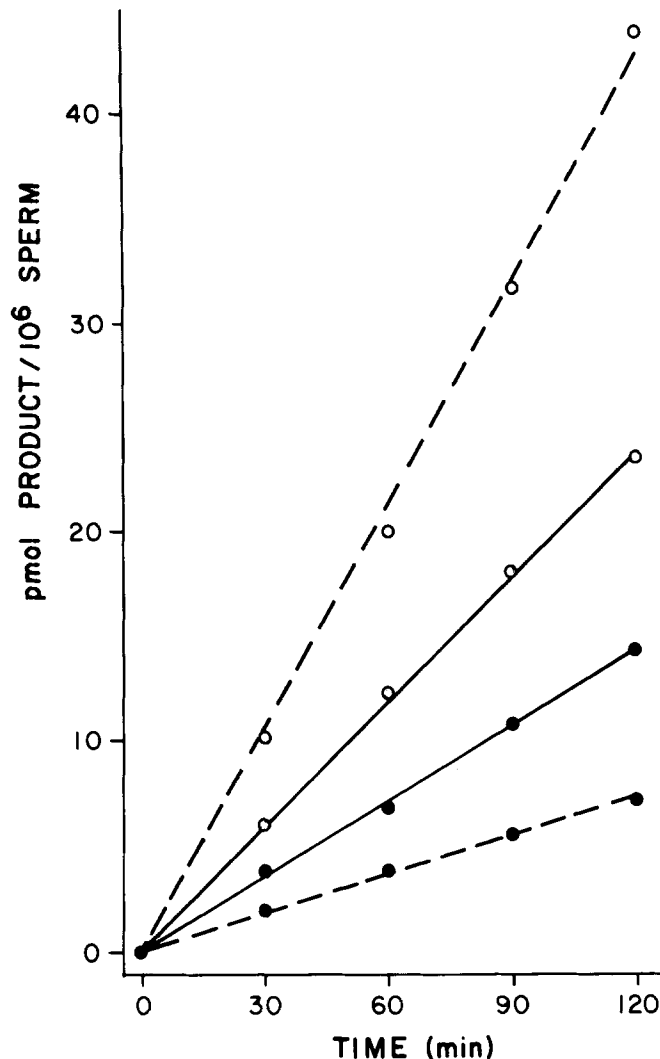


FIGURE 3 Sperm surface galactosyltransferase activity towards endogenous, cell-bound acceptors after either β -N-acetylglucosaminidase (●—●) or buffer (○—○) pretreatment. The effect of β -N-acetylglucosaminidase (○—○) or buffer (○—○) pretreatment on galactosyltransferase activity towards exogenous GlcNAc is also shown. Sperm preparation, enzymatic digestion, and galactosyltransferase assay were as in Materials and Methods.

TABLE I
Removal of Competitive Sperm Surface Polylactosaminyl Glycosides during Capacitation

Experiment	Sperm treatment	Number of eggs	Sperm bound/egg	pmol product/ 10^6 sperm, 90 min	
				Gal \rightarrow endogenous	Gal \rightarrow GlcNAc
1	Control	20	2.4	8.9 ± 0.6	17.2 ± 0.3
	Normal mouse serum (1/60 final dilution)	26	2.2	8.1 ± 0.7	15.5 ± 0.2
	Anti-F9 antiserum (1/60 final dilution)	31	5.0 ($P < 0.01$)	3.0 ± 0.1	32.3 ± 0.3
2	Control (Ca^{++} -free CM)	76	10.1	9.6 ± 0.3	10.3 ± 0.5
	2.0 mM Ca^{++}	59	29.2 ($P < 0.005$)	4.9 ± 0.5	17.2 ± 0.5
3	Control (unwashed)	25	9.1	46.1 ± 1.4	n.d.
	Washed, resuspended in original supernatant	22	8.6	n.d.	n.d.
	Washed, resuspended in fresh Ca^{++} -free CM	27	22.3 ($P < 0.005$)	6.6 ± 0.6	n.d.

Aliquots of sperm were assayed for capacitation and surface galactosyltransferase activities towards endogenous and exogenous (i.e., GlcNAc) substrates. Within any one experiment, sperm were taken from a common suspension to insure equal motility and concentration, thus avoiding inevitable variations between different sperm suspensions. Sperm capacitation was assessed by measuring the number of sperm bound/egg in the absence of Ca^{++} , except in Ex. 2. 90-min galactosyltransferase activities represent the average of four, 120-min time courses, \pm SEM. Galactosyltransferase assays contained 10 mM $MnCl_2$, except those in which the effect of Ca^{++} was examined (Ex. 2) thus producing lower endogenous activity compared to Ex. 3. Endogenous activity is low in Ex. 1, compared to Ex. 3, due to mild centrifugal washing (as in Figs. 2 and 3). Activity towards endogenous substrates has been subtracted from activity towards GlcNAc. n.d., not determined.

dependent (14). Thus, sperm binding to the egg zona pellucida was examined *in vitro* after preincubating epididymal sperm in Ca^{++} -containing or Ca^{++} -free medium for 1 h. As seen in Table I, sperm capacitation was accentuated by preincubation in 2.0 mM Ca^{++} , since binding to the zona increased by 2.9-fold. Simultaneously, 2.0 mM Ca^{++} pretreatment stimulated sperm glycosylation of exogenous GlcNAc by 1.7-fold and concomitantly inhibited glycosylation of endogenous substrates by 0.5, all relative to sperm incubated in the absence of Ca^{++} . The addition of 4 mM EDTA eliminated virtually all sperm binding to the zona pellucida (<1.0 sperm bound/egg).

We examined whether sperm capacitation could be mimicked, in the absence of Ca^{++} , by simply centrifugally washing away the sperm surface endogenous galactosyl acceptors. Results show (Table I) that washing uncapacitated sperm and resuspending them in fresh Ca^{++} -free CM mimicked capacitation, since there was a 2.5-fold increase in binding to the zona pellucida. Sperm motility was identical in both washed and unwashed preparations so long as CM was present. Parallel galactosyltransferase assays showed a concomitant 86% decrease in endogenous acceptor galactosylation.

In the absence of Ca^{++} , anti-F9 antiserum pretreatment also mimicked sperm capacitation since sperm-zona binding increased 2.1 times as a result of anti-F9 antiserum pretreatment (Table I). Under identical conditions, normal mouse serum had no effect. Similarly, the results described above show that anti-F9 antiserum, but not normal mouse serum, inhibited sperm galactosylation of endogenous acceptors while stimulat-

ing activity towards exogenous acceptors. Again, no motility differences could be detected, and no antiserum-mediated sperm agglutination could be found.

Released Polylactosaminyl Glycosides Serve as Competitive Decapacitation Factors

To determine whether either size class of epididymal fluid glycosides (see Fig. 1A) could decapacitate sperm (i.e., inhibit capacitated sperm binding to the zona pellucida), we added both size classes of epididymal glycosides back to *in vitro* fertilization assays. Epididymal glycosides were prepared from CD1 males as follows. Fresh epididymal sperm was pelleted by centrifugation and the resulting supernatant was exhaustively pronase-digested, boiled to denature the pronase, and chromatographed on Sephadex G-50 with 0.05 M ammonium bicarbonate, pH 7.7. The void volume containing the polylactosaminyl glycosides (Fig. 1A) was lyophilized and resuspended in BSA-free CM. Similarly, the included volume containing the low mol wt epididymal fluid galactosyltransferase substrates was lyophilized and resuspended. Results show that epididymal polylactosaminyl glycosides, which serve as the preferential substrate for uncapacitated sperm surface galactosyltransferases, inhibited binding by 63% relative to controls (Table II). In three separate experiments, polylactosaminyl glycoside inhibition of sperm binding was proportional to the amount of glycoside added, i.e., glycoside extracted from 1.2×10^7 sperm produced 39% inhibition, extract from 2.2×10^7

TABLE II
Effect of Polylactosaminyl Glycoconjugates on Sperm Binding to the Zona Pellucida

Glycoside source	Assay additions	Eggs	Sperm bound/egg
Epididymal fluids	Medium (control)	29	16.8
	Polylectosaminyl glycosides	35	6.2 ($P < 0.005$)
	Low mol wt "conventional" glycosides	29	15.3
F9 Cells (embryonal carcinoma)	Medium (control)	52	26.3
	Polylectosaminyl glycosides	54	2.5 ($P < 0.005$)
Retinoic acid-treated F9 cells (endo-derm)	Medium (control)	20	41.2
	"Conventional" glycosides	23	43.6

Epididymal fluids were pronase-digested, boiled, and chromatographed on Sephadex G-50. The void volume (polylactosaminyl glycosides) and included volume (low mol wt glycosides) were lyophilized and added to sperm zona binding assays. F9 cell polylectosaminyl glycoconjugates and retinoic acid-treated cell surface glycoconjugates were added to sperm-zona binding assays at equal concentrations, when normalized to the number of cells extracted (1.2×10^6 cells equivalents/440 μl assay). See reference 19 for details of F9 cell glycoconjugate extraction and characterization.

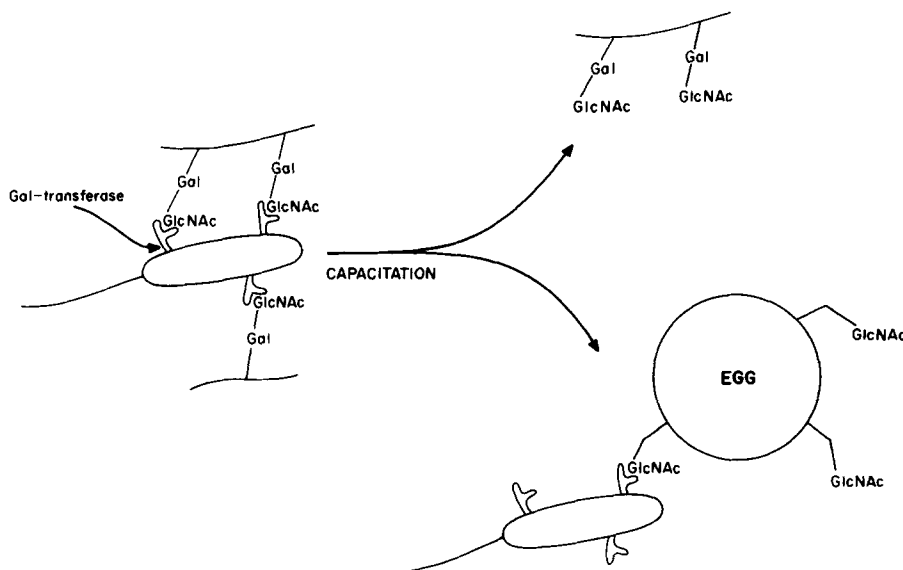


FIGURE 4 Diagram illustrating the release of poly-N-acetyllactosamine glycosides from the sperm surface during capacitation. Glycoconjugate release could be facilitated by either dilution in the oviduct, increased ionic strength, glycosidase digestion or UDPGal-mediated catalysis of the galactosyltransferase reaction. In this figure, only the terminal disaccharides of the "decapacitation factors" are illustrated. (Figure adapted from reference 12).

sperm gave 63% inhibition, extract from 2.9×10^7 sperm gave 94% inhibition. On the other hand, the low mol wt glycosides, which are substrates for soluble galactosyltransferases, did not inhibit binding significantly in any experiment (Table II).

To assess further the specificity of poly-lactosaminyl decapacitation activity, we examined the decapacitation activity of poly-lactosaminyl glycosides extracted from F9 embryonal carcinoma (EC) cells relative to "conventional" low mol wt glycosides extracted from differentiated F9 cells (19). F9 poly-lactosaminyl glycosides are similar, if not identical, to sperm poly-lactosaminyl glycosides, since both glycoconjugates show similar precipitation with anti-F9 antiserum, similar susceptibility to endo- β -galactosidase digestion, and similar elution from Sephadex G-50 after pronase-digestion. In addition, the use of F9 glycosides allowed us to examine whether decapacitating poly-lactosamines are substrates for sperm surface galactosyltransferases.

When F9 cell poly-*N*-acetyl-lactosamine glycoconjugates were added back to in vitro fertilization assays containing capacitated sperm, binding to the zona was inhibited by up to 91% (Table II), similar to that seen with epididymal poly-*N*-acetyl-lactosamines. Exhaustive pronase-digestion and boiling of the poly-lactosaminyl glycoconjugate did not affect its inhibitory activity. Equivalent concentrations of heated extracts from retinoic-acid-treated F9 cells, which do not synthesize these poly-lactosaminyl glycosides (19), did not inhibit sperm-zona binding, thus demonstrating the specific inhibitory nature of the poly-lactosaminyl glycoconjugate. The solubilized, decapacitating glycoconjugates competed for the sperm surface galactosyltransferase, since 6.2 pmol were galactosylated by 10^6 sperm in the presence of UDP[3 H]Gal, and they simultaneously inhibited sperm galactosylation of exogenous GlcNAc by 5.1 pmol (Table III). In addition, enzymatic removal of terminal GlcNAc residues by β -*N*-acetylglucosaminidase reduced the decapacitation factor activity of the competitive poly-lactosaminyl glycoside (no additions: 31.8 ± 3.1 sperm/egg, undigested glycoside: 3.2 ± 0.6 sperm/egg, enzyme digested glycoside: 15.4 ± 3.1 sperm/egg, all differences $P < 0.01$). These results suggest that solubilized poly-lactosamines inhibited sperm binding to the zona pellucida by competing for the surface galactosyltransferase. However, we do not know what effects, if any, poly-lactosamines may have on other events during capacitation.

In summary, the results presented in this paper show that surface galactosyltransferases on uncapacitated sperm are

TABLE III
Glycosylation of Poly-lactosaminyl Glycoconjugates by Sperm Surface Galactosyltransferases

Acceptor	Additions	pmol product/ 10^6 sperm, 90 min	
		min	Control
Endogenous	Buffer	3.1 ± 0.2	100
	Poly-lactosaminyl glycosides	9.3 ± 0.4	302
GlcNAc	Buffer	12.4 ± 0.2	100
	Poly-lactosaminyl glycosides	7.3 ± 0.2	59

Sperm were prepared and assayed for surface galactosyltransferase activity as described in Materials and Methods. Activity towards endogenous acceptors has been subtracted from activity towards exogenous GlcNAc. F9 cell poly-lactosaminyl glycoconjugates were extracted as described (19), and 3.0×10^5 F9 cell equivalents were added to each 50 μ l assay. Data are the average of quadruplicate determinations, \pm SEM.

loaded with high mol wt poly-lactosaminyl substrates (Fig. 4). Sperm capacitation in vitro involves the requisite release of these poly-lactosaminyl substrates from the surface, thereby exposing the galactosyltransferase. Furthermore, these substrates serve as "decapacitation factors" when added back to in vitro fertilization assays, and do so by binding back to the sperm surface galactosyltransferase. These results are interesting in light of evidence implicating the sperm surface galactosyltransferase as at least one of the sperm receptors for binding to the zona pellucida (see accompanying paper, and 12, 23, 24). Therefore, one result of capacitation may be the exposure of previously cryptic sperm receptors due to the spontaneous release of competitive substrates.

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