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Binding of Mouse Sperm to β-Galactose Residues on Egg Zona Pellucida and Asialofetuin-Coupled Beads

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Mouse eggs fixed with paraformaldehyde were incubated with various exoglycosidases and their sperm-binding activities were examined. The number of sperm bound per egg was increased by sialidase treatment and decreased by β-galactosidase treatment. No prominent reduction of sperm-binding was observed after α-galactosidase treatment. Mouse sperm also bound to asialofetuin-coupled agarose beads but not to fetuin-coupled beads. The sperm-binding was abolished when asialofetuin-gel was treated with β-galactosidase specific to the β1→4 linkage or *N*-Glycanase. Furthermore asialofetuin, but not β-galactosidase-treated asialofetuin, competitively inhibited the binding of sperm to the zona pellucida of live eggs. These results suggest that mouse sperm recognize β-galactose residues of the zona pellucida at the initial stage of the binding.

Mammalian eggs are coated with an extracellular matrix called the zona pellucida (ZP). In the course of fertilization, sperm bind to and penetrate through the ZP before fusing with the egg plasma membrane. It has been suggested in many mammalian species that sugar moieties of the ZP are involved in sperm-binding. In mice, sperm with the intact acrosomal membranes bind to the ZP and undergo the acrosome reaction, which is an exocytotic release of the components in the acrosomal vesicle. The ligand for acrosome-intact sperm has been suggested to be an *O*-glycan of ZP3, the smallest component of ZP glycoproteins (1). However, the structures of the functional sugar chains still remain controversial. One candidate serving as the ligand is a sugar chain bearing α-galactose (2) and the other is that bearing *N*-acetylglucosamine at the nonreducing end (3). A peripheral membrane protein, sp56, has been proposed as the receptor molecule on sperm for α-galactose-bearing sugar chain (4) and the possible counterpart for the *N*-acetylglucosamine-bearing sugar chain is a β1, 4-galactosyltransferase which binds to a substrate sugar chain on the ZP in a lectin-like manner (5). Importance of these sugar chains as the ligands of sperm has been suggested on the basis of the results of the inhibition assay of sperm-egg binding using chemically and enzymatically modified soluble ZP glycoproteins or oligosaccharides, and inhibitors for the possible sugar recognition molecules on sperm. But the experimental data were incompatible each other (2,3,6,7). Considering that pronase digest of ZP3 showed inhibition of sperm-egg binding at much higher concentrations than used with intact ZP3 (8), and that ZP glycoproteins are arranged into interconnected filaments to form the three-dimensional ZP matrix (9), the density and tertiary arrangement of sugar chains on the ZP matrix seem to be important factors in sperm-binding. In this study, we have applied glycosidase treatment to the ZP matrix without solubilization and examined the sperm-binding activity by means of the direct sperm-egg binding assay.We have also examined sperm-binding activities to exogeneous glycoproteins coupled to agarose beads.

MATERIALS AND METHODS

Collection of mouse gametes. Ovulated eggs were collected from oviducts of super ovulated B6C3F1 female mice and washed with m-KRB buffer (10) containing 25 mM Hepes, pH 7.3 and 0.4% BSA (m-KRBH) following hyaluronidase treatment. Sperm were collected from caudae epididymae of sexually matured B6C3F1 male mice and incubated in m-KRBH for 60 min in a CO_2 incubator at 37°C for capacitation.

Glycosidase treatment of eggs. Eggs were fixed in PBS containing 4% paraformaldehyde and PVP-360 (Sigma, 4 mg/ml) at room temperature for 30 min followed by incubation with 0.5 M Tris/HCl, pH 7.0 containing PVP-360 (4 mg/ml) for 30 min. Then eggs were washed three times with respective buffers for various glycosidase treatments; 200 mM sodium acetate, pH 5, for neuraminidase, 50 mM sodium acetate, pH 5.5, for β-galactosidase and 50 mM sodium ace-

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tate, pH 6.5, for α-galactosidase. All of these buffers contained 0.15 M NaCl, 10 mM $MgSO_4$ and 4 mg/ml PVP-360. Glycosidase treatment was performed using 0.1 μ/20 ul of sialidase from *Arthrobacter urefaciens* (Nacalai Tesque Inc., Kyoto) or 20 mU/20 μl of β-galactosidase from *Streptococcus 6646K* (Seikagaku Kogyo Co., Tokyo) at 37°C, or 0.7 U/20 μl of α-galactosidase from coffee bean (Boehringer Mannheim Yamanouchi, Tokyo) at 25°C. For α-galactosidase treatment 1 mg/ml D(−)-galactono-1,4-lactone (Nacalai Tesque Inc., Kyoto) was added. Each glycosidase treatment was continued for 24 h.

Sperm-binding assay. For the direct sperm-binding assay to eggs, fixed eggs with or without glycosidase treatment were washed with m-KRBH medium. One hundred μl drops of capacitated sperm suspension were added to 100 μl drops of the medium containing 10-15 eggs to make a sperm concentration of 1×10^6/ml and incubated under paraffin oil in a CO_2 incubator at 37°C for 15 min. Then unbound sperm suspension was removed with a pipette leaving eggs in the droplets. Then eggs were gently washed twice by adding and removing 200 μl PBS. Finally, eggs were fixed with PBS containing 1% glutaraldehyde and 4 mg/ml PVP-360 and the numbers of sperm bound per egg were determined by counting sperm tails in one plane of focus under a phase contrast microscope. For the sperm-binding assay to glycoprotein-coupled Sepharose beads, 50 μl drops of capacitated sperm suspension were added to 50 μl drops of 2% gel suspension to make a sperm concentration of 5×10^5/ml and incubated for 15 min at the room temperature followed by gentle washing and fixation. The numbers of sperm bound to beads were counted in the same manner as described above. For the sperm-binding inhibition assay to live eggs, 50 μl drops of the medium containing various amounts of glycoproteins were incubated for 30 min under paraffin oil in a CO_2 incubator and 50 μl drops of capacitated sperm suspension were added to make a sperm concentration of 1×10^6/ml and further incubated for 15 min. Then 10-15 ovulated eggs were added and incubated for 15 min. After removal of unbound sperm, eggs were washed, fixed and the numbers of sperm bound to eggs were counted in the same manner as described above.

Lectin staining of eggs. Fixed eggs with or without glycosidase treatment were washed with PBS and pre incubated in PBS containing 5% BSA at 37°C for 60 min. Then eggs were incubated with 5 μg/ml each of biotinylated *Maackia amurensis* lectin (MAM), *Sambucus sieboldiana* agglutinin (SSA), *Ricinus communis* agglutinin (RCA 120) (all from Seikagaku Kogyo Co., Tokyo) for 60 min at 4°C, or 20 μg/ml of biotinylated *Griffonia simplicifolia* agglutinin (GS-IB4, E. Y Labs Inc. CA) at 4°C for 16 h. Staining with 1 μg/ml of biotinylated *Psathyrella velutina* lectin (PVL, a kind gift from Dr. N. Kochibe, Gunma University) was performed at 4°C for 60 min after pre incubation of eggs in PBS containing 5% fetal calf serum previously dialyzed against PBS instead of BSA. Then eggs were washed, incubated with Vecstatin ABC reagents (Vector Laboratories Inc., CA) according to the manufacturer's protocol and visualized with diaminobenzidine. For dilution of reagents and washing, PBS containing 0.1% Tween-20 was used. In some experiments, the following inhibitors were added to lectin solutions; 0.1 M lactose in the solutions of MAM, SSA and RCA120, 0.1 M *N*-acetylglucosamine in the PVL solution and 0.1 M methyl α-galactoside in the GS-IB4 solution.

Preparation of glycoprotein-coupled Sepharose beads. Fetuin and asialofetuin from fetal calf serum or BSA, fraction V (all from Sigma, St. Louis) was conjugated to CNBr-activated Sepharose 4B (Pharmacia LKB, Uppsala) according to the manufacturer's protocol at a protein concentration of 5 mg/ml of packed gel. An aliquot of asialofetuin-Sepharose was treated with β-galactosidase from *Diplococcus pneumoniae* (Boehringer Mannheim Yamanouchi, Tokyo) at 25 mU/75 μl in 50 mM sodium acetate buffer, pH 6. Another aliquot was treated with *N*-Glycanase (Seikagaku Kogyo, Co. Tokyo) at 1.5 U/75 μl in 0.2 M sodium phosphate, pH 8.6. Glycosidase treatments were continued at 37°C for 48 h.

Determination of sugar components of asialofetuin-Sepharose beads. Asialofetuin-Sepharose beads before and after glycosidase treatments were extensively washed with 0.1 M ammonium bicarbonate and incubated with trypsin from bovine pancreas (10 mg/ml). Glycopeptides released from beads were subjected to acid hydrolysis with 4 N trifluoroacetic acid at 100°C for 3 h followed by evaporation. Sugar components were analyzed by high-pH anion-exchange chromatography (HPAEC) using a CarboPac PA-1 column and pulse amperometric detection as described (11).

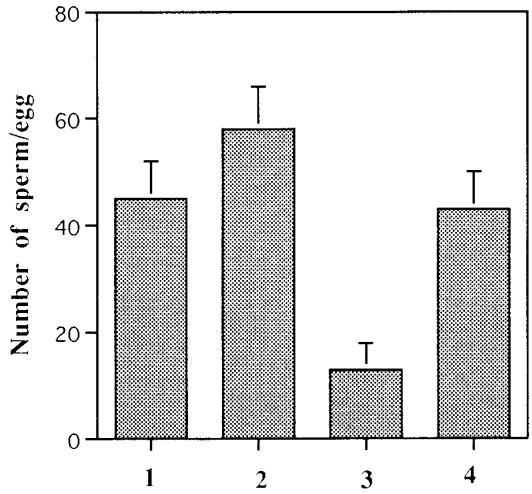

FIG. 1. Effect of glycosidase-treatment of fixed egg on sperm-binding. The average numbers of sperm bound per egg (± s.d.) were determined by counting 10 eggs. Lane 1, control without glycosidase treatment; lane 2, digested with sialidase; lane 3, digested with β-galactosidase from *Streptococcus 6646K;* lane 4, digested with α-galactosidase.

Preparation of inhibitors for sperm-binding assay. β-Galactosidase-treated asialofetuin was prepared by incubating with β-galactosidase from *Streptococcus 6646K* at 75 mU/75 μl in 50 mM sodium acetate, pH 5.5 at 37°C for 48 h, followed by boiling for 3 min at pH 2 with addition of HCl. Periodate-oxidized asialofetuin was prepared as follows; 30 mg asialofetuin was incubated with 80 mM $NaIO_4$ in 2 ml PBS at 4°C for 1 h in the dark followed by addition of 50 μl of ethylene glycol and reduced with $NaBH_4$. The removal or destruction of galactose residues in asialofetuin was ascertained by EIA using biotinylated RCA120 and PVL as probes. All inhibitors were buffer-exchanged into m-KRBH before use.

RESULTS AND DISCUSSION

Sperm-binding to glycosidase-treated eggs. When mouse intact eggs were incubated with various glycosidases, matrix structure of the ZP was easily destroyed by prolonged incubation. Therefore, eggs were first fixed with paraformaldehyde and then digested with glycosidases. This fixation kept the matrix structure unchanged under the microscope and the fixed control eggs without glycosidase digestion retained the sperm-binding ability. Then eggs were incubated with capacitated murine sperm for 15 min and fixed with glutaraldehyde. Fig. 1 shows the means of the sperm numbers bound per egg. The mean of the sperm numbers bound per egg without glycosidase treatment was shown as control (Fig. 1, lane 1). By sialidase treatment, binding

of sperm was increased up to 130% of the control (Fig. 1, lane 2), while decreased to 30% of the control by β-galactosidase treatment (Fig. 1, lane 3). The binding activity of the α-galactosidase-treated eggs remained almost unchanged (Fig. 1, lane 4). Removal of sialic acid residues should expose more galactose residues on the ZP and resulted in the increased sperm-binding, suggesting that sperm recognize β-galactose residue. On the other hand, removal of β-galactose residues should expose the penultimate GlcNAc residues leaving α-galactose residues intact, and resulted in the prominent reduction of sperm-binding, again suggesting that β-galactose residues are required for sperm-binding. A possible explanation for the α-galactosidase-insensitive binding is that even if sperm can recognize α-galactose residues, removal of α-galactose residues from the Galα1→3Galβ1→4GlcNAc group in the sugar chains newly exposes β-galactose residues which are recognized by the sperm, and therefore the sperm-binding remained unchanged. It is also possible that because of much higher amount of β-galactose than α-galactose residues as indicated by the previous structural analysis (12), the presence or the absence of small amounts of α-galactose residues does not make much difference in sperm-binding. To estimate the effectiveness of each glycosidase treatment, paraformaldehyde-fixed eggs were stained with RCA120, GS-IB4, PVL, MAM or SSA before and after each glycosidase digestion. It is known that RCA120 and GS-IB4 recognize β-galactose and α-galactose residues at the non reducing termini, respectively (13, 14). PVL recognizes *N*-acetylglucosamine residue with or without substitution at C-6 position (15). MAM and SSA recognize α2→3 and α2→6 linked sialic acids, respectively (16, 17). As shown in Fig. 2, A, D, G and J, the fixed eggs were stained positively by all of these lectins, suggesting that β-galactose, α-galactose, *N*-acetylglucosamine and α2→3 and α2→6 linked sialic acid residues are all expressed at non reducing termini of sugar chains of mouse ZP, although the quantitative ratio of them was not clear. These stainings were specific because they were completely inhibited by the addition of the inhibitory saccharides specific for each lectin (Fig. 2, C, F, I and L). By β-galactosidase treatment the positive staining with RCA120 became faint (Fig. 2B), while that with PVL became more dense (Fig. 2E). The staining with GS-IB4 turned to be negative after α-galactosidase treatment (Fig. 2H) and those with MAM (Fig. 2K) and SSA (data not shown) became faint by sialidase treatment. These results indicate that each glycosidase treatment was performed effectively.

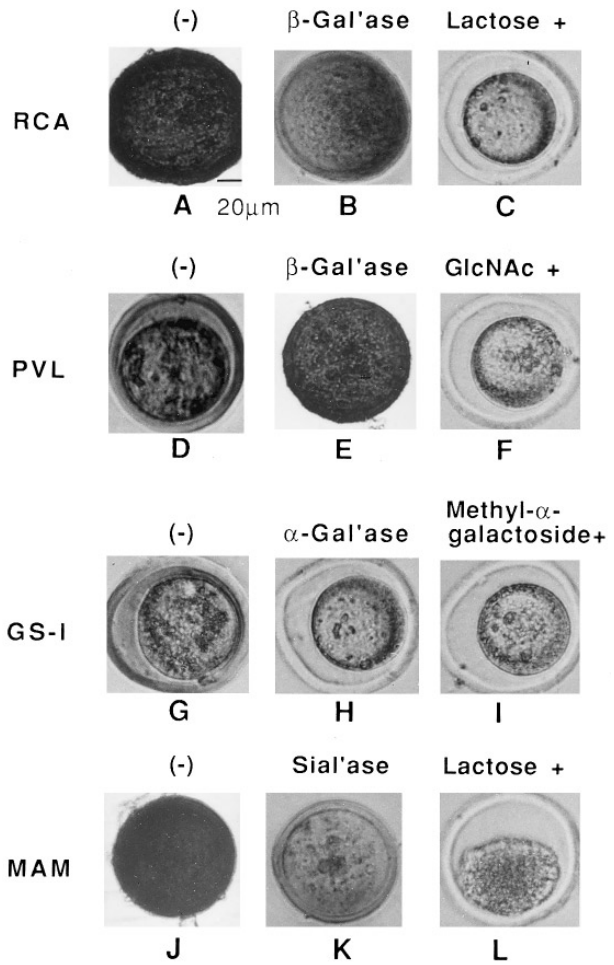

FIG. 2. Lectin-staining of eggs. Paraformaldehyde-fixed eggs were incubated with or without glycosidases and stained with biotinylated lectins followed by visualization with the ABC reagents and diaminobenzidine. A∼C, RCA120-staining in the absence (A, B) or presence (C) of 0.1 M lactose; D∼F, PVL-staining in the absence (D, E) or presence (F) of 0.1 M *N*-acetylglucosamine; G∼I, GS-1B4-staining in the absence (G, H) or presence (I) of 0.1 M methyl α-galactoside; J∼L, MAM-staining in the absence (J, K) or presence (L) of 0.1 M lactose. Eggs were pre incubated in buffers without glycosidase (A, C, D, F, G, I, J and L), with β-galactosidase (β-Gal'ase, B and E), α-galactosidase (α-Gal'ase, H) or sialidase (Sial'ase, K).

Sperm-binding to asialofetuin-coupled Sepharose beads. To know whether mouse sperm can recognize β-galactose-exposed glycoproteins, their binding to various glycosidase-treated fetuin-coupled Sepharose beads were examined under the microscope. Fetuin is known to contain *N*-linked triantennary oligosaccharides and *O*-linked oligosaccharides with the core 1 as main components (18, 19). The capacitated sperm were incubated with the beads, fixed with glutaraldehyde and the sperm numbers bound per bead were scored. As shown in Fig. 3A, the sperm binding to asialofetuin-coupled beads (lane 2) was remarkably higher than that to fetuin-coupled beads (lane 1). The binding to asialofetuin-beads was completely abolished after treatment of the beads with β1→4 linkage-specific galactosidase from *Diplococcus pneumoniae* (lane 4) (20), or with *N*-Glycanase (lane 3) (21). A typical figure of sperm-binding to asialofetuin-Sepharose beads was

shown in Fig. 3B. These results indicate that sperm can recognize the Galβ1→4GlcNAc group of *N*-linked sugar chains of asialofetuin. To confirm the observation, the validity of each glycosidase treatment was also examined. The results shown by HPAEC indicated that digestion with β-galactosidase from *Diplococcus pneumoniae* removed 65% of the total galactose residues in asialofetuin (data not shown), which correspond to 93% of β1→4 linked galactose residues. It was also calculated from the reduction of mannose residues that 80% of *N*-linked sugar chains of asialofetuin were released by *N*-Glycanase digestion (data not shown).

Inhibition of sperm-egg binding by glycoproteins. Finally, inhibition of sperm-binding to live eggs by various fetuin derivatives was examined. As shown in Fig. 4, addition of asialofetuin inhibited the sperm-binding in a dose-dependent manner and the binding was reduced to 30% of the control at the dose of 100 μg/ml. When β-galactosyl residues were removed from asialofetuin by β-galactosidase from *Streptococcus 6646K*, or destroyed by periodate oxidization, its inhibitory effect in sperm-binding was abolished. The enzymatic release or chemical destruction of β-galactose residues of asialofetuin was almost complete when examined by EIA using RCA-120 as a probe. Thus, the presence of terminal galactose residues is required for asialofetuin to inhibit the binding of sperm to live eggs, suggesting that sperm can recognize sugar chains with the Galβ1→4GlcNAc group included in the ZP as well as those in asialofetuin.

Requirement of relatively higher amounts of asialofetuin for inhibition of the sperm-egg binding might be due to the increased affinity by clustering of functional sugar chains on the egg ZP and/or the presence of additional binding mechanisms between sperm and the egg ZP. Recently, it has been shown that the polypeptide region encoded by *mZP3* gene exon 7 containing the amino acid sequence -NCSNSSSS-, which is expected to be highly glycosylated with *O*-linked as well as *N*-linked oligosaccharides, is required for both sperm-binding and acrosome reaction-inducing activities (22). It will be interesting to know whether the sugar chains having Galβ1→4GlcNAc group are actually included in this region.

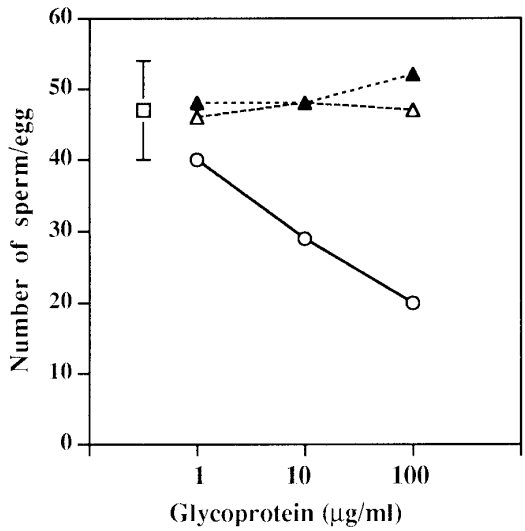

FIG. 4. Inhibitory effect of various fetuin preparations on sperm-binding to live eggs. The s.d. value was shown only for the mean of sperm-binding in the absence of inhibitors to avoid complication of figure. All other s.d. values were nearly the same or less than the value shown in the figure. The mean of sperm-binding in the absence (square) or presence of 1 to 100 μg/ml each of asialofetuin (open circle), β-galactosidase-treated asialofetuin (closed triangle) or periodate-oxidized asialofetuin (open triangle) was determined by counting 10 eggs.

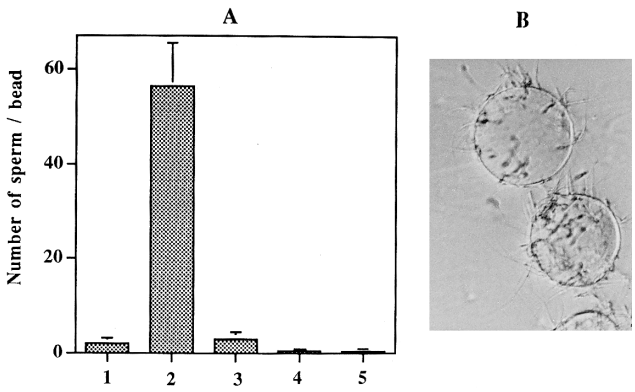

FIG. 3. (A) Sperm-binding to various fetuin-coupled Sepharose beads. The average numbers of sperm bound to a bead were determined by counting 20 beads. Lane 1, fetuin-Sepharose beads; lane 2, asialofetuin-Sepharose beads; lane 3, *N*-Glycanase-treated asialofetuin-Sepharose beads; lane 4, β1→4 galactosidase-treated asialofetuin-Sepharose beads; lane 5, BSA-Sepharose beads (negative control). (B), Binding of sperm to asialofetuin-beads.

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