

Lewis X-Containing Glycans are Specific and Potent Competitive Inhibitors of the Binding of ZP3 to Complementary Sites on Capacitated, Acrosome-Intact Mouse Sperm¹

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ABSTRACT

Mammalian fertilization requires a cascade of interactions between sperm and the egg's zona pellucida (ZP). *O*-linked glycans on mouse glycoprotein ZP3 have been implicated in mediating one step of the fertilization process, the firm adhesion of acrosome-intact sperm to the ZP. Experiments to identify structural requirements of a sperm-binding glycan have demonstrated that a Lewis X (Le^x)-containing glycan (Galβ4[Fucα3]GlcNAc-R) was a potent, competitive inhibitor of *in vitro* sperm-ZP binding (Johnston et al. *J Biol Chem* 1998; 273: 1888–1895). However, those experiments did not define the particular step in the fertilization pathway that was blocked. The experiments described herein test the hypothesis that Le^x-containing glycans are specific, competitive inhibitors of the binding of Alexa Fluor 568 fluorochrome (Alexa₅₆₈)-labeled ZP3 to sperm and, thus, bind the same sperm surface sites as ZP3. Dose-response analyses demonstrated that these glycans are potent inhibitors (IC₅₀ ~180 nM), which at saturation, reduced Alexa₅₆₈-ZP3 binding by ~70%. A Lewis A (Le^a)-capped glycan (Galβ3[Fucα4]GlcNAc) was also a potent inhibitor (IC₅₀ ~150–200 nM), but at saturation, it reduced Alexa₅₆₈-ZP3 binding by only 30%. In contrast, nonfucosylated glycans with nonreducing GlcNAcβ4 or Galβ4 residues did not compete; neither did sialyl-Le^x (Neu5Acα 3Galβ4[Fucα3]GlcNAc-Lewis X) nor sulfo-Le^x (3'-O-SO₃-Lewis X). However, at saturation, Galα3Galβ4GlcNAcβ3Galβ4Glc reduced Alexa₅₆₈-ZP3 binding by ~70% but with moderate apparent affinity (IC₅₀ ~3000 nM). Fluorescence microscopy revealed that Alexa₅₆₈-labeled Le^x-Lac-BSA, Le^a-Lac-BSA, and ZP3 bound to the same sperm surface domains. However, Le^a-Lac did not inhibit binding of Alexa₅₆₈-Le^x-Lac-BSA, and Le^x-Lac did not inhibit binding of Alexa₅₆₈-Le^a-Lac-BSA. Finally, Le^x-Lac and Le^a-Lac had an additive inhibitory effect on Alexa₅₆₈-ZP3 binding. Thus, Le^x is a ligand for a major class of ZP3 binding sites on mouse sperm, whereas Le^a binding defines a different but less-abundant class of sites.

fertilization, gamete biology, sperm

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INTRODUCTION

Mammalian fertilization is the consequence of a cascade of interactions between a male gamete and a female gamete. The first half of this cascade encompasses interactions between sperm and the egg's extracellular matrix, the zona pellucida (ZP). In the mouse, the principal mammal studied, two glycoproteins in this matrix, ZP3 and ZP2, form fibrils that are cross-linked by ZP1 (reviewed in [1]). Mouse sperm-ZP interactions commence with loose attachment of sperm to the ZP [1]. Subsequent higher-affinity interactions between the plasma membrane of the spermatozoon and ZP3 result in firm adhesion of the male gamete [1]. Then, the binding of ZP3 to its complementary receptors on the spermatozoon stimulates the acrosome reaction, whereby the plasma membrane overlying the acrosome and outer acrosomal membrane form vesicles that are shed [2]. This process exposes another ZP3 binding protein within the underlying acrosomal matrix, which may mediate sperm-ZP adhesion until the acrosomal matrix disperses [3, 4]. Adhesion continues because lower-affinity interactions occur between ZP2 and binding sites on the exposed, inner acrosomal membrane [5, 6]. Weakly tethered, the spermatozoon penetrates the ZP. Once a spermatozoon enters the perivitelline space it begins the second half of the fertilization cascade, in which the sperm binds to and fuses with the egg's plasma membrane (for a review, see [2, 7]). Thus, the fertilization cascade encompasses multiple steps, with each step potentially mediated by a distinct pair of binding partners expressed by sperms and eggs. Characterization of all of these binding partners is required for a full understanding of the molecular mechanisms responsible for fertilization. To that end, the experiments described in this paper focus on ZP3 binding sites on the plasma membrane of capacitated, acrosome-intact mouse sperm and the identification of model compounds that mimic the structures of their natural ligands, glycans, on ZP3.

Current models of the fertilization pathway propose that adhesion of acrosome-intact mouse sperm to the ZP is mediated by a subset of *O*-linked glycans of ZP3 [8, 9]. Experiments to assess structural features of these functional glycans have relied mainly on a competitive sperm-ZP binding assay (for details, see [10]). In this assay, capacitated sperm are incubated with ZP-enclosed eggs in the presence of potential competitors and bound sperm are enumerated. The first competitors tested were purified ZP3 and partially purified mixtures of glycans released from ZP3 that had been modified by the addition or removal of a specific monosaccharide [11]. Modifications that altered inhibitory activity were interpreted to reveal important struc-

	Abbreviation	Structure	Glycans /BSA	Manufacturer & Catalog No.
Neoglycoproteins				
i	Le ^x -Lac-BSA	Galβ4[Fucα3]GlcNAcβ3Galβ4Glc-3C-BSA	19	Dextra NGP0502
ii	Le ^a -Lac-BSA	Galβ3[Fucα4]GlcNAcβ3Galβ4Glc-3C-BSA	6.8	Dextra NGP0501
Glycans				
iii	Le ^x -Lac	Galβ4[Fucα3]GlcNAcβ3Galβ4Glc	na	Dextra L504
iv	Le ^a -Lac	Galβ3[Fucα4]GlcNAcβ3Galβ4Glc	na	Dextra L503
v	Le ^x	Galβ4[Fucα3]GlcNAc	na	Dextra LN303
vi	Galα3-Lac	Galα3Galβ4GlcNAcβ3Galβ4Glc	na	Dextra GN334
vii	[GlcNAc]3	GlcNAcβ4GlcNAcβ4GlcNAc	na	Dextra C8003
viii	βGal	Galβ4GlcNAc	na	Dextra GN204
ix	Sialyl-Le ^x	Neu5Acα3Galβ4[Fucα3]GlcNAc	na	Dextra SLN403
x	Sulfo-Le ^x	3'-O-SO ₃ Galβ4[Fucα3]GlcNAc	na	Dextra SSN303

FIG. 1. A list of the glycans and neoglycoproteins used in these studies, their structures, and the suppliers and catalog numbers of these reagents. The backbones of all neoglycoproteins are BSA, and their oligosaccharides are coupled to lysine residues via alkyl spacers. The average levels of substitution (moles of glycan per mole of BSA) were determined by matrix-assisted laser/desorption ionization, time-of-flight (MALDI-TOF) mass spectrometry by the manufacturer.

tural features of a sperm-binding glycan. Subsequently, glycans with defined structures were analyzed for their potential inhibitory activity [10, 12–14]. The most potent inhibitor was assumed to best mimic the essential structure of a sperm-binding glycan on mouse ZP3.

To gain insight into the essential structures of sperm-binding glycans on ZP3, we previously asked whether a related series of glycans of defined structures could inhibit the *in vitro* binding of sperm to ZP-enclosed eggs [10]. Results identified a glycan with a terminal Lewis X (Le^x) structure, (Galβ4[Fucα3]GlcNAc-R), as a specific and potent inhibitor (IC₅₀ ~500 nM). This result suggested that Le^x was a good mimic for the intrinsic sperm-binding glycans on ZP3 implicated in sperm-ZP adhesion. We recognized, however, that the competitive sperm-ZP binding assay did not prove that Le^x was a ligand for a ZP3 binding site. Neither did this assay identify the specific step in the first half of the fertilization cascade that was inhibited. It was possible that Le^x did not bind a ZP3 binding site, but rather a site mediating other steps in the cascade, ranging from loose attachment of sperm to the ZP, to ZP2-mediated sperm adhesion. Indeed, this potential problem is shared by all other studies employing the competitive sperm-ZP binding assay and may explain why different laboratories have proposed different glycan structures for a sperm-binding ligand on ZP3 [13]. Clearly, a more direct experimental strategy was needed to determine whether a glycan was a ligand for a ZP3 binding site on capacitated, acrosome-intact mouse sperm. The strategy used in the experiments described herein tested whether Le^x was a potent and specific inhibitor of the binding of purified ZP3 to complementary sites on acrosome-intact mouse sperm. A positive outcome of this test would directly support the conclusion that Le^x is a structural mimic for a subset of sperm-binding glycans on ZP3, which, based on current models, mediates the firm adhesion of sperm to the ZP.

The experiments in this study employ a recently described quantitative assay of the binding of ZP3 to live, acrosome-intact mouse sperm [6]. The ligand for this assay is purified ZP3 labeled with the fluorochrome Alexa Fluor 568 (Alexa₅₆₈). Fluorescence microscopy in combination with image analysis of bound Alexa₅₆₈-ZP3 allows simultaneous localization of ZP3 binding sites and measurement of the amount of bound fluorescent ligand. Using this assay, we previously identified ZP3 binding sites on two plasma membrane domains of capacitated mouse sperm. One domain overlies the acrosomal crest and the other overlies the posterior sperm head. Dose-response analysis demonstrated saturable, high-affinity (K_m ~63 nM) binding of Alexa₅₆₈-

ZP3 to the sites on both membrane domains. Inhibition by ZP3 but not by ZP2 or ZP1 demonstrated that this binding was specific. Additionally, it was shown that capacitation of sperm and extracellular calcium was required for this specific, high-affinity binding to occur. Thus, the binding of Alexa₅₆₈-ZP3 to sperm is specific, saturable, and, like fertilization, requires calcium and capacitation of sperm. It follows that a glycan that is a specific and potent competitive inhibitor of sperm-ZP3 binding must share essential structural features with one or more intrinsic sperm-binding glycans on ZP3.

Results of the experiments described herein demonstrate that Le^x-containing glycans are potent, competitive inhibitors of approximately 70% of ZP3 binding sites on sperm. A glycan that terminates in the structure of Lewis A (Le^a; Galβ3[Fucα4]GlcNAc) was also a potent, competitive inhibitor of a second class of ZP3 binding sites not recognized by Le^x. In contrast, a series of glycans with other nonreducing termini implicated in sperm-ZP binding were not inhibitors [12, 15]. We conclude that Le^x is a structural mimic of a glycan on ZP3 that binds to a major class of ZP3 binding sites on capacitated, acrosome-intact mouse sperm. Le^a is a structural mimic of a different glycan on ZP3 that binds a second but less-abundant class of sperm surface sites.

MATERIALS AND METHODS

Glycans and Neoglycoproteins

The structures of the unconjugated glycans and neoglycoproteins, and their corresponding abbreviations are provided in Figure 1. Both neoglycoproteins and glycans were dissolved in water as 2 mg/ml stock solutions and stored at -20°C.

Isolation of ZP3

ZP3 was separated from ZP2 and ZP1, and purity was established as previously described [6]. In brief, ZPs were isolated from frozen ovaries of 6- to 7-wk-old ICR mice (Harlan BioProducts for Science, Indianapolis, IN) using Percoll (Amersham-Pharmacia Biotech, Piscataway, NJ) gradient centrifugation. ZP3 was isolated from heat-solubilized ZP by high-performance liquid chromatography using a 250 Bio-Sil Select size exclusion column (Bio-Rad Laboratories, Hercules, CA). Proteins in selected chromatographic fractions were resolved by SDS gel electrophoresis. Purity of ZP3 in these fractions was established by silver staining and by Western blot analysis as previously described [6]. Chromatographic fractions containing only ZP3 were pooled, dialyzed exhaustively against water, concentrated, and stored at -20°C.

Conjugation of the Fluorochrome Alexa₅₆₈ to ZP3 and to Neoglycoproteins

Alexa₅₆₈ was conjugated to ZP3 and to neoglycoproteins according to the manufacturer's instructions (Molecular Probes, Eugene, OR). The level of substitution was approximately 1 mole of fluorochrome per mole of protein. Formulas for determining both protein concentrations and levels of substitution with Alexa₅₆₈ fluorochrome were provided by the manufacturer and used as previously described [6].

Culture Media

The medium used for all experiments was Medium 199 (M199; Life Technologies, Gaithersburg, MD) supplemented with 4 mg/ml crystalline BSA and 273 μ M sodium pyruvate (M199-M; Sigma, St. Louis, MO) [6]. During incubation with either ZP3 or neoglycoproteins, this medium was supplemented with 50 μ M 2-aminoethoxydiphenylborate (2-APB; Calbiochem, San Diego, CA), a cell-permeable inhibitor of inositol 1,4,5-triphosphate-induced Ca²⁺ release [16]. This inhibitor blocks the ability of sperm to undergo an agonist-induced acrosome reaction [17]. Double-strength M199-M was prepared from powdered M199 (Life Technologies) to which was added 8 mg/ml BSA and 546 μ M sodium pyruvate. Prior to each experiment, 2 \times M199-M was mixed 1:1 (vol:vol) with the stock solutions of glycans, neoglycoproteins, or ZP3.

Isolation and Capacitation of Sperm

Sperm were collected from the cauda epididymides of two 8- to 12-wk-old proven-breeder CD-1 mice (Charles River Breeding Labs, Wilmington, MA) and allowed to swim up for 45 min into 2 ml of M-199M. This procedure and all incubations of sperm were conducted at 37°C in an atmosphere of 95% air, 5% CO₂. The capacitated sperm in the upper 0.5 ml of medium were collected and used for the experiments described herein [6]. As measured by the ability of ionophore (50 μ M A23187) to induce sperm to undergo the acrosome reaction, 87% \pm 1% of the sperm were capacitated following this incubation (see [6] for methods used to distinguish acrosome-intact from acrosome-reacted sperm). The use of animals for these experiments was approved by the Animal Care and Use Committee of the Johns Hopkins University Bloomberg School of Public Health.

Incubations of Sperm with Alexa₅₆₈-Labeled ZP3 and Neoglycoproteins in the Presence or Absence of Unlabeled Glycans

Experiments described herein had three objectives. The first objective was to determine whether Le^x-containing glycans or nine other glycans were dose-dependent competitive inhibitors of the binding of Alexa₅₆₈-ZP3 to capacitated mouse sperm. The other nine unconjugated glycans that were tested were either structurally related to Le^x or had nonreducing termini found on other glycans implicated in sperm-ZP binding [12, 15]. Approximately 75 000 live capacitated mouse sperm were incubated for 1.5 h with 36 nM Alexa₅₆₈-ZP3 in the presence or absence of 2-, 5-, 10-, 50-, 100-, or 1000-fold molar excess of unlabeled glycan. The sperm were then washed, fixed in 4% formaldehyde, and dried onto glass slides as previously described [6]. The fixed sperm were then examined microscopically and fluorescence was quantified (see *Fluorescence Microscopy and Quantitative Analysis*, below).

The second objective was to determine whether Alexa₅₆₈-Le^x-Lactose (Lac)-BSA and Alexa₅₆₈-Le^a-Lac-BSA bound to one or both of the sperm surface domains bound by Alexa₅₆₈-ZP3 [6]. In the first of two experiments, we examined neoglycoproteins bound to live sperm surrounded by an intact membrane. This experiment did not discriminate between the possibilities that the acrosomal crest was covered by the plasma membrane or by the inner acrosomal membrane. Live capacitated sperm were incubated for 1 h with 180 nM Alexa₅₆₈-Le^x-Lac-BSA, Alexa₅₆₈-Le^a-Lac-BSA, or Alexa₅₆₈-ZP3. Next, sperm were washed, pelleted, and resuspended in M199-M supplemented with 50 nM SYTOX Green (Molecular Probes, Eugene, OR), a membrane-impermeant fluorescent nucleic acid dye. Sperm were then allowed to adhere to CellTak-coated glass coverslips (BD Biosciences, Bedford, MA), and the live sperm were immediately examined by fluorescence microscopy [6]. The objective of the second experiment was to confirm that the neoglycoproteins were binding to acrosome-intact sperm. Capacitated sperm were incubated for 1 h with 180 nM Alexa₅₆₈-Le^x-Lac-BSA or Alexa₅₆₈-Le^a-Lac-BSA, washed, fixed in 70% ethanol, and the acrosome was stained with fluorescein isothiocyanate-conjugated peanut (*Arachis hypogea*) agglutinin (FITC-PNA; Vector Labs,

Burlingame, CA) [6]. Sperm were then dried onto glass slides and examined by fluorescence microscopy as described below.

The third objective was to determine whether Le^x-Lac or Le^a-Lac were ligands for the same or different sites on sperm. The first of two experiments tested whether Le^x-Lac or Le^a-Lac were competitive inhibitors of the binding of Alexa₅₆₈-Le^x-Lac-BSA or Alexa₅₆₈-Le^a-Lac-BSA to capacitated mouse sperm. Sperm were incubated for 1.5 h with 36 nM of one of the two fluorescent neoglycoproteins and in the presence or absence of 100-fold or 1000-fold molar excess of Le^x-Lac or Le^a-Lac. The sperm were then washed, fixed in 4% formaldehyde, and dried onto glass slides [6]. The fixed sperm were then examined microscopically and the fluorescence was quantified. The second of two experiments tested whether there was an additive inhibitory effect of Le^x-Lac or Le^a-Lac on binding to sperm of Alexa₅₆₈-ZP3. Sperm were incubated for 1.5 h with 36 nM Alexa₅₆₈-ZP3 with and without a 5-fold molar excess of Le^x-Lac, a 50-fold and 100-fold molar excess of Le^a-Lac, or a 5-fold and 50-fold molar excess of Le^x-Lac and Le^a-Lac, respectively. Sperm were then washed, fixed, dried into glass slides, examined by fluorescence microscopy, and the fluorescence was quantified.

Fluorescence Microscopy and Quantitative Analysis

Fluorescence images were captured using a Nikon Eclipse E800 microscope (Nikon, Inc., Melville, NY) equipped with a 40 \times Plan Apo lens and a Princeton 5-MHz cooled interlined charge-coupled device (CCD) camera (Princeton Instruments, Trenton, NJ). Alexa₅₆₈ fluorescence was detected using a G2E-RHOD excitation filter, a 565-nm dichroic mirror, and a barrier filter with a bandwidth of 600–660 nm. The binding to sperm of FITC-PNA or of SYTOX-Green was detected using an FITC excitation filter, a 505-nm dichroic mirror, and a barrier filter with a bandwidth of 515–555 nm. Fluorescence filters were manufactured by Chroma, Inc. (Burlington, VT).

The binding of Alexa₅₆₈-Le^x-Lac-BSA, Alexa₅₆₈-Le^a-Lac-BSA, and Alexa₅₆₈-ZP3 to sperm was quantified using IPLab Spectrum analysis software (Scanalytics, Fairfax, VA) as previously described [6]. Published results document a linear response of the CCD camera over the range of fluorescence measurements made in these experiments (for details see [6]). For each experiment, all treatments were analyzed in duplicate, and every experiment was replicated two to three times. For each treatment, quantitative data were obtained from 200–900 sperm and presented as the percentage of the fluorescence emitted from positive control sperm that were incubated with only the Alexa₅₆₈-labeled ligand. Dose-response curves for individual oligosaccharides were analyzed as previously described [10]. Briefly, data were fit to a rectangular hyperbola, and regression analysis was performed using the SigmaPlot program (SPSS, Chicago, IL). This program estimated the IC₅₀ value (molar concentration of glycan producing a 50% inhibition in the binding of the Alexa₅₆₈-ZP3 to sperm), percent maximal inhibition, and fit of the data to the regression line (R² and *P* values). In experiments that compared effects of individual treatments on binding of fluorescent ligands to sperm, differences among means were established using analysis of variance, and effects of individual treatments were compared using the Fisher least significant difference test and the Statistical Analysis Systems Statistical Package version 6.1 (SAS Institute, Inc., Cary, NC). For all analyses, statistically significant differences were defined as *P* < 0.05.

RESULTS

Le^x-Containing Glycans are Potent, Competitive Inhibitors of ~70% of ZP3 Binding Sites on Capacitated, Acrosome-Intact Mouse Sperm

The hypothesis that Le^x-containing glycans are high-affinity ligands for ZP3 binding sites predicted that these glycans are potent, dose-dependent inhibitors of the specific and saturable binding of ZP3 to sperm. To test this prediction, a dose-response curve was generated by incubating capacitated mouse sperm with 36 nM Alexa₅₆₈-ZP3 alone or also with increasing concentrations of Le^x-Lac (72 nM to 36 000 nM). Regression analysis demonstrated that the data fit a rectangular hyperbola (R² = 0.94, *P* < 0.05). This statistical analysis estimated an IC₅₀ value of 105 \pm 18 nM Le^x-Lac with a saturating dose of glycan (1800 nM to 36 000 nM) reducing bound Alexa₅₆₈-ZP3 to 30% of control (Fig. 2A, closed circles). Identical results were obtained

when the Le^x trisaccharide was used as a competitive inhibitor (Fig. 2A, open squares). Taken together, these results indicate that oligosaccharides with a nonreducing terminal Le^x trisaccharide are high-affinity ligands for about ~70%, but not all, ZP3 binding sites on capacitated, acrosome-intact mouse sperm. Additionally, these data indicate that addition of lactose to the one carbon of the GlcNAc residue of Le^x, which stabilizes the ring structure of the GlcNAc and locks the number-one carbon of GlcNAc in the β position, does not affect the affinity of Le^x-containing glycans for ZP3 binding sites on sperm.

Complementary experiments tested the abilities of two nonfucosylated glycans, [GlcNAc]3 and Gal α 3-Lac to inhibit the binding of Alexa₅₆₈-ZP3 to sperm. [GlcNAc]3 at concentrations ranging from 72 nM to 36 000 nM did not inhibit this binding (data not shown). Additionally, Gal α 3-Lac at concentrations ranging from 72 nM to 1800 nM was an ineffective competitor. However, substantially higher concentrations (3600 nM and 36 000 nM; 100-fold and 1000-fold molar excess, respectively) reduced Alexa₅₆₈-ZP3 binding to 27% \pm 2% of control, producing an estimated IC₅₀ of ~3000 nM (data not shown). Results obtained with both glycans are consistent with data previously obtained from the in vitro competitive sperm-ZP binding assay [10].

Removal of the Fucosyl Residue from the Le^x Trisaccharide or Addition of Either an α 3-Sialyl or a 3'-Sulfo Group to the Galactosyl Residue Abolishes the Glycan's Ability to Act as a Competitive Inhibitor of ZP3 Binding

The results of the experiments described above support the conclusion that Le^x-containing glycans are potent, competitive inhibitors of ZP3 binding to sperm. What is the consequence of modifying the Le^x structure? To answer this question, we tested the effect of adding a negatively charged sialyl (Neu5Ac α 3) or sulfo (3'-O-SO₃) residue to Le^x or of removing the fucosyl residue. Neither sialyl-Le^x, sulfo-Le^x, nor β Gal, at concentrations as high as 36 000 nM (1000-fold molar excess), inhibited binding of Alexa₅₆₈-ZP3 to sperm. In contrast, Le^x reduced binding to ~30% of control (Fig. 2B).

A Lewis A (Gal β 3[Fuc α 4]GlcNAc)-Containing Glycan Is a Potent, Competitive Inhibitor of ~30% of ZP3 Binding Sites on Capacitated, Acrosome-Intact Mouse Sperm

Le^a is a structural isomer of Le^x in which the positions of the galactose and fucose residues are interchanged. Because both sialyl-Le^x- and sialyl-Le^a-containing glycans bind to the human cell surface lectin, L-selectin, Le^a-Lac (Fig. 1) might bind the same ZP3 binding sites as Le^x-Lac [18, 19]. To test this possibility, a dose-response curve was generated by incubating sperm with 36 nM Alexa-ZP3 alone or with increasing concentrations of Le^a-Lac (72 nM to 36 000 nM; 2-fold to 1000-fold molar excess). Results showed that this glycan was a dose-dependent inhibitor of Alexa₅₆₈-ZP3 binding (Fig. 3). Regression analysis demonstrated that the data fit a rectangular hyperbola ($R^2 = 0.97$, $P < 0.05$). This statistical analysis estimated an IC₅₀ value of 221 \pm 20 nM Le^a-Lac with a saturating dose of glycan (1800 nM to 36 000 nM) reducing bound Alexa₅₆₈-ZP3 to 70% of control. This result suggested that Le^a-Lac was recognized by a smaller fraction of sperm-surface ZP3 binding sites than Le^x-Lac. To confirm this conclusion statistically, data for both dose-response curves (Le^x-Lac and

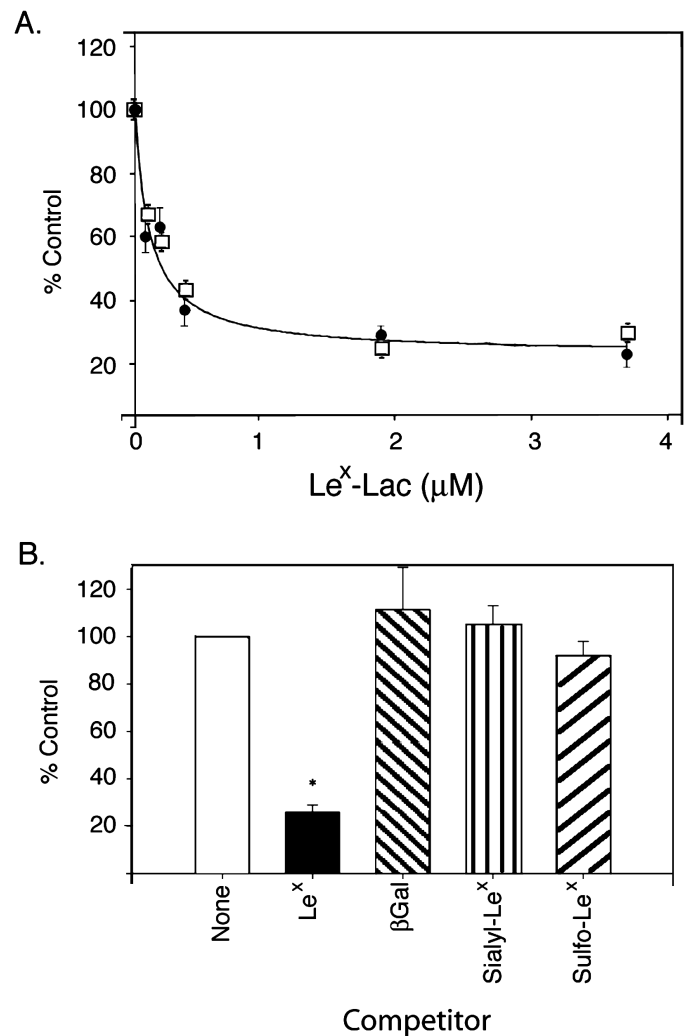


FIG. 2. Le^x and Le^x-Lac are potent and specific competitive inhibitors of the binding of Alexa₅₆₈-ZP3 to capacitated, acrosome-intact mouse sperm. **A**) Capacitated mouse sperm were incubated for 1.5 h with 36 nM Alexa₅₆₈-ZP3 alone (control sperm) or with 72 nM, 180 nM, 360 nM, 1800 nM, 3600 nM, and 36 000 nM (2-fold to 1000-fold molar excess) of Le^x-Lac or Le^x. Inhibition with 1000-fold molar excess competitor (not shown) was identical to inhibition with 100-fold molar excess competitor. **B**) Capacitated mouse sperm were incubated with 36 nM Alexa₅₆₈-ZP3 alone (control sperm) or also with a 1000-fold molar excess of Le^x, Lac, sialyl-Le^x, or sulfo-Le^x. Data (mean \pm SEM) are expressed as the percentage of Alexa₅₆₈-ZP3 bound by control sperm. In **(B)**, data marked with an asterisk differ from the control ($P < 0.05$).

Le^a-Lac vs. Alexa₅₆₈-ZP3) were compared by two-way analysis of variance. This analysis confirmed that Le^x-Lac recognized a larger fraction of ZP3 binding sites than Le^a-Lac.

Le^x-Lac-BSA and Le^a-Lac-BSA Bind to the Same Domains as ZP3 on the Plasma Membrane of Capacitated, Acrosome-Intact Sperm

Alexa₅₆₈-ZP3 binds to two distinct domains in the plasma membrane of the mouse sperm, the domain over the acrosomal crest and the domain over the posterior sperm head [6]. It was therefore possible that the ZP3 binding sites on a given domain would preferentially bind Le^x-Lac or Le^a-Lac. To assess this possibility we used as ligands Alexa₅₆₈-Le^x-Lac-BSA and Alexa₅₆₈-Le^a-Lac-BSA. Preliminary experiments demonstrated that the two fluorochrome-

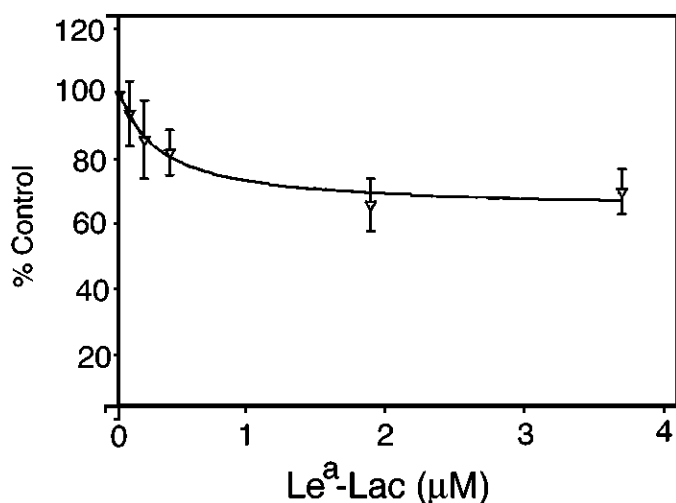


FIG. 3. Le^a-Lac is a potent, competitive inhibitor of 30% of ZP3 binding sites on capacitated, acrosome-intact mouse sperm. Capacitated sperm were incubated for 1.5 h with 36 nM Alexa₅₆₈-ZP3 alone (control sperm) or also with 72 nM, 180 nM, 360 nM, 1800 nM, 3600 nM, and 36000 nM (2-fold to 1000-fold molar excess) of Le^a-Lac. Inhibition with 1000-fold molar excess competitor (not shown) was identical to inhibition with 100-fold molar excess. Data (mean \pm SEM) are expressed as the percentage of Alexa₅₆₈-ZP3 bound by control sperm.

labeled neoglycoproteins bound sperm in a saturable manner with half-maximal saturation (K_d) achieved at approximately 150 nM Alexa₅₆₈-Le^x-Lac-BSA and 175 nM Alexa₅₆₈-Le^a-Lac-BSA (data not shown).

The first experiment examined the binding of these fluorochrome-labeled neoglycoproteins to live sperm. Capacitated sperm were incubated with 180 nM of Alexa₅₆₈-Le^x-Lac-BSA, Alexa₅₆₈-Le^a-Lac-BSA, or Alexa₅₆₈-ZP3, washed, and incubated with SYTOX Green, a membrane-impermeant nucleic acid dye. Living sperm were examined immediately thereafter. Microscopic analysis revealed that Alexa₅₆₈-Le^x-Lac-BSA and Alexa₅₆₈-Le^a-Lac-BSA bound to the same two membrane domains as ZP3, the membrane overlying the acrosomal crest and the posterior sperm head (Fig. 4, A, C, and E). These sperm were not labeled with SYTOX Green and thus, were alive and surrounded by an intact membrane (Fig. 4, B, D, and F). In contrast, dead or dying cells were intensely stained with this fluorescent nucleic acid dye (Fig. 4G).

To confirm that Alexa₅₆₈-Le^x-Lac-BSA and Alexa₅₆₈-Le^a-Lac-BSA were binding to acrosome-intact sperm, live, capacitated sperm were incubated with these two neoglycoproteins, washed, fixed with 70% ethanol, and then stained with FITC-PNA, which binds to acrosomal glycoproteins [6]. Results confirmed that Alexa₅₆₈-Le^x-Lac-BSA and Alexa₅₆₈-Le^a-Lac-BSA were binding to acrosome-intact sperm (compare Fig. 4, H and J with Fig. 4, I and K). Thus, the data indicate that the entire sperm head, including the acrosomal crest, was covered by the plasma membrane. Taken together, these results indicate that Le^x-Lac-BSA, Le^a-Lac-BSA, and ZP3 bind to the same plasma membrane domains of capacitated, acrosome-intact mouse sperm. These observations raised the issue of whether Le^x and Le^a are ligands for different classes of ZP3 binding sites that were present on these two domains.

Le^x-Lac and Le^a-Lac Recognize Different Classes of ZP3 Binding Sites on Sperm

If Le^x-Lac and Le^a-Lac are recognized by two distinct classes of ZP3 binding sites, then the two glycans would

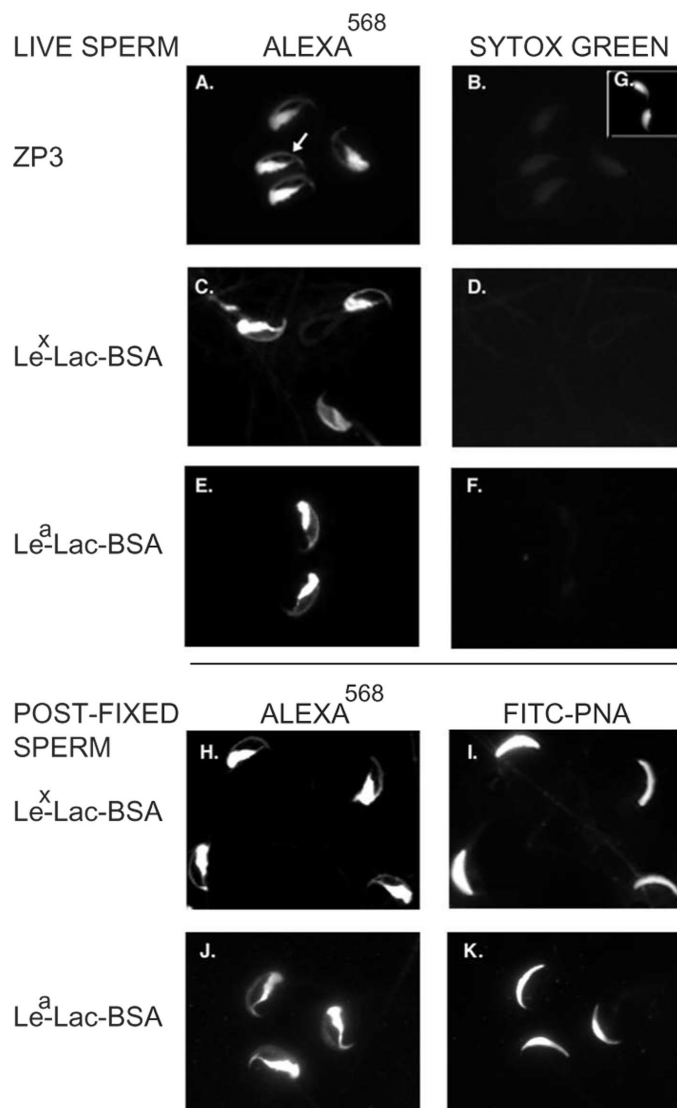


FIG. 4. Alexa₅₆₈-ZP3, Alexa₅₆₈-Le^x-Lac-BSA, and Alexa₅₆₈-Le^a-Lac-BSA bind to the same membrane domains of capacitated, acrosome-intact mouse sperm. Live sperm were incubated for 1 h with 180 nM of either Alexa₅₆₈-ZP3 (A) Alexa₅₆₈-Le^x-Lac-BSA (C), or Alexa₅₆₈-Le^a-Lac-BSA (E), washed, mounted in M199-M with 50 nM SYTOX Green nucleic acid stain, and examined immediately by fluorescence microscopy. Viability of sperm in (A, C, and E) was demonstrated by the absence of SYTOX Green staining (B, D, and F, respectively). In contrast, the heads of dead or dying sperm stained intensely (G). Note that Alexa₅₆₈-Le^x-Lac-BSA and Alexa₅₆₈-Le^a-Lac-BSA bind to the same two membrane domains as Alexa₅₆₈-ZP3, the domain over the acrosomal crest (see arrow in A) and the domain over the posterior sperm head. To confirm that Alexa₅₆₈-Le^x-Lac-BSA and Alexa₅₆₈-Le^a-Lac-BSA were binding to acrosome-intact sperm, capacitated sperm were incubated for 1 h with 180 nM Alexa₅₆₈-Le^x-Lac-BSA (H and I) or with 180 nM Alexa₅₆₈-Le^a-Lac-BSA (J and K). Sperm were then fixed and stained with FITC-PNA. Colabeling of the same sperm with Alexa₅₆₈-Le^x-Lac-BSA (H) and with FITC-PNA (I) is shown, as is colabeling of the same sperm with Alexa₅₆₈-Le^a-Lac-BSA (J) and with FITC-PNA (K). Magnification $\times 725$.

be predicted not to compete with each other for their respective sites. Additionally, the two glycans would be predicted to have an additive inhibitory effect on the binding of Alexa₅₆₈-ZP3. To test the first prediction, sperm were incubated with 36 nM Alexa₅₆₈-Le^x-Lac-BSA alone or with either a 100-fold or 1000-fold molar excess of Le^x-Lac or Le^a-Lac. Results (Fig. 5A) showed that both concentrations of Le^x-Lac displaced 60% of the binding of Alexa₅₆₈-Le^x-

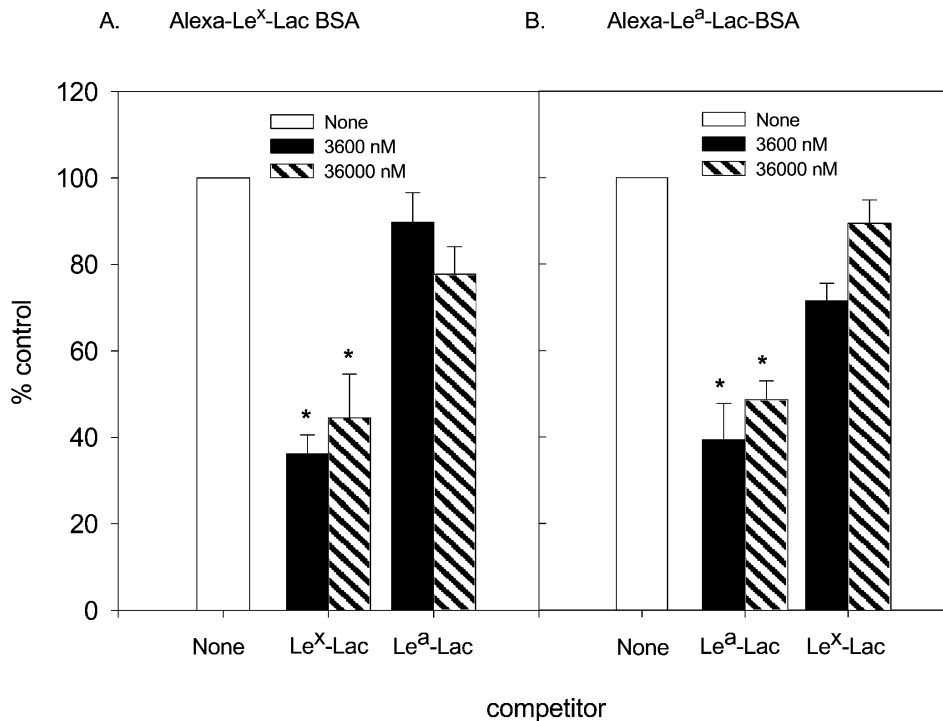


FIG. 5. Alexa₅₆₈-Le^X-Lac-BSA and Alexa₅₆₈-Le^a-Lac-BSA identify distinct ZP3 binding sites on capacitated, acrosome-intact mouse sperm. **A**) Capacitated sperm were incubated for 1.5 h with 36 nM Alexa₅₆₈-Le^X-Lac-BSA alone or with a 100-fold (3600 nM) or 1000-fold (36000 nM) molar excess of Le^X-Lac or Le^a-Lac. Le^X-Lac but not Le^a-Lac produced a substantial and statistically significant decrease in binding of Alexa₅₆₈-Le^X-Lac-BSA (* $P < 0.05$). **B**) Sperm were incubated for 1.5 h with 36 nM Alexa₅₆₈-Le^a-Lac-BSA alone or with a 100-fold (3600 nM) or 1000-fold (36000 nM) molar excess of Le^X-Lac or Le^a-Lac. Data (mean \pm SEM) are expressed as the percentage of Alexa₅₆₈-ZP3 bound by control sperm, which were incubated in the absence of competitor. Le^a-Lac but not Le^X-Lac produced a substantial and statistically significant decrease in the binding of Alexa₅₆₈-Le^a-Lac-BSA (* $P < 0.05$).

Lac-BSA, whereas Le^a-Lac did not compete. Sperm were also incubated in 36 nM Alexa₅₆₈-Le^a-Lac-BSA alone or with a 100-fold or 1000-fold molar excess of Le^X-Lac or Le^a-Lac. Similar to the results shown in Figure 5A, signif-

icant inhibition of Alexa₅₆₈-Le^a-Lac binding was observed only with Le^a-Lac (Fig. 5B).

To test the second prediction, capacitated sperm were incubated with 36 nM Alexa₅₆₈-ZP3 and in the presence or absence of 180 nM Le^X-Lac (5-fold molar excess), 1800 nM or 3600 nM Le^a-Lac (50-fold or 100-fold molar excess), or 180 nM Le^X-Lac plus 1800 nM Le^a-Lac (Fig. 6). We used saturating concentrations of Le^a-Lac but not of Le^X-Lac because of the low level of fluorescence from sperm incubated with 36 nM Alexa₅₆₈-ZP3 plus a saturating concentration of Le^X-Lac. Thus, in the presence of a saturating concentration of Le^X-Lac, the effect of adding a second competitor would be difficult to detect.

In this experiment, the addition of 180 nM Le^X-Lac to sperm reduced the binding of Alexa₅₆₈-ZP3 binding to 45% of control (Fig. 6). Additionally, both concentrations of Le^a-Lac reduced Alexa-ZP3 binding to 75% of control, confirming that 1800 nM Le^a-Lac was saturating. However, addition of 180 nM Le^X-Lac to a saturating dose of Le^a-Lac further reduced the binding of Alexa₅₆₈-ZP3 to 20% of control. This additive inhibitory effect of Le^X-Lac and Le^a-Lac on Alexa₅₆₈-ZP3 binding supports the conclusion that these two glycans recognize distinct classes of ZP3 binding sites on capacitated mouse sperm.

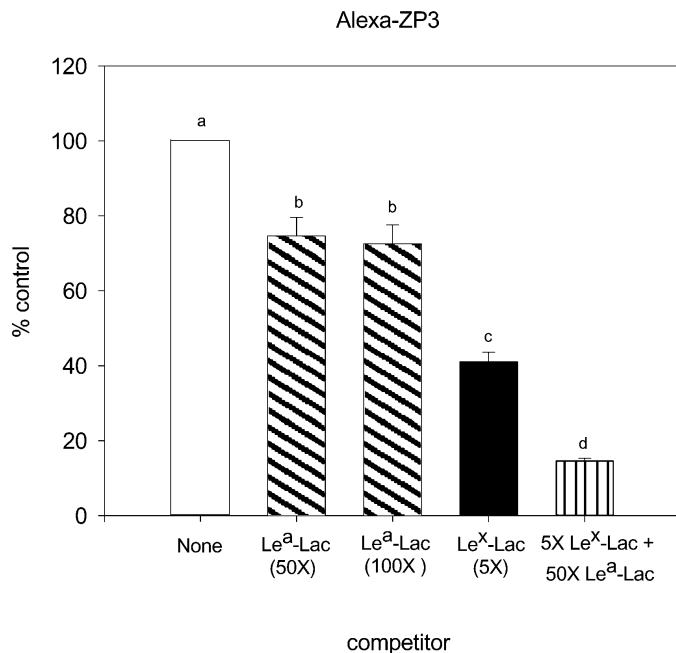


FIG. 6. Le^X-Lac and Le^a-Lac are ligands for distinct classes of ZP3 binding sites on capacitated, acrosome-intact mouse sperm. Capacitated sperm were incubated for 1.5 h with 36 nM Alexa₅₆₈-ZP3 alone (None), or with a 50-fold molar excess of Le^a-Lac (Le^a-Lac, 50X); a 100-fold molar excess of Le^a-Lac (Le^a-Lac, 100X); a 5-fold molar excess of Le^X-Lac (Le^X-Lac, 5X); or a 50-fold molar excess of Le^a-Lac plus 5-fold molar excess of Le^X-Lac (5X Le^X-Lac + 50X Le^a-Lac). Data (mean \pm SEM) are expressed as the percentage of Alexa₅₆₈-ZP3 bound by control sperm, which were incubated in the absence of competitor. Bars marked by different letters differ statistically ($P < 0.05$).

DISCUSSION

The main objective of the experiments described in this paper was to test the hypothesis that Le^X-containing glycans are specific and potent, competitive inhibitors of ZP3 binding sites on capacitated, acrosome-intact mouse sperm. The basis for this hypothesis was our previous report that these glycans were potent, competitive inhibitors of the binding of mouse sperm to ZP-enclosed eggs [10]. The current study adapted a widely used experimental strategy to determine whether a glycan with specific structures binds a mammalian cell-surface lectin. This strategy has identified essential structural features of glycans on the natural ligand for lectins, including the selectins [20–24]. Results of our

experiments identify the Le^x-containing glycans as specific and potent, competitive inhibitors of the binding of ZP3 to sperm. This finding supports the conclusion that Le^x is a structural mimic of one or more functional sperm-binding glycans on ZP3.

Evidence that Le^x Is a Structure-Specific Ligand for a Major Class of ZP3 Binding Sites on Capacitated, Acrosome-Intact Mouse Sperm

Our results demonstrate that at saturating concentrations, Le^x and Le^x-Lac reduced binding of Alexa₅₆₈-ZP3 to sperm by 60% to 70%. It was noteworthy that the IC₅₀'s of these glycans (~180–300 nM) were in the same range as Le^x in the competitive sperm-zona binding assay (~500 nM) and the affinity of Alexa₅₆₈-Le^x-Lac-BSA (K_d ~150 nM) for mouse sperm [10]. Thus, collectively, these results support the conclusion that Le^x and Le^x-Lac bind the same sites on sperm as ZP3. Furthermore, the apparent high affinity of these glycans supports the conclusion that glycans on ZP3 mediate tight adhesion of sperm to the ZP.

Our experiments also provide some insight into the structural requirements of the ligands for ZP3 binding sites. Consistent with its ability to inhibit the binding of sperm to ZP-enclosed eggs, our current studies identify αGal-Lac as a moderate-affinity (IC₅₀ ~3 μM) competitive inhibitor of Alexa₅₆₈-ZP3 binding. However, although Johnston et al. [10] identified a βGal-capped glycan as a low-affinity competitive inhibitor (IC₅₀ ~42 μM) of sperm-zona binding, βGal did not inhibit the binding of Alexa₅₆₈-ZP3 to sperm. A potential explanation for this apparent discrepancy comes from preliminary results indicating that βGal-BSA inhibits the binding of Alexa₅₆₈-ZP2, but not Alexa₅₆₈-ZP3 to mouse sperm (unpublished results).

The conclusion that βGal is not a ligand for ZP3 binding sites emphasizes the requirement of the α3-fucosyl residue of Le^x for producing a high-affinity ligand. Our results also indicate, however, that the molecular context of the fucose residue determines whether a fucosylated glycan is a ligand for these sites. Neither sialyl-Le^x nor sulfo-Le^x are ligands for ZP3 binding sites. However, Johnston et al. [10] demonstrated that α3-galactosyl-Le^x and Le^x are equally effective inhibitors of sperm-zona binding. Thus, we suggest that the reason sialyl-Le^x and sulfo-Le^x are not ligands for a ZP3 binding site is due to their negative charges.

Although Le^x is a ligand for about 60%–70% of all ZP3 binding sites on acrosome-intact mouse sperm, results in Figures 5 and 6 indicate that those sites do not recognize the structural isomer of Le^x, Le^a. However, our data indicate that Le^a is recognized by a second class of ZP3 binding sites that have an apparent low affinity, if any, for Le^x. Thus, an interesting conclusion from these studies is that mouse sperm has two distinct ZP3 binding sites that can be distinguished by their capacity to recognize Le^x and Le^a. On capacitated mouse sperm, the sites that recognize Le^x appear to be twice as abundant as the sites that recognize Le^a.

What Is the Structural Basis for the Specific Binding of Le^x and Le^a to Distinct Classes of ZP3 Binding Sites?

The apparent high affinity of Le^x, Le^x-Lac, and Le^a-Lac for ZP3 binding sites on sperm supports the conclusion that these glycans are structural mimics of sperm-binding glycans on ZP3. Our data also indicate that Le^x-containing glycans and Le^a-containing glycans are ligands for distinct sperm-surface ZP3 binding sites. What are the structural

differences between Le^x and Le^a that would allow them to be differentially recognized by these two sites? As pointed out by Bush and colleagues, the minimal energy conformations of Le^x and Le^a, determined by nuclear magnetic resonance, reveal that these two trisaccharides have remarkably overall similar structures [22, 23]. Both glycans are compact, rigid structures with essentially identical stacking between the fucose and galactose rings. The only major structural difference between these two trisaccharides is the orientation of the GlcNAc residue relative to the stacked rings. The GlcNAc residue of Le^x is rotated approximately 180° relative to the GlcNAc residue of Le^a [23, 24]. Consequently, the C2 acetoamido group and the C6 hydroxymethyl group of GlcNAc are in opposite orientation in the two trisaccharides. Thus, the opposite orientations of the GlcNAc residue of Le^x and Le^a may explain at the molecular level why these two glycans are ligands for distinct ZP3 binding sites on mouse sperm.

While the orientation of the GlcNAc residue potentially confers the specificity of Le^x and Le^a for their respective sperm-surface sites, it is the fucosyl residue that confers high affinity. This requirement of the fucosyl residue has also been observed in the binding of both sialyl-Le^x and sialyl-Le^a to P, E, and L selectins [18, 19, 25–27]. Solution structures of sialyl-Le^x and sialyl-Le^a are similar to their nonsialylated counterparts with the exception of the addition a more flexible sialic acid residue [24]. Crystallographic studies of sialyl-Le^x bound to either P or E selectin reveal that the GlcNAc residue does not form hydrogen bonds with the selectin's glycan-binding pocket. Rather, the fucose residue provides a substantial amount of the binding energy by coordinating the required calcium ion and forming hydrogen bonds with amino acids in the calcium-binding pocket [28]. This raises the possibility that the fucose residue in Le^x and in Le^a may play a similar role in the binding of these glycans to their respective sites on mouse sperm.

What are the Functions of the ZP3 Binding Sites Recognized by Le^x and Le^a?

While our experiments indicate that Le^x and Le^a can serve as ligands for distinct ZP3 binding sites, those experiments do not address the biological functions of those sites. Neither do they address whether the more abundant sites, which recognize Le^x, play a more important role in the fertilization cascade than the sites recognized by Le^a, it is possible that one or both of these sites are adhesion molecules whose primary functions are to tether sperm to the ZP. In that model, these sites are functionally analogous to L-selectin, which mediates the tethering of lymphocytes to high endothelial venules, whereas different molecules, acting as receptors and ligands, generate the signal transduction cascade that regulates the migration of these cells out of the vascular compartment (reviewed in [26, 29]). Alternatively, the sperm surface binding sites that recognize Le^x or Le^a may be functional receptors, which both tether acrosome-intact sperm to the ZP and mediate the responses of sperm to ZP3, culminating in the acrosome reaction. Because the glycans on ZP3 must be clustered on a polypeptide backbone to trigger the acrosome reaction, and because unconjugated Le^x does not trigger the acrosome reaction, testing this proposal requires that Le^x or Le^a be clustered on a polypeptide backbone, such as BSA [30]. The next paper in this series [17] employs neoglycoproteins with glycans of defined structures to address whether the ZP3 bind-

ing sites recognized by Le^x-Lac and Le^a-Lac are, in fact, functional receptors.

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