Oligosaccharide Constructs with Defined Structures That Inhibit Binding of Mouse Sperm to Unfertilized Eggs *in Vitro*†

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ABSTRACT: During fertilization in mice, free-swimming sperm bind to mZP3, an 83-kDa glycoprotein present in the egg extracellular coat, the zona pellucida [Wassarman, P. M. (1990) *Development 108*, 1−17]. Mouse sperm recognize and bind to a specific class of serine/threonine-linked (O-linked) oligosaccharides present on mZP3. After binding to mZP3, sperm undergo a form of cellular exocytosis, the acrosome reaction, thereby enabling them to penetrate the zona pellucida and fertilize the egg. Thus, gamete interactions in mice are carbohydrate-mediated. In this context, we tested 15 O-linked-related oligosaccharide constructs with defined structures for their ability to inhibit binding of mouse sperm to ovulated eggs and to induce sperm to undergo the acrosome reaction *in vitro*. Thirteen of the oligosaccharides were constructed and characterized in our laboratory [Seppo, A., Pentillä, L., Niemelä, R., Maaheimo, H., Renkonen, O., & Keane, A. (1995) *Biochemistry 34*, 4655−4661]; two were obtained commercially. We found that, while none of the oligosaccharides induced sperm to undergo the acrosome reaction, a few of them inhibited binding of sperm to eggs at relatively low concentrations (ID_{50} < 5 μM). In certain cases, sperm formed head-to-head aggregates in the presence of the oligosaccharides. The results suggest that the ability of oligosaccharides to inhibit binding of sperm to eggs is dependent on several parameters, including the size and branching pattern of the oligosaccharide, as well as on the nature of the sugar residue at the nonreducing end of the oligosaccharide.

For fertilization to occur in mammals, free-swimming sperm must bind to and then penetrate the ovulated egg extracellular coat, the zona pellucida (ZP)[1] (Gwatkin, 1977; Wassarman, 1987a,b; Yanagimachi, 1994). Binding of sperm to the ZP takes place in a relatively species-specific manner (Adams, 1974; Yanagimachi, 1977; Gulyas & Schmell, 1981; O'Rand, 1988; Roldan & Yanagimachi, 1989). In mice, sperm recognize and bind to Ser/Thr-(O-)-linked oligosaccharides associated with mZP3, an 83-kDa glycoprotein present in the ZP (Bleil & Wassarman, 1980; Florman & Wassarman, 1985; Wassarman, 1988, 1990; Wassarman & Litscher, 1995). Apparently, these O-linked oligosaccharides are located in a small region of the carboxy-terminal third of the mZP3 polypeptide (Rosiere & Wassarman, 1992; Kinloch et al., 1995; Wassarman and Litscher, 1995). A highly homologous glycoprotein has been identified in the ZP of eggs from a variety of mammalian species, including hamsters, marmosets, and humans (Kinloch et al., 1990; Chamberlin & Dean, 1990; van Duin et al., 1992; Thillai-Koothan et al., 1993; Wassarman, 1993; Wassarman & Litscher, 1995). Shortly after binding to mZP3, sperm undergo the acrosome reaction (Bleil & Wassarman, 1983; Wassarman, 1990; Kopf & Gerton, 1991) which enables them to penetrate the ZP and fuse with egg plasma membrane to form a zygote (Yanagimachi, 1994).

Involvement of carbohydrates in mammalian gamete adhesion is supported by a variety of experimental evidence, including the ability of various saccharides to inhibit binding of sperm to eggs *in vitro* (Wassarman, 1992; Litscher & Wassarman, 1993). In mice, small glycopeptides and O-linked oligosaccharides, isolated from purified mZP3 following either extensive digestion by pronase or alkaline reduction, respectively, inhibit binding of sperm to eggs (Florman et al., 1983; Florman & Wassarman, 1985; Wassarman, 1989). Although the structures of the active mZP3 O-linked oligosaccharides have not been determined, it is clear that at least the monosaccharide at the nonreducing end is essential for sperm binding (Bleil & Wassarman, 1988; Miller et al., 1992). However, there is disagreement as to whether this monosaccharide is Gal in α-linkage (Bleil & Wassarman, 1988; Shalgi et al., 1990) or GlcNAc in β-linkage (Shur & Hall, 1982; Miller et al., 1992) with the penultimate sugar.

In an attempt to assess parameters that may influence binding of sperm to mZP3 oligosaccharides, we tested 15 oligosaccharide constructs with defined structures for their effect on mouse gamete adhesion *in vitro*. All but two of these oligosaccharides were constructed and characterized in our laboratory (Seppo *et al.*, 1995). Whereas none of the oligosaccharides induced sperm to undergo the acrosome reaction, a few inhibited binding of sperm to eggs at

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[1] Abbreviations: ZP, zona pellucida; Glc, glucose; Gal, galactose; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannose; Ser, serine; Thr, threonine; Asn, asparagine, DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; PVP, polyvinylpyrrolidone.

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relatively low concentrations, and these oligosaccharides have certain features in common. A preliminary report of some of these results has appeared (Litscher et al., 1993).

MATERIALS AND METHODS

Sources of Monosaccharides and Oligosaccharides. Oligosaccharide constructs possessing defined structures were synthesized enzymatically *de novo* and characterized in our laboratory (Seppo et al., 1995). Blood group type B-related trisaccharide and pentasaccharide (purity >95% by HPLC, as determined by the supplier) were purchased from Accurate Chemical and Scientific Corp., and galactose (Gal), *N*-acetylglucosamine (GlcNAc), and melibiose (Galα1-6Glc) were purchased from Sigma Chemical Co. Stock solutions of oligosaccharides were prepared by addition of double-distilled, sterilized water, and solutions were stored at 4 °C. Saccharide concentrations were estimated by UV absorption at 205 nm and/or by analysis of samples of known specific radioactivity; the concentrations are accurate to within ±20%.

Purification of mZP3. Mouse ZPs were isolated by centrifugation of ovarian homogenates through Percoll, and mZP3 was purified to homogeneity from the ZP preparations by HPLC, essentially as previously described (Bleil & Wassarman, 1986; Rosiere & Wassarman, 1992).

Collection and Culture of Mouse Gametes. Ovulated eggs and two-cell embryos were collected from oviducts of superovulated female Swiss albino mice (CD-1; Charles River Breeding Labs) into Earle's modified medium 199 (Gibco) containing 25 mM Hepes, pH 7.3 (M199), supplemented with BSA (Sigma, fraction V; 4 mg/mL) and pyruvate (30 μg/mL) (M199-M), essentially as previously described (Bleil & Wassarman, 1980; Florman & Wassarman, 1985; Moller et al., 1990). Eggs were treated with hyaluronidase (1 mg/mL) to remove cumulus cells, fixed in 30 mM sodium phosphate, pH 7.2/150 mM NaCl/PVP-40 (4 mg/mL)/0.02% sodium azide (PBS−PVP) containing 1% formaldehyde, and stored for up to 1 week at 4 °C in M199, supplemented with 50 mM Tris-HCl, pH 7.5/PVP-40 (4 mg/mL)/0.02% sodium azide. Two-cell embryos were stored in a similar manner for up to 1 month. Sperm were collected from caudae epididymae of sexually mature male CD-1 mice into M199-M containing 4 mM EGTA, pelleted by low-speed centrifugation, and capacitated for 1 h in M199-M at 37 °C prior to incubation in the presence of test substances. Sperm concentration was adjusted to approximately 10^6 sperm/mL for each experiment by addition of 4−10 mL of M199-M to the pellet.

Sperm Binding Assay. All assays were carried out in M199-M at 37 °C in a humidified atmosphere of 5% CO_2 in air. Ovulated eggs and two-cell embryos were washed through 3 drops of M199-M before they were added to capacitated sperm. In each assay, an appropriate volume of saccharide was pipetted into a 500-μL Eppendorf tube, lyophilized, and redissolved in 2 μL of water to which 8 μL of warm (37 °C) M199-M was added. mZP3 was dissolved in warm (65 °C) water, and then 2 μL was added to 8 μL of M199-M. The 10-μL drops of saccharide and mZP3 ("test substances") were incubated at 37 °C under oil for 30−40 min, after which 10 μL of capacitated sperm were added and incubated in the presence of the test substances for 15 min at 37 °C. In parallel experiments, assays were performed with Gal (2 μM−5 mM), GlcNAc (2 μM−5 mM), mZP3 (5−10 ng/μL), and M199-M as controls. Twelve to 15 ovulated eggs and 1−3 two-cell embryos were then added to the sperm and were incubated for an additional 30−40 min at 37 °C. Sperm motility was monitored continuously, and preparations with less than 70% highly motile sperm were discarded. At the end of the incubation period, eggs and embryos with associated sperm were removed and washed through drops of M199-M, by using a wide-bore, mouth-operated micropipet, until no more than two sperm remained associated with the two-cell embryos, essentially as previously described (Bleil & Wassarman, 1980; Florman & Wassarman, 1985; Moller et al., 1990). Eggs with bound sperm were fixed immediately in 50% M199-M/50% PBS−PVP containing 3% glutaraldehyde, and the number of sperm bound per egg was determined by counting sperm tails in one plane of focus (typically 30−50 sperm/egg at the largest egg diameter) using dark-field phase microscopy. It should be noted that the assays using oligosaccharide constructs often were carried out in a "blind" fashion; i.e., the nature of the oligosaccharide was revealed only after the assays had been carried out a minimum of two times. The percent inhibition of sperm binding was calculated by comparing the number of sperm bound per egg in the presence of the test substance with the number bound in M199-M alone.

Acrosome Reaction Assay. To assess the status of the sperm acrosome, sperm were collected from the 20-μL test drops described above and were fixed in 5% formaldehyde−PBS, pH 7.2, overnight at 4 °C; washed by centrifugation at 5000 rpm for 5 min in 0.1 M ammonium acetate, pH 9.0; resuspended in the same buffer; and dried onto gelatin-coated slides. After the slides were washed in water, methanol, and water for 5 min each, sperm were stained with 0.04% Coomassie blue G-250 in 3.5% perchloric acid for 4 min, as previously described (Moller et al., 1990). Sperm were then observed under a coverslip, using PBS−30% glycerol as mounting medium, and were scored for the presence or absence of an intact acrosome by light microscopy. In some experiments, capacitated sperm were incubated in the presence of 10 μM ionophore A23187 (Calbiochem; 40 mM stock in DMSO, diluted 1:40 in PBS, pH 7.2, and made up in M199-M to 20 μM) and then scored for the presence or absence of an acrosome, as previously described (Moller et al., 1990).

RESULTS

Experimental Rationale. Mouse sperm preincubated in the presence of purified egg mZP3, mZP3-derived glycopeptides, or mZP3-derived O-linked oligosaccharides are prevented from binding to ovulated eggs *in vitro* in a dose-dependent manner (Bleil & Wassarman, 1980, 1988; Florman et al., 1983; Florman & Wassarman, 1985). The ID_{50} for mZP3 is approximately 100 nM, whereas it is approximately 10−100-fold higher for mZP3 glycopeptides and O-linked oligosaccharides. Previous results suggest that either Gal in α-linkage (Bleil & Wassarman, 1988) or GlcNAc in β-linkage (Miller et al., 1992) with the penultimate sugar at the nonreducing end of mZP3 O-linked oligosaccharides is essential for sperm binding. In view of these findings, here we examined the effect of various oligosaccharide constructs with defined structures on binding of mouse sperm to ovulated eggs *in vitro*. In each case, capacitated sperm were preincubated in the presence of oligosaccharides and chal-

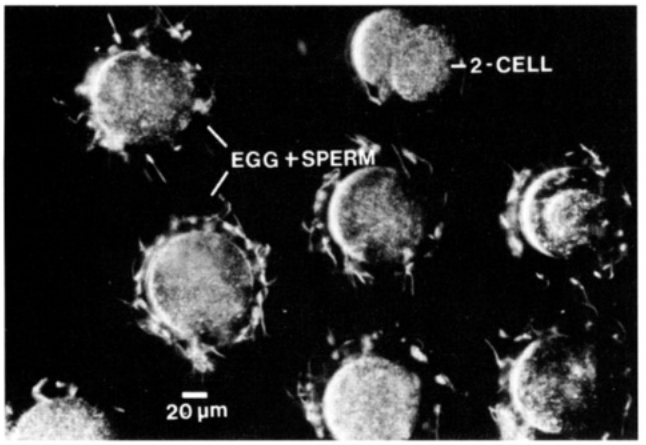

FIGURE 1: Photomicrograph of mouse gametes and two-cell embryos used in the competition assays. In this sample there are an average of 35 ± 5 sperm bound per egg ($n = 10$) in the largest plane of focus. The micrograph was taken using dark-field phase microscopy. The magnification bar equals 20 μm.

lenged with ovulated eggs and two-cell embryos, and the number of sperm bound to eggs was determined ("competition assay"; Figure 1), as described in Materials and Methods.

Effect of Blood Group Type B-Related Oligosaccharides. In view of previous results indicating the importance of a terminal α-Gal in mZP3 function, two blood group type B-related oligosaccharides (Watkins, 1980), a trisaccharide (**I**), and a pentasaccharide (**II**) (Figure 2), were tested for their ability to inhibit binding of sperm to eggs. Both oligosaccharides possess Gal in α-linkage at the nonreducing end and Fuc in α-linkage with the penultimate Gal residue. The effect of these oligosaccharides on sperm binding was compared with that of a disaccharide, melibiose (Galα1−6Glc), and two monosaccharides, Gal and GlcNAc, at concentrations ranging from 1 μM to 5 mM.

Whereas melibiose, Gal, and GlcNAc had no significant effect on the number of sperm bound to eggs, the blood group type B-related trisaccharide (**I**) and pentasaccharide (**II**) inhibited binding by 60 ± 34% ($n = 4$) and 96 ± 2% ($n = 4$), respectively, at 5 mM (ID_{50} of approximately 2 mM and 0.5 mM, respectively; determined from experiments carried out in the presence of 1 μM to 5 mM oligosaccharide) (Figure 3). In these experiments, sperm remained highly motile, but formed head-to-head aggregates in the presence of 5 mM pentasaccharide (Figure 4). Thus, binding of pentasaccharide to sperm resulted in aggregation and contributed to the relatively high level of inhibition of sperm binding to eggs observed at 5 mM (i.e., due to a significant reduction in the number of individual, free-swimming sperm). However, it should be noted that, even at lower concentrations of pentasaccharide, under conditions in which aggregation of sperm did not occur, binding of sperm to eggs also was significantly inhibited, albeit to a lesser extent (e.g., at 1 mM, 55 ± 8, $n = 4$, inhibition) (Figure 3). Similarly, no aggregation of sperm occurred in experiments carried out with the blood group type B-related trisaccharide, even at 5 mM.

Effect of Unbranched Oligosaccharides. Three unbranched oligosaccharide constructs were tested for their ability to inhibit binding of sperm to eggs: (i) a trisaccharide with GlcNAc in β-linkage (**III**), (ii) a tetrasaccharide with Gal in β-linkage (**IV**), and (iii) a pentasaccharide with Gal in α-linkage (**V**), all at the nonreducing end (Figure 2). At the relatively low concentrations employed, none of these

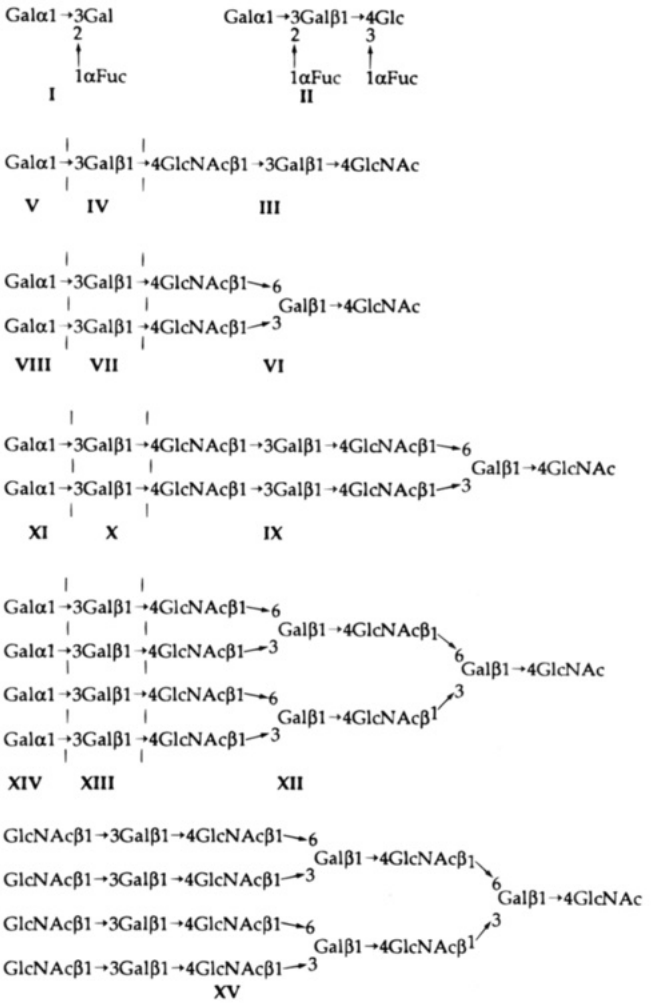

FIGURE 2: Structures of oligosaccharides used in the biological assays. Shown are the structures of two oligosaccharides obtained commercially (**I**, **II**) and 13 oligosaccharides (**III**−**XV**) constructed by Seppo et al. (1995), as described in Materials and Methods.

oligosaccharides had a significant effect on the number of sperm bound to eggs, even at 10 μM (Figure 5A).

Effect of Biantennary Blood Group I-Related Oligosaccharides. Two different classes of biantennary oligosaccharides were constructed for these experiments. One class included (i) a tetrasaccharide with GlcNAc in β-linkage (**VI**), (ii) a hexasaccharide with Gal in β-linkage (**VII**), and (iii) an octasaccharide with Gal in α-linkage (**VIII**), all at the nonreducing end (Figure 2). As with the unbranched oligosaccharides, none of these biantennary oligosaccharides had a significant effect on the number of sperm bound to eggs at the concentrations employed (Figure 5B).

A second class of biantennary oligosaccharides included (i) an octasaccharide with GlcNAc in β-linkage (**IX**), (ii) a decasaccharide with Gal in β-linkage (**X**), and (iii) a dodecasaccharide with Gal in α-linkage (**XI**), all at the nonreducing end (Figure 2). Whereas the oligosaccharide terminating in GlcNAc and Gal in β-linkage did not affect sperm binding (as above, **VI** and **VII**), the oligosaccharide terminating in α-Gal significantly inhibited binding of sperm to eggs (Figure 5C). For example, the latter inhibited sperm binding by 55 ± 23% ($n = 5$) at 10 μM. Therefore, increasing the chain length of the oligosaccharide branches from three (**VIII**) to five (**XI**) sugar residues significantly increased the affinity of the biantennary oligosaccharide for sperm.

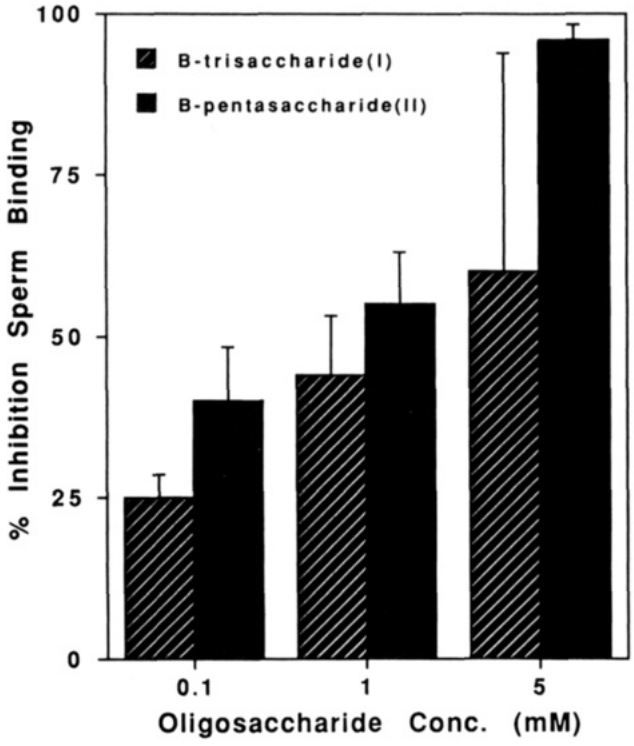

FIGURE 3: Effect of blood group type B-related oligosaccharides on binding of sperm to eggs. The competition assays were carried out as described in Materials and Methods in the presence of 0.1, 1, and 5 mM blood group type B-related trisaccharide (**I**) and pentasaccharide (**II**). Shown is the average percent inhibition of sperm binding (±SD) for three or four individual experiments at each oligosaccharide concentration.

Effect of Tetraantennary Blood Group I-Related Oligosaccharides. A set of three lactosaminoglycan-type tetraantennary oligosaccharides was constructed and tested *in vitro*. The set included (i) a decasaccharide with GlcNAc in β-linkage (**XII**), (ii) a tetradecasaccharide with Gal in β-linkage (**XIII**), and (iii) an octadecasaccharide with Gal in α-linkage (**XIV**), all at the nonreducing end (Figure 2). Oligosaccharides terminating in Gal, in either α- or β-linkage, inhibited binding of sperm to eggs in the concentration range 1−10 μM (Figure 6). The oligosaccharides terminating in α-Gal and β-Gal inhibited binding by 71 ± 14% ($n = 3$) and 76 ± 18% ($n = 3$), respectively, at 10 μM (ID_{50} of ~2.7 and ~4.0 μM, respectively, calculated from experiments carried out at six oligosaccharide concentrations, from 0.5 to 10 μM; for comparison, mZP3 has an $ID_{50} \leq 0.1$ μM). On the other hand, the oligosaccharide terminating in β-GlcNAc did not significantly affect the number of sperm bound to eggs (calculated $ID_{50} > 50$ mM) (Figure 6). [Note: As with the pentasaccharide (**II**) described above, head-to-head aggregates of sperm occassionally were observed with the tetradecasaccharide terminating in β-Gal (**XIII**) and octadecasaccharide (**XIV**) terminating in α-Gal; however, the oligosaccharides did not visibly affect sperm motility.]

In view of the major difference in effectiveness of tetraantennary oligosaccharides terminating in Gal and GlcNAc to inhibit binding of sperm to eggs, the contribution of chain length to the effect was evaluated. A tetraantennary octadecasaccharide with GlcNAc in β-linkage at the nonreducing end was constructed (**XV**) (Figure 2), and its ability to inhibit sperm binding was compared directly with that of the tetraantennary octadecasaccharide terminating in α-Gal (**XIV**). As seen in Figure 6, the oligosaccharide terminating in GlcNAc did not inhibit binding of sperm to eggs, even at 10 μM, and aggregation of sperm did not take place. Therefore, the nature of the sugar residue at the nonreducing end, rather than the oligosaccharide chain length, accounts for the differences in inhibition observed with tetraantennary oligosaccharides terminating in α-Gal and β-GlcNAc.

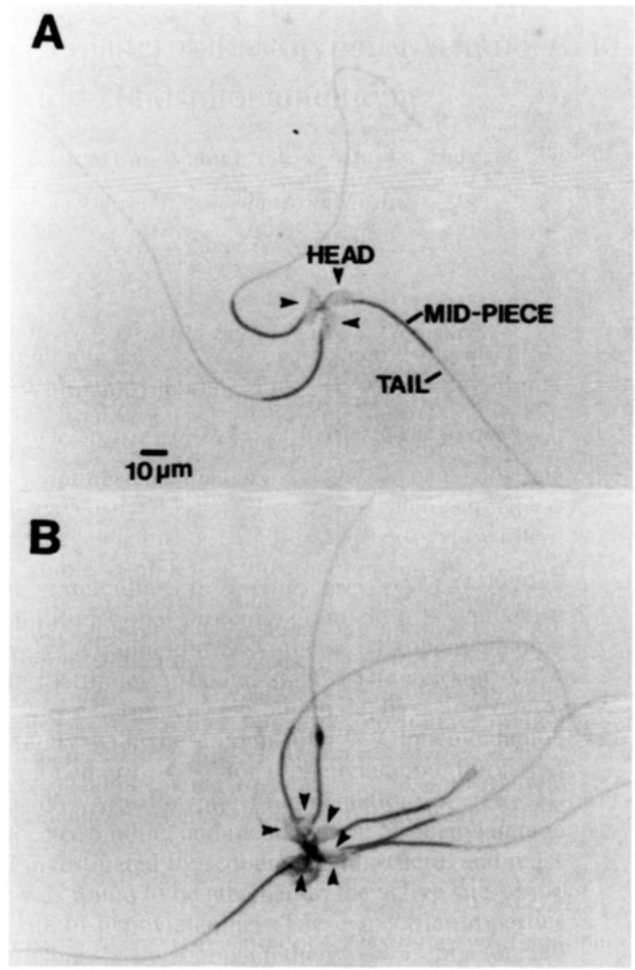

FIGURE 4: Photomicrographs of head-to-head aggregates of mouse sperm. Shown are two representative examples of sperm incubated in 5 mM blood group type B-related pentasaccharide (**II**) followed by fixation with formaldehyde and staining with Coomassie blue G-250, as described in Materials and Methods. Note that the sperm associate with each other by their heads (arrowheads), not by the mid-piece or tail. The magnification bar equals 10 μm.

Effect of Oligosaccharides on the Acrosome Reaction. Capacitated sperm that had been exposed to M199-M alone ("negative control"), either ionophore A23187 (10 μM) or mZP3 (~4−8 ZP/μL) ("positive controls"), Gal (20−80 μM), melibiose (5 mM), or four oligosaccharide constructs that inhibited binding of sperm to eggs [i.e., two blood group type B-related oligosaccharides (**I**, **II**; 5 mM) and two oligosaccharide constructs possessing Gal in α-linkage at the nonreducing end (**XI**, **XIV**; 10 μM)] were scored for the presence or absence of an intact acrosome. In these experiments, only ionophore A23187 and mZP3 induced sperm to undergo the acrosome reaction significantly above background levels (Figure 7). Therefore, although a few oligosaccharide constructs inhibit sperm binding, unlike mZP3, they do not induce sperm to undergo the acrosome reaction.

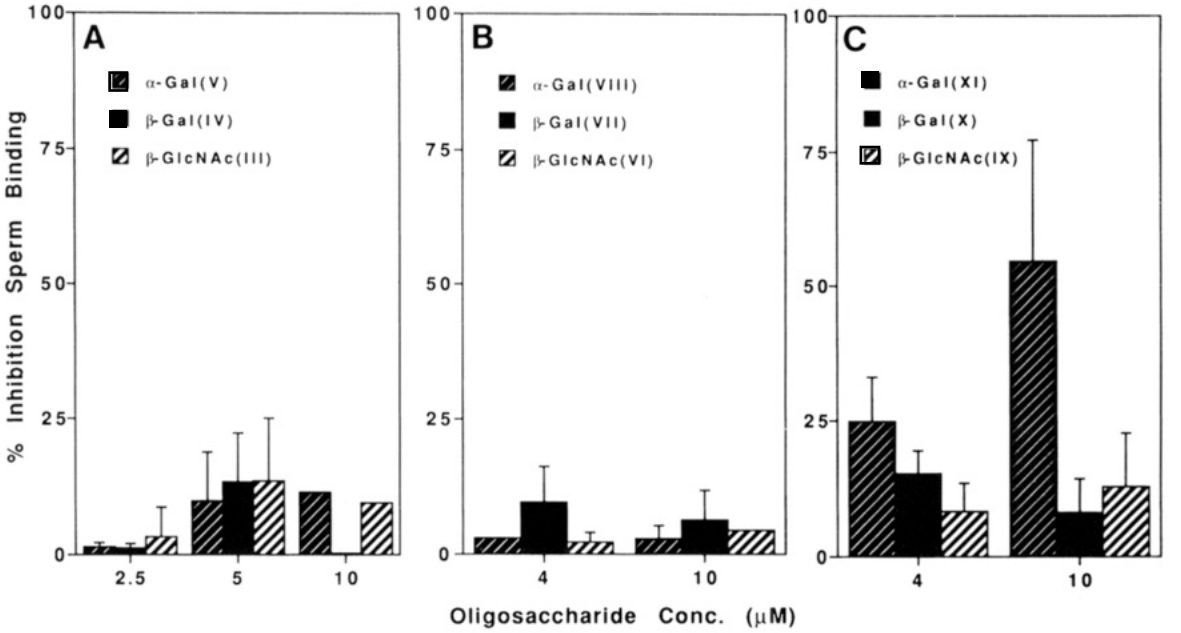

FIGURE 5: Effect of biantennary blood group I-related oligosaccharides on binding of sperm to eggs. The competition assays were carried out as described in Materials and Methods in the presence of 2.5, 5, and 10 μM unbranched oligosaccharides (panel A; **III**−**V**) and in the presence of 4 and 10 μM biantennary blood group I-related oligosaccharides (panels B and C; **VI**−**XI**). In most cases, shown is the average percent inhibition of sperm binding (±SD) for three to five individual experiments at each oligosaccharide concentration. In a few cases, the average of two experiments is shown without a calculated SD.

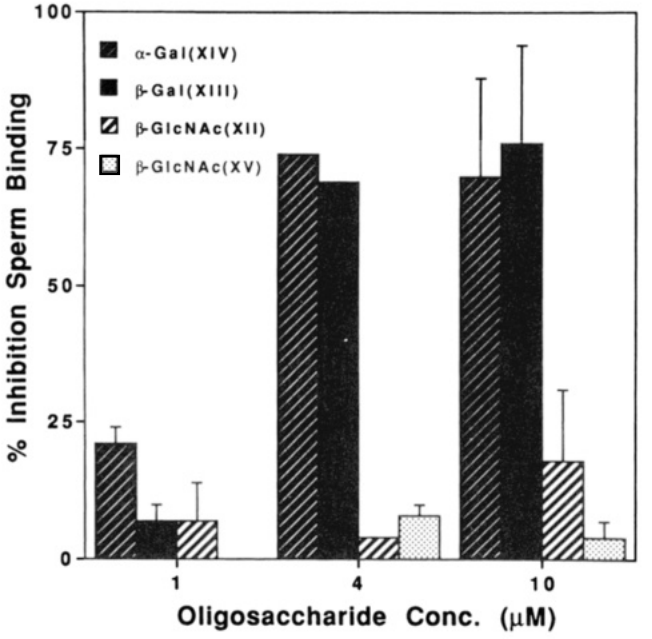

FIGURE 6: Effect of tetraantennary blood group I-related oligosaccharides on binding of sperm to eggs. The competition assays were carried out as described in Materials and Methods in the presence of 1, 4, and 10 μM tetraantennary blood group I-related oligosaccharides (**XII**−**XV**). In most cases, shown is the average percent inhibition of sperm binding (±SD) for three to five individual experiments at each oligosaccharide concentration. In a few cases, the average of two experiments is shown without a calculated SD.

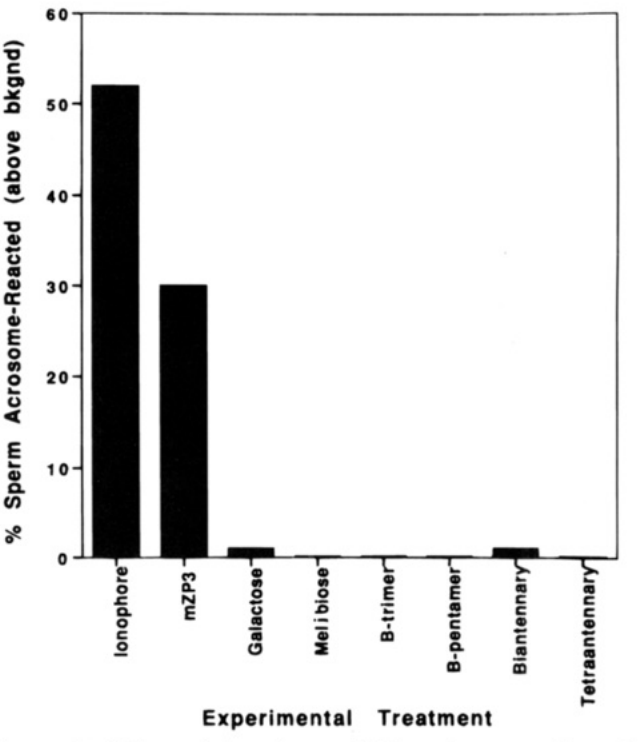

FIGURE 7: Effect of ionophore, mZP3, and mono-, di-, and oligosaccharides on the acrosome reaction. Samples stained with Coomassie blue G-250 were scored for the presence or absence of an intact acrosome by light microscopy, as described in Materials and Methods. In each case, a minimum of 200 sperm were evaluated ($n = 2-6$). Shown is the percent sperm acrosome-reacted (above background; i.e., the value for sperm incubated in M199-M alone) for sperm incubated in the presence of ionophore A23187 (20 μM), mZP3 (4−8 ZP equiv/μL), Gal (20−80 μM), melibiose (5 mM), B-trisaccharide (**I**; 5 mM), B-pentasaccharide (**II**; 5 mM), biantennary blood group I-related oligosaccharide (**XI**; 10 μM), and tetraantennary blood group I-related oligosaccharide (**XIV**; 10 μM).

DISCUSSION

Several lines of evidence suggest that gamete adhesion in mammals is carbohydrate-mediated (Macek & Shur, 1988; Wassarman, 1992; Litscher & Wassarman, 1993). This evidence includes reports that various monosaccharides, disaccharides, and fucoidan, a polysaccharide consisting primarily of sulfated fucose, inhibit binding of sperm to eggs *in vitro*. For example, GalNAc, GlcNAc, D-mannose, and α-methylmannoside at 50 mM, and fucoidan at 1 mg/mL, inhibit binding of rat sperm to eggs (Shalgi et al., 1986).

Similarly, GlcNAc, GalNAc, and ManNAc, at concentrations between 250 μM and 1 mM, and fucoidan at 50 μg/mL, inhibit binding of hamster sperm to eggs (Ahuja, 1982). Fucoidan at 500 μg/mL also inhibits binding of guinea pig sperm to eggs (Jones et al., 1988) and at 1 mg/mL inhibits binding of human sperm to eggs (Oehninger et al., 1990). In each of these cases, inhibition of gamete adhesion is attributable to binding of the monosaccharide or polysaccharide to sperm.

At concentrations in the range 50−100 nM, purified mZP3 binds to the sperm head and significantly inhibits binding of sperm to eggs *in vitro* (Bleil & Wassarman, 1980, 1986, 1988). However, in experiments reported here, most oligosaccharide constructs (**III**−**XV**; Figure 3) were tested at 4 and 10 μM (i.e., 40−200-fold higher concentrations than used with mZP3) for their ability to inhibit binding of sperm to eggs. While relatively high compared to mZP3 concentrations used in analogous experiments, these concentrations of oligosaccharides were chosen in view of the fact that small mZP3 glycopeptides (produced by extensive digestion with pronase) and O-linked oligosaccharides released from mZP3 (released by alkaline reduction) inhibit binding of sperm to eggs in this concentration range (Florman et al., 1984; Florman & Wassarman, 1985; Bleil & Wassarman, 1988). Apparently, the affinity of mZP3 glycopeptides and oligosaccharides for the mZP3-binding protein on sperm is substantially less (by 10−100-fold) than that of the intact glycoprotein. This situation is not unlike that found when K_as for binding of plant lectins to cells are compared with their K_as for binding to oligosaccharides (Lis & Sharon, 1986; Sharon & Lis, 1989). There are several possible explanations for this behavior, most of which relate to the conformation or the number of oligosaccharides (i.e., the valency) recognized at the mZP3 combining site for sperm. For example, since a glycoprotein's polypeptide can significantly affect the conformation and, hence, the presentation of bound oligosaccharides (Carver et al., 1989), the difference in affinities for bound and free mZP3 oligosaccharides is not unexpected. Free mZP3 oligosaccharides, as well as the oligosaccharide constructs used in the present study, might be expected to be more flexible in solution than when covalently linked to polypeptide (Carver et al., 1989; Homans, 1993) and, as a result, exhibit lowered affinity for binding sites on sperm.

Results presented here suggest that several parameters can influence the effectiveness of oligosaccharides as inhibitors of gamete adhesion in mice. These parameters include the nature of the monosaccharide at the nonreducing end of the oligosaccharides, the number of sugars in the oligosaccharides, and the branching pattern of the oligosaccharides. In view of the fact that sulfated or sialylated oligosaccharides were not used in this study, it should be noted that neither sulfate nor sialic acid appears to be essential for binding of mZP3 to sperm (C. Liu, E. Litscher, and P. Wassarman, unpublished results). This is unlike the situation in several analogous cellular adhesion systems, such as binding of L-selectin to its ligand GlyCAM-1 (Imai et al., 1992, 1993), where sulfate or sialic acid plays an essential role in binding.

The biantennary dodecasaccharide (**XI**) and tetraantennary octadecasaccharide (**XIV**) with Gal in α-linkage at their nonreducing end were very effective inhibitors of sperm binding to eggs (Figures 5C and 6). Similarly, the tetraantennary tetradecasaccharide (**XIII**) with Gal in β-linkage at its nonreducing end was a very good inhibitor, although the closely related biantennary decasaccharide (**X**) was not. Interestingly, the analogous oligosaccharide constructs (**IX**, **XII**, **XV**) with GlcNAc at their nonreducing end did not inhibit binding of sperm to eggs at equivalent concentrations. These findings emphasize the great importance of the sugar at the nonreducing end of oligosaccharides in determining their affinity for sperm. Such is the case for several other systems involving protein−carbohydrate interactions. The results provide further support for the proposal that Gal at the nonreducing end of oligosaccharides present at the mZP3 combining site plays an essential role in sperm binding (Bleil & Wassarman, 1988; Shalgi et al., 1990), but do not rule out a role for terminal GlcNAc residues (Shur & Hall, 1982; Miller et al., 1992) or other sugar residues as well. The finding that oligosaccharides with Gal in either α- or β-linkage are effective inhibitors is somewhat surprising. However, this could be due to oligosaccharides in solution (i.e., not bound to polypeptide) having enough flexibility to adopt some common topographical features (Spohr et al., 1985).

The monosaccharides Gal and GlcNAc and the disaccharide melibiose did not inhibit binding of sperm to eggs, even at 5 mM. Similarly, an unbranched oligosaccharide consisting of five sugars (**V**) and a biantennary oligosaccharide consisting of either six (**VII**) or eight (**VIII**) sugars, all with Gal at their nonreducing end, had no effect on sperm binding at 10 μM (Figure 5A,B). This is in contrast to a biantennary oligosaccharide consisting of 12 sugars (**XI**) and tetraantennary oligosaccharides consisting of either 14 (**XIII**) or 18 (**XIV**) sugars, all with Gal at their nonreducing end (in either α- or β-linkage), that significantly inhibited sperm binding at 4 and 10 μM (Figures 5C and 6). In this context, while the blood group type B-related trisaccharide (**I**) and the pentasaccharide (**II**) inhibit binding of sperm to eggs, they are only effective at relatively high concentrations ($\geq$1 mM and $\geq$50 μM, respectively) (Figure 3) as compared to the larger oligosaccharides (Figures 5 and 6). However, the fact that blood group type B-related tri- and pentasaccharides inhibit binding of sperm to eggs, albeit at relatively high concentrations, is consistent with previous results obtained using monoclonal antibody LA4, as well as a monoclonal antibody directed against blood group B antigens (Shalgi et al., 1990). In any case, overall, these findings suggest that the number of sugars in an oligosaccharide affects its ability to bind to sperm and, consequently, to interfere with binding of sperm to eggs.

While none of the unbranched oligosaccharides (**III**−**V**), even with Gal at the nonreducing end, inhibited sperm binding at 10 μM, a biantennary oligosaccharide (**XI**) and two tetraantennary oligosaccharides (**XIII**, **XIV**) with Gal at the nonreducing end were inhibitory at this concentration (Figures 5 and 6). Furthermore, a comparison of the biantennary (**XI**) and tetraantennary (**XIV**) oligosaccharides with Gal in α-linkage at their nonreducing end reveals that the latter is a slightly more effective inhibitor than the former. In this context, it has been reported that mammalian hepatic Gal/GlcNAc receptors, which recognize nonreducing terminal sugars, much prefer triantennary oligosaccharides over biantennary and unbranched oligosaccharides (Lee et al., 1983, 1984; Lee, 1989, 1992). For these receptors, the K_d values for unbranched oligosaccharides are generally in the millimolar range, whereas those for biantennary and trian-

tennary oligosaccharides are in the micro- and nanomolar range, respectively. Interestingly, there is very little difference in binding affinities between triantennary oligosaccharides and their tetraantennary counterparts. Finally, it should be noted that the extent of oligosaccharide branching can affect a variety of biological activities (Rasmussen, 1991). For example, it has been reported that the activity of recombinant erythropoietin containing tetraantennary N-linked oligosaccharides is significantly higher than that of erythropoietin containing biantennary oligosaccharides (Takeuchi et al., 1989).

Following binding of mZP3 to plasma membrane overlying the heads of acrosome-intact sperm (Saling et al., 1979; Saling & Storey, 1979; Bleil & Wassarman, 1986; Mortillo & Wassarman, 1991), sperm undergo the acrosome reaction (Bleil & Wassarman, 1983; Kopf & Gerton, 1991). Results of acrosome reaction assays reported here (Figure 7) are consistent with previous findings that, while small (1.5−6 kDa) mZP3 glycopeptides and O-linked oligosaccharides released from mZP3 inhibit binding of sperm to eggs, they do not induce sperm to undergo the acrosome reaction *in vitro* (Florman et al., 1983; Florman & Wassarman, 1985; Wassarman et al., 1985; Leyton & Saling, 1989a). It has been proposed that the acrosome reaction fails to take place due to the inability of small mZP3 glycopeptides and released O-linked oligosaccharides to aggregate the sperm plasma membrane protein(s) to which they bind (Wassarman et al., 1985; Leyton & Saling, 1989a; Bleil, 1991; Kopf & Gerton, 1991). In this context, there is ultrastructural evidence to suggest that massive lateral displacement of transmembrane glycoproteins on the sperm surface, overlying the acrosome, is an early step in the acrosome reaction (Aguas & Pinto da Silva, 1989).

The significance of results presented here will be further enhanced when the question of the nature of the mZP3-binding protein on sperm is finally resolved (Ward & Kopf, 1993; Litscher & Wassarman, 1993). At present there are several candidate mZP3-binding proteins, including sp56 (Bleil & Wassarman, 1990; Cheng et al., 1994), β-1,4-galactosyltransferase (Shur & Hall, 1982; Miller et al., 1992), p95 (Leyton & Saling, 1989b), and PH20 (Primakoff et al., 1985; Lathrop et al., 1990). Two of these candidates, sp56 and β-1,4-galactosyltransferase, are reportedly sperm head plasma membrane proteins that recognize and bind specifically to certain mZP3 O-linked oligosaccharides; sp56 recognizes terminal Gal residues, whereas β-1,4-galactosyltransferase recognizes terminal GlcNAc residues. The two other candidates, p95 and PH20, have recently been shown to be a unique form of hexokinase that is phosphorylated on tyrosine residues (Kalab et al., 1994) and hyaluronidase (Gmachi & Kreil, 1993), respectively. Thus, at present it is unclear how many subunits make up the mZP3-binding protein, how many sugar-binding sites are present per subunit, and whether or not the binding protein has a consensus carbohydrate-recognition domain (CRD; Drickamer & Taylor, 1993).

In conclusion, results presented here are consistent with previous evidence indicating that free-swimming sperm bind to mZP3 O-linked oligosaccharides possessing a Gal residue in α-linkage at their nonreducing end (i.e., related to blood group B-type oligosaccharides). The binding is relatively tight as compared to some analogous adhesion systems, such as the binding of selectins to their ligands (van der Merwe & Barclay, 1994). Previous estimates of the apparent molecular weight of the essential mZP3 oligosaccharides (~3.9 kDa; Florman & Wassarman, 1985; Bleil & Wassarman, 1988; Miller et al., 1992) suggest that they could consist of as many as 20 sugar residues. However, in view of the aberrant behavior of O-linked oligosaccharides on gel-exclusion chromatography (Kobata, 1994; E. Litscher and P. Wassarman, unpublished results), it is likely that the mZP3 oligosaccharides are significantly smaller than initially thought. Thus, the oligosaccharide constructs used in this study appear to be reasonable analogues of the native oligosaccharides and should prove to be effective probes of the carbohydrate-binding domain of the mZP3-binding protein associated with acrosome-intact sperm.

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