Direct evidence for the involvement of carbohydrate sequences in human sperm–zona pellucida binding

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Several lines of evidence indicate that mammalian fertilization is initiated via a binding process that is dependent upon the recognition of oligosaccharide sequences associated with zona pellucida (ZP) glycoproteins. Here, specific chemical and enzymatic methods were employed to modify human ZP and to test their effects on sperm binding in the hemizona assay system (HZA). Periodate oxidation of human ZP under very mild conditions (10 min, 0°C, 1 mM sodium m-periodate) that attacks only terminal sialic acid resulted in a 30% loss of human sperm binding in the HZA (hemizona index (H2I) = 70.2 ± 10.9, n = 22; P < 0.05). Periodate oxidation under mild conditions (1 h, 23°C, 10 mM sodium m-periodate) caused a 40% decrease in binding (H2I = 60.8 ± 10.3; n = 24; P < 0.01). Treatment of human ZP with neuraminidase caused a substantial increase in sperm binding to human ZP (H2I = 297 ± 45, n = 22; P < 0.01). These findings indicate that there are sialic acid dependent binding sites coexisting with binding sites that are obscured by sialic acid. To determine the periodate sensitivity of these obscured sites, hemizons were first digested with neuraminidase and subsequently subjected to mild periodate oxidation. The combined enzymatic and chemical treatments caused a 79% decrease in sperm binding compared to control hemizona (H2I = 20.7 ± 4.4, n = 16; P < 0.001). Human sperm–ZP interaction was also increased by digestion of human ZP with endo-β-galactosidase (H2I = 710 ± 232, n = 14; P < 0.01), indicating that potential binding sites for spermatozoa are also obscured by lactosaminoglycan sequences. These studies support a definitive role for the involvement of ZP-associated glycans in the binding of human spermatozoa to oocytes.

Key words: carbohydrates/glycoconjugates/hemizona/sperm–zona binding/zona pellucida

Introduction

It has been proposed that mammalian gamete binding requires the appropriate recognition of specific carbohydrate sequences expressed on the zona pellucida (ZP) by lectin-like proteins associated with the plasma membranes of mature spermatozoa (Macek and Shur, 1988; Miller and Ax, 1990; Wassarman, 1990). The best characterized mammalian sperm–oocyte binding system is the mouse model (reviewed in Wassarman, 1990), although the human interaction is also being investigated by several groups (Chapman and Barratt, 1996; Clark et al., 1996a; Whitmarsh et al., 1996; Benoff et al., 1997).

Two models have been proposed to define the molecular basis for the gamete interaction in the murine system. Initial studies by Wassarman and colleagues indicates that the O-linked ZP oligosaccharides terminating with Galβ1–3Galα1–4GlcNAC– sequences are responsible for mediating sperm–oocyte binding in this system (Bleil and Wassarman, 1988). Consistent with this hypothesis, mouse spermatozoa will bind very tightly to rabbit erythrocytes known to express substantial amounts of terminal Galβ1–3Galβ1–4GlcNAC– sequences (Sandow and Clark, 1993; Clark et al., 1996b). This result provides further evidence in support of the carbohydrate binding specificity that was previously proposed (Wassarman, 1990). However, recent knockout mouse studies indicate that there is not an obligatory requirement for the expression of terminal α1–3 galactose sequence for fertility (Thall et al., 1995). Nevertheless, previous studies indicate that the removal of terminal α-linked galactose from rabbit erythrocytes reduces but does not eliminate their binding affinity for murine spermatozoa (Clark et al., 1996b). Similarly, removal of terminal α-linked galactose from synthetic branched polylactosamine type glycans did not completely abrogate their ability to inhibit murine sperm–oocyte binding (Litscher et al., 1995).

In the second model, Shur and colleagues have suggested that murine gamete adhesion occurs when a specific β-galactosyltransferase expressed on the surface of the mouse spermatozoon interacts with oligosaccharides bearing terminal β-linked N-acetylgalactosamine (GlcNAc) or N-acetylgalactosamine (GlcNAC) sequences associated with the ZP (reviewed in Macek and Shur, 1988; Miller et al., 1994). Therefore data regarding the identity of the terminal (mono)saccharides involved in murine sperm–oocyte binding is equivocal.

The scarcity of available human oocytes poses a major hurdle for investigating the human gamete binding interaction at the molecular level. To circumvent this problem, the human hemizona assay (HZA) system has been used to evaluate and characterize the potential oligosaccharide sequences respons-
Carbohydrate dependent human sperm–zona binding

Human spermatozoa were collected by masturbation from six healthy fertile male donors and separated from semen by using an established swim-up procedure (reviewed in Oehninger et al., 1992a). Motion parameters were assessed using a computer analyser with fixed parameter settings (Hamilton–Thorne, Danvers, MS, USA). Only samples with progressive motility >75% after swim-up were employed in this study (% progressive motility = 80 ± 1.5, n = 24) (Oehninger et al., 1992a). The sperm concentration employed in all studies was 1×10⁶/ml.

Oocyte microbisection and HZA

Oocytes were removed from salt storage and rinsed 5–10 times in phosphate-buffered saline (PBS; 20 mM sodium phosphate buffer, pH 7.4 containing 150 mM sodium chloride). Micromanipulators (Narishige Tokyo, Japan) were mounted on a phase contrast inverted microscope (Nikon Diaphot, Garden City, NY, USA) and used for cutting the oocytes. Detailed descriptions of the HZA procedure have been reported (Burkman et al., 1988; Franken et al., 1989). The results observed with the binding are presented both as the total number of spermatozoa tightly bound for the test hemizona (treated hemizona) and the control hemizona (untreated matching hemizona) as well as the hemizona index or HZI (calculated as follows: number of spermatozoa bound for test hemizona/control×100). Values associated with a HZI <100 indicate inhibition of binding whereas a HZI >100 demonstrates stimulation of binding.

Statistical analysis of comparison of sperm–ZP binding for test conditions was performed using the paired t-test. The HZI results obtained under test conditions were compared to the HZI for interassay variation (100 ± 8.2, n = 46) to determine significance. P < 0.05 was considered significant.

Periodate oxidation of human hemizona

For mild periodate oxidation, a test hemizona was incubated in 25 μl of freshly made 10 mM sodium m-periodate in PBS for 1 h at 23°C in the dark. The reaction was terminated by adding 5 μl of 0.5 M ethylene glycol dissolved in PBS. The matching control hemizona was incubated under identical conditions except that the ethylene glycol solution was added immediately at the beginning of the reaction to inactivate the periodate. This type of control was performed to assure that at the end of the assay there would be exposure of both the control and test hemizona to the same chemicals, except that the test would contain active periodate for the period indicated. After this reaction, the periodate-oxidized hemizona and its matching control were washed three times in PBS and transferred to a freshly made solution of 0.1 M sodium borohydride. After 3 h incubation, the hemizona were washed five times in PBS. The matching treated and control hemizonae were either used in FITC–lectin binding studies or transferred to Ham’s F-10 medium containing 0.3% human serum albumin (w/v) for the HZA.

Conditions for very mild periodate oxidation specific for terminal sialic acid involved incubation of test and control hemizonae in 1 mM sodium m-periodate for 10 min at 0°C in the dark. The hemizonae were subsequently washed and reduced with sodium borohydride exactly as described for the mild periodate oxidation.

Neuraminidase and endo-β-galactosidase digestion of human ZP

Human hemizonae or whole human oocytes were digested in 1 unit/ml neuraminidase for 10 h in PBS. Digestion of whole oocytes or hemizona with endo-β-galactosidase was carried out for 10 h under the

Materials and methods

Lectins, chemicals and enzymes

Fluorescein isothiocyanate (FITC)-labelled lectins from Maackia amurensis (FITC-MAA), wheat germ agglutinin (FITC-WGA) and pokeweed mitogen (FITC-MAA) were purchased from E-Y Laboratories (San Mateo, CA, USA). Human serum albumin was obtained from Irvine Scientific (Santa Anna, CA, USA). Human serum albumin from Gibco Laboratories (Grand Island, NY, USA). Ham’s F-10 medium was obtained from Hyclone (Logan, UT, USA). Neuraminidase from Clostridium perfringens (Type X), α-sialyllactose, sodium m-periodate and all other chemicals and reagents were obtained from Sigma (St Louis, MO, USA).

Biological materials and reagents

Human oocytes used in this study were obtained from patients undergoing in-vitro fertilization therapy following signed consent under institutional review board approved guidelines at this institution. The oocytes were obtained from antral follicles >12 mm in diameter and were fully grown at the time of collection. The zona-intact, immature oocytes (prophase I, with a germinal vesicle present or that had not undergone germinal vesicle breakdown upon 24 h in culture) were denuded of granulosa cells by pipetting, placed in small plastic vials containing 0.5 ml of 1.5 M MgCl₂ plus 0.1% polyvinylpyrrolidone (w/v) in 40 mM HEPES buffer, pH 7.4, and stored at 4°C for 10–60 days. This hypotonic salt solution has been shown to retain both biochemical and biological properties associated with the ZP when tested in sperm–ZP binding tests including the HZA (Yangamichi et al., 1979; Oehninger et al. 1992a).

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(Continued)
same buffer conditions described previously (Litscher and Wassarman, 1996). However, the concentration of this endoglycosidase was boosted to 4 units/ml to facilitate digestion of both linear and branched polylactosaminoglycans (Fukuda et al., 1984). The matching control hemizonas were treated identically except that the neuraminidase and endo-β-galactosidase were inactivated by boiling for 3 and 5 min, respectively. The glycosidase-treated hemizona and matching control were washed three times in PBS and subjected to analysis as indicated. In a separate pilot study, it was found that the inclusion of the boiled enzyme alone in the test incubations yielded no significant difference from normal intra-assay variation defined previously (HZI = 98 ± 6.5, n = 6).

**Table I.** Effect of different chemical and enzymatic treatments on sperm binding in the hemizona assay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hemizona index</th>
<th>No. of spermatozoa bound to control hemizona&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of spermatozoa bound to test hemizona&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. of eggs</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild periodate oxidation</td>
<td>61 ± 10</td>
<td>73 ± 23</td>
<td>26 ± 4</td>
<td>22</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Very mild periodate oxidation</td>
<td>70 ± 11</td>
<td>42 ± 16</td>
<td>36 ± 11</td>
<td>24</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>297 ± 45</td>
<td>60 ± 10</td>
<td>150 ± 22</td>
<td>22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neuraminidase + mild periodate oxidation</td>
<td>207 ± 4.4</td>
<td>76 ± 10</td>
<td>12 ± 3</td>
<td>16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endo-β-galactosidase</td>
<td>710 ± 232</td>
<td>28 ± 10</td>
<td>104 ± 25</td>
<td>14</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Number of spermatozoa bound is rounded to the whole number.

<sup>a</sup>Based on a statistical comparison of the conditional hemizona index (HZI) with the HZI obtained for inter assay variation.

**Labelling of ZP with FITC-labelled lectins**

The interaction of human oocytes with FITC-labelled lectins was analysed as previously described (Patankar et al., 1997). Salt-stored human oocytes were transferred with a micropipette to 50–100 µl of FITC-labelled lectin dissolved in PBS (final lectin concentration: 5 µg/ml). After 10 min at 23°C, the oocytes were washed five times in PBS, fixed on a slide, immersed in a drop of Antiquench, and covered with a glass coverslip. The oocytes were immediately viewed and photographed under a Nikon Microphot FX microscope equipped with a 627 nm bandpass filter and a FX-35WA camera. The results of lectin binding studies were analysed by direct visualization under the microscope to obtain a score [no binding (–); maximum binding (+ + + +)]. This score was also confirmed by determining the amount of exposure time in seconds required for photography when the microscope was set to the automatic mode to take pictures. This method gives an estimate of the relative fluorescent intensity of the object being viewed and could be correlated by visual inspection.

**Results**

**Effect of periodate oxidation**

Mild oxidation of human ZP was performed in the presence of 10 mM sodium m-periodate at 23°C for 1 h to attack all vicinal hydroxyl groups on either ring structures or straight chains. Comparison of binding using the HZA approach indicated that there was an ~40% loss of sperm binding to hemizonae after this chemical treatment (Table I). This loss of binding was also reflected in the average number of spermatozoa bound to the test hemizona and its matching half (Table I). Oxidation of the ZP under very mild conditions that attack only terminal sialic acid residues resulted in a 30% loss in sperm binding based on HZA data (Table I). These results demonstrated that periodate oxidation under either mild or very mild conditions led to significant inhibition of human sperm binding. However, the data did not prove that the majority of the binding sites were carbohydrate dependent.

To confirm that periodate oxidation had chemically modified the carbohydrate sequences on the surface of the hemizonae, the effect of periodate oxidation on the binding of lectin binding to oocytes was determined (Table II). MAA lectin was chosen because it has affinity for glycoconjugates terminated with α2–3-linked sialic acid. WGA has also been reported to interact with sialylated glycans, although it can also bind to other glycoconjugates (reviewed in Osawa and Tsuji, 1987). Both lectins were bound to untreated oocytes, although WGA was slightly more fluorescent (Table I).

Both mild and very mild periodate oxidation of human oocytes led to a very substantial decrease in the binding of FITC-MAA, but did not influence FITC-WGA binding at all (Table II). The reversibility of FITC-MAA binding with a specific oligosaccharide inhibitor (10 mM 3'-sialyllactose) indicated that the interaction was carbohydrate dependent (Table II).

**Effect of glycosidase treatments**

The susceptibility of human sperm–ZP binding to very mild periodate oxidation suggested that terminal sialic acid could also be involved in some of the binding sites that mediate human sperm–ZP interaction. Therefore digestion of human ZP with neuraminidase should also result in a similar loss in sperm binding. To ensure that digestion with neuraminidase actually occurred, the binding of FITC-MAA to oocytes was also monitored after digestion with neuraminidase. In a pilot study, human eggs were digested with 1 unit/ml of neuraminidase for 5, 10 and 18 h. Digestion of terminal sialic acid was monitored by the loss of binding of FITC-MAA. The loss of binding was optimal at 10 h, reducing lectin binding to the level shown in Table II. Addition of another unit/ml of fresh neuraminidase after 10 h of digestion did not further reduce FITC-MAA binding analysed at the 18 h time point, indicating that optimal digestion had taken place. Confidence in the result of neuraminidase digestion was confirmed by the observation that the effect of neuraminidase treatment on FITC-MAA binding was basically equivalent to the reduction in FITC-
MAA binding obtained by either mild or very mild periodate oxidation (Table II). However, instead of decreasing human sperm–ZP binding as anticipated, neuraminidase digestion increased this interaction by roughly 3-fold (Table I). This result indicated that not only did sialic acid residues mediate initial human sperm–ZP binding but that they also obscured potential binding sites for spermatozoa.

To determine if these binding sites exposed by neuraminidase treatment were also carbohydrate dependent, both test and control hemizonae were digested with neuraminidase. The test hemizonae were subjected to mild periodate oxidation to attack all monosaccharides expressing vicinal hydroxyl groups on surface glycans. When this experiment was performed, there was a 79% reduction in human sperm binding, indicating that not only did sialic acid residues mediate human sperm–ZP binding as anticipated, neuraminidase digestion increased this interaction by roughly 3-fold (Table I). This result indicated that not only did sialic acid residues mediate initial human sperm–ZP binding but that they also obscured potential binding sites for spermatozoa.

Previous studies have clearly indicated the presence of lactosaminoglycan sequences on murine and porcine ZP (Hokke et al., 1994; Nagdas et al., 1994). Therefore human hemizona were digested with a specific enzyme that degrades lactosaminoglycans (endo-β-galactosidase) by cleaving internal β1-4-linked galactose residues within these repeating co-polymers (Fukuda et al., 1984). Digestion with this endoglycosidase also caused a 4–7-fold increase in human sperm–ZP binding (Table I). Previous studies indicate that WGA can also bind to lactosaminoglycan type chains (Clark and Ivatt, 1984; Gallagher et al., 1985). However, FITC-WGA binding to the human ZP was not altered by digestion with endo-β-galactosidase (Table II). PWM is another lectin that can specifically bind to lactosaminoglycans bearing β1–6-linked N-acetyllactosamine branches (Irimura and Nicolson, 1983). No binding of this lectin to intact human oocytes was observed either before or after endo-β-galactosidase digestion (Table II).

### Discussion

The functions of the human ZP include: (i) mediation of the initial binding with spermatozoa; (ii) triggering of the acrosome reaction; (iii) mediation of the secondary binding with the inner acrosomal membrane once it is exposed; (iv) block to polyspermy; and (v) physical protection of the developing embryo from potential immune or inflammatory responses via expression of carbohydrate sequences that are jointly recognized by the immune and gamete binding systems (Patankar et al., 1993; Clark et al., 1995, 1996a, 1997; Clark and Patankar, 1997). The ZP-specific expression of glycans linked to suppression of NK cell-mediated responses (Patankar et al., 1997) and the association of glycoproteins that regulate complement-mediated cytolsis with this extracellular matrix (Roberts et al., 1992; reviewed in Vince and Johnson, 1995) suggest the possibility that specific immunoprotective effects are also associated with the human ZP.

Chemical treatment with sodium m-periodate under carefully controlled conditions has been useful for determining if carbohydrate sequences are required in biological processes (Van Lenten and Ashwell, 1971; Vacquier and Moy, 1977; Kalyan et al., 1982). In this study, only relatively low concentrations (1–10 mM) of sodium m-periodate were used to ensure specific oxidation of sugars. Periodate oxidation under mild conditions (10 mM, 1 h, 23°C) selectively cleaves C–C bonds presenting vicinal hydroxyl groups within carbohydrate chains, generating aldehydes on both sides where the original C–C bond existed. Aldehydes are highly reactive, so in this study they were converted back to alcohols by reduction with sodium borohydride. Since proteins do not present vicinal hydroxyl groups, periodate oxidation under controlled conditions does not affect amino acid sequences.

Treatment with very mild periodate (1 mM, 0°C, 10 min) preferentially oxidizes the C–C bond between C7 and the C8 group of sialic acids, splitting off C-8 and C-9 as glycoaldehyde (Van Lenten and Ashwell, 1971). Vicinal hydroxyl groups attached to ring sugars are left intact (Van Lenten and Ashwell, 1971). Therefore this chemical treatment is highly specific for terminal sialic acids.

The results indicate that periodate treatment under both mild and very mild conditions led to a loss of ~30–40% of control sperm binding to hemizona. The most significant implication

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### Table II. Effect of different treatments on lectin binding to human oocytesa

<table>
<thead>
<tr>
<th>Lectin tested</th>
<th>Treatment</th>
<th>Control ZP fluorescence</th>
<th>Test ZP fluorescence</th>
<th>Mean exposure time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC-MAA</td>
<td>Mild periodate oxidation</td>
<td>+++</td>
<td>+/-</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Very mild periodate oxidation</td>
<td>+++</td>
<td>+/-</td>
<td>7</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>3′-Sialyl lactose</td>
<td>+++</td>
<td>+/-</td>
<td>6</td>
</tr>
<tr>
<td>FITC-WGA</td>
<td>Mild oxidation</td>
<td>++++</td>
<td>++++</td>
<td>5</td>
</tr>
<tr>
<td>Endo-β-galactosidase</td>
<td>–</td>
<td>–</td>
<td>&gt;45</td>
<td>&gt;45</td>
</tr>
<tr>
<td>FITC-PWM</td>
<td>Endo-β-galactosidase</td>
<td>–</td>
<td>–</td>
<td>&gt;45</td>
</tr>
</tbody>
</table>

*aScale followed for measuring fluorescence of the bound lectin was determined by the visual inspection and the exposure time required by the fluorescence scope when set on the automatic mode to take photographs. The scale for visual inspection correlated with the exposure time as follows: (+ + + + +) 1–5 s; (+ + + ) 6–10 s; (+ +) 11–20 s; (+) 21–30 s; (+/−) 31–40 s; (−) >40 s. The data obtained from visual inspection and mean exposure time were obtained with five separate oocytes for each condition tested. ZP = zona pellucida; FITC = fluorescein isothiocyanate; MAA = *Maackia amurensis*; WGA = wheat germ agglutinin; PWM = pokeweed mitogen.*
of these studies is that terminal sialic acid may participate in human sperm–ZP binding. However, the term ‘sialic acid’ is actually a broad term encompassing over 30 different molecular forms based upon specific modifications like O-acetylation, sulphation, phosphorylation and N-glycolylation (Reuter and Schauer, 1994). The sensitivity of sperm binding to very mild periodate oxidation does indicate that the hydroxyl groups at C-7 and C-8 of these terminal sialic acid residues are not acetylated, sulphated, phosphorylated or N-glycolylated.

The precise form of sialic acid that participates in this interaction has not been determined. Biophysical analysis was not pursued because of the small amount of eggs available. The logistics that preclude such an analysis are as follows. Exact figures are not available, but, based on comparisons with mouse ZP, it is likely the human ZP contains no more than 10 ng of glycoprotein. If 50% of the mass is carbohydrate (not an unreasonable expectation based on gel electrophoresis and molecular analyses), then only 5 ng of carbohydrate is associated with each ZP. If sialic acid constitutes 5–10% of the total mass of the glycans, then each human ZP contains 0.25–0.5 ng (0.75–1.5 pmol) of this monosaccharide. Since the lower limit for sialic acid detection by the most sensitive biophysical methods is ~100 ng, such an analysis could not be performed on a single egg or hemizona. Therefore in this study a lectin probe specific for terminal α2–3-linked sialic acid (FITC-MAA) was used to demonstrate the oxidation of sialic acid under both mild and very mild oxidative conditions. In addition, this lectin was also employed to demonstrate enzymatic removal of terminal sialic acid residues (Table II).

The inability of periodate oxidation to inhibit human sperm binding completely could be due to a variety of different factors. First, the majority of the binding sites may be present on internal carbohydrate sequences that are resistant to periodate treatment because they lack vicinal hydroxyl groups. Secondly, ZP glycans that act as ligands for sperm binding could be sulphated or phosphorylated, thus presenting a strong negative charge that blocks periodate action. Oligosaccharides not involved in human sperm–ZP binding could also inhibit periodate action if they are in close proximity to the binding glycans. Finally, the possibility exists that there is protein–protein interaction that works in conjunction with the carbohydrate interaction to mediate sperm binding. The existence of a zona receptor kinase (ZRK) with a putative domain that interacts with recombinant human ZP3 has been proposed (Burks et al., 1995).

Digestion of human ZP with neuraminidase caused a substantial increase in human sperm binding. A previous study indicated that a 10-fold lower digestion time with neuraminidase obtained from another commercial supplier (Oxford Glycosystems, UK) did not affect sperm–ZP binding in the HZA (Clark et al., 1995). However, the appropriate control studies were not undertaken to define the efficacy of the neuraminidase treatment using lectin probes as performed in this study. It is now apparent that either removal or chemical modification of terminal sialic acid residues greatly influences human sperm–ZP binding.

The results of the periodate oxidation and neuraminidase digestion indicate that there may exist two modes for human sperm–ZP binding. The first mode depends in part upon the binding of terminal sialic acid, probably α2–3-linked sialic acid based on the very strong binding of FITC-MAA. The second mode is independent of this terminal sugar. Binding in the presence of terminal sialic acid is weaker, either because of lower affinity or decreased numbers of binding sites of this type.

The effect of desialylation is particularly significant when considered in the context of the results obtained in other studies. Desialylation of human spermatozoa has been shown to lead to a 3-fold increase in their binding to the ZP (Lassalle and Testart, 1995). Recent evidence also indicates that there is a sialic acid binding protein of uterine origin that induces desialylation of the human sperm surface, probably by activating a neuraminidase activity on the plasma membrane (Banerjee and Chowdhury, 1997). Thus desialylation of either spermatozoa or ZP leads to substantial increases in sperm–ZP binding.

The majority (79%) of the sperm binding sites exposed by neuraminidase digestion are sensitive to mild periodate oxidation. This result combined with other studies suggests a new carbohydrate dependent model for human gamete interaction. When the spermatozoon encounters the oocyte, the neuraminidase located on the plasma membrane of spermatozoa interacts first with sialylated oligosaccharides, providing sufficient affinity for initial weak ‘attachment’ of the spermatozoon. In this model, the sperm surface neuraminidase then removes terminal sialic acid, enabling access of the other lectin-like protein(s) that can bind glycan sequences located beneath the sialylated sequences. This process may be facilitated by oviductin, a glycoprotein that binds to the ZP in the oviduct and greatly enhances this interaction (O’Day-Bowman et al., 1996). Thus the transition between loose sperm ‘attachment’ and subsequent ‘tight’ primary binding could be explained in the human by this ‘active digestion’ model combined with other glycoproteins that promote adhesion.

There is some indirect evidence that supports this model. Optimal sperm binding to the ZP requires an extended (4 h) incubation (Burkman et al., 1988). The HZA when applied as it is in our studies is considered to be a measure of tight binding (Burkman et al., 1988; Oehninger et al., 1992a) that leads to the induction of the acrosome reaction (Franken et al., 1991). Thus the kinetics of sperm binding to ZP are consistent with the ‘active digestion’ model. However, further study will be required to confirm the validity of this new paradigm.

The results with endo-β-galactosidase are also significant. Litscher and Wassarman (1996) isolated a restricted 55 kDa lactosaminoglycan-containing glycopeptide from mouse ZP3 glycoprotein that blocked murine sperm–ZP binding. Digestion of this glycopeptide with endo-β-galactosidase resulted in a substantial loss of glycan mass but did not affect its capacity to inhibit gamete interaction. By contrast, the results of our digestion studies indicate that lactosaminoglycan chains block potential binding sites for human spermatozoa. These rather bulky extensions of glycans could obscure binding sites either directly by being attached to the potential binding glycans or indirectly by being in close proximity to them.

Other investigators have suggested that initial human gamete
binding is dependent upon the expression of terminal mannose residues (Mori et al., 1989; Tulsiani et al., 1990; Benoff et al., 1997). This possibility cannot be ruled out at this time, but the data presented in this study indicate that it is very unlikely that terminal mannose alone is required for human sperm–ZP binding. The sensitivity of a subset of binding sites to chemical conditions that specifically oxidizes terminal sialic acid residues strongly argues against such terminal mannosyl residues being solely responsible for this binding interaction. In addition, if terminal mannose residues play any role in the adhesive process, they would probably have to be adjacent to highly charged sulphate or phosphate groups to be protected from periodate oxidation. Mannose 6-phosphate is found at the terminal ends of some lysosomal proteins (reviewed in Dahms et al., 1989), but it is unlikely that such a sequence could be associated with ZP glycoproteins.

Lucas et al. (1994) have also implicated the Lewisβ sequence as a putative ligand for human gamete binding. However, it is clear from our studies that this sequence could not be the only ligand mediating sperm binding, because such an oligosaccharide should be exquisitely sensitive to mild periodate oxidation. It is possible that terminal sialic acid could block periodate oxidation of the Lewisβ glycans. However, it is difficult to understand how desialylation could make such binding sites more accessible when an anti-Lewisβ antibody was capable of blocking this interaction (Lucas et al., 1994). In addition, a Lewisβ-conjugated neoglycoprotein was also incapable of inhibiting human sperm–ZP binding in the HZA at a final concentration of 1 mg/ml (M. Patanek and G. Clark, unpublished data).

Considerable work will be needed to determine exactly how human spermatozoon bind to the human ZP. However, the evidence indicates that this interaction may be considerably more complex than previously thought. The involvement of sialic acid, the evidence for a sperm-associated neuraminidase, and other considerations discussed in this report support this conclusion. However, both direct and indirect methods will undoubtedly be useful for determining the molecular basis underlying human sperm–ZP binding.

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