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# Mannose-binding molecules of rat spermatozoa and sperm–egg interaction

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## Summary

We have previously reported the occurrence and partial characterisation of an α-D-mannosidase activity on plasma membranes of rat, mouse, hamster and human spermatozoa. A soluble isoform of the rat sperm surface mannosidase was purified and polyclonal antibody raised. Since several reports have suggested that mannosyl residues on the rat, mouse and human zona pellucida may be involved in sperm–zona binding, studies were undertaken to examine the receptor-like role of mannose-binding molecules on rat spermatozoa. Sprague-Dawley rats (25–30-days old) were superovulated and eggs collected from the oviduct were treated with 0.3% hyaluronidase to remove the cumulus cells. Spermatozoa, collected from the cauda epididymis were capacitated for 5 h at 37 °C in 5% $CO_2$ in air. The sperm–zona binding assay was performed in the presence of increasing concentrations of several sugars as well as preimmune and immune (anti-mannosidase or anti-mannose binding protein) IgG. Data from these studies show that: (1) significantly fewer sperm bound per egg in the presence of competitive inhibitors of mannosidase; (2) among the sugars examined, D-mannose was the most potent inhibitor causing 70% reduction in the number of sperm bound per egg; (3) anti-mannosidase or anti-mannose binding protein (but not preimmune) IgG showed a dose-dependent reduction in the number of sperm bound per egg; (4) anti-mannosidase IgG (but not anti-mannose binding protein IgG) showed a dose-dependent inhibition of sperm surface mannosidase activity; (5) the competitive inhibitors of mannosidase or the immune IgG had no effect on sperm motility or the sperm acrosome reaction. These results suggest that mannose-binding molecule(s) such as α-D-mannosidase or mannose-binding protein on the spermatozoa may recognise mannosyl residues on zona pellucida, and play a receptor-like role in sperm-egg interaction in the rat.

Keywords: Carbohydrates and fertilisation, Mannose-binding proteins, Rat, Sperm surface mannosidase, Zona pellucida

## Introduction

It is generally accepted that sperm-egg interaction in mammals is a carbohydrate-mediated event (Yanagimachi, 1994; Wassarman, 1994; Miller & Shur, 1994; Tulsiani *et al.*, 1997; Benoff, 1997; Shur, 1998). In the past two decades, several carbohydrate-binding proteins (glycosyltransferases, glycohydrolases and lectin-

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like molecules) present on the sperm plasma membrane and their complementary sugar molecules on zona pellucida (ZP) have been proposed to be involved in sperm–egg interaction (Wassarman, 1994; Miller & Shur, 1994; Tulsiani *et al.*, 1997; Benoff, 1997; Shur, 1998). However, because of the difficulty in purifying ZP glycoproteins, classical structure–function studies on ZP glycoproteins have not been possible. Therefore, alternative indirect approaches have been attempted to assess the role of sugar residues. These approaches include use of lectins, monosaccharides, oligosaccharides, and endo-/exo-enzyme-treated ZP glycoproteins to assess their inhibitory effect on sperm–egg interaction (Oikawa *et al.*, 1973, 1974; Yamagata, 1985; Florman & Wassarman, 1985; Shalgi *et al.*, 1986; Cornwall *et al.*, 1991; Wassarman, 1995; Yonezawa *et al.*, 1995).

Previously, our group has identified and biochemically characterised an α-D-mannosidase on the surface of rat, mouse, hamster and human spermatozoa (Tulsiani *et al.*, 1989, 1990, 1993). Since several reports have suggested that mannosyl residues on ZP may be involved in sperm–egg binding (Shalgi *et al.*, 1986; Lambert, 1984; Mori *et al.*, 1989), studies were undertaken to examine the role of mannosidase during fertilisation in the mouse *in vitro* (Cornwall *et al.*, 1991). These studies demonstrated that incubation of mouse spermatozoa in the presence of the inhibitory sugars, such as α-methyl mannoside, α-methyl glucoside, D-mannose or D-mannitol, resulted in a dose-dependent decrease in the number of spermatozoa bound per egg and a dose-dependent inhibition of mouse sperm mannosidase. Although the sperm plasma membrane α-D-mannosidase was initially identified and characterised in rat spermatozoa (Tulsiani *et al.*, 1989), little or no information is available concerning its role in this species. In addition, recent studies have shown the presence of a mannose-binding protein on human spermatozoa as a mannose lectin for ZP (Benoff *et al.*, 1993; Benoff, 1997). This molecule was reported to be initially stored in the subplasmalemmal space of the sperm. Following capacitation, it is translocated to the sperm surface. Subsequently, sperm–ZP attachment causes it to aggregate and change its localisation to the equatorial/post-acrosomal segment of the sperm head, followed by acrosomal exocytosis. The macromolecule has also been suggested to have a receptor-like role in binding to mannose-containing oligosaccharides on the ZP, since decreased expression of the molecule on spermatozoa caused unsuccessful fertilisation in human. It is noteworthy that the numbers of spermatozoa capable of binding to mannose residues were increased following capacitation *in vitro* (Benoff *et al.*, 1993; Benoff, 1997).

The present studies were undertaken to examine the receptor-like role of α-D-mannosidase and mannose-binding protein on rat spermatozoa. It should be noted that throughout the literature the complementary molecules on the spermatozoa and ZP have been interchangeably referred to as ligands and receptors. The convention of cell biology dictates that the small functional group should be designated the ligand, and the sperm surface protein(s) binding to the ligand should be designated the receptor(s). Therefore, throughout this report we refer to the sperm surface protein(s) that bind to the sugar moiety(ies) on the ZP as receptor(s). Data presented in this report seem to suggest a receptor-like role for the sperm surface mannosidase and perhaps the mannose-binding protein.

## Materials and methods

### Animals and chemicals

Sprague-Dawley rats and female golden hamsters purchased from Japan SLC (Hamamatsu, Japan) were given free access to food and water. They were maintained at the Animal Center of Yamagata University under 12L:12D conditions. Galactose, mannose, fucose, methyl-α-D-galactoside, methyl-α-D-mannoside, methyl-β-D-mannoside, methyl-α-D-glucoside, mannitol, eCG, hCG, BSA (fraction V), hyaluronidase (type IV from bovine testes), trypsin and Hoechst 33342 were purchased from Sigma Chemical Co. (St Louis, MO). Sperm plasma membrane α-D-mannosidase antibody was prepared as described previously (Tulsiani *et al.*, 1993). This polyclonal antibody was shown to cross-react with the α-D-mannosidase activity present in epididymal fluid and detergent-solubilised spermatozoa from the rat and the mouse (Tulsiani *et al.*, 1993). Anti-mannose binding protein IgG was purified from goat anti-mannose receptor antiserum (Lennartz *et al.*, 1987) (a generous gift from Dr Philip Stahl, Washington University School of Medicine, St Louis, MO) by affinity chromatography on a column of immobilised protein G. Silicon oil was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other reagents used were of the highest grade available.

### Preparation of rat gametes

Human tubal fluid (HTF) medium and Hepes-buffered HTF medium (pH 7.4) (Quinn *et al.*, 1985) supplemented with 5 mg bovine serum albumin (BSA)/ml were used as previously described (Nohara *et al.*, 1998). To prepare rat gametes, we followed the protocol used for the preparation of mastomys gametes as reported previously (Nohara *et al.*, 1998) with slight modification. Briefly, the caudae epididymides were removed from 3- to 6 month-old male rats and approximately

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10 µl of cauda epididymal contents was overlaid with 1 ml HTF medium. After incubation for 15 min at 37 °C in 5% $CO_2$ in air, the top layer of the medium (10–20 µl) containing motile (>95%) sperm was carefully transferred into 100 µl of HTF medium, then adjusted to a final concentration of $1 \times 10^6$ sperm/ml and capacitated for 5 h at 37 °C in 5% $CO_2$ in air. Sperm capacitation was evaluated by a heterologous sperm penetration assay (see below) as previously described (Yanagimachi, 1972; Hanada & Chang, 1976; Nohara *et al.*, 1998). For egg collection, female Sprague-Dawley rats (25–30 days old) were superovulated by injection of 15 IU eCG followed by injection of 15 IU of hCG 50 h later. Fifteen to sixteen hours after hCG injection, the rats were killed and the oviducts removed. For the sperm–ZP binding assay, each oviduct was pricked with a needle and the cumulus masses, isolated from each oviduct, were suspended in HTF medium containing 0.3% hyaluronidase to remove cumulus cells. The cumulus-free eggs were transferred into a droplet of HTF medium containing the capacitated spermatozoa under silicon oil for further studies.

### *In vitro* sperm–ZP binding assay

The sperm–ZP binding assay was performed in the presence of various concentrations of sugars, immune IgG (anti-mannosidase or anti-mannose binding protein) as well as preimmune IgG. To determine whether various sugars or antibodies could affect the binding of spermatozoa to the ZP, stock solutions (500 mM) of specific sugars were prepared in HTF medium and a small volume (2–10 µl) added to the droplet of sperm in HTF medium to yield a final concentration of 10–50 mM 15 min before mixing with the eggs. In the case of the antibodies, the appropriate antibody or preimmune IgG was added to the droplet of sperm in HTF medium to give a final concentration of 10–50 µg protein/ml and incubated with spermatozoa for 15 min before mixing with the eggs. Incubation of eggs with sugar (or antibody)-treated spermatozoa was carried out for 90 min at 37 °C in 5% $CO_2$ in air. After incubation, the eggs were removed from each droplet and washed twice in Hepes-buffered HTF. They were fixed with 2.5% glutaraldehyde/0.1 M cacodylate buffer (pH 7.4), washed once with Hepes-buffered HTF and mounted on glass slides. The number of spermatozoa bound per egg was determined using phase-contrast microscopy.

### Sperm α-D-mannosidase assay

Oligosaccharide-cleaving mannosidase activity was assayed by our published procedure (Tulsiani *et al.*, 1982). Briefly, the incubation mixture (100 µl) contained mannose-containing oligosaccharide (~4000 cpm of [$^3$H]$Man_9GlcNAc$), 50 mM sodium cacodylate buffer (pH 6.4), 0.2% (w/v) Triton-X100 and spermatozoa in the absence or presence of sugars or preimmune/immune IgG. After incubation for 2 h at 37 °C, the reaction was stopped by heat treatment at 100 °C for 5 min. The released [$^3$H]mannose was separated from the oligosaccharide by gel filtration on a column of Bio-Gel P2 (Bio-Rad, Richmond, CA) and the radioactivity measured by a scintillation counter as described previously (Tulsiani *et al.*, 1982).

### Sperm motility test

For evaluation of sperm motility, epididymal spermatozoa were capacitated as described above. Various amounts of sugar or antibody were added to the spermatozoa (100 µl droplet containing $10^5$ capacitated sperm) to a final concentration of 50 mM (sugar) or 50 µg protein/ml (antibody), and incubated at 37 °C in 5% $CO_2$ in air. Ten microlitres from each suspension was aspirated after 15 min and examined for sperm motility at 45 min intervals under light microscopy as previously described (Nohara *et al.*, 1998). A total of 100 spermatozoa were scored for each determination.

### Sperm penetration assay

To determine whether the sugars or the antibody affected fertilising ability, including capacitation and the acrosome reaction of spermatozoa, a heterologous sperm penetration assay was performed using zona-free hamster eggs according to the method described by Chaudhuri & Yanagimachi (1984). Capacitated spermatozoa (see above) were incubated with the zona-free hamster eggs in the presence of various sugars (50 mM) or antibodies (50 µg protein/ml). After incubation for 6 h, sperm penetration was assessed by observation of the enlargement of the sperm head as stained with Hoechst 33342 (Nohara *et al.*, 1998).

### *In vitro* fertilisation procedure for the rat

For the rat *in vitro* fertilisation (IVF) in HTF medium, we applied the IVF procedure for mastomys as reported previously (Nohara *et al.*, 1998) with slight modification. Briefly, capacitated spermatozoa (see above) were transferred into 100 µl of HTF medium to a final concentration of $1–2 \times 10^5$ sperm/ml, then incubated with 5–30 cumulus-intact rat eggs prepared by the method described above for 8 h at 37 °C in 5% $CO_2$ in air. To determine whether various sugars could affect the fertilisation rate, stock solutions (500 mM) of specific sugars were prepared in HTF medium and a small volume (2–10 µl) added to the droplet of sperm in HTF medium to yield 10 or 50 mM final concentra-

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tion 15 min before mixing with the eggs. After incubation, the eggs were mounted on slides, fixed with 2.5% (w/v) glutaraldehyde for 20 min at room temperature, stained with 1% (w/v) orcein, then examined under a phase-contrast microscope. Fertilisation was assessed according to the formation of the female and male pronuclei, a fertilising sperm tail and a second polar body.

### Statistical analysis

A repeated measures ANOVA test was utilised to examine the significance of differences in sperm motility in the presence of various sugars or antibodies. In the sperm penetration assay, the number of penetrated sperm per egg was analysed by Welch's *t*-test. A probability of $p < 0.05$ was considered statistically significant. Data from IVF in the presence of various sugars were analysed by chi-squared test and Fisher's exact test after applying the Bonferroni correction.

## Results

### Effect of various sugars on sperm–ZP binding

We investigated the effect of various sugars (galactose, mannose, fucose, methyl-α-D-galactoside, methyl-α-D-mannoside, methyl-β-D-mannoside, methyl-α-D-glucoside and mannitol) on rat sperm–ZP binding *in vitro*. In the absence of sugars in the binding assay system, many sperm bound to the ZP-intact unfertilised egg (31.4 ± 6.3 sperm/egg). However, the presence of mannose, methyl-α-D-galactoside, methyl-α-D-mannoside, methyl-α-D-glucoside or mannitol inhibited the number of sperm bound per egg in a dose-dependent manner as shown in Fig. 1. Among the sugars examined, mannose was the most potent inhibitor, causing 70% reduction at 10 mM and higher concentrations. Methyl-α-D-mannoside at 50 mM also caused a 70% reduction; however, inhibition observed at the 10 mM concentration was quite low compared with mannose. Although mannitol, methyl-α-D-glucoside and methyl-α-D-galactoside inhibited sperm–ZP binding by

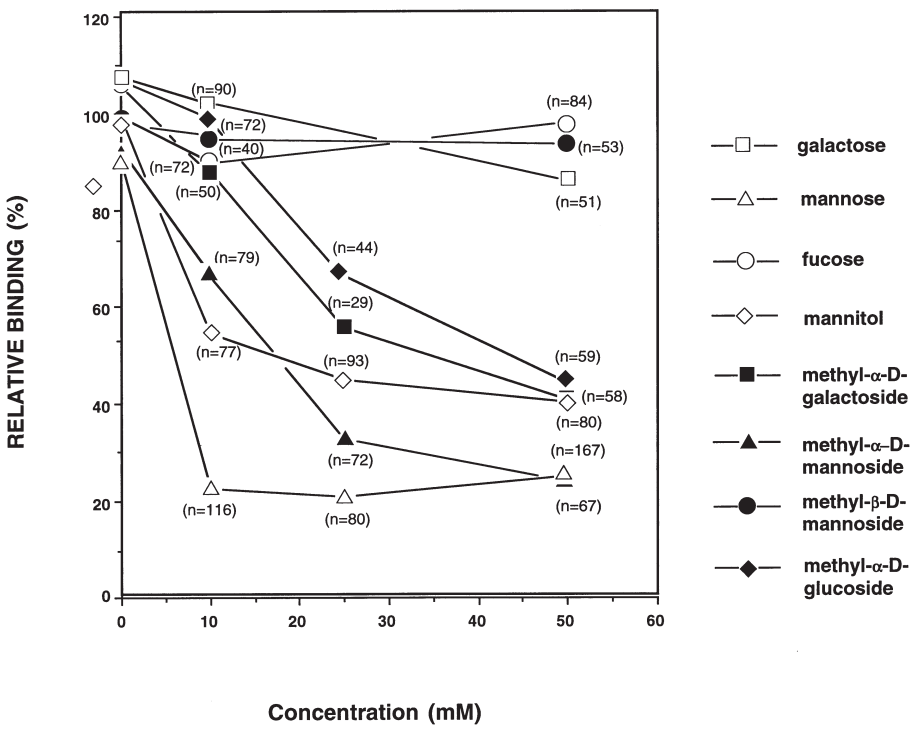



**Figure 1** Effect of various sugars on sperm–ZP binding *in vitro*. Cauda spermatozoa ($1 \times 10^5$) were incubated for 90 min at 37 °C in 5% $CO_2$ in a 100 µl droplet of HTF medium containing zona-intact eggs and 5–50 mM concentrations of sugars. The eggs were washed once in HTF-Hepes buffer and the number of spermatozoa bound per egg was determined for each sugar at each concentration (mean ± SE): control (galactose), 32.8 ± 5.7; 10 mM, 31.1 ± 5.9; 50 mM; 26.4 ± 5.22; control (mannose), 27.5 ± 6.0; 10 mM, 6.89 ± 3.5; 25 mM, 6.36 ± 3.8; 50 mM, 7.63 ± 3.4; control (fucose), 30.3 ± 5.4; 10 mM, 27.4 ± 6.0; 50 mM, 29.7 ± 5.8; control (methyl-α-D-galactoside), 31.9 ± 6.3; 10 mM, 26.8 ± 7.4; 25 mM, 26.8 ± 7.4; 50 mM, 12.4 ± 4.2; control (methyl-α-D-mannoside), 28.2 ± 6.6; 10 mM, 20.4 ± 7.5; 25 mM, 9.8 ± 3.3; 50 mM, 7.29 ± 2.8; control (methyl-β-D-mannoside), 29.1 ± 4.1; 10 mM, 27.5 ± 6.1; 50 mM, 27.9 ± 7.0; control (methyl-α-D-glucoside), 32.9 ± 7.5; 10 mM, 30.1 ± 7.1; 25 mM, 20.3 ± 5.6; 50 mM, 13.5 ± 4.5; control (mannitol), 30.0 ± 7.7; 10 mM, 16.6 ± 5.6; 25 mM, 13.7 ± 5.0; 50 mM, 12.3 ± 4.4.

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50–60% at 50 mM concentration, galactose, fucose and methyl-β-D-mannoside had no significant effect on the sperm–ZP binding (Fig. 1). These data were quite similar to our earlier studies with mouse except that mannose appears to be a more effective inhibitor of rat sperm–ZP binding than methyl-α-D-mannoside as reported for the mouse (Cornwall *et al.*, 1991).

### Effect of anti-sperm mannosidase antibody on sperm–ZP binding *in vitro* and sperm mannosidase activity

Since mannose was a potent inhibitor of rat sperm–ZP binding, attempts were made to determine whether the sperm surface mannosidase (Tulsiani *et al.*, 1989, 1995) or mannose-binding protein (Lennartz *et al.*, 1987) may be involved in sperm–ZP recognition and binding. In these studies, various concentrations of preimmune or immune IgG were incubated with cauda epididymal spermatozoa to examine the effect on sperm mannosidase activity and sperm–ZP binding. In the presence of anti-α-D-mannosidase antibody (Tulsiani *et al.*, 1993), the number of sperm bound per egg was significantly decreased in a dose-dependent manner as shown in Fig. 2. At 25 µg protein/ml, the antibody caused 50% inhibition in the number of sperm bound to ZP; a 55% reduction was observed at a concentration of 50 µg protein/ml. The preimmune IgG had no effect on the number of spermatozoa bound per egg (Fig. 2). The antibody also caused a similar inhibitory effect on rat sperm surface mannosidase in a concentration-dependent manner (see below).

### Effect of anti-mannose binding protein IgG on sperm–ZP binding and sperm surface mannosidase activity

To examine whether anti-mannose-binding protein has any effect on sperm–ZP binding *in vitro*, rat cauda epididymal spermatozoa were incubated in the presence of preimmune or immune IgG and the number of sperm bound per egg was quantitated. The immune IgG but not preimmune IgG caused a 50% inhibition in the number of sperm bound per egg at 50 µg protein/ml (Fig. 3). However, unlike anti-mannosidase IgG, this antibody did not show any inhibitory effect on the sperm surface mannosidase activity, as shown in Fig. 4. These results demonstrate that anti-mannose-binding protein IgG has an effect on sperm–ZP binding *in vitro* without affecting mannosidase activity.

### Effect of sugars and antibodies on rat sperm motility, capacitation and acrosome reaction

To confirm that inhibition of sperm–ZP binding was not due to the decrease in sperm motility, the effect of sugars or antibodies on sperm motility was examined. Cauda epididymal spermatozoa were incubated in the presence of the highest concentration of sugars (50 mM) or antibodies (50 µg protein/ml). However, no statistical difference was observed on sperm motility during the course of incubation in the presence of sugars or antibodies, as shown in Fig. 5. We also examined whether the presence of these sugars or antibodies had

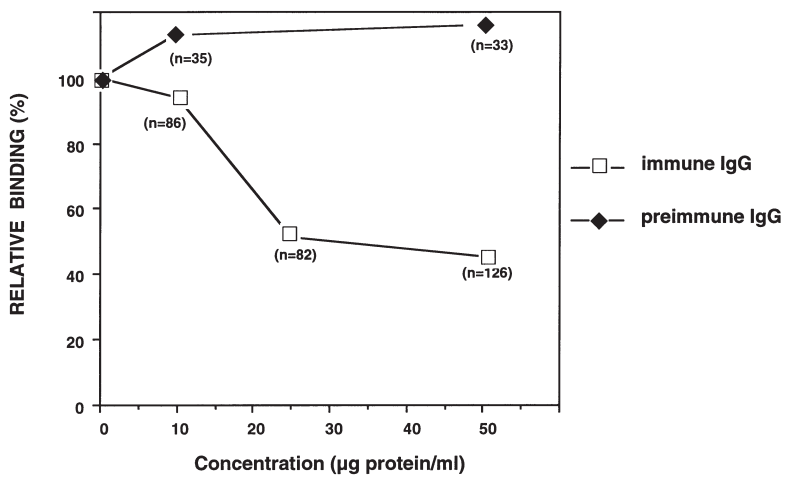



**Figure 2** Effect of preimmune or anti-sperm surface α-D-mannosidase IgG on sperm–zona binding *in vitro*. Cauda spermatozoa ($1 \times 10^5$) were incubated for 90 min at 37 °C, 5% $CO_2$ in a 100 µl droplet of HTF medium containing zona-intact eggs and 0–50 µg IgG protein/ml. The eggs were washed once in HTF-Hepes buffer and the number of spermatozoa bound per egg determined: Mean ± SE: control, 32.9 ± 5.8; immune IgG 10 µg/ml, 30.9 ± 7.5; 25 µg/ml, 16.9 ± 4.9; 50 µg/ml, 14.9 ± 5.5; preimmune IgG 10 µg/ml, 43.1 ± 7.9; 50 µg/ml, 41.3 ± 7.4.

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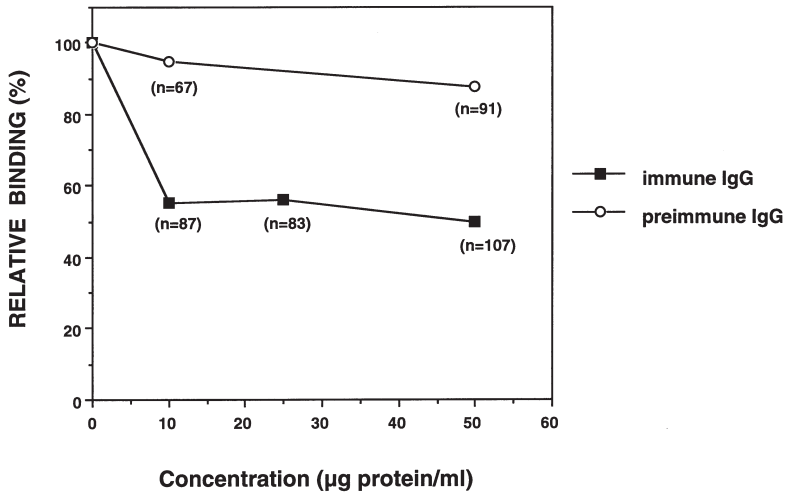



**Figure 3** Effect of pre-immune or anti-mannose binding protein IgG on sperm–zona binding *in vitro.* Cauda spermatozoa ($1 \times 10^5$) were incubated for 90 min at 37 °C in 5% $CO_2$ in a 100 µl droplet of HTF medium containing zona-intact eggs and 0-50 µg IgG protein/ml. The eggs were washed once in HTF-Hepes buffer and the number of spermatozoa bound per egg determined (mean ± SE): control, 35.7 ± 6.0; immune IgG 10 µg/ml, 19.7 ± 5.8; 25 µg/ml, 20.1 ± 5.9; 50 µg/ml, 17.7 ± 5.0; preimmune IgG 10 µg/ml, 33.9 ± 6.5; 50 µg/ml, 31.1 ± 6.3.

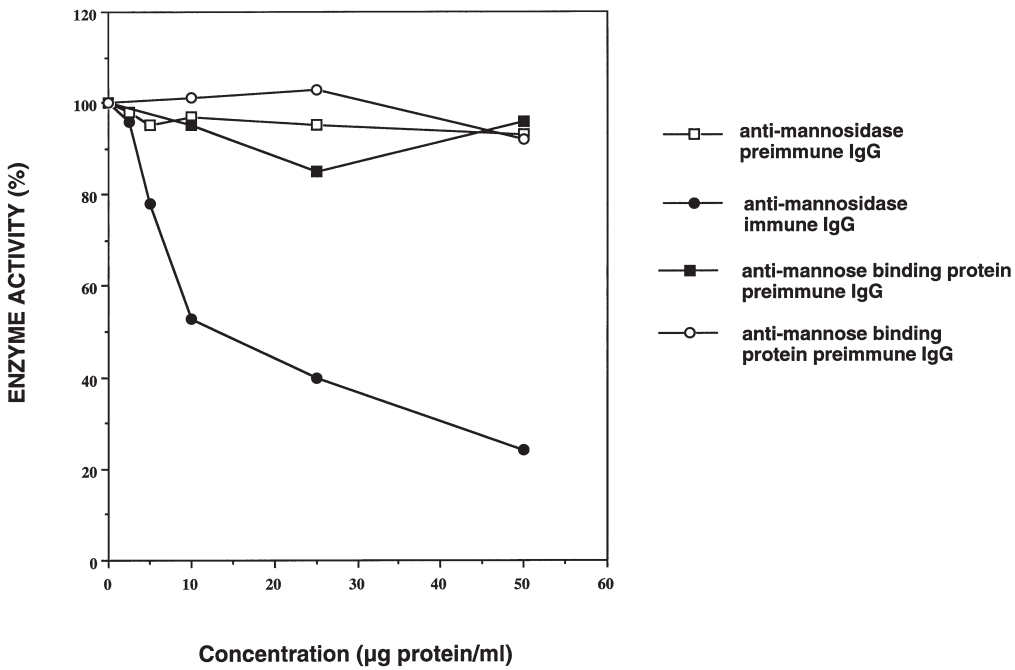



**Figure 4** Effect of pre-immune, anti-mannosidase IgG or anti-mannose binding protein IgG on mannosidase activity of rat cauda spermatozoa. Each point represents the mean of two independent experiments. The duplicate values agreed within 10%.

an effect on the acrosome reaction. Since the rat sperm acrosome is relatively small, it is difficult to distinguish acrosome-reacted from acrosome-intact sperm by light microscopy directly. Therefore, sperm capacitation and the acrosome reaction were assessed indirectly by following the rate of sperm penetration into ZP-free hamster eggs as previously reported (Yanagimachi, 1972; Hanada & Chang, 1976; Nohara *et al.*, 1998). As shown in Tables 1 and 2, there was no significant difference in the absence or presence of sugars and antibodies, suggesting that inhibition of sperm–egg binding in the presence of sugars or antibodies (Figs. 1–3)

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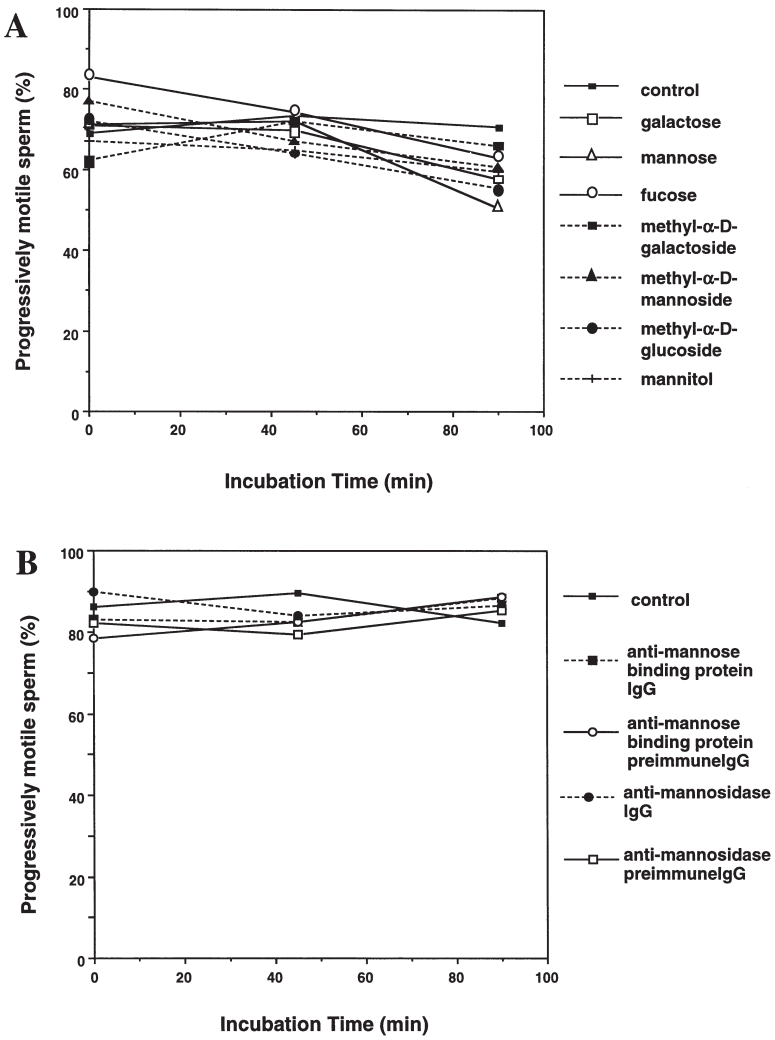



**Figure 5** The percentage of progressively motile spermatozoa after incubation in the presence of various sugars (*A*) or antibody (*B*). Fifty millimolar concentrations of the sugars or 50 µg IgG protein/ml were added to $1 \times 10^5$ spermatozoa and the motility examined over 90 min. Values represent the mean of three experiments using galactose, fucose, methyl-α-D-galactoside, methyl-α-D-mannoside, methyl-α-D-glucoside, mannitol, five experiments using mannose and two experiments using antibodies. No statistical difference was observed during incubation with sugars or antibodies.

was not due to an alteration in sperm motility or a change in sperm acrosome integrity.

### Effect of various sugars on rat IVF

Data presented above suggest that mannose-recognising molecules such as α-D-mannosidase or mannose-binding protein on the rat sperm surface probably recognise mannosyl residues on the ZP, and play an important role in sperm–egg interaction. Attempts were then made to examine the effect of various sugars on rat IVF. Data obtained from IVF in the presence of various sugars were statistically analysed after applying the Bonferroni correction. After this correction, statistical significance should be set at $p = 0.0056$. As shown in Table 3, the $p$ values of the mannose (50 mM) group and methyl-α-D-mannoside (50 mM) group were 0.0058 and 0.019, respectively. Although not statistically significant, these $p$ values were very low compared with other groups. Therefore, we suppose that D-mannose and methyl-α-D-mannoside, the sugars which caused maximum inhibition of sperm–ZP binding, also had an effect on the decrease in fertilisation rate.

## Discussion

In previous studies, our group has presented evidence for the occurrence of α-D-mannosidase activity on the

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**Table 1** Effect of sugars on the ability of spermatozoa to penetrate the zona-free hamster egg

| Additions | No. of test replicates | No. of eggs penetrated/ no. of eggs examined (%) | Average no. of penetrated sperm/egg ± SD |
|---|---|---|---|
| Control | 3 | 29/30 (96.7) | 20.3 ± 6.0 |
| Galactose | 3 | 32/34 (94.1) | 23.4 ± 9.2 |
| Mannose | 3 | 30/30 (100) | 21.5 ± 9.3 |
| Methyl-α-D-mannoside | 3 | 26/26 (100) | 22.6 ± 9.3 |

**Table 2** Effect of preimmune or immune IgG on the ability of spermatozoa to penetrate the zona-free hamster egg

| Additions | No. of test replicates | No. of eggs penetrated/ no. of eggs examined (%) | Average no. of sperm/egg ± SD |
|---|---|---|---|
| Control | 2 | 10/10 (100) | 21.8 ± 3.5 |
| Anti-mannose binding protein | | | |
| Immune IgG | 2 | 35/35 (100) | 19.8 ± 5.4 |
| Preimmune IgG | 2 | 17/17 (100) | 24.1 ± 3.4 |
| Anti-mannosidase | | | |
| Immune IgG | 2 | 55/55 (100) | 22.8 ± 5.8 |
| Preimmune IgG | 2 | 29/29 (100) | 20.9 ± 4.5 |

**Table 3** *In vitro* fertilisation rates in the presence of various sugars

| Additions (concentration) | No. of replicates | No. of fertilised eggs/ no. of eggs examined | Relative fertilisation rate (%)[a] | *p* value[b] |
|---|---|---|---|---|
| Control | 6 | 46/54 | – | – |
| Galactose (50 mM) | 3 | 34/40 | 99.8 | 0.98 |
| Fucose (50 mM) | 3 | 31/37 | 98.5 | 0.86 |
| Methyl-α-D-galactoside (50 mM) | 5 | 35/40 | 102.7 | 0.50 |
| Mannose (10 mM) | 3 | 23/32 | 84.3 | 0.13 |
| Mannose (50 mM) | 5 | 34/55 | 74.6 | 0.0058 |
| Methyl-α-D-glucoside (50 mM) | 4 | 43/54 | 93.4 | 0.44 |
| Methyl-α-D-mannoside (10 mM) | 3 | 29/40 | 85.1 | 0.13 |
| Methyl-α-D-mannoside (50 mM) | 4 | 32/49 | 76.6 | 0.019 |
| Methyl-β-D-mannoside (50 mM) | 2 | 24/29 | 97.1 | 0.50 |

[a] Relative fertilisation rate (%) = [% of fertilisation in the presence of sugar / % of fertilisation in the absence of inhibitory sugars (control)] × 100.
[b] *p* value compared with the fertilisation rate in control.

sperm plasma membranes of several species including man (Tulsiani *et al.*, 1989, 1990). Several lines of evidence suggested that the enzyme may have a receptor-like role in sperm–egg interaction. First, the initial characterisation studies demonstrated that the enzyme is optimally active near neutral pH and its catalytic site is oriented towards the sperm surface (Tulsiani *et al.*, 1989). This result suggested that the enzyme would be functional at the site of fertilisation. Second, inclusion of known inhibitors of sperm surface mannosidase, namely methyl-α-D-mannoside, methyl-α-D-glucoside, D-mannose or mannitol during *in vitro* sperm–egg binding assay in mouse resulted in a dose-dependent decrease in the number of spermatozoa bound per egg (Cornwall *et al.*, 1991). Finally, the mouse ZP2 and ZP3 were shown by us to contain high mannose/hybrid-type oligosaccharides (Tulsiani *et al.*, 1992). These haptens are believed to be the ligand site(s) for the mouse sperm surface mannosidase. Taken together, these lines of evidence are consistent with the proposed receptor-like role for sperm surface mannosidase.

The studies reported here were undertaken to exam-

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ine *in vitro* sperm–egg binding events and a receptor-like role for the rat sperm surface mannosidase. Data presented demonstrate that inclusion of several sugars during *in vitro* sperm–egg binding assay significantly decreased the number of spermatozoa bound per egg in a concentration-dependent manner (Fig. 1). The most potent inhibitor of rat sperm–ZP binding appears to be D-mannose rather than methyl-α-D-mannoside as reported for mouse sperm–ZP binding *in vitro* (Cornwall *et al.*, 1991). This difference between the mouse and rat suggests that different receptors and their complementary molecules (ligand) may be important in the interaction of the opposite gametes in different species.

In an earlier report, Shalgi *et al.* (1986) presented evidence suggesting that methyl-α-D-mannoside was a potent inhibitor of sperm–egg interaction in the rat. Although results presented in this report are somewhat different, we believe that differential treatment of spermatozoa in the two reports may be responsible for these differences. For instance, whereas Shalgi and coworkers (1986) used ejaculated spermatozoa in the uterine horn of the female, the present study used epididymal spermatozoa capacitated in a chemically defined medium. We used HTF medium (Quinn *et al.*, 1985), which is formulated following the composition of human tubal fluid, for the sperm–ZP binding assay. Although this medium was originally developed for human IVF (Quinn *et al.*, 1985), it was shown by several research groups to be quite suitable for IVF in rodents (Quinn, 1995; Nakazawa *et al.*, 1997; Lee *et al.*, 1997). Indeed, a recent study from our laboratory has demonstrated a successful IVF procedure in the mastomys (a small African rodent) using sperm preincubated for 6–10 h in HTF medium (Nohara *et al.*, 1998). Therefore, the different conditions used in the two reports may have caused different effects on various sperm functions including capacitation and the acrosome reaction.

In earlier studies, our group reported that the properties of human (Tulsiani *et al.*, 1990) and rat (Tulsiani *et al.*, 1989) sperm surface mannosidase were quite similar. First, the enzyme from rat and human spermatozoa was mainly active towards high mannose oligosaccharides, cleaving α1,2-, α1,3- (α1,6-)linked mannosyl residues. Second, the enzyme present on the spermatozoa from the two species was inhibited by $Cu^{2+}$ and $Zn^{2+}$ but stimulated by $Co^{2+}$ and $Mn^{2+}$. Other divalent cations, such as $Ca^{2+}$, and $Mg^{2+}$, had little or no effect on the enzyme activities present on spermatozoa from the two species. Third, the rat and human sperm surface mannosidase showed a similar inhibition to a number of other sugars, including some used in this report. Finally, the anti-rat epididymal α-D-mannosidase IgG cross-reacted with the human sperm surface mannosidase. It would be of interest to examine whether the anti-rat sperm mannosidase IgG has an adverse effect on human sperm–egg interactions.

In addition to sperm surface mannosidase (Tulsiani *et al.*, 1990), human spermatozoa are reported to possess mannose-binding protein (Benoff *et al.*, 1993; Benoff, 1997). This molecule exhibited an apparent molecular mass of 180 kDa on SDS-PAGE and was characterised as binding to glycoproteins containing D-mannose with high affinity and requiring $Ca^{2+}$ and neutral pH (Haltiwanger & Hill, 1986). It has been suggested that each of these macromolecules may have a receptor-like role in binding to mannose-containing glycans on the ZP. Therefore, we examined sperm–egg binding in the absence or presence of anti-mannosidase or anti-mannose binding protein IgG. Interestingly, significantly fewer spermatozoa bound per egg in the presence of the two antibodies (Figs 2, 3). This result implies that the sperm surface mannosidase may not be the only receptor recognising mannosyl residues. Since only the anti-sperm mannosidase IgG caused a concentration-dependent inhibition of the enzyme activity (Fig. 4), it is reasonable to suggest that the observed inhibition of the number of sperm bound per egg in the presence of anti-mannose-binding protein IgG is by a mechanism other than the inhibition of sperm mannosidase activity. This result, in conjunction with recent reports (Thaler & Cardullo, 1996; Loeser & Tulsiani, 1999), strongly suggests the occurrence of multiple receptors on mammalian spermatozoa. It should be noted that although the presence of inhibitory sugars (mannose and methyl-α-D-mannoside) during the binding assay caused a significant decrease in the number of spermatozoa bound per egg (Fig. 1), the observed reduction in the rate of fertilisation in the presence of these sugars was not as great (Table 3). These data seem to suggest that sperm–egg binding and rate of fertilisation may not be directly proportional. However, the fact that these sugars caused a reduction in the binding as well as fertilisation rate may suggest a role for the mannosyl residue(s) on rat ZP and its complementary binding molecules (i.e. α-D-mannosidase and mannose-binding protein) on rat sperm surface in sperm–egg binding leading to fertilisation.

In most mammalian species studied, only the capacitated (motile) acrosome-intact spermatozoa bind to the ZP (Yanagimachi, 1994; Tulsiani *et al.*, 1997). Therefore, studies were carried out to determine whether the presence of saccharides or immune IgG during the *in vitro* binding assay had any adverse effect on sperm motility or acrosomal integrity. Data from these studies presented in Table 1 allow us to conclude that the observed inhibitory effect of the sugars or antibodies on the binding of the opposite gametes is not due to an alteration in sperm motility or a change in acrosome status. We cannot, however, rule out the possibility

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that the inhibitory effects caused by the sugars or antibodies were due to steric hindrance in sperm–egg interaction at this time. Further studies to detemine the effects of oligosaccharides such as high-mannose or complex type glycans as well as monovalent antibodies (Fab fragment) are needed to clarify these points.

It should be noted that sperm surface mannosidase is a glycohydrolase and belongs to a class of enzymes responsible for hydrolytic cleavage of glycosidic bonds. The catalytic mechanism of action of these enzymes is generally accepted to follow the model proposed for lysozyme (Ichikawa *et al.*, 1992). In this model, there are two carboxylic acid moieties in the active site: one moiety stabilises the resulting oxocarbonium ion, either by ion pair interaction or by covalent bonding, whereas the other facilitates departure of the leaving group. Thus, the catalytic mechanism of the hydrolytic enzymes is thought to have a common feature in that the enzyme:glycosyl intermediate is formed before hydrolytic cleavage of the glycosidic bond. Since the mannose-containing glycoprotein is a poor substrate (Tulsiani *et al.*, 1993), it is reasonable to hypothesise that the sperm surface mannosidase interacts with mannosyl residues on rat ZP forming enzyme:substrate complexes which leads to sperm–egg adhesion and the induction of the acrosome reaction before hydrolytic cleavage of mannosyl residues occurs. The finding that treatment of ZP-intact eggs with jack bean α-D-mannosidase inhibits sperm–egg interaction in rat (Shalgi *et al.*, 1986) is consistent with the presence of mannosyl residues on the ZP glycoconjugates.

It is of interest to mention that with the exception of a few studies from our laboratories (Araki *et al.*, 1992; Akatsuka *et al.*, 1998), the chemical characterisation of rat ZP glycoproteins has never been carried out. Our studies provide evidence that although rat ZP, like mouse ZP, is composed of three glycoproteins (rZP1, rZP2 and rZP3), the rZP3 (perhaps the primary ligand for homologous spermatozoa) is much larger than mouse ZP3 (115 kDa vs 83 kDa). In a recent report we have used molecular biological approaches to demonstrate that the amino acid sequence of rZP3 shares high homology with mouse (90%), hamster (78%) and human (65%) ZP3 (Akatsuka *et al.*, 1998). This study indicates that the primary structures of the rZP3 and mZP3 are very similar, including the potential glycosylation sites. Thus, it is reasonable to assume that the glycan units present on the ZP3 from these species are structurally and functionally different. Interestingly, recent studies using gene disruption of the candidate molecules thought to be responsible for sperm–egg interaction did not always result in the production of a fully infertile mouse (Baba *et al.*, 1994; Thall *et al.*, 1995). These results imply that although the candidate molecules may have a role, they are not essential for sperm–egg interaction, at least in the mouse *in vivo*. Since conditions of *in vitro* and *in vivo* sperm–egg interaction remain unclear, further careful studies will be necessary to clarify these points.

The identification of several receptor and ligand molecules on the spermatozoa and ZP, respectively, suggests that probably several receptor-ligand molecules must be involved in sperm–egg interaction (see reviews by Wassarman, 1994; Miller & Shur, 1994, Tulsiani *et al.*, 1997; Benoff, 1997; Shur, 1998). Despite numerous recent advances, the identity of the sperm surface protein(s) responsible for sperm–ZP binding as well as the characteristics of the complementary oligosaccharide units on ZP3 remain unknown. The precise order of these interactions or the dominant receptor–ligand interaction may vary among species and may contribute to relative species-specificity. A recent study reported the replacement of mouse ZP3 gene with the human ZP3 gene (Rankin *et al.*, 1998). Eggs from these animals interacted with the mouse (rather than human) spermatozoa. Although the rescued ZP3 in the mouse has human ZP3 core peptide, its carbohydrate moieties are expected to be similar to the mouse (rather than human) ZP3 glycoconjugate. This result is consistent with earlier reports suggesting the importance of glycan portion of the ZP3 glycoprotein in species-specific interaction of the opposite gametes.

The potential involvement of the carbohydrate portion on ZP glycoproteins in sperm–egg interaction *in vitro* was first suggested almost a quarter of a century ago (Oikawa *et al.*, 1973). At present, conflicting results from several groups have made interpretation of the current data difficult. However, further studies using a combination of both *in vitro* and *in vivo* systems (using various gene-disrupted animals that lack the candidate molecule(s) responsible for sperm–egg interaction) will provide important information concerning the molecular mechanism of mammalian fertilisation. Understanding the mechanisms underlying sperm–egg interaction in different species, however, awaits the development of more sensitive methods for characterisation of glycan residues on ZP glycoproteins from various species.

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