Carbohydrate analysis of the zona pellucida and cortical granules of human oocytes by means of ultrastructural cytochemistry

María Jiménez-Movilla1, Manuel Avilés1,4, María José Gómez-Torres2, Pedro José Fernández-Colom3, María Teresa Castells1, Joaquín de Juan2, Alberto Romeu3 and José Ballesta1,4

1Department of Cell Biology, Faculty of Medicine, University of Murcia, Murcia, 2Department of Biotechnology, University of Alicante, Alicante and 3Servicio de Ginecología (Reproducción Humana), Hospital Universitario La Fe, Valencia, Spain
4To whom correspondence should be addressed at: Department of Cell Biology, Faculty of Medicine, University of Murcia, Espinardo, 30071 Murcia, Spain. E-mail: maviles@um.es and ballesta@um.es

BACKGROUND: The zona pellucida (ZP), the mammalian oocyte coat, consists of a restricted number of highly glycosylated proteins. In vitro sperm binding studies suggest a higher binding affinity for the outer region of the ZP compared to its inner region in different species. However, the reason for this difference in binding distribution remains unresolved. Many studies suggest that the carbohydrate sequences linked to ZP glycoproteins act as ligands for sperm binding to this matrix. METHODS: Lectins and antibodies that recognize different carbohydrates were employed to perform an ultrastructural analysis of human ZP and cortical granule glycosylation. RESULTS: This study reveals variable glycosylation of the human ZP throughout its thickness, with pronounced differences between the most external and internal regions of this matrix. The binding studies also indicate that ZP glycoproteins express some carbohydrate sequences not previously detected in other species. Finally, cytochemical analysis of human cortical granules suggests similarities in glycosylation to ZP glycoproteins but not to cortical granules from other mammalian species. CONCLUSION: A heterogeneous carbohydrate composition was observed in the thickness of the human ZP that could be responsible for the different sperm binding affinity detected between the outer and inner regions of the ZP.

Key words: carbohydrate/cortical granule/cytochemistry/oocyte/zona pellucida

Introduction

During fertilization, sperm initially bind to the oocyte, undergo the acrosome reaction, penetrate the zona pellucida (ZP), and fuse with the oocyte to form a zygote. The entry of a single sperm into the oocyte initiates a cascade of reactions known as oocyte activation. During this process, cortical granules lying just beneath the cytoskeleton of the oocyte fuse with the plasma membrane and undergo exocytosis. Factors released from the granules induce specific biochemical changes in the ZP, leading in part to the block to polyspermy (Hoodbhoy and Talbot, 1994; Yanagimachi, 1994; Green, 1997; Wassarman et al., 2001; Sun, 2003).

The interaction between the sperm and the oocyte is often considered to be species specific (Yanagimachi, 1994). However, Bedford and co-workers reported that, with the exception of the human and the guinea-pig, there was a surprising degree of interspecies cross-reactivity in mammalian sperm–oocyte binding (Bedford, 1977). Human sperm are somewhat unique because they apparently manifest order-specific binding (i.e. they bind only to the oocytes of higher primates) (Bedford, 1977; Lanzendorf et al., 1992). Remarkably, sperm from rabbit, mice and hamsters avidly bind to human oocytes (Bedford, 1977). The results of recent genetic manipulations suggest that the substitution of murine ZP glycoproteins with their corresponding human homologues (human ZP2, human ZP3, or both human ZP2 and huZP3) in mice yields oocytes that bind murine but not human sperm (i.e. retain their taxon binding specificity) (Rankin et al., 1998, 2003). Thus, it is generally accepted that the specific interaction between the sperm and the ZP is a carbohydrate-mediated event in different species including humans (Chapman and Barratt, 1996; Benoff, 1997; Ozgur et al., 1998; Dell et al., 1999; Primakoff and Myles, 2002; Talbot et al., 2003). This binding is probably mediated by oocyte binding proteins located in the head region of capacitated sperm that bind to specific oligosaccharides expressed on the surface of the ZP (Nixon et al., 2001). Previous studies indicate a substantial difference in the binding of sperm to the inner and outer regions of
isolated ZP in different mammalian species including humans (Phillips and Shalgi, 1980a,b; Ahuja and Bolwell, 1983; Burkman et al., 1988; Fazeli et al., 1997). However, the molecular mechanism responsible for this process remains unsolved. The determination of the precise composition of the carbohydrate sequences contained in the human ZP glycoproteins could provide valuable insight into the identity of the specific oocyte binding protein(s) expressed in the sperm plasma membrane. However, these studies are severely limited in the human due to the difficulties in both the availability and manipulation of human oocytes.

In this study, the carbohydrate composition of the human ZP and cortical granules was analysed by employing lectin- and immunocytochemistry at the ultrastructural level in combination with enzymatic treatment and quantitative analysis. The precise identification of the carbohydrate distribution in these compartments should provide new information about the roles played by these glycanes during both sperm binding interactions and the cortical reaction. This information could also be useful for future studies related to idiopathic human infertility encountered in IVF programmes.

### Materials and methods

#### Reagents

Rabbit anti-mouse IgG polyclonal antibody, rabbit anti-horseradish peroxidase polyclonal antibody, unlabelled (DBA, LTA, MPA and SBA), horseradish peroxidase (HRP)-labelled lectins (Ala, BSA-I-B₄, ConA, E-PHA, RCA-I, STA and UEI-I), bovine serum albumin (BSA), neuraminidase (type V from Clostridium perfringens), galactose oxidase (from Dactylium dendroides), D-galactose (Gal), L-fucose (Fuc), N-acetylgalactosamine (GalNAc), D-N-acetylgalactosamine (GlcNAc) and N-acetylenuraminic acid (Neu5Ac) were purchased from Sigma (Madrid, Spain). Digoxigenin (DIG)-labelled lectins (AAA, DSA, GNA, L-PHA, PNA, MAA, SNA, and WGA) and mouse monoclonal IgG anti-DIG antibodies were purchased from Roche (Spain). HRP-labelled LFA was from Calbiochem (Madrid, Spain). Goat anti-mouse IgG + IgM–gold complex (15 nm) were purchased from Biocell Research Ltd (UK). Rabbit unconjugated anti-mouse immunoglobulin was from Dako (Spain). Protein A-colloidal gold conjugate (15 nm) was from the Department of Cell Biology (Utrecht University, Utrecht, The Netherlands). The taxonomic names and specificity of lectins used in the present study are shown in Table I. Antibodies used are shown in Table II. A mouse monoclonal antibody against the human ZP3

### Table I. Lectin characteristics

<table>
<thead>
<tr>
<th>Taxonomic name</th>
<th>Abbreviation</th>
<th>Carbohydrate binding specificity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bandeiraea simplicifolia I-B₄</td>
<td>BSA-I-B₄</td>
<td>Terminal α-linked Gal residues</td>
<td>Hayes and Goldstein, 1974</td>
</tr>
<tr>
<td>Ricinus communis</td>
<td>RCA-I</td>
<td>Terminal Galβ1, 4GlcNAc disaccharides</td>
<td>Baenziger and Fiete, 1979; Green and Baenziger, 1987</td>
</tr>
<tr>
<td>Datura stramonium</td>
<td>DSA</td>
<td>Bi-, tri- and tetrantennary sugar chains in which at least one N-acetyllactosamine is present</td>
<td>Yamashita et al., 1987</td>
</tr>
<tr>
<td>Arachis hypogaea</td>
<td>PNA</td>
<td>Galβ1,3GalNAc</td>
<td>Lotan et al., 1975</td>
</tr>
<tr>
<td>Galanthus nivalis</td>
<td>GNA</td>
<td>Terminal Manα1,3 in high mannose N-linked oligosaccharides</td>
<td>Shibuya et al., 1988</td>
</tr>
<tr>
<td>Canavalia ensiformis</td>
<td>Con A</td>
<td>Trimannosyl core of high mannose and bi-antennary complex type N-linked oligosaccharides</td>
<td>Bhattacharyya et al., 1987; Brewer and Bhattacharyya, 1986, 1988</td>
</tr>
<tr>
<td>Artocarpus integrifolia jacin (jacin)</td>
<td>AIA</td>
<td>GalNAc (Tn antigen)</td>
<td>Wu et al., 2003</td>
</tr>
<tr>
<td>Helix pomatia</td>
<td>HPA</td>
<td>Terminal α- and β-linked GalNAc residues</td>
<td>Piller et al., 1990; Wu and Sugii, 1991</td>
</tr>
<tr>
<td>Dolichos biflorus</td>
<td>DBA</td>
<td>βGalNAc</td>
<td>Piller et al., 1990; Wu and Sugii, 1991</td>
</tr>
<tr>
<td>Glycine max</td>
<td>SBA</td>
<td>GalNAc</td>
<td>Piller et al., 1990; Wu and Sugii, 1991</td>
</tr>
<tr>
<td>Maclura pomifera</td>
<td>MPA</td>
<td>Galβ1,3GalNAc, GalNAc (Tn antigen)</td>
<td>Goldstein and Poretz, 1986</td>
</tr>
<tr>
<td>Maackia amurensis</td>
<td>MAA</td>
<td>Neu5Aco2,3Galβ1,4GlcNAc, NeuGcα2,3</td>
<td>Wang and Cummings, 1988; Knibbs et al., 1991; Brinkman-Van der linden et al., 2002</td>
</tr>
<tr>
<td>Sambucus nigra</td>
<td>SNA</td>
<td>Neu5Aco2,6Gal/GalNAc, NeuGcα2,6</td>
<td>Shibuya et al., 1987; Mandal and Mandal, 1990; Brinkman-Van der linden et al., 2002</td>
</tr>
<tr>
<td>Linus flavus</td>
<td>LFA</td>
<td>NeuGC, Neu5Ac</td>
<td>Knibbs et al., 1993</td>
</tr>
<tr>
<td>Triticum vulgaris</td>
<td>WGA</td>
<td>Sialylated glycan, polylactosamine sequences</td>
<td>Bhavanandan et al., 1977; Debray et al., 1981; Gallagher et al., 1985</td>
</tr>
<tr>
<td>Solanum tuberosum</td>
<td>STA</td>
<td>β-GlcNAc</td>
<td>Allen and Neuberger, 1978; Debray et al., 1981</td>
</tr>
<tr>
<td>Tetragonolobus purpureas</td>
<td>LTA</td>
<td>Type 2 chains containing Fuc residues on C-2 of the Gal of Galβ1,4GlcNAc, with or without a second Fuc on the GlcNAc</td>
<td>Pereira and Kabat, 1974; Sugii and Kabat, 1982</td>
</tr>
<tr>
<td>Ulex europaeus</td>
<td>UEA-I</td>
<td>H type 2 antigen and Lewis®</td>
<td>Sugii and Kabat, 1982; Baldus et al., 1996</td>
</tr>
<tr>
<td>Aleuria aurantia</td>
<td>AAA</td>
<td>Complex-type N-linked oligosaccharides with a α1,6 fucosyl residue at the innermost GlcNAc</td>
<td>Osawa and Tsuji, 1987; Yamashita et al., 1985</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>PHA-E</td>
<td>Complex type N-linked glycans bearing a bisecting GlcNAc linked β1–4 to the β-linked mannose</td>
<td>Cummings and Kornfeld, 1982; Yamashita et al., 1983; Green and Baenziger, 1987</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>PHA-L</td>
<td>Complex type N-linked glycan bearing β1,6 linked lactosamine to the β-linked mannose</td>
<td>Cummings and Kornfeld, 1982; Green and Baenziger, 1987</td>
</tr>
</tbody>
</table>
peptide (amino acid residues 335–350) was kindly provided by Dr Jurrien Dean (NIH, USA) (Rankin et al., 1998). Different antibodies directed against Lewis and sialyl–Lewis antigens were also used. Human polyclonal anti-α-Gal IgG antibodies were kindly supplied by Dr Uri Galili (Allegheny University, Philadelphia, PA, USA). Mouse monoclonal antibody TEC-02, specific for GalNAcβ1,4-Galβ1,4 disaccharide, was kindly provided by Dr Peter Dräber (Institute of Molecular Genetic, Czech Republic).

### Human oocyte fixation and embedding procedure

Human oocytes used in this study were donated by patients from the IVF programme of the Reproduction Unit of the Hospital La Fe (Valencia, Spain). Informed consent was obtained from all subjects participating in the study as approved by the ethics committee at the Hospital La Fe in Valencia. These oocytes represent material that is normally discarded.

In most cases, metaphase II (MII) oocytes that failed to be fertilized by IVF technique were fixed 48–72 h after oocyte retrieval. However, it is important to take into consideration, as previously described, that there exists the possibility of fertilization arrest at different stages that is undetectable using light microscopy (Asch et al., 1995). Germinal vesicle (GV) oocytes were fixed 2 h after oocyte retrieval. The GV oocytes were included in this study to discard a possible modification of the carbohydrate composition of the ZP due to the effect produced by the sperm during the IVF process or by a partial cortical reaction of the unfertilized MII oocytes after the 48–72 h culture period.

GV (nine oocytes from five patients) and MII (28 oocytes from 16 patients) human oocytes were fixed in 2% glutaraldehyde buffered in sodium cacodylate pH 7.4 for 2 h at 4°C. After extensive washing in the cacodylate buffer, the oocytes were embedded in 2% agarose. The samples were then processed for embedding in Lowicryl medium as previously described (Avilés et al., 1994, 1996, 1997).

### Lectin- and immunocytochemistry

Colloidal gold particles were employed as marker for cytochemistry at the ultrastructural level. For DIG- and HRP-labelled lectins, a three step-method was used (Avilés et al., 1996, 1997a, 2000a). The grids were floated on a drop of phosphate-buffered saline (PBS) supplemented with 1% BSA for 10 min, and then transferred to a drop of the labelled lectins in PBS for 1 h. After washing in buffer, the grids were floated on a drop of anti-HRP polyclonal antibody from rabbit (1:500) and anti-DIG mouse monoclonal antibody for HRP- and DIG-labelled lectins respectively diluted in PBS for 1 h. Grids were washed in PBS and floated on a drop of protein A- and goat anti-mouse IgG + M–gold conjugate antibodies (15 nm) for HPR- and DIG-labelled lectins respectively, for 1 h.

For immunocytochemistry, a three step method was used for the mouse anti-human ZP3 monoclonal antibody. The ultrathin sections were incubated with the anti-ZP3 antibody (1:500) for 1 h at room temperature. After several washings in PBS, the grids were floated in a drop of an unconjugated rabbit anti-mouse IgG antibody (1:400). After washing, ultrathin sections were incubated with a protein A–colloidal gold conjugate (1:60) (15 nm).

For the mouse monoclonal antibodies against Lewis and sialyl–Lewis antigens, a two step method was used. Briefly, the grids were incubated with the primary antibody (1:30) and, after the corresponding washes, the sections were incubated with the goat anti-mouse IgG + M–gold conjugate antibodies (15 nm).

After washing in PBS buffer and twice in distilled water, the grids were counterstained with uranyl acetate and lead citrate. The ultrathin sections were observed in a Phillips Tecnai 12 electron microscope (The Netherlands).

### Controls

#### Negative controls

The following controls were used. (i) Substitution of the anti-ZP3 and anti-sialyl–Lewis antibodies by the corresponding buffer. (ii) Preincubation of the lectins with the corresponding hapten–sugar inhibitor used at 0.2 mol/l: D-GlcNAc (for WGA), N-acetyllactosamine (for DSA), L-Fuc (for AAA), D-Gal (for PNA and RCA I). (iii) Preincubation of the grids with neuraminidase for MAA, LFA and anti-sialyl–Lewis antibodies. (iv) For the anti-sialyl–Lewis antibodies, the ZP from mice ovarian oocytes were used. No antibody binding was observed in the ZP from this species as previously described (Avilés et al., 2000b) and these carbohydrate sequences were also not detected in the mouse ZP glycoproteins using biophysical methodology (Easton et al., 2000).

#### Positive controls

Mouse ZP for BSAI-B4, DBA, HPA, PNA, SBA and anti-α-Gal antibody and rat oviduct for GNA, LTA and UEA I were used as previously described (Avilés et al., 1997a, Avilés et al., 2000a,b). Rat stomach (Toma et al., 2001) and bovine ZP (unpublished results) were used for SNA. Reed sternberg cells of Hodgkin’s disease and circulating human granulocytes were used as positive controls for anti-Lewis a antibody binding. Human colon adenocarcinoma was also used as a positive control for the binding of anti-Lewis a, anti-Lewis b and anti-Lewis x antibodies as previously described (Avilés et al., 2000b). An additional control was employed for the anti-Lewis a and Lewis x antibodies consisting of the incubation of the ultrathin sections of human oocytes with neuraminidase.

### Enzymatic treatment

#### Neuraminidase treatment

WGA shows affinity for polylactosamine sequences (Gallagher et al., 1985), polyvalent sialylated O-linked T antigen (Bhavanandan et al., 1985).
1977) and terminal GlcNAc residues (Debray et al., 1981). Digestion of ultrathin sections with neuraminidase followed by WGA staining was performed to establish the presence of sialylated glycans. Neuraminidase treatment was employed to remove terminal sialic acid residues, thus exposing potential ligands for lectins or antibodies masked by these acidic sugars. This treatment was used with some lectins (DBA, HPA, LFA, LTA, PNA, SBA and UEAI) and antibodies (Lewis α, Lewis β, Lewis γ, sialyl–Lewis α, sialyl–Lewis γ). Grids carrying the sections were treated with neuraminidase type V from Clostridium perfringens (1 IU/ml) in acetate buffer pH 5.0 at 37°C for 3 h to remove sialic acid residues.

**Galactose oxidase treatment**

This treatment was performed as previously described (Avilés et al., 1997b). Briefly, ultrathin sections were incubated with 50 IU/ml of galactose oxidase in PBS (pH 7.2) for 12 h at 37°C in a moist chamber. This enzymatic treatment was employed in combination with the following lectins: AIA, MPA and PNA. For the enzymatic controls, the different samples were incubated in the appropriate buffer lacking the enzyme.

**Quantitative analysis**

In the present study, the ZP was divided into two zones of approximately equal thickness: inner and outer zones. The inner zone is normally located close to the oocyte cell itself while the outer zone faces the cumulus cells and mediates sperm binding. These two regions show different morphological and cytochemical characteristics. Because the human ZP is relatively thick compared to other species such as the mouse, rat and hamster, the inner and outer zone were subdivided into two regions that were designated inner 1, inner 2, outer 1 and outer 2 according to their proximity to the oocyte cell. For quantitative analysis, separation of the ZP into inner and outer zones was indicated on photomicrographs by drawing a line separating the ZP into two equal halves concentrically. At least five randomly selected areas of ZP of each unfertilized MII oocyte (five oocytes from four patients) were photographed on the electron microscope at × 4500 magnification. The negatives were enlarged at a final magnification of ×13 500. Fifteen to twenty fields (squares of 2.5 μm side) of the different regions of the ZP from each oocyte were used for the quantitative evaluation. Density of labelling was assessed with a computer-assisted image analyser Q500MC (Leica, Spain).

The density of labelling was determined by automatic counting of the gold particles enclosed in manually delineated areas. A statistical comparison was performed for lectin and antibody labelling in the four regions of the ZP previously described. Labelling densities were compared using analysis of variance and T3 de Dunnet and Games-Howell test with \( P < 0.05 \) performed with SPSS v10.

**Results**

**Cytochemistry of the human zona pellucida**

**Lectin- and immunocytochemistry**

No qualitative differences in the lectin- and immunolabelling pattern was observed between the GV and MII oocytes.

Staining with anti-ZP3 antibodies revealed an intense immunoreactivity that was observed over the thickness of the ZP of both GV and MII oocytes (Figure 1). The human ZP associated with different oocytes also stained intensely with anti-sialyl–Lewis α and anti-sialyl–Lewis γ antibodies (Figure 2). No immunolabelling was detected with the other antibodies employed in the present study (Table II).

The human ZP of GV and MII oocytes displayed affinity for the following lectins: AAA, ConA, DSA, LFA, Jacalin, MAA, MPA, E-PHA, L-PHA, RCA I and WGA (Figures 3–7). However, no labelling was detected in the human ZP with BSA-I-B4, DBA, HPA, GNA, LTA, PNA (Figure 9a), SBA, SNA, STA or UEAI.

**Neuraminidase treatment**

The human ZP stained intensely with LTA, PNA and SBA lectins, but only after the removal of terminal sialic acid by neuraminidase treatment (Figures 8, 9b). This treatment also exposed binding sites for the anti-Lewis α and anti-Lewis γ antibodies. However, WGA binding was reduced but not eliminated by digestion with this glycosidase. LFA, MAA, anti-sialyl–Lewis α and anti-sialyl–Lewis γ antibody labelling was completely abolished after neuraminidase digestion. No

![Figure 1](image)

**Figure 1.** Metaphase II oocyte. Anti-human-ZP3 antibody. An intense immunolabelling is observed throughout the zona pellucida (ZP). Note an unreactive cortical granule (arrow) in the ooplasm. O = oocyte. Scale bar = 1 μm.
modifications in the binding of antibody, DBA, HPA, UEAI and anti-Lewis b were observed following neuraminidase treatment.

Quantitative analysis
Quantitative analysis was restricted to a subset of the lectins and antibodies that reacted with the human ZP using MII oocytes due to the limitation of the number of human oocytes available for study. The results of this study are summarized in Table III. We observed colloidal gold particles indicating AAA and WGA lectin binding primarily in the inner region of the ZP proximal to where the oocyte cell would normally be. A similar colloidal gold distribution was observed with anti-ZP3 antibody. However, immunolabelling with anti-sialyl–Lewisa and anti-sialyl–Lewis x antibodies was denser in the outer region of the ZP. The differences observed between the different regions of the human ZP were statistically significant.

Cytchemistry of the human cortical granules
Cortical granules showed a typical localization just beneath the oocyte plasma membrane. No qualitative difference in the lectin- and immunolabelling pattern was observed between the GV and MII oocytes. The granules were intensely labelled with the following lectins: AAA, AIA, DSA, LFA, MPA, E-PHA and WGA (Figures 4a, 5, 7, 11). Both the MAA lectin and anti-sialyl Lewis x antibody reacted weakly with the granules (Figure 11e). However, the labelling of the cortical granules was not uniform. Thus, most of the cortical granules observed in one ultrathin section were labelled with these lectins but others were not reactive (Figure 11c).

No labelling was observed with the BSA1-B4, ConA, DBA, GNA, HPA, LTA, L-PHA, PNA (Figure 10a), RCA I, SBA, SNA, STA, UEA I or the other antibodies employed in this study. After neuraminidase treatment, an intense PNA reactivity was observed over the cortical granules (Figure 10b); however, no changes were observed in the binding of DBA, HPA, LTA and SBA (Figure 8).

Figure 2. Metaphase II oocyte. Anti-sialyl–Lewis a antibody. Most of the zona pellucida (ZP) is intensely immunolabelled; however, a weak labelling is observed in the ZP close to the oocyte. Cortical granules (arrows) are unlabelled. O = oocyte. Scale bar = 1 µm.

Figure 3. Metaphase II oocyte. WGA lectin. An intense labelling is observed throughout the zona pellucida (ZP). O = oocyte. Scale bar = 1 µm.
Discussion

Carbohydrate composition of the human zona pellucida

Recent studies using transgenic mice indicate that oocytes expressing human ZP2 and ZP3 instead of their murine analogues bind murine but not human sperm (Rankin et al., 1998, 2003). These results suggest that murine sperm–oocyte binding is carbohydrate dependent. These results also suggest that human sperm bind to specific carbohydrate sequences expressed on the native human ZP that are not present in the mouse ZP.

Due to a critical involvement of carbohydrate in the sperm–ZP interaction, a detailed description of the carbohydrate composition of the human ZP is necessary. The ZP properties are modified by the release of the cortical granules after oocyte activation due to fertilization (Sun, 2003). A previous study reported that a considerable number of oocytes thought to be unfertilized have actually initiated the fertilization process; however, they are arrested at specific stages (Asch et al., 1995). It is therefore possible that in some cases the cortical granules could be released. To address this specific point, we have investigated only MII oocytes that showed abundant cortical granules in the cortex region of the oocyte. Moreover, GV oocytes were also included in this study to compare the lectin and antibody labelling patterns with the MII oocytes. No qualitative differences between these two groups of oocytes were observed. However, a detailed quantitative study including these oocytes and others such as MI and in vitro-matured MII oocytes is necessary to clarify the molecular mechanism of the zona maturation previously described (Oehninger et al., 1991).

Sialic acid residues

Clark and co-workers first suggested the possibility that the interaction between human sperm and the ZP could be mediated by a selectin-like interaction (Patankar et al., 1998). Figure 4. (a) Metaphase II oocyte. AAA lectin. (a) The zona pellucida (ZP) shows a homogeneous labelling. Note the intense reactivity in the cortical granules (arrows). (b) Control section incubated with AAA in the presence of 0.2 mol/l fucose. Labelling is abolished in the ZP and cortical granule (arrow). m = mitochondria; O = oocyte; PVS = perivitelline space. Scale bar = 1 μm.

Figure 5. Metaphase II oocyte. DSA lectin. A uniform and strong labelling is observed in the zona pellucida (ZP). Cortical granule (arrow) seen immediately below the oocyte plasma membrane is also labelled. O = oocyte. Scale bar = 1 μm.
In this study, we confirm that some of the human oocyte glycans are terminated sialyl–Lewis^a^ and sialyl–Lewis^x^, consistent with previous results (Lucas et al., 1994). The observation that neuraminidase digestion led to the loss of these sequences coincident with the exposure of binding sites for anti-Lewis^a^ and Lewis^x^ antibodies provided complementary evidence for sialyl–Lewis^a/x^ expression. Therefore known selectin ligands are associated with the human ZP. The binding of the lectin from Maackia amurensis (MAA) indicates that terminal Neu5Acα2-3Galβ1,4GlcNAc sequences are also expressed on the human ZP.

Other sialylated sequences were also detected by using a combination of the lectins PNA, SBA and SNA in conjunction with neuraminidase treatment. Thus, sialylated T-antigen (Galβ1,3GalNAc-Ser/Thr) and the terminal sequence Neu5Ac-GalNAc were detected by demonstrating PNA and SBA binding following neuraminidase digestion. Moreover, the lack of binding observed with the SNA lectin, specific for Neu5Acα2,6Gal/GalNAc (Table I), strongly suggests the presence of α2,3 linkage between the sialic acid and the penultimate carbohydrate of the T and Tn antigens. Some of the lectins used in this study also bind to N-glycolylneuraminic acid (NeuGc) residues. NeuGc is abundantly expressed in the mouse ZP (Easton et al., 2000). However, this carbohydrate residue is not a normal constituent of human glycoproteins due to the genetic inactivation of the enzyme required for its synthesis (Irie et al., 1998).

Fucose residues

Fucose residues have been suggested to be involved in the sperm–oocyte interaction in different species from invertebrate to human (Ahuja, 1982; Tesarik et al., 1993; Miranda et al., 1997). A recent study suggests that epitopes containing fucose in the Lewis-like structure are involved in the human gamete interaction due to the ability of human sperm to bind to oocytes from the mollusc bivalve Unio elongatulus (Focarelli et al., 2003). In this study, we employed three different lectins (AAA, LTA, UEA I) and several monoclonal antibodies (Table II) to detect fucosylated oligosaccharides in...
Specific labelling was observed with the AAA lectin. This lectin reacts with N-glycans expressing fucose-linked $\alpha 1,6$ linked by GlcNAc residues proximal to the Asn linkage site (Yamashita et al., 1985; Osawa and Tsuji, 1987). This type of N-linked oligosaccharide was previously identified in the mouse and pig ZP glycoproteins (Easton et al., 2000; Nakano and Yonezawa, 2001). The fact that LTA reactivity and anti-Lewis x antibody binding is observed only after neuraminidase treatment suggests that this lectin could be recognizing the $\alpha 1,3$-linked fucose associated with the Lewis x antigen. The high affinity observed by the LTA lectin for oligosaccharides containing the Lewis x determinant was previously demonstrated (Pereira and Kabat, 1974; Yan et al., 1997). As noted previously, other fucose residues present in the sialyl–Lewis antigen context were also detected (see Table II).

**N-Acetylgalactosamine residues**

A previous study suggests the presence of a galactosyl binding protein in the sperm plasma membrane in both the rat and human (Goluboff et al., 1995; Rivkin et al., 2000). Recently, this C-type lectin was cloned in the rat sperm and shown to have a higher affinity for the glycans terminated with $\beta$-linked GalNAc than $\beta$-linked Gal (Rivkin et al., 2000). In this study, we have used five different lectins (Table I) with different affinity for $\alpha$- and $\beta$-linked GalNAc residues. Only AIA (jacalin) and MPA lectins displayed affinity for the human ZP. No labelling was observed with the other lectins employed, consistent with previous studies (Bar-Shira Maymon et al., 1994; Lucas et al., 1994; Talevi et al., 1997). AIA is specific for the Tn antigen (GalNAC$\alpha$-Ser), demonstrating the presence of this carbohydrate in the human ZP. Furthermore, MPA lectin binding is also consistent with the expression of the Tn antigen. It is known that MPA also reacts strongly with the T antigen (Gal$\beta$1,3GalNAc) (Goldstein and Poretz, 1986; Wu and Sugii, 1991). However, the lack of PNA binding to native human ZP indicates that this matrix does not express the Gal$\beta$1,3GalNAc.
The ZP was divided into four regions that we call inner 1, inner 2, outer 1, and outer 2 according to their proximity to the oocyte. The ZP was divided into four regions that we call inner 1, inner 2, outer 1, and outer 2 according to their proximity to the oocyte.

**Antigens**

- **Anti-ZP**
  - 49.1
  - SLXα: 20.7
  - SLXc: 23.3
  - AAAb: 79.8

**Values are mean ± SEM.**

The ZP was divided into four regions that we call inner 1, inner 2, outer 1, and outer 2 according to their proximity to the oocyte.

- **WGA**
  - 58.3 ± 1.3
- **AAA**
  - 79.8 ± 2.4
- **SLA**
  - 23.3 ± 1.5
- **SLX**
  - 20.7 ± 0.7
- **Anti-ZP**
  - 49.1 ± 1.5

**Statistical analysis**

- No statistically significant differences between o1 and o2 regions of the ZP.
- Statistically significant differences between the o1 region and the other regions of the ZP (P < 0.05).
- No statistically significant differences between i2 and o2 regions of the ZP.
- Statistically significant differences between the rest of the combinations (P < 0.05).

**Disaccharide**

In this study, we obtained a similar reactivity in both unfertilized GV oocyte and failed fertilization MII oocytes. This result is in partial disagreement with a previous study (Talevi et al., 1997). These authors observed that the MPA lectin reacted only with the ZP of the failed fertilization MII oocytes. The reason for this discrepancy is not yet apparent but may be due to the different source of the lectin and to the different methodology employed (i.e. electron microscopy versus light microscopy).

This study demonstrates the differential expression of GalNAc residues on the human ZP compared with other mammalian species. Thus, oligosaccharides terminated with GalNAc residues in the context of the Sdα antigen (NeuAcα2,3GalNAcβ1,4Gal) expressed on the mouse ZP and implicated in the secondary binding and/or sperm penetration in this species (Cahová and Dráber, 1992; Avilés et al., 1999; Easton et al., 2000) are not observed in the human ZP. However, Tn antigen detected in the human ZP has not been described in the ZP of other species. This carbohydrate epitope is not usually found in normal human tissue except in early human embryos (Springer, 1984). Tn antigen is a pancarcinoma antigen expressed on many tumour cells (Springer, 1984; Kim et al., 1996). It is generally accepted that elevated Tn antigen expression is correlated with increased cell adhesion, invasion and metastasis of cancer cells (Cao et al., 1995). The role played by this carbohydrate epitope in human fertilization remains to be determined.

### Mannose residues

Mannose residues have been proposed to play an important role in the human sperm–oocyte interaction (Mori et al., 1989; Tulsiani et al., 1990; Tesarik et al., 1991; Benoff et al., 1993a–c; Miranda et al., 1997; Maegawa et al., 2002). Previous studies suggest the presence of mannose-containing glycans in many different species including the human using the ConA lectin (Bar-Shira Maymon et al., 1994; Lucas et al., 1994; Talevi et al., 1997). GNA is a much more specific lectin for detecting high mannose N-linked oligosaccharide than ConA (Shibuya et al., 1988). This latter lectin binds both high mannose and biantennary complex type N-glycans (Brewer and Bhattacharyya, 1986, 1988; Bhattacharyya et al., 1987). High mannose type N-glycans are expressed in the rat and mouse ZP based on both biochemical and biophysical methods (Easton et al., 2000; Tulsiani, 2000) but not by cytotoxic techniques using GNA (Avilés et al., 1994, 1996, 1997a). Similar results were observed in the human ZP. These data suggest to us that there are other modifications (i.e. sulphation or phosphorylation) of the high mannose type N-glycans that render them unrecognizable by GNA but not by ConA. More precise analysis will be necessary to confirm this proposed expression.

### Galactose residues

Terminal galactose residues have been implicated in sperm–oocyte binding in many different mammalian species (Benoff, 1997; Tulsiani, 1997; Shalgi and Raz, 1997). In this study, the lack of specific lectin (BSAI-B4) and a polyclonal anti-α-Gal antibody binding indicate that the Galα1–3Gal sequence is not expressed on human oocytes. Human sperm–

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**Figure 10.** Metaphase II oocyte. PNA lectin. (a) No labelling is observed in cortical granules (arrows). (b) After neuraminidase treatment, a clear labelling was observed in the cortical granules (arrows). O = oocyte; PVS = perivitelline space. Scale bar = 0.5 μm.
ZP binding is inhibited in vitro in the presence of high concentrations of Gal or RCA-I, a lectin that normally reacts with terminal β1,4-linked Gal residues (Mori et al., 1989; Miranda et al., 1997). Human oocytes react well with RCA-I, consistent with the expression of terminal β-linked Gal residues. Human ZP also binds DSA, a lectin that can also bind to polylactosamine chain extensions (Cummings and Kornfeld, 1984) or terminal Galβ1,4GlcNAc sequences β1,6-linked to the core mannose residue of complex type N-glycans (Yamashita et al., 1987). This reactivity with

Figure 11. Cortical granules. (a) AAA lectin. Metaphase II oocyte. An intense reactivity was observed in the cortical granules (arrows). (b) AIA lectin. MII oocyte. A clear labelling was observed in the cortical granules (arrows). (c) AIA lectin. MII oocyte. After galactose oxidase treatment the labelling is abolished. (d) MPA lectin. MII oocyte. An intense affinity for cortical granules is observed (arrow); however, other cortical granules (arrowheads) are devoid of labelling. (e) MPA lectin. GV oocyte. Cortical granules are also intensely labelled. (f) DSA lectin. MII oocyte. Cortical granules (arrows) are moderately labelled. (g) MAA lectin. MII oocyte. A weak labelling is observed in the cortical granules (arrows). (h) PHA-E lectin. MII oocyte. The cortical granules (arrows) are labelled. (i) WGA lectin. Cortical granules (arrows) are specifically bound by this lectin. m = mitochondria; PVS = perivitelline space; O = oocyte. Scale bar = 0.5 μm.
The strong binding of E-PHA is consistent with the expression of biantennary and triantennary bisecting type N-glycans. It was previously suggested that the carbohydrate residues recognized by E-PHA could be involved in the protection of the human oocyte from immune responses mediated by natural killer cells in the female genital tract (Patankar et al., 1997). This sequence is also expressed on human sperm (Patankar et al., 1997). The binding of L-PHA strongly suggests that complex type N-glycans terminated with Galβ1,4GlcNAc sequences β1,6-linked to a core mannoside residue are also expressed on the human ZP. This result is also consistent with the expression of DSA binding sites, which can also detect the same sequence.

N-acetylglucosamine residues

Previous studies using WGA lectin, GlcNAc residues and β-hexosaminidase digestion suggest that terminal β-linked GlcNAc residues are required for human sperm binding using the hemizona assay (Mori et al., 1989; Miranda et al., 1997, 2000). In this study, we demonstrate the presence of the GlcNAc residues in the context of the polylactosamine sequence in the human ZP by the sequence neuraminidase–WGA (Gallagher et al., 1985). We have used another GlcNAc-specific lectin, STA, but no labelling was detected. This lectin reacts with ovine, mouse and rat ZP (unpublished results). These data suggest that GlcNAc is a common carbohydrate of the ZP of different species; however, they are present in a specific and not yet determined context as demonstrated by the different binding observed with different GlcNAc-specific lectins.

Heterogeneity of the human zona pellucida

A previous study indicates a substantial difference in the binding of sperm to the inner and outer regions of the human ZP (Burkman et al., 1988). Ultrastructural cytochemistry studies suggest a heterogeneous carbohydrate distribution throughout the thickness of the mouse and rat ZP (Avilés et al., 1996, 2000b). We observed a similar type of different distribution in this study. Our quantitative analyses indicate that some carbohydrate sequences like sialyl-Lewis α and Lewis x are mainly located in the outer region of the ZP. The immunolabelling observed with the anti-ZP3 antibody and with the lectins WGA and AIA indicate that these components are primarily localized to the inner region of the ZP. A previous scanning electron microscopy study demonstrated that the more compacted region of the ZP is proximal to the oocyte cell in mammalian oocytes. By contrast, the more porous region of the ZP is in contact with follicular cells in different species and constitutes the contact area for sperm binding (Phillips and Shalgi, 1980a,b; Familiari et al., 1992). The heterogeneous distribution of the carbohydrate sequences observed in this study is not due to the different structural characteristics of the inner and outer region of the human ZP previously reported in a scanning electron microscopy study. The distribution of human ZP (the putative sperm receptor glycoprotein) was higher in the inner region of the ZP proximal to the oocyte with a more compact structure compared with the outer region of the ZP that is more porous. We consider that this different distribution of the human ZP glycoproteins probably occurs during ZP synthesis as we have previously reported in the mouse and rat ZP (Avilés et al., 2000a,b). However, we cannot discard the hypothesis that a different supramolecular structure of the ZP is present in the outer and inner region, producing some kinds of steric problems that affect the accessibility of the lectins or antibodies and sperm binding as suggested previously (Rankin et al., 2003).

The different composition of the ZP through its thickness could also be related to sperm penetration as previously suggested in the mouse (Cahová and Dráber, 1992; Avilés et al., 1999).

Previous scanning electron microscopical reports provide information about the inner and outer region of the ZP only, unlike the current study. Our ultrastructural cytochemical findings using anti-human ZP3 antibodies suggest that the porous region of the ZP is limited to the 25% of the external region of the human ZP, whereas the compacted region constitutes ~75% of the total ZP. This hypothesis is consistent with the accessibility of the anti-ZP3 antibody to the entire thickness of the ZP in an ultrathin section. The origin of this different structural organization in the compact versus porous zones of the human ZP remains unresolved.

Composition of the cortical granules from human oocytes

Cortical granules are specialized secretory granules located just beneath the plasma membrane of the oocyte and are involved in the block to polyspermy. However, their direct role in this process is still unknown due to the lack of detailed data about cortical granule composition and their effect on the ZP (Hoodbhoy and Talbot, 1994; Yanagimachi, 1994; Gross et al., 2000; Hoodbhoy et al., 2000; Sun, 2003).

Several lines of evidence suggest that the WGA and LCA lectins specifically recognize cortical granule contents in different human oocytes by means of fluorescence microscopy (Talevi et al., 1997; Ghetler et al., 1998; Sengoku et al., 1999). In this study, we demonstrate the presence of the following carbohydrate residues by means of lectin–gold cytochemistry GlcNAc (WGA), Fuc (AAA), Galβ1,4GlcNAc (DSA), GalNAc (MPA and AIA), Neu5Acα2,3Galβ1,4 GlcNAc (MAA), Neu5Acα2,3Galβ1,3GalNAc (neuraminidase treatment followed by PNA binding) and biantennary and/or triantennary bisecting type N-glycans (E-PHA). However, other lectins and antibodies that react with human ZP do not bind to the cortical granules. Thus the carbohydrate composition of the cortical granules does not completely overlap with the human ZP. We consider that the carbohydrate composition similarities observed between the cortical granules and the ZP are not due to a partial or total cortical granule exocytosis as previously described in some species including the human. In this study, all the oocytes analysed showed a large number of cortical granules in
the oocyte cortex avoiding the possibility that the cortical reaction was produced due to the culture period. A quantitative analysis of the modification of carbohydrate composition using fertilized human oocytes that failed to develop or activated human oocytes in vitro using a calcium ionophore should be performed to determine the effect of the cortical granules in the ZP carbohydrate composition and its implication in the zona reaction.

The intensity of lectin binding to the cortical granules was not uniform, however. Some cortical granules are completely unreactive. This suggests that a different population of cortical granules exists in the human oocyte, an observation previously reported using conventional electron microscopy (Nicosia et al., 1997) and lectin cytochemistry at ultrastructural level in other species (Avilés et al., 1994, 1996, 1997a). The functional significance of the heterogeneous cortical granule population described in human species and other species remains unresolved.

In summary, we have demonstrated that the human ZP possesses a unique carbohydrate composition that is quite different from other mammalian species. This suggests that the glycosylation machinery is different in human oocytes. These findings are consistent with the hypothesis that differential glycosylation could be responsible for the unique specificity that human sperm manifest in their binding to the oocytes of higher primates (Bedford, 1977).

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References


Carbohydrate composition of the human ZP

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