Variations in modifications of sugar residues in hamster zona pellucida after *in vivo* fertilization and *in vitro* egg activation

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Lectins were used as probes in conjunction with quantitative analysis to investigate the distribution of different carbohydrate residues in hamster zona pellucida and their possible modification patterns after *in vivo* fertilization and *in vitro* egg activation. Several lectins including HPA, WGA, RCA-I, PNA, DSA, BSAIB₄, DBA, AAA and MAA were used to label the zona pellucida of both unfertilized and fertilized eggs. With the exception of PNA and BSAIB₄, the same lectins were also used to label the zona pellucida of oocytes activated *in vitro*. A multicomparison quantitative analysis of the density of labelling in the inner and outer regions of the zona pellucida before and after fertilization *in vivo*, as well as after *in vitro* egg activation, was performed. Of all the lectins studied, preferential localization of labelling by RCA-I and DSA to the inner zona pellucida of unfertilized eggs was observed. After *in vivo* fertilization, there was an increase in labelling in the inner region of the zona pellucida when thin sections of fertilized oocytes were incubated with HPA, BSAIB₄ and AAA. Although increased labelling by RCA-I was observed, a significant decrease in labelling intensity was obtained with WGA and the sequence Neu-WGA in both the inner and

outer zona pellucida of fertilized oocytes. A significant increase in the density of labelling with WGA was also observed after digestion with neuraminidase. In parallel, when hamster oocytes activated *in vitro* were compared with those fertilized *in vivo*, a difference in lectin–gold labelling was observed in both the inner and outer region of the zona pellucida. Labelling with HPA, WGA, DSA and MAA increased significantly in both the inner and outer regions of the zona pellucida, whereas labelling by DBA significantly decreased in the inner portion of the zona pellucida. After neuraminidase treatment, a significant increase in labelling density was observed when thin sections of *in vitro*-activated oocytes were incubated with WGA. These results demonstrate: (i) the post-fertilization modifications of major saccharidic determinants that may play a role in the sperm–egg interaction process of fertilization *in vivo*; and (ii) that the modified properties of zonae pellucidae of fertilized and *in vitro*-activated eggs resulting from the action of hydrolytic enzymes, as well as glycoproteins released through exocytosis of cortical granules, are not identical.

Introduction

The zona pellucida is an amorphous layer of glycoproteins that surrounds mammalian oocytes. It plays a critical role during mammalian oogenesis, fertilization and preimplantation development. The composition of the zona pellucida has been characterized in several mammalian species and the zona pellucida matrix comprises mainly three major glycoproteins (ZP1, ZP2 and ZP3) that show extreme molecular mass and charge heterogeneity as a result of extensive post-translational modifications including glycosylation and sulphation (Wassarman, 1988a; Moller *et al.*, 1990; Dunbar *et al.*, 1994). Both the initial sperm binding activity and acrosome inducing activity have been ascribed to a class of O-linked oligosaccharides that are linked covalently to ZP3 (Florman and Wassarman, 1985; Bleil and Wassarman, 1988; Litscher *et al.*, 1995). Another major

glycoprotein, ZP2, is probably responsible for the secondary binding of the acrosome-reacted spermatozoa and its post-fertilization modification may play a role in the block to polyspermy (Bleil *et al.*, 1988; Wassarman, 1988a). ZP1, a homodimer in mice, may serve as a cross-linker of copolymers of ZP2 and ZP3 and, thus, adds structural integrity to the zona pellucida to facilitate the protection of the embryo as it passes down the oviduct (Wassarman, 1988b; Rankin *et al.*, 1999). However, results from other species (rabbits, pigs) indicate that there may be a role for ZP1 homologues in sperm binding (Yonezawa *et al.*, 1995; Prasad *et al.*, 1996). On fusion of a spermatozoon with the egg plasma membrane, a series of events in the egg referred to as 'egg activation' is initiated. During activation, a transient increase in intracellular Ca²⁺ concentration evokes exocytosis of cortical granule exudate into the perivitelline space, thereby altering the biological properties of the zona pellucida glycoproteins (zona reaction) and establishing a block to polyspermy (Ducibella, 1991). The nature of the

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Table 1. Lectins used for identification of sugar residues in the zona pellucida of unfertilized, fertilized and *in vitro*-activated hamster eggs

Source of lectin	Acronym	Carbohydrate binding specificity	Inhibitory sugar
<i>Helix pomatia</i> (Roman snail)	HPA	Terminal α - and β -linked GalNAc	D-GalNAc
<i>Dolichos biflorus</i> (horse gram)	DBA	Terminal α -linked GalNAc	D-GalNAc
<i>Triticum vulgaris</i> (wheatgerm)	WGA	Terminal non-reducing GlcNAc or Neu5Ac	D-GlcNAc and Neu5Ac
<i>Galanthus nivalis</i> (snowdrop bulb)	GNA	Terminal Man α 1,3 in high mannose <i>N</i> -linked oligosaccharides	Methyl- α -mannose
<i>Ricinus communis</i> (castor bean)	RCA-I	Terminal Gal β 1,4 GlcNAc disaccharides	D-Galactose
<i>Arachis hypogea</i> (peanut)	PNA	Terminal Gal β 1,3 GalNAc disaccharides	D-Galactose
<i>Datura stramonium</i> (Jimson weed)	DSA	Bi-, tri- and tetra-antennary sugar chains with at least one <i>N</i> -acetylglucosamine present	<i>N</i> -Acetylglucosamine
<i>Bandaeria simplicifolia</i> (seeds)	BSAIB ₄	Terminal α -linked galactose sugar residues	Methyl- α -galactose
<i>Ulex europaeus</i> (asparagus pea)	UEA-I	Terminal α -L-fucose	L-Fucose
<i>Lotus tetragonolobus</i> (asparagus pea)	LTA	Type 2 chains with fucose on C-2 of the galactose of Gal α 1,4GlcNAc	L-Fucose
<i>Aleuria aurantia</i> (fresh water snail)	AAA	Complex-type <i>N</i> -linked oligosaccharides with an α 1,6 fucosyl residues at the innermost GlcNAc > terminal α 1,2 fucose	L-Fucose
<i>Maackia amurensis</i> (seeds)	MAA	Neu5Ac α 2,3Gal β 1,4GlcNAc	<i>N</i> -acetylneuraminylactose
<i>Sambucus nigra</i> (elderberry)	SNA	Neu5Ac α 2,6Gal	<i>N</i> -acetylneuraminylactose

GalNAc: *N*-acetyl-D-galactosamine; GlcNAc: *N*-acetyl-D-glucosamine; Gal: galactose; Man: mannose; Fuc: fucose; Neu5Ac: *N*-acetylneuraminic acid.

biochemical alterations occurring in the zona pellucida during fertilization or egg activation is only partially understood. ZP2 undergoes a limited proteolytic cleavage and is converted to a form called ZP2f which is associated with the block to polyspermy (Bleil *et al.*, 1981; Moller *et al.*, 1990), whereas ZP3 loses both sperm receptor activity and the ability to induce the acrosome reaction (Bleil and Wassarman, 1988; Moller *et al.*, 1990); however, the migration of ZP3 on SDS–polyacrylamide gels was unchanged after the cortical granule reaction (Miller *et al.*, 1992). Although the role of at least a portion of ZP3 polypeptide backbone has been implicated in induction of the sperm acrosome reaction (Wassarman, 1988a), the above data imply clearly the crucial role of the carbohydrate moiety of ZP3-linked oligosaccharides in such post-fertilization changes. Several monosaccharide residues on ZP3, including *N*-acetylglucosamine, *N*-acetylgalactosamine, mannose, fucose, galactose and sialic acid have been implicated as the complementary sperm receptors, mediating the primary binding between the spermatozoon and the zona pellucida (Bleil and Wassarman, 1988; Miller *et al.*, 1992; Litscher *et al.*, 1995; Thaler and Cardullo, 1996; Benoff, 1997; Tulsiani *et al.*, 1997; Johnston *et al.*, 1998). However, the exact oligosaccharide sequence or sequences that mediate initial sperm–egg binding is not known. In the present study, high resolution lectin–gold cytochemistry was used with electron microscopy to characterize the distribution of various terminal oligosaccharide sugar residues of zona pellucida glycoproteins in both unfertilized and fertilized hamster eggs, as well as in hamster eggs activated *in vitro*. The multicomparison quantitative analysis of the density of labelling obtained with various lectin probes provides a means to evaluate the anticipated modifications of the different sugar residues in the bilaminar zona matrix after

fertilization *in vivo* and presents further insights into the role of cortical granule exudate in bringing about these modifications.

Materials and Methods

Reagents

Calcium ionophore A23187, polyethylene glycol (molecular mass 20 000), eCG, hCG, sodium citrate, tetrachloroauric acid ($\text{HauCl}_4 \cdot 2\text{H}_2\text{O}$), hyaluronidase (type VI, from bovine testis), neuraminidase (type V, from *Clostridium perfringens*), BSA and galactose oxidase (from *Dactylium dendroides*) were purchased from Sigma (St Louis, MO). Colloidal gold-conjugated lectins (DSA and DBA), unlabelled lectins (HPA, RCA-I, LTA, UEA-I, BSAIB₄ and WGA), ovomucoid, D-galactose, L-fucose, *N*-acetylglucosamine, *N*-acetyl-D-galactosamine, *N*-acetylneuraminic acid, *N*-acetylneuraminylactose, methyl- α -D-mannopyranoside, methyl- α -galactopyranoside and *N*-acetylglucosamine were obtained from EY laboratories, Inc. (San Mateo, CA). Digoxigenin (DIG)-labelled lectins (AAA, MAA, SNA, GNA and PNA) and mouse monoclonal IgG anti-DIG antibodies were purchased from Roche Diagnostics (Quebec). Goat anti-mouse IgG + IgM–gold complex (15 nm) was purchased from Cedarlane Research Ltd (Ontario). The taxonomic names and specificities of the lectins used in the present study are shown (Table 1).

Collection and preparation of oocytes and fertilized eggs for lectin cytochemistry

Twenty sexually mature female golden hamsters (*Mesocricetus auratus*), aged 7–8 weeks, were purchased

from Charles River Laboratories (St Constant). The hamsters were tested for the typical vaginal secretion that is found in abundance at metoestrus to obtain oviductal oocytes. The oestrous cycles of the hamsters were observed for 2–3 consecutive weeks to ascertain their regularity. Four animals were superovulated by i.p. injection of 25 iu eCG the day before the expected oestrus followed by another injection of 25 iu hCG 48 h later (Flemming and Yanagimachi, 1980). The animals were killed by cervical dislocation 17 h after injection with hCG to obtain cumulus masses (made up of unfertilized oocytes and the associated cumulus cells). The ventral abdominal wall of each animal was immediately cut open and the oviducts were excized. The ampullary portion of the oviduct was identified and torn open under a dissecting microscope using fine tweezers, and the cumulus masses were collected in PBS, pH 7.4, washed briefly in PBS and fixed by immersion at room temperature for 2 h in 2.5% (v/v) glutaraldehyde in 0.1 mol cacodylate buffer l^{-1} , pH 7.4. For collection of fertilized eggs, four female hamsters were placed with fertile males on the evening before oestrus. Fertilized eggs were collected from the oviducts of females killed by cervical dislocation on day 1 after the animals were mated. The eggs were fixed at room temperature by immersion in 2.5% (v/v) glutaraldehyde in 0.1 mol cacodylate buffer l^{-1} (pH 7.4) for 2 h. At the end of fixation, the cumulus masses and fertilized eggs were washed three times in 0.1 mol cacodylate buffer l^{-1} and left overnight at 4°C in the same buffer. For post-embedding labelling, fertilized eggs were embedded in a 3% (w/v) gelatin solution, trimmed into small cubes, dehydrated in a series of graded ethanols, infiltrated and embedded in LR White (Electron Microscopic Sciences, Fort Washington, PA) according to routine procedures. The cumulus masses were processed in the same way without trimming. Areas of interest were first located in sections of 1 μm thickness of LR White-embedded specimens by light microscopy. Thin sections of cumulus masses and fertilized eggs were cut with a diamond knife on an LKB ultramicrotome and mounted on formvar-carbon-coated nickel grids.

Ionophore activation of eggs

Cumulus masses were collected from the oviductal ampullae of 12 superovulated female hamsters prepared as described above. Cumulus cells were removed by digestion for 5 min in 1 mg testicular hyaluronidase ml^{-1} . Cumulus-free eggs were washed by consecutive transfer through at least six droplets of dmKRBT buffer (120 mmol NaCl l^{-1} , 2 mmol KCl l^{-1} , 2 mmol CaCl_2 l^{-1} , 10 mmol NaHCO_3 l^{-1} , 1.2 mmol MgSO_4 l^{-1} , 5.6 mmol glucose l^{-1} , 1.1 mmol sodium pyruvate l^{-1} , 25 mmol TAPSO (3-[N-Tris (hydroxymethyl) methylamino]-2-hydroxy propane-sulphonic acid) l^{-1} , 18.5 mmol sucrose l^{-1} , 6 mg BSA ml^{-1} , pH 7.3; Neill and Olds-Clarke, 1988; Miller *et al.*, 1993) covered with mineral oil to ensure the removal of hyaluronidase and contaminating glycosidases. The eggs ($n = 100$) were activated parthenogenetically within 1 h of

collection to avoid the deleterious effects of egg ageing, by incubation in medium containing 10 $\mu\text{mol l}^{-1}$ (final concentration) of the calcium ionophore A23187 in dmKRBT, under mineral oil, for 30 min at 37°C in a chamber acclimatized with 5% CO_2 (Cherr *et al.*, 1988; Ducibella *et al.*, 1988). Stock solution of calcium ionophore A23187 was prepared at 10 mmol l^{-1} in dimethylsulphoxide (DMSO) and kept at 4°C until used. For controls, a total of 50 eggs was placed in a medium containing dmKRBT only and incubated under the same conditions. Both ionophore-activated and control eggs were washed extensively at least six times (5 min each) by consecutive transfer through droplets of dmKRBT covered with mineral oil. The eggs were finally transferred to beem capsules (CANEMCO, St Laurent) and fixed for 2 h in 2.5% (v/v) glutaraldehyde in 0.1 mol cacodylate buffer l^{-1} at room temperature. After fixation, both control and ionophore-activated eggs were washed three times in 0.1 mol cacodylate buffer l^{-1} . For post-embedding lectin cytochemistry, activated and control eggs were processed further in the same way as unfertilized and fertilized oocytes.

Preparation of colloidal gold, lectin-gold and glycoprotein-gold complexes

Colloidal gold solutions with a mean particle diameter of 15 nm were prepared by the sodium citrate method as described by Frens (1973). Ovomucoid-gold and lectin (HPA, RCA-I, LTA and UEA-I)-gold complexes were prepared as described by Geoghegan and Ackerman (1977). The minimal amounts of lectins or protein needed for stabilization of colloidal gold and the optimal pH were estimated by the method of Geoghegan and Ackerman (1977).

Cytochemical labelling

Colloidal gold was used as a marker for lectin cytochemistry carried out at the ultrastructural level. Cytochemical labelling was performed by the one-step (Roth, 1983; Benhamou, 1986), two-step (Geoghegan and Ackerman, 1977; Benhamou, 1986) or three-step (Martínez-Menárguez *et al.*, 1992) methods. The one-step method was used for HPA, RCA-I, UEA-I, LTA, DBA and BSAIB₄. Ultrathin tissue sections were first incubated on a drop of 0.5% (v/v) BSA in 0.01 mol PBS l^{-1} (pH 7.4) for 5 min and transferred on to a drop of the lectin-gold complex for 1 h. The sections were washed with PBS followed by bidistilled water and dried on a filter paper. For WGA, the two-step method was applied. In brief, tissue sections were floated for 5–10 min on a droplet of 0.5% (v/v) BSA in PBS and transferred to a drop of unlabelled WGA diluted in PBS for 1 h. After PBS washing, the sections were floated on a droplet of ovomucoid-gold complex for 30 min followed by rinsing with PBS and double-distilled water. For lectin-DIG labelling (AAA, MAA, PNA, GNA and SNA), a three-step method was used as described by Martínez-Menárguez *et al.* (1992) and Avilés *et al.* (1996).

The above incubations were all performed at room temperature. The sections were counterstained with uranyl acetate and lead citrate before examination on a Hitachi 7000 electron microscope operated at 75 kV.

Cytochemical controls

The labelling specificities of the lectins were assessed by the following controls (incubations were all carried out at room temperature unless indicated otherwise): (i) substitution of conjugated or unconjugated lectins by the corresponding buffer; (ii) reincubation of the lectins with the corresponding hapten-sugar inhibitor at concentrations ranging from 0.1 mol l⁻¹ to 0.2 mol l⁻¹: *N*-acetyl-D-galactosamine (GalNAc) (for HPA and DBA), D-galactose (for RCA-I and PNA), D-*N*-acetyl-D-glucosamine (GlcNAc) and/or *N*-acetylneuraminic acid (Neu5Ac) (for WGA), methyl- α -mannose (for GNA), *N*-acetylglucosamine (for DSA), L-fucose (for AAA, UEA-I and LTA), methyl- α -galactose (for BSAIB₄) and *N*-acetylneuraminylactose (for MAA and SNA); (iii) preincubation of tissue sections with neuraminidase (1 iu ml⁻¹) for WGA and MAA, and with galactose oxidase for HPA and PNA.

Enzymatic treatment and controls

WGA has an affinity for GlcNAc and Neu5Ac residues. For neuraminidase treatment, tissue sections were digested with 1 iu neuraminidase in acetate buffer ml⁻¹, pH 5.0, for 3 h at 37°C before labelling with the lectin to remove Neu5Ac residues. For galactose oxidase treatment, ultrathin sections were incubated with 50 iu galactose oxidase in PBS ml⁻¹, pH 7.4, for 24 h at 37°C in a moist chamber before labelling with the corresponding lectin.

Controls for both neuraminidase and galactose oxidase treatments were performed by substitution of the enzymes with their corresponding buffers.

Quantitative analysis

Quantitative evaluation of the lectin-gold labelling was performed as described by Avilés *et al.* (2000a). For tissue sections that showed positive reactivity to lectins, the labelling densities over the zona pellucida of both unfertilized and fertilized, as well as of *in vitro*-activated, oocytes were evaluated on positive electron micrographs enlarged to $\times 22\,500$. For each of the lectins under study, the density of labelling, evaluated as the number of gold particles per μm^2 square surface area, was determined by use of a Carl Zeiss MOP-3 modular system equipped for quantitative digital image analysis.

The heterogeneous nature of the hamster zona pellucida has been revealed by lectin cytochemistry (Yanagimachi and Nicolson, 1976; Ahuja and Bolwell, 1983). More recently, the hamster zona pellucida has been shown to present a multilaminar structure by non-invasive polarized light microscopy and digital image processing (Keefe *et al.*, 1997). As such, the hamster zona pellucida appeared to

consist of two birefringent layers separated by an anisotropic layer. In the present study, for the purposes of quantitative analysis, the zona pellucida was separated into inner and outer zones on photomicrographs by drawing a line separating the zona pellucida into two equal halves concentrically. Electron micrographs were taken at random of ten different regions of the zona pellucida of unfertilized and fertilized oocytes ($n = 4$ animals in each case, 1–2 oocytes per animal), as well as of activated oocytes (five randomly selected oocytes in total). Fifteen to twenty fields (3–5 μm^2 each) of both the outer and inner zona pellucida from each egg were used for the quantitative evaluation (Table 2). Labelling densities were compared using one-way analysis of variance with $P < 0.001$ and a Student–Newman–Keuls method with $P < 0.05$ performed with a SigmaStat program (Jandel, San Rafael, CA), respectively. A multicomparison statistical analysis of the differences in the mean labelling densities for the lectin-gold labelling was undertaken for: (i) lectin labelling in the outer and inner zona pellucida within each of the three experimental groups (unfertilized, fertilized and *in vitro*-activated oocytes); (ii) lectin labelling in the inner and outer zona pellucida of oocytes before and after fertilization; (iii) lectin labelling in the inner and outer zona pellucida of fertilized and *in vitro*-activated oocytes; and (iv) lectin labelling before and after neuraminidase digestion for each of the three experimental groups.

In the present study, the number of gold particles present in control sections of unfertilized, fertilized and *in vitro*-activated oocytes incubated with various lectins in the presence of their corresponding blocking sugars was negligible and, therefore, was not included in the quantitative analysis.

Results

Distribution of specific sugar residues of oligosaccharide side chains in the oocyte proper and in the zona pellucida of unfertilized, fertilized and in vitro-activated oocytes

Electron microscope analysis of thin sections of hamster ovulated oocytes (unfertilized oocytes) labelled with HPA (Fig. 1a), WGA (Fig. 2a), PNA, BSAIB₄, DBA, AAA and MAA, including the sequence Neu-WGA, presented a homogeneous distribution throughout the zona matrix. However, labelling of ovulated eggs with DSA (Fig. 3a) and RCA-I revealed an asymmetric distribution of the corresponding binding sites in the zona pellucida. The density of labelling varied among different lectins ranging from moderate to intense. In the oocyte proper, various structures, including the Golgi apparatus and its associated vesicles, and the vesicular aggregates (made up of several large vesicles embedded in a mass of much smaller and smooth vesicles) displayed a moderate to strong labelling by gold particles. Below the oolemma, cortical granules reacted strongly to labelling by AAA, RCA-I and PNA, but

Table 2. Labelling densities in the zona pellucida of unfertilized, fertilized and *in vitro*-activated hamster eggs after incubation with different lectins

Lectins	Zona pellucida	Unfertilized eggs	Fertilized eggs	Activated eggs
HPA	Inner	93.66 ± 5.33	135.54 ± 8.40 ^{ab}	275.05 ± 8.61 ^{ac}
	Outer	118.55 ± 5.42	92.87 ± 7.71	169.90 ± 8.83 ^c
DBA	Inner	8.66 ± 1.31	12.48 ± 1.27	6.58 ± 0.98 ^c
	Outer	10.45 ± 1.70	8.06 ± 1.29	5.23 ± 0.45
WGA	Inner	79.09 ± 4.05	47.79 ± 3.26 ^b	66.91 ± 3.51 ^c
	Outer	68.32 ± 4.79	47.03 ± 2.30 ^b	58.69 ± 5.79 ^c
Neu-WGA	Inner	97.11 ± 5.08 ^d	72.75 ± 4.91 ^{bd}	92.68 ± 4.50 ^d
	Outer	92.57 ± 7.93 ^d	65.05 ± 4.32 ^{bd}	84.59 ± 9.04 ^d
RCA-I	Inner	122.91 ± 4.77 ^a	177.53 ± 4.32 ^{ab}	164.31 ± 12.87 ^a
	Outer	102.67 ± 2.88	145.58 ± 3.20 ^b	128.56 ± 7.15
PNA	Inner	17.71 ± 1.31	15.65 ± 2.11	na
	Outer	12.09 ± 1.39	12.70 ± 1.03	na
DSA	Inner	37.05 ± 2.31 ^a	28.66 ± 2.10 ^a	75.47 ± 7.74 ^{ac}
	Outer	8.13 ± 0.85	7.95 ± 0.64	22.84 ± 2.30 ^c
BSAIB ₄	Inner	20.71 ± 0.95	34.85 ± 2.61 ^{ab}	na
	Outer	18.46 ± 1.08	21.38 ± 1.46	na
AAA	Inner	77.92 ± 3.62	99.78 ± 3.57 ^b	91.16 ± 4.60
	Outer	80.36 ± 3.60	84.14 ± 1.86	83.32 ± 2.48
MAA	Inner	25.58 ± 1.92	27.26 ± 2.30	76.45 ± 3.67 ^{ac}
	Outer	19.46 ± 1.18	18.02 ± 1.02	87.23 ± 6.03 ^c

Values are gold particles per μm^2 (mean ± SEM). na: not assessed.

^aSignificant difference between inner and outer zones of the zona pellucida ($P < 0.05$).

^bSignificant difference between unfertilized and fertilized eggs ($P < 0.05$).

^cSignificant difference between fertilized and activated eggs ($P < 0.05$).

^dSignificant difference after neuraminidase treatment ($P < 0.05$).

only weakly to HPA, DBA, BSAIB₄, WGA and MAA. El-Mestrah and Kan (2001) reported similar lectin labelling results in the cortical granules. Other structures inside the oocyte, such as mitochondria, rough endoplasmic reticulum and stacks of annulate lamellae were not labelled. The perivitelline space, located between the inner boundary of the zona pellucida and the surface of ovulated oocytes, was virtually unlabelled by any of the lectins used. A representative example is shown (Fig. 1a).

Noticeable differences in the lectin binding pattern of some of the lectins used in this study were observed in the zona pellucida of fertilized eggs. Unlike unfertilized oocytes, in which the binding pattern of HPA, BSAIB₄ and AAA was homogeneous in the zona pellucida, fertilized eggs labelled with the same lectins and DSA (Fig. 3b), as well as RCA-I, displayed a heterogeneous distribution of gold particles over the entire zona pellucida (see Table 2 for statistical analysis). However, no significant difference was noted in the distribution of labelling by DBA, PNA and MAA, as well as by Neu-WGA, between the inner and outer regions of the zona pellucida. Tissue sections prepared from activated oocytes displayed a heterogeneous distribution in colloidal gold labelling over the zona pellucida with HPA (Fig. 1b), DSA (Fig. 3c), RCA-I and MAA (Table 2). A noticeably higher concentration of gold labelling was detected in the inner zona pellucida with DSA (Fig. 3c) and RCA-I, as well as in both the inner and outer zona pellucida with HPA

(Fig. 1b). When tissue sections of both fertilized and *in vitro*-activated oocytes were labelled with each of the aforementioned lectins, the pattern of labelling by gold particles over the various cellular organelles in the oocyte proper was similar to that observed in the unfertilized oocytes. Of particular interest was the detection of a specific association of intense labelling over the oolemma and in the perivitelline space of fertilized and *in vitro*-activated eggs with almost all the lectins used in this study (Figs 1b and 3c). No labelling was observed with UEA-I, LTA, GNA and SNA.

Control tissue sections of unfertilized, fertilized and *in vitro*-activated oocytes showed a negative reaction to all lectins examined (see Materials and Methods) in the zona pellucida and over previously labelled subcellular structures, thereby demonstrating the specificity of the labels. However, after neuraminidase treatment there was a noticeable increase in WGA labelling in the zona pellucida, whereas an abolition of MAA labelling was observed over the same structure (see Table 2 for quantitative results).

Comparative quantitative analysis of the density of labelling in the zona pellucida of unfertilized and fertilized oocytes, and in vitro-activated oocytes

Considering the heterogeneous nature of the zona pellucida matrix, a multicomparison quantitative analysis was undertaken between the inner and outer zona pellucida

Fig. 1. *Helix pomatia* agglutinin (HPA)-gold labelling. (a) In the unfertilized hamster oocyte, gold labelling of HPA occupies the entire thickness of the zona pellucida (ZP) with no reactivity observed in the perivitelline space (PVS). (b) An increase in HPA labelling in the inner and outer layers of the zona pellucida is evident after *in vitro* activation. Note that the perivitelline space of this *in vitro*-activated egg is heavily labelled with gold particles. O: ooplasm. Scale bars represent (a) 150 and (b) 130 μm .

within each of the experimental groups under study (unfertilized, fertilized and *in vitro*-activated oocytes). A similar multicomparison test was also conducted between the inner and outer zones of the zona pellucida of unfertilized and fertilized oocytes, as well as between the inner and outer layer of the zona pellucida of fertilized and activated eggs, to determine whether there was any modification in the distribution of various sugar residues after *in vivo* fertilization and *in vitro* activation. The quantitative evaluation revealed significant differences in the distribution of some sugar residues, thereby confirming our electron microscope observations. As such, there was a significant difference in the density of labelling between the inner and outer layers of the zona pellucida with DSA and RCA-I in unfertilized oocytes, as well as with HPA, RCA-I, DSA and BSAIB₄ in fertilized oocytes (see Table 2 for quantitative results). After fertilization, the lectin-binding of HPA, BSAIB₄ and AAA increased significantly in the inner portion of the zona

pellucida, whereas that of RCA-I showed a significant increase in both the inner and outer zona pellucida. However, labelling by WGA and the sequence Neu-WGA was reduced significantly in both the inner and outer zona pellucida of fertilized eggs. No significant differences were detected with PNA, DBA, DSA and MAA in both the inner and outer zones of unfertilized and fertilized eggs. In contrast, a significant difference in the density of gold labelling was obtained between the inner and outer zona pellucida when tissue sections of activated oocytes were incubated with HPA, RCA-I, DSA and MAA. Comparative analysis of the density of gold labelling in the inner and outer zona pellucida of fertilized and *in vitro*-activated oocytes revealed significant variations between the two experimental groups. Labelling by HPA, WGA, DSA and MAA showed a significant increase in both the inner and outer zona pellucida of activated oocytes, whereas DBA labelling showed a significant decrease in the inner layer of

Fig. 2. *Triticum vulgaris* agglutinin (WGA) labelling followed by incubation with ovomucoid-gold. (a) In the unfertilized hamster oocyte, a homogeneous distribution of gold labelling is observed throughout the zona pellucida (ZP). A cortical granule (arrowhead) is labelled with a few gold particles. (b) After *in vivo* fertilization, a decrease in WGA labelling over both the inner and outer layer of the zona pellucida is noted. The perivitelline space (PVS) is weakly labelled. (c) After *in vitro* activation, an increase in WGA labelling is detected in the inner and outer layers of the zona pellucida. Many gold particles are also found in the perivitelline space of this *in vitro*-activated egg. O: ooplasm. Scale bar represents 150 μ m.

the zona pellucida (see Table 2 for quantitative results). However, no significant differences were found between the inner and outer zonae pellucidae when tissue sections of fertilized and *in vitro*-activated oocytes were incubated with RCA-I, AAA and the sequence Neu-WGA.

Discussion

The results obtained in the present study are in agreement with previous studies performed in several other mammals, including rats and mice, using lectin cytochemistry to demonstrate modifications in various carbohydrate residues after fertilization (Raz *et al.*, 1996; Avilés *et al.*, 1997).

Recently, a study using confocal scanning laser microscopy for the detection of lectin-binding glycoconjugates in hamster cortical granules also showed the binding of RCA, PNA, DBA and WGA to the zona pellucida of unfertilized hamster oocytes (Hoodbhoy and Talbot, 2001). However, these authors did not attempt to quantify the density of lectin labelling in the inner and outer regions of the zona pellucida before and after fertilization or after egg activation. Previous studies in several mammalian species demonstrated the occurrence of GalNAc residues as a component of zona pellucida glycoproteins (Shimizu and Ito, 1986; Kimura *et al.*, 1991; Yurewicz *et al.*, 1991; Avilés *et al.*, 1996). Cahova and Draber (1992) showed that pre-

Fig. 3. *Datura stramonium* (DSA)-gold labelling. (a) An unfertilized hamster oocyte showing preferential distribution of labelling in the inner layer of the zona pellucida (ZP). (b) No apparent change in the distribution of DSA-gold labelling is observed in the inner and outer layers of the zona pellucida of the fertilized oocyte. Labelling is absent in the perivitelline space (PVS) of this fertilized oocyte. (c) An *in vitro*-activated oocyte showing an increase in DSA labelling in both the inner and outer layers of the zona pellucida. The perivitelline space of this *in vitro*-activated egg is devoid of any gold labelling. O: ooplasm. Scale bar represents 150 μm .

incubation of eggs with a monoclonal antibody (TEC-02) directed against the disaccharide GalNAc β 1,4Gal β 1,4, which is present in mouse ZP2 and ZP3, inhibits fertilization *in vitro*; however, the antibody does not interfere with the initial attachment of the spermatozoon to the zona pellucida but inhibits maintenance of the spermatozoon–zona pellucida binding (secondary binding). Other studies using the TEC-02 antibody to demonstrate the localization of GalNAc β 1,4Gal β 1,4 oligosaccharide sequence to the inner region of unfertilized mouse eggs indicate that the

TEC-02 epitope does not play a role in secondary binding (Avilés *et al.*, 1999). Results from cytochemical studies using HPA lectin (specific for GalNAc sugar residues) in both unfertilized and fertilized mouse eggs indicate that, in mice, non-reducing GalNAc sugar residues are probably not involved in the initial recognition between spermatozoa and the zona pellucida (Avilés *et al.*, 1997). In the present study, the occurrence of non-reducing GalNAc sugar residues over the entire thickness of the zona pellucida in both unfertilized and fertilized hamster oocytes is reported

using two different lectins: HPA, which preferentially binds non-reducing α - and β -linked GalNAc residues, and DBA, which predominantly recognizes α -GalNAc residues (Piller *et al.*, 1990; Wu and Sugii, 1991). The occurrence of HPA labelling in the outer portion of the zona pellucida indicates a possible role for the non-reducing terminal GalNAc sugar residues in the initial spermatozoon–zona pellucida interaction. This finding is supported further by results from a previous study in hamsters, which showed that spermatozoa mainly recognize the outer region of the zona pellucida (Phillips and Shalgi, 1980).

In the present study, cortical granules were found to react weakly to both HPA and DBA. However, the significant increase in the density of labelling by HPA in the inner region of the zona pellucida and the localization of labelling of both HPA- and DBA- binding receptors at the cell surface of fertilized eggs can be attributed to the incorporation of the enzymatic content of cortical granules into the zona pellucida. Previous studies have implicated enzymes such as proteinases and glycosidases contained in the cortical granules in altering the carbohydrate residues of the zona pellucida glycoproteins, thereby establishing a block to polyspermy (Ducibella, 1991; Miller *et al.*, 1992, 1993). Further studies are required to unravel the anticipated involvement of GalNAc sugar residues in post-fertilization hardening of the zona pellucida in addition to their possible roles in sperm–egg recognition and secondary binding.

The presence of both non-reducing α - and β -galactose residues in both the inner and outer zona pellucida of unfertilized and fertilized eggs was also demonstrated using BSAIB₄ and the group RCA-I, PNA and DSA, respectively. However, an increase in BSAIB₄ (specific for α -galactose) in the inner zona pellucida and an increase in RCA-I (specific for β -galactose) labelling of both the inner and outer zona pellucida were noted after fertilization. Reactivity to RCA-I revealed the occurrence of Gal β 1,4GlcNAc disaccharide throughout the zona pellucida, whereas the presence of the disaccharide Gal β 1,3GalNAc was revealed using PNA lectin. Therefore, these results indicate that galactose sugar residues with both α -linkage (recognized by BSAIB₄) and β -linkage (recognized by RCA-I, DSA and PNA) may also be involved in the initial recognition between spermatozoa and the zona pellucida in hamsters. In particular, the results of the present study indicate that α - and β -galactose sugar determinants may play a role in later stages of sperm penetration. It is possible that, after fertilization, an alteration in the distribution of either α -galactose or β -galactose sugar residues, presumably caused by the release of specific enzymes or other glycoproteins contained in the cortical granules, is necessary as a secondary barrier to prevent additional spermatozoa from entering the fertilized egg. Previous cytochemical studies in mouse and rat ovarian oocytes excluded the role of α -galactose in primary sperm–egg binding, as the latter epitope was confined to the inner portion of the zona pellucida (Avilés *et al.*, 2000b). Therefore, the results of the present study indicate that distribu-

tion of α -galactose in the zona pellucida varies among rodent species.

GlcNAc sugar residues contained in the zona pellucida of several mammals have been implicated to play a key role in the initiation of fertilization (Berger *et al.*, 1989; Miller *et al.*, 1992, 1993). In mice, it was suggested that the enzyme β -1,4-galactosyltransferase (GalTase), an integral plasma membrane component of the sperm surface, serves as the sperm receptor by binding to terminal GlcNAc residues on mouse ZP3 (Miller *et al.*, 1992; Shur, 1993; Gong *et al.*, 1995). Removal of ZP3 terminal GlcNAc residues by *N*-acetylglucosaminidase digestion caused a loss of ZP3 sperm receptor activity (Miller *et al.*, 1992). It has been proposed that the enzyme, contained predominantly in the cortical granules of mouse eggs, acts on ZP3 to remove terminal GlcNAc residues, thereby inactivating ZP3 sperm receptor activity and establishing the block to polyspermy (Miller *et al.*, 1993). In the present study, a decrease in labelling with WGA and the sequence Neu-WGA was detected in both the inner and outer portions of the zona pellucida after fertilization. This occurrence of GlcNAc residues in the outer portion of the zona pellucida in both fertilized and unfertilized eggs, and the subsequent decrease in WGA labelling in the outer zona pellucida after fertilization, indicates that GlcNAc is a sugar component that is likely to be involved in sperm–zona pellucida interaction. Our results also demonstrated labelling by Neu-WGA in the cortical granules of unfertilized eggs (results not shown) and the occurrence of a high intensity of labelling in the perivitelline space of fertilized eggs after labelling with WGA and Neu-WGA. Therefore, it is likely that the decrease in the binding of both WGA and Neu-WGA in both the inner and outer zona pellucida of fertilized eggs is caused by the release of specific glucosaminidase, similar to that reported in mice (Miller *et al.*, 1992), into the perivitelline space during the cortical reaction. Further evidence for this contention is provided by results from previous studies that demonstrated structural and functional similarities between hamster and mouse zona pellucida glycoproteins (Moller *et al.*, 1990).

In the present study, the zona pellucida in both fertilized and unfertilized hamster eggs showed no affinity for UEA-I and LTA, both of which recognize the external non-reducing fucose residues (Debray *et al.*, 1981; Sugii and Kabat, 1982). However, fucose residues were detected in the zona pellucida of oocytes under all three experimental conditions when AAA was used as a probe. AAA, which preferentially binds α 1,6-fucosyl residue linked to the core region of *N*-linked glycoproteins (Yamashita *et al.*, 1985), showed an increase in the density of labelling in the inner portion of zona pellucida after fertilization. Previous *in vitro* fertilization and cytochemical studies demonstrated the role of fucose residues in sperm–zona pellucida recognition in guinea-pig, hamster, rat and human eggs (Huang *et al.*, 1982; Shalgi *et al.*, 1986; Mahony *et al.*, 1991). In mice, Bleil and Wassarman (1988) reported that mouse zona pellucida treated with α -L-fucosidase inhibits *in vitro*

fertilization. The modifications of fucose residues detected by AAA after fertilization may be attributed to the release of specific enzymes or other glycoproteins into the perivitelline space of fertilized eggs. The alteration in the distribution of fucose residues in the inner layer of the zona pellucida of fertilized eggs probably reflects an alteration in the sperm binding sites, thus participating in the establishment of a block to polyspermy. However, more accurate tools are needed to support this hypothesis. In the present study, modifications of terminal Neu5Ac sugar residues, as recognized by MAA, were not detected after fertilization. Labelling by MAA is indicative of the occurrence of $\alpha(2,3)$ -linked Neu5Ac in hamster zona pellucida glycoproteins; however, the presence of $\alpha(2,6)$ -linked Neu5Ac was not detected with the lectin SNA. The other carbohydrate residue that may have a role as ZP3 sperm receptor is D-mannose. Pretreatment of human spermatozoa with D-mannose inhibited sperm penetration through the zona pellucida completely (Mori *et al.*, 1989) and α -methylmannoside and D-mannose are potent inhibitors of sperm penetration in rats (Shalgi *et al.*, 1986) and mice (Cornwall *et al.*, 1991). In the present study, the occurrence of $\alpha(1,3)$ -mannosyl residues in high mannose N-linked oligosaccharides was not detected with GNA in fertilized, unfertilized and activated eggs.

In the present study, activation of cumulus-free eggs with the calcium ionophore A23187 was undertaken to obtain further insights into the anticipated post-activation modifications of various sugar residues in the zona pellucida. The cytochemical results of *in vitro*-activated eggs showed a different pattern of modifications with respect to the distribution of various lectin-binding sites over both the inner and outer portions of the zona pellucida compared with that of fertilized oocytes. Previous studies have demonstrated significant differences between parthenogenetic activation and *in vivo* fertilization (Raz *et al.*, 1998). In addition, morphological studies in several species, including humans and hamsters, revealed the presence of two kinds of cortical granules with different electron densities (Ducibella *et al.*, 1988; Okada *et al.*, 1993). As such, *Lens culinaris* (LCA) agglutinin, a marker of cortical granule content, also showed two different types of binding after induction of cortical reaction by parthenogenetic activators (Raz *et al.*, 1998). On the basis of the aforementioned studies, it is possible that the discrepancies in the density of labelling by the various lectins in the bilaminar zona pellucida between the fertilized and *in vitro*-activated eggs can be attributed to the differences in the composition of hydrolytic enzymes and glycoproteins contained in the two types of cortical granules, as well as in the timing of their discharge. In a dose-dependent study, Raz *et al.* (1998) demonstrated the occurrence of a full cortical reaction with the use of 2 μmol calcium ionophore ionomycin l^{-1} as opposed to the partial cortical reaction resulting from the use of a lower concentration (0.5 μmol l^{-1}) of the same artificial activator. Therefore, we are unable to rule out at this point that the concentration of calcium ionophore

A23187 used in the present study (10 μmol l^{-1}) is a possible effector in bringing about a pattern of post-activation modifications, at least for the lectins used in the present study, that differ from those observed in both the inner and outer zona pellucida of fertilized oocytes. It is also possible that even a full cortical reaction induced parthenogenetically does not emulate the *in vivo* activation process by sperm penetration (Raz *et al.*, 1998).

In conclusion, the results of the present study demonstrate that sugar residues contained in the zona pellucida glycoproteins of hamster eggs are modified after fertilization as well as after *in vitro* activation. They also provide further information about the asymmetric nature of the zona pellucida as demonstrated by quantitative analysis of lectin-gold labelling. The topographical distribution of various lectin-binding sites on the zona surface before and after fertilization indicates a possible role for different sugar residues in the process of sperm-zona pellucida binding, as acrosome-reacted spermatozoa are first observed at the outer surface of the zona pellucida. It has been argued that the initial sperm-egg interaction may involve components of low- and high-binding affinity, thereby indicating a hierarchy of binding events at the sperm-zona pellucida interface (Thaler and Cardullo, 1996), or possibly a different set of effectors including one or more sugar-recognizing proteins (Benoff, 1997). Modifications of sugar residues in the outer portion of the zona pellucida after fertilization, as is the case with WGA, Neu-WGA and RCA-I, may indicate a direct role of GlcNAc and galactose sugar residues, respectively, in the initial stages of recognition and binding between the spermatozoon and the zona pellucida. Of particular interest is the modification in the distribution of lectin-binding sites (HPA, RCA-I, WGA, Neu-WGA, BSAIB₄ and AAA) in the inner zona pellucida of fertilized eggs. It is likely that these modifications of the zona pellucida are required by the fertilized egg to prevent the lethal state of polyspermy from occurring. The detection of a high concentration of labelling with various lectins over the cortical granules of unfertilized eggs, as well as over the oolemma and perivitelline space of fertilized eggs, strongly supports the hypothesis for an important role of the cortical granules in contributing their contents of various enzymes towards the establishment of a block to polyspermy. Of particular importance are the differences in the pattern of lectin-gold labelling between the *in vivo*-fertilized and *in vitro*-activated eggs. Although the results obtained from our *in vitro* study confirm findings from previous studies regarding the role of cortical granule contents in modifying the biochemical structure of the zona pellucida after fertilization, it is possible that the results from our *in vivo* study represent a more accurate account of the distribution and modification of various sugar residues that might be involved in the various events of fertilization.

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