

Interspecific variation of zona pellucida glycoconjugates in several species of marsupial

J. A. Chapman¹, O. W. Wiebkin² and W. G. Breed^{1*}

Departments of ¹Anatomical Sciences and ²Medicine, University of Adelaide, South Australia 5005, Australia

The zona pellucida glycoconjugate content of several marsupial species was investigated using differential lectin histochemistry. Ovaries from fat-tailed dunnarts, a southern brown bandicoot, grey short-tailed opossums, brushtail possums, ringtail possums, koalas and eastern grey kangaroos were fixed, embedded in paraffin wax, sectioned and stained with ten fluorescein isothiocyanate-conjugated lectins. Sections were also incubated with either neuraminidase or saponified, respectively, before incubation with the lectins to identify saccharide residues masked by sialic acids or O-acetyl groups on sialic acids. The zonae pellucidae surrounding the oocytes of the marsupials demonstrated interspecific variation in glycoconjugate content, with mannose-containing glycoconjugates exhibiting the greatest variation. Some of the zona pellucida glycoconjugates of all species, except those of the opossums, were masked by sialic acid with an increase in fluorescence with lectins from *Arachis hypogea* (PNA), and *Glycine max* (SBA), after desialylation. The disaccharide β -galactose(1-4)*N*-acetyl-D-glucosamine appeared to be conformationally masked by O-acetyl groups of sialic acids in the zonae pellucidae of all species, with an increase in fluorescence with the lectin from *Erythrina cristagalli* (ECA), after saponification. Similar intensity and localization of β -(1-4)-*N*-acetyl-D-glucosamine, as shown by staining of the lectin from *Triticum vulgare* (WGA), to the inner and outer regions of the zona pellucida, were found to those reported in eutherian species. WGA fluorescence became uniform throughout the zonae pellucidae after saponification, indicating differential O-acetylation of sialic acids on the internal compartment of the zonae pellucidae.

Introduction

The eutherian mammalian zona pellucida (ZP) is composed of three sulphated glycoproteins: ZPA (or ZP2), ZPB (or ZP1) and ZPC (or ZP3), each of which is encoded by a gene that is highly conserved among species (Harris *et al.*, 1994). Although the protein components of the zona pellucida glycoproteins are highly homologous among species, interspecific differences in the sugar components of the *N*- and *O*-linked oligosaccharides are evident, and these may relate to species specificity of sperm-zona pellucida binding where this occurs. The importance of the oligosaccharides for sperm binding is well established and supported by the finding that addition of various saccharides and lectins to the *in vitro* fertilization environment can prevent sperm-zona pellucida binding and fertilization (Shalgi *et al.*, 1986; Cornwall *et al.*, 1991; Mori *et al.*, 1993; Tulsiani *et al.*, 1997).

In mice, the *O*-linked oligosaccharides of ZPC facilitate primary sperm binding (Florman and Wassarman, 1985), either through a terminal α -galactose (α -Gal) (Bleil and Wassarman, 1988) or *N*-acetylglucosamine (GlcNAc) (Miller

et al., 1992). In pigs, an *N*-linked oligosaccharide attached to Asn220 of ZPB in a ZPB-ZPC heterocomplex appears to be the primary binding site for spermatozoa (Kudo *et al.*, 1998; Yurewicz *et al.*, 1998) while, in humans, sperm-zona pellucida binding may be mediated through a selectin-like ligand on the zona pellucida which recognizes fucose (Fuc)-rich oligosaccharides (Patankar *et al.*, 1993; Clark *et al.*, 1996; Oehninger *et al.*, 1998). Removal of terminal sialic acid residues (desialylation) from both the human zona pellucida and sperm surface results in an increase in sperm binding (Lassalle and Testart, 1994; Banjeree and Chowdhury, 1997; Ozgur *et al.*, 1998).

Lectin histochemistry has been used to identify the saccharides and their distribution within eutherian zonae pellucidae (Nicolson *et al.*, 1975; Skutelsky *et al.*, 1994). Observational studies on the zonae pellucidae of rats (Shalgi *et al.*, 1991), humans (Bar-Shira Maymon *et al.*, 1994) and river buffalo (Parillo *et al.*, 1998) have demonstrated interspecific variation in the intensity of lectin-binding patterns and the distribution of various saccharide components.

The zonae pellucidae of marsupials appear to be thinner and more readily solubilized by proteases than those of eutherians (Bedford, 1991, 1996; Bedford and Breed, 1994). The three genes encoding the zona pellucida proteins in

*Correspondence.

Revised manuscript received 19 November 1999.

brush-tail possums have been cloned and the putative amino acid sequences deduced (Mate and McCartney, 1998; Haines *et al.*, 1999; McCartney and Mate, 1999; Voyle *et al.*, 1999). As yet, no study has characterized the glycoconjugate composition of marsupial zonae pellucidae. In the present study differential lectin binding, together with enzyme elimination and saponification, is used to investigate the saccharide components of the zonae pellucidae of follicular oocytes of seven marsupial species from different families within this infra-class.

Materials and Methods

Animals

The following Australian species were used in this study; fat-tailed dunnarts (*Sminthopsis crassicaudata*; family: Dasyuridae; $n = 4$); a southern brown bandicoot (*Isodon obesulus*; family: Peramelidae; $n = 1$); brush-tail possums (*Trichosurus vulpecula*; family: Phalangeridae; $n = 4$); common ringtail possums (*Pseudocheirus peregrinus*; family: Pseudocheiridae; $n = 2$); koalas (*Phascolarctos cinereus*; family: Phascolarctidae; $n = 3$); and eastern grey kangaroos (*Macropus giganteus*; family: Macropodidae; $n = 2$). All Australian species, except the fat-tailed dunnarts, were obtained from wild populations. Fat-tailed dunnarts were obtained from a colony bred at the Division of Animal Services of the University of Adelaide. Grey short-tailed opossums (*Mondelphis domestica*; family: Didelphidae; $n = 2$) originating from the colony bred at the South West Foundation for Biomedical Research, San Antonio, Texas, were also used. All animals were adults and had never been subjected to treatments that could affect natural follicular growth. Staining with each lectin, and each disruptive treatment, was performed at least twice on ovaries of all species.

Ovary collection

Excised ovaries from the above animals, except opossums, were immediately fixed in Rossman's fluid (90% ethanol

saturated with picric acid and 10% formalin) for 12–24 h, washed in 95% alcohol for 24–48 h, routinely processed, embedded in paraffin wax and sectioned at 7 μm . Opossum ovaries were also fixed and then stored in Rossman's fluid for several weeks before processing as above.

Lectin histochemistry

Sections were deparaffinized by immersion in Histoclear (National Diagnostics, Atlanta, GA), rehydrated by passing through a series of alcohols (100–50%) and brought to Tris-buffered saline (TBS, pH 7.6). The sections were then covered with 1% BSA in TBS for 30 min, excess solution was shaken off, and the slides were blotted with tissue paper. Sections were then incubated with one of ten fluorescein isothiocyanate (FITC)-conjugated lectins (Vector Laboratories, Burlingame, CA) (Table 1). These lectins were used at a concentration of 20 $\mu\text{g ml}^{-1}$, except for WGA, which was used at a concentration of 10 $\mu\text{g ml}^{-1}$, for 30 min at room temperature in a humidified light-safe chamber. The sections were then rinsed three times in TBS, one drop of antifade solution was added (Slowfade Antifade kit, Molecular Probes, USA), and then a coverslip was applied. Sections were viewed with an Olympus BH epifluorescent and phase-contrast microscope, using a 515 nm excitation filter and an IFK90 nm barrier filter with an absorption wavelength of 535 nm and an emission wavelength of 617 nm. The intensity of lectin staining was determined qualitatively using a scoring system adapted from the light microscopical lectin histochemistry investigations of zonae pellucidae by Skutelsky *et al.* (1994), Legge (1995), Parillo *et al.* (1996, 1998) and Parillo and Verini-Supplizi (1999). A positive result was indicated by (+) and a negative result by (–), with the intensity of fluorescence being defined as mild (+), strong (++) or intense (+++).

Only the zonae pellucidae of antral follicular oocytes were compared to maintain standardization among species, while exposure times for photography of fluorescence results were kept identical at 51.2 s and photographed on T-Max 400 ASA black and white film.

The sugar specificities and the inhibiting sugars for the lectins used in this study are listed (Table 1).

Table 1. Lectins used and their corresponding saccharide specificities

Origin of lectin	Common name	Acronym	Sugar specificity	Sugar inhibitor
<i>Arachis hypogea</i>	Peanut	PNA	$\beta\text{Gal-(1-3)-GalNAc}$	Lactose
<i>Erythrina cristagalli</i>	Coral Tree	ECA	$\beta\text{Gal-(1-4)-GlcNAc}$	D-GlcNAc
<i>Concanavalina ensiformis</i>	Jack bean	Con A	$\alpha\text{-D-Man, } \alpha\text{-D-Glc}$	D-Man, D-Glc
<i>Pisum sativum</i>	Garden pea	PSA	$\alpha\text{-D-Man}$	D-Man
<i>Ricinus communis II</i>	Castor bean	RCA-II	$\beta\text{-D-Gal}$	Lactose
<i>Triticum vulgare</i>	Wheat germ	WGA	$[\beta\text{(1-4)-D-GlcNAc}]^2, \text{NeuNAc}$	D-GlcNAc*, NeuNAc
<i>Sambucus nigra</i>	Elderberry	SNA	$\alpha\text{-NeuNAc(2-6)Gal/GalNAc}$	NeuNAc
<i>Glycine max</i>	Soybean	SBA	$\alpha\text{-D-GalNAc, } \alpha\text{-D-Gal}$	$\alpha\text{-D-GalNAc}$
<i>Ulex europaeus I</i>	Gorse	UEA-I	$\alpha\text{-L-Fuc}$	$\alpha\text{-L-Fuc}$
<i>Lotus tetragonolobus</i>	Winged Pea	LTA	$\alpha\text{-L-Fuc}$	$\alpha\text{-L-Fuc}$

*Sugar inhibitor used in this study.

Fuc: fucose; Gal: galactose; GalNAc: N-acetylgalactosamine; Glc: glucose; GlcNAc: N-acetylglucosamine; Man: mannose; NeuNAc: N-acetylneuraminic acid (sialic acid) (from Wu *et al.*, 1988).

Enzymatic and saponification treatments

Sections were incubated at 37°C for 1 h with 1 U neuraminidase ml⁻¹ (from *Clostridium perfringens*, Sigma, St. Louis, MO) in 0.05 mol acetate buffer l⁻¹ (pH 5.5) before incubation with SBA, PNA, SNA, RCA-II and WGA (Riley and Elhay, 1994) to determine sugar residues masked by terminal sialic acid residues. In addition, sections were subjected to mild alkali hydrolysis (saponification) in a 1% (w/v) potassium hydroxide solution in 70% ethanol at room temperature for 1 h before incubation with the lectins (Wiebkin, 1994) to detect the presence of sugar residues masked by O-acetyl groups.

Controls

Incubation of the lectins with 0.2–0.4 mol l⁻¹ of their inhibitory sugar made up in distilled water for 30 min before the application of the lectins to the sections served as specific controls. For the desialylation treatments, control sections were incubated in acetate buffer alone while, for the saponification treatment, control sections were incubated in 70% ethanol.

Results

Ovaries from all individuals had follicles at various stages of development, with oocytes surrounded by a zona pellucida from the early primary follicular stage up to tertiary follicles. No variation in lectin staining was noted among the zonae pellucidae surrounding oocytes of secondary and tertiary follicles in the same animal, and there was no observable difference in lectin binding between individuals of the same species. The intensity of fluorescence of ten lectins with the zonae pellucidae around the antral follicular oocytes of fat-tailed dunnarts, the southern brown bandicoot, grey short-tailed opossums, brushtail possums, ringtail possums, koalas and eastern grey kangaroos is summarized (Table 2).

Arachis hypogea (peanut) lectin (PNA)

The staining of the marsupial zonae pellucidae with PNA varied from negative in the dunnarts to intense fluorescence in the ringtail possums. However, after neuraminidase treatment, there was an increase in fluorescence of the zonae pellucidae in all species, except for those of the opossums

Table 2. Lectin binding to zonae pellucidae of marsupial follicular oocytes

Lectin	Fat-tailed dunnart (Dasyuridae)	Southern brown bandicoot (Peramelidae)	Grey short-tailed opossum (Didelphidae)	Brushtail possum (Phalangeridae)	Ringtail possum (Pseudocheiridae)	Koala (Phascolarctidae)	Eastern grey kangaroo (Macropodidae)
PNA	–	+	+	++	+++	++	++
NEU-PNA ^a	++	++	+	+++	+++	+++	+++
KOH-PNA ^b	+	++	++	+++	+++	++	+++
ECA	–	++	+	–	–	+	+
KOH-ECA ^b	+++	+++	++	+++	+++	++	++
Con A	–	–	–	–	–	–	–
KOH-Con A ^b	++	–	–	–	–	–	–
PSA	–	++	–	–	++	++	–
KOH-PSA ^b	+++	++	++	++	++	++	++
RCA-II	+	+	–	–	+++	+	–
NEU-RCA-II ^a	++	++	–	–	+++	+	+
KOH-RCA-II ^b	+	++	–	–	+++	+	+
WGA	++	+++	+++	+++	++	+++	+++
NEU-WGA ^a	+++	+++	+++	+++	++	+++	+++
KOH-WGA ^b	++	+++	+++	+++	+++	+++	+++
SNA	+	+	–	–	++	++	–
NEU-SNA ^a	++	++	–	+	+	++	+
KOH-SNA ^b	+	+	++	–	+	++	++
SBA	–	+	–	–	–	–	–
NEU-SBA ^a	+	++	–	+	+	++	++
KOH-SBA ^b	++	+	–	–	–	–	–
UEA-I	–	–	–	–	–	–	–
KOH-UEA-I ^b	–	–	–	–	–	–	–
LTA	–	–	–	–	–	–	–
KOH-LTA ^b	–	–	–	–	–	–	–

^aNeuraminidase treatment before binding study with respective lectin.

^bSaponification with alcoholic KOH before binding study with respective lectin.

and ringtail possums. Saponification also increased the fluorescence of the zonae pellucidae of all the species (Fig. 1a–c), except those of the ringtail possums and koalas.

Erythrina cristagalli lectin (ECA)

Before the saponification treatments, no fluorescence was detected with ECA on the zonae pellucidae in fat-tailed dunnarts, brushtail possums and ringtail possums, while there was mild fluorescence in the zonae pellucidae of opossums, koalas and kangaroos, and strong fluorescence in the zonae pellucidae of the bandicoot. However, after removal of *O*-acetyl groups, intense fluorescence with ECA was found in the zonae pellucidae of the dunnarts, bandicoot, brushtail possums and ringtail possums and there was an increase in fluorescence in the zonae pellucidae of the other species.

Concanavalia ensiformis lectin (Con A)

Apart from the saponified zonae pellucidae of the fat-tailed dunnarts, the zonae pellucidae of other species did not stain with Con A. Specific controls showed that this fluorescence was inhibited by α -D-mannose but not α -D-glucose.

Pisum sativum lectin (PSA)

Before saponification, only the zonae pellucidae of the bandicoot, ringtail possums and koalas strongly fluoresced with PSA and there was no change in intensity of fluorescence after saponification. The zonae pellucidae of the other species exhibited fluorescence only after saponification, and then fluoresced strongly or intensely (Fig. 1d–f).

Ricinus communis II lectin (RCA-II)

Intense fluorescence of RCA-II was found in the zonae pellucidae of ringtail possums, while mild fluorescence was observed in the zonae pellucidae of dunnarts, the bandicoot and koalas. The zonae pellucidae of the opossums and brushtail possums did not fluoresce with RCA-II before, or after, either of the treatments, while the zonae pellucidae of the kangaroos stained mildly only with RCA-II after they were desialylated or saponified. After neuraminidase treatment, the fluorescence of the zonae pellucidae of the dunnarts, the bandicoot and kangaroos increased while, after saponification, only the zonae pellucidae of the bandicoot and kangaroos increased in fluorescence, with the zonae pellucidae of the bandicoot fluorescing intensely with this lectin.

Triticum vulgare (wheat germ) lectin (WGA)

The zonae pellucidae of all species exhibited strong to intense fluorescence with WGA which was localized to the

inner and outer regions of the zonae pellucidae before desialylation or saponification treatments (Fig. 2a–d). There was an increase in fluorescence with this lectin after neuraminidase treatment of the zonae pellucidae of fat-tailed dunnarts, and an increase in fluorescence of the zonae pellucidae of the ringtail possums after saponification. Although there were no changes in the intensity of fluorescence of this lectin to the zonae pellucidae of the other species after either treatment, saponified zonae pellucidae of all species exhibited a loss of inner and outer zona pellucida localization and demonstrated a uniform fluorescence throughout the entire zona pellucida.

Sambucus nigra lectin (SNA)

Fluorescence with SNA was demonstrated in the zonae pellucidae of the fat-tailed dunnarts, bandicoot, ringtail possums and koalas, before exposure to neuraminidase or mild alkali hydrolysis. An increase in the intensity of fluorescence was evident after pre-treatment with neuraminidase in the zonae pellucidae of fat-tailed dunnarts, the bandicoot, brushtail possums and kangaroos, while a decrease in fluorescence was noted in the desialylated and saponified zonae pellucidae of ringtail possums. An increase in fluorescence with this lectin was noted in the zonae pellucidae of the opossums and kangaroos, from an absence of evident fluorescence before saponification to strong fluorescence after removal of *O*-acetyl groups.

Glycine max (soybean) lectin (SBA)

Only the zonae pellucidae of the bandicoot fluoresced with SBA before desialylation or saponification treatments. An increase in fluorescence with this lectin was evident in the zonae pellucidae of all species, except those of the opossums, after incubation with neuraminidase, while only the zonae pellucidae of the dunnarts demonstrated an increase in fluorescence with this lectin after saponification (Fig. 3a–c).

Ulex europeus I lectin (UEA-I) and Lotus tetragonolobus lectin (LTA)

No fluorescence with either UEA-I or LTA was evident in any of the zonae pellucidae tested before, or after, any of the treatments.

Controls

Incubation of the lectins with their complementary sugar before incubation with the sections resulted in elimination of fluorescence in the zonae pellucidae of all species (Fig. 4a,b), except for that of WGA, which still demonstrated mild fluorescence after incubation with D-GlcNAc.

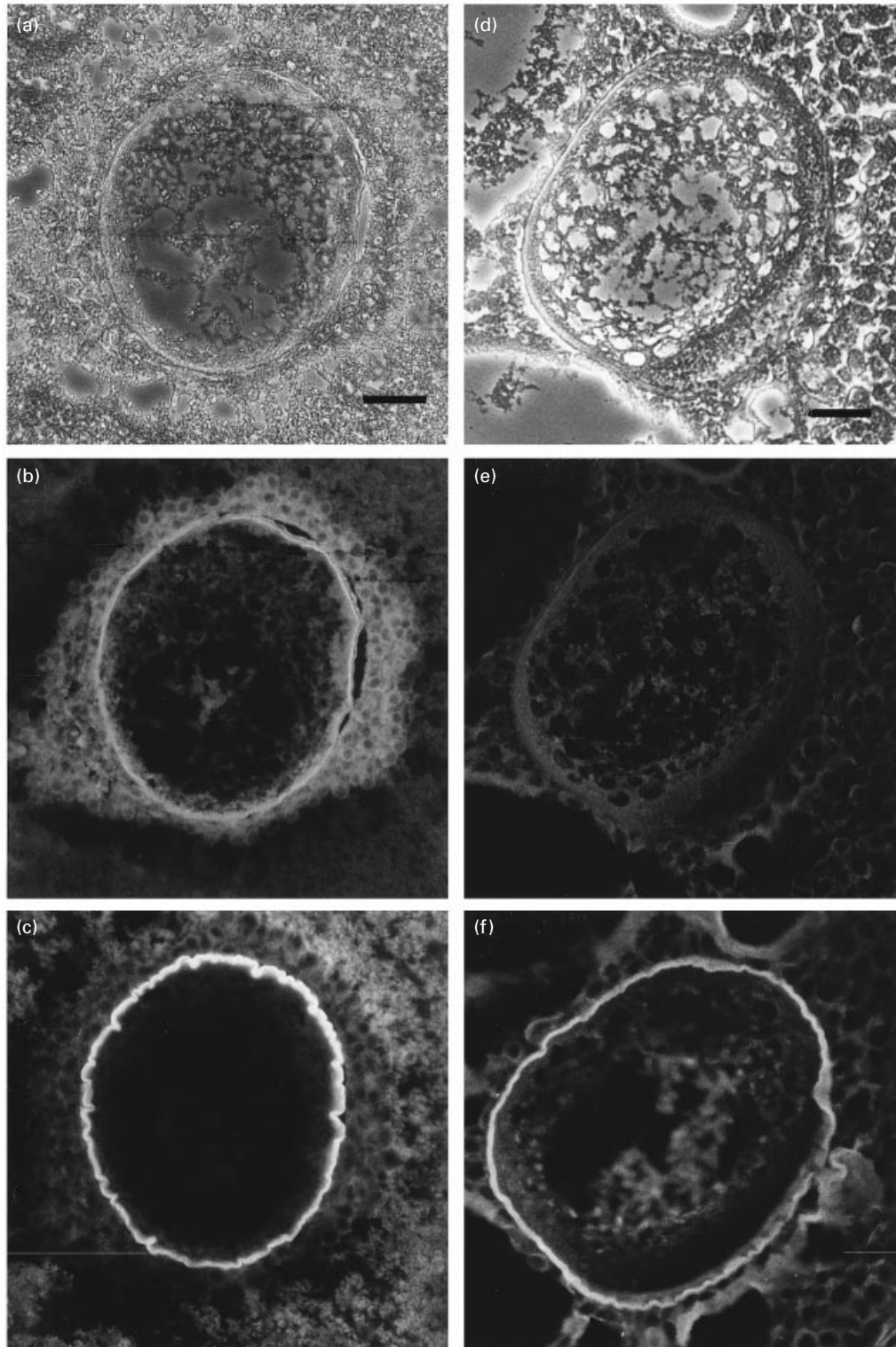


Fig. 1. Lectin histochemistry of antral follicular oocytes and surrounding zonae pellucidae from the brushtail possum (a–c) and fat-tailed dunnart (d–f) showing greater fluorescence intensity after saponification. Phase-contrast (a) and fluorescent (b,c) micrographs of brushtail possum zona pellucida stained with fluorescein isothiocyanate–*Arachis hypogea* (peanut) lectin (FITC–PNA) before (b) and after (c) saponification. Note the increased fluorescence in (c). Phase-contrast (d) and fluorescent (e,f) micrographs of a fat-tailed dunnart zona pellucida stained with FITC–PSA (*Pisum sativum* lectin) before (e) and after (f) saponification. Note the absence of fluorescence before, and intense fluorescence after, saponification. The lectin PNA is specific for β Gal (1-3) *N*-acetylgalactosamine and PSA for α -D-mannose. Scale bars represent 45 μ m.

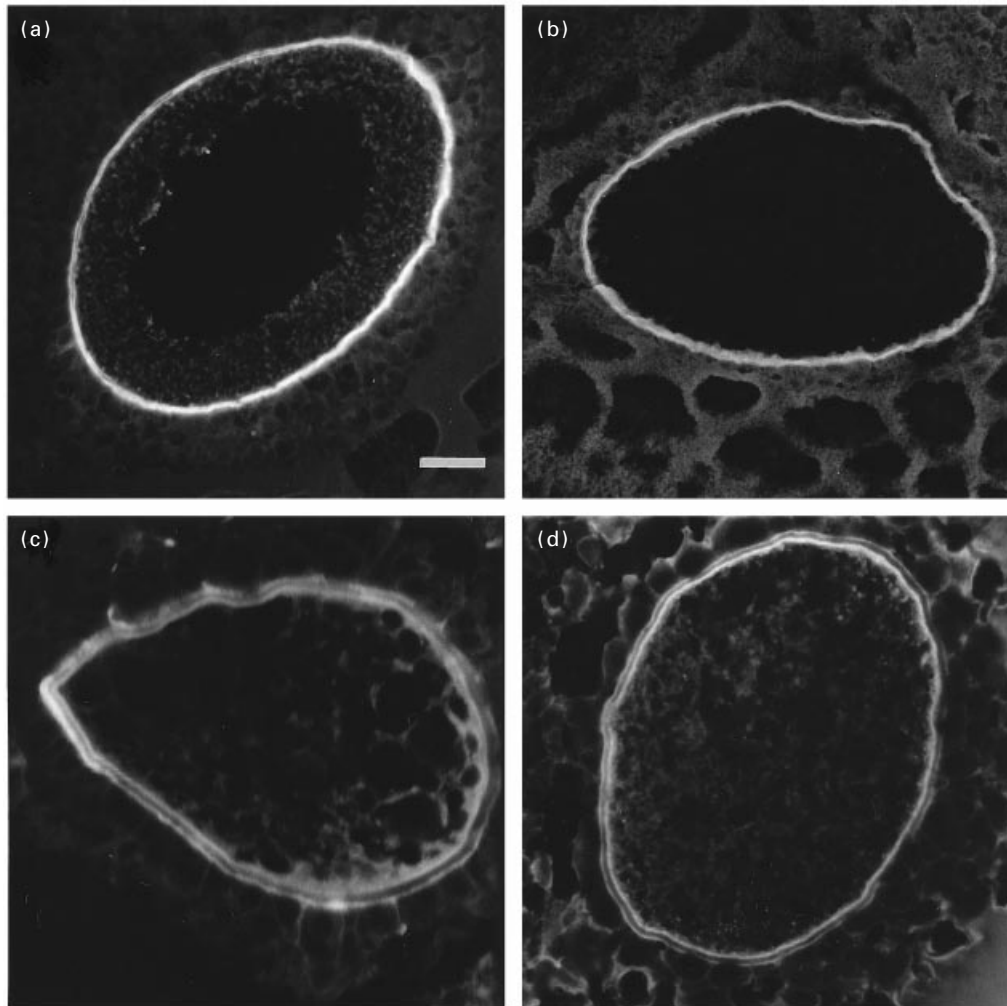


Fig. 2. Lectin histochemistry of oocytes and surrounding zona pellucidae from several different marsupial families. Oocytes are from (a) grey short-tailed opossum, (b) brushtail possum, (c) koala and (d) eastern grey kangaroo, and are stained with fluorescein isothiocyanate-*Triticum vulgare* (wheat germ) lectin (FITC-WGA) and show intense staining of the inner and outer regions of the zona pellucidae, particularly in the koala (c) and eastern grey kangaroo (d). WGA binding is specific for β (1-4) *n*-acetylglucosamine, *N*-acetylneuraminic acid. Scale bar represents 45 μ m.

Discussion

Eutherian zona pellucidae are composed of highly glycosylated glycoproteins, and both *N*- and *O*-linked oligosaccharides are present (Benoff, 1997; Shalgi and Raz, 1997). Where it has been possible to obtain large numbers of zona pellucidae, detailed analysis of their oligosaccharides has been performed by high performance liquid chromatography (HPLC) and such studies have demonstrated interspecific differences in the carbohydrate components. For example, mouse ZPA and ZPC have β -GlcNAc and α -galactose at the non-reducing ends of *N*-linked chains which are absent from pig ZPC (Noguchi and Nakano, 1993), whereas bovine zona pellucidae contain high-mannose-type neutral oligosaccharides which are absent from both mouse and pig zona pellucidae (Katsumata *et al.*, 1996).

For species in which large amounts of zona pellucidae are unavailable, or where glycoconjugate localization is required, lectin histochemistry has been used widely for identification of the saccharides (Shalgi *et al.*, 1991; Bar-Shira Maymon *et al.*, 1994; Avilés *et al.*, 1999). Staining with lectins has shown interspecific variation of sugar residues within eutherian zona pellucidae (Nicolson *et al.*, 1975; Skutelsky *et al.*, 1994; Parillo *et al.*, 1996) and, in the present study on the zona pellucidae of seven marsupial species, differential lectin histochemistry has also demonstrated interspecific variation in saccharide components. For example, in the present study interspecific differences in α -D-mannose were detected. Before saponification, α -D-mannose could not be identified with Con A but, after saponification, the zona pellucidae of only the fat-tailed dunnarts fluoresced with Con A. However, staining with PSA, which is also specific for α -D-mannose, resulted in fluorescence of the zona

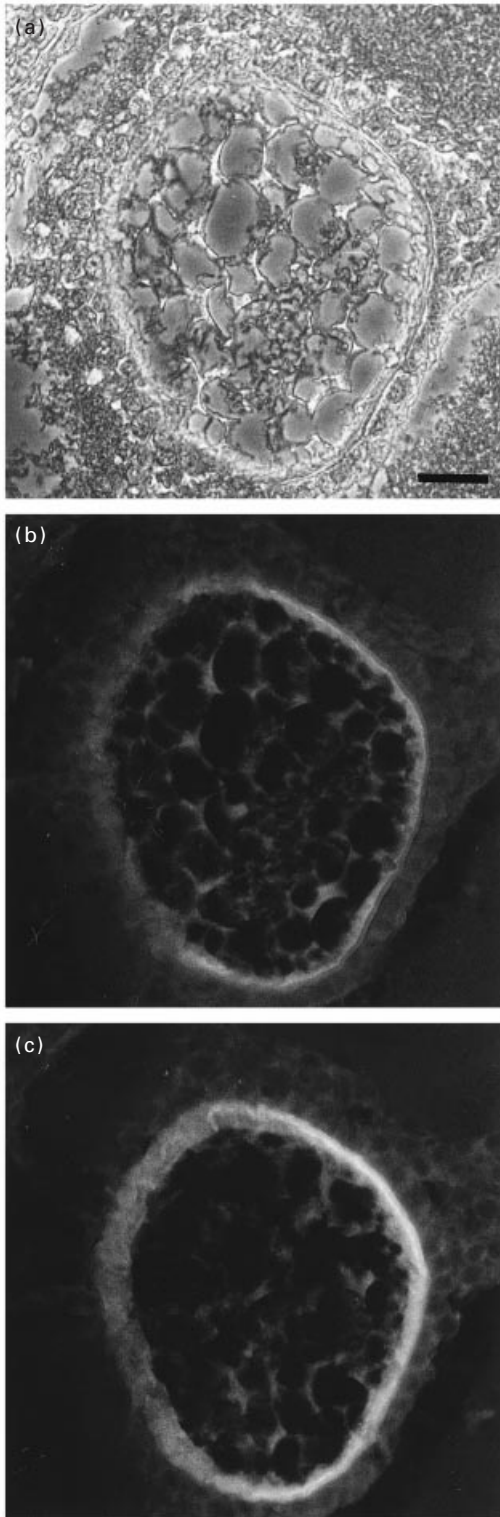


Fig. 3. Phase-contrast (a) and lectin histochemistry (b,c) of oocyte and surrounding zona pellucida from southern brown bandicoot. Control (b) and pre-treatment with neuraminidase (c) followed by staining with fluorescein isothiocyanate-*Glycine max* (soybean) lectin (FITC-SBA). Note that before neuraminidase treatment (b) the bandicoot zona pellucida fluoresces less intensely with SBA than it does after removal of sialic acids (c). SBA binding is specific for galactose and α -D-N-acetylgalactosamine. Scale bar represents 45 μ m.

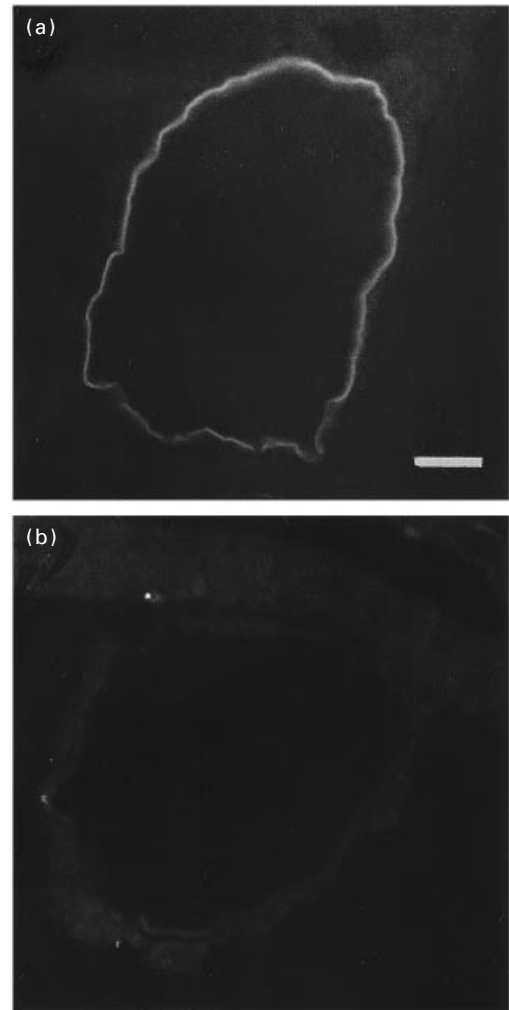


Fig. 4. Lectin histochemistry of brushtail possum oocyte and surrounding zona pellucida after saponification stained with fluorescein isothiocyanate-*Erythrina cristagalli* lectin (FITC-ECA) showing fluorescence of the saponified possum zona pellucida (a), with no fluorescence when prior incubation in 0.4 mol D-N-acetylglucosamine was carried out (b). ECA binding is specific for β -galactose (1-4) N-acetylglucosamine. Scale bar represents 45 μ m.

pellucidae of the southern brown bandicoot, ringtail possums and koalas before saponification and in the zonae pellucidae of all species after saponification. These results not only highlight the need to stain with more than one lectin specific for the same saccharides but also show that the differences in staining between Con A and PSA may be due to an interspecific variation in glycoconjugates. While both lectins are specific for the tri-mannosidic core of N-linked oligosaccharides, PSA has a greater affinity for those that also possess α -L-fucose linked to GlcNAc at their reducing ends (Debray *et al.*, 1981; Kornfeld *et al.*, 1981; Wu *et al.*, 1988). While α -L-fucose was not positively identified in the zonae pellucidae of the marsupials investigated in the present study, both of the fucose-specific lectins used recognize di- and tri-saccharides through an L-fucose- α (1-2) linkage to

galactose, whereas PSA recognizes α -L-fucose through a (1-6) linkage (Wu *et al.*, 1988).

The identification of mannose indicates, for the first time, the presence of *N*-linked oligosaccharides in the zona pellucida glycoproteins of all the marsupial species used in the present study. This finding is in agreement with studies on the cDNA and deduced amino acid sequences of the three zona pellucida glycoproteins in brushtail possums, which indicate that the zona pellucida polypeptides contain several potential *N*-linked glycosylation sites (Mate and McCartney, 1998; Haines *et al.*, 1999; McCartney and Mate, 1999). The most likely number of potential *N*-linked glycosylation sites is eight in ZPA, seven in ZPB and two in ZPC (Mate and McCartney, 1998; Haines *et al.*, 1999; McCartney and Mate, 1999; Voyle *et al.*, 1999), after discounting one site in ZPA and ZPC in which proline was in the X position of the determining sequon N-X-S/T. Several authors have noted that sugar substitution at this site is extremely unlikely (Mononen and Karjalainen, 1984; Kornfeld and Kornfeld, 1985; Shakin-Eshleman *et al.*, 1996). Moreover, the potential for *N*-linked sites to be glycosylated varies according to factors that determine glycosylation efficiency. For example, sequons with Thr in the hydroxy position are more likely to be glycosylated than those with Ser (Kasturi *et al.*, 1995). The amino acid in the X position of N-X-S/T also determines glycosylation efficiency (Shakin-Eshleman *et al.*, 1996). The third, fourth and fifth potential *N*-linked glycosylation sites in both ZPA and ZPB in brushtail possums contain Thr in the hydroxy position, while the fifth site of both glycoproteins also contain Ser in the X position, with a core glycosylation efficiency of approximately 95% (Shakin-Eshleman *et al.*, 1996). Taken together, these factors indicate that the possum zona pellucida may contain *N*-linked oligosaccharides.

While a number of interspecific differences were observed in glycoconjugate content, major variation or similarities appeared to relate to a large extent to the sialic acids of the glycoproteins. For instance, all the zonae pellucidae of the marsupials tested, except for those of the opossum, appeared to contain masking residues of sialic acid since neuraminidase treatment increased fluorescence with PNA and SBA. Sialic acid is commonly found as a capping sugar of the terminal saccharide units of oligosaccharides and may affect the ability of the internal sugar residues to interact with lectins. Removal of these sialic acid residues by neuraminidase before incubation of the zonae pellucidae with the lectins has demonstrated that terminal β -Gal (1-3) *N*-acetylgalactosamine (β -Gal (1-3)GalNAc) and α -D-Gal(NAc) residues in the zonae pellucidae are indeed masked by sialic acid. This also appears to be the case for zonae pellucidae of eutherian mammals in which increases in binding to PNA and SBA after desialylation occur in the zonae pellucidae of sheep, goats, pigs (Parillo *et al.*, 1996), rats (Avilés *et al.*, 1997) and river buffalo (Parillo *et al.*, 1998).

Marsupial zonae pellucidae also appear to contain masking sialic acids that relate to their *O*-acetylation, some of which are common across all species. The disaccharide β Gal(1-4)GlcNAc appears to be commonly masked by *O*-acetylated sialic acids in all marsupial zonae pellucidae, with increases to strong and intense fluorescence with ECA after saponification. In the fat-tailed dunnarts and brushtail

possums, this disaccharide was not evident before saponification, indicating that the *O*-acetyl groups on sialic acids are responsible for a conformational masking of these residues. In marsupial zonae pellucidae, *O*-acetylated sialic acids are also found to mask other glycoconjugates conformationally, including the aforementioned mannose in the dunnarts, opossums, brushtail possums and kangaroos, β Gal(1-3)GalNAc in all species except the ringtail possums and koalas, and various other residues consistent with interspecific variation.

Another apparent conservation of glycoconjugate content of the zonae pellucidae across mammals is the presence and distribution of the disaccharide [β -(1-4)-D-GlcNAc]₂, to which WGA binds. In all the marsupial zonae pellucidae studied, there was strong-to-intense fluorescence with this lectin, and this was localized to the inner and outer regions. Similar intensities and localizations have been described in the zonae pellucidae of almost all eutherian species studied, except for those of cats (Skutelsky *et al.*, 1994), although this finding has received some equivocation (Parillo and Verini-Supplizi, 1999). While WGA is also specific for sialic acid, binding intensity in the present study remained either unchanged or increased after removal of the sialic acid with neuraminidase, indicating that the specificity of WGA is for [β -(1-4)-D-GlcNAc]₂. The localization of WGA binding to the inner and outer regions of the zonae pellucidae and the loss of such localization after saponification indicates that the sialic acids of the internal glycoproteins may be *O*-acetylated, whereas the residues of the glycoproteins of the inner and outer regions appear not to be. This apparent complexity of structure supports a re-evaluation of the model of zona pellucida structure proposed by Wassarman (1988), at least as it applies to marsupials.

The role of *O*-acetylated sialic acids in zona pellucida structure and function has not been discussed previously. *O*-acetylated sialic acids play a major role in increasing the lifespan of a glycoprotein by preventing or retarding degradation of glycoconjugates (Schauer, 1982, 1988). An *O*-acetyl residue at the O-4 position of a sialic acid has been found to completely block the action of a neuraminidase which, in turn, prevents further glycosidases from acting on internal saccharide residues (Schauer, 1988). The inability to detect masking sialic acid residues in the opossum zona pellucida may relate to this, as saponification increased the reactivity of the zonae pellucidae of this species to various lectins, indicating that *O*-acetylated sialic acids are, in fact, present. The apparent disparity of sialic acid *O*-acetylation between the inner and outer parts of the zonae pellucidae (in which there is little or none), from the internal compartment (in which there is much), may relate to the function and longevity of the zona pellucida. The high ratio of *O*-acetylated sialic acids at the core of the zonae pellucidae may make this area extremely resistant to degradation and, thereby, help to maintain its structural integrity throughout the lifetime of the egg and early embryo. Alternatively, the lack of *O*-acetylated sialic acids on the outer surface of the zona pellucida may allow selective glycolysis and proteolysis by the acrosomal enzymes of the spermatozoon.

The viscoelasticity and protease resistance of a glycoprotein has been related to the extent of glycosylation of the protein.

Mucins with long oligosaccharide side-chains have been found to have more pronounced viscoelastic properties (Sellers *et al.*, 1988; Jentoft, 1990), whereas the more glycosylated a protein, the more resistant it may be to proteases (Semino *et al.*, 1985; Jentoft, 1990). The reported inability of marsupial zonae pellucidae to retain their spherical shape after removal of the oocyte and their increased sensitivity to dilute proteases (Bedford, 1991; Bedford and Breed, 1994) may relate to their zona pellucida glycoproteins being less glycosylated, with shorter O-linked oligosaccharides, than those of eutherian zonae pellucidae. While the observations of Bedford (1991) pertained mainly to the zonae pellucidae of the American opossums, and there is some evidence of differences between the zonae pellucidae of American and Australian marsupials, further biochemical analyses are needed to elucidate the specific glycoconjugate structure and function of the zonae pellucidae of these species.

This work was partly supported by an ARC grant (A09531986) to W. G. Breed. J. A. Chapman was in receipt of an University of Adelaide Postgraduate Award. The authors would like to thank C. Leigh for his technical assistance, and I. Hough, G. Johnsson and employees of Cleland National Park for the supply of the koala and kangaroo ovaries. J. VanderBerg is also thanked for access to the opossum material.

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