Glycosylation in the near-term epitheliochorial placenta of the horse, donkey and camel: a comparative study of interbreeding and non-interbreeding species

C. J. P. Jones¹, F. B. P. Wooding², M. M. Abd-Elnaeim³, R. Leiser⁴, V. Dantzer⁵
and R. W. Stoddart⁶

¹Academic Unit of Obstetrics and Gynaecology, School of Medicine, University of Manchester, St Mary's Hospital, Whitworth Park, Manchester M13 0JH, UK; ²AFRC Babraham Institute, Babraham Hall, Cambridge CB2 4AT, UK; ³Faculty of Veterinary Medicine, University of Assiut, Assiut, Egypt; ⁴Institute for Veterinary Anatomy, Histology and Embryology, University of Giessen, D-35392 Giessen, Germany; ⁵Institute of Anatomy and Physiology, Royal Veterinary and Agricultural University, DK-1870 Frederiksberg C, Denmark; and ⁶School of Medicine, Faculty of Medicine, Dentistry and Nursing, University of Manchester, Oxford Road, Manchester M13 9PT, UK

Studies from this laboratory have shown great diversity in the glycosylation of tissues comprising the interhaemal barrier of species with different placental types. This diversity may be one of the factors preventing interbreeding between species. Glycan expression within the uterine epithelium and trophoblast of the interhaemal barrier was examined to test this proposition in three species with similar diffuse, microcotyledonary, epitheliochorial allantochorionic types of placenta: the horse (*Equus caballus*) and donkey (*Equus asinus*), which can interbreed with each other, and the camel (*Camelus dromedarius*), which cannot interbreed with either of the other two species. A panel of 14 lectins was used and it was found that glycosylation patterns were generally similar between placental tissues of the horse and donkey, except for the expression of non-bisected complex *N*-glycan and some sialic acids, whereas those of the camel showed striking differences in the binding of lectins to many structures carrying terminal residues of fucose, *N*-acetyl galactosamine and β -galactose, as well as to complex *N*-glycans and sialic acids. These results are consistent with the proposition that interbreeding species carry similar glycans in tissues forming the interhaemal barrier whereas glycodiversity is one of the factors preventing implantation and subsequent placental development in interspecific hybrids.

Introduction

Glycan expression in the tissue layers comprising the interhaemal barrier has been shown to vary in different placental types (Jones *et al.*, 1997a) and this may be one of the factors preventing interbreeding between species. Glycosylation patterns of the maternal epithelium and microcotyledonary trophoblast of three species possessing epitheliochorial placentation were studied to examine this proposition. Two of the species studied (horse and donkey, *Equidae*) can interbreed, while the third species, the camel (*Camelidae*), cannot interbreed with either of the other two species. The placentae of horses and donkeys are structurally very similar, being diffuse, microcotyledonary and villous (Allen, 1982; Steven, 1982; Leiser and Kaufmann, 1994; Wooding and Flint, 1994; Allen and Short, 1997) while the camel placenta is also diffuse and microcotyledonary, but the degree of villous branching is much less pronounced, producing rather squat, knob-like villi, especially in early pregnancy (Van Lennep, 1961, 1963; Abd-Elnaeim, 1998;

Leiser *et al.*, 1998). The camel placenta also differs from that of the horse and donkey in having occasional giant cells in the trophoblast (Van Lennep, 1961, 1963; Skidmore *et al.*, 1996; Abd-Elnaeim *et al.*, 1999).

In epitheliochorial placentation, there is close interdigitation of the apposed microvilli covering the luminal surface of the maternal uterine epithelium and the fetal trophoblast (Björkman, 1970). The initial association of the apical surface of the cells of the trophoblast with the uterine epithelium is somewhat irregular in the horse (Enders and Liu, 1991); the trophoblast cells have long microvilli whereas the uterine epithelial cells are stubby, and interdigitation is absent initially, although it is established by day 46 of gestation (Samuel *et al.*, 1976). In camels, the process of attachment is more gradual and complex (Skidmore *et al.*, 1996; Abd-Elnaeim *et al.*, 1999), with variation in the type of intercellular adhesion, ranging from the meeting of trophoblastic microvilli with flat uterine ectoplasmic pads to full interdigitation of trophoblastic and uterine microvilli separated by a gap of only about 20 nm. As these microvilli are heavily glycosylated (Jones *et al.*, 1997a),

their intercellular contact is probably mediated via glycan-glycan interactions (Aplin, 1997).

Evolutionary divergence of the phylogenetic groups containing horses (Perissodactyla) and camels (Artiodactyla) is estimated to have occurred 60–80 million years ago (Miyamoto *et al.*, 1993; Kumar and Hedges, 1998) while the genus *Equus* invaded the Old World no more than 2.5 million years ago, so that divergence of horses and donkeys can be no older than this (Churcher and Richardson, 1978). Hence, the opportunity for molecular evolution and mutation of placental glycans is considerable between horses and camels and minimal between horses and donkeys. The present study is an examination of the materno-fetal interface of the horse, donkey and camel near-term epitheliochorial placenta and a comparison of the glycans expressed therein.

Materials and Methods

Near-term placentae from a horse (*Equus caballus*, 80 cm crown-rump (C–R) length), donkey (*Equus asinus*, 80 cm C–R length) and camel (*Camelus dromedarius*, 100 cm C–R length) were perfuse-fixed and diced into full-depth pieces. The horse placenta, from the TBA Equine Fertility Unit (Newmarket, UK) via the Babraham Institute (Cambridge, UK), was perfusion-fixed from both fetal and maternal sides with 3% (w/v) paraformaldehyde–1% (v/v) glutaraldehyde and embedded in Araldite epoxy resin. The camel placenta was obtained from the abattoir at Cairo (Egypt) whereas the donkey placenta was given by the Assiut Veterinary Hospital (Egypt). Both placentae were available within 15 min of the death of the animal and fixed by perfusion with 3% (v/v) glutaraldehyde in 0.1 mol phosphate buffer l^{-1} , pH 7.3, with 2% (w/v) polyvinylpyrrolidone through a small branch of the uterine artery on the maternal side and a small branch of the umbilical artery on the fetal side. The placentae were then immersion fixed for 2 h in the same fixative. After washing in buffer, full depth slices of placental tissue were dehydrated in an ascending alcohol series, treated with propylene oxide and infiltrated with Taab epoxy resin (Taab Laboratories Equipment Ltd, Aldermaston) before being embedded in flat-bottomed capsules and polymerized at 60°C for 72 h. Sections 1.5 μm thick were cut from selected areas of all three specimens, mounted on 3-aminopropyltriethoxysilane (APES)-coated slides and stained with a panel of 14 biotinylated lectins and an avidin–peroxidase revealing system as described by Jones *et al.* (1999). Resin was removed with saturated sodium ethoxide diluted 1:1 with absolute ethanol for 15 min and, after washing in ethanol and then in distilled water, endogenous peroxidase was blocked with 10% (v/v) hydrogen peroxide (BDH, Poole) before exposure to 0.03% (w/v) trypsin (Type II crude, Sigma) in 0.05 mol Tris-buffered saline (TBS) l^{-1} , pH 7.6, for 4 min at 37°C. After washing, sections were incubated with 10 μg biotinylated lectin ml^{-1} (Sigma; SNA and MAA from Boehringer Mannheim, Lewes; LFA from EY Laboratories, San Mateo, CA; see Table 1 for the major sugar specificities of the lectins) in 0.05 mol TBS l^{-1} containing 1 mmol calcium chloride l^{-1} for 1 h at 37°C, washed in the same buffer then treated with 5 μg avidin peroxidase ml^{-1}

(Sigma) in 0.125 mol TBS l^{-1} , pH 7.6, with 0.347 mol sodium chloride l^{-1} for 1 h at 37°C (Jones *et al.*, 1987). Sections were washed and sites of lectin binding revealed with 0.05% (w/v) diaminobenzidine tetrahydrochloride dihydrate (Aldrich Chemical Co., Gillingham) in 0.05 mol TBS l^{-1} , pH 7.6 and 0.015% (v/v) hydrogen peroxide (30 volumes) for 5 min at 18 $\pm$ 0.5°C. Sections were rinsed, air-dried and mounted in neutral synthetic mounting medium (BDH). Controls were carried out as described by Jones *et al.* (1997a,b). Sections were assessed using a ranking system of analysis whereby staining intensity was allocated a grade from 0 (negative) to 4 (intensely stained) and intracellular granule density was allocated a grade from + (sparse) to ++++ (densely packed). Inter- and intra-observer error was tested to assess the reproducibility of the system.

Owing to the great difficulty in obtaining carefully dated and perfuse-fixed specimens for this study, only one placenta from each species was studied. The problem of obtaining material from large species such as these has been addressed by Jones *et al.* (1999). The main limiting factor in the present study was that access to suitable donkey material was severely limited, with only two specimens available to date, one of which was near-term. Therefore, the horse and camel placentae were selected to match as closely as possible to the gestational age of the donkey.

Results

Maternal uterine epithelium

PSA bound more strongly to camel uterine epithelium than to that of horse or donkey, while e-PHA showed only weak, diffuse staining of the camel tissue compared with moderate to strong staining of both horse and donkey epithelia (Fig. 1a–c). L-PHA (Fig. 1d–f) was bound by a subpopulation of epithelial granules in the horse only. MPA, SBA, ECA and WFA bound well to all three epithelia, while staining with AHA was strongest in the camel and weakest in the donkey epithelia (Fig. 1g–i). There was no detectable binding of DBA to the camel tissue, but strong to intense staining of donkey and horse epithelia (Fig. 2a–c). In contrast, LTA (Fig. 2d–f) and UEA-1 both bound to camel epithelium, whereas the epithelia of horse and donkey gave no staining with LTA, and weaker staining with UEA-1. No staining with SNA was observed in any of the species and MAA stained only occasional granules in the donkey epithelium. LFA bound to subpopulations of intracellular granules in horse and donkey epithelia but no staining was observed in that of the camel (Fig. 2g–i).

Microcotyledonary trophoblast

All three species showed some degree of binding of PSA and e-PHA (Fig. 1a–c), but this was weakest in the camel tissues, which also showed diffuse staining with e-PHA and nuclear staining within the trophoblast cells (Fig. 1a). However, L-PHA did not stain the camel trophoblast but, in both horse and donkey trophoblast, it was strongly bound (Fig. 1d–f). Both AHA and ECA bound more to horse and

Table 1. Lectins used in this study and their major specificities

Acronym	Source	Major specificity	Reference
PSA	<i>Pisum sativum</i> (garden pea)	α -D-mannose in non-bisected bi/tri-antennary, complex N-linked sequences	Trowbridge, 1974; Debray <i>et al.</i> , 1981
e-PHA	<i>Phaseolus vulgaris</i> (kidney bean) (erythroagglutinin)	Bi/tri-antennary bisected complex N-linked sequences	Cummings and Kornfeld, 1982; Yamashita <i>et al.</i> , 1983
I-PHA	<i>Phaseolus vulgaris</i> (kidney bean) (leukoagglutinin)	Tri/tetra-antennary, non- bisected complex N-linked sequences	Hammarström <i>et al.</i> , 1982; Green and Baenziger, 1987
AHA	<i>Arachis hypogaea</i> (peanut)	Gal β 1,3GlcNAc β 1- >Gal β 1,4GlcNAc β 1-	Lotan and Sharon, 1978; Sueyoshi <i>et al.</i> , 1988
ECA	<i>Erythrina cristagalli</i> (coral tree)	Gal β 1,4GlcNAc β 1-	Iglesias <i>et al.</i> , 1982; De Boeck <i>et al.</i> , 1984
MPA	<i>Maclura pomifera</i> (osage orange)	Gal β 1,3GalNAc α 1- >GalNAc α 1-	Sarkar <i>et al.</i> , 1981
SBA	<i>Glycine max</i> (soybean)	Terminal GalNAc α 1- >Gal α 1	Pereira <i>et al.</i> , 1974; Bhattacharyya <i>et al.</i> , 1988
WFA	<i>Wisteria floribunda</i> (wisteria)	GalNAc α 1,6Gal β 1->GalNAc α 1,3Gal β 1-	Sugii and Kabat, 1980; Baker <i>et al.</i> , 1983
DBA	<i>Dolichos biflorus</i> (horse gram)	GalNAc α 1,3(L-Fuc α 1,2)Gal- β 1,3/4GlcNAc β 1-	Etzler and Kabat, 1970; Baker <i>et al.</i> , 1983
UEA-1	<i>Ulex europaeus-1</i> (gorse)	L-Fuc α 1,2Gal β 1,4-GlcNAc β 1-	Hindsgaul <i>et al.</i> , 1985; Debray <i>et al.</i> , 1981
LTA	<i>Tetragonolobus purpureus</i> (lotus)	α -L-fucosyl termini (especially where clustered)	Pereira and Kabat, 1974; Debray <i>et al.</i> , 1981
SNA	<i>Sambucus nigra</i> (elderberry bark)	NeuNAc α 2,6Gal/GalNAc-	Shibuya <i>et al.</i> , 1987
MAA	<i>Maackia amurensis</i>	NeuNAc α 2,3Gal β 1-	Wang and Cummings, 1988; Knibbs <i>et al.</i> , 1991
LFA	<i>Limax flavus</i> (yellow slug)	Certain sialyl termini	Miller <i>et al.</i> , 1982; Knibbs <i>et al.</i> , 1993

donkey trophoblast than to camel trophoblast (Fig. 1g–i), while SBA showed a converse pattern, staining only a subpopulation of granules in the camel cells. DBA did not stain the camel trophoblast, but occasional granules were evident in horse and donkey trophoblast (Fig. 2a–c), whereas neither LTA (Fig. 2d–f) nor UEA-1 bound to horse or donkey trophoblast, although a subpopulation of intracellular granules was stained in the camel. MPA and WFA showed little difference in staining among the three species, with MPA staining moderately to strongly and WFA more intensely. SNA and MAA failed to bind to the trophoblast in all three species and there was only weak staining of occasional intracellular granules by LFA in horse trophoblast (Fig. 2h).

Interdigitating microvillous membrane

In the horse placenta, the presence of granules in either the maternal or fetal compartment was matched by staining of the interdigitating microvillous membrane. Those lectins that bound moderately or weakly to the membrane included

PSA, ePHA (Fig. 1b), I-PHA (Fig. 1e), UEA-1, MPA and WFA, while LTA (Fig. 2e), SNA and MAA showed no staining. The other lectins (AHA (Fig. 1h), DBA (Fig. 2b), ECA, SBA and LFA (Fig. 2h)) bound strongly. The pattern of binding in donkey placenta was similar except that staining with UEA-1 was absent and AHA and LFA showed only moderate binding (Figs 1i and 2i). All the other lectins exhibited strong to intense staining of the microvillous membrane. The camel placenta differed markedly in the lectin binding pattern of this membrane. L-PHA, DBA (Figs 1d and 2a), SNA, MAA and LFA (Fig. 2g) did not bind, while e-PHA (Fig. 1a), UEA-1, AHA (Fig. 1g) and ECA showed weak to moderate staining. All the other lectins (PSA, LTA (Fig. 2d), MPA, SBA, and WFA) bound strongly or intensely. These results have been summarized graphically (Fig. 3).

Controls

Substitution of the buffer for the lectin resulted in complete absence of stain, while incubation of the tissue with lectin in

Fig. 1. (a–c) *Phaseolus vulgaris* erythroagglutinin (e-PHA) binding to camel (a) is weak and diffuse while moderate to strong in horse (b) and donkey (c). (d–f) *Phaseolus vulgaris* leucoagglutinin (l-PHA) binding to camel (d), horse (e) and donkey (f), with absence of staining in the camel and donkey uterine epithelia and in the camel trophoblast. (g–i) *Arachis hypogaea* agglutinin (AHA) binds to subpopulations of granule in camel (g), horse (h) and donkey (i) epithelia and trophoblast. E, maternal uterine epithelium; T, trophoblast. Scale bar represents 20 μ m.

the presence of its competing sugar resulted in a marked reduction or elimination of staining, as described by Jones *et al.* (1997a,b, 1999). Testing of inter- and intraobserver error showed a very high degree of consistency in assessing the degree of staining within tissues.

Discussion

The lectin-binding patterns of horse and donkey uterine epithelium and trophoblast show a striking resemblance, implying close similarities between their glycans, while

Fig. 2. (a–c) *Dolichos biflorus* agglutinin (DBA) shows absence of binding in the camel (a) and strong staining of horse (b) and donkey (c) maternal epithelia; these species also show staining of occasional granules in the trophoblast. (d–f) *Tetragonolobus purpureus* agglutinin (LTA) stains only the camel placenta (d); that of the horse (e) and donkey (f) do not bind. (g–i) *Limax flavus* agglutinin (LFA) does not bind to the camel placenta (g) but some granules are stained in the uterine epithelia of horse (h) and donkey (i). Scale bar represents 20 μ m.

camel tissues are often quite different. This finding was especially evident in the microcotyledonary trophoblast, affecting particularly those glycans carrying terminal residues of fucose (LTA), the fucosylated structures GalNAc α 1,3(Fuc α 1,2)Gal β 1,3/4GlcNAc β 1- (DBA) and L-Fuc α 1,2Gal β 1,4GlcNAc β 1- (UEA-1), *N*-acetyl galactosamine (SBA), β -galactose (AHA, ECA) and non-bisected tri/tetra-antennary complex *N*-glycan (I-PHA). In the maternal

uterine epithelium, the interspecific differences were less marked, being most prominent with DBA, LTA and LFA, which last interacts with sialic acid. In camels, there were also more bi-antennary and bisected structures. The presence of fucosylated termini is regulated by fucosyl transferases, and Sidhu and Kimber (1999) describe steroidally modulated alterations in the expression of the FUT1 (α (1-2) fucosyl transferase) gene, which is involved in the biosynthesis of

Fig. 3. Graphic representation of lectin binding to maternal uterine epithelium and fetal trophoblast in the camel, horse and donkey, with granule density ranging from 1 (sparse) to 4 (numerous) and staining intensity from weak to intense: □, weak; ▨, moderate; ▩, strong; ■, intense. PSA, *Pisum sativum* agglutinin; e-PHA, *Phaseolus vulgaris* erythroagglutinin; l-PHA, *Phaseolus vulgaris* leucoagglutinin; AHA, *Arachis hypogaea* agglutinin; ECA, *Erythrina cristagalli* agglutinin; MPA, *Maclura pomifera* agglutinin; SBA, *Glycine max* agglutinin; WFA, *Wisteria floribunda* agglutinin; DBA, *Dolichos biflorus* agglutinin; UEA-1, *Ulex europaeus-1* agglutinin; LTA, *Tetragonolobus purpureus* agglutinin; SNA, *Sambucus nigra* agglutinin; MAA, *Maackia amurensis* agglutinin; LFA, *Limax flavus* agglutinin.

$\alpha(1-2)$ fucosylated structures, in the mouse endometrium in early pregnancy. In the present study, it is evident that changes in the distribution of fucosyl residues are one of the most striking variations among species, as has also been shown in a comparison between human and baboon endometria (Jones *et al.*, 1998).

Comparison with an earlier study on the pig placenta

(Jones *et al.*, 1995) showed that the near-term pig trophoblast expressed glycans similar to those found in the camel, with PSA, l-PHA, MPA, SBA, DBA, LTA and LFA showing similar patterns (e-PHA staining was not carried out), while the maternal epithelium was more like that of the horse and donkey, for example, in the binding of AHA, MPA, SBA, WFA, DBA, LTA and LFA. These findings follow the trend

observed in the present study, with some conservation of glycans on the maternal side and more interspecies variation in the trophoblast. The pig placenta is epitheliochorial, diffuse and folded, resembling a thin, undulating membrane, in which maternal perpendicular folds create bulbous, villous-like structures on the fetal side (Dantzer, 1984), consistent with the fact that pigs are considered to be the most primitive living artiodactylae (Prothero, 1993).

Although it could be argued that the glycosylation patterns reported here are those seen near term, and so bear little relation to those expressed during early implantation, the previous study of placentation in horses showed that there are no major changes in the nature of the glycans present on either the uterine epithelium or the fetal trophoblast from 35 days to term, apart from a decrease in the amount of α 2,6- and α 2,3-linked sialic acid (Jones *et al.*, 1999). In the interdigitating microvillous membrane, there is a slight decrease in staining with some lectins after 200 days possibly caused by steric hindrance (Jones *et al.*, 1999). In camels, there are variations in the degree of fucosylation (data not shown) but major changes with increasing gestational age are not found (C. J. P. Jones, unpublished). No early material was available from the donkey for comparison.

As glycans are so abundant at the two cell surfaces, they may be functionally significant in allowing initial attachment and subsequent implantation, and any aberration of this recognition system may be a cause of the failure of embryos to implant (Whyte and Allen, 1985). Despite the superficial similarities between the glycosylation of horse and donkey interhaemal barrier, in the case of the transfer of a pure donkey embryo to the uterus of a mare, there is a failure of interdigitiation of allantochorionic villi with endometrial crypts, in contrast with conventional intraspecific equine pregnancy (Allen and Short, 1997). In about 70% of donkey-in-horse pregnancies, implantation fails completely (Allen, 1982), apparently by a cell-mediated, cytotoxic response. In 30% of donkey-in-horse pregnancies, attachment and interdigitiation occur eventually and development proceeds relatively normally until term. Fetal genotype and uterine microenvironment also exert marked, interacting influences on endometrial cup development and subsequent equine chorionic gonadotrophin (eCG) secretion rates in equids (Allen, 1982; Allen *et al.*, 1987, 1993; Enders *et al.*, 1996). Therefore, it appears that both genetic and immunological components influence the success of these pregnancies, and it seems likely that the glycans of the implanting trophoblast, partially controlled by the genotype of the embryo, may be contributory factors. Schlafke and Enders (1975) pointed out that the crucial, initial regulated step of the implantation process is the interaction of the trophoblast with the apical cell surfaces of uterine epithelial cells. This phenomenon has been described as a 'cell biological paradox' (Denker, 1990; Thie *et al.*, 1996) as apical plasma membranes are generally non-adhesive, while basolateral domains normally express adhesion molecules. Attachment appears to be under steroid control (Carson *et al.*, 1990; Aplin, 1997). A stage of apposition of the trophoblast to the uterus is followed by a progressive process of adhesion, as has been illustrated in the epitheliochorionic placenta of the camel (Skidmore *et al.*, 1996; Abd-Elnaeim *et al.*, 1999) and horse (Samuel *et al.*, 1976;

Enders and Liu, 1991) with increasing interdigitiation of apposing microvilli. As well as molecules such as cytokines, growth factors and integrins being implicated in cell-cell interactions of the placenta, it has been suggested that changes from a non-adhesive to adhesive state involve steroid-modulated alterations in the nature or distribution of glycoproteins (Schlafke and Enders, 1975; Denker, 1990; Cross *et al.*, 1994; Wegner and Carson, 1994; Tabibzadeh and Babaknia, 1995; Thie *et al.*, 1996; Aplin, 1997), with many different types of structure (glycans, lectins, cadherins, extracellular matrix, glycosyl transferases) acting as linking molecules.

Experiments with interspecific hybridization have also implicated the trophoblastic glycocalyx in successful implantation, whereas no close correlation has been found between the chromosome count or the duration of gestation and the ability of two species to hybridize (Gray, 1972). Fehilly *et al.* (1984) manipulated embryos *in vitro* so that the trophoctoderm consisted of cells from the host, and this increased the probability of successful pregnancy. There are very few instances in nature of successful hybridization, but one of the more common man-made ones is the horse-donkey cross. The present findings indicate that if crossbred species, even when closely related, have different glycans on the surface membranes, then inappropriate or inadequate cell-cell interactions may occur resulting in a failure of implantation.

Jones *et al.* (1997a) compared the glycosylation patterns of different types of placenta from unrelated species and discussed the possible existence of species-specific 'glycotypes'. The purpose of such diversity, and the forces driving its development were unclear. Short (1976) suggested that natural selection must operate to minimize reproductive wastage and, therefore, there is probably evolutionary pressure to prevent the development of sterile hybrids, for example, mules and the hinnies resulting from donkey-horse crosses. This natural selection is best effected at an early stage, that is, by failure of implantation (or even fertilization), rather than by producing an offspring that reduces the reproductive potential of its mother by committing her to a long and fruitless gestation, and competing for limited resources to the detriment of fertile members of the herd.

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