Expression of carbohydrate antigens in the rat uterus during early pregnancy and after ovariectomy and steroid replacement

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Monoclonal antibodies were used to examine the expression of a number of carbohydrate antigens in the rat endometrial epithelium from day 1 to day 8 of pregnancy and in ovariectomized rats supplemented with ovarian steroids. Carbohydrate antigens based on the Gal β 1–GlcNAc backbone structure were expressed and some of these (Le^y, Le^x, B antigen) were present at all stages of pregnancy and independent of ovarian steroids. The H-type-2 antigen was stimulated by progesterone and expressed in the sensitized and receptive uterus, but was not detected after implantation or, in ovariectomized rats, in the refractory phase. The H-type-1 antigen, which is stimulated by oestrogen in the mouse, appeared to be stimulated by progesterone in rats. It was expressed throughout the period of pregnancy but maximal expression was found on days 4–5. The histo-blood group A antigen appeared in ovariectomized rats only after treatment with progesterone followed by three daily injections of progesterone and oestrogen, and in the corresponding post-implantation period of pregnant rats. Its appearance corresponded to the loss of detectable H-type-2 antigen. This study shows that the rat endometrial epithelium expresses some carbohydrate antigens not expressed in mice (A and B antigen) or under completely different steroidal regulation (H-type-1). Moreover, the T antigen was expressed on the endometrial epithelium adjacent to decidua on days 7 and 8 of pregnancy, but not in rats given ovarian steroids to mimic the sensitized, receptive or refractory phase. Differences in expression between glandular and luminal epithelia indicated differences in steroidal regulation, as found in mice.

Introduction

In vivo, blastocysts can attach only to uteri that have undergone maturational changes induced by an appropriate sequence of ovarian steroids (Finn and Martin, 1970; Psychoyos, 1973). In rodents, increased titres of ovarian progesterone convert the uterus to a sensitized organ that, when subjected to a small transitory increase in nidatory oestrogen between day 3 and day 4 of pregnancy (McCormack and Greenwald, 1974; Watson *et al.*, 1975), becomes receptive to embryo attachment (Psychoyos, 1973; Glasser and McCormack, 1979a; Glasser 1990). Neither RNA nor protein synthesis is required for receptivity (Finn and Bredl, 1973). Indeed, nidatory oestrogen suppresses RNA initiation sites (Glasser and McCormack, 1979b). The receptive period (window of implantation; Psychoyos, 1986) appears to last about 24 h in rodents, after which the uterus will no longer support blastocyst attachment (refractory period; Psychoyos, 1973, 1986).

However, attachment *in vitro* to extrauterine sites takes place regardless of the hormonal environment (Colwell, 1969; Enders

et al., 1981). These data suggest that, under certain conditions, functional and structural changes occur at the apical surfaces of the uterine epithelial cells that allow attachment, and the luminal epithelium becomes receptive. Under different conditions, the uterine epithelial cells either fail to produce adhesion molecules or produce ligands that prevent attachment (Glasser, 1990; Mulholland and Glasser, 1991).

Until recently little was known about the identity of molecules specifically involved in blastocyst implantation. Molecular markers for the sensitized, receptive and refractory uterus have not been available. Consistent changes are documented, such as the thinning of the glycocalyx around the time of implantation (receptive period) in rodents and rabbits (Enders and Schlafke, 1974; Chavez and Anderson, 1985) and the parallel decrease in surface charge (Hewitt *et al.*, 1979; Morris and Potter, 1984; Anderson and Hoffman, 1984; Jansen *et al.*, 1985). Changes in the expression of apical cell surface carbohydrate epitopes during the peri-implantation period are indicated by alterations in the binding of certain lectins (Chavez and Anderson, 1985).

Our knowledge of the differential effects of ovarian steroids on the synthesis, assembly, and secretion of apparently stage-specific uterine epithelial glycoproteins is becoming more

Table 1. Regimen for steroid treatment of ovariectomized rats

Group	Days (treatment begins 10 days after ovariectomy)										Physiological status of rat uterus
	1	2	3	4	5	6	7	8	9	10	
a	O	O	—	—	—	—	—	K			Vehicle control
b	O	O	—	—	O	O	O	K			Oestrogen control
c	O	O	—	—	P	P	P	K			P sensitized
d	O	O	—	—	P	P	P + o	K			Receptive
e	O	O	—	—	P	P	P + o	P + o	P + o	K	Refractory

O: primary dose of 100 ng oestradiol.

o: nidatory dose of 50 ng oestradiol.

P: 5 mg progesterone.

K: day rats killed.

detailed. Oestrogen stimulates sugar metabolism and glycosylation in the uterus, preferentially stimulating branched N-linked lactosaminoglycans in the murine uterus (Dutt *et al.*, 1986; Carson *et al.*, 1987). Synthesis of a number of endometrial proteins and glycoproteins is increased by oestrogen (Kuivanen and De Sombre, 1985; Teng *et al.*, 1986; Anderson *et al.*, 1986; Brown *et al.*, 1990). While progesterone decreases cell surface glycosylation and expression of specific glycoproteins (Anderson and Hoffman, 1984; Mulholland and Leroy, 1989; Svalander *et al.*, 1990; Brown *et al.*, 1990), it stimulates the expression of other glycoproteins (Anderson *et al.*, 1986; Mulholland and Leroy, 1989; Svalander *et al.*, 1990). Investigators have had difficulty in resolving the interplay between these two steroids on the expression of molecules by endometrial epithelial cells and with identifying specific roles for particular molecules in implantation.

There is increasing interest in the role of carbohydrates in cell–cell adhesion in mammalian reproductive processes. They have been implicated in fertilization (Wassarman, 1990) and compaction of the early embryo (Kimber, 1988; Fenderson *et al.*, 1990). The role of the selectin family of carbohydrate binding molecules has been defined in the initial adhesion of leucocytes to endothelial cells at sites of inflammation, and in lymphocyte homing (Lasky, 1992; Bevilacqua and Nelson, 1993). The possibility of a carbohydrate–selectin-like interaction has been suggested to explain the specific carbohydrate sensitivity of mouse embryo attachment to endometrial epithelial cells (Kimber *et al.*, 1993, 1994). The purified oligosaccharide lacto-N-fucopentannose 1, carrying the histo-blood group H-type-1 antigen at its nonreducing terminal, blocks blastocyst attachment to endometrial epithelial monolayers *in vitro* (Lindenberg *et al.*, 1988). Synthesis of the H-type-1 antigen in mouse endometrial epithelium is stimulated by oestrogen (Kimber and Lindenberg, 1990; Kimber *et al.*, 1994; White and Kimber, 1994) and exhibits a marked change in expression around the peri-implantation period. Expression becomes low or negligible after implantation. It follows that endometrial carbohydrates carrying H-type-1 may be involved in initial adhesion of the mouse embryo (Lindenberg *et al.*, 1988; Kimber *et al.*, 1993, 1994). The H-type-1 epitope is also expressed on the endometrial epithelium of other species (for example, cows and humans: Kimber, 1994; S. Kimber and S. Lindenberg, unpublished), suggesting it could be a widespread

component in the implantation process. In this study, we examined whether expression of this epitope in rats showed similar ovarian steroidal control to that demonstrated in mice. Our goal was also to identify other carbohydrate epitopes, expressed by the endometrial epithelium under the control of ovarian steroids, that may be markers of particular (sensitized, receptive, refractory) endometrial epithelial states.

Materials and Methods

Rats

Sprague–Dawley rats were obtained from either Harlan Industries (Houston, TX) or Harlan Olac (Bicester). Females were housed individually with males of their own strain and mating established by checking for spermatozoa in the vagina (day 1 of pregnancy). On the day required, rats were killed between 14:00 and 15:00 h by i.p. injection of avertin (50 mg kg⁻¹) or inhalation of chloroform, and the uterine horns dissected and cut into four pieces. They were either immersed in OCT embedding compound (Miles, Elkhart) and snap frozen in liquid nitrogen, or directly snap frozen in liquid nitrogen and stored at –80°C until required for sectioning. Pregnancy was confirmed by flushing embryos from either the oviduct or part of one uterine horn, up to day 5 as appropriate.

Ovariectomy and hormone supplementation

Female rats were bilaterally ovariectomized and left for 10 days to recover and for endogenous hormone concentrations to subside. The rats were injected (Table 1) with two daily priming s.c. injections of 100 ng oestradiol benzoate, left for 2 days and then injected according to one of the regimens below. In each of groups a–d, rats were killed 18 h after the final injection. Injections were given daily on days 5, 6 and 7 as follows: (a) controls, three injections of sesame oil only; (b) three injections of 100 ng oestradiol benzoate; (c) three injections of 5 mg progesterone (sensitized); (d) two injections of 5 mg progesterone followed by 5 mg progesterone and 50 ng oestradiol on day 3 (receptive); (e) as in (d) but on days 4 and 5, two further injections of 5 mg progesterone and 50 ng

Table 2. Specificity of mouse monoclonal antibodies used in this study

mAb	Isotype	Specificity	Reference
619/1D2	IgM	LNT	MonoCarb product information
P001	IgM	P ₁ -antigen	Brodin <i>et al.</i> (1988)
P ^k 002	IgM	P ^k -antigen	Brodin <i>et al.</i> (1988)
T001, BM22	IgM	T-antigen	Steuden <i>et al.</i> (1985)
H001	IgM	Le ^b /Le ^y	Brodin <i>et al.</i> (1987); Enblad <i>et al.</i> (1986)
H004	IgM	Le ^y /H-type-2	MonoCarb product information
672/7E3	IgM	Le ^y	MonoCarb product information
647/9A2	IgM	H-type-2	MonoCarb product information
667/9E9	IgM	H-type-1	Kimber <i>et al.</i> (1988); Lindenberg <i>et al.</i> (1990)
630/7H1	IgM	Le ^x and dimeric Le ^x	Kimber <i>et al.</i> (1988); MonoCarb information
A003/5	IgM	Blood group A	Chen and Kabat (1985); Bouhours <i>et al.</i> (1987)
B005/6	IgM	Blood group B	MonoCarb product information

Table 3. Carbohydrate structures investigated

LNT	Galβ1-3GlcNAcβ1-3Galβ1-4Glc
P ₁ -antigen	Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glc
P ^k antigen	Galα1-4Galβ1-4Glc
T-antigen	Galβ1-3GalNAcα1-
Le ^b	Fuca1-2Galβ1-3[Fuca1-4]GlcNAcβ1-
Le ^y	Fuca1-2Galβ1-4[Fuca1-3]GlcNAcβ1-
H-type-1	Fuca1-2Galβ1-3GlcNAcβ1-
H-type-2	Fuca1-2Galβ1-4GlcNAcβ1-
Le ^x	Galβ1-4[Fuca1-3]GlcNAcβ1-
Blood group A	Galα1-3[Fuca1-2]Galβ1-3/4GlcNAc
Blood group B	GalNAcα1-3[Fuca1-2]Galβ1-3/4GlcNAc

oestradiol were given and the rats killed 48 h later (refractory). All hormone injections were given at 16:00 h in a 0.1 ml volume of sesame oil.

Immunocytochemistry

A double-layer immunofluorescence technique was used as reported previously (Kimber *et al.*, 1988; White and Kimber, 1994). A minimum of three rats was used for staining for each steroid treatment, or two from each stage of pregnancy. When staining varied between days of pregnancy, a minimum of three rats was used for each day. Primary antibodies were mouse monoclonals (mAbs) from MonoCarb AB (formerly BioCarb AB), Lund, recognizing a number of type 1 and type 2 carbohydrate antigens as shown in Tables 2 and 3. Transverse sections, 7 µm thick, of uterus from a minimum of two different areas at least 20 µm × 7 µm apart were stained with each antibody. On days 7 and 8 of pregnancy, areas adjacent to the embryo within the decidualized swelling and between implantation sites were examined. No variation between these regions was detected except for the T and A antigens (see Results). A fluorescein isothiocyanate (FITC) goat anti-mouse antibody (Sigma, Poole) specific for the isotype of the primary antibody was used for the second layer. Antibody binding was observed in a Leitz Dialux epifluorescence microscope and photographed.

Results

Pregnancy

The staining patterns detected with different mAbs during pregnancy are summarized in Table 4. The P¹ and P_k antigens were not detected on the epithelium or in stromal cells on any day of pregnancy. Similarly, no staining was observed in the uterus with a mAb to the unfucosylated backbone structure Galβ1-3GlcNAcβ1-3Galβ1-4Glc (LNT) used routinely as a negative control (Table 3).

T antigen. The T antigen was not detected on the endometrial epithelium between days 2 and 6 of pregnancy, but on days 7 and 8 moderate apical staining was observed on approximately 75% of luminal cells (Fig. 1a, b) adjacent to the decidualized stroma. The epithelium in the interimplantation site was negative as were most glands, although a few showed apical staining similar to that found in the lumen. Individual scattered cells in the stroma were stained at all stages but were particularly marked on day 2, and many were found immediately basal to the epithelium (Fig. 1a).

Le^y and Le^b. Antibodies to the difucosylated antigens Le^b and Le^y (H001, H004, 672/7E3) stained the endometrial epithelium throughout pregnancy (Fig. 1c–h), although the appearance of staining on day 7 was different from that observed earlier (Fig. 1h). While up to day 6 the epithelial cells reacted uniformly with the three mAbs, on day 7 the staining pattern was heterogeneous, with some cells heavily stained but others only dimly fluorescent or unstained. This appearance was marked with H001 and least noticeable with 672/7E3. The antibodies stained both the cytoplasm and the cell surface but the resolution was insufficient to ascertain whether the membrane itself was being stained. Faint filamentous fluorescence in the lumina of glands and the cavity of the lumen, not seen in the controls, indicated reaction with secreted material. Stromal cells were unstained at all stages.

H-type-2. Staining was negative on glands and lumen on day 2 of pregnancy (Fig. 1i). It was first observed on the

Table 4. Summary of expression of carbohydrate antigens in the rat endometrial epithelium during pregnancy

Antigen	E/S	Day of pregnancy						
		2	3	4	5	6	7	8
LNT	E/S	—	—	—	—	—	—	—
P _i	E/S	—	—	—	—	—	—	—
P _k	E/S	—	—	—	—	—	—	—
T	E	—	—	—	—	—	+ ^{ab}	+ ^{ab}
	S	++	+	+	+	+	+	+
Le ^x /Le ^b	E	++	++	++	++	++	++ or —	+
	S	—	—	—	—	—	—	—
H-type-2	E	—	+ ^a or —	+ ^a	+ ^a	—	—	—
	S	—	—	—	—	—	—	—
H-type-1	E	+	+	++	++	+ or —	++	++
	S	—	—	—	—	—	—	—
Le ^x	El	+	+ or —	+ or —	+ or —	++	++	++
	Eg	—	—	—	—	—	—	—
	S	—	—	—	—	—	—	—
A	El	—	—	—	—	++ or —	++ or —	+/- or — ^b
	Eg	—	+ or —	++	++	++	++	++
	S	—	—	—	—	—	—	—
B	E	++	++	++	++	++	++	++
	S	+ or —	++ or —	++ or —	+ or —	+ or —	+ or —	+ or —

E/S: Epithelium or stroma; Eg: glands; El: luminal epithelium; ^apredominantly apical; ^badjacent to decidua.

luminal and glandular epithelium on the morning of day 3 of pregnancy (Fig. 1j). From late day 3 to day 5 of pregnancy, the apical surface of the luminal epithelium stained strongly (Fig. 1j, k). The fluorescence was localized in the apical cytoplasm, with weaker lateral staining. Staining in the glands was very weak but reactivity was detected on apical surfaces. The pattern of luminal fluorescence was patchy; some cells were unstained, particularly late on day 3 of pregnancy. On days 6–8 of pregnancy, the antigen was absent (Fig. 1l). Staining of secretions within the lumen could not be detected and no antibody binding to cells of the stroma was observed.

H-type-1. The mAb 667/9E9 stained the luminal epithelium throughout pregnancy. On day 2 of pregnancy, fluorescent staining was predominantly apical in both glands and lumen (Fig. 2a). By day 3 all surfaces and the cytoplasm were stained (Fig. 2b) and there was a marked increase in staining intensity on days 4 and 5. The fluorescence was slightly reduced and variable by day 6 and became patchy on the glands. However, on day 7 of pregnancy, staining was stronger and more uniform on all aspects of the cell surface and cytoplasm (Fig. 2c), although unreactive areas remained in both glands and lumen. Filamentous material within the cavity of the lumen reacted with the mAb (Fig. 2b) except on days 5 and 6. The stroma did not react at any stage.

Le^x. Expression of the Le^x antigen on the luminal epithelium depended on the day of pregnancy. On day 2, the cytoplasm and all cell surfaces except basal were stained (Fig. 2d). Most luminal epithelial cells were still strongly stained apically on days 3–5 (Fig. 2e), although there were

now patches of unstained cells. Basal staining was low or absent and cytoplasmic staining was weaker than on day 2. The intensity of staining was high on all surfaces and cytoplasm on days 6–8 (Fig. 2f). Staining of glands was negligible on all days of pregnancy. mAb-reactive material within the lumen could sometimes be detected but the stroma was always negative.

A antigen. Staining with the antibodies to the A antigen showed a stage-specific pattern. The luminal epithelium was negative on all surfaces (Fig. 2g, i, j) until day 6, when fluorescence became strong on the apical surface and cytoplasm (Fig. 2k). The lumen had a striped appearance, with strongly stained cells interspersed with unstained cells. This appearance was retained on day 7 while on day 8, staining was weaker and practically negative adjacent to the decidua (Fig. 2l). In some cases, faintly fluorescent material in the lumen was detectable on days 6 and 7. On day 2 of pregnancy, the glands were negative and some remained negative on day 3. In contrast to the lumen, most glands on day 3 and all glands from day 4 onwards were strongly stained on all surfaces (Fig. 2h, i). No staining was detected in the stroma.

B antigen. The B histo-blood group antigen was present throughout pregnancy on the luminal and glandular epithelium. The fluorescence intensity was high at all stages with little variation (Fig. 3a, b). Scattered stromal cells, particularly those adjacent to the lumen on days 3 and 4 of pregnancy, also stained for the B antigen. No preference for a particular (for example, mesometrial or antimesometrial) region of the stroma could be detected.

Fig. 1. Staining of the rat endometrium during early pregnancy with mAbs recognizing carbohydrate antigens (Tables 2 and 3). (a) Staining with mAb T001 on day 2 of pregnancy (T antigen); (b) mAb T001 on day 7 of pregnancy, note staining of luminal epithelium; (c) day 2 of pregnancy stained with mAb H001 (Le^a, Le^b); (d) staining on day 3 of pregnancy with mAb H004 (Le^a, H-type-2); (e) staining on day 4 of pregnancy with mAb 672/7E3 (Le^a); (f) staining on day 5 of pregnancy with mAb H004; (g) staining on day 6 of pregnancy with mAb H001; (h) staining on day 7 of pregnancy with mAb 672/7E3; (i) staining on day 2 of pregnancy with mAb 647/9A2 (II-type-2); (j) staining on day 3 of pregnancy with mAb 647/9A2; (k) staining on day 4 of pregnancy with mAb 647/9A2; (l) staining on day 6 of pregnancy with mAb 647/9A2. Arrows indicate luminal epithelium; g indicates gland. Scale bars represent 40 μm.

Fig. 2. Staining of rat endometrium during early pregnancy with mAbs recognizing various carbohydrate antigens (Tables 2 and 3). (a) Staining with mAb ce7/9E9 (H-type-1) on day 2 of pregnancy; (b) staining with mAb ce7/9E9 on day 3 of pregnancy; (c) staining with mAb ce7/9E9 on day 7 of pregnancy; (d) staining with mAb e30/7H1 (Le^x) on day 2 of pregnancy; (e) staining with mAb e30/7H1 on day 4 of pregnancy; (f) staining with mAb e30/7H1 on day 7 of pregnancy; (g) staining with mAb A003 (A histo-blood group antigen) on day 2 of pregnancy; (h) staining of glands with mAb A003 on day 4 of pregnancy; (i) staining with mAb A003 on day 4 of pregnancy; (j) staining with mAb A003 on day 5 of pregnancy; (k) staining with mAb A003 on day 6 of pregnancy; (l) staining with mAb A003 on day 8 of pregnancy. Arrows indicate luminal epithelium; g indicates gland. Scale bars represent 40 μ m.

Fig. 3. Staining of rat endometrium during early pregnancy with mAb B006 to the histo-blood group B antigen (Tables 2 and 3). (a) Staining on day 4 of pregnancy; (b) staining on day 6 of pregnancy. Arrows indicate luminal epithelium; g indicates gland. Scale bars represent 40 μ m.

Ovariectomized rats

The staining patterns detected with different mAbs after ovariectomy are summarized in Table 5. No staining was observed with antibodies to the T, P^k, P₁ or LNT antigens on the endometrial epithelium in ovariectomized rats after any treatment. Unlike the situation in pregnant animals, scattered cells that reacted with antibody to the T antigen were never detected in the stroma (Fig. 4a), independent of the type of treatment (Table 1).

Le^a and Le^b. The antibodies to Le^a and Le^b (Table 2) stained the luminal and glandular epithelia after all treatments (Fig. 4b–f). Staining of the luminal epithelium appeared not to be altered by any of the hormonal regimens (Table 1). Antigen was detected in the cytoplasm, particularly apically, and at all cell surfaces. Stained material within the lumen was also detected (Fig. 4c). Staining was reproducibly high and showed little intracellular variation.

These antibodies also stained the glands, but in rats treated with progesterone (sensitized) or progesterone followed by a single injection of progesterone and oestrogen (receptive), staining was greatly reduced or absent in the apical region of the glandular epithelium (Fig. 4e). Progesterone followed by three daily injections of progesterone plus oestrogen (refractory) gave more variable glandular staining with strong apical fluorescence for all three mAbs in only one rat (Fig. 4f). Deep in the endometrium (but not adjacent to the lumen), lateral glandular cell surfaces and cytoplasm were unstained and basal surfaces faintly stained in all animals. Although mAbs to these difucosylated structures did not stain most of the stroma, the fluorescence sometimes appeared to have bled from the basal surface of luminal or glandular epithelium into the adjacent stroma, suggesting either basal secretion or diffusion.

H-type-2. In animals treated with sesame oil or oestrogen alone there was generally no staining (Fig. 4g). However, after treatment with progesterone alone, patches of apical cytoplasmic fluorescence were present (Fig. 4h), varying in extent and intensity between animals. Intervening stretches of epithelium were negative. After progesterone treatment followed by one or three injections of progesterone plus oestrogen, areas of lumen were again found in which apical surfaces and cytoplasm were brightly stained (Fig. 4i, j). These stretches were often more extensive than after progesterone alone. The glands were unstained or occasionally reacted weakly on the apical aspect.

H-type-1. In vehicle or oestrogen treated rats, weak punctate, patchy staining was detectable on apical, but not lateral or basal surfaces of the luminal and glandular epithelium (Fig. 4k, l). However, in rats given progesterone alone, the cells stained on all surfaces (Fig. 5a), the intensity being greatest at apical surfaces and in the cytoplasm. This pattern was little changed in rats given progesterone followed by a single injection of progesterone plus oestrogen (Fig. 5b), but the fluorescence was more uniform and all surfaces and cytoplasm were brightly stained. Staining of glandular, but not luminal, epithelium was diminished after three injections of progesterone plus oestrogen (refractory, Fig. 5c).

Le^x antigen. The luminal epithelium stained for Le^x after all hormone treatments or injection of the vehicle (Fig. 5d–g). The apical region of the luminal epithelium always stained most strongly. Fluorescence was consistently brightest in rats treated with progesterone followed by three injections of oestrogen and progesterone (Fig. 5g). However, the difference between this and other treatments was only noticeable at the lateral and basal surfaces and in the cytoplasm. The glands stained little after any treatment, although occasional fluorescence was detected at the apices of individual cells, suggesting secretory material. This was most marked after progesterone followed by a single, or more often three, injections of progesterone plus oestrogen. Little mAb-reactive secreted material was detected, except after the latter treatment, and no reactivity was detected in the stroma.

A antigen. Both luminal and glandular epithelia were negative in rats treated with vehicle or oestrogen (Fig. 5h, i). In rats treated with progesterone alone, the luminal epithelium was also almost always negative (Fig. 5j) but the glands were strongly stained on all surfaces and in the cytoplasm. After progesterone followed by a single dose of progesterone plus oestrogen, the luminal epithelium was again negative (Fig. 5k). In contrast, in rats given progesterone followed by three injections of progesterone plus oestrogen, the luminal epithelial staining had a striking, reproducible, banded appearance (Fig. 5l). This suggested apical cytoplasmic reactivity, possibly of mucous material, which was not present in every epithelial cell. In contrast, the glands were uniformly brightly stained, while the stroma was negative.

B antigen. As for pregnant animals, the B antigen was expressed uniformly on the endometrial epithelium in all animals (Fig. 6a, b). There was a slight reduction on the

Table 5. Summary of expression of carbohydrate antigens in the rat endometrial epithelium after ovariectomy and hormone replacement

Antigen	E/S	Oil	O	Treatment		
				P	P + O	R
LNT	E/S	—	—	—	—	—
P ¹	E/S	—	—	—	—	—
P ^k	E/S	—	—	—	—	—
T	E/S	—	—	—	—	—
Le ^v /Le ^b	El	++	++	++	++	++
	Eg	++	++	+ or — ^c	+ or — ^c	+ or — ^c
	S ^d	—	—	—	—	—
H-type-2	El	—	—	+ ^a or —	+ ^a or —	+ ^a or —
	Eg	—	—	—	—	—
	S	—	—	—	—	—
H-type-1	El	+ ^a	+ ^a	+	++	++
	Eg	+ ^a	+ ^a	+	++	+
	S	—	—	—	—	—
Le ^x	El	+	+	+	+	++ ^b
	Eg	—	—	—	— or + ^a	— or + ^a
	S	—	—	—	—	—
A	El	—	—	—	—	++ or —
	Eg	—	—	+	+	++
	S	—	—	—	—	—
B	E	++	++	++	++	++
	S	+ or —	+ or —	+ or —	+ or —	+ or —

Oil: vehicle control (treatment a in Table 1); O: oestrogen (treatment b in Table 1); P: progesterone (treatment c in Table 1); P + O: progesterone followed by a single injection of progesterone + oestrogen (receptive, treatment d in Table 1); R: progesterone followed by three injections of oestrogen + progesterone (refractory; treatment e in Table 1).

E/S: Epithelium or stroma; Eg: glands; El: luminal epithelium; ^apredominantly apical; ^bglands less intense; ^cdeep glands unstained except basally; ^dsome staining of stroma adjacent to epithelium.

basal-lateral surface and in general cytoplasmic staining after the vehicle or oestrogen. Cells in the stroma with various morphologies also stained (Fig. 6a).

Discussion

Several carbohydrate epitopes based on Galβ1-GlcNAc chains were previously demonstrated as markers of endometrial epithelium in mice (Kimber *et al.*, 1988, 1994). Similar antigens have now been examined on rat endometrium and those specific to different stages of pre- and peri-implantation pregnancy have been identified. These will help define changes in the endometrium in response to hormones and other regulatory agents and expand our understanding of ovariectomy.

Two types of sequence may be formed from repeat Galβ1-GlcNAc units: lacto (type 1) sequences based on Galβ1-3GlcNAcβ1-3Galβ1-3, and neolacto (type 2) sequences based on Galβ1-4GlcNAcβ1-3Galβ1-3 chains. These structures form linear or branched chains attached to lipids or proteins and, together with globo-series carbohydrates, constitute backbone chains for the attachment of terminal histo-blood group antigens (Hakomori, 1989; Holgersson *et al.*, 1992). In humans these are both tissue and blood group antigens, but in rodents they exist as tissue antigens (Oriol *et al.*, 1992). The staining pattern generated by a mAb recognizing a carbohydrate

antigen may result from the summed distribution of the same structure on several different molecules. In addition, changes in the detection of an antigen may reflect its availability to antibody binding as a result of masking or unmasking.

In rat endometrium, expression of histo-blood group antigens was generally detected exclusively on epithelial cells, as in mice (Kimber *et al.*, 1988; Babiarz and Hathaway, 1988). However, the T and B antigens were expressed on both epithelial and stromal cells in rats. The T antigen was detected on luminal epithelium only between days 7 and 8 of pregnancy adjacent to decidualized stroma, 1.5–2.5 days after implantation. No staining was detected in ovariectomized rats treated to give a uterus refractory to embryo implantation (Psychoyos, 1973). Thus, expression may occur only after implantation, possibly triggered by an embryonic factor, or transiently as the epithelium passes from the receptive to refractory state. By examining ovariectomized rats relatively late in the refractory period (approximately 64 h after the end of receptivity), we may have missed the transient period of T antigen expression. The P₁ and P^k antigens and unsubstituted terminal Galβ1-3GlcNAc were detectable on endometrial epithelia, as in the mouse (Kimber *et al.*, 1988 and unpublished data). These antigens are probably substituted with fucose or sialic acid, which mask mAb binding.

Antigens expressed throughout pregnancy and irrespective of hormone regimen after ovariectomy include those recognized by H001, H004 and 672/7E3, which stained with a

Fig. 4. Staining of rat endometrium after ovariectomy and hormone supplementation with mAbs recognizing various carbohydrate antigens (Tables 2 and 3). (a) mAb T001 (T antigen) staining after treatment with oestrogen; (b) staining with mAb H004 (Le^b, H-type-2) after treatment with the vehicle only; (c) staining with mAb 672/7E3 (Le^b) after treatment with oestrogen; (d) staining with mAb H001 (Le^b, Le^h) after progesterone treatment; (e) staining with 672/7E3 after progesterone and a single injection of progesterone + oestrogen (receptive); (f) staining with H004 after treatment with progesterone followed by three daily injections of oestrogen + progesterone (refractory); (g) staining with mAb 647/9A2 (H-type-2) after treatment with the vehicle only; (h) staining with mAb 647/9A2 after treatment with progesterone; (i) staining with mAb 647/9A2 after progesterone and a single injection of progesterone + oestrogen (receptive); (j) staining with mAb 647/9A2 after treatment with progesterone followed by three daily injections of oestrogen + progesterone (refractory); (k) staining with mAb 667/9E9 (H-type-1) after treatment with vehicle alone; (l) staining with 667/9E9 after treatment with oestrogen. Arrows indicate luminal epithelium; g indicates gland. Scale bars represent 40 μm.

Fig. 5. Staining of rat endometrium after ovariectomy and hormone supplementation with mAbs recognizing various carbohydrate antigens (Tables 2 and 3). (a) Staining with mAb 667/9E9 (H1-type-1) after treatment with progesterone; (b) staining with mAb 667/9E9 after progesterone and a single injection of progesterone + oestrogen (receptive); (c) staining with mAb 667/9E9 after treatment with progesterone followed by three daily injections of oestrogen + progesterone (refractory); (d) staining with mAb 630/7H1 (Le) after treatment with oestrogen; (e) staining with 630/7H1 after treatment with progesterone; (f) staining with 630/7H1 after treatment with progesterone followed by an injection of progesterone + oestrogen; (g) staining with 630/7H1 after treatment with progesterone followed by three daily injections of oestrogen + progesterone (refractory); (h) staining with mAb A003 (A histo-blood group antigen) after treatment with the vehicle alone; (i) staining with mAb A003 after treatment with oestrogen; (j) staining with mAb A003 after treatment with progesterone; (k) staining with mAb A003 after progesterone and a single injection of progesterone + oestrogen (receptive); (l) staining with mAb A003 after treatment with progesterone followed by three daily injections of oestrogen + progesterone (refractory). Arrows indicate luminal epithelium; g indicates gland. Scale bars represent 40 μ m.

Fig. 6. Staining of rat endometrium after ovariectomy and hormone supplementation, with mAbs recognizing various carbohydrate antigens (Tables 2 and 3). (a) Staining with mAb B006 (B histo-blood group antigen) after treatment with progesterone; (b) staining with mAb B006 after treatment with progesterone followed by three daily injections of oestrogen + progesterone (refractory). Arrows indicate luminal epithelium; g indicates gland. Scale bars represent 40 μ m.

similar pattern. These mAbs have overlapping specificity for Le^y (Table 2), making it probable that they all recognize Le^y in rat endometrial epithelium. On the luminal epithelium of mice, staining with H001 and H004 varied little during the pre-implantation period, or after ovariectomy and different steroid treatments (Kimber *et al.*, 1988; Kimber and Lindenberg, 1990). However, some progesterone-mediated downregulation occurred in the glands of ovariectomized rats. The B histo-blood group antigen was also expressed uniformly in endometrial epithelium irrespective of stage. Stromal reactivity was marked in rats, in contrast to mice, where this antigen is not expressed in endometrium but is a marker of trophoblast and visceral endoderm (Brown *et al.*, 1993).

Similarly the A histo-blood group antigen is absent from the endometrial epithelium of mice (Kimber *et al.*, 1988) but present on that of the rat. Progesterone stimulated expression in the glands of pregnant and ovariectomized rats. Luminal epithelial reactivity correlated with the refractory period, being strong in rats given progesterone followed by three injections of progesterone plus oestrogen. It was undetectable on day 5 of pregnancy, strong on days 6 and 7 and negligible on day 8. Thus, this antigen seems to be a useful marker for initiation of the refractory period in rats, although it is absent after implantation in pregnant animals.

The H-type-1 antigen appeared to be stimulated by progesterone on the ovariectomized rat endometrial epithelium, rather than oestrogen as in mice (Kimber and Lindenberg, 1990; Kimber *et al.*, 1994; White and Kimber, 1994). Nevertheless, the expression patterns in pregnant rats and mice are not dissimilar. Maximum staining in rats occurred between days 3 and 5 of pregnancy, before implantation on day 5.5, while in the mouse it occurred between days 2 and 4 with implantation on day 4.5–5. However, the less patchy, non-punctate staining pattern in rats around the time of implantation suggests the epitope may be carried on different types of molecules. Furthermore, in

rats, the antigen could still be demonstrated up to day 8, or in refractory ovariectomized animals, while in mice, staining was weak or negligible by day 6 of pregnancy (Kimber *et al.*, 1988). Thus, there are clear differences in control of H-type-1 expression between these two rodents. Whether this antigen functions in the attachment of the embryo at implantation in rats, as appears to be the case in mice (Lindenberg *et al.*, 1988; Kimber *et al.*, 1993, 1994), remains unresolved.

Expression of the H-type-2 antigen was stimulated by progesterone in ovariectomized rats and correlated with progesterone sensitization in pregnancy. Retention of detectable antigen in the refractory luminal endometrial epithelium suggests it is not a reliable marker of progression from a receptive to refractory epithelium. Rather, its absence on day 6 of pregnancy may indicate that implantation has been successfully initiated. Addition of terminal GalNAc to an H precursor produces the A histo-blood group antigen (Table 3) that appears on the luminal epithelium between days 5 and 6 of pregnancy, when the H-type-2 antigen is absent. Thus, disappearance of H-type-2 may result from masking by terminal GalNAc.

Strong Le^x staining on the luminal epithelium on day 2 of pregnancy and in oestrogen-treated ovariectomized rats suggests oestrogen stimulation. Only a subtle enhancement of luminal epithelial Le^x expression by progesterone plus oestrogen was observed in ovariectomized mice (Kimber and Lindenberg, 1990), although Babiarz and Hathaway (1988) reported that glandular Le^x expression required both oestrogen and progesterone, while luminal expression required oestrogen alone.

The interactions between oestrogen and progesterone in the uterus are complex and, although the glycosylation machinery is stimulated by oestrogen (Dutt *et al.*, 1986; Carson *et al.*, 1987, 1990), the response to oestrogen and progesterone is molecule- and tissue-specific. Tissue variation in hormonal regulation of antigens cannot be accounted for by steroid receptor modulation alone (Wheeler *et al.*, 1987). Differences in steroidal control of various molecules occur between glandular and luminal epithelia (Babiarz and Hathaway, 1988; Svalander *et al.*, 1990; Kimber *et al.*, 1994). Luminal and glandular epithelia also differ in their proliferative response to ovarian steroids (Martin and Finn, 1968; Martin *et al.*, 1973). Our results indicate that, in rats, glandular and luminal expression of a number of carbohydrate antigens are controlled independently. For example, the A antigen is present from day 3 of pregnancy and is stimulated by progesterone on glands, but it is expressed on the luminal epithelium from day 6 of pregnancy and in refractory ovariectomized animals. The clear distinction between glandular and luminal staining, seen in sections through the glandular–luminal junction, suggests precise control at the level of individual cells. Possibly, shared carbohydrate epitopes on glands and lumen are carried on different molecules. Alternatively, glandular and luminal epithelia may differ in response to ovarian steroids owing to differences in receptors or interaction between ligand–receptor complexes and nuclear genes. However, regulatory molecules such as growth factors may exert disparate influences on these two epithelia. Information is needed on the control of carbohydrate antigens by epidermal growth factor (EGF) family members, particularly heparin binding EGF-like growth factor (HB-EGF;

Abraham *et al.*, 1993; Das *et al.*, 1994b; Zhang *et al.*, 1994), and their common receptor, implicated in many effects of oestrogen in the uterus (Mukku and Stancel, 1985; Nelson *et al.*, 1991; McLachlan *et al.*, 1991; Ignar-Trowbridge *et al.*, 1992; Das *et al.*, 1994a).

Although a number of endometrial epithelial (glyco)proteins are stimulated by oestrogen (Kuivanen and De Sombre, 1985; LeJeune *et al.*, 1985; Anderson *et al.*, 1986), there are few molecular markers of specific phases in the endometrial cycle. In ovariectomized rats, three endometrial epithelial proteins detected in the pre-receptive period disappeared within 12 h of injection with nidatory oestrogen (receptive period), but no new proteins could be detected. In the refractory period, a further protein was lost but two proteins reappeared and two appeared for the first time (Mulholland and Leroy, 1989). Two new *Ricinus communis* agglutinin-II-reactive glycoproteins reported to be expressed on days 3–4 of pregnancy in mice (Horvat, 1993) may indicate pre-receptive epithelium. Although few proteins specific to the pre-receptive or refractory phases have been identified, and none specific to the receptive period, it is suggested that differentiation of a receptive luminal epithelium is linked with changes in the expression or distribution of cell surface components (Denker, 1990; Glasser and Mulholland, 1993; Kimber *et al.*, 1994). Vital attachment epitopes for the embryo may become exposed apically on the luminal epithelium and anti-adhesive moieties may be removed at this time. These changes are thought to be reversed in the refractory phase, implying the fine modulation of exposed antigens by the masking and unmasking of carbohydrate epitopes. Our study suggests that expression of A histo-blood group antigen is a constitutive part of these changes and a useful marker of initiation of the refractory period in rats.

In conclusion, there are differences between rats and mice in the carbohydrate epitopes expressed by the endometrial epithelium and in their steroidal control. Histo-blood group A and B antigens, undetectable in the endometria of mice, are present in the rat, while H-type-I, expressed in both species, is stimulated by oestrogen in mice and by progesterone in rats. These differences reflect subtle variations in the control of glycosylation machinery, cell surface molecules and, possibly, implantation mechanisms. Identified stage-specific markers can be used *in vivo* and *in vitro* to investigate the response of the endometrial epithelium to regulatory agents. The possible role of these antigens in implantation awaits further investigation.

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