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Effects of monoclonal antibody directed to Le^Y on implantation in the mouse

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The role of carbohydrates in embryo implantation in the mouse was investigated using an embryo transfer model and a blastocyst–uterine epithelial cell co-culture system. The monoclonal antibody (mAb) AH_6 directed to Le^Y oligosaccharide (Fuc α1-2 Gal β 1-4 [Fuc α1-3] GlcNAc) and other three mAbs directed to carbohydrates whose structures are closely related to Le^Y were used to show the effect of carbohydrate specificity on implantation. In the embryo transfer model, donor blastocysts (4 days post-coitus) were pretreated with mAb AH_6 (experimental) or other mAbs (control) and transferred into one uterine horn of a recipient. The implantation rate was checked after 5 days. Implantation was significantly inhibited by mAb AH_6 pretreatment, and inhibition was not observed in control groups. In the co-culture system, the attachment and outgrowth rate of blastocysts on the surface of uterine epithelial cells was significantly inhibited when monolayer epithelial cells or blastocysts were pretreated with mAb AH_6. The most obvious effect of mAb AH_6 was obtained during 2–4 h co-incubation. No inhibition was observed in the control groups. It was, therefore, concluded that oligosaccharide Le^Y recognized by mAb AH_6 plays an essential role at the initial stage of implantation. It may act as a mediator molecule for adhesion between the surface of blastocyst and epithelial cell, and its function is carbohydrate-specific.

Key words: co-culture/embryo transfer/implantation/monoclonal antibody/oligosaccharide

Introduction

Implantation is a complex developmental process and a key step of reproduction, which occurs only if there is complete synchronization between the development of the embryo and the response of the hormonally-primed uterus to hormones and embryonic signals (Cross *et al.*, 1994). Direct interaction between the embryo and the uterus is initiated by recognition of the hatched, free embryo, followed by membrane apposition, and adhesion between the trophectoderm–trophoblast and endometrial luminal epithelium. Finally the embryo passes through the luminal epithelium and epithelial basement membrane and implants in the endometrial stroma.

Recognition, apposition and attachment of the blastocyst to the surface of endometrium involves complex cell interactions. Although the molecular mechanisms are unclear, growing evidence indicated that cell membrane glycoconjugate may play an important role in this process. Cell surface carbohydrates have been implicated in cellular interaction such as cell–cell recognition, adhesion, fusion and differentiation (Karlsson, 1989; Andrews *et al.*, 1990; Carson *et al.*, 1991; Sharon and Lis, 1993). Monoclonal antibodies directed to oligosaccharides have been used extensively to monitor changes in the composition of endometrial glycoconjugates during the peri-implantation period (Zhu *et al.*, 1995a,b). Several fucosylated oligosaccharides such as Le^Y, Le^X and H type 1 chain have been found on uterine epithelial cells. Expression of these glycans is under control of oestrogen and progesterone (Babiarz and Hathaway, 1988; Kimber *et al.*, 1988; Kimber and Lindenberg, 1990) and their expression and distribution changes at the time of implatantion (Kimber *et al.*, 1988; Zhu *et al.*, 1992, 1995a). The embryo also undergoes a series of glycosylation changes prior to implantation (Fenderson *et al.*, 1986).

The presence of Le^Y on peri-implantation mouse embryos and the fate of expressed Le^Y antigen during hatching and trophoblast outgrowth were described previously (Zhu *et al.*, 1995b). Embryonic Le^Y expression is regulated by differentiation or coating factors within the uterine lumen (Fenderson *et al.*, 1986). Le^Y was detected on endometrial glycoproteins in both pregnant and non-pregnant mice. However, certain species, with a molecular mass of ~12 and 16 kDa, appeared in the endometrium on the fourth day post-coitus coincident with the onset of uterine receptivity. We also demonstrated that anti-Le^Y monoclonal antibody (AH_6) injected in the uterine lumen significantly inhibits implantation in mice (Zhu *et al.*, 1995b). In the present study, the effects of the monoclonal antibody (mAb) AH_6 on implantation were further tested using an embryo transfer model. The effects of mAb AH_6 on blastocyst to uterine epithelium adhesion and blastocyst outgrowth were also investigated by an in-vitro co-culture system.

Materials and methods

Animals

Mature female mice of the outbred Kunming white strain weighing 25–30 g and aged 5–6 weeks were purchased from the Experimental Animal Center of the Institute of Animal Science. The animals were housed in the animal facility of the State Key Laboratory of Reproductive Biology having 14 h:10 h light:dark cycle and mated at night. Females were stimulated to ovulate with 5 IU pregnant mare's serum gonadotropin (PMSG) i.p. and human chorionic gonadotrophin (HCG) was administered 48 h later before mating. Fertilization was assumed to take place around midnight (5–7 h after dark). The presence of a copulatory plug the next day morning was considered to define the first day of pregnancy. Pseudopregnant mice were obtained by mating female mice with ligated male mice and were used as recipients for embryo transfer experiments.

Culture of uterine epithelial cells

Uteri from day 4 pregnant mice were split longitudinally to expose the epithelial cell surface and placed into solution containing 6 mg/ml trypsin (Sigma, St Louis, MO, USA) in 10 mM phosphate-buffered 0.9% saline (PBS, pH7.2) without Ca^{2+} and Mg^{2+}. Tissues were incubated at 4°C for 2 h followed by another 0.5 h at 25–30°C. The trypsin digestion was terminated by washing in Ham's F-12 medium (Gibco; Life Technologies (Pacific) Ltd., Tsuenwan, Hong Kong). Tissues were gently shaken to dislodge the epithelium from the endometrial bed, and the epithelial cells and fragments were collected by centrifugation at 500 *g* for 5 min. Cells were washed four times with Ham's F-12, resuspended in Ham's F-12 containing 2.20 mM calcium lactate, 2.05 mM glutamine, 10% fetal calf serum (FCS) and antibiotics (pH 7.4). The cell suspension adjusted to 1×10^6 cells/ml (>95% were viable as determined by Trypan Blue dye exclusion) was placed in 24-well Falcon plates, in the volume of 0.5 ml/well. The cells were incubated at 37°C under 5% CO_2 in air in a humidified chamber. The culture medium was changed on the next day to remove unattached cells and cell debris (Zeng and Cao, 1996).

Embryo collection

Preimplantation embryos were flushed from the uterus on the fourth day of pregnancy (82 h post-coitus) with PBS (as above), transferred in droplets of preheated medium [the same as that used for cell culture, except that 0.4% bovine serum albumin (BSA) was replaced with 10% FCS], and incubated at 37°C as described above.

Embryo transfer

Preimplantation embryos were flushed from the uterus on the fourth day of pregnancy (82 h post-coitus) with Ham's F-12. Normally-developed blastocysts were selected and transferred in 0.1 ml droplets of preheated embryo culture medium as control, and others were in 1:100 of mAb AH_6 or mAb anti-Le^b (αLe^b) or mAb FE-A_5 respectively [immunoglobulin (Ig)M = 10 μg/ml]. This dilution was based on preliminary experiments using dilutions ranging from 1:50 to 1:200. After 6 h incubation, 6–9 blastocysts from each group were washed with PBS (the same as above) to remove excess antibodies and transferred to one uterine horn of day 3 pseudopregnant mice in 0.1–0.3 μl total volume. The mice were killed 5 days later and the fetuses in the uterus were counted. The implantation rate was defined as the number of fetuses/the number of transferred blastocysts ×100.

Embryos and uterine epithelial cell co-culture

Epithelial cells harvested as described above were placed in 24-well sterile plastic plates and cultured under the same conditions as above. After ~18 h, the formation of a monolayer of epithelial cells was monitored under a phase-contrast microscope (Olympus; Taiyokoek Co. Ltd, Tokyo, Japan). Blastocysts (3 h) or epithelial cells (6 h) were pre-incubated in either culture medium alone or in monoclonal antibodies, AH_6, FE-A_5, αLe^b, αLe^a at a dilution of 1:100 monoclonal antibody:culture medium (IgM = 10 μg/ml). Following pre-incubation, the blastocysts were placed in corresponding wells of monolayer of uterine epithelial cells respectively (6–9 hatched blastocysts were used in each batch of experiments) and their attachment and outgrowth were observed.

Criteria for attachment and outgrowth of blastocysts

At 2, 4, 8, 24 and 48 h intervals, batches of embryo–epithelial cell co-cultures were monitored to examine extent of blastocyst attachment by rotating the culture plate along a circle of 30 cm diameter at a speed of one rotation per second for ~20 s. Blastocysts found to float in the medium at the end of rotation were judged as not attached (Figure 1A), otherwise, they were judged as attached (Figure 1B). Blastocyst outgrowth was identified when primary giant trophoblast cells were visible around the attachment site (Figure 1C) following examination of the co-cultures under phase-contrast microscopy.

Antibodies

The following monoclonal antibodies were used: mAb AH_6 directed to Le^Y and mAb FE-A_5 directed to the lactoseries type 2 chain, which were kindly supplied by Dr S.Hakomori of The Biomembrane Institute (Seattle, WA, USA); mAb anti-Le^a (αLe^a) and mAb anti-Le^b (αLe^b) directed to blood antigen Le^a or Le^b respectively were the gifts from Dr N.H.Guo in NIH, MD, USA. The carbohydrate structures recognized by these mAbs are listed in Table I. The carbohydrate specificity of these reagents was reported previously (Abe *et al.*, 1983; Fenderson *et al.*, 1986).

Statistical analysis

Data are given as mean ± SE and were analysed using the *t*-test. $P < 0.05$ was considered to be significant.

Results

Embryo implantation

An embryo transfer model was applied to test the effect of mAb AH_6 on implantation. Blastocysts were flushed from donors on the fourth day post-coitus, incubated with monoclonal antibodies (10 μg/ ml) (experimental) or incubated in medium only (control), and then transferred to different uterine horns of the same pseudopregnant mice. The implantation rate on both sides was checked on the eighth day post-coitus. Blastocysts pretreated with mAb AH_6 were associated with a dramatic decline in implantation rate, from 63.6% (control) to 28.4% ($P < 0.001$). Inhibition was not obtained in embryo groups that were pretreated with mAbs FE-A_5 or αLe^b ($P > 0.1$, Table II).

Embryo and epithelial cell co-culture

An in-vitro co-culture system was used to test the effect of mAb AH_6 on attachment and outgrowth of mouse blastocysts to the surface of monolayers of uterine epithelial cells (Figures 2 and 3). Results were observed at different times (2, 4, 8, 24 and 48 h) after hatched embryos were transferred onto the monolayer cells. When epithelial cells were pretreated with

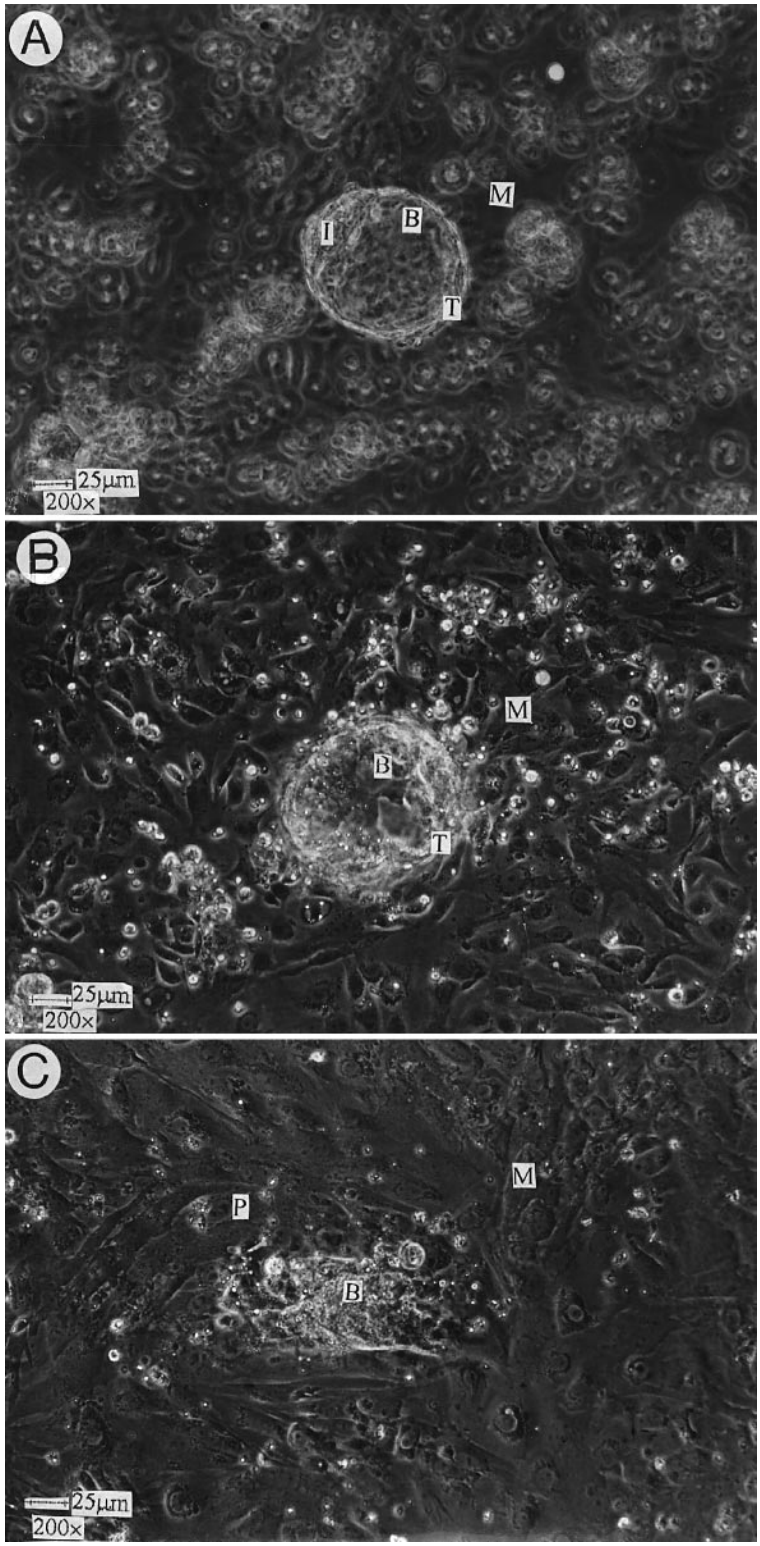

Figure 1. Attachment and outgrowth of mouse blastocysts on monolayers of uterine epithelial cells. (**A**) Non-attached blastocysts which floated over the monolayer; (**B**) attached blastocysts; and (**C**) trophectoderm outgrowth. M = monolayer of uterine epithelial cells; B = blastocyst; I = inner cell mass; T = trophoblastocyst; P = primary trophoblast giant cell.

mAb AH6 (37°C, 6 h), the attachment and outgrowth rates of cultured blastocysts decreased significantly during the period of 2–24 h co-culture time (CT). The greatest inhibition occurred at 2 h CT ($P < 0.001$). After 2 h, the difference between the mAb AH6 pretreated group and the control group gradually diminished, until no significant difference was present at 48 h CT ($P > 0.05$). The effect of mAb AH6 on outgrowth of blastocysts was most obvious at 2–4 h CT. Essentially, no blastocyst outgrowth occurred. The outgrowth rate gradually increased at 8 and 24 h, but it was still significantly different

Table I. Carbohydrate structures recognized by monoclonal antibody used in this study

Antibody	Antigen	Carbohydrate structure
AH_6	Le^Y	Fucα1→2Galβ1→4GlcNAcβ1→3Galβ1→R 3 ↑ Fucα1
$FE\text{-}A_5$	Lacto (Type 2)	Galβ1→4GlcNAcβ1→3Gal
αLe^a	Le^a	Galβ1→3GlcNAcβ1→4Galβ1→R 4 ↑ Fucα1
αLe^b	Le^b	Fucα1→2Galβ1→3GlcNAcβ1→4Galβ1→R 4 ↑ Fucα1

Table II. Effect of monoclonal antibodies on implantation rate of mouse blastocysts

Embryo transfer model		No. embryos transferred	No. implantation sites	Implantation rate (%)	*P* value
AH_6	Control	280	178	63.6 ± 0.3	<0.001
	AH_6	261	74	28.4 ± 0.2	
$FE\text{-}A_5$	Control	177	112	62.6 ± 0.3	NS
	$FE\text{-}A_5$	175	120	68.6 ± 0.3	
αLe^b	Control	118	75	63.6 ± 0.2	NS
	αLe^b	116	75	64.7 ± 0.2	

NS = not significant.

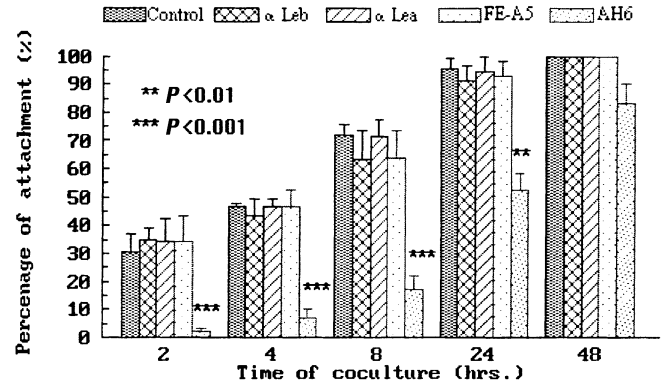

Figure 2. Effects of monoclonal anti-carbohydrate antibodies on blastocyst attachment to cultured uterine epithelial cells. Epithelial cell monolayers were pre-treated with monoclonal antibodies (mAbs) at a dilution of 1/100 and washed prior to blastocyst addition and co-culture. The effects of mAbs on attachment rate were determined by comparing the total number of blastcysts co-cultured with epithelial cells (TB) to the total number of attached blastocysts (TA). The attachment rate is defined as TA/TB×100. Results represent the mean ± SE of four replicates (n = 35 blastocysts for each). **Significantly different from controls (P <0.01); ***significantly different from controls (P <0.001).

from the control. At 48 h CT, no significant difference between the two groups was observed. However, inhibition of attachment and outgrowth were not obtained with three other control mAbs (Figures 2 and 3).

In a related set of experiments, blastocysts were pretreated

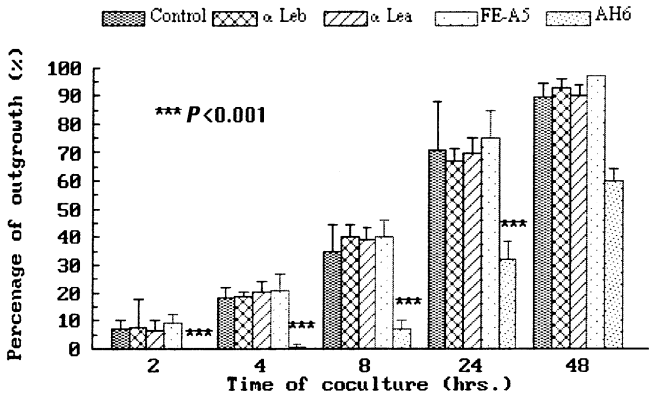

Figure 3. Effects of monoclonal anti-carbohydrate antibodies on blastocyst outgrowth on cultured uterine epithelial cells. Epithelial cell monolayers were pre-treated with monoclonal antibodies (mAbs) at a dilution of 1/100 and washed prior to blastocyst addition and co-culture. The effects of mAbs on outgrowth rate were determined by comparing the total number of blastcysts co-cultured with epithelial cells (TB) to the total number of attached blastocysts outgrowths (TA). The outgrowth rate is defined as TA/TB×100. Results represent the mean ± SE of four replicates (n = 35 blastocysts for each). ***Significantly different from controls (P <0.001).

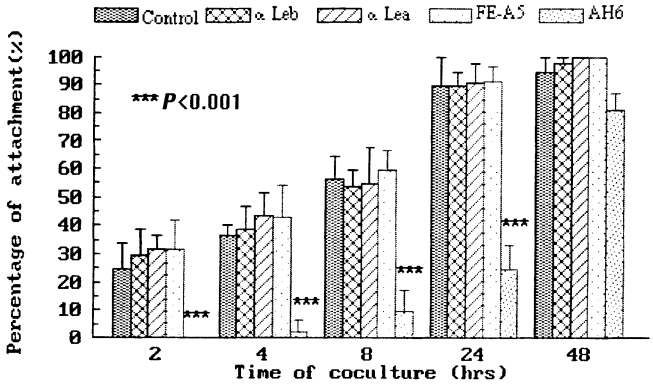

Figure 4. Effects of pre-treating mouse blastocysts with monoclonal anti-carbohydrate antibodies at a dilution of 1/100 on blastocyst attachment to monolayer cultures of uterine epithelial cells. The effects of monoclonal antibodies (mAbs) on attachment were determined by comparing the total number of blastcysts co-cultured with epithelial cells (TB) with the total number of attached blastocysts (TO), and the attachment rate is defined as TO/TB×100. Results represent the mean ± SE of four replicates (n = 35 blastocysts for each). ***Significantly different from controls (P <0.001).

(37°C, 3 h) with mAb AH6 or three other control mAbs, washed and then co-cultured with monolayer epithelial cells. Both attachment and outgrowth were significantly inhibited by mAb AH6, during the period of 2–24 h CT (P <0.001). At 48 h CT there was still a significant difference (P <0.05) between the two groups. The inhibitory effect was not observed with embryo groups pretreated with the three control mAbs (Figures 4 and 5).

Discussion

Implantation is the outcome of interaction of blastocyst with endometrium. During the process, the morula enters the

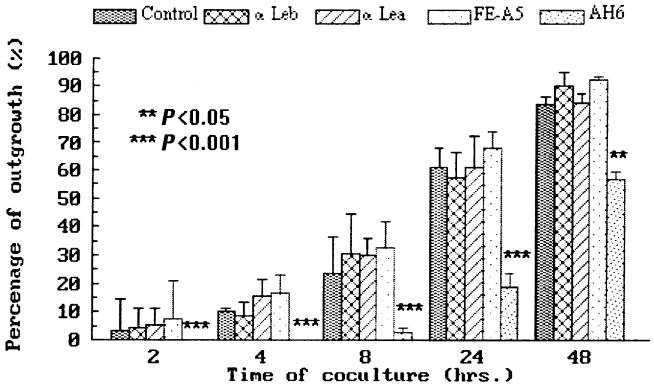

Figure 5. Effects of pre-treating mouse blastocysts with monoclonal anti-carbohydrate antibodies at a dilution of 1/100 on outgrowth of blastocyst on monolayer cultures of uterine epithelial cells. The effects of monoclonal antibodies (mAbs) on outgrowth were determined by comparing the total number of blastcysts co-cultured with epithelial cells (TB) with the total number of attached blastocyst outgrowths (TO), and the outgrowth rate is defined as TO/TB×100. Results represent the mean ± SE of four replicates (n = 35 blastocysts for each). **Significantly different from controls (P <0.05); ***significantly different from controls (P <0.001).

endometial cavity and a blastocyst is formed (Narlow and Lane, 1988). At first, the blastocyst is free within the cavity and has not directly interacted with the surface of epithelium. During this time it matures and loses its zona pellucida (hatching) (Narlow and Lane, 1988). After the blastocyst hatches, it adheres to and penetrates the surface epithelium and is subsequently embedded in the underlying stroma.

It is known that Le^Y oligosaccharides (see Table I) are present on the surface of embryos after entering the uterine cavity (Fenderson *et al.*, 1986), and Le^Y is present on the surface of uterine epithelial cells and uterine secretion prior to implantation (Zhu *et al.*, 1995a,b). We have previously reported that implantation of mouse embryos was blocked by intrauterine injection of anti-Le^Y mAb. The results here corroborate and extend these findings and are summarized as follows: (i) implantation was inhibited in embryo transfer experiments by pretreating blastocysts with mAb AH_6 *in vitro*; (ii) in a blastocyst and epithelial cell co-culture system, mAb AH_6 pretreatment of epithelial cells or blastocysts inhibited adhesion and penetration between the surfaces of blastocysts and epithelial cells; and (iii) inhibition of implantation and attachment was carbohydrate-specific.

According to these results and our previous work, the time window for the inhibitory effect of mAb AH_6 on implantation is narrow, ~87–93 h post-coitus. After 105 h post-coitus, no inhibition is observed. Therefore, we postulate that Le^Y may play a role as a mediator at the initial stage of implantation, i.e. prior to the adhesion between the embryo and endometrium. Blockade of Le^Y may cause steric hindrance of receptors involved in the attachment of the blastocyst to the uterine luminal epithelium or decrease the immunosuppression induced by Le^Y oligosaccharide antigen and induce a maternal immune response in a temporal manner (Clark *et al.*, 1997). Direct immunofluorescence analysis showed that after intrauterine injection, mAb AH_6 could be detected for ~18 h on the surface of uterine endometrium (data not published) and during this time the inhibitory effect of mAb AH_6 on blastocyst attachment gradually declined. After 48 h, the effect almost disappeared. Blastocyst penetration rate recovery was not so obvious, but the tendency was the same. These results suggest that mAb AH_6 which blocks Le^Y antigen could be removed by endocytosis or phagocytosis or that the antigen could be resynthesized: at that time, the adhesion between blastocyst and epithelium occurs again. However, *in vivo*, successful implantation of the blastocyst depends on synchronous developmental changes between the embryo and pregnant uterus. Therefore, even if Le^Y is able to reappear later, the earlier blockade by mAb AH_6 will have already resulted in an unsuccessful implantation.

Targeted mutation in β 1,4-galactosyl transferase has recently been shown to cause fetal growth retardation and neonatal lethality with variable penetrance (Asano *et al.*, 1997; Lu *et al.*, 1997). Surviving females, presumably lacking Le^Y oligosaccharides on their uterine epithelium are fertile, but litter size in these females is reduced. Galactosyl transferase-deficient embryos may continue to express carbohydrate differentiation antigens such as Le^X and Le^Y during the peri-implantation period due to stored maternal message. These results suggest that Le^Y oligosaccharides on the surface of the blastocyst are most critical in establishing recognition between the embryo and the uterine endometrium. Efforts to map the expression of Lewis antigens in galactosyl transferase knockout mice are in progress.

Recently, several growth factors/cytokines have been implicated in the implantation process. These include leukaemia inhibitory factor (LIF), epidermal growth factor (EGF), interleukin (IL)-6, transforming growth factor (TGF) and colony stimulating factor (CSF), and an attempt to assemble some of the events in the implantation cascade into a model has been reported (Tabibzadeh and Babaknia, 1995). Maternal uterine production of LIF appears to be a key component of the implantation process (Harvey *et al.*, 1995). Interestingly, the time window for the inhibitory effect of mAb AH_6 and the stage-specific expression of low molecular weight Le^Y carried glycoproteins is coincident with LIF expression on the endometrium on the fourth day of pregnancy (Stewart *et al.*, 1992). The study of the role of Le^Y in implantation and its relationship to LIF and other cytokines may provide insight into the signals and molecular pathways involved in the complex process of implantation.

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