The milk oligosaccharide, lacto-*N*-fucopentaose I, inhibits attachment of mouse blastocysts on endometrial monolayers

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Summary. Seven oligosaccharides isolated from human milk were tested for their effect in an in-vitro model of mouse blastocyst adhesion and trophoblast outgrowth on endometrial epithelial monolayers. One compound, lacto-*N*-fucopentaose I (LNF I), produced a significant reduction in the percentage of attached and outgrown blastocysts after co-culture for 72 h ($P < 0.001$). No significant effect of any other tested oligosaccharide was obtained.

Keywords: trophectoderm; uterine-epithelial cells; blastocyst; oligosaccharides; attachment; *in vitro*

Introduction

Several studies have demonstrated morphological changes in the uterine epithelial cells preceding the initial contact of the blastocyst with the uterine surface (Nilsson, 1974; Enders, 1976; Enders *et al.*, 1983). Well defined ultrastructural alterations in the apical cytoplasm and on the cell surface have been observed which are believed to be crucial for initial adhesion and implantation (Ferenczy & Richart, 1973; Parkening, 1976; Jansen *et al.*, 1985). Specifically, it has been reported that a decrease in the thickness of the uterine epithelial glycocalyx precedes the time of implantation in rodents (Enders & Schlafke, 1974; Chavez & Anderson, 1985) and parallels a decrease in the cell surface charge (Hewitt *et al.*, 1979). Lectin binding studies indicate that modulation of cell surface glycoconjugates of both the uterine epithelium and the trophectoderm accompanies these changes (Wu & Chang, 1978; Johnson & Calarco, 1980; Chavez & Enders, 1981, 1982; Chavez & Anderson, 1985; Chavez, 1986).

The appearance of new glycoproteins at the apical surface of the uterine luminal epithelium during the period of receptivity has been demonstrated biochemically in the rabbit (Anderson *et al.*, 1986), but it has not been shown that glycosylated cell surface components participate in the implantation of the embryo.

However, cell-cell interactions have been observed to be dependent on homo- or hetero-philic interaction of carbohydrate receptors (Harrison & Chesterton, 1980; Barondes, 1981; Bozzaro, 1985; Edelman *et al.*, 1985; Misevic & Burger, 1987).

One way to demonstrate such a mechanism is to utilize competing oligosaccharides, which may specifically interfere with the interaction of cells by hapten-inhibition (reviews: Harrison & Chesterton, 1980; Barondes, 1981; Bozzaro, 1985). In particular, a specific fucosylated pentasaccharide purified from human milk (Bird & Kimber, 1984) or its lysyl-lysine conjugate (Fenderson *et al.*, 1984) has been shown to reverse close intercellular contacts and cause decompaction in the 8–16-cell stage of the mouse morula.

To study further the role of uterine surface glycoconjugates in implantation, we have investigated the early apposition and adhesion phase between the trophectoderm and uterine epithelium. In this present study we report the effect of some milk oligosaccharides on adhesion of mouse blastocysts to uterine epithelial monolayers *in vitro*.

Materials and Methods

Animals and methods

Animals. The mice were F1 B6D2F1/BOM hybrids (from the Laboratory of Animal Breeding and Research Center, Gl. Bomholdt Gård, Ltd, 8680 Ry, Denmark). Females aged 21 days and weighing 13–18 g and mature 6-month-old males of previously proven fertility were used. They were housed in air-conditioned quarters with pelleted food and water available *ad libitum*, and light between 07:30 and 19:30 h.

The female mice were injected intraperitoneally with 5 i.u. PMSG (Antex, Leo Pharmaceuticals, Copenhagen, Denmark) at 09:00 h followed 48 h later by injection of 2.5 i.u. hCG (Phsysix, Leo) to induce superovulation. They were caged with males 4 h after the last injection at 13:00 h and left overnight. Mating was confirmed on the following morning by the presence of a vaginal plug. The day of observation of the vaginal plug was designated as Day 1 of pregnancy.

Embryo culture. Females were killed at 09:00 h on Day 2 of pregnancy and 2-cell embryos were flushed from the isolated oviducts. Handling the 2-cell embryos outside the CO₂ incubator was carried out in Ham's F-10 medium supplemented with Hepes buffer, 20 mM (Flow Labs, Irvine, U.K.; cat. no. 16-884-49). The embryos were cultured in Earle's balanced salt solution supplemented with 2% Ultrose (LKB, cat. no. 2216-100) in 4-well culture dishes (Nunc A/S, DK-4000, Roskilde, Denmark). All media used had previously been found to support more than 80% blastocyst formation from 2-cell mouse embryos after 72 h in culture (Trownson & Wood, 1981). Moreover, the media were endotoxin-free as assessed by the *Limulus* test (Sigma Chemical Company, St Louis, MO, U.S.A.). After culture for 72 h at 37°C in an humidified atmosphere of 5% CO₂ in air, the embryos to be used had reached the hatching blastocyst stage and were transferred to the culture dishes containing uterine monolayers.

Preparation of endometrial epithelial monolayers. The uterus of each mouse was isolated at the same time as the oviducts and opened longitudinally. Endometrial epithelial cells were collected from the uterine lumen by scraping with a rubber policeman (Sherman & Wudl, 1976). These cells were suspended in the Hepes-buffered medium. After 15 min in Earle's minimum essential medium (MEM) (Flow Labs) with 300 i.u. collagenase type 4/ml (Gibco) the cells were seeded in 4-well culture dishes (Nunc) containing MEM medium substituted with D-valine (Flow Labs) and supplemented with penicillin (0.0075 g/l), streptomycin (0.0075 g/l), glutamine (1 mM), 2% ultrose (LKB) and 5% fetal calf serum (Biochrom, Beteiligungs GmbH & Co., Berlin, FRG).

A number of culture dishes had coverslips placed on the bottom before seeding the cell suspension and were used for staining with antibodies (see below).

The cultures were maintained in an humidified atmosphere with 5% CO₂ in air and medium was changed every other day for 7 days before they had reached confluency and could receive embryos. The medium used here has been shown to be selective in supporting proliferation of endometrial epithelial cells in preference to stromal cells (Gilbert & Migeon, 1975; Lindenberg *et al.*, 1985).

Test system

Oligosaccharides. For the test system two control media and one or more test media were used in parallel. Each test medium was always supplemented with only one oligosaccharide at a time. The control media consisted of: (1) Medium MEM (Flow Labs) supplemented with 0.0075 g penicillin-G/l, 0.0075 g streptomycin/l, 1 mM-glutamine, 5% fetal calf serum and 2% Ultrose-G (hereafter named MEM+ when used as a control), and (2) the same medium supplemented with LNF II (see Table 1 for structure) at the same concentration as the sugar to be tested. In a pilot study this oligosaccharide did not have any effect on adhesion and subsequent trophoblast outgrowth.

To investigate the potential effect of oligosaccharides (see Table 1) on the attachment of blastocysts on monolayers the oligosaccharide to be tested was added to Medium MEM+. The oligosaccharides, 2–5 batches of each, were supplied by BioCarb AB (Lund, Sweden) under a code and all tests took place blind. The identity of the oligosaccharides was only revealed after completion of the study. If possible more than one oligosaccharide was tested in parallel with the controls. Addition of oligosaccharides to the medium did not alter the osmolality or the pH.

Oligosaccharide-containing medium and blastocysts were added simultaneously to the monolayers and pooled blastocysts were allocated without preference to wells containing control or test medium. No well contained more than 30 blastocysts.

The mouse blastocysts and endometrial monolayer cells were observed 24, 48 and 72 h after the start of the co-culture and the numbers of embryos which had adhered and outgrown, as well as non-adhesive blastocysts, were counted. Adhesion was defined as tandem motion of the embryo and uterine monolayer during gentle tapping of the culture vessel, as observed under the inverted phase contrast microscope. When adhering embryos were observed after

Table 1. Structure of the oligosaccharides used in this study

Trivial name	Abbreviation	Structure
2-Fucosyllactose	2 FL	Gal β (1-4)Glc α (1-2) Fuc
Lactodifucotetraose	LDFT	Gal β (1-4)Glc α (1-2) α (1-3) Fuc Fuc
Lacto- <i>N</i> -tetraose	LNT	Gal β (1-3)NAcGlc β (1-3)Gal β (1-4)Glc
Lacto- <i>N</i> -fucopentaose I	LNF I	Gal β (1-3)NAcGlc β (1-3)Gal β (1-4)Glc α (1-2) Fuc
Lacto- <i>N</i> -fucopentaose II	LNF II	Gal β (1-3)NAcGlc β (1-3)Gal β (1-4)Glc α (1-4) Fuc
Lacto- <i>N</i> -difucohexaose I	LND I	Gal β (1-3)NAcGlc β (1-3)Gal β (1-4)Glc α (1-2) α (1-4) Fuc Fuc
Lacto- <i>N</i> -fucopentaose III	LNF III	Gal β (1-4)NAcGlc β (1-3)Gal β (1-4)Glc α (1-3) Fuc

Gal = D-galactose; Glc = D-glucose; Fuc = L-fucose; NAcGlc = *N*-acetyl-D-glucosamine.

Table 2. Murine monoclonal antibodies used to detect oligosaccharide determinants on epithelial monolayers and blastocysts or in the attachment inhibition studies

Code	Isotype	Specificity
667/9E9	IgM	LNF I (H type I chain)
630/7HI	IgM	LNF III (X-antigen, SSEA 1)
HOOI	IgM	LND I (difucosylated type I and II chains)
		LNneoDI*

*Lacto-*N*-neo-difuco-hexaose I.

24 h in co-culture a mark was placed on the bottom of the dish so that further development after adhesion could be monitored. Implantation of a blastocyst was considered to have occurred when outgrowth and displacement of endometrial cells by trophectoderm was visible. Blastocysts retaining the zona pellucida were not scored as adhesive or as outgrown. Trials in which one or both the controls had an implantation rate (no. of implanted blastocysts/total no. of blastocysts at start of co-culture) of less than 70% were excluded from this study, as these cultures were possibly accidentally subjected to deleterious pH or temperature changes during preparation.

The morphology of the blastocysts with trophectoderm outgrowth was studied by light and transmission electron microscopy (TEM) to assess the health of the blastocysts and the monolayer.

The blastocyst attachment sites were fixed in 70% Karnovsky's fixative and processed for TEM as previously described (Lindenberg *et al.*, 1986).

Antibodies. Mouse monoclonal antibodies (IgM), were provided as supernatants with an approximate concentration of 100 μ g immunoglobulin/ml by BioCarb AB (Lund, Sweden) (see Table 2). The supernatants containing the antibodies (667/9E9 and 630/7HI) were added in equal volume to Medium MEM+ to examine their effect on blastocyst attachment and adhesion.

To investigate whether the oligosaccharides used above could be identified on the uterine surface or on the blastocysts, blastocysts and monolayers were stained as follows. Epithelial monolayers were grown on coverslips in parallel with those used for in-vitro co-culture with blastocysts. They were fixed in acetone for 10 min during the time that the blastocysts attached in parallel cultures. The coverslips with monolayers were hydrated in Dulbecco's phosphate buffer (pH 7.2), containing 0.1% bovine serum albumin (Behringwerke AG, Marburg, FRG) and 0.05% Tween 20 at 20°C. All antibodies and sera were diluted in the same buffer. The sections were incubated for 30 min in a 1/20

dilution of normal goat serum ($C \times 907$; Dakopatts, Copenhagen, Denmark) at 20°C to prevent non-specific binding. Monoclonal antibody supernatants were diluted to 1/10 or 1/20. The coverslips were overlaid with 50 µl of the primary antibody and incubated overnight in an humidified atmosphere at 4°C, rinsed in the buffer and again incubated for 30 min in 1/20 normal goat serum at 20°C. They were then overlaid with the second antibody, a fluorescein isothiocyanate (FITC) conjugate of goat anti-mouse IgM (Southern Biotechnology Associates, Inc., Birmingham, Alabama, U.S.A.) at a final dilution of 1/80 for 60 min. The coverslips were finally rinsed in the buffer and mounted in 2.5% DABCO (Boehringer, Mannheim, FRG) in 80% glycerol.

Blastocysts were stained after culture from the 2-cell stage or after flushing directly from the uteri on Day 4 or 5 of pregnancy. When necessary the zona pellucida was removed with acid Tyrode's solution pH 2.5. They were washed 3 times in Medium M2 + 4 mg BSA/ml (Quinn *et al.*, 1982) and transferred to 1/20 normal goat serum for 20 min. Embryos were then incubated with the antibodies at a 1/2 dilution in Medium M2 + BSA + 0.02% sodium azide but without phenol red for 30 min at 20°C. They were washed in Medium M2 + BSA + azide and stained with FITC-goat anti-mouse IgM (1/80 dilution) for 1 h at 20°C. After washing in Medium M2 + BSA + 0.02% azide they were mounted in optically true microslides (Camlab, Cambridge, U.K.).

Monolayer cultures and blastocysts were viewed in a Zeiss or Leitz epi-fluorescence microscope with a mercury source for illumination and excitation filter BP 450–490 and suppression filter LP 515 and photographed.

Statistical evaluation of the results

The endpoint of the tests was the number of blastocysts which were adhesive or had trophectoderm outgrowth compared to the total number of blastocysts in the culture chamber. To examine the possible effect of an oligosaccharide in the culture system a trial always comprised (1) the sugar (or sugars) under test, (2) the medium supplemented with a sugar, which had previously been shown to be without influence (LNF II) and unsupplemented medium. For statistical evaluation we used the Mantel–Haenszel χ^2 test for paired data, and tests having *P* values of <0.01 were regarded as statistically different from controls.

Results

During 72 h culture of the blastocysts in co-culture with monolayers only 2% of the blastocysts were lost.

As the medium supplemented with LNF II did not differ from the other control medium (MEM +) the comparison is restricted to data for which Medium MEM + was the control.

Incubation with 0.1 mM-oligosaccharides

After 24 h of co-culture some of the blastocysts had adhered to the monolayer in medium with and without oligosaccharides. By 48 h outgrowth of trophectoderm was clearly visible. Over 90% of the adhered blastocysts had trophoblast outgrowth 24 h later (after 48 h of co-culture). Blastocysts additional to those found attached at 24 h had also adhered and outgrown by 48 h of co-culture.

There was no significant difference in adhesion or outgrowth between blastocysts in any of the oligosaccharide-containing media and controls after 24 h and 48 h in co-culture. A significant difference ($P < 0.001$) was seen at 72 h in the test with LNF I but not with any of the other oligosaccharides. At 72 h the mean percentage of attachment for LNF I was reduced to 59%, which was 62% of the controls ($P < 0.001$) (Table 3).

Blastocysts incubated with various concentrations of LNF I and LNF III

When blastocysts were incubated with 0, 0.1, 1, 2.5 and 5 mM LNF I, the number of attached blastocysts compared to LNF III, the controls, was significantly reduced ($P < 0.01$) in each case. When the concentration of LNF I in the medium was increased from 0.1 to 1 mM there was a significant decrease in attachment and blastocysts with trophectoderm outgrowth. Increasing the concentration further to 2.5 and 5 mM did not produce any greater effect (Table 4).

Table 3. The effect of oligosaccharides at 0.1 mM on attachment and outgrowth of mouse blastocysts on uterine epithelial monolayers 24, 48 and 72 h in co-culture with endometrial epithelial monolayers

Saccharide	Total no. in test/ total no. in control	Implantation (% attached in test/ % attached in control)			Significance after 72 h
		24 h	48 h	72 h	
2 FL	130/115	38/37	74/61	91/97	NS*
LDFT	91/73	38/37	77/62	76/74	NS
LNT	129/146	35/30	66/66	71/70	NS
LNF I	228/175	21/17	38/51	59/95	$P < 0.001$
LNF II	134/178	37/26	78/72	88/86	NS
LND I	194/162	38/27	74/72	75/81	NS
LNF III	153/160	21/26	60/73	72/86	NS

*NS = not significant.

Table 4. The effect of LNF I and LNF III at 1.0, 2.5 and 5.0 mM on attachment of mouse blastocysts on uterine epithelial monolayers 72 h after co-culture

	LNF	Conc. (mM)				
		0	0.1	1.0	2.5	5
No. attached/total no. (%)	I	154/195 (79)	135/228 (59)	36/76 (47)	56/104 (54)	62/136 (43)
No. attached/total no. (%)	III	84/110 (76)	166/175 (95)	32/34 (94)	39/50 (78)	60/70 (86)
Significance		NS	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$

Morphology

There were no morphological differences detected by light and electron microscopy between attachment sites formed in the presence of any oligosaccharide (including LNF I) and those formed in medium without oligosaccharide (Fig. 1).

Blastocysts incubated with monoclonal antibodies

When blastocysts were incubated for 72 h with Mab 667/9E9 there was a significant ($P < 0.01$) reduction in attachment sites compared to blastocysts incubated with Mab 630/7HI (see Table 5).

Staining of blastocysts and monolayer cultures

Monolayers that had been cultured concurrently with those used to examine implantation were stained with the three monoclonal antibodies (see Table 2) and all three of them bound to the epithelial cells. The majority of cells present were fluorescent after staining with Mab 630/7HI and Mab H001. Binding of Mab 667/9E9 was less intense but at least 50% of the cells in culture were positive for the determinant recognized by this antibody (Fig. 2).

Neither blastocysts, which were expanded but not hatched 48 h after the 2-cell stage (23 embryos examined), nor hatching blastocysts 72 h after the 2-cell stage (44 embryos examined)

Fig. 1. Semi-thin section through a blastocyst adhering to a uterine epithelial monolayer after 3 days of co-culture in the presence of 0.1 mM-LNF I. The appearance of the attachment site is identical to that observed in control medium. Note the confluency of the monolayer. Scale bar = 20 μ m.

Table 5. The effect of monoclonal antibodies against LNF I and LNF III on attachment of mouse blastocysts on uterine epithelial monolayer after 72 h in co-culture

Code	Antibody specificity	No. attached/ total no.	(%)
667/9E9	LNF I	57/149	(38)
630/7H1	LNF III	113/160	(71)
Control	—	93/115	(81)

bound Mab 667/9E9. This was true whether we used blastocysts which had been cultured from the 2-cell stage or flushed directly from the uterus. Morulae (40 embryos) stained with Mab 630/7H1 in parallel acted as positive controls.

Discussion

The present results demonstrate that an oligosaccharide from human milk, lacto-*N*-fucopentaose I, is able significantly to inhibit blastocyst attachment and trophectodermal outgrowth *in vitro*. The oligosaccharide, which is more than 95% pure, was tested in a well defined in-vitro model (Sherman & Wudl, 1976; Kubo *et al.*, 1981; Van Blerkom & Chavez, 1981; Lindenberg *et al.*, 1986). At 0.1 mM the pentasaccharide LNF I, containing fucose $\alpha(1-2)\text{Gal-}\beta(1-3)\text{GlcNAc}$, reduced attachment and

Fig. 2. A monolayer of uterine epithelial cells grown on a coverslip in parallel with those used for blastocyst co-cultures and stained with 667/9E9. Note the punctate cell-surface fluorescence.

trophoblast outgrowth to 62% of that in control medium. This difference was statistically significant ($P < 0.001$) and none of the other 6 related fucosylated and unfucosylated oligosaccharides had any significant effect in this study. The results indicate that the inhibition of attachment and trophoblast outgrowth by LNF I is specific since the other inactive oligosaccharides used were closely related compounds.

The reason that a statistically significant effect of LNF I was not detected until 72 h of co-culture is probably due to the fact that blastocysts are asynchronous. They were pooled from up to 20 female mice and it is well known that embryos from individual females develop asynchronously during the preimplantation period. Further, the lower number of embryos scored as adhering or outgrown at 24 and 48 h co-culture militates against detecting an earlier effect. When adhering blastocysts were marked on the bottom of the dish after 24 h in co-culture, so that they could be identified subsequently, they were always found to give rise to trophoblast outgrowth in all oligosaccharide-containing and control media.

These results suggest that LNF I interferes with the initial adhesion of mouse blastocysts to endometrial monolayers, and consequently outgrowth of trophectoderm is prevented. Those embryos that escaped the influence of LNF I were able to adhere to the monolayers and form normal attachment sites which were ultrastructurally indistinguishable from the controls. Therefore, the presence of LNF I in culture at this concentration has no morphologically recognizable toxic effect on embryos or endometrial cells. Neither was there any reduction in the total number of blastocysts present in the culture system between the beginning and the end of the experiment compared to controls.

The mechanism by which this pentasaccharide acts has yet to be determined. However, there are indications that it may involve a cell surface event. Kimber *et al.* (1988) show that the monoclonal antibody 667/9E9, which recognizes LNF I, binds in frozen sections to the apical surface of the uterine endometrial epithelium during pregnancy. Binding of this antibody is inhibited by LNF I. In the present paper we report that Mab 667/9E9 also binds to the surface of

cells in the endometrial monolayers but not to blastocysts. Moreover, the antibody 667/9E9 was able specifically to inhibit attachment and trophoblast outgrowth of blastocysts on monolayers. It is therefore possible that LNF I is able to inhibit attachment by binding to a cell surface receptor present on the trophectoderm of the mouse blastocyst. We propose that this cell surface receptor normally interacts with glycoconjugates carrying LNF I-like determinants present on the endometrial surfaces. Such glycoconjugates might form part of integral membrane proteins or the glycocalyx of the endometrial epithelium. It has been reported that high molecular weight lactosaminoglycans are a major class of the cell surface glycoconjugates synthesized by epithelial but not stromal cells of the mouse uterus (Dutt *et al.*, 1987). Dutt *et al.* (1987) were able to inhibit cell-cell and cell-substrate adhesion in primary cultures of mouse uterine epithelium with alpha-lactalbumin or UDP-Gal, which they interpret to indicate that cell adhesion may depend on alpha galactosyltransferase activity. However, these substrates had no effect on attachment and trophoblast outgrowth of blastocysts on the primary cultures. Conversely, LNF I used in this study had no effect on the cell-cell or cell-substrate adhesion of the uterine epithelial cells in culture. Therefore, cell adhesion between uterine epithelial cells and between the uterine epithelium and the blastocyst clearly relies on quite different mechanisms even if related oligosaccharide chains may be involved.

Since antibody binding studies show that neither LNF I (this study) nor the related H type 2 structure (Fenderson *et al.*, 1986) are detectable on the trophectoderm surface of the blastocysts, the interaction of uterine LNF I with trophectoderm presumably involves heterophilic rather than homophilic binding. How far such a mechanism is applicable to other species has yet to be determined, but fucosylated cell surface components have been identified in the endometrium of pregnant domestic animals (Whyte & Robson, 1984; Whyte & Allen, 1985) and women (Bychkov & Toto, 1986). There is also considerable supporting evidence for both quantitative (Enders & Schlafke, 1974; Chavez & Anderson, 1985) and qualitative (Hewitt *et al.*, 1979; Chavez & Anderson, 1985) changes in the endometrial glycocalyx of the mouse preceding the period of implantation, which may reflect the new expression or rearrangement of glycoconjugates at the interphase between blastocysts and endometrial cells. Such changes could contribute to the reported temporal restriction in the receptivity of the uterus (McLaren & Michie, 1956; Finn & Martin, 1974; Psychoyos & Casimiri, 1980) controlling blastocyst implantation. There are also changes in the cell surface of the trophectoderm (Jenkinson & Searle, 1977; Chavez & Enders, 1981; Carollo & Weitlauf, 1981; Chavez, 1986) around the time of implantation which presumably include those directed towards adhesion of the blastocysts to the endometrium. Antibodies recognizing a group of these glycoproteins, which appear to be involved in cell-substratum adhesion for other cell types, have been demonstrated to inhibit outgrowth of mouse trophoblast *in vitro* (Richa *et al.*, 1985). However, in this study outgrowth on tissue culture plastic was investigated. The relationship of outgrowth on this substrate to the normal complex cell-cell interaction of blastocyst-uterine apposition and adhesion *in utero* is still unclear.

We were never able to obtain total inhibition of adhesion and trophoblast outgrowth using physiological concentrations of oligosaccharides, although we observed a significantly better inhibition using higher concentrations of the oligosaccharide (Table 4). This may reflect the fact that the structure on the endometrium that carries the LNF I-like determinant is a more complex entity than the pentasaccharide used in this study. To investigate this we are currently evaluating the effect of multivalent LNF I conjugates in our *in-vitro* system.

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