Original article

Effect of immunisation against leukaemia inhibitory factor on the establishment of pregnancy in sheep

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Summary — Leukaemia inhibitory factor (LIF), a pleiotropic cytokine, is implicated in blastocyst implantation in mice and maintains the development of ovine embryos in culture. Previously, LIF mRNA and protein were demonstrated in the endometrium throughout the oestrous cycle and early pregnancy in the ewe. In this study pregnant ewes were passively immunised against human recombinant LIF with polyclonal antibodies raised in cows by active immunisation. Ewes were immunised during two stages of early pregnancy: blastocyst development to hatching, and blastocyst elongation to implantation. Only animals passively immunised against LIF showed detectable LIF antibodies in their sera and in uterine lumina flushings by radioimmunoassay and Western blot analysis. Pregnancy was confirmed by ultrasound on day 55 and a 33.5% non-significant decrease in pregnancy rate of anti-LIF treated animals was observed, when compared to animals in control groups (untreated or treated with bovine anti-keyhole limpet hemocyanin). Cows actively immunised with recombinant human LIF and exhibiting high levels of LIF antibodies in their sera at the time of blastocyst implantation also showed a reduced pregnancy rate in comparison to control animals. Although LIF may not be obligatory for implantation in ruminants it does appear to have a role during the establishment of pregnancy.

LIF / immunisation / early pregnancy / implantation

Résumé — Effet de l'immunisation contre le LIF sur l'établissement de la gestation chez la brebis et la vache. Le rôle du LIF dans l'implantation chez la souris et dans le développement du blas-

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tocyste ovin est bien établi. Les messagers codants pour le LIF et cette protéine sont présents dans l'endomètre de la brebis cyclique et en début de gestation. Ce travail rapporte les effets de l'immunisation passive contre le LIF chez des brebis gestantes. Du sérum de vaches immunisées activement contre le LIF a été injecté au moment de l'éclosion du blastocyste et entre l'élongation du blastocyste et l'implantation. Des anticorps anti LIF ont été détectés dans le sang (RIA) et dans des flushings utérins (Western Blot) des femelles immunisées. Par rapport aux contrôles, il a été observé une réduction du taux de gestation de 33.5% (échographie à j 55), non significative, chez les femelles immunisées. De même, les vaches immunisées activement contre le LIF ont présenté un taux de gestation réduit par rapport aux contrôles. Le LIF paraît donc jouer un rôle dans l'établissement de la gestation même s'il n'est pas obligatoire.

LIF / immunisation / début de gestation / implantation

INTRODUCTION

Leukaemia inhibitory factor (LIF) is a pleiotropic cytokine that acts on many different cells (Hilton 1992; Metcalf 1992). When mouse blastocysts were cultured in the presence of LIF they had a greater mass of trophectoderm in comparison to control blastocysts (Robertson et al, 1991) and LIF significantly increased the number of embryos hatching and exhibiting trophoblast outgrowth (Lavranos et al, 1995). Furthermore, recombinant human LIF improved the development of ovine embryos in culture (Fry et al, 1992) indicating that LIF may be one of many factors involved in improving embryo viability during sustained in vitro culture.

In the mouse uterus, LIF is expressed in the endometrial glands coincident with the time of blastocyst implantation (Bhatt et al, 1991) and mice that lack a functional LIF gene have normal blastocysts that fail to implant (Stewart et al, 1992). Furthermore, the low affinity LIF receptor (LIFR) is an integral component of the functional LIF receptor complex and interacts with gp130 to form a high affinity receptor (Ip et al, 1992; Taga and Kishimoto, 1992; Gearing et al, 1992; Davis et al, 1993). Disruption of LIFR leads to perinatal death as mice homozygous for the mutation die shortly after or during birth (Ware et al, 1995), while

embryos homozygous for the gp130 mutation die between 12.5 days postcoitum and term (Yoshida et al, 1996). In both cases normal implantation of embryos occurs, suggesting that endometrial LIF may not act directly through LIFR or gp130 on the blastocyst. Since both mutations are lethal their effects in adult mice cannot be investigated.

In human endometrium, LIF mRNA is present transiently with the highest expression observed during the mid and late secretory phases of the menstrual cycle (Charnock-Jones et al, 1994; Kojima et al, 1994; Arici et al, 1995; Vogiagis et al, 1996a; Cullinan et al, 1996). Immunoreactive LIF has been localised in the endometrial epithelium and stroma throughout the menstrual cycle (Vogiagis et al, 1996a), indicating that LIF may have a role in endometrial function. We have demonstrated previously that LIF mRNA and protein are present in the ovine uterus during early pregnancy (Vogiagis et al, 1996b), although these are not confined to the time of blastocyst implantation as in the mouse (Bhatt et al, 1991).

The sheep, unlike the human and mouse, has a characteristically long intrauterine blastocyst development before implantation (Bindon, 1971). Whereas in the mouse the blastocyst hatches from the zona pellucida and implants on the uterine wall on the

fourth day of pregnancy (Finn and McLaren, 1967; Orsini and McLaren, 1967), in the sheep hatching and implantation occur about eight days apart (Bindon, 1971; Rowson and Moor, 1976). Thus the effects of LIF on preimplantation growth, development and subsequent implantation of the ovine blastocyst can be investigated.

In this study, we passively immunised pregnant ewes against LIF in an attempt to temporarily reduce or block the amount of LIF available to target cell receptors of the uterus and/or embryo. Two important stages of early pregnancy were examined: 1) blastocyst development to hatching; 2) blastocyst elongation to implantation. The large quantities of polyclonal antibodies against LIF utilised in the passive immunisation study, were obtained by actively immunising pregnant cows against LIF and collecting colostral immunoglobulin G (IgG). As a secondary objective, the effect of active immunisation against LIF on embryo implantation in these cows was assessed by re-immunising and re-mating the animals to boost their antibody levels during the early stages of a second pregnancy.

MATERIALS AND METHODS

All animal experimentation was approved by the animal ethics committee at the Victorian Institute of Animal Science, Werribee.

Active immunisation of cows with LIF

Pregnant Friesian cows were actively immunised with either human recombinant glycosylated LIF from Chinese hamster ovary [CHO] cells (AMRAD Pharmacia Biotech; $n = 3$), or keyhole limpet hemocyanin (KLH, control; $n = 2$), which was emulsified in Freund's complete or incomplete adjuvant. Animals received a 50 µg dose at 10 (sub-cutaneously, behind the ear), 6 and 2 (intramuscular, hind leg) weeks prior to calving. Blood samples were collected from the tail vein prior to each immunisation and were assayed for anti-LIF. Colostrum was harvested after calving.

Purification and preparation of bovine colostral IgG for immunisation

Bovine colostral IgG was isolated by ammonium sulphate precipitation (Fang and Mukkur, 1976) then reconstituted (67 mg/mL, saline, 0.01% sodium azide) and centrifuged. The supernatant was filtered (Suporcap-50, 0.45 µm), dialysed (phosphate buffered saline [PBS] 3 days, 4 °C) and filtered (Acrocap 0.2 µm; Gelman Sciences, MI, USA). Injection material administered to sheep at days 12–16 was purified further to remove possible endotoxin using Affi-Prep polymixin beads (Bio-Rad, CA, USA).

Western blot analysis

Human recombinant non-glycosylated LIF (50 ng, AMRAD) was subjected to polyacrylamide gel electrophoresis on a 12% gel and the protein transferred to nitrocellulose. Non-specific binding was blocked with 5% ovalbumin (Sigma) and the filter probed with bovine serum (collected before third immunisation of LIF or KLH; 1:5000). Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-bovine IgG using a chemiluminesence kit (Amersham International).

LIF radio-immunoassay

Radio-iodinated recombinant human non-glycosylated LIF (^{125}I-LIF) was provided by Dr DJ Hilton (Walter and Elisa Hall Institute, Melbourne, Australia). LIF immunoassay was established to test antibody binding to ^{125}I-LIF. Samples were diluted in PBS (with 0.1% [v/v] Triton X-100, 0.1% [w/v] BSA, 0.02% [w/v] sodium azide; Sigma), assayed in duplicate with ^{125}I-labelled LIF 10 000 counts per minute (4 days; 4 °C). Separation of bound from free ^{125}I-LIF was achieved by donkey anti-sheep serum (1:160, 1 h) before addition of 3.3% PEG and 0.05% NaCl (1 h; 4 °C). Tubes were centrifuged (4 000 rpm, 30 min, 4 °C) and the pellet was counted using a gamma counter. Non-specific binding was performed for each sample using PBS.

LIF bioassay

The biological activity of the bovine anti-human-LIF antibody was assessed in a bioassay using

murine myeloid leukaemic (M1) cells and performed by Dr DJ Hilton. In the absence of LIF, the M1 cells proliferate while in the presence of LIF, the cells differentiate into macrophages. M1 cells were cultured in agar with 500 U/mL of E coli derived non-glycosylated human LIF (tenfold higher than half maximal dilution). Doses of the antibody, (0-50 µg/mL) in two-fold dilutions were added to cultures. The assay was performed twice.

Passive immunisation of ewes with LIF antibodies

The oestrous cycles of 100 parous Corriedale ewes were synchronised as described (Salamonsen et al, 1986). Ewes were mated at the subsequent oestrus and divided into five groups: group C1: untreated controls (n = 24); groups L1 and L2: treated with anti-LIF IgG (n = 19/group); and groups K1 and K2: treated with anti-KLH IgG (n = 19/group). Passive immunisation coincided with either blastocyst development to hatching (days 6–10; groups L1 and K1) or blastocyst elongation to implantation (days 12–16; groups L2 and K2).

Passive immunisation

Ewes received 30 mL (67 mg/mL of IgG; 3 min) administered into the jugular vein. Second and third injections of 15 mL were similarly administered at 2-day intervals.

Blood sampling and testing of antiserum levels

Blood samples were collected: 15 min after first injection, prior to the second, third injections and every alternate day up to day 35. Undiluted serum was assayed.

Confirmation of pregnancy

Ewes were run with fertile rams bearing crayon mating markers and checked daily to determine which returned to service. Ultrasounds were performed at day 55.

Collection of tissue and uterine flushings

Six animals from control (C1 and K1) groups that had returned to service were used for this second experiment. Three were passively immunised with anti-LIF (days 6–10), while three animals, were re-injected with anti-KLH (days 6–10). Animals were killed (day 12), their uteri flushed and the presence of a blastocyst was determined microscopically. Uterine flushings were concentrated 20-fold (Centricon-10 tubes Micron, MA, USA) and tested for anti-LIF by radioimmunoassay.

Statistical analysis

Logistic regression analysis was used to test which factors: 1) condition (immunised or non-immunised), 2) treatment type (anti-LIF, anti-KLH or untreated) or 3) schedule (immunised; days [6–10] or [12–16]) significantly ($P < 0.05$) reduced the pregnancy rate.

Active immunisation of cows with LIF: effect on early pregnancy

Immunisation and synchronisation of the oestrous cycle were performed, so that anti-LIF titres would be maximal around the time of implantation (fig 1). Cows actively immunised with LIF and KLH (experiment 1, 12 months earlier), were subjected to a booster injection of 50 µg of LIF or KLH in Freund's incomplete adjuvant. Cows had their oestrous cycles synchronised and were artificially inseminated at 12 and 24 h after oestrus. Blood was sampled (fig 1) and diluted serum (1:2000) binding to ^{125}I-LIF was determined. Ultrasounds were performed on day 36.

RESULTS

Active immunisation of cows with LIF

Western blot analysis (fig 2) demonstrated that by third injection, all cows actively immunised with LIF had developed antibodies (bovine anti-human LIF antiserum), which recognised human LIF (approximately 20 kDa). Control animals showed
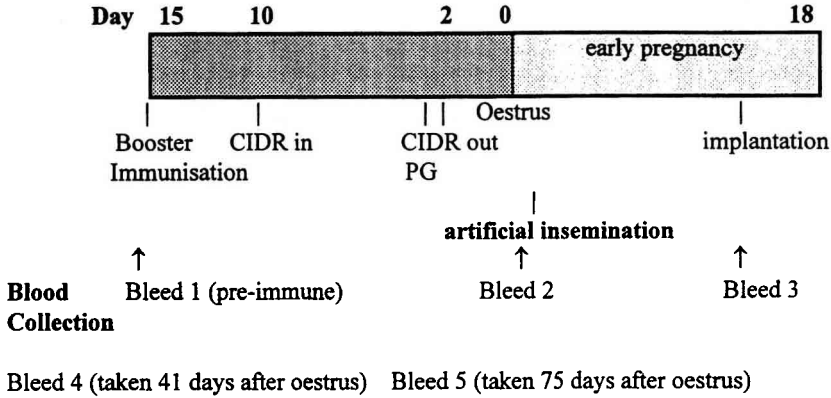
Fig 1. Diagram showing the sequence of events involved in synchronisation of the bovine oestrous cycle, artificial insemination and active immunisation with LIF or KLH of cows prior to early pregnancy.
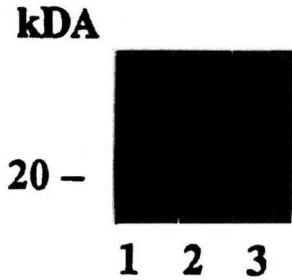

Fig 2. Western blot analysis. Lanes 1, 2 and 3 represent sera from cows 1, 2 and 3, respectively, which recognise human recombinant non-glycosylated LIF (20 kDa).

Table I. Activity of bovine anti-human LIF antibody in LIF bioassay using M1 cells.

[Antibody] (µg/mL)	Experiment 1		Experiment 2	
	No colonies undifferentiated	No colonies differentiated	No colonies undifferentiated	No colonies differentiated
0	0	8	0	13
1.62	0	7	0	11
3.12	0	13	0	23
6.25	11	82	0	34
12.5	41	49	58	54
25	92	16	106	51
50	97	0	134	0

Bioactivity of the antibody was assessed by its ability to inhibit the LIF induced differentiation of cultured M1 cells.

no binding. When this antibody was combined with LIF at 50 µg/mL (table I) it inhibited the ability of LIF to induce the differentiation of M1 colonies. The activity of the antibody was half maximal at about 12.5 µg/mL. The antibody had no inhibitory effect below 6.25 and 12.5 µg/mL, in experiment 1 and 2, respectively.

## Passive immunisation of ewes with LIF antibodies

During the passive immunisation, seven animals died (six immunised with anti-LIF and one with anti-KLH). Post-mortem examinations (University of Melbourne, School of Veterinary Science) indicated that the deaths may have been due to toxic shock. In view of the possibility that endotoxins may have been present in the IgG preparations, IgG awaiting injection on days 12–16 was treated to remove endotoxin. However, four of the seven deaths occurred subsequently.

## ^{125}I-LIF binding to sera of passively immunised ewes

All ewes receiving anti-LIF IgG had measurable serum titres of LIF antibody while all pre-immune sera and sera of animals injected with anti-KLH contained no detectable anti-LIF. Data for four ewes in groups L1 and L2 and one in each of groups K1 and K2 are represented in figure 3. All animals injected with anti-LIF had measurable levels in serum until at least day 35. Bleeds taken prior to the secondary and tertiary injection usually showed a slight increase in LIF antibody titre compared to those taken 15 min after the primary injection. Both groups of animals (fig 3a, b) showed the highest anti-LIF titres 6 days after the tertiary injection, on day 16 (fig 3a) and day 22 (fig 3b), respectively. By day 22 (fig 3a) and day 35 (fig 3b) anti-LIF titres began to decrease.

^{125}I-LIF binding in uterine luminal flushings of passively immunised ewes

^{125}I-LIF binding was demonstrated in uterine luminal flushings of ewes injected with antiserum in the second phase of experiment 2 and killed on day 12 of pregnancy (fig 4). Flushings from animals immunised
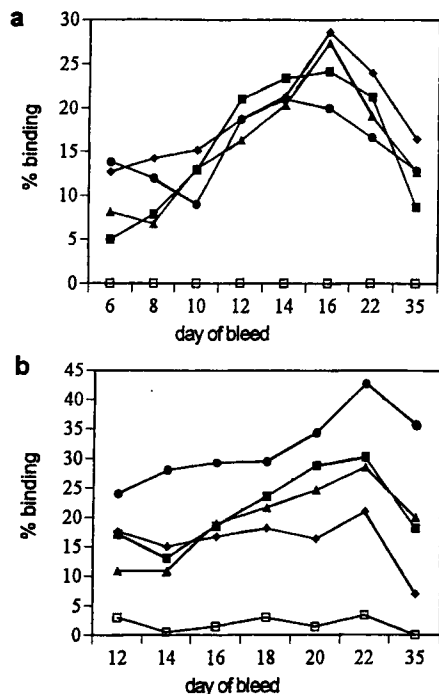
Fig 3. Binding of ^{125}I-LIF to serial serum samples from individual ewes, between the day of the first injection of anti-LIF and day 35 of pregnancy. Data is expressed as % specific binding, (a) animals injected on days 6, 8 and 10 of pregnancy, (b) animals injected on days 12, 14 and 16 of pregnancy. Shaded boxes = four representative animals injected with anti-LIF, and open boxes = one representative animal injected with anti-KLH.
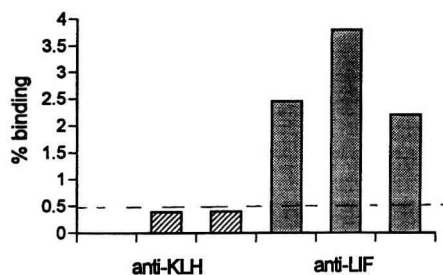
Fig 4. Binding of ^{125}I-LIF to anti-LIF in uterine flushings of individual animals receiving anti-LIF and anti-KLH on day 12 of pregnancy. Specific binding of antibody is shown above the dotted line.

Table II. Pregnancy rates following passive immunisation of pregnant ewes against LIF and KLH.

Group	Total/group	Total pregnant	Total non-pregnant	Pregnancy rate
C1 Control	24	15	9	62.5%
L1 Anti-LIF	16	7	9	40.6%
L2 Anti-LIF	16	6	10	
K1 Anti-KLH	18	11	7	59.5%
K2 Anti-KLH	19	11	8	

C1 = untreated ewes; L1 = anti-LIF treated ewes on days 6–10; L2 = anti-LIF treated ewes on days 12–16; K1 = anti-KLH treated ewes on days 6–10; K2 = anti KLH treated ewes on days 12–16.

with anti-LIF showed approximately 3% specific binding to iodinated LIF compared with a mean binding of 0.27% to flushings from three animals injected with anti-KLH.

Comparison of pregnancy rates in passively immunised ewes

Pregnancy rates in the groups of ewes are given (table II). There were no significant differences between groups L1, L2 or K1, K2, data were pooled for final analysis. Anti-LIF animals showed a 33.5% decrease

in pregnancy rate, compared with control and anti-KLH groups, but this was not statistically significant ($P > 0.05$).

Active immunisation of cows with LIF: effect on early pregnancy

The effect of active immunisation against LIF was assessed during early pregnancy in the cow. Binding to ^{125}I-LIF was detected in serum of animals actively immunised with LIF (fig 5) and antibody levels were elevated in all three cows at the
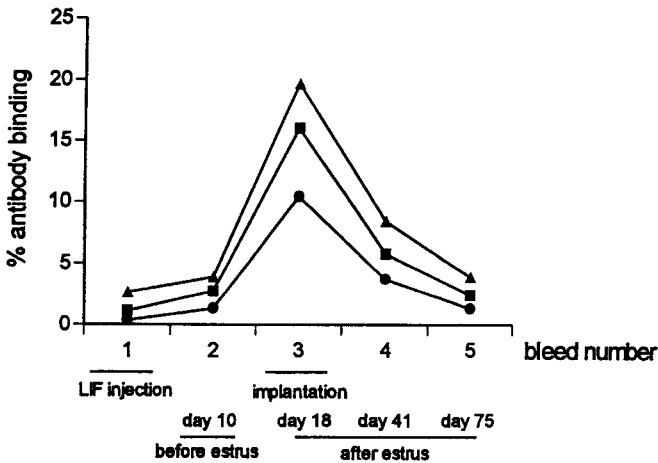
Fig. 5 Percentage binding of ^{125}I-LIF to cow sera following booster injections to actively immunised cows prior to artificial insemination. The bleed numbers correspond to those shown in figure 1 and in the text, all sera were diluted 1:2000. ■ cow 1, ● cow 2, ◆ cow 3.

expected time of implantation. No binding was detected in serum of the cows immunised with KLH. Two of the animals actively immunised with LIF were not pregnant, whereas all three control animals were pregnant.

DISCUSSION

A decrease in pregnancy rate in ewes that were passively immunised against LIF was observed in comparison to animals in control groups, although this was not statistically significant. Furthermore, cows actively immunised with recombinant human LIF and exhibiting high levels of LIF antibodies in their serum at the time of blastocyst implantation also showed a reduced pregnancy rate in comparison to control animals.

Purified bovine IgG, which was shown to be biologically active owing to its ability to inhibit LIF induced differentiation of cultured M1 cells, was injected into each ewe. Anti-LIF was detected in serum as early as 15 min after the primary injection into the jugular vein, but the highest antibody titres occurred 6 days after the final injection. This could indicate that anti-LIF may be sequestered within tissues, possibly by binding to its ligand, and slowly released back into the circulation. When LIF binds in vitro to cells containing high affinity receptors (bone marrow, resident peritoneal cells, hepatocytes and M1 cells) less than 50% dissociation occurs after 20 h at 4 °C, while dissociation of LIF from transgenic peritoneal cells is biphasic with a rapid ($t^{1/2}$ = 2 min) and a slow component ($t^{1/2}$ > 20 h) corresponding to the numbers of low and high affinity receptors, respectively (Hilton et al, 1992). In this study the highest concentration of anti-LIF binding to ^{125}I-LIF in serum occurred at least 4 days after the final immunisation, while studies in vitro have indicated that the half-life of LIF bound to high affinity LIF receptors in certain cells is about 1 day at 4 °C. Locally produced

LIF and injected anti-LIF may be complexed in tissues where there are high local concentrations of the LIF ligand, and as anti-LIF is released from these complexes, it may re-enter the circulation and become accessible to bind to ^{125}I-LIF in the assay.

The important question in passive immunisation studies is whether or not the antiserum binds the antigen to a sufficient extent to prevent its action. In the present study where production and actions of LIF are within the embryo-endometrial axis, it was important to show that the antiserum reaches the uterine lumen. In this study, anti-LIF was detected in the uterine flushings of animals treated with anti-LIF, but not in control animals. The similar rates of pregnancy between the two groups injected with LIF antibodies suggest that in the sheep LIF may be involved in both blastocyst development and implantation, but may not be obligatory for either process. Animals passively immunised with KLH antibodies showed the same pregnancy rate as untreated ewes, indicating that the immunisation regimen itself, was not the cause of the decrease in pregnancy. Previous studies have also demonstrated that specific antibodies can be detected in the uterus following passive immunisation. Parr and Parr (1986) demonstrated that IgG in mouse serum is taken up by uterine luminal and glandular epithelial cells (Wang et al, 1989). Furthermore, serum proteins selectively enter the uterine lumen. Sheep uterine flushings contain a large number of serum proteins, which include IgG (Salamonsen et al, 1984).

During the passive immunisation six ewes all immunised with anti-LIF and one immunised with anti-KLH died within 24 h of antibody administration. Both sets of IgG preparations were raised and purified under identical conditions. Therefore the greater mortality in the anti-LIF treated groups suggests a direct toxic effect of anti-LIF by binding to locally produced LIF; a diverse range of cells bind and/or are

affected by LIF (Hilton, 1992; Metcalf, 1992).

When cows that had been actively immunised with LIF or KLH had their antibody levels boosted during the early stages of pregnancy, the pregnancy rate of the LIF immunised animals was reduced with only one in three cows pregnant in comparison to all cows ($n = 3$) pregnant in the control groups. These preliminary findings indicate that LIF may play a role in early pregnancy in the cow. Active immunisation studies with larger numbers of animals were not possible owing to the quantity of antigen required and the cost of the animals.

Passive immunisation against LIF during early pregnancy did not prevent 60% of ewes from establishing that pregnancy. One possible explanation is that the actions of uterine LIF were not completely blocked in some animals, thus allowing embryonic development and implantation to take place. Alternatively, as the extent of implantation varies between species (Wooding and Flint, 1994), physiological differences could explain why LIF is not obligatory for implantation in ruminants. Furthermore, other cytokines such as ciliary neurotrophic factor (CNTF) and oncostatin M (OSM), which are related to LIF and utilise the signalling system involving the LIF receptor complex (LIFR and gp130), may function when LIF is absent or reduced in the uterus during early pregnancy (Ip et al, 1992; Taga and Kishimoto, 1992; Gearing et al, 1992; Davis et al, 1993). To date, these cytokines have been demonstrated in the human, however sheep and cow tissue have not been examined. Overall, these results suggest that while LIF may have a role in embryo development, implantation and pregnancy in ruminants, it is not critical as it appears in the mouse. Further, they highlight the possibility that LIF may have different or lesser roles in these processes in different species.

ACKNOWLEDGMENTS

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