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Effect of interleukin-3 on ovine trophoblast interferon during early conceptus development

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The effect of interleukin-3 (IL-3) on conceptus production of ovine interferon tau (oIFNτ) was examined using two dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D SDS-PAGE), western blot, northern/slot blot, *in situ* hybridization and immunohistochemical methodologies. Ovine conceptuses (day 17, $n = 14$) were cultured *in vitro* for 24 h in Eagle's Minimum Essential Medium (MEM) containing various doses of human recombinant interleukin-3 (hIL-3). At 75 and 150 colony forming units (units)/ml hIL-3, amounts of oIFNτ released into the culture media increased 3-8-fold over controls while at 300 units/ml (60 pM), hIL-3 did not enhance oIFNτ production. These variations in production of oIFNτ paralleled amounts of oIFNτ mRNA detected by northern and slot blot analyses. Ovine IL-3 mRNA was primarily localized at luminal and glandular epithelia with minor staining at stromal regions of uterine endometria collected from day 17 pregnant ewes but its polypeptide was localized in the luminal epithelial region. However, such signals were not detected in day 17 conceptuses. Ovine IL-3 mRNA and its polypeptide were also localized at the same regions of the endometrium obtained from cyclic ewes on days 8 and 12. The effect of hIL-3 on the enhancement of oIFNτ production *in vitro* could be mimicked by the addition of phorbol 12-myristate 13-acetate (PMA). The present observations demonstrate that the hemopoietic cytokine, IL-3, stimulates conceptus production of oIFNτ *in vitro* and that ovine IL-3 and its mRNA are localized in the maternal endometrium. These results suggest that a cytokine, IL-3, produced by the maternal endometrium, is involved in conceptus production of oIFNτ which may result from the activation of a second messenger system, protein kinase C.

Keywords: ovine; endometrium; IL-3; conceptus; IFNτ; PKC

Introduction

Normal progression of early conceptus development which leads to successful implantation requires concomitant hormonal and metabolic adjustment by the mother. Ovine trophoblast interferon (oIFNτ), formerly recognized as ovine trophoblast protein-1 (oTP-1), is implicated as a signal that proceeds the process of implantation (Martal *et al.*, 1979; Godkin *et al.*, 1982). However, physiological and developmental signals that regulate conceptus production of oIFNτ are not clearly understood.

There is ample evidence that the conceptus may benefit from the local abundance of inflammatory cytokines and a role for endometrial cytokines in maternal-fetal communications has been proposed (Armstrong & Chaouat, 1989; Brown *et al.*, 1989; Paria & Dey, 1990; Simmen & Simmen, 1991). These cytokines include epidermal growth factors (EGF, Brown *et al.*, 1989), heparin-binding EGF-like growth factor (Das *et al.*, 1994), granulocyte-colony stimulating factor (G-CSF; Nicola *et al.*, 1979), granulocyte macrophage-colony stimulating factor (GM-CSF; Wegmann *et al.*, 1989; Robertson *et al.*, 1992), macrophage-colony stimulating factor (M-CSF or CSF-1; Pollard *et al.*, 1987), and interleukin-6 (IL-6, Robertson *et al.*, 1992). Recently, GM-CSF was found to promote production of oIFNτ *in vitro* and its mRNA was localized at luminal and glandular epithelia of the uterus (Imakawa *et al.*, 1993).

Interleukin-3 (IL-3), a potent hemopoietic growth factor, stimulates proliferation and differentiation of various lineages of hemopoietic cells (Schrader, 1986). Both IL-3 and GM-CSF have shown cross competition for high affinity binding sites in human hemopoietic cell lines (Gesner *et al.*, 1988). Although polypeptide structures of GM-CSF and IL-3 are different, their receptors consist of α and βc subunits and their βc-subunits share the same amino acid composition (Kitamura *et al.*, 1991). The α subunit binds to its ligand with low-affinity and is unable to stimulate a signal. In association with the βc subunit, a high-affinity receptor capable of signal transduction is formed (Kitamura *et al.*, 1991). Despite the fact that these receptors lack an intrinsic tyrosine kinase activity, one of the earliest events to occur after IL-3 or GM-CSF stimulation is the induction of protein tyrosine phosphorylation (Isfort & Ilhe, 1990; Kanakura *et al.*, 1990). Several tyrosine kinases have been reported to become activated in IL-3 or GM-CSF sensitive cells (reviewed by Mui & Miyajima, 1994).

Through the activation of protein kinase C (PKC), IL-3 and GM-CSF increase the expression of nuclear protooncogenes, c-*fos* and c-*jun* (Adunyah *et al.*, 1991; Garland *et al.*, 1992). These nuclear proteins form a heterodimer which interacts with a transcriptional enhancer element, AP-1, resulting in the enhancement of gene transcription (Bohmann *et al.*, 1987). One of oIFNτ genes, the predominant oIFNτ expressed between day 13 and 20 of gestation in sheep, contains an AP-1 site in the 5'-flanking region (Nephew *et al.*, 1993) and the amounts of c-*fos* mRNA parallel that of oIFNτ during the same period (Xavier *et al.*, 1991). These results suggest that the production of oIFNτ by the trophoblast during pregnancy establishment are mediated at least in part by the activation of PKC which in turn enhances the expression of nuclear protooncogenes, c-*fos* and c-*jun*.

Although conceptus production of oIFNτ as an antiluteolysin is well studied, molecular events that regulate oIFNτ expression have not been elucidated. More importantly, physiological events that take place at the endometrium during the period of oIFNτ expression have not been well characterized. Previously this laboratory has demonstrated that when various cytokines and growth factors are examined, only GM-CSF enhances oIFNτ production *in vitro* (Imakawa *et al.*, 1993). Because of the structural similarities between GM-CSF and IL-3 receptors, it is suspected that IL-3 may promote production of oIFNτ. Objectives of this study were to determine the effect of hIL-3 on oIFNτ expression *in vitro* and to localize ovine IL-3 polypeptide and mRNA at maternal-fetal compartments dur-

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ing the period of early conceptus development. Attempts were also made to determine if the activation of protein kinase C enhanced oIFNτ production.

Results

Effect of hIL-3 on oIFNτ mRNA

Amount of DNA in each tissue mass (approximately one quarter of a conceptus/culture dish) was similar ($P>0.1$) among treatment groups and doses (Table 1). Therefore, various doses of hIL-3, hGM-CSF or PMA did not affect DNA contents of tissue masses during the 24 h culture period. In addition, amount of RNA extracted from each conceptus tissue averaged 109 ± 7 μg. Correlation coefficients for standard curves in slot blot analyses averaged 0.991 ± 0.004. Northern and slot blot analyses of RNA extracted from day 17 conceptuses revealed that maximum increases in oIFNτ mRNA were detected with 75 units hIL-3 (Figure 1) and 150 units hGM-CSF (Table 2). Conceptuses cultured with PKC activator, phorbol 12-myristate 13-acetate (PMA), had enhanced oIFNτ mRNA levels.

Effect of hIL-3 on oIFNτ production in vitro

Tissue masses of cultured conceptuses as determined by DNA contents and protein concentrations as measured by nondialyzable radioactivity in each culture media sample were similar ($P>0.1$; Table 1). Therefore, amount of oIFNτ released into the media during a 24 h culture period was comparable. Figure 2 shows fluorographs of 2D SDS–PAGE consisting of nondialyzable macromolecules released by day 17 conceptus tissues. When spots corresponding to oIFNτ on fluorographs and radioactivities of oIFNτ on dried 2D SDS–PAGE were analysed, both densitometric and [^{3}H]-leucine concentration values, respectively, were highly correlated ($r = 0.986$, $P<0.002$). Therefore, amounts of oIFNτ produced by conceptus tissues that were cultured with various doses of hIL-3, hGM-CSF or PMA was expressed as radioactive counts (Figure 3). *In vitro* oIFNτ production was enhanced by the addition of 75 units/ml hIL-3 (Figure 3). Increase in oIFNτ production detected by 2D SDS–PAGE was confirmed by western blot analysis using recombinant oIFNτ and oIFNτ antiserum (Figure 4). Level of oIFNτ released into the culture media during a 24 h period, when incubated with 75 units/ml hIL-3, was determined to be 74 ± 6 μg/7 ml culture ($n = 3$). When hIL-3 (75 units/ml) and hGM-CSF (75 units/ml) were analysed within the same experiment for their ability to stimulate oIFNτ production *in vitro*, hGM-CSF was not as effective as hIL-3 in the stimulation of oIFNτ (Figure 3). In addition, there were no additive effects on oIFNτ production when combinations of hIL-3 and hGM-CSF were tested (Figure 3). Conceptuses cultured with PMA also had enhanced oIFNτ production as analysed by western blot and 2D SDS–PAGE (Figure 3).

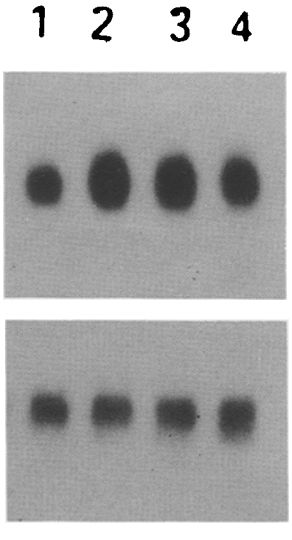

Figure 1 Representative northern blot analysis of oIFNτ mRNA in day 17 conceptus tissues after 24 h culture. Ten μg RNA were subjected to the analysis ($n = 24$, 4 doses × 6 replicates). Top panel: 1, control no hIL-3; 2, culture treated with 75 units/ml (15 pM) hIL-3; 3, 150 units/ml hIL-3; 4, 300 units/ml hIL-3. Bottom panel: Northern blot analysis with γ-actin probe (Gunning *et al.*, 1983)

Localization of ovine IL-3 polypeptide and mRNA

An antipeptide antiserum prepared from a portion of ovine IL-3 amino acid sequence identified the presence of the polypeptide at the luminal epithelial region of uterine endometria (Figure 5A). Specificity of this antibody was confirmed by a western blot analysis using culture media and hIL-3. In addition, normal rabbit IgG used in place of the antipeptide antiserum did not give any signals when serial sections of the same tissues were examined. For the *in situ* hybridization, probe specificity was provided by the observations that sense ovine IL-3 cRNA or tRNA substitution did not provide any signals when uterine endometria were examined. Antisense ovine IL-3 cRNA bound primarily to the luminal and glandular epithelia (Figure 5B) and weakly to stromal cells. Ovine IL-3 mRNA was not localized at the trophoblast cells of day 17 conceptuses (Figure 5F), suggesting that oIFNτ production *in vivo* is regulated by maternal IL-3 in a paracrine manner. In days 8 and 12 cyclic ewes, ovine IL-3 polypeptide and mRNA were also localized at the same regions of the endometria when compared to those of day 17 pregnant ewes (Figure 5E).

Discussion

Coordination of conceptus development and endometrial preparation during the preattachment period is believed to be necessary for successful implantation. Although these physiological events are mediated by ovarian estrogen and pro-

Table 1 DNA content and nondialyzable radioactivity of conceptus tissues and culture media after 24 h[a]

Doses[b]	*DNA Content μg/200 mg tissues*	*Nondialyzable radioactivity DPM × 10⁶/200 mg tissue/7 ml MEM*
1	46.3±5.7	5.3±0.8
2	43.5±3.0	5.1±0.9
3	46.0±4.2	4.9±0.7
4	51.7±8.5	5.2±0.9

[a]Mean ± SE, values within a column were not different ($P>0.1$). [b]There are four treatment groups; IL-3 ($n = 6$), GM-CSF ($n = 2$), PMA ($n = 2$) and a combination of IL-3 and GM-CSF ($n = 2$). Each treatment consists of four doses of the treatment. IL-3 or GM-CSF treatment: Dose 1, 0 units/ml; dose 2, 75 units/ml; dose 3, 150 units/ml; dose 4, 300 units/ml. PMA treatment: Dose 1, 0 nM; dose 2, 0.1 nM; dose 3, 1 nM; dose 4, 10 nM. IL-3/GM-CSF treatment: Dose 1, 0 units/ml; dose 2, 75 units/ml IL-3 only; dose 3, 75 units/ml GM-CSF only; dose 4, 75 units/ml each of IL-3 and GM-CSF

Table 2 Amounts of oIFNτ mRNA (pg/μg RNA) determined by slot blot analysis[a]

Doses[b]	*IL-3* n = 5	*GM-CSF* n = 1	*PMA* n = 2	*IL-3/GM-CSF* n = 2
1	72 ± 9	46	65 ± 22	95 ± 14
2	157 ± 7*	105	83 ± 26	139 ± 0
3	77 ± 14	145	147 ± 13*	109 ± 0
4	68 ± 9	66	92 ± 51	160 ± 49

[a]Mean ± SE, *$P<0.05$ *vs* dose 1. [b]See Table 1 for treatments and doses

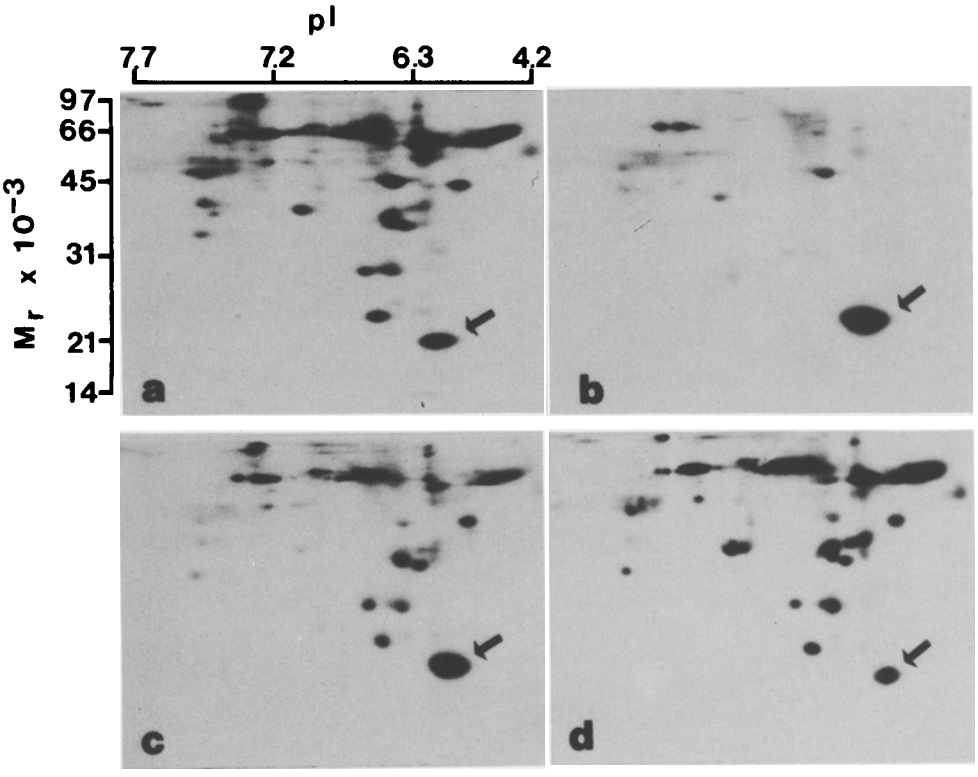

Figure 2 Representative fluorograph of 2D SDS–PAGE gels of proteins secreted in culture by day 17 conceptus tissues. Amounts of nondialyzable radioactivity subjected to the analysis ($n = 6$) were 250 000 dpm: Fluorograph (a), control no hIL-3; (b), conceptus tissues treated with 75 units/ml (15 pM) hIL-3; (c), 150 units/ml hIL-3; (d), 300 units/ml hIL-3. Arrow indicates oIFNτ

gesterone (Psychoyos, 1973), the mechanism for this process is not clearly understood. At least some effects of ovarian estrogen and progesterone in the processes of implantation may be mediated by cytokines because several cytokines and their receptors are expressed in the uterus and embryo during early pregnancy (Paria & Dey, 1990; Robertson *et al.*, 1992).

Hemopoietic cytokines such as IL-3 and GM-CSF are pleiotropic, and exhibit various biological effects depending on the target cells. More than one cytokine can interact with a single cell or a subset of cytokines often elicits a similar biological effect from the same target cells (Sato & Miyajima, 1994). Both IL-3 and GM-CSF induce the proliferation, differentiation and activation of hemopoietic cells. Various signal transduction systems for these cytokines have been identified: PKC (Kanakura *et al.*, 1990; Yanagisawa *et al.*, 1994), JAK2 (Matsuda & Hirano, 1994; Quelle *et al.*, 1994), cAMP (Sakamoto *et al.*, 1994), and Ras, Raf and mitogen activated protein (MAP) kinase (Matulonis *et al.*, 1993; Carroll & May, 1994). All of the signal transduction systems described above in hemopoietic cells are directed to phosphorylation and subsequent proliferation of target cells. Data presented do not necessarily show the growth of trophoblastic cells, rather, they elucidate molecular mechanisms which influence oIFNτ production. PKC represents one of the signal transduction systems of the conceptus that may be induced by maternal IL-3 and GM-CSF. Binding of IL-3 to hemopoietic cells has been reported to be partially inhibited by GM-CSF while high affinity binding of GM-CSF is inhibited by IL-3 (Gesner *et al.*, 1988; Kitamura *et al.*, 1991). Whether hIL-3 exerts its effect on oIFNτ production through its own receptor or through the GM-CSF receptor was not determined in this study.

Although minute amounts of bIFNτ, the bovine counterpart of oIFNτ, are produced by *in vitro* fertilized conceptuses cultured without maternal influences (Hernandez-Ledezma *et al.*, 1992), bIFNτ production is increased about 2000-fold when bovine conceptuses are exposed to the uterine environment. Induction of oIFNτ may be regulated by autocrine factors and may require no uterine factors; however, the sufficient production of oIFNτ required for maternal recognition of pregnancy appears to be supported by uterine factors. In addition, the presence of ovine IL-3 polypeptide and mRNA at the endometrium of a cyclic animal (day 8) suggests that maternal IL-3 exists near the time or even before oIFNτ production is initiated, and that conceptus production of oIFNτ is partially regulated by IL-3. To understand physiological mechanisms by which maternal IL-3 production is regulated, experiments using ovariectomized ewes with steroid replacements are underway (Christenson, R.K., Imakawa, K., unpublished observations). Figure 6 represents the working hypothesis: Maternal IL-3 and GM-CSF are expressed constitutively and possibly in cyclic nature. In pregnancy, these factors act on the conceptus presumably through their receptors in a paracrine manner and various second messenger systems are activated. In response to these signals nuclear proto-oncogenes, c-*fos* and c-*jun*, are activated and form a heterodimer which binds to the AP-1 site, resulting in the enhancement of oIFNτ gene transcription. In support of this hypothesis, gel-shift assays with a portion of oIFNτ gene and nuclear proteins isolated from ovine conceptuses demonstrated that both c-*fos* and c-*jun* polypeptide bind to the AP-1 site of the oIFNτ gene (Taylor, A., Imakawa, K., unpublished observation).

It has become apparent that cytokine/growth factors of endometrial and possibly conceptus origins are involved in maternal-fetal communication during pre- and peri-implantation periods. It is possible, however, that individual cytokines may play different roles during the processes of implantation and placentation. GM-CSF has been shown to stimulate proliferation of choriocarcinoma cell lines JEG, JAR and BeWo (Wegmann & Guilbert, 1991), suggesting that in addition to the regulation of oIFNτ expression GM-CSF and possibly IL-3 are involved in trophoblast cell growth and differentiation. Furthermore, GM-CSF and IL-3 may also participate in local regulation of some other factors essential for the process of implantation.

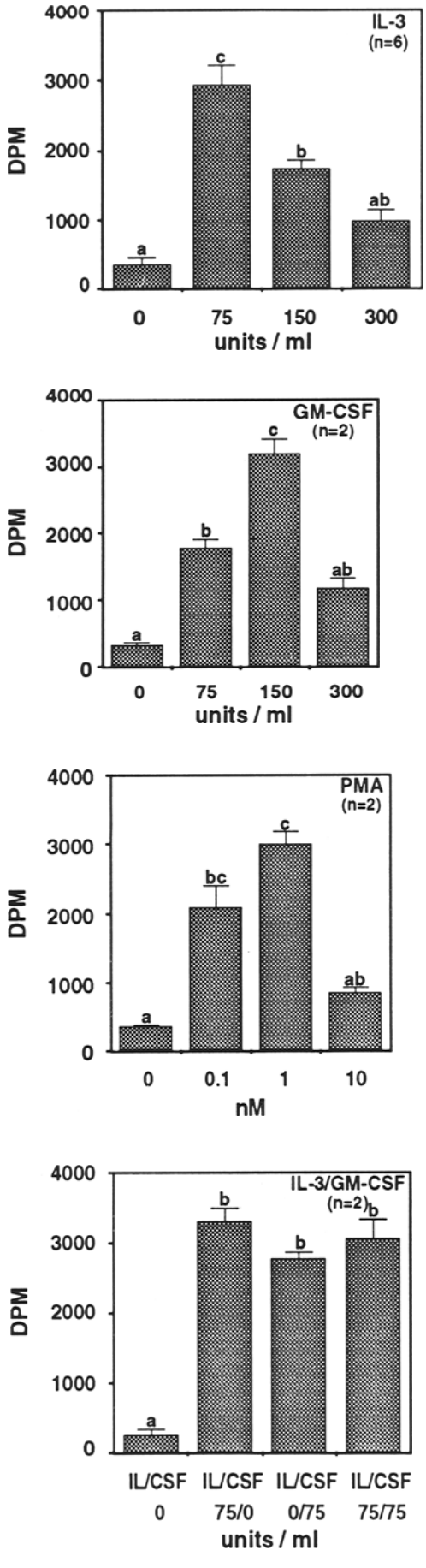

Figure 3 Levels of oIFNτ produced by day 17 conceptuses *in vitro*. Culture media (250 000 dpm) were subjected to 2D SDS–PAGE and fluorography. Spots corresponding to oIFNτ were punched out from dried gels and their radioactivities were counted by a β-counter. Doses with different superscripts represent significant differences ($P<0.01$)

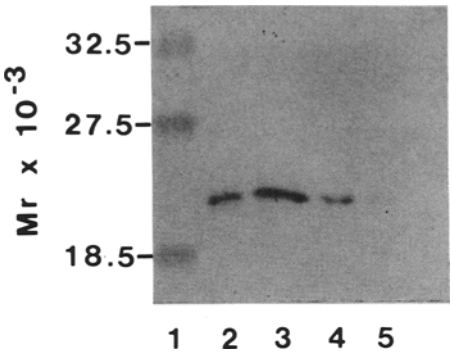

Figure 4 Representative western blot analysis showing oIFNτ production *in vitro* by day 17 ovine conceptuses in the presence of various doses of hIL-3. The same amounts of nondialyzable radioactivity (7500 dpm/treatment) were analysed. 1, molecular size marker; 2, control no hIL-3; 3, conceptus tissues treated with 75 units/ml (15 pM) hIL-3; 4, 150 units/ml hIL-3; 5, 300 units/ml hIL-3

Materials and methods

Materials

L-[3,4,5-^{3}H (N)] Leucine (specific activity, 179.60 Ci/mmol), [^{35}S]-dATP (specific activity, 1422 Ci/mmol), [^{32}P]-dCTP (specific activity, 3000 Ci/mmol) and [^{3}H]-CTP (specific activity, >20 Ci/mmol) were purchased from NEN-Dupont Research Products (Boston, MA). Sequenase DNA sequencing system was obtained from U.S. Biochemical Corp. (Cleveland, OH). Random primed DNA labeling kit to prepare ^{32}P-labeled oIFNτ cDNA for northern and slot blot analyses was from Boehringer Mannheim (Indianapolis, IN). Restriction endonucleases (EcoRI, HindIII, PstI and Sma) were obtained from Promega (Madison, WI). For cRNA probes and cRNA standard used in *in situ* hybridization and slot blot studies, respectively, *in vitro* transcription kit including T3 and T7 RNA polymerases and plasmid vector pBS M13 were purchased from Stratagene (LaJolla, CA). Human recombinant (h) IL-3 and hIL-3 cDNA were generous gifts from Genetics Institute (Cambridge, MA). For immunohistochemistry experiments, an antiserum against oligopeptide of ovine IL-3 was prepared at Research Genetics (Huntsville, AL) and Streptavidin-biotin system was from Zymed Laboratories, Inc. (South San Francisco, CA). Recombinant oIFNτ and its antibody used for western blot analysis were generous gifts from Dr. R.M. Roberts, University of Missouri-Columbia. Agarose and nylon membranes were purchased from Bio-Rad Laboratories (Richmond, VA), and calf thymus DNA was obtained from Hoefer Scientific Instruments (San Francisco, CA). X-ray film was from Eastman Kodak (Rochester, NY). Trichloroacetic acid (TCA), phorbol 12-myristate 13-acetate (PMA), G-50 sephadex and yeast RNA were purchased from Sigma Chemical Co. Nitroblue tetrazolium and bromo chloro indoyl phosphate were obtained from Promega, and Levamisole was purchased from Vector Labs (Burlingame, CA). All other reagents were the highest quality commercially available.

Animals

All crossbred ewes used in this study were housed at Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, NE and the protocol for sheep experimentation has been approved by the animal care committee at the USDA. Behavioral estrus of these animals was synchronized with intravaginal sponges of 40 mg 17-α acetoxy-9α-fluoro-11β-hydroxy progesterone for 13 days and an *i.m.* injection of 10 mg progesterone on the day of sponge removal, followed by an *i.m.* injection of 10 mg progesterone and a single *s.c.* injection of 500 IU PMSG on the subsequent day. Estrous

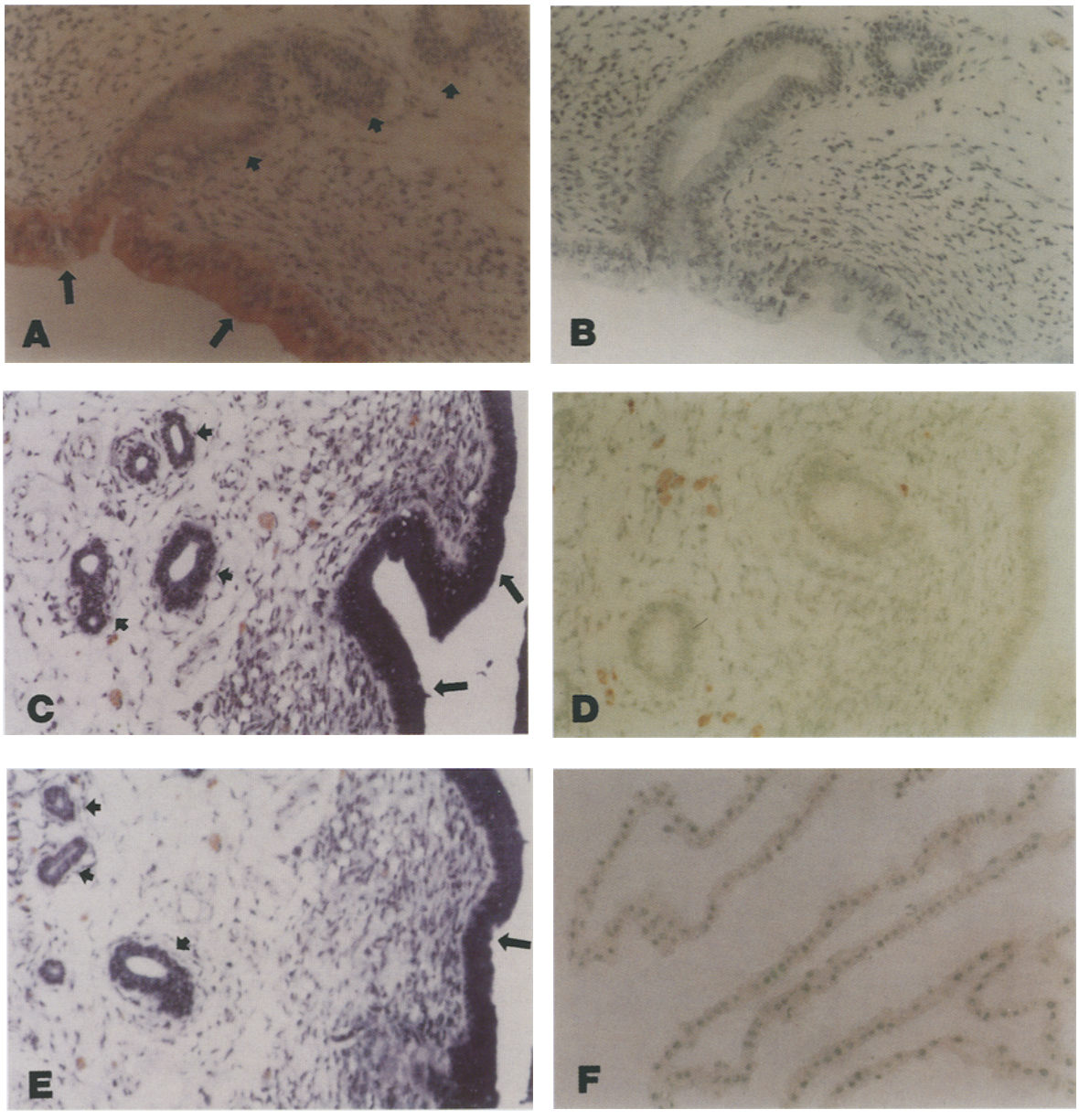

Figure 5 Localization of ovine IL-3 and its mRNA by immunohistochemistry and *in situ* hybridization. (**A**) Using anti-ovine IL-3 peptide antiserum, ovine IL-3 was detected mainly at luminal (large arrow) and glandular (small arrow) epithelia cells obtained from day 17 pregnant ewe. (**B**) Negative control in a semiserial section of the same tissue. (**C**) Cellular localization of IL-3 mRNA in the uterine endometrium. hIL-3 *antisense* cRNA probe hybridized to RNA in luminal (large arrow) and glandular (small arrow) epithelium cells in cross sections of uterine endometrium obtained from day 17 pregnant ewe. (**D**) hIL-3 *sense* cRNA probe in a semiserial section of the same tissue. No hybridization was observed. (**E**) hIL-3 *antisense* cRNA probe hybridized to RNA to the same regions of uterine endometrium obtained from the day 12 cyclic ewe. (**F**) Day 17 conceptuses along with day 17 endometrium were fixed in the same blocks and subjected to *in situ* hybridization studies using the hIL-3 *antisense* probe. No hybridization, except green counterstain, was observed

activity was recorded twice daily and the ewes were group-mated with two fertile rams fitted with marking harnesses (day of estrus = day 0). After breeding, vasectomized rams with marking harnesses were used to monitor subsequent estrous behavior; ewes not showing behavioral estrus during the next 17 day period were subjected to the study.

On day 17 ($n = 10$) of gestation, the selected ewes were anesthetized 15–20 min before surgery with xylazine hydrochloride (0.1 mg/lb) and lidocaine. The reproductive tracts were removed through mid-ventral incision after double clampings of uterine and ovarian blood vessels. Special care was taken to insure that the uteri were removed within 5 min after the blood supply was terminated in order to prevent possible hemolysis and subsequent RNA degradation. Each reproductive tract was immediately placed into a plastic bag covered with ice and transported to a sterile laminar flow hood; conceptuses were flushed gently with 20 to 30 ml sterile phosphate buffered saline (PBS, pH 7.2). Endometrial tissues from days 8 and 12 cyclic ewes as well as from day 17 pregnant ewes were obtained, weighed, immediately frozen in liquid nitrogen and stored at −80°C (Imakawa *et al.*, 1993; Nephew *et al.*, 1993).

In vitro *culture of conceptuses*

A total of 14 conceptuses were obtained from 10 pregnant ewes of which eight conceptuses were subjected to *in vitro* culture studies examining the effects of various doses of hIL-3 or hGM-CSF on conceptus production of oIFNτ. The remaining conceptuses ($n = 6$) were subjected to the culture experiments involving hGM-CSF, PMA and combination of hIL-3 and hGM-CSF. Each conceptus was dissected into

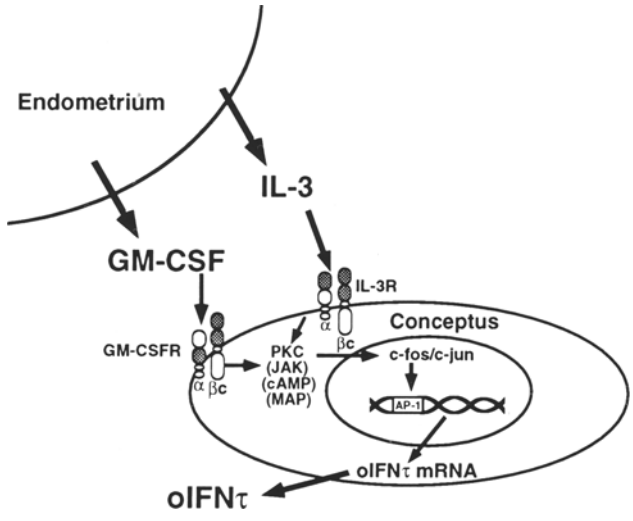

Figure 6 A model of maternal-fetal communication during peri-implantation period in the ewe. Maternal GM-CSF and/or IL-3 bind to the receptors and possibly activate one of second messengers, PKC. These signal transductions activate oIFNτ gene transcription, resulting in the enhanced production of oIFNτ

four approximately equal tissue masses (200 mg, wet weight) which were placed in 60 mm culture dishes containing 7 ml Eagle's Minimum Essential Medium (MEM), 50 μCi[^{3}H]-leucine (Imakawa *et al.*, 1993) and one of four doses of hIL-3; 0, 75, 150 or 300 units/ml (0, 15, 30 or 60 pM, respectively; units = colony forming units, Nicola *et al.*, 1979). Conceptuses were cultured with various doses of hGM-CSF as previously described (Imakawa *et al.*, 1993). Combination of hIL-3 (75 units/ml) and hGM-CSF (75 units/ml) was also tested. Furthermore, conceptuses were cultured in the absence or presence (0.1 nM, 1 nM or 10 nM) of PMA which stimulates intracellular PKC activity. All culture dishes were placed in a controlled atmosphere chamber (50% O_2, 45% N_2 and 5% CO_2) on a rocking platform (6 cycles/min) and were incubated at 37°C for 24 h. The culture media and conceptus tissues were then collected separately and frozen in liquid nitrogen. Medium samples were dialyzed against 10 mM Tris (pH 8.2) and incorporation of ^{3}H-leucine into nondialyzable macromolecules was determined by scintillation counting. Dialyzed samples (250 000 dpm) were lyophilized and were subjected to 2D SDS–PAGE analysis and fluorography (Imakawa *et al.*, 1993) for examination of proteins synthesized by the conceptus tissue in culture. After 4 weeks, spots on fluorographs corresponding to oIFNτ were analysed densitometrically. Spots of oIFNτ on dried 2D SDS–PAGE gels were then punched out and their radioactivities were determined by scintillation counting. Furthermore, the dialyzed samples (7500 dpm) along with various amounts of recombinant oIFNτ (12.5, 25, 50 and 100 ng) were subjected to western blot analysis. Amounts of oIFNτ produced by the conceptus tissues during a 24 h *in vitro* culture were determined from densitometric values. Cultured conceptus tissues were used to extract RNA for northern and slot blot analyses (Sambrook *et al.*, 1989).

RNA preparation, northern and slot blot analyses

Cultured ovine conceptuses were homogenized in 400 ul 4 M guanidine isothiocyanate (GTC) solution. In order to estimate tissue masses (approximately one quarter of a conceptus) within treatment groups, 2 μl of the homogenized GTC solution were subjected to DNA content determination. Amounts of DNA were determined by the fluorometer (460 nm, TKO 100, Hoefer Scientific Institute, San Francisco, CA). The remaining samples were subjected to RNA isolation using the method reported by Chomczynski & Sacchi (1987) and to northern blot analysis as previously described by Imakawa *et al.* (1993). Amounts of oIFNτ mRNA were determined by slot blot analysis using oIFNτ cRNA as a standard. In brief, cRNA corresponding to a oIFNτ cDNA (Imakawa *et al.*, 1987) was prepared as previously described (Nephew *et al.*, 1993). Ten μg conceptus RNA along with 15.6, 31.3, 62.5, 125, 250 and 500 pg oIFNτ cRNA were spotted onto nylon membranes and hybridized with ^{32}P-labeled oIFNτ cDNA (Nephew *et al.*, 1993). After washing and autoradiography (24–48 h), amounts of oIFNτ mRNA were determined densitometrically from a standard curve included on each membrane.

In situ *hybridization*

For *in situ* hybridization procedures, endometrial tissues were obtained from days 8 ($n = 1$) and 12 ($n = 1$) cyclic ewes and day 17 ($n = 4$) pregnant ewes. Conceptus tissues (day 17, $n = 2$) were also studied. Endometrial explants and conceptus tissues (approximately 125 mm^3 and 60 mm^3, respectively) were fixed overnight at 4°C in freshly prepared 4% (wt/vol) paraformaldehyde-PBS buffer (pH 7.2). Slides of serial 5 μm sections were carefully prepared with DEPC-treated H_20 at the Department of Histopathology, Wesley Medical Center, Wichita, KS (Imakawa *et al.*, 1993).

Human IL-3 cDNA was digested with restriction endonucleases PstI and Sma and the 3' portion of this 190 base pair cDNA, which has higher specificity to detect the oIL-3 mRNA, was subcloned into the plasmid vector pBS M13 for *in vitro* transcription. The orientation of the cDNA in the pBS M13 was confirmed by DNA sequencing (Sanger *et al.*, 1977; Chen & Seeburg, 1985).

To obtain the sense-cRNA (T3) and antisense-cRNA (T7), the plasmid was digested with restriction endonucleases, EcoRI and HindIII, respectively. After linearization of the plasmid, sense and antisense IL-3 cRNA probes were synthesized by *in vitro* transcription with digoxigenin 11-UTP using T7 and T3 RNA polymerases and [^{3}H]-CTP as tracer. A portion of the newly synthesized cRNA was precipitated with trichloroacetic acid (TCA) and amounts of TCA precipitable counts were used to estimate the yield of the probe. Purified digoxigenin cRNA probes were used in the hybridization mixture that contained 50% deionized formamide, 10% dextran sulfate, 2 × SSC, 300 μg/ml yeast tRNA and RNase inhibitor (hybridization cocktail). The final hybridization cocktail contained sense or antisense probe with equal cpm and equal amounts of RNA (0.2 to 0.6 μg/ml). *In situ* hybridization was performed using the method described by Lawrence & Singer, 1885 as modified by Hunt *et al.*, (1992) and Imakawa *et al.* (1993).

Immunohistochemistry

Serial sections of the tissues tested for ovine IL-3 mRNA were evaluated for the presence of its polypeptide. A rabbit polyclonal antibody to an oligopeptide representing amino acid 53 to 64 (GSLNSDGKNILA) of ovine IL-3 (McInnes *et al.*, 1993) was prepared commercially. This antiserum was used at a dilution of 1:50 in PBS. Binding was detected with an avidin-biotin immunoperoxide staining kit (Hunt *et al.*, 1992). The use of 3-amino-9-ethylcarbazole in N, N-dimethylformamide as a substrate for the peroxidase yielded a red brown coloration of positive cells. An equivalent concentration of normal rabbit IgG was used on serial sections of the same tissues in each experiment to assess nonspecific binding of the avidin-biotin immunoperoxidase staining system. The tissue sections were counterstained with hematoxylin.

Statistical analysis

Amounts of DNA in conceptus tissues and levels of non-dialyzable macromolecules were analysed by one-way anal-

ysis of variance. Effects of various doses of hIL-3, hGM-CSF and PMA on levels of oIFNτ and their mRNA were also analysed by one-way analysis of variance. When significant F-ratios were observed, Duncan's multiple range test and *t*-test were used to differentiate the effects of various doses of hIL-3, hGM-CSF or PMA on the levels of oIFNτ and their mRNA. Linear regression analysis was applied to establish standard curves that were used to determine amounts of oIFNτ mRNA on slot blot analysis. Densitometric data and radioactive counts (DPM) were used to estimate oIFNτ produced by conceptuses in culture and associations of these two values were determined by correlation analysis.

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Proprietary or brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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