AN ABSTRACT OF THE THESIS OF

Chandra Sekhara Reddy Meka for the Master of Science Degree in Biology presented on May 15, 1993

Title: A Novel Role for the Cytokine Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) in the Induction of mRNA for Ovine Interferon tau (oIFNτ).

Abstract Approved: Gaylen Frenfeld

During pregnancy the sheep conceptus produces various proteins, most notably oIFNτ (ovine trophoblast protein-1, oTP-1). Their production is enhanced by various cytokines/growth factors. To study the role of GM-CSF in the reproductive tract of non-pregnant and pregnant ewes, the temporal expression and cellular sites of synthesis of GM-CSF mRNA were determined. GM-CSF mRNA was detected by *in situ* hybridization in luminal and glandular epithelium of uterine endometrium of both non-pregnant and pregnant ewes. GM-CSF expression was restricted to the uterine luminal and glandular epithelial cells at all stages of gestation. GM-CSF mRNA was also detected in the cytoplasm of trophoblastic cells of the conceptus. Higher levels of GM-CSF mRNA expression were observed in pregnant ewes compared to that of non-pregnant ewes and the conceptus. Total cellular RNA extracted from conceptuses cultured with various cytokines was characterized for relative amounts of oIFNτ mRNA by northern blot analysis. GM-CSF increased oIFNτ mRNA in a dose dependent fashion with maximal induction at 300 U/ml hrGM-CSF. IL-3 increased conceptus oIFNτ mRNA in a dose dependent manner with maximal induction in conceptuses treated with 75 U/ml IL-3. IL-6 increased oIFNτ mRNA regardless of doses tested. Retinoic acid

(RA) alone had no effect on oIFNτ mRNA, but when combined with GM-CSF added to cultures simultaneously, conceptus oIFNτ mRNA was not increased suggesting that RA down regulated GM-CSF induction of oIFNτ *in vitro*. These results document that the GM-CSF gene is expressed in non-pregnant and pregnant ewes, specifically in uterine luminal and glandular epithelial cells. The presence of GM-CSF transcripts in trophoblastic cells of the conceptus suggests a major role for GM-CSF in the dynamic development of the conceptus and the induction of oIFNτ in a paracrine manner. Collectively, these results suggests that a well established communication exists between the developing conceptus and the endometrium during pregnancy establishment.

A Novel Role for the Cytokine Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) in the Induction of mRNA for Ovine Interferon tau (oIFNτ)

A Thesis

Submitted to

the Division of Biological Sciences

Emporia State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Chandra Sekhara Reddy Meka

May 1993

Approved by Major Adviser

Approved by Co-Major Adviser

Approved by Committee Member

Approved by Committee Member

Approved for Major Department

Approved for Graduate Council

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to the following for their contribution towards the completion of this work.

I am indebted to Dr. Kazuhiko Imakawa (Kaz), my research adviser, for his criticism which was "usually" constructive, and for his motivation and patience. His friendship and financial support for this project were greatly appreciated. My appreciation is also extended to his wife, Atsuko, and to his children, Akiko and Ayako Turner, for their continuous love and support.

The help, friendship, and encouragement of Dr. Gaylen J. Neufeld, my major adviser, has made this work possible. I shall always be grateful for the opportunity he has given me to work at The Women's Research Institute. My appreciation is also extended to his family for their warmth and friendship.

A special note of thanks goes to the members of my advisory committee, Drs. Katherine N. Smalley and David R. Edds for their time, patience and guidance; to Dr. Ronald K. Christenson, USDA, Nebraska, for his vital contribution in providing the animals used in this study; to Dr. Brooks A. Keel, President and CEO, The Women's Research Institute, for his support and encouragement throughout this project. Appreciation is extended to Drs. Jeffrey V. May, John S. Davis, Stephen J. Zimniski, and Bhushan K. Gangrade for broadening the depth of my program.

A special note of thanks goes to Ms. Arleene Moore for her friendship and help in the preparation of this thesis as well as to Ms. Kendra Hall for her support and encouragement during this work.

I wish to express my special thanks to the Post-Doctoral Research Associates at The Women's Research Institute, Drs. Aruna Chakravorty, Sarita Chary, Thomas He, Yun-Hwa Mau, Craig Turczynski and Shafiq Khan for their invaluable assistance and friendship and to Lisa A. Harbison for her outstanding technical assistance.

DEDICATION

This thesis is dedicated with love and appreciation to my uncle, Ramamohana R. Bommareddy, and to my parents, Dr. Nageswara Reddy Meka and Krupavathi Meka. I am eternally grateful for their love, support and encouragement which has made it possible for me to achieve my goals and to be what I am today. Their inspiration and faith in me have enabled me to fulfill my dreams.

It is further dedicated to my brothers, Raja Sekhara Reddy Meka and Venugopala Reddy Meka, and my sister "Lilly", whose help, caring support and encouragement was instrumental in my pursuit of this degree.

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INTRODUCTION

Maternal Recognition of Pregnancy

Corpus luteum (CL) function is fundamental to the process of reproductive cyclicity and maintenance of pregnancy in all mammalian females. The CL primarily synthesizes and secretes the steroid hormone, progesterone (P_4), which is involved in uterine function through augmentation of uterine endometrial secretions, promoting a suitable environment for normal embryonic development. The normal life span of the CL should be extended in order to interrupt the regular pattern of ovarian cyclicity and to establish continuous production of P_4. This phenomenon whereby maternal CL functions continuously beyond the estrous cycle is termed "maternal recognition of pregnancy" (Short, 1969) and is mediated by a series of biochemical interactions that occur between the conceptus (defined as the embryo plus its surrounding membranes) and the maternal uterine endometrium. It has been known for quite some time that "maternal recognition of pregnancy" in sheep occurs around 12-13 days after the estrus.

Considerable evidence has accumulated that uterine prostaglandin $F_2\alpha$ ($PGF_2\alpha$) is the primary substance released in the late luteal phase of the estrous cycle and is responsible for inducing regression of the CL in sheep and cattle (McCracken et al., 1984). In ewes, the uterine endometrium begins to produce $PGF_2\alpha$ in a pulsatile manner on days 13 and 14 of a 17-day estrous cycle. At about the same time, P_4 levels begin to fall and the animal returns to behavioral estrus. In contrast, if the animal is pregnant, the release of $PGF_2\alpha$ is nonpulsatile and the CL continues to function at least for the initial 60 days of a 149-day pregnancy.

Ovine Trophoblast Protein-1/Ovine Interferon tau

Roles of substances (i.e., proteins) produced by the blastocyst in maternal recognition of pregnancy in sheep and cattle have been studied intensively. In sheep during the period of maternal recognition of pregnancy, a major ~20,000 Mr acidic protein known as ovine trophoblast protein-1 (oTP-1) is secreted by the developing conceptuses (Godkin et al., 1982) and the highest level of oTP-1 mRNA is found in day 16 conceptuses (Hansen et al., 1988). oTP-1 is the primary factor responsible for prolongation of CL function during early pregnancy in sheep (Godkin et al., 1984). It acts via receptors present on the surface of the uterine endometrium (Godkin et al., 1984) and attenuates endometrial secretion of $PGF_2\alpha$ from the uterus (Vallet et al., 1988). Thus, oTP-1 has been generally recognized as the primary substance responsible for eliciting the maternal recognition of pregnancy in sheep.

Recently the N-terminal amino acid (Charpigny et al., 1988) and cDNA sequences (Imakawa et al., 1987) have revealed that oTP-1 is related to the interferon-alpha (IFN-α) family. More specifically its 172 amino acid sequence places it in the IFN-αII subfamily (Capon et al., 1985 and Charlier et al., 1989). oTP-1 has antiviral (Pontzer et al., 1988), anti-proliferative (Roberts et al., 1989) and immunosuppressive (Fillion et al., 1991) properties, known characteristics of functional IFN-αs. However, oTP-1 is antigenically dissimilar from typical IFN-αs and is produced by non-lymphatic, trophoblast cells; this IFN is named as ovine interferon tau (oIFNτ, Roberts et al., 1992). Several lines of evidence suggest that oIFNτ is coded by different genes (Klemann et al., 1990) and that various oIFNτ genes are differently expressed during early pregnancy

(Nephew et al., 1993). Nephew et al. (1993) also demonstrated that one of the oIFNτ genes contained an AP-1 sequence (5'-TGACTCA-3'), transcription enhancer element, in its 5'-flanking region and this gene was highly expressed during day 13-20 of pregnancy.

In situ hybridization and immunohistochemical experiments have shown that oIFNτ is a product of the embryonic trophectoderm (Farin et al., 1989; Guillomot et al., 1990). These studies have also demonstrated that oIFNτ synthesis begins as early as day 11 and that massive production of oIFNτ starts when the conceptus changes morphologically from spherical to filamentous form. It is well established that a single day 16 embryo produces up to 100 μg of oIFNτ during a 24 h culture period *in vitro* (Ashworth et al., 1989).

Maternal-Fetal Interactions

In the absence of maternal factors, preimplantation mouse embryos grow and differentiate into blastocysts, suggesting that endogenous factors (in an autocrine manner) must support their early development (Brinster, 1973). In human placenta, platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF-I, IGF-II) genes are actively expressed during first trimester of gestation (Goustin et al., 1985; Wang et al., 1988; Ohlsson et al., 1989). Expression of these growth factor genes is temporally and spatially associated with increased c-myc proto-oncogene expression (Goustin et al., 1985) suggesting that growth factors and proto-oncogenes are functionally linked. Maternal growth factors (serum or uterine origin) could, therefore, influence growth and/or differentiation of fetal membranes.

Colony Stimulating Factors: Colony stimulating factors (CSFs) belong to cytokine families that function as autocrine, paracrine and endocrine factors. These factors modulate physiologically important functions such as the specific (antibody formation and T-cell receptor formation) and aspecific, i.e., immunogen independent, host defense mechanisms. Various cytokines/growth factors are known to be involved in embryonic development and they greatly affect the endometrium necessary for pregnancy establishment. Recent findings show the involvement of growth factors/cytokines at the maternal-fetal annexes; epidermal growth factors (EGF, Brown et al., 1989), granulocyte-colony stimulating factor (G-CSF, Nicola et al., 1979), granulocyte-macrophage colony stimulating factor (GM-CSF, Wegmann et al., 1989; Robertson and Seamark, 1990) and interleukin-6 (IL-6, Robertson et al., 1992).

GM-CSF, a 22 KDa polypeptide hormone, can stimulate the growth of a single cell precursor to mature eosinophils, granulocytes or macrophages (Opdenakker et al., 1989). In addition, exogenous GM-CSF can stimulate the proliferation of trophoblast cells *in vitro*, particularly ectoplacental cone trophoblast cells derived from 7.5 day mouse embryos (Grough et al., 1984; Armstrong and Chaouat, 1989). Moreover, effects of GM-CSF on human placental and choriocarcinoma cells *in vitro* suggest that GM-CSF action is of an autocrine nature within the reproductive tissues themselves (Wegmann and Guilbert, 1991).

GM-CSF activates the transcription of number of protooncogenes including c-fos (Colotta et al., 1987) and c-jun (Adunyah et al., 1991), although it remains unclear how binding of GM-CSF to its cell surface receptor (Adunyah et al., 1991) induces changes

in transcription of specific genes. Recently, Adunyah et al. (1991) have demonstrated that the binding of GM-CSF to its receptor stimulates an increase in c-jun mRNA and its protein activates the AP-1 enhancer sequence. The protein products of c-fos and c-jun mRNA, Fos and Jun, contain a leucine repeat region, leucine zipper, which allows Fos and Jun to dimerize (Chiu et al., 1988). The basic amino terminal region of the leucine zipper contains the DNA-binding portion and allows it to bind to the AP-1 nucleotide sequence, thereby initiating transcription of the gene(s) containing AP-1 sites. In addition, it was hypothesized (Figure 1, Imakawa K, Helmer SD, and Christenson RK, personal communication) that GM-CSF enhances transcription of one of five oIFNτ genes containing AP-1 in its 5' flanking region (Nephew et al., 1993). This oIFNτ gene is highly expressed between days 13-20 pregnancy. Also, these investigators have demonstrated that GM-CSF increases both oIFNτ (Figure 2) and oIFNτ mRNA in 24 h *in vitro* conceptus culture and that GM-CSF transcripts are present at the endometrium of day 17 pregnant ewe (Imakawa et al., 1993a).

Interleukins (ILs): IL-3 is a potent hemopoietic growth factor which stimulates proliferation and differentiation of various lineages of hemopoietic cells (Schrader, 1986). Both IL-3 and GM-CSF induce similar intracellular signals and biological activities: tyrosine phosphorylation of a similar set of proteins (Kanakura et al., 1990; Isfort and Ilhe, 1990). In addition, both IL-3 and GM-CSF show cross competition for high affinity binding sites in human hemopoietic cell lines (Kitamura et al., 1991).

IL-6 is a cytokine that mediates different host responses and regulates multiple cell types. On hemopoietic cells and stem cells, IL-6 can stimulate the growth of

hemopoietic colonies composed of granulocytes and macrophages. IL-6 is secreted *in vitro* primarily by uterine epithelial and glandular epithelial cells (Jacobs et al., 1991; Robertson et al., 1992). Mathialagan and co-workers (1992) found the expression of IL-6 in porcine, bovine and ovine preimplantation conceptuses, suggesting that IL-6 stimulates conceptus growth in an autocrine manner.

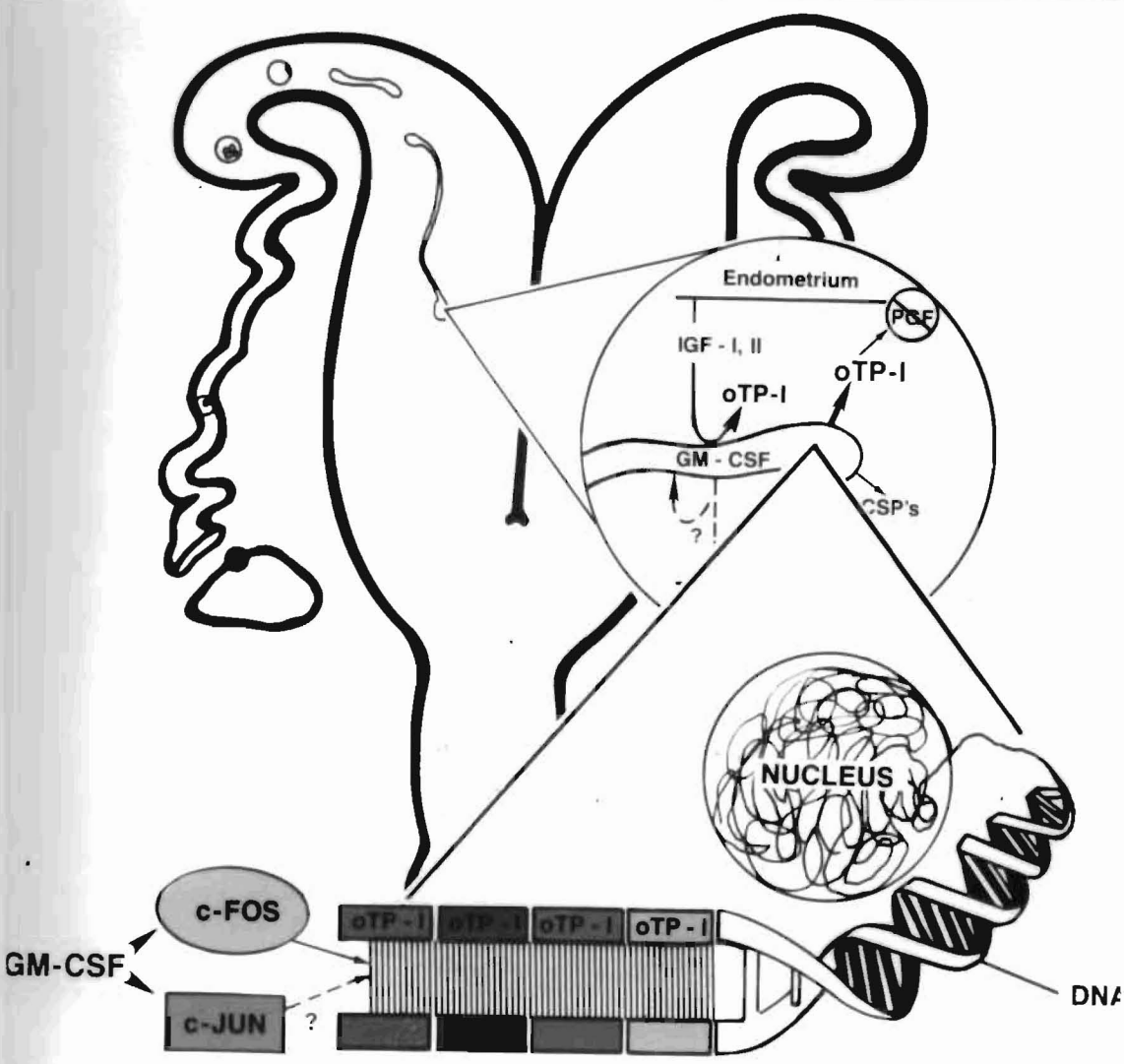


Figure 1: An illustration of working hypothesis showing GM-CSF induction of oIFNτ (oTP-1) gene(s): GM-CSF of maternal and/or conceptus origin stimulates the expression of nuclear protooncogenes c-fos and c-jun of which protein products bind to the AP-1 site present in the oIFNτ o10 gene, resulting in the enhancement of oIFNτ gene transcription and subsequent translation at the trophectoderm of developing ovine conceptuses.

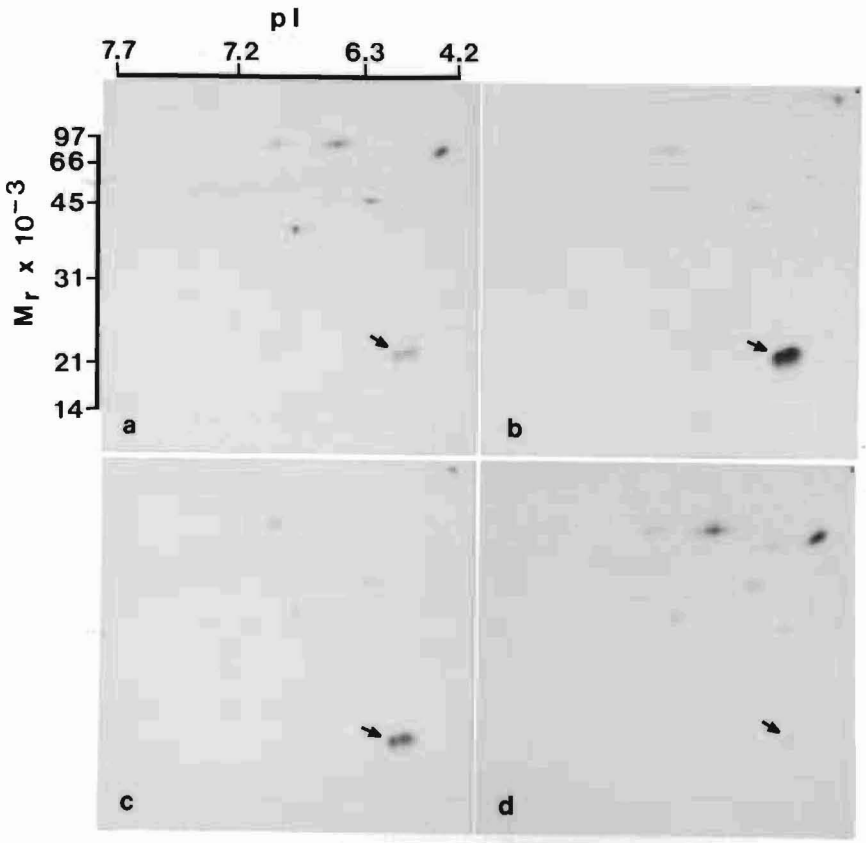


Figure 2: Fluorography illustrating the result of two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D SDS-PAGE) used to identify enhanced production of oIFNτ by GM-CSF from day 17 ovine conceptus (24 h) culture media: Fluorograph a, control no hrGM-CSF; b, conceptus tissues treated with 150 U/ml hrGM-CSF; c, 300 U/ml hrGM-CSF; d, 600 U/ml hrGM-CSF. Arrow indicates oIFNτ. (Data are from Imakawa et al., 1993a.)

Retinoic Acid: Vitamin A and its analogs (retinoids) play an essential role in embryonic development and possess properties of an endogenous morphogen, capable of differential regulation of pattern formation in the developing embryo (Thaller and Eichele, 1987). It is well defined that retinoic acid (RA) affects gene transcription, steroidogenesis, hematopoiesis and interferon production. RA has also been shown to suppress the protooncogenes, c-myc, N-myc, c-fos and c-myb, and to down regulate growth factors in human choriocarcinoma cell lines (Miller et al., 1990). In addition, RA regulates c-fos expression in simian sarcoma virus-transformed normal rat kidney cells at the transcriptional initiation site (Jaffey et al., 1992). It was recently discovered that retinal binding protein (RBP) was synthesized by ovine placental membranes from day 23 through mid pregnancy (Liu et al., 1992), suggesting a possible role of RA in sheep pregnancy.

Objective of the Study

Much of the research has focused on how oTP-1/IFN prevents luteal regression, however, the molecular events which maintain the apparently high levels of oIFNτ needed for pregnancy establishment have not been studied. This study has mainly focused on the temporal and spatial expression of the endometrial cytokine GM-CSF which contributes to the massive production of oIFNτ during the period of maternal recognition of pregnancy. Attempts were made to characterize the effect of other cytokines/growth factors, such as IL-3 and IL-6 on oIFNτ expression during the conceptus development in ewes. Finally, the *in vitro* effect of RA on oIFNτ production, as a potential substance which may terminate IFN production, was determined.

MATERIALS AND METHODS

Materials

Radiochemicals: 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).

Kits: *In vitro* transcription kit was purchased from Stratagene (La Jolla, CA), Sequenase DNA sequencing system was obtained from U.S. Biochemical Corp. (Cleveland, OH), and Random primed DNA labeling kit was from Boehringer Mannheim (Indianapolis, IN).

Enzymes and plasmid vector(s): Restriction endonucleases (EcoRI, HindIII, Pst I and Sma) were obtained from Promega (Madison, WI). T3 and T7 RNA polymerases and plasmid vector pBS M13 were purchased from Stratagene.

Recombinant Proteins: Human recombinant (hr) GM-CSF, hrIL-3 and hrIL-6 were generous gifts from Genetics Institute (Cambridge, MA), and Retinoic Acid (RA) was purchased from Sigma Chemical Co. (St.Louis, MO).

Probe: cDNA of oGM-CSF was a generous gift from Dr. Colin J. McInnes, Modern Research Institute (Edinburgh, England).

Reagents and chemicals: 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), 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 utilized in this study were housed at Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, NE. Behavioral estrus of these animals was synchronized with the administration of intravaginal sponges of 40 mg 17-α acetoxy-9α-fluoro-11ß-hydroxy progesterone for 14 days and a single *im* injection of 500 IU PMSG on the day of withdrawal of the sponges. Estrous activities in these ewes were observed approximately 48 h later and the ewes were mated with proven fertility rams twice on that day (day of estrus = day 0). After breeding, vasectomized rams with fitted paint were introduced to monitor the behavioral estrus. The ewes which did not show behavioral estrus during the next 10-17 day period were subjected to the studies.

On days 13 (n = 6), 15 (n = 8), 17 (n = 10), 19 (n = 2) and 22 (n = 2) of gestation, the selected ewes were not fed for 24 h before surgery, but water was supplied *ad libitum*. The ewes were anesthetized 15-20 min before surgery with xylazine (0.1 mg/lb) and lidocaine. The reproductive tracts including uterine horns and body and ovaries were exposed via mid-ventral laparotomy and obtained by hysterectomy under aseptic conditions when functional corpora lutea were present. Special care was taken to insure that after the blood supply was terminated the uteri were removed within 5 min in order to prevent possible hemolysis causing RNA degradation.

The uteri were immediately placed in ice, transported to a sterile laminar flow

hood, and conceptuses from days 13, 15, and 17 were flushed gently with 20 to 30 ml sterile phosphate buffered saline (PBS, pH 7.2). Conceptuses older than day 17 were collected after uterine horns were dissected longitudinally. Endometrial tissues from days 8 and 12 of cyclic ewes as well as from days 13, 15, 17, 19 and 22 of pregnant ewes were obtained, weighed, frozen immediately in liquid nitrogen and stored at -80°C.

In-vitro Culture of Conceptuses

Conceptuses from ewes on days 13, 15, 17, 19 and 22 of gestation (day = 0 estrus; n = 3, 3, 4, 2 and 2, respectively) were subjected to *in vitro* culture studies examining the effects of various doses of GM-CSF on conceptus production of oIFNτ. Each conceptus was dissected into four approximately equal tissue masses (200 mg, wet weight) and placed in 60 mm culture dishes containing 7 ml Eagle's Minimum Essential Medium (MEM), 50 μCi ^{3}H-leucine and one of four different doses; 0, 150, 300 or 600 U/ml, of hrGM-CSF (Imakawa et al., 1993a). All culture dishes were placed in a controlled atmosphere chamber (50% O_2, 45% N_2, and 5% Co_2) which was placed on a rocking platform (6 cycles/min), and incubated at 37°C for 24 h (Figure 3).

In the second set of experiments conceptuses from day 17 (n = 6) were dissected into four approximately equal tissue masses (200 mg, wet weight) and cultured in 7 ml MEM containing 50 μCi ^{3}H-Leucine and one of four different doses of hrIL-3 (n = 2) or hrIL-6 (n = 2), 0, 75, 150, or 300 U/ml respectively. Effects of RA and hrGM-CSF (n = 2) on oIFNτ production were also tested; i. no treatment, ii. 150 U/ml hrGM-CSF, iii. 1 μM RA and iv. a combination of 150 U/ml hrGM-CSF and 1 μM RA, respectively. Cultures were terminated after 24 h and the culture media and conceptuses tissues were

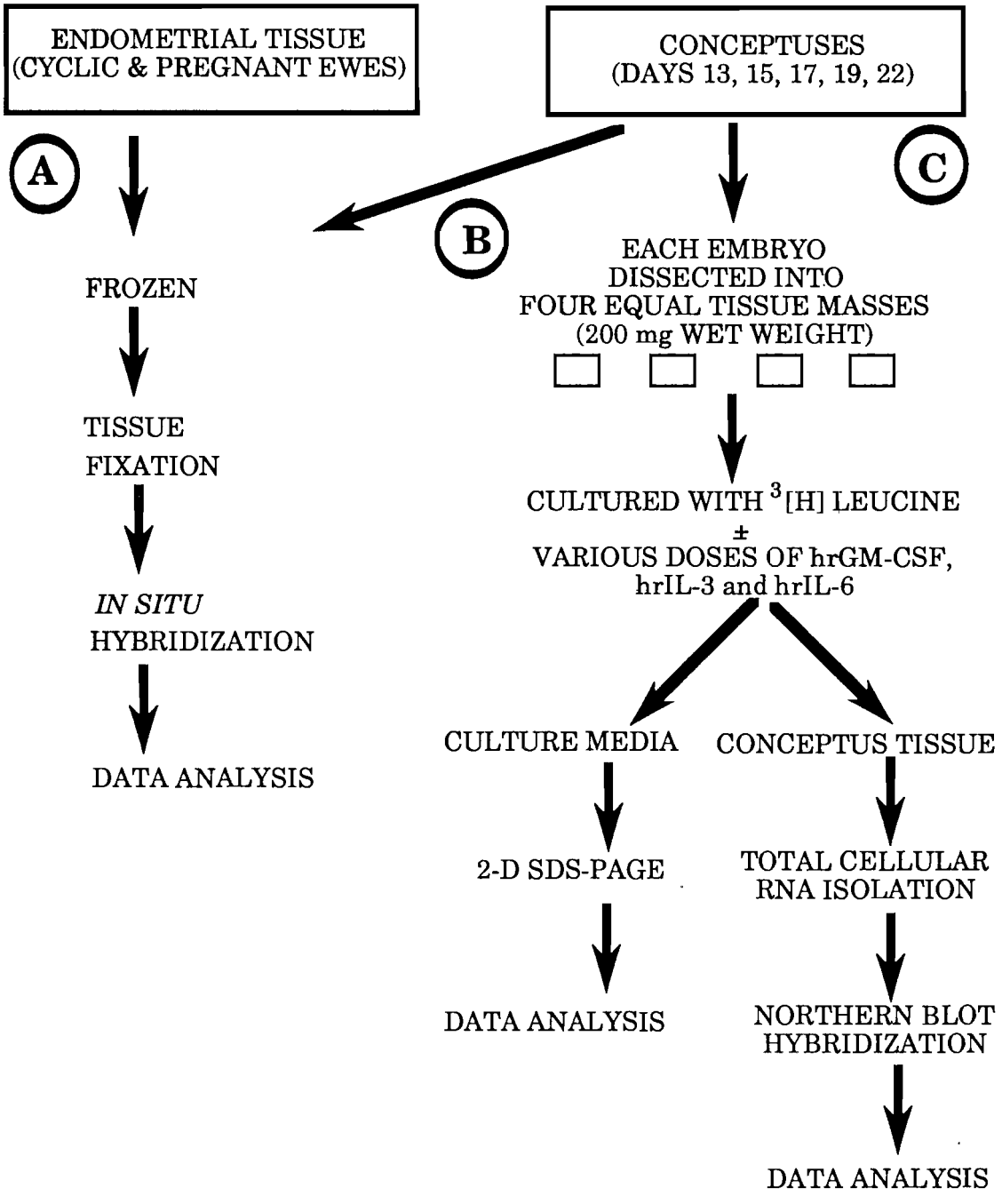


FIGURE. 3 : A schematic presentation of the present study in which the *in vitro* conceptus cultures and *in situ* hybridization methodologies were utilized. A. Endometrial tissue (125 mm^3 mass) processed for *in situ* hybridization; B. Conceptus tissues (60 mm^3 mass) subjected to *in situ* hybridization; C. Each dissected embryo (200 mg wet weight) was cultured *in vitro* with 7.0 ml MEM supplemented with 50 μ Ci ^{3}[H] Leucine and 0, 150, 300 and 600 U/ml of hrGM-CSF.

collected separately and flash frozen in liquid nitrogen. Medium samples were subjected to two dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D SDS-PAGE) analysis and fluorography. Conceptus tissues cultured were utilized to extract total cellular RNA (tcRNA) for northern blot analyses.

RNA Preparation

Frozen ovine conceptuses were homogenized in 400 ul 4 M guanidium isothiocyanate solution (GTC solution). Two μl of the homogenized solution were subjected to DNA concentration determination and were used for the estimation of tissue masses. Amounts of DNA were determined by the fluorometer (460 nm, TKO 100, Hoefer Scientific Institute, San Francisco, CA) which detected fluorescent signals of DNAs. Briefly, the fluorometer was standardized by using the dye solution: Heochst 33258 stock (fluorescent dye) solution, 10 $\times$ TNE (100 mM Tris, 10 mM EDTA, 1.0 M NaCl, pH 7.4) and sterilized distilled H_2O. The standard curves were constructed by measuring 1 μl (50 ng/ml), 3μl (150 ng), 5μl (250 ng), 7 μl (350 ng), and 10 μl (500 ng) of calf thymus DNA, and by plotting standard DNA concentration, ng/ml on X axis and fluorescence intensity, nm on Y axis. Later a 2 ul aliquot from each tissue homogenate in GTC solution was subjected to fluorometer analysis and concentrations of DNAs were determined from the standard curve.

After the DNA measurements, the remaining samples were subjected to tcRNA isolation using the method described by Chomczynski and Sacchi (1987). To individual conceptus tissues homogenized in GTC solution, 28 μl of 3 M NaAc (pH 5.2), 1 volume of phenol (water saturated, pH 4.5), and 0.2 volumes of chloroform-isoamyl alcohol

(24:1) were added simultaneously and placed on ice for 15 min. Samples were centrifuged (10,000 × g, 20 min, 4°C), the aqueous phase containing tcRNA was transferred to a fresh tube and the residual proteins were eliminated by further extraction with 1 volume of 50%/50% (vol/vol) phenol/chloroform. tcRNA was precipitated fromthe aqueous phase with 1 volume of isopropanol at -80°C. After centrifugation (10,000 × g, 10 min, 4°C), the resulting RNA pellets were dissolved in 0.3 volumes of GTC solution and precipitated again with 1 volume of isopropanol. After centrifugation (10,000 × g, 3 min, 4°C), the RNA pellets were washed with 75% ethanol, sedimented, vacuum-dried, dissolved in 0.1% (wt/vol) diethylpyrocarbonate (DEPC) treated water and stored at -80°C. The concentration of the isolated tcRNA was determined spectrophotometrically and amounts of RNA were calculated by using the following equation; λA 260 × 40 (co-factor) × dilution factor.

Northern Blot Hybridization

Ten μg tcRNA from each cultured conceptus on days 13, 15, 17, 19 and 22, respectively, were placed in 1.5 ml Eppendorf tubes containing 10 × MOPS (200mM 3-N-Morpholino-Propanesulfonic acid, 50mM NaAc and 10mM EDTA), 2.2 M formaldehyde, 50% (vol/vol) formamide and 10 × dye (25% glycerol and 0.025% bromophenol blue). Mixed samples were denatured at 65°C for 10 min and were subjected to constant voltage electrophoresis (100 volts) in a 1.5% (wt/vol) agarose gel that contained 1.1 M formaldehyde (Sambrook et al., 1989). HindIII/EcoRI digested lambda DNA was used as a size marker determining molecular sizes of tcRNA during electrophoresis. After the completion of electrophoresis, the gels were washed in

20 × SSC (1 × SSC: 0.15 M sodium chloride and 0.015 M sodium citrate) for 30 min and the tcRNA was transferred overnight to nylon membranes by capillary action through 10 × SSC transfer buffer. The following morning, membranes were baked for 2 h at 80°C and subjected to a 4 h prehybridization at 42°C in order to reduce binding of nucleotides that were not specific. Prehybridization buffer consisted of 50% (vol/vol) formamide, 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) polyvinylpyrrolidone (PVP), 0.02% (wt/vol) BSA, 5 × SSC, 0.05 M sodium phosphate (pH 6.5), 0.1% (wt/vol) SDS and 200 μg/ml sheared, denatured herring sperm DNA. Nylon membranes were then hybridized with ^{32}P-labeled oIFNτ cDNA probes in freshly prepared hybridization buffer, which contained the same components as in prehybridization buffer except sperm DNA, for 18 h at 42°C. The probe labeling was performed by the random priming method using (α-^{32}P)dCTP (3000 Ci/mmol) and the specific activity of cDNA probes was approximately 1-2 × 10^8 cpm/μg cDNA. The probes were purified by filtration on G-50 sephadex columns. After hybridization, nylon membranes were washed twice for 10 min each at 42°C in 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) PVP, 0.02% (wt/vol) BSA, 5 × SSC, 0.05 M sodium phosphate and 0.1% (wt/vol) SDS, and in 5 × SSC/0.1% SDS for 5 min followed by the 3 min-wash in 2 × SSC/0.1% SDS at room temperature. The nylon membranes were exposed to X-ray film for 4-16 h in the presence of an intensifying screen at -80°C. After autoradiographic analyses, the ^{32}P-labelled oIFNτ cDNA probes were stripped from the nylon membranes by washing twice in 0.1 × SSC/0.5% SDS buffer for 20 min each at 95°C. Later the nylon membranes were prehybridized and hybridized with ^{32}P-labelled gamma (γ) actin cDNA probes (Gunning

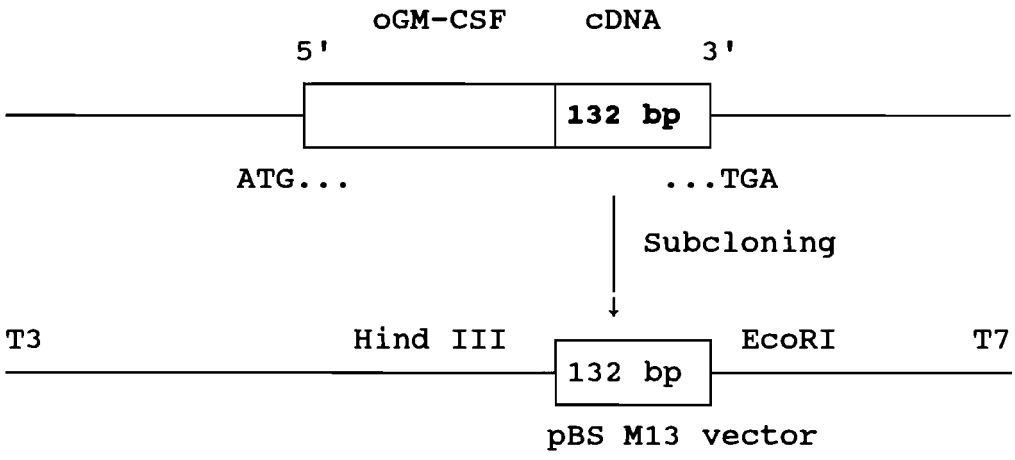
et al., 1983). Actin was considered to be a "housekeeping gene" whose amounts were consistent from cell to cell, providing the tool to assess amounts of tcRNA from sample to sample. The specific activity of the cDNA probes was approximately 1-2 $\times$ 10^8 cpm/μg cDNA. The conditions for hybridization and autoradiography were the same as aforementioned.

Paraffin Sections

For *in situ* hybridization experiments, endometrial tissues were obtained from day 8 and 12 cyclic ewes and days 13, 15, 17, 19 and 22 pregnant ewes (n = 1 per day). Conceptus tissues (day 15, n = 2) were also subjected to the analyses. Endometrial and conceptus tissue explants approximately 125 mm^3 (5 $\times$ 5 $\times$ 5 mm) and 60 mm^3, respectively, were fixed at 4°C in freshly prepared 4% (wt/vol) paraformaldehyde PBS buffer (pH 7.2) overnight, and in 70% ethanol, the following morning. Slides of serial 5-μm sections were carefully prepared with DEPC-treated H_20 at the Department of Histopathology, Wesley Medical Center, Wichita, KS. Slides were stored at -80°C until use.

Preparation of Digoxigenin Labeled cRNA Probes

Ovine GM-CSF cDNA was digested with restriction endonucleases Pst I and Sma and the 3' portion of this 132 base pair cDNA, which has higher specificity to detect the oGM-CSF mRNA, was subcloned into the plasmid vector pBS M13 for *in vitro* transcription. The orientation of the cDNA in the pBS M13 was determined by DNA sequencing (Dideoxy chain termination method) using DNA polymerase (sequenase).



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 GM-CSF 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 30 units of 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

In situ hybridization experiments were performed using the method described by Lawrence and Singer, as modified by Hunt et al. (1992) and Imakawa et al. (1993a). Before experiments, the 5-μm tissue sections were deparaffinized in xylene for 30 min

and hydrated with graded ethanol for 10 min. These sections were prehybridized for 10 min at room temperature in PBS containing 5 mM $MgCl_2$, and later incubated for 10 min at 65°C in a buffer containing 50% formamide and 2 × SSC. The sections were overlaid with 40-50 μl of a hybridization cocktail and coverslipped, then incubated overnight at 37°C in a humidified chamber. The tissue sections were washed for 30 min at 37°C in the buffer of 50% formamide and 2 × SSC, then in 50% formamide/1X SSC buffer at 37°C for 30 min, followed by 1 × SSC and by 0.1 × SSC for 30 min at RT. The sections were blocked with normal sheep serum containing 0.3% Triton X-100 for 30 min. The presence of oGM-CSF mRNA was detected immunologically using an anti-digoxigenin antibody prepared from sheep Fab fragments conjugated with alkaline phosphatase. After 3 h incubation with anti-digoxigenin antibody, the unbound antibody was removed by washing tissue sections with 1M Tris/3M NaCl buffer. Hybridization signals were detected with color reactions by adding a mixture of nitroblue tetrazolium/bromo chloro indoyl phosphate substrate and levamisole (to inhibit endogenous non-intestinal phosphatase activity) to tissue sections. The tissues were counterstained with methyl green. In other control preparations, tRNA was substituted for the digoxigenin incorporated cRNA probes.

RESULTS

Effects of hrGM-CSF on oIFNτ Production *In Vitro*

Northern blot analyses of tcRNA extracted from days 13, 15, 17, 19 and 22 conceptuses showed that the amounts of oIFNτ mRNA were increased by hrGM-CSF treatment in a dose dependent manner (data from day 17 conceptus are shown in Figure 4). In particular the relative amounts of oIFNτ mRNA were increased with 150 and 300 U/ml of hrGM-CSF, whereas the highest dose of hrGM-CSF at 600 U/ml seemed to be inhibitory to the transcription of oIFNτ genes. Autoradiographic signals (Figure 4) indicated increased amounts of mRNA for oIFNτ, as detected by ^{32}P-dCTP labelled oIFNτ cDNA probe. Increase in oIFNτ mRNA was similar on days 13, 15, 17, 19 and 22 of gestation at concentrations 150 and 300 U/ml of hrGM-CSF. Degrees of hybridization signals associated with actin mRNA indicated that amounts of tcRNA were similar, if not the same, among treatments (Figure 4).

Effects of hrIL-3, hrIL-6 and RA/hrGM-CSF on *In Vitro* Cultured Ovine Conceptuses

Northern blot analyses of tcRNA extracted from day 17 conceptuses revealed that when conceptuses were cultured with hrIL-3, oIFNτ mRNA was increased in a dose dependent manner with maximal induction at 75 U/ml (Figure 5). The hybridization signal for oIFNτ on day 17 conceptuses in the presence of 75 and 150 U/ml of hrIL-3 was higher than the one without hrIL-3 (Figure 5). In contrast, day 17 conceptuses cultured with hrIL-6 had increased amounts of oIFNτ mRNA regardless of doses tested (Figure 6). Northern blot analysis of day 17 conceptuses tcRNA revealed that conceptuses treated with RA had no effect on oIFNτ mRNA induction, however, conceptuses cultured together with hrGM-CSF and RA, the oIFNτ mRNA level was decreased (Figure 7).

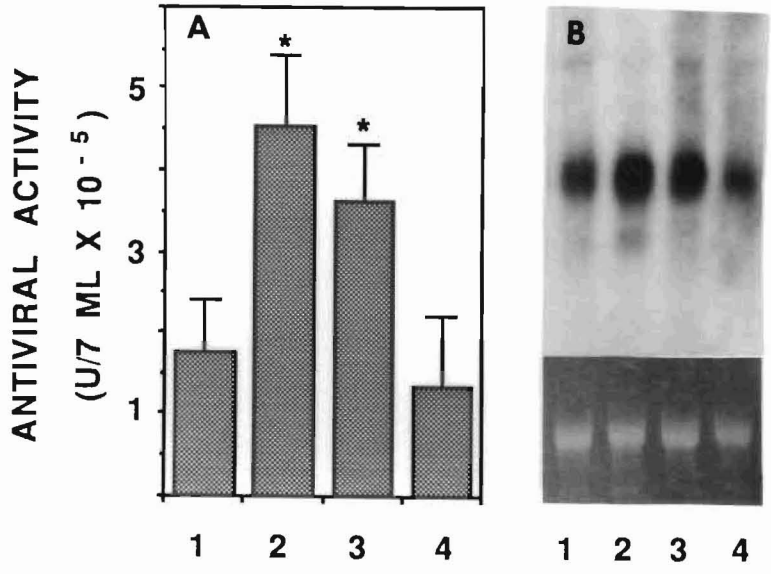


Figure 4: IFN associated antiviral activity and amounts of oIFNτ mRNA detected by northern blot analysis in day 17 conceptus. A. Antiviral activity in culture media; levels of antiviral activity are mean $\pm$ SEM for three experiments. Difference ($P < 0.05$) from control (0 U/ml hrGM-CSF) values by t-test are indicated by asterisks (data are from Imakawa et al., 1993a). B. Northern blot analysis detecting levels of oIFNτ in mRNA conceptus tissues after 24-h *in vitro* culture with various doses of hrGM-CSF. Lane 1 = conceptus cultured with 0 U/ml of hrGM-CSF. Lane 2 = 150 U/ml. Lane 3 = 300 U/ml. Lane 4 = 600 U/ml. Bottom B. Ethedium bromide-stained 18s rRNA illustrating the equal amounts of tcRNA used for the analysis.

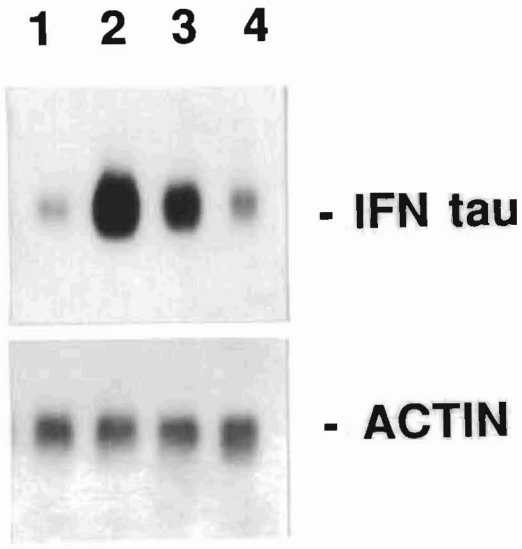


Figure 5: Northern blot analysis of oIFNτ mRNA levels after day 17 conceptus tissues were cultured for 24 h with various doses of hrIL-3: Top panel: (1) control no hrIL-3; (2) culture treated with 75 U/ml hrIL-3; (3) 150 U/ml hrIL-3; (4) 300 U/ml hrIL-3. Bottom panel: Northern blot analysis with γ-actin probe.

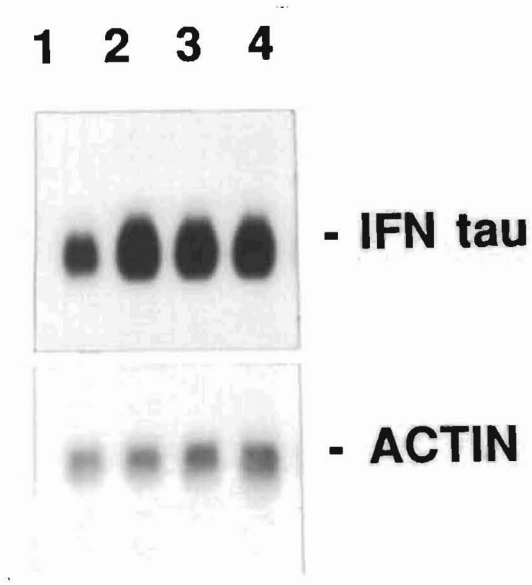


Figure 6: Northern blot analysis of oIFNτ mRNA levels after day 17 conceptus tissues were cultured with various doses of IL-6: Top panel: (1) control no hrIL-6; (2) culture treated with 75 U/ml hrIL-6; (3) 150 U/ml hrIL-6; (4) 300 U/ml hrIL-6. Bottom panel: Northern blot analysis with γ-actin probe.

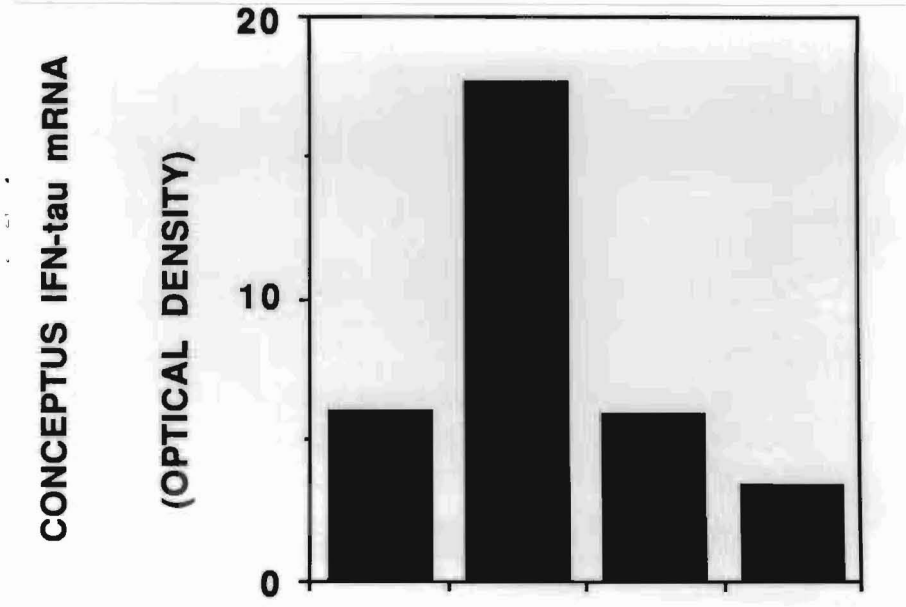


Figure 7: Densitometric analysis of oIFNτ mRNA levels detected by northern blot analysis: Conceptuses were cultured for 24 h with (1) no hrGM-CSF, no RA; (2) 150 U/ml hrGM-CSF; (3) 1 μM RA; (4) a combination of 150 U/ml hrGM-CSF and 1 μM RA.

Collectively these results demonstrated that hemopoietic cytokines GM-CSF, IL-3 and IL-6 induced increase in oIFNτ gene(s) transcription, as indicated by the increased amounts of oIFNτ mRNA, but RA when combined with GM-CSF decreased oIFNτ mRNA.

GM-CSF mRNA in Endometrium of Non-Pregnant/Cyclic Ewes

In situ hybridization experiments demonstrated that GM-CSF mRNA was present in all samples of endometrial tissues tested. Figure 8 shows the hybridization patterns of the antisense oGM-CSF cRNA probe to the tissue sections of endometrium taken from two different stages of estrous cycles. oGM-CSF mRNA was found in the luminal and glandular epithelium of endometrium in early luteal phase (Day 8) (Figure 8A) and late luteal phase (Day 12) (Figure 8C), and the levels of specific message as determined by the intensity of staining were minimal. No positive signals were obtained when semiserial sections of the tissues were incubated with the sense oGM-CSF cRNA probe (Figure 8 B and D). Other control experiments in which tRNA was substituted for the oGM-CSF probes also revealed negative results (not shown).

GM-CSF mRNA in Endometrium of Pregnant Ewes

Unlike the endometrium of cyclic ewes, where weak hybridization signals were observed in the luminal and glandular epithelium, distinct signals for GM-CSF mRNA were detected in the endometrium of pregnant ewes. GM-CSF transcripts appeared to increase as the pregnancy progressed, and the mRNA for GM-CSF was present in the luminal and glandular epithelium of endometrium on days 13 (Figure 9A), 15 (Figure 9C), 17 (Figure 9E), 19 (Figure 9G) and 22 (Figure 9I) of gestation. Tissue sections

hybridized with sense oGM-CSF cRNA probe showed no positive signal (negative control); figures 9B, 9D, 9F, 9H, and 9J. In other control experiments where tRNA was substituted for the oGM-CSF probe, no signals were detected (not shown).

GM-CSF mRNA in Conceptuses

In situ hybridization experiments were conducted on paraffin sections prepared from day 15 conceptuses (Figure 10). GM-CSF mRNA was localized specifically within the cytoplasm of the trophoblastic cells (Figure 10A and 10C). The embryonic disc and extraembryonic endoderm did not respond to the antisense probe (not shown). Positive signals were not detected either by using sense oGM-CSF cRNA probe (Figure 10B and 10D) or by tRNA (not shown) substituted for the oGM-CSF probe.

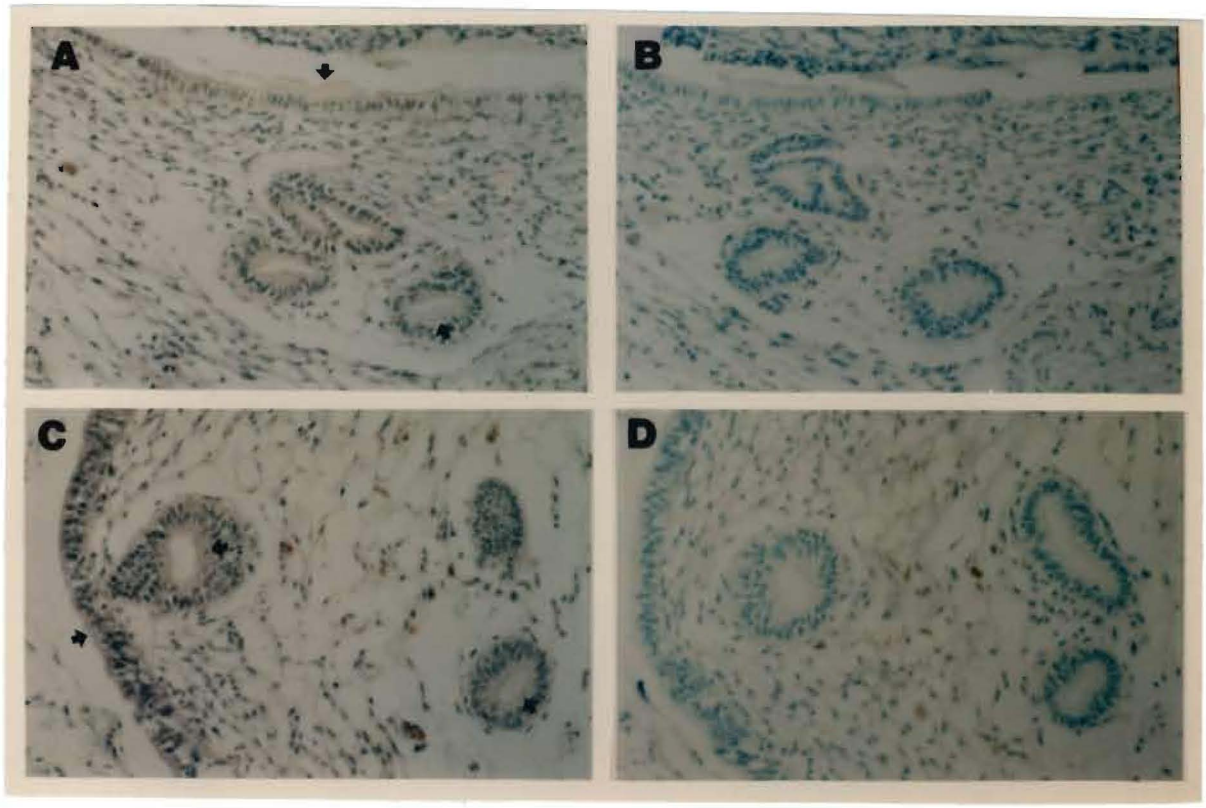
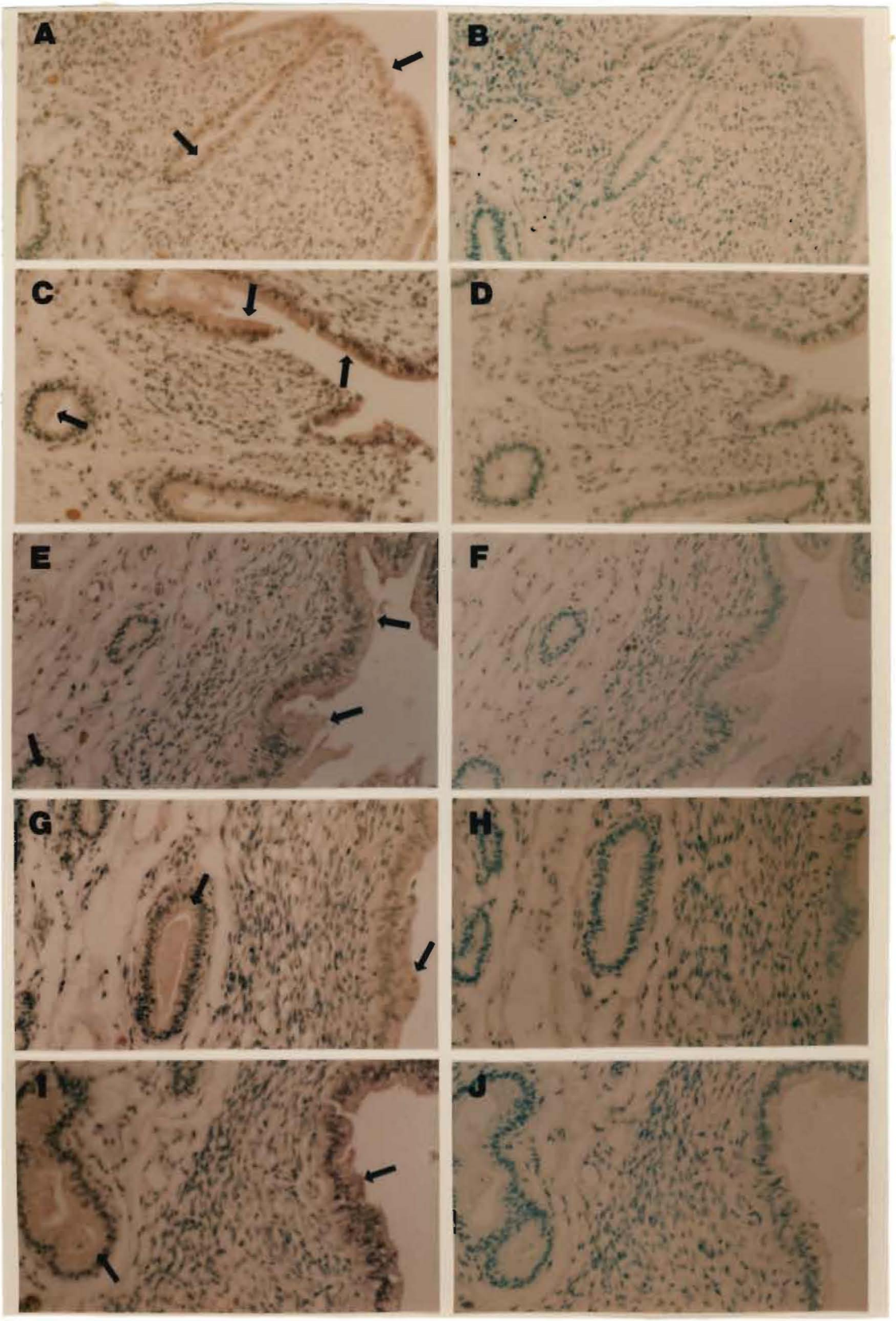


Figure 8: Cellular localization of GM-CSF mRNA in the endometrium of cyclic ewes: GM-CSF mRNA in the endometrium identified by hybridization with a digoxigenin labeled GM-CSF antisense cRNA probe; Figure 8A, luteal phase (Day 8) and Figure 8C, luteal phase (Day 12) show that the mRNA for GM-CSF was localized specifically at the luminal and glandular epithelial cells (arrows). Figures B and D show that no hybridization was detectable with sense version of the GM-CSF cRNA probe used as a control on semiserial sections of the same tissues.

Figure 9: Cellular localization of GM-CSF mRNA in the endometrium of pregnant ewes: Hybridization patterns obtained with the 132bp oGM-CSF probe are shown. GM-CSF transcripts were localized at both luminal and glandular epithelial cells (arrows) in the endometrium of pregnant ewes; days 13 (Figure A), 15 (Figure C), 17 (Figure E), 19 (Figure G), and 22 (Figure I). No hybridization signal was detected with the sense version of the GM-CSF cRNA probe; Figures B, D, F, H, and J.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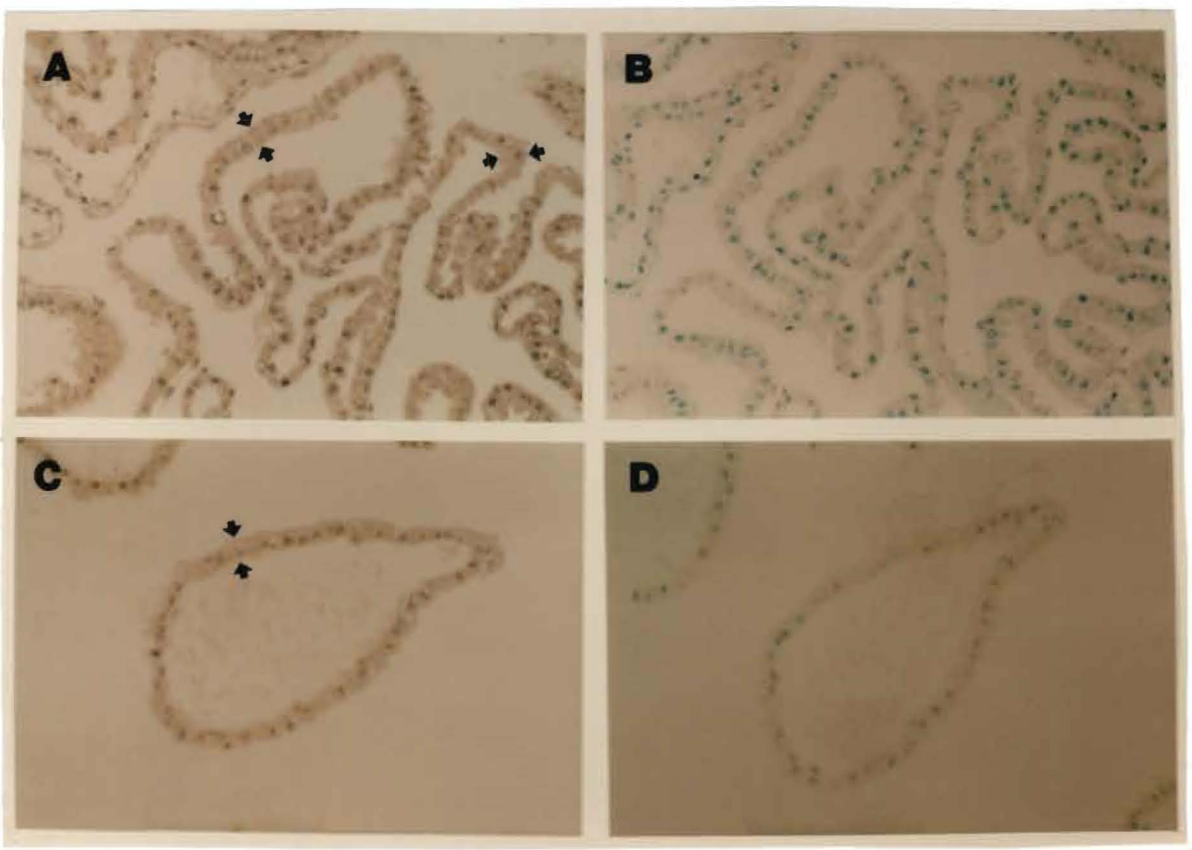

Figure 10: Cellular localization of GM-CSF mRNA in day 15 conceptuses: Figures A and C; GM-CSF mRNA detected with the antisense GM-CSF cRNA probe is localized in the cytoplasm of the extra-embryonic trophoblast (arrows). No hybridization signal is detectable using sense version of GM-CSF cRNA probe on the semiserial sections of the same tissue; Figures B and D.

DISCUSSION

A growing body of evidence has accumulated that hemopoietic cytokines, previously unsuspected, are expressed *in utero*. One of such cytokines GM-CSF has been detected in epithelial cells of murine endometrium (Robertson and Seamark, 1991). These investigators also show that uterine production of GM-CSF is abrogated in ovariectomized mice and that E_2 but not P_4 treatment in ovariectomized mice induces GM-CSF secretion at the endometrium. These results suggest that GM-CSF synthesis by the endometrial cells in mice is regulated by E_2, however, whether GM-CSF expression in sheep is controlled only by E_2 remains to be determined. In addition, GM-CSF has also been detected in decidual cells of murine endometrium (Wegmann et al., 1989), however, endometrial derived T-cells are not a major source of GM-CSF synthesis (Athanassaki et al., 1987; Wegmann et al., 1989). Futhermore, conventional and unique sizes of GM-CSF transcripts have been detected in tcRNA extracted from murine decidua (Crainie et al., 1990).

Using *in situ* hybridization methodology, the present study demonstrated that GM-CSF transcripts are localized exclusively at the luminal and glandular epithelial cells of uterine endometrium in days 13-22 pregnant and cyclic ewes. However, based on the intensity of the signals, the amounts of mRNA for GM-CSF are much higher in the endometrial tissues of pregnant ewes as compared to those in cyclic animals. Recently, Robertson et al. (1992) have shown that the endometrial epithelial cells isolated from both pregnant and non-pregnant mice are a potent source of GM-CSF and IL-6 *in vitro*, and that the epithelial cells harvested from both within and between implantation sites secrete high levels of GM-CSF comparable to the cells from earlier stages of pregnancy.

Results of the present study are consistent with those observations and suggest that GM-CSF of the maternal origin enhances oIFNτ production at the embryonic trophectoderm of the developing conceptus in a paracrine manner. Higher amounts of GM-CSF mRNA detected in the pregnant endometrium further suggest that the presence of conceptus is required for the full expression of maternal GM-CSF *in utero*.

GM-CSF treatment has been shown to induce transcription of nuclear protooncogenes c-fos and c-jun, which in turn initiates activation of various genes containing a transcription enhancer element, AP-1 (Adunyah et al., 1991). One of five oIFNτ genes recently isolated contains an AP-1 binding site in its 5' flanking region and this gene is highly expressed between days 13-20 of pregnancy (Nephew et al., 1993). Xavier et al. (1991) demonstrated that c-fos mRNA was specifically localized in cytoplasm and nuclei of trophoblastic cells in sheep and its existence paralleled that of oIFNτ. Apart from these observations GM-CSF was shown to enhance production of oIFNτ and oIFNτ mRNA *in vitro* (Imakawa et al., 1993a). These results strongly suggest that GM-CSF induces oIFNτ production at the trophectoderm through c-fos and AP-1 site, and that maternal GM-CSF acts on the conceptus in a paracrine manner. The present study further demonstrates that GM-CSF transcripts are also localized in the cytoplasm of the trophoblastic cells. Results of the present study and the fact that higher amounts of GM-CSF mRNA were detected in the pregnant endometrium suggest that conceptus expression of GM-CSF implicates the full expression of maternal GM-CSF *in utero*.

The present study confirmed the previous finding that IL-3 enhanced oIFNτ

mRNA *in vitro* and the degree of oIFNτ enhancement by IL-3 was very similar to that of GM-CSF (Imakawa et al., 1993b). It has been reported that binding of IL-3 to hemopoietic cells is partially inhibited by GM-CSF and the high affinity binding of GM-CSF is inhibited by IL-3 (Gesner et al.,1988). In addition, both IL-3 and GM-CSF induce similar intracellular signals and biological activities (Kanakura et al.,1990). However, it is not possible to determine whether IL-3 exerted its effects on oIFNτ production through its own receptor or by GM-CSF receptor (Kitamura et al.,1991).

Interleukin-6 is a multifunctional cytokine with diverse biological properties including regulation of hemopoiesis and participation in acute-phase reactions (Van Snick, 1990). Our study has clearly demonstrated that IL-6 enhances oIFNτ mRNA regardless of doses tested. Nishino et al.(1990) have shown that IL-6 derived from human placental trophoblast cells regulates the synthesis of human chorionic gonadotrophin (hCG) through its receptor in an autocrine fashion. In addition, Mathilagan et al. (1992) found the expression of IL-6 in porcine, ovine and bovine pre-implantation conceptuses. These findings suggest that IL-6 plays a role on trophoblast cell growth during conceptus development. It is interesting to note that IL-6 is also produced *in vitro* by mouse uterine glandular and luminal epithelial cells (Robertson et al., 1992). Therefore, it is reasonable to speculate that IL-6 produced by the endometrial tissue may enhance oIFNτ in a paracrine manner.

Results from *in vitro* cultures of conceptuses with a combination of GM-CSF/RA treatment revealed that RA down regulated the induction of oIFNτ mRNA by GM-CSF while RA alone did not affect amounts of oIFNτ mRNA. As described earlier, the

expression of one of five oIFNτ genes containing an AP-1 site in the 5' flanking region may in fact be controlled by GM-CSF through protooncogene products, Fos and Jun. Although RA is known to reduce c-jun expression (Busam et al., 1992), it is possible that RA inhibits protooncogene expression only when c-fos and c-jun genes are actively transcribed. Recently, it was shown that RBP was expressed in a transient manner during bovine estrous cycles (21 days/cycle); minute amounts on days 2-10, progressive increase toward late luteal phase (days 13-17), and barely detectable levels at the end of the luteal phase (day 20) (Liu and Godkin, 1992). In addition, Doré et al. (1992) demonstrated that maternal RBP expression was highest before embryonic expression and that once the embryonic RBP gene was activated maternal expression was reduced. These results suggest that RA, when transported to conceptuses by maternal and/or conceptus RBP, is actively involved in the regulation of oIFNτ gene expression *in utero*. Moreover, RA/retinoids modulate the expression of transforming growth factor-ß (TGF-ß) and epidermal growth factor/TGF-α receptor in certain embryonic derived cell lines (Jetten, 1980; Mummery et al.,1990), further suggesting a role of RA during conceptus/placental development.

In conclusion, it has become apparent that hemopoietic growth factors are involved in trophoblast/placental growth. In fact, GM-CSF has been shown to stimulate proliferation of choriocarcinoma cell lines JEG, JAR and BeWo (Wegmann and Guilbert, 1991). Co-expression of hemopoietic cytokines and trophoblast IFN (oIFNτ) during the period of pregnancy establishment suggest that GM-CSF, IL-3 and IL-6 participate in local regulation of trophoblast cell growth and differentiation, and possibly in local immuno-regulation *in utero*. Differences in temporal patterns of GM-CSF synthesis and

other cytokines expression strongly suggest that the individual cytokines may target conceptus growth as well as maternal endometrium proliferation at different stages of pregnancy. The expression of GM-CSF transcripts both in maternal-fetal compartments suggest the idea of autocrine and paracrine networks of cytokines during early pregnancy. Finally, uterine RA/retinoid with maternal and/or conceptus RBP participates in the regulation of oIFNτ, GM-CSF as well as other cytokines.

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A Novel Role for the Cytokine Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) in the induction of mRNA for Ovine Interferon tau (oIFN$_\tau$).

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