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THE IMMUNOBIOLOGY OF THE PORCINE PLACENTA

by

JAGDEECE J. RAMSOONDAR

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSPHY

IN

ANIMAL REPRODUCTION

DEPARTMENT OF ANIMAL SCIENCE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE IMMUNOBIOLOGY OF THE PORCINE PLACENTA submitted by JAGDEECE RAMSOONDAR in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in ANIMAL SCIENCE (ANIMAL REPRODUCTION).

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Date: Sept. 8, 1994

Dedicated to my parents John and Rajkumarie Ramsoondar and to my supervisor Dr. Thomas Wegmann. Although you contributed the most towards this work you could not oversee its completion. The memories of your love and support will always be with me.

ABSTRACT

The pig, an animal with an epitheliochorial placenta, was used as a model in experiments to investigate various aspects of fetal-maternal immune interactions. The objective of the *in-vivo* study was to determine whether immunization of gilts with leucocytes from the sires can improve their reproductive performance. Except for an increase in placental weights in animals treated with leucocytes, other parameters including the numbers of embryos and embryo mortality were not improved. Our results confirm that under optimal husbandry conditions this treatment offers no advantage in improving reproductive performance of gilts.

Trophoblast tissue constitutes the major barrier between maternal and fetal circulations. To characterize this cell type in the pig, we have isolated a cell line, designated Jag-1, from the trophoblastic tips of day 14 blastocysts. It was found to possess morphological characteristics unique to trophoblast. In addition, the Jag-1 cell line secretes a paracrine macrophage growth factor(s; MGF) as well as autocrine factor(s). Regulated expression of MHC antigens on trophoblast appears to be one of the evasive strategies employed in mammalian pregnancies to prevent rejection of the semiallogeneic fetus. To determine whether the simple non-invasive trophoblast of the pig also regulates placental MHC antigens, we determined the pattern of expression of Class I MHC antigens and mRNA in peri-implantation blastocysts and term placental tissues. Class I MHC antigens were not detected on the surfaces of these tissues by immunohistochemistry. However, they were detected in the vascular mesoderm of term placental tissues. The levels of Class I MHC mRNA, detected by Northern analysis and in-situ hybridization, was also considerably down-modulated.

The final study was undertaken to determine the expression of mRNA for cytokines (CSF-1, GM-CSF, IGF-1, IFN-γ, IL-2 and TGF-β1) at the maternal-fetal interface and to characterize the MGF secreted by the Jag-1 cells. The cytokine mRNAs were found to be temporally and spatially variable in their expression. All exhibited relatively strong expression in day 14-16 blastocysts and uterine epithelial cells. This coincides with the period of implantation when considerable changes are taking place in the conceptus and uterus in the pig. The MGF activity secreted in a protein free culture medium by the Jag-1 cells was concentrated by ultrafiltration, and the proteins separated by RP-FPLC, followed by SDS-PAGE. The active fraction contained at least four peptides, two of which appeared to be unique to this fraction with molecular weights around 45Kd. This size is similar to that of CSF-1 under reducing conditions. In addition, Jag-1 cells strongly expressed mRNA for CSF-1. Therefore, CSF-1 is a possible candidate for this MGF, however, it could be a novel pig trophoblast-derived cytokine. In conclusion, convincing evidence is presented that implicates a dynamic bidirectional interaction between the maternal immune system and the conceptus during pregnancy in the pig.

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LIST of ABBREVIATIONS

AI	Artificial Insemination
BEWO	Human Choriocarcinoma Cell Line
CL	Corpus Luteum
CSF-1	Colony Stimulating Factor-1
FCS	Fetal Calf Serum
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GTC	Guanidine Thiocyanate Salt
^{3}HTdR	Tritiated Thymidine
IFN-γ	Interferon-γ
IGF-1	Insulin-Like Growth Factor-1
IL-2	Interleukin-2
IP	Intraperitoneal
IU	Intrauterine
Jag-1	Porcine Trophoblast Cell Line
JAR	Human Choriocarcinoma Cell Line
JEG	Human Choriocarcinoma Cell Line
mAB	Monoclonal Antibody
mRNA	messenger Ribonucleic Acid
MGFs	Macrophage Growth Factors
MHC	Major Histocompatibility Antigens
NK	Natural Killer Cells
PBL	Peripheral Blood Leucocytes
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
P450 SCC	Cytochrome P450 Cholesterol Side Chain Cleavage Enzyme
RP-FPLC	Reverse Phase-Fast Protein Liquid Chromatography
RSA	Recurrent Spontaneous Abortion
rBoGM-CSF	Recombinant Bovine GM-CSF
SDS-PAGE	Sodium Dodecyl Sulphate -Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SLA	Swine Leucocyte Antigens
TEM	Transmission Electron Microscopy
TGF-β	Transforming Growth Factor-β

CHAPTER I

INTRODUCTION

A. Preimplantation Blastocyst Development in the Pig

Embryos enter the uterus primarily at the 4-cell cleavage stage approximately 2-3 days after ovulation (Perry & Rowlands 1962; Hunter, 1974). This delay appears to be necessary for the conditioning of the uterine environment (Hunter,1977). Activation of the embryonic genome and RNA synthesis commences at or about the 4-cell stage (Freitag *et al.*, 1988). A process of compaction then follows at the 8-cell cleavage stage on or about day 4, to form the morula (Hunter, 1974). The blastocyst stage is reached at day 5-6 upon formation of the blastocoele (Papaioannou & Ebert, 1988). This marks the beginning of the protective and nutritive functions of the trophectoderm (trophoblast). At this stage, the trophoblast is already developed into a layer of polarized cells surrounding the inner cell mass (ICM; Stroband and Van der Lende, 1990). From this point onwards the pig blastocyst undergoes a remarkable series of developmental changes.

Extensive expansion of the blastocyst takes place between days 6 to 11 resulting in a several thousand fold increase in volume (Geisert *et al.*, 1982). This expansion is most likely effected by the influx of fluid from the uterine lumen, with the trophectoderm as a selectively permeable barrier playing a major role (Barends *et al.*, 1989). The zona pellucida is lost (hatching) on day 6 (Hunter, 1977). The exact mechanisms responsible for the hatching of blastocysts are unknown. However, embryonic and/or uterine enzymes and the mechanical forces of expansion may be involved

(Stroband and Van der Lende, 1990). The newly hatched blastocyst is about 0.2 mm in diameter (Geisert *et al.*, 1982) and contains between 65-120 cells; 25% of which belongs to the inner cell mass (Stroband and Van der Lende, 1990).

After hatching there is a growing dependence on uterine secretions (Stroband and Van der Lende, 1990). At day 7, hypoblast cells proliferate from the base of the ICM and migrate along the inner surface of the trophectoderm to form a continuous but fenestrated layer of endoderm by day 8 (Barends *et al.*,1989). At this stage the blastocyst is bilaminar and migrates along both uterine horns. Transcornual migration is common in the pig (Dziuk *et al.*, 1964). The blastocysts are evenly spaced by day 10 as a direct result of myometrial peristalsis (Perry & Rowlands, 1962). Geisert *et al.* (1982) found that at day 10 spherical blastocysts increased about 0.25 mm/h in diameter, up to 9 mm. This is followed by a rapid increase in length, at a rate of approximately 30-45 mm/h, and is accompanied by a reduction in diameter. This rapid growth phase does not simultaneously occur in all blastocysts, and as a result, spherical, tubular and elongating blastocysts are present in the uterus on day 11 of pregnancy (Perry and Rowlands, 1962; Anderson, 1978).

Between days 11-12 blastocysts changed from a spherical shape of about 10 mm in diameter to a filamentous structure exceeding lengths of up to 1m (Geisert *et al.*, 1982). The early change in shape is apparently through cellular remodelling with later hyperplasia to produce the filamentous form (Geisert *et al.*, 1982). The ICM maintains a central position and a constant size (approximately 300 μm in diameter) throughout the changes in shape (Mattson *et al.*, 1990). In contrast, the

trophectoderm cells undergo changes in shape, size and organization of their cytoskeletal elements (Albertini *et al.*, 1987; Mattson *et al.*, 1990).

Mattson *et al.*, (1990) suggested that the trophectoderm may be actively involved in blastocyst morphogenesis. From day 11-12 onwards, mesoderm cells separate from the ICM and migrate between the trophectoderm and endoderm reaching about 20 cm from the ICM by day 15 (King & Ackerly, 1985; Barends *et al.*, 1989). Formation of the amnion by folding is completed by day 18 of gestation (Friess *et ,al.*, 1980). The allantois (a saclike extension of the hind gut) appears around day 14 and grows rapidly reaching the size of the embryo by day 17 (Fries *et al.*, 1980). The pig blastocyst remains free within the uterine lumen up to day 13 of gestation (Dantzer, 1985).

B. Blastocyst Implantation (Attachment) and Development of the Epitheliochorial Placenta

Preimplantation development, attachment and placentation represents a continuum of events. The elongated blastocysts become aligned along the mesometrial side of both uterine horns. They occupy almost the entire length of each horn, usually with no overlap, however ends do sometimes touch and adhere (Perry and Rowlands,1962; Anderson, 1978). The elongated blastocysts are accommodated within the uterine lumen by becoming very convoluted between the numerous folds of the endometrial mucosa (Anderson, 1978).

Implantation is superficial in the pig and occurs through a process of attaching to, rather than invasion of, maternal tissue. The process of attachment begins around day 13-14 (Dantzer, 1985). The blastocyst

becomes loosely adherent to the uterine lumen first in the region of the embryoblast and then attachment progresses to the tips (King *et al.*, 1982).

Adhesion is facilitated by the tacky nature of the blastocyst surface, probably due to changes in composition of the outer membranes of trophectoderm cells (Amoroso, 1952). Also, knob-like protrusions of the uterine lumen, produced in response to the presence of a conceptus aids in its anchorage (King *et al.*, 1982; Dantzer, 1985). In addition, the accumulation of fluid within the developing allantois eventually distends the entire vesicle intensifying contact and adhesion (King *et al.*, 1982). Soon after, junctional complexes form between the chorionic and uterine epithelial cells, and by day 18 attachment by interdigitating microvilli is evident (Perry *et al* ., 1976). This process of attachment is completed over the entire surface of the conceptus by day 26 (Amoroso 1952).

Chorionic cytoplasmic processes were found inserted between the endometrial epithelial cells but never reached the underlying basal lamina (Amoroso, 1952; King *et al.*, 1982). The mesoderm covering the allantois makes contact with the chorion on day 19 and by day 30 the chorion is well vascularized by allantoic blood vessels (Fries *et al.*, 1980). The placenta thus formed is simple, diffuse and functional. However, further modifications take place as gestation progresses. The chorionic membrane develops secondary folds to increase the surface area of contact with the endometrial epithelium. Furthermore, around day 30 the chorionic membrane overlying uterine glands develops into specialized structures called regular and irregular aereolae because of their shape and organization of microvilli around these glandular openings (Amoroso, 1952).

As pregnancy progresses, the secondary chorionic ridges become larger and considerably modified (King *et al.*, 1982). The position of the underlying capillary network within these ridges also changes (Fries *et al.*, 1980). These capillary plexuses deeply indent the epithelium on the lateral sides and tips of the ridges (Amoroso, 1952; Fries *et al.*, 1980). The interepithelial protrusion of the capillaries continues throughout pregnancy. The basal lamina of the capillaries usually becomes interfaced with that of the trophoblast to form a single lamina in the areas of indentation (Fries, *et al.*, 1980). The height of the trophoblast cells covering the ridges is progressively reduced. However, this is more pronounced at the sides and the tips. Only about 2 μm of trophoblast cytoplasm covers the interepithelial fetal capillaries at term (Amoroso, 1952). On the maternal side, uterine capillaries also project between the endometrial epithelial cells which are also generally reduced in height (Fries *et al.*, 1980). At term the maternal and fetal circulation are separated by 2 μm or less of placental tissue (Amoroso, 1952, Fries *et al.*, 1980). The development of the embryo proper is beyond the scope of this review.

C. Maternal-Embryo/Fetal Communications in the Establishment and Maintenance of Pregnancy: An Endocrinological/Physiological Perspective

C.1. Maternal Recognition of Pregnancy

The evolution of viviparity among mammals not only required a unique uterine microenvironment, but also, a system of maternal-fetal communication that is synchronous and efficient. There is evidence for the initiation of maternal recognition events soon after fertilization.

Apparently in some species, fertilized and unfertilized oocytes direct discriminate signals to the oviduct resulting in differential transport to the uterus. For example, in the mare, unfertilized oocytes are retained in the oviduct while fertilized ova are transported to the uterus (Betteridge and Mitchel, 1972). In addition, an early pregnancy factor (EPF) is detected in the plasma of several species within 24 h of fertilization (Koch, 1985). However, far more concrete evidence of maternal-embryonic communication is apparent during the establishment of pregnancy. The process by which the embryo signals its presence within the uterine lumen resulting in extension of the lifespan of the corpus luteum and maintenance of the pregnant state is known as "Maternal Recognition of Pregnancy." This process involves a complex sequence of morphological and biochemical events that are not mutually exclusive and are controlled by both local and systemic mechanisms. In some species, the functions of the corpus luteum (CL) are assumed by the placenta at later stages. However in the pig, functional CLs are necessary throughout pregnancy (Bazer and First, 1983).

The embryonic signal for pregnancy recognition in the pig is believed to be estrogen produced by the rapidly elongating blastocysts beginning on or about day 11-12 post estrus (Bazer and Thatcher, 1977). Embryos must be present in both uterine horns (at least 2 per horn) between days 10-12 to prevent luteolysis (Polge *et al.*, 1966; Dhindsa and Dziuk, 1968). The timing of migration and spacing to coincide with blastocyst elongation and estrogen production underscores the requirement for local stimulation over a large area of the uterus. Utilizing both *in vivo* and *in vitro* studies, it was found that the successful establishment and survival of blastocysts depend on rapid elongation and estrogen production (Bazer *et al.*, 1987; Geisert *et*

al., 1990). Convincing evidence has been accumulated for estrogens of conceptus origin as the antileuteolytic agent in the pig (see reviews, Ford and Young, 1993; Roberts *et al.*, 1993; Thatcher *et al.*, 1986).

However, there is an ongoing debate over the mechanisms by which estrogen effects maintenance of the CL and whether it is the sole antileuteolytic agent in the pig (Geisert *et al.*, 1990; Roberts *et al.*, 1993). Estrogen production by the blastocysts is transient and reaches a maximum during the period of elongation (Heap *et al.*, 1979). Evidence obtained from exogenous administration of estrogen at different times during the oestrous cycle prompted Geisert *et al.*, (1987) to suggest that the maintenance of prolonged CL functions requires two phases of estrogen stimulation, the first at day 11 and the second more prolonged phase between days 14-18. This pattern closely parallels natural estrogen production. The increase in conceptus estrogen synthesis is detected in the utero-ovarian vein (Ford *et al.*, 1982) and the lymphatic vessels draining the uterus (Magness and Ford, 1982), making it possible for both local and systemic effects during pregnancy.

C.2. Mechanisms of Action of Estrogen

The earliest sign of the effects of estrogen is an increase in blood flow to the uterus on day 11 (Ford *et al.*, 1982; Ford and Christensen, 1979). This increase in blood flow to the uterus coincides with the two phases of estrogen synthesis. In addition, there is an increase in blood flow to the ovaries and intraovarian to the CLs (see, Thatcher *et al.*, 1986). Conceptus estrogens on day 11 stimulate a rapid increase in Ca^{++} released into the uterine lumen coincidental with a downloading of secretory products (histotrophe) stored within vesicles in the endometrial epithelial cells (see,

Roberts *et.al.*, 1993). This significantly alters the uterine environment for the elongating conceptus (Geisert *et al.*, 1982). De novo protein synthesis and secretion is under the influence of progesterone and substantial activity is not realized until the late luteal phase (day 14-16) of the estrous cycle (Geisert *et al.*, 1982). The source of the luteolytic factor is the uterus, as hysterectomy before day 14 of the estrous cycle extends CL lifespan beyond 30 days (Melamphy and Anderson, 1968).

Prostaglandin F2α (PGF2α) secreted by the uterine endometrium is the luteolysin responsible for CL regression in the pig (for excellent reviews see, Bazer *et al.*, 1986, 1989; Geisert *et al.*, 1990). PGF2α is luteolytic in the pig and it is produced by the uterine endometrium (Bazer *et al.*, 1982). Bazer and Thatcher (1977) have proposed a hypothesis based on experimental information to explain how the antiluteolytic mechanism of estrogen operates in the pig. They proposed that estrogen produced by the blastocysts interacts with the uterine endometrium to alter the pattern of PGF2α secretion from an endocrine to an exocrine pathway. This would result in the reduction of PGF2α entering the uterine venous drainage and reaching the CL in amounts sufficient to cause luteolysis. A key assumption is that the direction of movement of PGF2α is determined by local concentration of estrogen produced by the elongating blastocysts.

However, the precise mechanism whereby estrogen prevents secretion of PGF2α in an endocrine direction is not known. Johnson *et al.*, (1988), found that changes in the tight junctional structures of the endometrial epithelium are under the influence of progestrone but do not directly play a role in rescue of the CL nor in the establishment of pregnancy. All cell types of the uterus and the conceptus are capable of secreting prostaglandins (Davis and Blair, 1993).

Similar dynamic changes were found for estrogen and prostaglandins in the uterine milieu during early pregnancy (Geisert *et al.*, 1982). Other luteolytic, luteoprotective or luteotrophic mechanisms for maintenance of the CL may exist in tandem with that of the antiluteolytic, anti-PGF2α effects of estrogen, and the relative importance of each may change during pregnancy (Thatcher *et al.*, 1986). In the sheep and cow a Type I interferon (IFN-τ) has been found to protect the CL from the luteolytic effect of PGF2α produced by the uterus (see, La Bonnardiere, 1993). Although pig blastocysts have been found to secrete both a Type I and Type II (IFN-τ, IFN-γ respectively) interferons (La Bonnardiere, 1993) an antiluteolytic protein has not been found in the pig.

C.3. Embryo Survival and the Need for Synchrony between Embryo Development and a Permissive Uterine Environment

In this polytocous species, it is apparent that the term "survival of the fittest" aptly describes the outcome of pregnancy. Prenatal mortality is relatively high with a range of 20-50% loss occurring naturally (Flint *et al.*, 1982; Pope and First, 1985; Boiet, 1986). This loss is usually higher in sows than in gilts (Bolet, 1986) and it is independent of fertilization rates (Anderson,1978; Polge, 1982). Genetic abnormalities account for about 10% of this loss and is unavoidable; the remainder is of unknown etiology (Flint *et al.*, 1982). The majority of prenatal mortality is embryonic and occurs within the first 40 days of gestation, followed by a 10-20% loss realized between days 50 and term (Pope and First, 1985). The second phase of loss is better understood, and is usually a manifestation of restricted placental development arising from overcrowding due to limited

uterine capacity (Pope and First, 1985). Little or no loss is incurred prior to days 7-10, at which time the numbers of developing embryos are represented by corresponding numbers of corpora lutea (Pope and First 1985). Not much is known about the early embryonic loss which evidently is clustered between days 12-18; very few embryos are lost between days 18-40 (Ford and Young, 1993).

It appears to be more than coincidental that the majority of embryonic loss occurs within the same window of time when migration, elongation and attachment of the blastocysts, and maternal recognition of pregnancy events are simultaneously taking place. A characteristic of embryo development in the pig is the considerable variation in morphological stages among littermates (Perry and Rowlands, 1962; Anderson, 1978). This is most evident between days 11-12 when blastocysts varying between 1 mm - 800 mm in size can be found within the same uterus (Anderson, 1978). Although the cause of this extensive diversity is not clear, and may have evolved to accommodate environmental changes and still ensure that a litter is produced, it is widely believed to be the root of embryonic loss in the pig. One group of workers have reported a similar diversity in follicular development and time of ovulation (Foxcroft and Hunter, 1985; Hunter and Wiesak, 1990). Because of this, ovulation is protracted over a 6h period. One school of thought is that the asynchronous embryo development evident at day 12 is a direct result of the asynchrony in follicular development and ovulation, and hence, the cause of embryonic mortality (Pope *et al.*, 1988; 1990). In contrast, when the prolific Chinese Meishan pig was used in comparative studies with European commercial breeds, no association was found between littermates asynchrony and litter size (see, Ford and Young, 1993). It was found that the prolificacy of the

Chinese Meishan pigs, which has 3-5 more piglets/litter, was due to lower embryo mortality (Haley and Lee, 1990). The explanation was that the growth rate and estrogen secretion was markedly reduced up to day 12. This allowed for a more gradual change in the uterine environment which was beneficial for survival (Ford and Young, 1993).

Little is known of the cause of early embryonic loss. An attractive hypothesis is that the more advanced blastocysts commence estrogen secretion earlier, and as a result, alters the uterine environment making it intolerable to the lesser developed blastocysts (Xie *et al.*, 1990; Geisert *et al.*, 1991). Evidence indicates that it is these lesser developed blastocysts that are lost (Roberts *et al.*, 1993). The secretory pattern of the endometrium must be synchronized with the developmental stage of the embryo in order for it to implement its own genetic program of development (Pope *et al.*, 1990). Bazer *et al.*, (1969) proposed that blastocysts compete for some unknown biochemical factor(s) that is in limited supply. Pope and First, (1985) aptly described the transient nature of uterine secretion as a "shifting window of uterine development." The asynchony between uterine and embryonic development is believed to be the major cause of embryonic loss in the pig (Anderson, 1978, Polge, 1982).

C.4. Components of the Uterine Luminal Fluid that are Important for Conceptus Development and Survival.

The conceptus depends on factors secreted by the uterine endometrium for its development and survival. The extent and duration of this dependence, however, varies among species and tends to correlate with the degree of invasiveness of the trophoblast during implantation

(Amoroso, 1952). The conceptus of the pig, which has a non-invasive form of implantation, is dependent on uterine secretions (histotrophe) for its nutrition and protection throughout pregnancy (Amoroso, 1952; Roberts and Bazer, 1988). The nature of endometrial support is poorly understood. Furthermore, the identification and characterization of proteins secreted by the endometrium and the conceptus is imperative for understanding the processes involved in embryo/fetal development and survival.

The uterus is under the influence of progesterone during the luteal phase of the estrous cycle and throughout pregnancy. Most proteins synthesized *de novo* and secreted by the endometrium are regulated by progesterone. However, beginning around day 9-11 blastocyst estrogens may synergize with progesterone to modify the quantity if not the quality of proteins secreted (Simmen and Simmen, 1990; Roberts and Bazer 1988). As mentioned in the previous section, the uterine environment places strong selection pressures on the conceptus population, accentuating the need for synchrony between the developmental stages of the blastocyst and uterine secretions. This becomes apparent around day 11 when there is a massive downloading of endometrial proteins into the uterine lumen that is coincidental with the onset of blastocyst estrogen synthesis (Anderson, 1978, Geisert *et al.*, 1982).

Before day 11 the amount of protein in uterine flushings are low (Geisert *et al.*, 1982). Endocytotic activity in trophoblast becomes upregulated around day 11 coincidental with the increase in uterine luminal protein content (Stroband 1984; Geisert *et al.*, 1982). Specialized chorionic structures (aerolae, described previously) develop around day 30 over the openings of secondary glands in the endometrium to absorb macromolecules and other products that are not easily diffusible (Amoroso

1952). Secretory activity of the endometrium is increased after day 30 reaching a maximum around day 60-75 then gradually decreases to term (reviewed by Roberts and Bazer, 1988).

C.4.1. Secretory products of the Uterine Endometrium

The best characterized protein secreted by the uterine glandular epithelium and the most abundant is uteroferrin (see, Roberts and Bazer, 1988; Roberts *et.al.*, 1993). It is a 35 Kd purple acid phosphatase that carries two atoms of iron per molecule. Its proposed function is the transport of iron via the chorionic aerolae into the fetal circulation (Roberts and Bazer, 1988). Protease inhibitors that bind to plasmin and trypsin are secreted by the epithelial cells of the endometrium (Roberts and Bazer, 1988). The proposed functions of these protease inhibitors include inhibition of the high amounts of Plasminogen Activator produced by blastocysts, and to preserve the integrity of the uterine epithelium from breakdown of the extracellular matrix by proteases. This was suggested to be one mechanism utilized by the endometrium to prevent trophoblast invasion (Roberts *et.al.*, 1993). Protease inhibitors may also protect against the degradation of proteins needed for blastocyst nutrition (Bazer and Roberts, 1988).

Other better characterized proteins secreted by the endometrial epithelium include lysozyme and retinol binding protein (RBP). RBP, like uteroferrin, is a transport protein and appears to regulate the supply of vitamin A to the conceptus (Bazer and Roberts, 1988). Growth factors such as TGF-β1, TGF-β2 and a 4-8 Kd mitogenic factor termed uterine luminal fluid mitogen that is distinct from epidermal growth factors (EGF), are produced by the endometrium (reviewed by Simmen and

Simmen, 1990). Other small molecular weight mitogens (<10 Kd) are also produced by the endometrium. One appears to be an analogue of EGF (Simmen and Simmen, 1990). Furthermore, there are a number of proteins produced in moderate amounts that remain to be characterized (Roberts *et al.*, 1993). In addition, all cell types of the uterus secrete protaglandins (PGs) and apart from PFG2α as the luteolytic agent in the pig, the other functions of PGs have not been elucidated (Roberts *et al.*, 1993).

C.4.2. Secretory Products of the Conceptus

The conceptus contributes towards the composition and modification of the surrounding uterine luminal milieu throughout its program of development. As early as 4 h after mating early pregnancy factor (EPF), a pregnancy associated protein, is detectable in the serum of female pigs and is present throughout pregnancy (reviewed by Koch, 1985). It is detected by its inhibition of rosette formation between T lymphocytes and heterologous erythrocytes (Koch,1985). As such, an immunosuppressive function was proposed for this factor. EPF is not species specific and was originally detected in the sera of mice and later humans, sheep, cattle and pigs (see Koch, 1985).

The conceptus of the pig secretes interferon-like molecules during implantation that are similar to IFN-α (Type-1 IFN; Mirando *et.al.*, 1990) and IFN-γ (Type II IFN; Lefevre *et.al.*, 1990). Maximum secretion of these interferons occurs around day 16 of pregnancy and in addition to the possible antiviral activity, these cytokines are known to exert multiple effects on cells of the immune system in a paracrine manner (see review, La Bonnardiere,1993). Type I conceptus interferons called trophoblast protein - 1 or interferon tau (IFN-τ) are also produced by the sheep (oTP-

1), cow (bTP-1) and goat (cTP-1). In these species, unlike that of the pig, IFN-τ is the antiluteolytic signal for maternal recognition of pregnancy. It interacts with receptors on the uterine endometrium to suppress the release of luteolytic amounts of PGF2α (see reviews, Bazer and Johnson, 1991). Retinol binding proteins are also produced by pig conceptuses beginning at day 10-12 (see, Roberts et.al., 1993). The culture of blastocyst tissue has revealed that a number of proteins are produced that remain to be identified and characterized (Godkin *et al.*, 1982). These may include growth factors, cytokines, hormones and carrier proteins that are hormonally regulated. In the present study, a trophoblast cell line isolated from the bulbous tips of day 14 blastocysts, together with primary day 14 blastocyst tissue, have been shown to produce a factor that stimulates the proliferation of monocyte/macrophages (Ramsoondar *et al.*, 1993). As previously mentioned, pig blastocysts produce considerable amounts of estrogens that are responsible for maternal recognition of pregnancy and that synergises with progesterone to maintain this state to term. Prostaglandins are also produced by all cells of the conceptus and there is a ten fold increase in uterine luminal fluid content of PGs during attachment (Davis and Blair, 1993). The role of PGs in the process of attachment and blastocyst development is unelucidated.

D. Materno-Embryo/Fetal Communications in the Establishment and Maintenance of Pregnancy: An Immunological Perspective

D.1 Maternal Immunological Responses during Pregnancy

The placenta of mammals presents a unique situation whereby genetically disparate epithelial tissues from the conceptus and the mother exist intimately in an immunological environment that defies the laws of transplantation. There is ample evidence that the maternal immune system recognizes and responds to this allogeneic tissue (Hunziker and Wegmann, 1986; Gill, 1988; Antczak and Allen, 1989). On the other hand, the maternal immune response to paternally derived antigens of the conceptus does not behave like a classical immune response to an allograft and, as such, is not destructive in normal pregnancy (Gill, 1988).

The earliest evidence for maternal immunological recognition of pregnancy is the presence of EPF (mentioned above); an immunosuppressive factor produced by the oviduct and ovary within 24-48h after fertilization in a number of species including the pig (Koch, 1985). Evidence for maternal cell mediated responses to pregnancy includes the enlargement of lymph nodes draining the uterus in rodents and humans (McLean *et al.*, 1974; Maroni and de Sousa, 1978; Billingham, 1971). There are also numerous macrophages at the implantation site after the onset of implantation in the rat (Tachi and Tachi, 1989), and an influx of large numbers of lymphocytes into the placentas of mice at mid gestation (Lala *et al.*, 1980). These changes were more pronounced in outbred than inbred pregnancies.

In the pig and cattle the opposite effects are seen; the numbers of intraepithelial lymphocytes are significantly reduced during early gestation, possibly to reduce the numbers of potentially damaging cells at the placental interface (King, 1988). In addition, there is a considerable increase in the numbers of lymphocytes of T-suppressor phenotypes in the uterine draining lymph nodes and peripheral blood of the sow (Georgieva, 1984). Furthermore, natural killer cell activities gradually increase to levels greater than that found in blood during the post-attachment period of pregnancy (Croy *et al.*, 1988). Large numbers of lymphoid cells were located in the necrotic tips of the allantochorion and interlocular zone of the endometrium (Marble, 1968; Flood, 1973). The most convincing evidence of cell mediated immunity comes from the equine species in respose to the endometrial cup formation (Antczak and Allen, 1989). There is an accumulation of leucocytes around the endometrial cups before their eventual demise around day 100-120 of gestation. This rejection response is greatly increased in interspecific pregnancies (Antczak and Allen, 1989).

Humoral responses are also elicited within the uterus in recognition of the presence of the conceptus. Cytotoxic antibodies generated against paternally inherited antigens present on the conceptus are found in maternal blood during and after pregnancy in a number of species (Bell and Billington, 1980; 1981; Ghani *et al.*, 1984, Antczak and Allen, 1989). These antibodies are mainly directed against paternal strain MHC antigens.

In the pig, local antibody production does occur in the uterus (Hussein *et al.*, 1983). Anti-spermàtozoa antigens result in an increase in stillbirths, but only after the second pregnancy (Veselsky *et al.*, 1981). Antibodies against paternal antigens may also be present in the sow as male karyotypes are found in 1-4% of metaphases from peripheral blood (Rudek and

Kwiatkowska, 1983). In mice anti-MHC cytotoxic antibodies were found to be of the non-complement fixing IgG_1 isotype (Bell and Billington, 1980). There is evidence that the maternal immune response is biased towards humoral rather than cell mediated immunity during pregnancy (Lin *et al.*, 1993). Other aspects of involvement of the immune system in the reproductive process include evidence for the generation of immunotrophic and immunosuppressive effects, and their possible influence on events leading up to and including ovulation and CL formation are addressed in later sections of this review.

D.2. Immune-Endocrine Network in Reproduction

It is increasingly becoming apparent to endocrinologists that the "missing link" between the interaction of hormones with their target cells and the effector arm of the responses generated in most cases are likely to be cytokines. In mammals, progesterone is the primary hormone responsible for engendering and maintaining the pregnant state, however, its precise role in this process is not clear (Stites *et al,* 1979). Progesterone has been shown to specifically prolong xenogenic and allogenic skin grafts and have strong anti-inflammatory effects (Siiteri *et al.*, 1977). Fittingly, these workers raised the question; "is progesterone Nature's immunosuppressant"?

Progesterone can block DNA synthesis in both mitogenic and allogenic stimulated lymphocytes (Clemens *et al.*, 1979; Mendelsohn *et al.*,1977). Mouse trophoblast and its supernatants were shown to have profound suppressive effects in mixed lymphocyte reaction (MLR) and cell mediated lympholysis (CML) tests (Van Vlasselaer and Vanderputte, 1984). The source of this immunosuppression was apparently progesterone

produced by the trophoblast, since anti-progesterone serum abrogated this effect when added. Progesterone was also found to block *in vitro* T cell activation (Sities *et.al.*, 1983), inhibit IL-1 induced T cell proliferation (Stites and Siiteri; 1983) and to interfere with IL-2 receptor binding (Van Vlasselaer and Vanderputte, 1986). The percentage of progesterone receptor positive (PR^+) lymphocytes, which are mainly $CD8^+$, was found to increase throughout gestation in humans (Szekeras-Bartho *et al.*, 1993).

In the presence of progesterone, these $CD8^+PR^+$ lymphocytes produce a 34 Kd immunosuppressive protein that inhibits the release of arachidonic acid, NK mediated cell lysis and antigen induced proliferation (Szekeres-Bartho *et al.*, 1993). This protein is present in the serum of pregnant women and disappears at term. Standaert *et al.*, (1990) reported that pig lymphocytes and monocytes stimulated the production of progesterone by granulosa cells *in vitro*. These workers later found that in response to LHRH and con A porcine lymphocytes secrete a bioactive and immunoreactive LH-like factor that stimulated progesterone production in the *in vitro* assay with granulosa cells (Standaert *et al.*, 1991a). In addition, these workers found that there were dynamic changes in blood leucocytes in the ovary during the processes of follicle atresia, ovulation, luteinization, and CL regression (Standaert *et al.*, 1991b). They postulated that these immune cells are involved in an immuno-endocrine network that regulates these processes.

The trophoblast becomes a component of the host immune system during pregnancy, and shares a number of characteristics with macrophages, which it closely resembles (Guilbert *et al.*, 1993). Macrophages are abundant in the uterus during pregnancy and together with trophoblast secrete a number of proteins including cytokines, to which

they may also respond. These cell types appear to be involved in an autocrine, paracrine cytokine-network which includes cytokines produced by the uterine epithelium (Guilbert *et al.*, 1993; Ramsoondar *et al.*, 1993). The cytokines involved in this network are described in a later section of this review, however, of significance here is that the production of these cytokines are apparently regulated by estrogen, progesterone or a combination of both (see Guilbert *et al.*, 1993). It has been discussed in a previous section that the pig blastocysts produce copious amounts of estrogen during the periimplantation period. This estrogen stimulates the endometrial epithelium to secrete a vast array of proteins. Some of these as yet unknown proteins could be cytokines involved in the regulatory network just described.

D.3. Proposed Mechanisms for Survival of the Fetal Allograft

D.3.1. The trophoblast barrier hypothesis

Morphological characteristics of pig trophoblast including evidence for the lack of surface expression of MHC antigens, and how this compares with that of other species, are described in chapter IV and V. This section focuses on the features of trophoblast that encouraged the proposal that trophoblast tissue functions as an anatomic barrier for protection from various harmful microorganisms, and against the potentially harmful effects of the maternal immune responses, the net result of which is the development and survival of the fetal allograft. A number of paternal antigens are expressed on cells of the placenta that can potentially elicit an immune response, the most important of which are the MHC antigens. Apparently, due to the selective expression of surface

molecules, trophoblast are resistant to both cellular and humoral destructive immune responses. Results of the present study indicated that pig trophoblasts do not express MHC antigens throughout gestation. A similar situation was reported for the sheep (Low *et al*, 1990).

Selective expression of MHC antigens on trophoblast have been found in other species (see Chapter V). A general feature is the lack of expression of these antigens on trophoblast subpopulations in direct contact with maternal blood. Some species expressed forms of Class I MHC antigens that are nonpolymorphic. Class II MHC antigens are not expressed on trophoblast among the species studied. As mentioned in a previous section, there is evidence of humoral responses to paternal antigens in various species, which increase with successive pregnancies. These antibodies appear to be of the non-complement fixing IgG1 isotype (Bell and Billington, 1980).

In the case of the pig, this sensitization occurs only after delivery and is mainly against blood group antigens also carried by lymphocytes (Renard, 1987). Maternal immunoglobulins, although secreted locally in the uterus (Hussein *et al.*, 1983) do not appear to cross the placenta in farm animals (Renard, 1987; Gill, 1988). Similarly, few if any, sensitized maternal cells cross into the fetal side of the placenta due to the physical barrier created by the trophoblast tissue (Gill, 1988). There appears to be selective passage of immunoglobulins across the placentas of mouse and human. However, in these species the placenta appears to be able to remove maternal anti-fetal antibodies that can be harmful to the fetus (reviewed by Singh et.al., 1983).

Anti-paternal Class I MHC antigens can be eluted from the placenta (Voisin and Chaouat, 1974) and it appears that these "blocking" antibodies

when complexed with antigens on the placenta are internalized by trophoblast cells and digested (Wegmann *et al.*, 1979; Chaouat *et al.*, 1983). Many infectious agents do not cross the placenta to infect the fetus probably due to this trophoblast barrier. Among pregnant women infected with the HIV virus, only around 30% transmit the virus across the placenta to infect the fetus (Gwinn *et al.*, 1991). Trophoblast tissue is also poorly antigenic and this is indicated by the few trophoblast-specific molecules against which specific antibodies can be generated (Anderson *et al.*, 1987; Antczak and Allen, 1989). This was clearly demonstrated by the survival of mouse trophoblast transplants at ectopic sites compared to the rejection of fetal transplants (Simmons and Russel, 1962).

Another dramatic demonstration of the central role of trophoblast in the survival of the fetus was provided by the "trojan horse" experiments of Croy and coworkers using wild mouse strains (Rossant *et al*, 1982; Croy *et al.*,1982). They showed that chimeras composed of *Mus caroli* embryonic cells enclosed within a trophoblastic shell of *Mus musculus* are protected when the transfer recipient is *Mus musculus*. Xenogenic transfer of whole embryos results in death due to loss of trophoblast function rather than lymphocyte mediated destruction of the trophoblast (Crepeau *et al.*, 1989).

Sinov *et al.*, (1993) showed that the trophoblast of implanting mouse blastocysts protected the inner cell mass from destruction by LPS-activated macrophages. This was achieved by processes of nonadhesion and active repulsion. Finally, it appears that mouse trophoblasts are resistant to a variety of cell mediated effector mechanisms. Mouse trophoblast was resistant, to allospecific cytotoxic T lymphocytes (CTL) *in vitro* even after treatment with IFN-γ, to antibody-dependent cell-mediated cytotoxicity, or to naive or IFN-γ activated NK cells (Zuckermann and Head, 1987; 1988).

In these studies, trophoblast was also shown to be an effective cold target inhibitor of tumor target cell killing in a dose-dependent manner. However, murine trophoblast can be killed *in vitro* by lymphokine (IL-2) activated killer cells(Drake and Head, 1989) or by granulated metrial gland cells (Steward and Mukhtar, 1988). Mouse trophoblast was also found to be resistant to TNF-α (Drake and Head, 1990). Human trophoblast was also found to display impaired susceptibility to MHC nonrestricted killer cells (Saji *et al.*, 1989).

D.3.2 The Immunotrophism Hypothesis

In humans, equids, mice and pigs, the maternal immune response to paternal antigens can enhance the chances of fetal survival, especially when it would otherwise be compromised (see, Gill and Wegmann ,1986; Murray, 1983). The idea that maternal T cells play a positive role in tissue growth and regeneration including that of the placenta (Immunotrophism) have been around since the turn of the century (reviewed by Green and Wegmann, 1986). This idea died prematurely due to the lack of reproducible observations and techniques to evaluate it that are now available. Evidence for the phenomenon of immunotrophism began resurfacing again in the 1980's when two independent groups found that among women suffering from recurrent spontaneous abortion (RSA) of unknown aetiology, a significant percentage carried their pregnancy to term after immunization with their husbands, or pooled third party lymphocytes (McIntyre and Faulk, 1983; Beer *et al.*, 1985). This technique is now widely used as a treatment for RSA even though success is based only on a single double-blind clinical trial (Mowbray *et al.*, 1985).

The most convincing evidence for an immunotrophic effect of maternal immunity on reproduction comes from animal studies. In the mouse, a fetal resorption prone model was developed by mating CBA females with DBA/2 males. This mating combination consistently displayed a resorption rate of 30% to 50% compared to a normal rate of about 10 percent (Clark *et al.*, 1980). Using this model, Wegmann and colleagues found that immunization of CBA females with Balb/c spleen cells having a similar MHC haplotype (H-2^d) as that of DBA/2 males dramatically reduced fetal loss, while simultaneously increasing fetal and placental size (Chaouat *et al.*, 1983a). A similar effect was not found with DBA/2 spleen cells. NK cells were implicated in the resorptions observed in this model; an influx of NK cells into the resorbing units was found.

Furthermore, treating CBA/2 females with the anti-asialo GM-1 antibody which reduces NK activity, prevented fetal loss (Gendron and Baines, 1988). The high fetal resorption rates in this mating combination could be a consequence of microbial infection, since siblings reared under germ free conditions or in ordinary animal rooms have normal and high resorption rates, respectively (Hamilton and Hamilton, 1987). It appears that a strong immune signal is sufficient to reverse this resorption rate, and complete Freund's adjuvant was effective in doing so (Toder *et al.*, 1989). Maternal T lymphocytes are implicated to be involved in generating the immunotrophic effects observed. Removal of maternal T cells by injecting CBA females with either polyclonal or monoclonal antibodies directed against T cells resulted in a decrease in placental growth and function (Athanassakis *et al.*, 1987; 1990).

The initial immunotrophism proposal states that maternal T cells may enhance placental growth and function in a paracrine manner by the release

of cytokines (Wegmann, 1984). Additional evidence for T cell involvement in this phenomenon comes from studies with the MRL-1pr/1pr strain of autoimmune mice having a T cell disorder. The placentas of these mice are large and have a dramatically increased level of phagocytosis (Chaouat *et al.*, 1983b). Spleen cells from these mice, but not cells from normal MRL mice can adoptively transfer protection to mice with high resorption rates. In addition, treatment of the MRL-1pr/1pr mice with anti-T cell monoclonal antibodies significantly reduce placental size and phagocytosis (Athanassakis *et al.*, 1990). It is now clear that the cytokines involved in enhancing development and survival of the fetus are not exclusive to T cells but are produced by other non-T cell types at the materno-fetal interface, especially the endometrial epithelium. This is discussed in a later section. Neither T cells nor an intact immune system are absolutely required for successful pregnancy. Croy and Chapeau, (1990) showed that mice deficient in NK cells and lacking B cells and T cells (scid/scid. bg/bg) can reproduce successfully in a gnotobiotic environment. Further information on immunotherapy as a treatment for embryo loss in humans and animals can be found in Chapter III.

D.3.3. The Immunosuppression Hypothesis

The uterus has been suggested to be an immunologically privileged site based on survival of allograft transplants (Beer and Billingham, 1974), and this was proferred as the reason for the acceptance of the fetal allograft. However, there is little evidence to support this hypothesis (Stites *et al.*, 1979). Furthermore, immune responses with immunological memory are generated in the uterus, albeit in a modified manner compared to that following systemic antigen stimulation (Gill, 1988). Experiments in

mice carried out by Clark and coworkers led them to propose that suppressor cells in the maternal decidua are responsible for survival of the fetal allograft (see Clark et.al, 1987).

They found two types of non-specific T suppressor cells in the deciduas from a number of strains of laboratory mice that prevent the generation of cytotoxic T lymphocytes *in vitro*. They described a small type of lymphocytic cells that localized to the implantation site and secreted a factor that blocks IL-2 stimulation of T cells (Clark, *et al.*, 1985). These suppressor cells were absent from the implantation sites of xenogeneic *Mus caroli* embryos in *Mus musculus* uterus on day 9.5 of pregnancy and the embryos were subjected to resorption (see Clark *et al.*, 1987). The numbers of small suppressor cells were also low in the CBA x DBA/2 pregnancy associated with high resorption. There was an increase in numbers of these cells by immunization with paternal strain BALB/c lymphocyte(Clark *et al.*, 1987). These workers also postulated that the suppressor factor may create a functional barrier to invasion of the trophoblast by maternal cells (see Clark *et al.*, 1987).

Brierly and Clark, (1985) also described a type of large suppressor cell having no cytoplasmic granules, detected in the uterus and draining lymph nodes during the first half of mouse pregnancy. They suggested that these cells may only block the afferent arm of the immune response without preventing the expression of immunity. Several other types of T and non-T suppressor cell types have been described, however their phenotype and specificity remains controversial (Clark *et al.*, 1983; Voisin, 1983).

Apart from suppressor cells, a plethora of substances having suppressor activity have been reported. Beaman and Hoversland, (1988)

used a monoclonal antibody (mAb 14-30) which binds T-cell suppressor factors (Tsf) to show that this antibody induces abortion in mice when injected at various times after mating. They showed that Tsf was present only in the uterus of pregnant animals. Croy *et al.*, (1988) reported that medium conditioned by day 10-19 pre-attachment bovine blastocysts were highly suppressive in PHA-stimulated bovine leucocyte cultures. Implanting mouse blastocysts were also reported to produce factor(s) that induced suppressor T cells (Mayumi *et al.*, 1985). Immunotherapy in RSA patients was reported to induce blocking antibodies such as anti HLA class II and anti-idiotypic antibodies having specific immunosuppressive properties, (Sugi *et al.*, 1981).

Placental cells have been shown to produce immunosuppressive factors (Choaout and Kolb, 1984; Van Vlasseler and Vandeputte, 1984; Duc *et.al.*, 1985). Seminal plasma also appears to possess immunosuppressive properties (Bouvet *et.al.*, 1990). As described before, progesterone receptor positive T lymphocytes produce a 34 kd immunosuppressive factor in response to progesterone (Szekeras-Bartho *et al*,1993). This factor was found to be responsible for the reduced resorption rate in CBA x DBA/2 matings when treated with CFA (Szekeres-Bartho *et al.*,1991). It appears that the immunosuppressive properties of progesterone might be an indirect one, such as that described by Szekeres-Bartho *et al.*, (1993), because higher than normal concentrations of progesterone are needed for immunosuppression of lymphocyte proliferation *in vitro* (Yagel *et al.*, 1987).

Other factors reported to be immunosuppressive include, early pregnancy factor (previously described), and α-Fetoprotein (AFP), an albumin produced by the fetal liver and yolk sac which increases in amount

in maternal serum during pregnancy. AFP was also found to inhibit macrophage expression of Ia antigens (Lu *et al.*, 1984). However, controversy exists as no immunosuppressive properties were found with highly purified rat or human AFP (see Stites *et al.*, 1979). As mentioned previously, anti paternal "blocking" antibodies and antibody-antigen complexes can be eluted from the placenta, and it is believed that they may serve an immunosuppressive function (Voisin and Chaouat, 1974,). Finally, prostaglandin E2 (PGE_2) is produced in large amounts during implantation in the pig (Roberts and First, 1985) and may have physiologic as well as immunosuppressive roles during implantation, and decidua formation in rodents (reviewed by Clark, 1991). The immunosuppressive effects of TGF-β and TGF-β-like molecules are discussed below.

In summary, it is apparent that there is evidence to support each of the proposed mechanisms for the survival of the fetal allograft. On the other hand, this phenomenon is complex and cannot be accounted for by any one hypothesis. The most logical conclusion, is that each of these mechanisms may contribute in some way towards the regulation of this process.

E. Regulatory Cytokine Network at the Maternal Fetal-Interface

The reality that, after decades of research, survival of the fetal allograft remains an enigma underscores the complexity of this phenomenon. Nevertheless, the picture that is emerging indicates that an intricate network of cytokines are temporally and spatially produced at the maternal-fetal interface which act via autocrine and paracrine mechanisms to regulate growth and development of the feto-placental unit, and allow

fetal survival to term. Central to this regulatory network are the cells and tissues that produce these cytokines. The uterine epithelium and trophoblast have emerged as key players, but of no lesser importance are the immune cells of maternal origin present in high numbers in the uterus in response to the presence of the conceptus (reviewed in previous section).

Trophoblast and macrophages share many structural and functional features (Guilbert *et al.*, 1993). Macrophages (and evidently trophoblast) are known to secrete a plethora of factors, some of which they respond to in an autocrine fashion (Nathan, 1987). Studies in our lab and others, have indicated that the growth and function of trophoblast are regulated by a similar subset of cytokines as that of macrophages (Guilbert *et al.*, 1993), the CSF family of cytokines. Trophoblast and trophoblast-like cell lines from mouse placentas were shown to proliferate in response to the EL4 T-lymphoma cell supernatants and recombinant IL-3, GM-CSF and CSF-1 (Armstrong and Chaouat, 1989; Athanassakis *et al.*, 1987). Injection of IL-3 or GM-CSF significantly reduced the rate of resorption in the CBA x DBA/2 mated mice (Chaouat *et al.*, 1990).

When the BL-6T2 tumor was injected into mice of particular strain combinations, high resorption rates resulted, due to the constitutive production of GM-CSF and CSF-1 by the tumor (Tartakovsky, 1989). The high amount of CSF-1 was suggested to be the abortifacient which probably disrupted the delicate cytokine balance. The receptor for CSF-1 encoded by the *c-fms* proto-oncogene was found to be temporally and spatially regulated in the placentas of humans (Joki *et al.*, 1993), mice (Regenstrieff and Rossant, 1989; Arceci *et al.*, 1989) and cattle (Beauchamp and Croy., 1991). As expected, it is highly expressed on the trophoblast tissue of the placenta in these species. In the mouse, CSF-1 is synthesized by the uterine

epithelium throughout gestation and levels increased 1000-fold by term (Daiter *et al.*, 1992). The central role played by this cytokine is demonstrable in the op/op mice which lack biologically active CSF-1 and are subfertile (Pollard *et al.*, 1991).

GM-CSF was shown to facilitate implantation *in vitro* into uterine epithelium (Robertson and Seamark,1991). It stimulated the release of placenta lactogen (hPL) and chorionic gonadotropin (hCG) by human term cytotrophoblast and also their differentiation *in vitro* (Garcia-Lloret *et al.*, 1991; Guilbert *et al.*,1991). The receptor for GM-CSF is expressed in the placenta (Gearing *et al.*, 1989) and high levels of GM-CSF appears to be produced by the uterine epithelium (Robertson *et al.*, 1992). In addition, *in situ* hybridization studies have localized GM-CSF transcripts in the decidua and spongiotrophoblast of the mouse (Kanzaki *et al.*, 1991). Novel cytokine transcripts for GM-CSF were also found in the placenta of the mouse (Crainie *et al.*, 1990).

Interleukin-3, another member of the CSF family of cytokines, is produced almost exclusively by activated T cells and appears to be constitutively secreted in the mouse placenta (Lin *et al*, 1993). IL-3, like GM-CSF, can prevent resorption in the CBA x DBA/2 mated mice (Chaouat *et.al.*, 1990). In addition, an IL-3 like activity was detected in the sera of pregnant women in increasing levels before delivery. Furthermore, *in vitro* production of this IL-3-like activity by mononuclear cells was found to be stimulated by progesterone in a dose-dependent manner (Fishman et.al., 1992).

The CSF cytokines are not the only ones involved in this network and it appears that, in normal pregnancy, a delicate balance is set between cytokines that have positive and negative influences at the materno-fetal

interface. Of course, this is an overly simplified statement, since cytokines that have an enhancing influence at one stage of development may have an inhibitory action at another. An example of this is evident from the *in vitro* study of Haimovici *et al.*, (1991) in which, blastocyst attachment was inhibited by GM-CSF and IL-1, wheras IL-1 and not GM-CSF enhanced trophoblast outgrowth. Lea and Clark, (1993) also reported that physiological amounts of GM-CSF had no effect on blastocyst and ectoplacental-cone trophoblast proliferation while higher levels were toxic. Hill *et al.*, (1987) found that human and mouse CSF, IFN-γ, IL-4, IL-1 and TNF-α arrested preimplantation mouse embryo development *in vitro*.

There is also evidence for an acute inflammatory response after mating in mice, with an influx of granulocytes and mononuclear cells and high levels of GM-CSF, CSF-1, IL-1, IFN-γ and IL-6 on day 1, which subsided by day 3 (Sanford and Wood, 1992). A number of these cytokines are also produced by uterine epithelial cells, including GM-CSF, IL-6, TNF-α , TGF-β and LIF (reviewed by Guilbert *et al.*, 1993). There is also evidence for an IL-2 -like molecule in human syncytiotrophoblast and amnion (Soubiran *et al.*, 1987). Masuhiro *et al.*, (1991) reported that IL-1 produced by trophoblast stimulated the release of hCG by activating IL-6 and IL-6 receptor system in first trimester human trophoblast. TGF-α, an epidermal growth factor-like cytokine that also binds to the EGF receptor, was found to be produced by trophoblast and decidua in humans and stimulated first trimester trophoblast to proliferate in vitro (Lysiak *et.al.*, 1993). TGF-β, is also produced by human trophoblast and decidua *in vitro*, but instead, inhibited the proliferation and stimulated the differentiation of trophoblast (Graham *et.al.*, 1992). IFNs are also produced at the maternal-fetal interface (described in a previous section).

Recent advances in lympho-hematopoietic cytokine biology have indicated that the T helper type-2 cytokines (IL-4, IL-5, IL-6, IL-10) might predominate locally in the uterus to regulate the maternal immune response (Lin *et al.*, 1993). In this network, an equilibrium between the Th2, together with the CSF cytokines produced by the uterine epithelium, and the Th1 cytokines (IL-2, IFN-γ, TNF-α) is necessary for the regulation of fetal development and survival. Disruption of this cytokine network would ultimately lead to embryo/fetal loss.

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CHAPTER II

OBJECTIVES, RATIONALE AND EXPERIMENTAL APPROACH

The overall objective of this study was to investigate possible interactions between the immune and reproductive systems during pregnancy that would offer an insight into how the semiallogeneic fetus survives to term in the pig.

Rationale: The placenta of mammals is a unique chimeric organ comprising of genetically disparate tissues of fetal and maternal origin existing in an immunological environment that defies the laws of transplantation. Moreover the maternal immune system recognizes and responds to paternally derived antigens of the conceptus as indicated by studies in human, mouse and horse [Hunziker and Wegmann, 1986; Gill, 1988; Antczak and Allen, 1989]. In the pig local antibody production does occur in the uterus [Hussein *et al.*, 1983]. Antibodies against the sire's antigens may also be present in the sow since male karyotypes originating from cells of male conceptuses were found in 1 - 4% of metaphases from maternal peripheral blood [Rudek and Kwiatkowska, 1983]. Furthemore, natural killer cell activity gradually increases to levels greater than that found in blood during the post-attachment period [Croy *et al.*, 1988].

Why then are pig fetuses not rejected? What are the characteristics of these interactions between the conceptuses and the maternal immune system that ensure their survival? In normal pregnancies the humoral and cell-mediated immune responses against the conceptus are not destructive

because they do not behave like classical immune responses to an allograft [Gill, 1988]. In addition, trophoblast tissue constitutes the major barrier between the maternal and fetal circulations. They appear to possess unique characteristics that facilitate not only the nourishment of the developing fetus but also its protection against harmful microorganisms and that of the maternal immune effector response [Gill, 1988; see Chapter I].

The pig is an economically important animal that experiences a high level of prenatal mortality [Pope and First, 1985]. This loss occurs naturally and ranges between 20-50% [Flint *et al.*, 1982; Pope and First, 1985; Bolet, 1986]. The majority of prenatal mortality is embryonic [Pope and First, 1985] and of unknown etiology [Flint *et al.*, 1982]. Not much is known about this early embryonic loss which is clustered between days 12-18; very few embryos are lost between days 18-40 (Ford and Young, 1993). Attempts to minimize this loss have met with little success [Flint *et al.*, 1982; Bolet, 1986]. A percentage of this loss may be immunologic as indicated in humans and experimental animals experiencing recurrent pregnancy wastage [Mowbray, 1987].

Understanding the role of the immune system during pregnancy may aid in the development of methods to reduce embryo loss in the pig. The following objectives were addressed in an attempt to realize the overal objective of this study.

Objective 1: To determine whether the treatment of gilts with leucocytes from the sire can improve their reproductive performance.

Rationale: Improvement of embryo survival in order to maximize litter size is an important concern, especially to pig producers. A great deal of

attention have already been focused on optimizing the plane of nutrition, health and other husbandry practices in order to increase litter size. Presently, development of ways to improve embryo survival offers the greatest potential for increasing the numbers of live piglets per litter at term [Flint *et al.*, 1982; Bolet, 1986]. Reports have indicated that SLA haplotype can influence embryo survival [Mallard *et al.*, 1987; Conley *et al.*, 1988]. However the technology is not yet available for selection of SLA mating combinations among outbred pigs [Mallard *et al.*, 1987].

Leucocyte immunotherapy as a clinical treatment for recurrent spontaneous abortion (RSA) among women is widespread in use [Coulam and Coulam, 1992]. This treatment has also been studied in the mouse and horse [see Gill and Wegmann, 1987] and the pig [Almlid, 1981; Murray, 1983, 1986; Blichfeldt, 1984; Van der Lende *et.al.*, 1986]. Both the addition of leucocytes to semen to increase its antigenicity [Almlid, 1981] and presensitization of gilts to paternal antigens present in semen [Murray, 1983; 1986] were reported to improve embryo survival. On the other hand, studies utilizing similar appproaches could not reproduce the same results [Blichfeldt, 1984; Van der Lende *et.al.*, 1986]. Controversy also exists among human studies with respect to the success of this treatment [Clark and Daya, 19991; Coulam and Coulam, 1992].

Since not all experiments with pigs have shown a consistent improvement in reproductive capacity in response to immunization to paternal antigens, this form of treatment remains questionable.

Experimental Approach: The present study reexamined this form of treatment using a protocol that incorporated both presensitization to leucocyte antigens from the sire and the addition of sire's leucocytes to

semen to increase its antigenicity. Treatment of gilts with leucocytes from the sire, autologous leucocytes or phospate buffered saline were the three treatments tested.

For presensitization to paternal antigens leucocytes were isolated from the sire's blood and 2 x 10^8 cells injected intraperitoneally (I.P.) at the onset of the first estrous cycle. Similarly 2 x 10^8 autologous leucocytes or PBS were injected I.P. in the control animal. At the onset of the second estrous cycle 2 x 10^8 frozen/thawed leucocytes or PBS were infused into the uterus immediately prior to artificial or natural mating. The gilts were slaughtered on average 30 days after first mating and parameters to assess reproductive performance measured [see Chapter III for details].

Objective 2a: To develop a reproducible method for the isolation of primary trophoblast in sufficient quantity for study.

2b: To isolate and characterize a cell line from the trophectoderm of day 14 blastocysts.

Rationale: Trophoblast constitutes the major barrier between the maternal and fetal circulation. Its strategic position at the materno-fetal interface enables trophoblast to provide physical, immunological and physiological protection to the developing fetus [Wegmann, 1990; Loke and King, 1991; Head, 1991]. There is emerging evidence that a cytokine network exists at the materno-fetal interface, in which most of the diverse cell types of maternal and fetal origin are integrated [Hunt, 1989; Guilbert, *et al.*, 1993]. Key players in this network are the uterine epithelial cells, macrophages and trophoblast, all highly secretory cell types [Hunt, 1990; Guilbert, *et al.*, 1991; Pollard, *et al.*, 1991; Robertson, *et al.*, 1992]. A major dilemma in elucidating the role of trophoblast in the maintainace of

pregnancy is to procure, in sufficient quantities, a homogenous population of these cells.

The pig periimplantation blastocysts offer an opportunity to obtain primary trophoblast in quantity and the potential for isolating trophoblast cell lines because of its unique form of development [Anderson, 1978; King *et al.*, 1982]. Success in realizing this objective would make possible a number of studies directed towards understanding the role of this cell type in pregnancy maintenance.

Experimental Approach: The pig blastocysts undergo considerable elongation during the preimplantation period [Anderson, 1978; Mattson *et al.*, 1990]. Except for a few centimeters occupied by the embryonic disc region, most of the tissue is trophoblastic [King and Ackerly, 1985]. Blastocyst tissue in culture forms two types of vesicles one of which appears within 24 h and is composed of pure trophectoderm. These vesicles collapse soon after formation to grow as monolayer colonies at the bottom of the well. To obtain primary trophoblast in quantity for study, the approach was to harvest these colonies within 48 h, pool and grow them in large tissue culture flasks.

To isolate the trophoblast cell line the bulbous tips of day 14 blastocysts were used. The underlying endoderm has yet to migrate as a complete layer into the bulbous ends [Mattson *et al.*, 1987]. Therefore the ends are made up of pure trophectoderm. The ends of a pool of blastocysts from one pig were disected into ~ 1 mm pieces and cultured one piece per well. The resulting monolayer colonies were harvested, pooled and similarly expanded as above. The cell line that resulted was assessed for trophoblast characteristics by light and electron microscopy and

immunohistochemistry. A monocyte/macrophage proliferation assay was developed to assess for the production of macrophage growth factors.

Objective 3: To characterize class I MHC antigen expression at the maternal-embryo/fetal interface in the pig.

Rationale: Trophoblast cells appear to have developed unique mechanisms for protecting the fetus from maternal immunologic rejection responses, one of which is the regulation of MHC antigen expression. MHC antigens of domestic animals have been implicated in influencing reproductive performance [Mallard *et al.*, 1991] and therefore have been considered in selective breeding programs [Mallard *et al.*, 1991;Van der Zijpp and Egbert, 1989]. Class II MHC antigens have not been detected on the trophoblast of any species under normal conditions [see reviews Head *et al.*, 1987; Loke and King, 1991; Head, 1991; Hunt, 1992; Bulmer and Johnson, 1985].

In contrast class I MHC is expressed, but differently, among species [Low, *et al.*, 1990; Donaldson, et al,. 1990; Head *et al.*, 1987; Loke and King, 1991; Head, 1991; Hunt, 1992; Bulmer and Johnson, 1985; Stern, *et al.*, 1987]. In human, mouse and rat placentas either classical or monomorphic forms of class I MHC antigens are expressed on trophoblast and these are restricted to certain subpopulations [Low, *et al.*, 1990; Donaldson, et al,. 1990; Head *et al.*, 1987; Loke and King, 1991; Head, 1991; Hunt, 1992; Bulmer and Johnson, 1985; Stern, et al., 1987]. A common feature among species with haemochorial placentas is the lack of MHC on trophoblast that are in direct contact with the maternal

circulation, that is, in the exchange area of the placenta [Head *et al.*, 1987; Loke and King, 1991; Head, 1991; Hunt, 1992].

The few studies of MHC antigen expression in the epitheliochorial placentas of farm animals also indicated highly regulated expression [Crump, *et al.*, 1987; Donaldson, *et al.*, 1990; Templeton, *et al.*, 1987; Low, *et al.*, 1990]. Preimplantation pig blastocysts have been reported to express β2-microglobulin between days six and twelve of gestation [Meziou, *et al.*, 1983] and class I MHC antigen was serologically detected on preimplantation embryos between days two to six of gestation [Warner, *et al.*, 1986]. However, there has been no localization of MHC expression in pig embryos. The rather high resorption rate in pig pregnancies [Flint, *et al.*, 1982; Bolet, *et al.*, 1986] and the commercial importance of the species argues for a greater understanding of where and how MHC class I is expressed in the embryo.

Experimental Approach: Periimplantation blastocysts from days 14, 16 and 22 of pregnancy and extrafetal membranes at term were assessed for SLA class I MHC antigen expression at both the pre-(mRNA) and post-(antigen) translation levels, the former by Northern and *in situ* analyses with cross-reactive bovine cDNA probes and the latter immunohistochemically with a monoclonal antibody that recognizes a common determinant on all class I MHC antigens (see Chapter V).

Objective 4: To characterize the expression of cytokines at the maternal-embryo/fetal interface and the macrophage growth factor (s; MGF) produced by the Jag-1 trophoblast cell line.

Rationale: The identification of cytokines at the maternal-fetal interface in farm animals is lagging behind that of human and mouse [reviewed by Hunt 1989; La Bonnardiere, 1993, Guilbert *et al.*, 1993]. This underscores the need for more studies to address this problem in farm animals. Knowledge of the temporal and spatial expression of cytokines in the placenta of these species can help in elucidating the roles of cytokines in the development of feto-placental unit. The uterine epithelium, macrophages and trophoblast appear to be the sources of most cytokines produced within the placenta [Hunt, 1989; Guilbert *et al.*, 1993].

Macrophages share many morphological and functional characteristics with that of trophoblast including the production of factors to which they are mutually responsive [Guilbert, *et al.*, 1993]. The Jag-1 trophoblast cells produce an MGF that needs to be characterized. In determining the expression of mRNA for cytokines that are known MGFs in primary trophoblast and the Jag-1 cells, possible candidates for the MGF produced by these cells can be uncovered. This information together with data from the purification and characterization of this factor can help in its identification.

Experimental Approach: Total RNA from periimplantation blastocysts, extra fetal membranes at term, pooled day 14-16 uterine epithelial cells, the Jag-1 trophoblast cell line and unstimulated peripheral blood leucocytes, was analysed by Northern blotting for expression of the mRNA

of various cytokines including CSF-1 and GM-CSF. A protein free tissue culture medium was used to obtain medium conditioned by the Jag-1 cells (PFM-CM). The PFM-CM was concentrated by ultrafiltration and proteins separated by reverse phase- fast protein liquid cromatography (RP-FPLC). The fractions collected were tested for activity in the monocyte/macrophage proliferation bioassay. The proteins in all the fractions were then further separated by SDS-PAGE. The molecular weights of the bands in the active fraction were determined. These weights were compared to those of known cytokines, and together with the mRNA data a possible candidate for the MGF was suggested (reported in Chapter VI).

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CHAPTER III

TREATMENT OF GILTS WITH LEUCOCYTES FROM THE SIRE : Can it Improve Reproductive Performance?

INTRODUCTION

One of the primary objectives of research in pig reproduction is to develop techniques for improving embryo survival in order to maximize litter size. This is an important concern since embryonic mortality is relatively high in this species, and the majority of this loss occurs within the first 30 days of gestation [see, Pope and First, 1985]. Attempts to minimize this early loss have met with little success [Flint *et al.*, 1982; Bolet, 1986]. An association of swine leucocyte antigen (SLA) haplotype and embryo survival have been suggested from studies utilizing SLA defined miniature pigs (Mallard *et al.*, 1987; Conley *et al.*, 1988). However, selection of SLA mating combinations among outbred pigs in order to maximize litter size awaits the development of more sensitive typing reagents and the establishment of a universally accepted SLA nomenclature (Mallard *et al.*, 1987).

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Survival of the fetal allograft, an immunological paradox, has been the subject of numerous studies in various species [see reviews: Gill and Wegmann, 1987; Beer, 1988; Clark, 1991; Wegmann, 1990; Wegmann *et al.*, 1993]. Paternal antigens, present in semen during insemination [Thaler, 1989; Coulam and Coulam, 1992] and at the maternal-fetal interface, are recognized by the maternal immune system but the ensuing response is normally not deleterious to the developing fetus [see reviews: Beer, 1988; Loke, 1991; Head, 1991]. Under certain conditions, maternal immune recognition of paternal antigens can lead to the development of heavier feto-placental units and improved fetal survival [Hamilton and Hamilton, 1987; Wegmann *et al.*, 1989; Guilbert *et al.*, 1991].

Immunotherapy as a clinical treatment for recurrent spontaneous abortion (RSA) among women is widespread but controversial. However, trials are underway to resolve the existing controversy [see reviews: Clark and Daya, 1991; Coulam and Coulam, 1992]. Leucocyte immunotherapy has also been studied in the mouse, horse [see, Gill and Wegmann, 1987] and the pig [Almlid, 1981; Murray, 1983, 1986; Blichfeldt, 1984; Van der Lende *et al.*, 1986]. Almlid [1981] reported that the addition of leucocytes to semen at the time of artificial insemination (AI) improved its antigenicity and resulted in an increase in numbers of viable embryos by 12.5%. Murray, [1983, 1986] using a slightly different approach, based upon presensitization of gilts to paternal antigens present in semen (by exposure of the uterus to semen a few weeks prior to conception), reported an increase in litter size at birth. On the other hand, Blichfeldt, [1984] and Van der Lende *et al.*, [1986] found that there was no improvement in embryo survival when the antigenicity of AI semen was increased. Since not all experiments with pigs have shown a consistent improvement in

reproductive capacity in response to immunization to paternal antigens, this form of treatment remains questionable. The present study reexamined this issue using a presensitization approach in an attempt to establish a rationale for or against the use of this procedure in pig reproduction and as a parallel to *in vitro* studies of the porcine trophoblast.

MATERIALS AND METHODS

Animals

This study was conducted at the University of Alberta Swine Research facilities on outbred pigs of the Yorkshire breed. The recommendations of the Canadian Council on Animal Care were followed in the treatment and care of the animals. They were fed a ration composed of approximately 16.1 MJ ME per Kg and 13.42% crude protein ad libitum prior to mating and twice daily post-mating. A total of 24 second-estrous gilts were bred by artificial insemination in the first experiment and 45 second-estrous gilts were naturally mated in the second experiment.

Treatment Groups

Peripubertal littermate gilts (90-95Kg) were penned in groups of four to six within close proximity to a mature boar for the induction of puberty. Gilts were monitored daily for the onset of first estrous, at which time they were randomly assigned to one of the following three treatment groups:

Group A: Each gilt received leucocytes isolated from one of three boars (randomly selected) in experiment 1 and one of five boars in experiment 2 to which it was mated.

Group B: Each gilt received leucocytes isolated from its own blood (autologous leucocytes).

Group C: Each Gilt received Phosphate buffered saline (PBS) as a control.

Isolation of Peripheral Blood Leucocytes

When each gilt in group A came into first estrous, a single blood sample was collected from the prospective sire. Likewise, a single blood sample was collected from each gilt at first estrus in group B. Blood was collected by jugular venipuncture into heparinized sterile evacuated tubes for the isolation of leucocytes. The blood was then transfered to a 50 ml tube and centrifuged for 30 min at 4°C and a speed of 1500 x g. The clear plasma at the top was aspirated off and the buffy coat carefully collected. Red blood cell contamination was reduced to a minimum by hypotonic lysis. This was achieved by sequential suspension of cells in sterile double distilled water for 20-30 seconds, then in 2x PBS (equal volume) before centrifugation at 600 x g for 10 minutes. Isolated leucocytes were next pooled and passed through a double layer of gauze to remove cell clumps and debris. Live cells were enumerated in 0.4% (w/v) trypan blue using a haemocytometer. The cells were then divided into two aliquots of approximately 2 x 10^8 cells each. One aliquot was resuspended in 2 ml of PBS for the intraperitoneal injection at first estrous and the second aliquot frozen in liquid nitrogen (-196°C) for use just prior to mating at the onset of second estrous.

Treatment Regimen

Detection of standing heat was designated as day 0. At the onset of the first estrous cycle gilts in treatment group A were injected intraperitoneally (IP) with 2 ml PBS containing 2 $X10^8$ freshly isolated leucocytes from the prospective sire, group B gilts each received a similar IP dose of their own leucocytes while group C gilts were injected with 2 ml PBS (IP). The gilts were monitored for the onset of second estrous. On day 0 of the second estrous cycle, gilts in group A were given an intrauterine (IU) administration of 2 x 10^8 leucocytes in 25 ml PBS using sterile disposable AI spirettes. This second dose of leucocytes was thawed from stock frozen from blood collected at first estrus. Gilts in group B were similarly administered IU with their own frozen-thawed leucocytes while those in group C received IU 25 ml PBS .

Within 1 h after this second IU leucocyte treatment, the gilt was either artificially inseminated with 50 ml of whole semen collected by hand from the appropriate boar in experiment 1 or naturally mated to the appropriate boar in experiment 2. Artificial insemination or natural mating was repeated the following day. Gilts were slaughtered 28-32 days (average 30 $\pm$ 1.3) after first mating. The reproductive tracts were recovered and taken to the lab on ice where the following measurements were recorded: 1) number of corpora lutea 2) number of viable/nonviable embryos 3) weight of the uterus 4) weight of the ovaries 5) weight of the conceptus (the placenta without the chorioallantoic fluid but including the embryo and the amniotic fluid) 6) weight of the placenta (extraembryonic membranes) 7) weight of the embryo and 8) weight of the chorioallantoic fluid.

Data was collected from a total of 24 gilts in experiment 1 (Group A, 11gilts; Group B, 7; and Group C, 5) and semen was collected from

three boars randomly selected but all three represented in each treatment group. Instead of diluted semen, 50 ml of the freshly collected whole ejaculate was used for each artificial insemination in order to maintain the semen antigenicity. In experiment 2, data was collected from 45 gilts (15 gilts per treatment group). The same five boars were used for natural mating across all three treatment groups. Three gilts from each group were randomly selected and mated to the same boar for a total of nine gilts per boar. Gilts that did not come into first estrus within one week after selection and those that failed to return to second estrus within one week from their due date were replaced by new gilts in this experiment. Gilts bred but not pregnant at slaughter were also replaced. Two samples, one from each uterine horn comprising a small portion of the uterine wall with the chorioallantoic membrane still attached, were taken from each pig and fixed in Bouin solution and checked later for possible infection.

Statistical Analyses

The variables were analyzed using PROC GLM (SAS, 1990). A randomized block design was used: sires being treated as blocks in experiment 2. Gestation length was used as the covariate. One gilt from group A (experiment 2) was not included in the analysis due to the presence of only three implanting embryos with no evidence of any infection. At least four implanting embryos are required to maintain pregnancy (Polge, 1966).

RESULTS

The weight gains from the time of selection to that of slaughter were monitored and found to be uniform among treatments in both experiments. The proportion of gilts bred but not pregnant at slaughter (14 %) was similar for each treatment group. A summary of the data for experiment 1 is presented in Table 1. Except for placental weights there were no significant differences among the three treatment groups for the parameters measured. Placental weights for gilts treated with leucocytes, whether from the sire or autologous, were significantly different from the saline control group ($p < 0.05$) for animals bred by AI (Table 1). Embryo weights showed a similar trend where the differences observed bordered on significance ($0.05 < p < 0.07$). On the other hand, although embryo survival was within the normal range, there was a tendency towards reduced survival rates in the leucocyte treatment groups. The average chorioallantoic fluid weight also was slightly greater for the leucocyte treatment groups.

In experiment 2, a total of 44 pregnant gilts were assessed for their reproductive performance after the various treatments. A summary of the data is presented in Table 2. There was no significant difference among the three treatment groups for any of the parameters measured. The large variability within each group precluded any apparent evidence of statistical difference among the treatment groups. Although mean ovulation rates (number of CLs), number of viable embryos, embryo survival percentages (No viable embryos / No of CLs x 100) and uterine weights were not statistically different among groups ($p > 0.05$), they were skewed to favour the PBS control treated animals. On average, viable embryos in the PBS

control treated group were higher when compared to that of either of the two leucocyte treatment groups. This difference approached statistical significance between the boar leucocyte treatment group and the PBS treated animals ($0.05 < p < 0.06$). Figure 1, shows the distribution of the percent embryo loss per gilt within each group and to which of the five boars each gilt was bred. Mean embryo loss (calculated as, [(No. of CLs - No. of viable embryos) / No of CLs] x 100) for the boar leucocyte Group A was almost double that of the PBS treated animals. However, this loss was highly variable among the animals within each group.

A trend towards heavier conceptus and placenta weights was evident in the leucocyte treated groups when compared to those of the PBS controls in experiment 2 (Table 2). Gilts exposed to the sire leucocytes prior to mating had on average heavier conceptus and placenta weights. In addition, there were significant boar ($p< 0.05$) and boar by treatment interaction ($p< 0.05$) effects on embryo and placenta weights. Gilts receiving either the sire or autologous leucocytes showed increased placenta and embryo weights when mated to two of the boars and a decrease when mated to the other three.

The data for both leucocyte treatment groups from the two experiments were pooled and compared to those of the pooled PBS control group (Table 3). The difference in the number of viable embryos was now significant ($p < 0.05$) with the PBS control group having about a 10% better embryo survival. Placenta weights were higher for the leucocyte treated animals ($p < 0.05$).

DISCUSSION

The results shown here support other studies [see, Blichfeldt, 1984] in confirming that within an outbred population of pigs kept under optimum husbandry conditions, pretreatment of females with leucocytes from the sire prior to insemination offers no enhancement of subsequent reproductive ability. There was no evidence of improved reproductive performance in animals receiving this treatment. Overall, mean embryo survival was within the normal range reported for first parity animals during the fifth week, the stage of pregnancy looked at in this study [Flint *et al.*, 1982; Bolet, 1986]. However, embryo survival for animals treated with leucocytes, whether autologous or sire's, were on average lower than that of the PBS treatment group. The increase in placental, embryo and chorioallantoic fluid weights observed for the leucocyte treatment group was most likely as a result of fewer numbers of conceptuses within the uterus. However, an immunotrophic effect cannot be ruled out [Wegmann 1990, Wegmann *et al.*, 1989, 1993].

The main reason for the controversy among studies evaluating this procedure is that there are reports, including the present study, of an equally high success rate within the control (PBS or autologous leucocytes) treatment groups [see reviews: Clark and Daya, 1991; Coulam and Coulam, 1992]. This evidence prompted Clark and Daya [1991] to suggest that immunotherapy may act via a placebo effect rather than by an immunological mechanism. Further support for such an assumption came from studies showing that psychotherapy was equally as effective as leucocyte immunotherapy in increasing the rate of successful pregnancy among recurrent spontaneous abortion-prone (RSA) women [Stray-

Pederson and Stray-Pederson, 1988)]. In addition, the use of trophoblastic vesicles (Johnson *et al.*, 1988) or seminal plasma [(McIntyre *et al.*, 1989] were comparable to leucocyte immunotherapy in improving fertility in RSA women. In contrast, Clark *et al.*, [1993] used immunotherapy to treat stress-triggered abortion in mice and concluded that this treatment can protect against spontaneous abortions. Some of the explanations offered for the conflicting results included cointervention of the autologous leucocyte placebo treatments, too small sample size, heterogenous sample populations and suboptimal numbers of leucocytes used for immunization among these studies [Clark and Daya, 1991; Coulam and Coulam, 1992].

The present study used established routes of administration of leucocytes to sensitize the recipients to paternal leucocyte antigens. In fact, the justification for using seminal plasma in immunotherapy studies is that maternal sensitization to paternal antigens begins at insemination [McIntyre *et al.*, 1988; Thaler, 1989] and the uterus is apparently an effective route for immunization [Searle, 1986]. The number of leucocytes administered per dose was within the optimum concentration recommended for presensitization to paternal antigens in humans [Mowbray, 1986, Aoki *et.al.*, 1993].

A modified approach was taken in this study which encompassed both presensitization (IP injection of leucocytes at first estrus) and increased antigenicity of semen (infusion of leucocytes into the uterus immediately prior to insemination with whole semen). However, a similar enhancing effect was not evident either in our study or in that of Blichfield, [1984]. In fact, the opposite was true in our study where treatment with paternal leucocytes resulted in a decrease in embryo survival when compared to that of the PBS treatment group. The significant boar and

boar by treatment interaction effects observed in the present study indicated that better reproductive performance may be realized with certain boars. Murray and Grifo [1986] found that semen from any boar is as beneficial as that of the sire in presensitizing females.

Although the number of animals per treatment was relatively small in this trial, similar results were obtained when compared to studies that employed larger numbers of animals in which leucocytes have been added to semen at insemination [see Blichfield 1984]. Our results reaffirm the notion that the positive effect of leucocyte treatment on embryo survival is inconsistent. It appears that any putative involvement of the maternal immune system resulting in enhancement of the reproductive process only becomes evident under suboptimal husbandry conditions. The literature supports this assumption, since the subjects in human studies are all RSA women, and in the mouse and horse, abortion-prone models were utilized.

In conclusion, treatment of gilts with leucocytes from the sire does not improve reproductive performance in a healthy, well-managed, outbred population of pigs.

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The effect of treatment of gilts with leucocytes on reproductive performance after A.I.

Parameters measured	Treatments (means ± s.e.m.)[z]		
	Leucocytes		
	Boar (sire)	Gilt (autologous)	PBS
No of gilts examined	11	7	6
No. of corpora lutea	14.9 ± 1.2	15.4 ± 0.5	14.6 ± 0.5
No. of viable embryos	10.9 ± 0.9	13.0 ± 0.9	13.2 ± 0.9
Embryo survival (%)	77.9 ± 7.6	84.7± 6.2	89.6 ± 4.9
Placenta weight (g) [y]	21.52 ± 1.42[a]	23.46 ± 4.6[a]	16.98 ± 2.4[b]
Embryo weight (g)[x]	1.73 ± 0.1[c]	1.61 ± 0.28[d]	1.59 ± 0.2[d]
Uterine weight (Kg)	3.5 ± 0.24	4.2 ± 0.46	3.6 ± 0.23
Ovarian weight (g)	14.7 ± 0.5	15.2 ± 0.63	15.4 ± 0.93
Chorioallantoic fluid weight (g)	182.5 ± 50	184.2 ± 41	138.1 ± 42

z ($p > 0.05$ for all means except for those of placenta and embryo weights)
y ($p < 0.05$ for means with different superscripts within rows)
x ($0.05 < p < 0.07$ for means with different superscripts within rows)

Table III.1 The effects of treatment of gilts with leucocytes from the sire, autologous leucocytes (self) and phospate buffered saline on the reproductive performance of gilts at second estrous bred by artificial insemination.

The effect of treatment of gilts with leucocytes on reproductive performance after natural mating

Parameters measured	Treatments (means ± s.e.m.)[z]		
	Leucocytes		
	Boar (sire)	Gilt (autologous)	PBS
No of gilts examined	14	15	15
No. of corpora lutea	13.9 ± 0.7	13.5 ± 0.4	14.8 ± 0.5
No. of viable embryos	10.6 ± 0.9	10.8 ± 0.8	12.7 ± 0.5
Embryo survival (%) [y]	76.4± 4.5[a]	79.8 ± 4.2[ab]	86.0± 2.9[b]
Conceptus weight (g)	32.0 ± 1.3	30.0 ± 0.7	28.0 ± 1.0
Placenta weight (g)	27.7 ± 1.2	25.5 ± 0.1	22.9 ± 0.8
Embryo weight (g)	1.9 ± 0.1	2.0 ± 0.1	1.9 ± 0.1
Uterine weight (Kg)	3.5 ± 0.2	3.6 ± 0.2	4.3 ± 0.2
Ovarian wt. (g)	15.71 ± 0.5	15.02 ± 0.52	15.4 ± 0.52

z ($p > 0.05$ for all means)
y ($0.05 < p < 0.06$ for means with different superscript within rows)

Table III.2. The effects of treatment of gilts with leucocytes from the sire, autologous leucocytes (self) and phospate buffered saline on the reproductive performance of gilts at second estrous bred by natural mating.

The effect of treatment of gilts with leucocytes on reproductive performance : Data pooled from experiments one and two.

Parameters measured	Treatments (means ± s.e.m.)[z]	
	Leucocytes	PBS
No. of gilts examined	47	21
No. of corpora lutea	14.25 ± 0.4	14.7 ± 0.4
No. of viable embryos[y]	11.25 ± 0.4	13.27 ± 0.5
Embryo survival (%)	80.5 ± 2.7	90.15 ± 2.1
Placenta weight (g)[x]	24.8 ± 1.2	21.3 ± 1.4
Embryo weight (g)	1.86 ± 0.1	1.84 ± 0.12
Uterine weight (Kg)[w]	3.65 ± 0.14	4.15 ± 0.16
Chorioallantoic fluid weight (g)w	187.1 ± 7.2	164.8 ± 13

z ($p > 0.05$ for all means excep for that of the No. of viable embryos and placenta weights)
y ($p < 0.05$ for mean No. of viable embryos)
x ($p < 0.01$ for mean placenta weights)
w ($0.05 < p < 0.07$ for mean uterine weights)

Table III.3. Data for leucocyte treatments (Sire's and Gilt's leucocytes) from experiment I and II were pooled and compared to that of the phospate buffered saline controls, also pooled from both experiments.

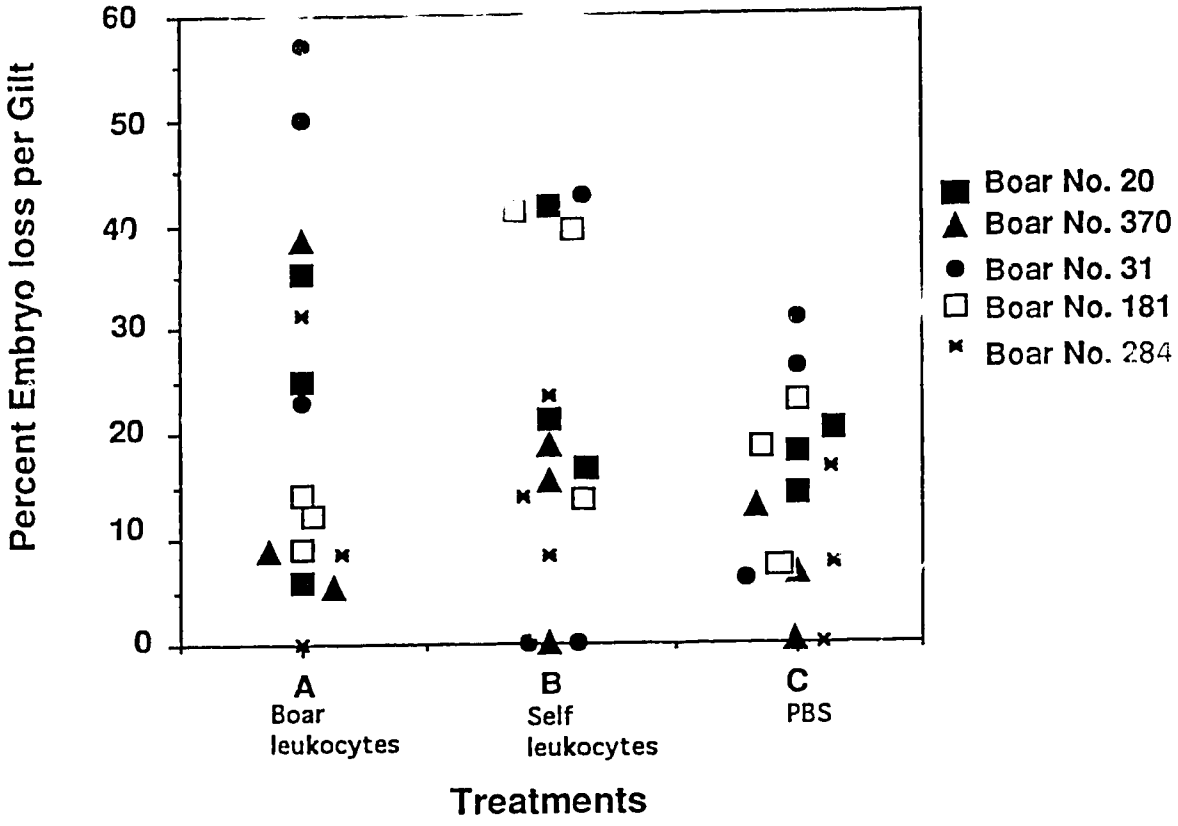

FIGURE III.1 **Percent Embryo Loss per Gilt bred to each of the five boars**

CHAPTER IV

A PORCINE TROPHOBLAST CELL LINE THAT SECRETES GROWTH FACTORS WHICH STIMULATE PORCINE MACROPHAGES

INTRODUCTION

Trophoblast tissue is derived from the ectodermal layer of the mammalian embryo [Perry, 1981]. In the placenta, it constitutes the major barrier between the maternal and fetal circulation. Comparative studies on placental morphology have revealed that trophoblast tissue can range from a single layer of cells in the simple epitheliochorial placenta of the pig to the complex multilayered tissue seen in the hemochorial placentas of mouse and humans [Amoroso, 1952; Perry, 1981, Enders, 1982; King, *et al.*, 1982; Steven, 1983]. Its strategic position at the maternal-fetal interface enables trophoblast to provide physical, immunological and physiological protection to the developing fetus [Wegmann, 1990; Loke and King, 1991; Head, 1991]. Recent studies have utilized *in vitro* systems to characterize trophoblast. The rationale is that within a simplified system it is easier to recognise some of the molecular signals involved in trophoblast regulation.

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Evidence from our lab and others has demonstrated both in mice [Guilbert *et al.*, 1991; Pollard *et al.*, 1987; Athanassakis *et al.*, 1987; Wegmann *et al.*, 1989; Armstrong and Chaouat, 1989;] and in humans [Garcia-Loret, 1990] that the macrophage growth factor (MGF) family of cytokines are involved in trophoblast growth and differentiation. Granulocyte-macrophage colony stimulating factor (GM-CSF) mRNA expression has been localized by *in situ* hybridization within the spongiotrophoblast zone of the mouse placenta [Kanzaki *et. al.*, 1991] and is produced by the uterine epithelium during pregnancy [Robertson *et al.*, 1992]. This source of GM-CSF could be unique since northern blotting of polyA+ placental RNA has revealed a novel form of GM-CSF mRNA expression at this site [Crainie *et al.*, 1990]. Another CSF cytokine, CSF-1, is produced by the uterine glandular epithelial cells of the mouse and large amounts are secreted during pregnancy [Pollard *et al.*, 1987]. The CSF-1 receptor, has been found on the trophoblast of the mouse [Arceci *et al.*, 1989], the cow [Beaucamp and Croy, 1991] and humans [Visvader and Verma, 1989].

In addition, GM-CSF and CSF-1 are autocrine growth factors for the human choriocarcinoma cell lines JEG, JAR and BEWO [Garcia-Loret, 1990]. These cytokines added exogenously to human term cytotrophoblast cultures stimulate syncytium formation and the production of human chorionic gonadotrophin and placental lactogen [Garcia-Loret, 1990]. Thus, there is emerging evidence that lymphohemopoietic cytokines may play autocrine and paracrine roles in the regulation of trophoblast growth and function.

A major dilemma faced by reproductive biologists attempting to elucidate the role of trophoblast in the maintenance of pregnancy is to

procure, in sufficient quantities, a homogenous population of these cells. The objective of the current study was to establish a long-term, stable porcine trophoblast cell line. To do so we took advantage of the fact that the pig periimplantation blastocyst offers an opportunity to obtain pure trophoblast in quantity. The porcine conceptus undergoes a remarkable form of development which has been studied both at the light and electron microscope level [Dempsey *et al.*, 1955; Friess *et al.*, 1980; Stroband *et al.*, 1984; Albertini *et al.*, 1987; Barends *et al.*, 1989; Richoux *et al.*, 1989; Mattson *et al.*, 1990]. The blastocyst undergoes considerable elongation during the prolonged preimplantation period to reach lengths of up to one meter [Anderson, 1978; Geisert *et al.*, 1982; Mattson *et al.*, 1990;]. Except for a few centimeters occupied by the embryonic disc region, most of this tissue is composed of trophoblast [King and Ackerly, 1985].

This report describes a method to obtain pure trophoblast cells, and the isolation and characterization of a trophoblast cell line, termed Jag-1, from the bulbous ends of pig blastocysts. In parallel we developed a bioassay for monocyte/macrophage growth factors to measure homologous and heterologous cytokine activity. The trophoblast cell line produces a blood monocyte/macrophage growth factor activity as determined by the bioassay. This study generalizes the observation that trophoblast can produce macrophage-like growth factors to a species with an epithelial-chorial placenta. It also indicates the direct importance of lympho-hematopoietic cytokines for reproductive success in an animal species that is an important food source, and provides the means for discovering cytokines beneficial to porcine reproductive performance.

MATERIALS AND METHODS

Blastocyst Collection and Processing.

Sexually mature crossbred gilts (6-8 months of age) maintained at the University of Alberta Swine Facility were used in this study. Blastocysts were surgically collected from gilts at their second estrous cycle on Day 14 post mating (day of onset of estrus = Day 0). A total of 14 corpora lutea were counted on the ovaries of the pig from which the Jag-1 cell line was isolated. Thirteen filamentous blastocysts were flushed from both uterine horns using phospate buffered saline solution (PBS) with 2% Fetal Calf Serum (FCS; Bockneck, Toronto, ON). The blastocysts were transfered to a sterile petri dish with calcium- and magnesium-free Hanks balanced salt solution (HBSS; Gibco laboratories, Grand Island NY, USA) and were gently teased apart to reveal the bulbous ends. A fine pair of scissors was used to cut only the extreme dilated tips to avoid contamination with endoderm. The ends were dissected into approximately 1mm pieces and cultured in 24 well tissue culture plates (3047; Falcon, CA, USA), one piece per well, in medium 199 (Gibco) with 10% FCS (M199).

Isolation and Culture of Jag-1 Trophoblast Cell Line.

The wells were monitored at 24h, 48h, and 72h after the onset of culture for trophoblastic vesicle formation. At the end of 72h, only those wells that had previously displayed the transparent type of vesicle growth were harvested. By this time, these vesicles had collapsed into monolayer growth at the bottom of the wells. The necrotic remnants of tissue were removed and the monolayer colonies were harvested by adding HBSS containing 0.5% trypsin and 5.3 mM EDTA (Gibco) at 37^0C for 10

minutes. Some wells had free floating and/or attached dense vesicles indicating contamination with endoderm. These were discarded. The monolayer cells were pooled and cultured to confluency in tissue culture flasks of increasing size; first in a 25 cm^2 flask (3013; Falcon) for seven days, then in a 75cm^2 flask (3024; Falcon) for a further seven days. From that time onwards, 175 cm^2 flasks (3028; Falcon) were used and the cells passaged every 6-7 days. A seeding rate of 2-4 x 10^4 cells /ml was used in a final volume of 50 ml/flask and the medium was changed every 2-3 days.

Light Microscopy.

To characterize Jag-1 cell morphology at the light microscopic level, cells were grown on collagen-coated glass slides and stained as follows: slides were placed in May-Grunwald Stain (Sigma, St Louis, MO, USA) for 5 min, then in Tris buffer (.05M, pH 7.2) for a further 5 min. The slides were next placed in Giemsa stain diluted 1 : 20 with deionized water for 30 minutes and then rinsed and dried. Observations were made under bright field using a Ziess photomicroscope.

Electron Microscopy.

Samples for electron microscopy included cells grown on collagen-coated coverslips, porous tissue culture membranes (3095; falcon) and monolayer cells resuspended and pelleted. All samples were prefixed in 2.5% glutaraldehyde in Millonig's buffer (pH 7.2) at 4^0C for 1h. Samples were postfixed in 1% osmium tetroxide for 1h and then passed through an ascending series of alcohol up to 100%. For scanning electron microscopy (SEM), the samples were dried to critical point (41^0C), mounted and sputter-coated with gold (Edwards, Model 5150B sputter coater). Specimens

for transmission electron microscopy (TEM) were next placed in propylene oxide for 30 min and embeded in araldite. Sections were stained with uranyl acetate and lead citrate. Electron micrographs were taken with a Hitachi H7000 (TEM) and a Hitachi S-2500 (SEM) microscopes.

Immunocytochemical Staining.

Jag-1 cells were grown to confluency on 22 mm^2 glass coverslips coated with collagen (10 *u*g/ml). The cells were then fixed with cold acetone for 20 minutes at -18^{0}C and air dried. Histostain-SP kits (Dimension laboratories, Mississauga, ON) were used for immunocytochemical staining with rabbit and mouse primary antibodies in a streptavidin-biotin system. Antibody detection was carried out according to the manufacturer's specifications. A rabbit polyclonal anti-bovine cytokeratin antibody (Dako, Santa Barbara, CA, USA) was used at a 1: 600 dilution to detect the cytokeratin intermediate filaments. Mouse placental cells and adherent monocyte/macrophages were used as positive and negative controls respectively. These cells were also grown on glass coverslips and fixed in a similar manner to that of the Jag-1 cells. As a reagent control normal rabbit serum was used. For the determination of expression of vimentin as an intermediate filament, a mouse monoclonal anti-porcine vimentin antibody (Dakopatts, Glostrup, Denmark) supplied as a tissue culture supernatant was used at a 1: 20 dilution. Adherent pig monocyte/macrophages were the positive control. Normal mouse serum and a mouse IgG1 isotype antibody (Zymed laboratories, CA, USA) were reagent controls for this mouse primary antibody. In order to determine whether Jag-1 cells express classical major histocompatibility antigens (MHC), the monoclonal antibodies PT85, MSA3 and TH16 [VMRD, Inc., WA; 31, 32] were used at

a 25 ng/ml (1:200) dilution. These mouse monoclonal antibodies recognize specific monomorphic determinants, the former on all classical class I swine leucocyte antigens (SLA), the latter two on all DRw and DQw class II SLA, respectively [Davis *et al.*, 1987; Hammerburg *et al.*, 1986]. The staining of Jag-1 cells and the bulbous tips of day 14 blastocysts was compared to that on porcine peripheral blood leucocytes (PBL) as positive controls. Nonimmune mouse serum was used as the reagent control. All specimens were counterstained with hematoxylin and observed under a light microscope.

Determination of Jag-1 Cell Growth Kinetics.

The cells were cultured in 75 cm^2 tissue culture flasks (3024; Falcon) in M199 with varying seeding densities. After 48 hours of culture the flasks were harvested and the cells enumerated using a haemocytometer. In addition, the cells were also assayed for their ability to incorporate tritiated thymidine (^{3}HTdR). This assay was carried out in 96 well microtitre tissue culture plates (3072; Falcon) and 10^4 cell/well (6 wells/treatment) were cultured with 1*u*ci ^{3}HTdR (Amersham, Oakville, ON) for 18 hours. The cells were harvested on fibreglass filters and counted for ^{3}HTdR incorporation in an LKB 1218 Rack beta counter. This procedure was repeated every 24 hours for 6 days.

Isolation of Peripheral Blood Leucocytes (PBL).

Blood samples were collected from randomly chosen nonpregnant animals into heparinized sterile tubes for isolation of PBL. The blood was then transfered to 50 ml tubes and centrifuged for 30 min at 4°C and a speed of 1500 x g. The clear plasma at the top was aspirated off and the buffy coat

carefully collected. Red blood cell (RBC) contamination was reduced by hypotonic lysis. This was achieved by sequential suspension of cells in sterile double distilled water for 20-30 seconds, then in 2x PBS (equal volume) before centrifugation at 600 x g for 10 minutes. Isolated PBL were next pooled and passed through Nitex screening (#102) to remove cell clumps and debris. Live cells were enumerated in 0.4% trypan blue using a haemocytometer. The cells were then diluted to 10^6/ml in RPMI-1640 (Gibco) supplemented with 10% FCS (RPMI) and aliquots of 50 mls were cultured overnight in 175 cm^2 flasks to allow for adherence.

Harvesting of Adherent Monocyte/Macrophages.

Culture flasks were shaken to resuspend loosely attached cells and the suspension aspirated. This was repeated again with cold PBS before the addition of PBS (without calcium or magnesium) with 1mM EDTA. All flasks were chilled on ice for 15-30 minutes and the attached cells gently resuspended using a rubber policeman. Resuspended cells were then pelleted and diluted to 10^5/ml in RPMI and pipetted into 96 well tissue culture plates at 10^4 cells/well.

Recombinant Bovine GM-CSF(r-BoGM-CSF) Assay.

Recombinant BoGM-CSF stock solution (0.75 mg/ml; Ciba Geigy, Basel, Switzerland) was diluted in RPMI in three-fold serial dilutions from 270 ng/ml-0.06 pg/ml and added (100 *u*l/well; 6 wells/dilution) into the microtitre plates containing the recently harvested monocyte/macrophages (see, culture conditions below).

Cell-Conditioned Medium Assay.

Jag-1 cells were cultured to confluency in 175 cm^2 tissue culture flasks. The culture medium was changed and allowed to be conditioned for 72 hours. The conditioned medium (Jag-1 CM) was then pooled and filtered through a 0.2 *u*m filter (Millipore, Bedford, Mass., USA) and stored at -20^0C. Jag-1 CM was diluted in two-fold serial dilutions with RPMI and aliquoted (100 *u*l/well; 6 wells/dilution) into tissue culture plates containing recently harvested monocyte/macrophage. The embryonic disc regions of day 14 blastocysts were removed by dissection and the remaining trophoblastic tissue minced and cultured for approximately 72 h to obtain blastocyst conditioned medium (B-CM). B-CM and mouse L929 cell conditioned medium (L-cell CM) were similarly assayed for pig monocyte/macrophage stimulating activity. Medium similarly incubated but in the absence of cells or tissue were used in the controls.

Culture Conditions.

Incubations were carried out at 37^0C, in an atmosphere of 5% CO_2 in air and 100% humidity for 48 hours. The medium was then decanted from all plates and replenished as described before. Incubations were allowed to continue for another 24 hours and then 1*u*ci ^{3}HTdR/well was added and cultured for an additional 18 hours. At the end of incubation cells were harvested and counted for ^{3}HTdR incorporation.

RESULTS

Isolation of the Trophoblast Cell Line (Jag-1) from the Bulbous Ends of Filamentous Pig Blastocysts.

The Jag-1 porcine trophoblast cell line was isolated from the extreme bulbous tips of day 14 pig blastocysts. The morphological changes in culture of tissue pieces taken from this region were characterized by formation within 24 h of transparent, multicellular vesicles (Fig. 1a). These trophoblastic vesicles ranged between 0.1-0.5 mm in diameter and were always anchored singly or in groups to tissue pieces. Under the inverted light microscope they appeared to be single layered with cells having very attenuated cytoplasm. These vesicles collapsed within a few hours after formation and then grew as monolayers of concentric colonies surrounding a nucleus of necrotic tissue remnants. There was no evidence of this transparent type of vesicle after 48 h of culture. In some wells dense vesicles, free floating or attached to necrotic tissue, were also formed by 48 hours (Fig. 1b) . This compact vesicle form persisted after 72 h of culture. These were discarded on the presumption that they contained endothelial cells. The Jag-1 cell line was isolated from the monolayer cells growing at the bottom of wells at 72 h of culture. This line was isolated in May of 1990. In culture, it is passaged once every five to seven days. It survives cryopreservation in liquid nitrogen (-196^{0}C) and thawing in a 37^{0}C water bath with subsequent viability between 60-80 percent.

Jag-1 Cell Expression of Morphological Features of an Epithelium Common to Trophoblast at the Light and Electron Microscope Levels.

Light microscopy.

Jag-1 cells are predominantly uninuclear with prominant staining single or multiple nucleoli (Fig. 1c). However, binucleate cells are also produced (Fig. 1d). Interspersed among these are the occasional, large (giant) cells that are sometimes bi- or multinucleated (Figs 1c and 1d), and are reminiscent of mouse giant trophoblast cells.. The cytoplasm of confluent growing Jag-1 cells contains numerous relatively small vacuoles. In contrast, those found in the giant cells are large and mostly perinuclear and may have formed by the coalesing of small vacuoles. When Jag-1 cells from earlier passages are compared to those in the very recent cultures, there are no obvious morphological changes under light microscopy.

Formation by Confluent Growing Cells of a Continuous Sheath Not Unlike a Simple Epithelium.

When cells were grown on porous tissue culture membranes inserted into 24 well plates there were fewer giant cells. Those giant cells found growing on these membranes also had fewer large perinuclear vacuoles (Fig. 2). This synthetic membrane appears to simulate a basement membrane allowing for better expression of the epithelial characteristics of Jag-1 cells. The apical surface of the cells is covered with relatively long microvilli. They persist at higher densities along the intercellular borders, thus revealing the outline of each cell. The large perinuclear vacuoles appear as "crater-like" depressions under SEM (Fig. 2). The microvilli

within these depressions are shorter and more widely spaced than those found elsewhere on the surface of these cells.

Ultrastructural Features of a Simple Epithelium Revealed by Transmission Electron Microscopy .

Ultrastructural studies show that confluent growing Jag-1 cells possess well developed jùnctional complexes at their lateral borders (Fig. 3a). These junctional complexes are intercellular structures that are unique to an epithelium. They are always situated near the apical membrane surface and are composed of a tight junction usually followed closely by a pronounced desmosome, sometimes two in tandem repeat (Figs 3a and 3b). Gap junctions are seldom seen. The adjacent lateral cell membranes can be very interdigitated and often open into large intercellular spaces or lumina sealed apically by desmosomes. Cytoplasmic projections of varying length are discernible within these intercellular spaces (Fig. 3a).

Cell division continues after reaching confluency, albeit at a slower rate. The newly formed cells do not go into suspension but form junctional complexes with the spread cells to maintain the epithelium (Fig. 3b). In general nuclei are ovoid in shape and are closer to the basal than the apical surface (Fig. 3b). The nucleoplasm contains mainly euchromatin with some margination of heterochromatin along the inner surface of the nuclear membrane. TEM shows the vacuoles to be scattered throughout the supranuclear cytoplasm. Some have coated membranes and others contain cellular material and therefore could be lysosomes. Large electron dense vacuoles apparently containing lipids are also discernable. Coated pits or caveolae also are present between the microvilli on the apical surface. Mitochondria are numerous and vary in size and shape from small round or

irregular to large round or elongated forms. Short strands of rough endoplasmic reticulum are seen and the smooth form appears even less developed. Scattered throughout the cytoplasm are aggregates of ribosomes. Cytoskeletal elements are prominent within the cytoplasm with filament bundles terminating at plaques on either side of desmosomes (Figs 3a and 3b). Immunocytochemical staining colocalized cytokeratin intermediate filaments to these structures (see below).

Cell polarity is very evident both at the surface and intracellularly. The apical surface of the cells is covered with microvilli of varying length while the basal surface is relatively smooth (Fig. 3b). This external surface polarity is maintained even after adherence is disrupted by trypsinization. The apical surface of these cells was very convoluted bearing long microvilli. The nucleus remains closely apposed to the basal surface and most of the organelles are found within the supranuclear cytoplasm (data not shown).

Cytokeratin Positivity and Vimentin, MHC Class I and Class II Negativity of Jag-1 cells.

All the cells stained positively for the intermediate filament cytokeratin. There was staining throughout the cytoplasm revealing intensely stained cytokeratin filaments terminating at the cell boundries (Fig. 4a). Electron microscopy showed these filaments terminate at well developed desmosomes. Adherent cells specifically stained with this rabbit polyclonal anti-bovine cytokeratin antibody were also seen in the mouse placenta which was used as a positive control (Fig. 4b). Adherent blood monocyte/macrophages of the pig, used as the negative control because of their mesodermal origin, showed no staining with this antibody (Fig. 4c).

When a normal rabbit serum control was used as the primary antibody there was no staining of Jag-1 cells (Fig. 4d), mouse placental cells, or monocyte/macrophages (not shown).

Jag-1 cells do not express the intermediate filament vimentin as part of their cytoskeleton. There was no staining with the mouse monoclonal anti-porcine vimentin antibody (Fig. 4e). In contrast the positive control, adherent blood monocyte/macrophages, were intensely stained with this antibody (Fig. 4f). No staining of Jag-1 cells nor monocyte/macrophages was observed with normal mouse serum or the IgG1 isotype control antibodies (not shown). Jag-1 cells and fresh trophoblast tissue from the tips of day 14 blastocysts (surgically opened to allow both peripheral and luminal exposure to antibodies) failed to stain with class I-(Fig. 5a and 5b respectively) or class II-specific primary antibodies (data not shown). In contrast, porcine PBL all stained with the class I-specific Mab (Fig.5c). As expected, a portion of these also stained with two class II specific Mabs, MSA3 (Fig.5d) and TH16 (data not shown). Normal mouse serum as reagent controls for these primary antibodies were negative (not shown).

Doubling Time of 48 Hours after Initial Lag Period.

There is a lag period of growth of about 48 hours after seeding of Jag-1 cells (Fig. 6). During this time, the cells adhere and spread but can still be seen as individual cells (Fig. 1b). After this initially static phase, proliferation accelerates to attain a doubling time of about 48 h. The length of time taken to reach confluent growth is dependent upon the seeding density. At seeding densities between 2-4 x 10^4 cells/ml the state of confluent growth is reached within 3.5-4.5 days. Cell proliferation, as

reflected by ^{3}HTdR uptake, peaks at day 3 for cultures that reach confluency within six days (not shown).

Secretion by Jag-1 Cells of Porcine Macrophage Growth Factor(s)

The lag period preceeding onset of population growth and its dependence on cell density suggested accumulation of trophoblast growth factors in Jag-1 cultures to be necessary. In the human and mouse, macrophages growth factors (MGFS) have been implicated in the regulation of trophoblast growth [Guilbert *et al.*, 1991; Pollard *et al.*, 1987; Athanassakis *et al.*, 1987; Wegmann *et al.*, 1989; Armstrong and Chaouat, 1989; Garcia-Loret, 1990; Kanzaki *et al..*, 1991; Robertson *et al.*, 1992]. We therefore developed an assay, based on the ability of porcine peripheral blood monocytes to be mitogenically stimulated in culture, for the detection of porcine MGFS. Supernatants from Jag-1 cultures strongly stimulated porcine macrophage proliferation (Fig. 7a). Maximal proliferation occured at 50% Jag-1 conditioned medium. In four independent experiments, this concentration of conditioned medium stimulated at least a five-fold increase in porcine macrophage proliferation. Experiments were performed to determine whether conditioned medium from primary day 14 blastocyst tissue produces a similar macrophage stimulatory activity to that of Jag-1 cells. Indeed, day 14 blastocyst conditioned medium produced a similar level of stimulation in this bioassay (Fig. 7d).

In order to determine whether the porcine MGF assay was detecting traditional MGFS (CSF-1, GM-CSF, or IL-3), we next asked whether these cytokines could stimulate porcine macrophage proliferation. Lacking defined preparations of porcine cytokines, we examined possible species

cross-reactivities. Both pure r BoGM-CSF (Fig. 7b) and crude mouse L929 conditioned medium (Fig. 7c), which contains CSF-1 but not GM-CSF or IL-3 [Stanley and Guilbert, 1981], strongly stimulate porcine macrophage proliferation. Thus, porcine macrophages appear to respond to the same cytokine subsets as do mouse and human macrophages, and in addition show a striking species cross-reactivity.

The Jag-1 cells secrete autocrine factors and are Steroidogenic in Origin

The Jag-1 cells secrete an autocrine factor(s; Fig. 8). This was only evident when the assay was done in a protein-free medium that was conditioned by the Jag-1 cells. Otherwise, the presence of fetal calf serum masks this stimulatory effect. The Jag-1 cells strongly expressed the mRNA for a cytochrome P450 side chain cleavage enzyme (Fig. 9). The 2.1 Kb band for this enzyme was also strongly expressed in periimplantation blastocysts. Day 14 blastocysts expressed a similar level of P450 SCC as that of the Jag-1 cells. Weak bands were also present in total RNA from days 14-16 uterine epithelial cells and the ovary. As expected it was absent from PBL.

DISCUSSION

In the early filamentous stages of the pig blastocyst the endoderm has yet to migrate as a complete layer into the bulbous ends [Mattson *et al*, 1987]. In addition, the ends do not become penetrated by blood vessels up to day 40 of pregnancy [Friess *et al.*, 1980]. Therefore, one can isolate pure trophectodermal cell lines from the bulbous tips of pig blastocysts. The method outlined in the present study has led to the isolation of the Jag-1 trophoblast cell line. Elongated pig blastocysts, cut into fragments or enzymatically dissociated, form multicellular vesicles and monolayer growth in culture [Whyte *et al.*, 1987; Kuzan and Wright , 1980; Selgrath and Wright, 1988]. Similar vesicles are also produced from elongated cow and ewe blastocysts [Heyman *et al.*, 1984]. The morphology [Selgrath and Wright, 1988] and ultrastructure [Whyte *et al.*, 1987] of these vesicles has been characterized and they have been used in studies involving maternal-fetal and cell-cell interactions in farm animals [Selgrath and Wright, 1988].

Surprisingly, only the dense, free floating or attached type of vesicles have been described in the literature. This study is the first to describe the evanescent, transparent, monolayer vesicles that form within 24 hours of culture and collapse soon after formation to grow as concentric colonies at the bottom of the dish. From our experience, monolayer culture growth resulting from tissue taken from the region between the dilated tips and the embryonic disc contains both trophectoderm and endoderm cells. A similar conclusion was reached by Whyte *et.al.*, [1987]. These cultures eventually become overgrown by fibroblastic endoderm cells and persist for only two to four passages. On the other hand, pure trophectoderm cells can be obtained from this region under the following conditions. It is imperative

that the monolayer cells be harvested by enzymatic dissociation within 48 hours from the onset of tissue culture. Up to this time, the monolayers are strictly from the transparent type of vesicles. Prior to harvesting, the dense type of vesicles and tissue remnants must be removed by moderate washing, leaving the monolayer cells intact. The trophoblast cells harvested this way can survive for a number of passages without fibroblast overgrowth.

Jag-1 cells appear to be trophectoderm in origin and display epithelial characteristics. For example, they stain positively for cytokeratin and not the vimentin intermediate filament, possess well developed junctional complexes at their lateral borders, similar to that found in trophectoderm of the preimplantation embryo [Ducibella *et al.*, 1975] and they display pronounced cell polarity. Cell polarity, described as "a striking and fundamental property of epithelia" by Griepp and Robbins, [Griepp and Robins, 1988] is very evident in the growth of Jag-1 cells. The ultrastructural observations concerning the apical, lateral and basal surfaces, cell-cell communication and organization and types of cytoplasmic organelles in Jag-1 cells correspond closely to those observed in the trophectoderm of preimplantation blastocysts [Stroband *et al.*, 1984; Albertini *et al.*, 1987; Barends *et al.*, 1989; Stroband *et. al.*, 1990] trophoblastic vesicles [Whyte *et al.*, 1987] and even in the postimplantation placenta [Dempsey et al., 1955; Friess et al., 1980].

A monoclonal antibody (SN1/38) which reacts specifically to an antigen on the surface of trophectoderm cells of the pig was produced by Whyte *et.al.* [1984]. Approximately 20% of the monolayer cells derived from trophoblastic vesicles produced by Whyte *et. al.* [1987] expressed the trophectodermal antigen recognized by SN1/38. However, after two weeks in culture they found that only 2% of these cells reacted with this antibody.

SNl/38 was used for immunocytochemical staining of Jag-1 cells and showed no reaction (C. La Bonnardière, personal communication). To circumvent this difficulty antibodies against class I and II SLA antigens was used for corroboration of the trophoblastic nature of Jag-1 cells. Additional evidence that Jag-1 cells are trophoblasts is that they do not express classical class I or class II MHC antigens on their surface. Also, trophectodermal tips of day 14 blastocysts (the region from which the Jag-1 cell line was isolated) were found to lack classical MHC antigen expression. Furthermore, no staining was evident on trophoblast from other regions of day 14 blastocysts, or on that of day 11-12 expanded spherical and tubular blastocysts with the anti SLA class I and II mMabs (data not shown). Therefore, the lack of MHC antigen expression on Jag-1 cells parallel that seen on day 14 trophoblasts. This finding is not unique to the pig, as trophoblast from several other species lack class II MHC antigens [Loke and King, 1991; Head, 1991]. The expression of class I antigens may be of a nonclassical nature and restricted to certain populations of trophoblast within the placenta [Loke and King, 1991; Head, 1991]. In the pig, β_2-microglobulin has been detected on preimplantation blastocysts, between days 6 - 12 postconception. However, it has not yet been shown to be associated with MHC class I heavy chains [Meziou *et al.*, 1981]. We are currently investigating the nature of MHC mRNA expression in the Jag-1 cell line and primary day 14-16 trophoblast tissue.

SEM reveals craterlike depressions on the surface of giant cells which appear as clear perinuclear vacuoles under the light microscope. A similar type of depression has been found in day 15 trophoblast cells of the sheep [Guilomot *et al.*, 1981]. Perinuclear vacuolation was also described by Whyte *et.al.*, [1987]. They suggested that these are features of senesence

with no corroborative evidence. Many of these giant Jag-1 cells are binucleate and even multinucleated. Whyte *et.al.*, [1987] did not find tritiated thymidine labeling in bi- nor multinucleated trophoblast cells. On the assumption that the appearance of binucleate and multinucleate cells are not *in vitro* artifacts, the observation of these cells in both this study and that by Whyte *et.al.*, [1987] contradicts the statement by King *et.al.*, [1982] that,"binucleate cells do not develop in the porcine chorion".

Trophoblasts are known to produce a number of factors. In ruminants and pigs different forms of interferon alpha are produced by trophoblast [Mirando *et al.*, 1990]. Pig trophoblast also appears to produce interferon gamma [Lefevre *et al.*, 1990]. In order to determine whether porcine trophoblast produce or respond to CSF cytokines, other sources have to be found because there is no available porcine CSF cytokines. Mouse CSF-1 has been shown to stimulate blood monocyte/macrophages from the pig [Genovesi *et al.*, 1989]. A modification of the former assay was used in this study to test the cross species reactivity of rBoGM-CSF before the determination of its effect on Jag-1 cells and to determine whether medium conditioned by Jag-1 cells could stimulate monocyte/macrophage proliferation.

The kinetics of Jag-1 population growth in culture suggests an autocrine regulation of proliferation. Growth is strongly and positively dependent on population density: proliferation does not occur at densities of less than 10^4 cells/ml and much higher densities are reached at higher seeding rates. Medium conditioned by confluent cultures of Jag-1 cells stimulates the proliferation of lower density Jag-1 cultures (data not shown). Whether the population growth dependency is due to humoral cross-talk between identical cells [public autocrine, Guilbert, 1985] or

between distinct culture subpopulations (paracrine) remains to be determined by the preparation of homogeneous clones from the parent line. The density dependence of Jag-1 cell proliferation has apparently made it difficult to clone this cell line. It is intriguing, since commonly used methods for cell cloning such as limiting dilution, use of irradiated feeder layers and cell conditioned medium have not resulted in successful cloning. The lack of cloning success appears unique and could be related to the fact that this cell line was isolated by the confluence of independent monolayer cell colonies. This situation warrants further investigation which is beyond the scope of this study.

Media conditioned by Jag-1 cells and day 14 fresh blastocysts also stimulate the proliferation of porcine macrophages (Fig. 7a and 7d). It is highly unlikely that this stimulatory effect was due to endotoxins in the medium as no stimulation of macrophage proliferation was evident in the controls. Since porcine macrophages mitogenically respond to both GM-CSF (Fig. 7b) and CSF-1 [7c; Lefevre *et al.*, 1990], it appears that porcine macrophage proliferation, like that of mouse macrophages, is regulated by classical macrophage growth factors. Presumably this would include some or all of either CSF-1, GM-CSF or IL3 [Guilbert, 1985]. Given the observations: that human and mouse trophoblast prolif ration and differentiation are regulated by macrophage growth factors [Guilbert *et al.*, 1991; Pollard *et al.*, 1987; Athanassakis *et al.*, 1987; Wegmann *et al.*, 1989; Armstrong and Chaouat, 1989; Garcia-Loret, 1990], that CSF-1 can be produced by human trophoblast [Daiter *et al.*, 1992] and that mRNA for GM-CSF is detectable within trophoblast of the mouse [Robertson et al., 1992], it is very likely that the autocrine agent in Jag-1 conditioned medium

is a macrophage growth factor. A conclusive answer awaits the preparation of suitable defined porcine cytokines and antibodies reactive to them.

Finally, porcine trophoblast is well known for its ability to produce steroid hormones, especially oestrogen which is the maternal recognition of pregnancy signal in the pig [Bazer and Thatcher, 1977]. P450 side-chain cleavage enzyme (P450 SCC) converts cholesterol to pregnenolone in the mitochondria of steroidogenic cells (Mulheron *et al.*, 1990). This is the first and rate-limiting reaction of steroid hormone biosynthesis [Schiff *et al.*, 1993]. This rate limiting steroidogenic enzyme is apparently regulated by a number of factors including estrogen, insulin-like growth factor, FSH and LH. [cited by Mulheron *et al.*, 1990]. Cytochrome P450 SCC mRNA has been detected in trophoblast tissue [Durkee *et al.*, 1992] and the decidua during early pregnancy in the rat. Northern blot analysis using a cDNA probe for P450 SCC has been used to determine the steroidogenic ability of cells (see Mulheron et al., 1990; Durkee *et al.*, 1992; Schiff *et al.*, 1992). The Jag-1 cells expressed the mRNA for this enzyme but does not produce steroid hormones (No evidence of progesterone nor estradiol production was found by RIA). This could be due to long term culture because primary pig trophoblast tissue in culture stops secreting oestrogens by 24 h.

In summary, the data strongly supports that Jag-1 cells are trophectoderm cells which originated from the bulbous tips of early filamentous pig blastocysts.A transparent monolayer type of trophoblastic vesicle forms within 24 hours and collapses to grow as concentric trophectoderm colonies from which Jag-1 cells were isolated. Further characterization of Jag-1 cells is ongoing and the immediate focus is to isolate and characterize the secreted monocyte/macrophage growth factor(s)

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Fig. IV.1. The two types of trophoblastic vesicles that develop within one week of culture of pig blastocyst fragments, and Jag-1 cells grown on collagen-coated glass coverslips and double stained with May Grunwald/Giemsa stains for light microscopy. Bars indicate fifty micrometers (50*um*).

a) Transparent multicellular, trophectoderm vesicles attached to tissue remnants. They are a single cell in thickness. The arrows show cell bodies with attenuated cytoplasm. These vesicles form within 24 h and collapse soon after to grow as monolayers.

b) Dense multicellular, bilaminar vesicles attached to tissue remnants. These vesicles are similar to the blastocyst in that they have an outer trophectoderm and an inner endoderm layer. They develop by 48 h in culture and persist for varying length of time as free-floating or attached vesicles.

c) Jag-1 cells at 48 h of culture. Cells have attached and spread but are not confluent. Bi- and multinucleated cells are also present (arrows).

d) Confluent growing Jag-1 cells at 96 h of culture form a continuous sheath. Giant cells with perinuclear vacuolation and nuclei with multiple nucleoli are evident (arrows).

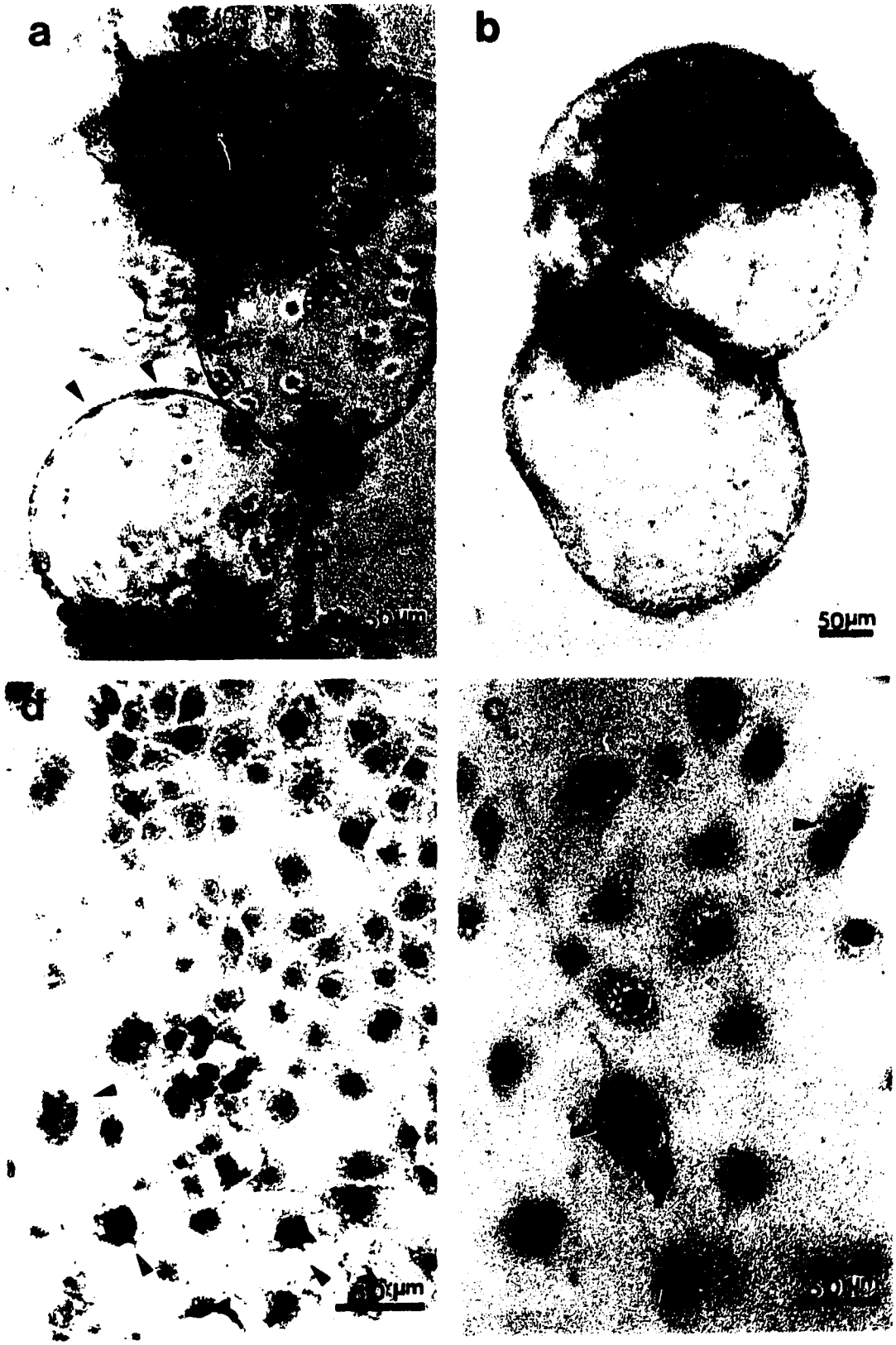

a
b
50μm
d

Fig. IV.2. SEM of confluent Jag-1 cells grown on tissue culture synthetic membranes. The cells grew as a confluent sheath with their apical surface covered with microvilli. These microvilli are most abundant along the intercellular borders (inset). Perinuclear vacuoles are seen as craterlike depressions (arrows) and are fewer compared to cells grown on collagen coated coverslips or in tissue culture flasks; N, nucleus. Bar indicates one micrometer [1*um*; inset, fifty nanometers (50*nm*)].

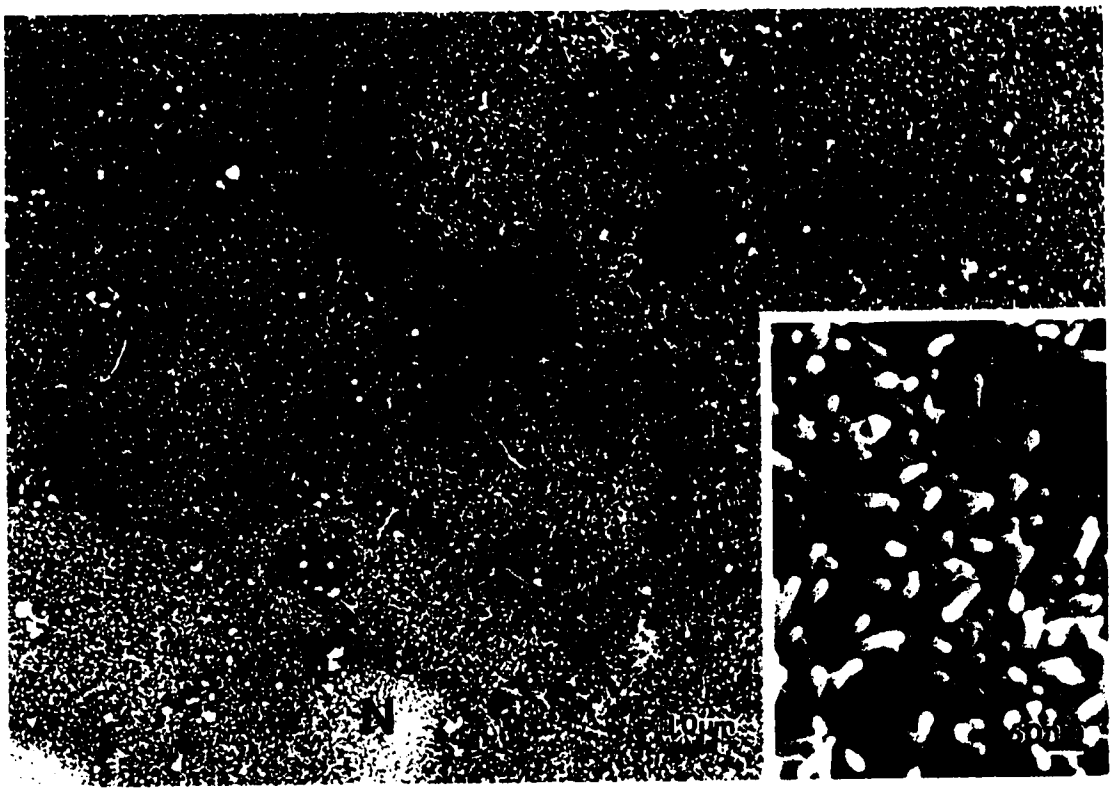

10μm
500

Fig. IV.3. TEM of tranverse sections of confluent growing Jag-1 cells. a) Junctional complexes that are unique to an epithelium can be seen between the lateral cell borders. These consist of a tight junction (TJ; enlarged in the lower left inset) usually followed closely by one or more desmosomes (D; enlarged in the upper right inset) in tandem repeat. These junctional complexes were always found towards the apical surface (AS). The lateral borders can be very convoluted and open into intercellular spaces within which cytoplasmic projections can be seen (arrow). Many types of vacuoles (V) were also found including lipid containing vacuoles (LV); microvilli (MV); mitochondria (M); rough endoplasmic reticulum (RER). Bar indicates 0.5 micrometer [0.5 *um*; insets, fifty nanometers (50*nm*)].

b) Cell division continued after reaching confluency. The new cells formed junctional complexes with the spread cells to maintain the epithelium. Tranverse section revealed the junctional complexes on both sides of one such cell. The junctional complex on the right was enlarged (inset) to show the tight junction (TJ) and the desmosome (D). The nucleus (N) is situated close to the basal surface (BS) which is relatively smooth, (G) golgi apparatus.

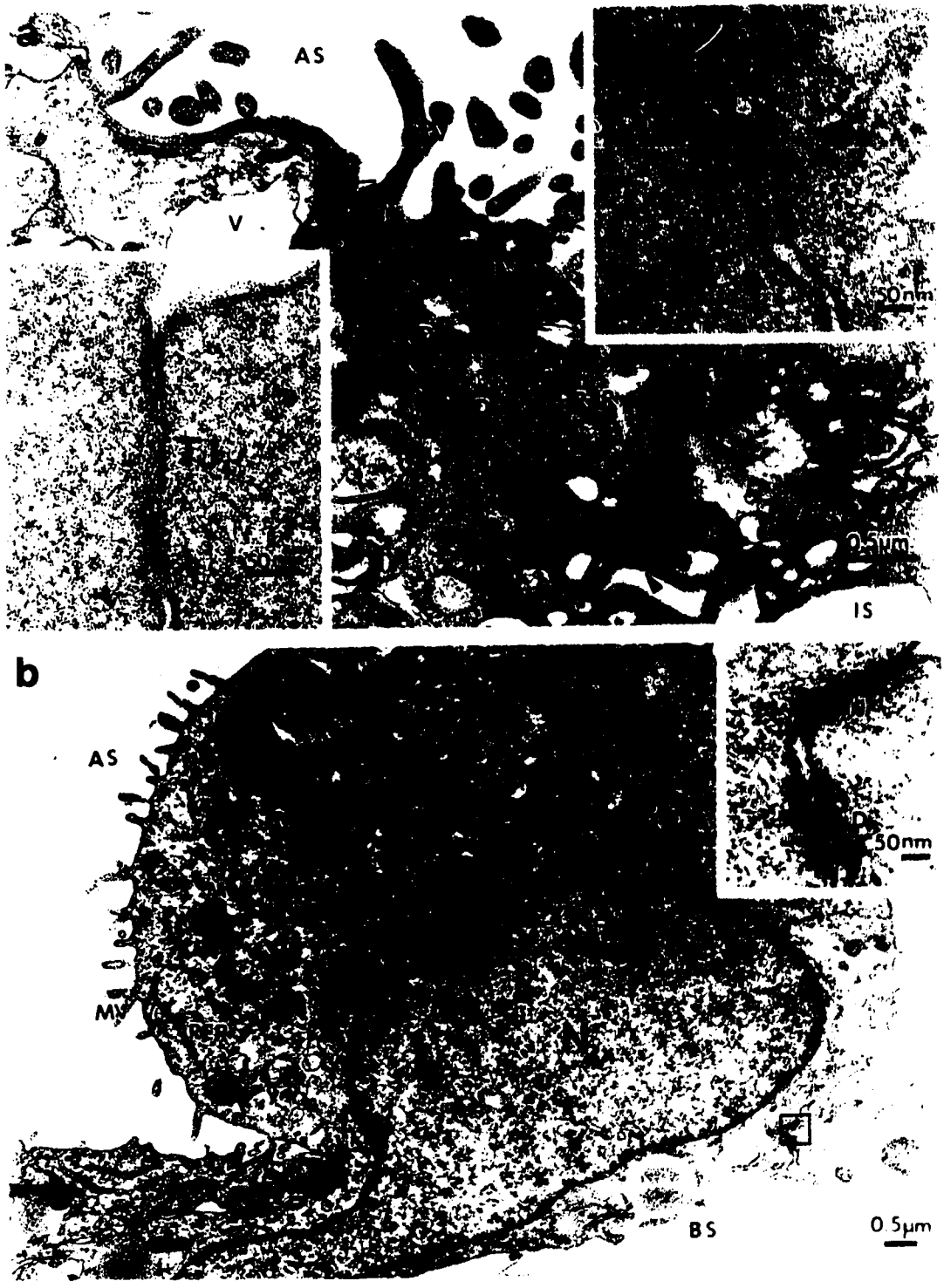

AS
V
50nm
0.5µm
IS
b
AS
50nm
MV
BS
0.5µm

Fig. IV.4. Identification of cytokeratin and vimentin intermediate filaments by immunocytochemistry. Bars indicate fifty micrometers (50*um*).

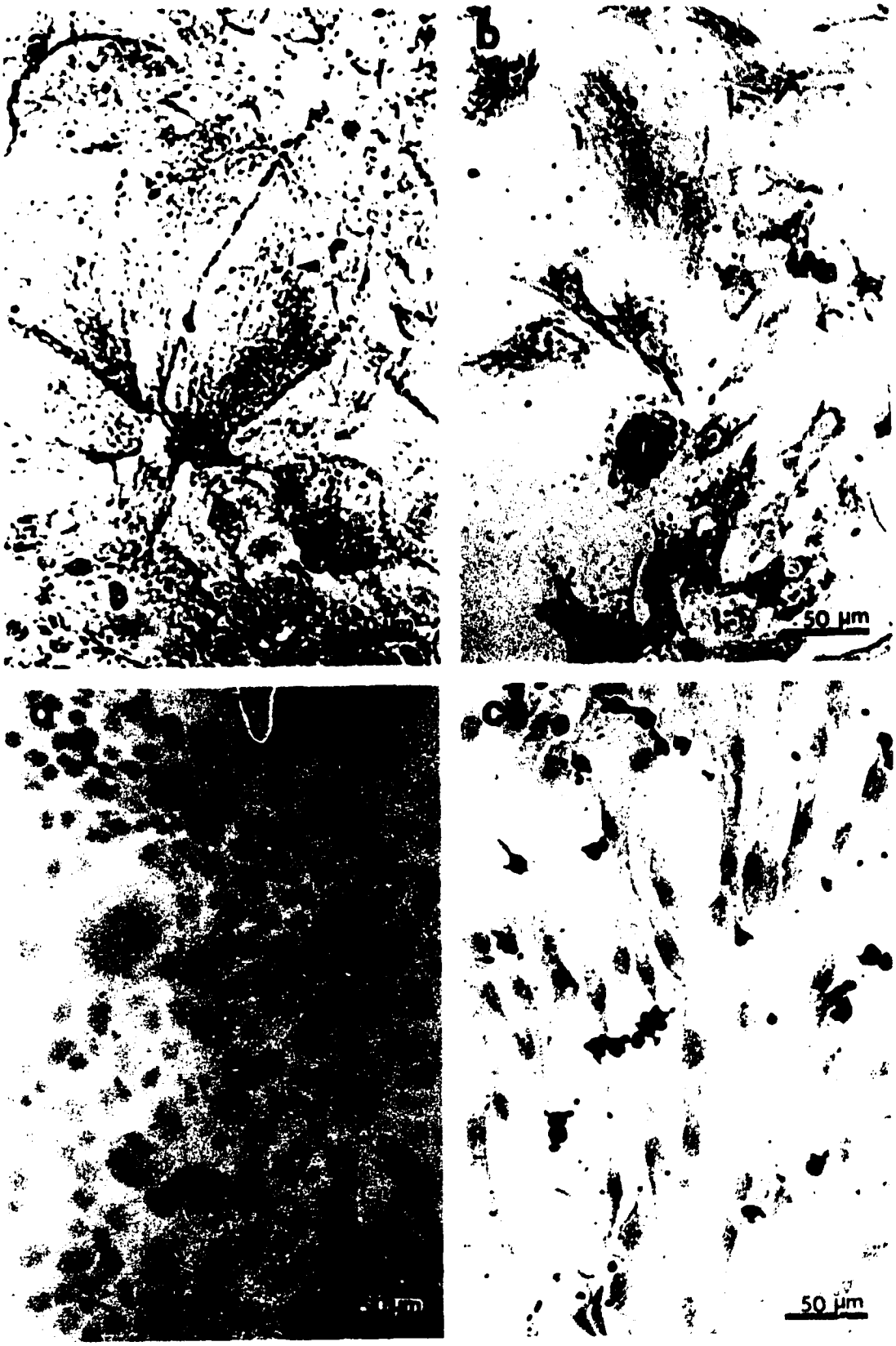
a) Using a polyclonal rabbit anti-cytokeratin primary antibody, Jag-1 cells at confluent growth are all positively stained. Cytokeratin filaments can be seen extending towards the cell boundaries (arrow).

b) Mouse trophoblast cells as the positive control were also stained with this antibody.

c) Pig monocyte/macrophages (mesodermal in origin) used as the negative control, did not react with this antibody. Confluent growing Jag-1 cells, mouse trophoblast and pig monocyte/macrophages did not stain with this nonimmune serum (not shown).

d) Identification of the vimentin intermediate filaments using a monoclonal mouse anti-vimentin primary antibody. Jag-1 cells are vimentin-negative.

e) Pig monocyte/macrophages, as the positive control, were strongly stained with this antivimentin antibody. Jag-1 cells and monocyte/macrophages were not stained when nonimmune mouse serum or the IgG1 isotype controls were used as the primary antibodies (not shown).


b
50 µm
c
d
50 µm

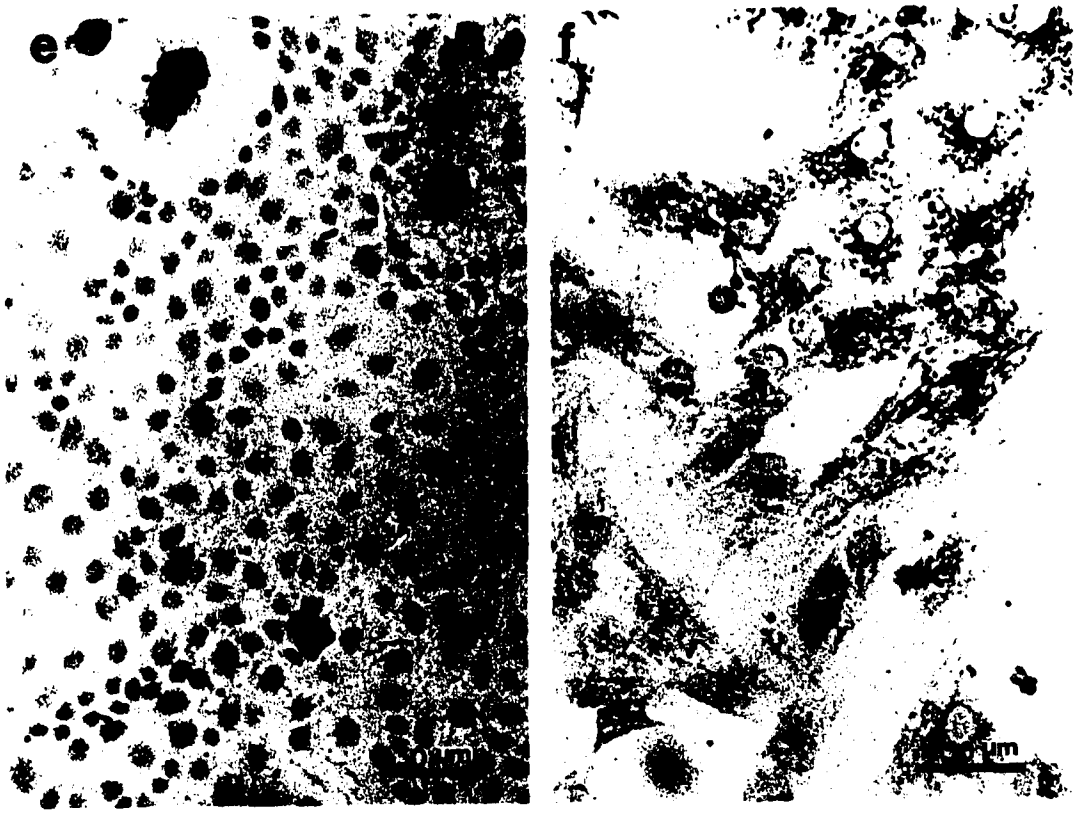

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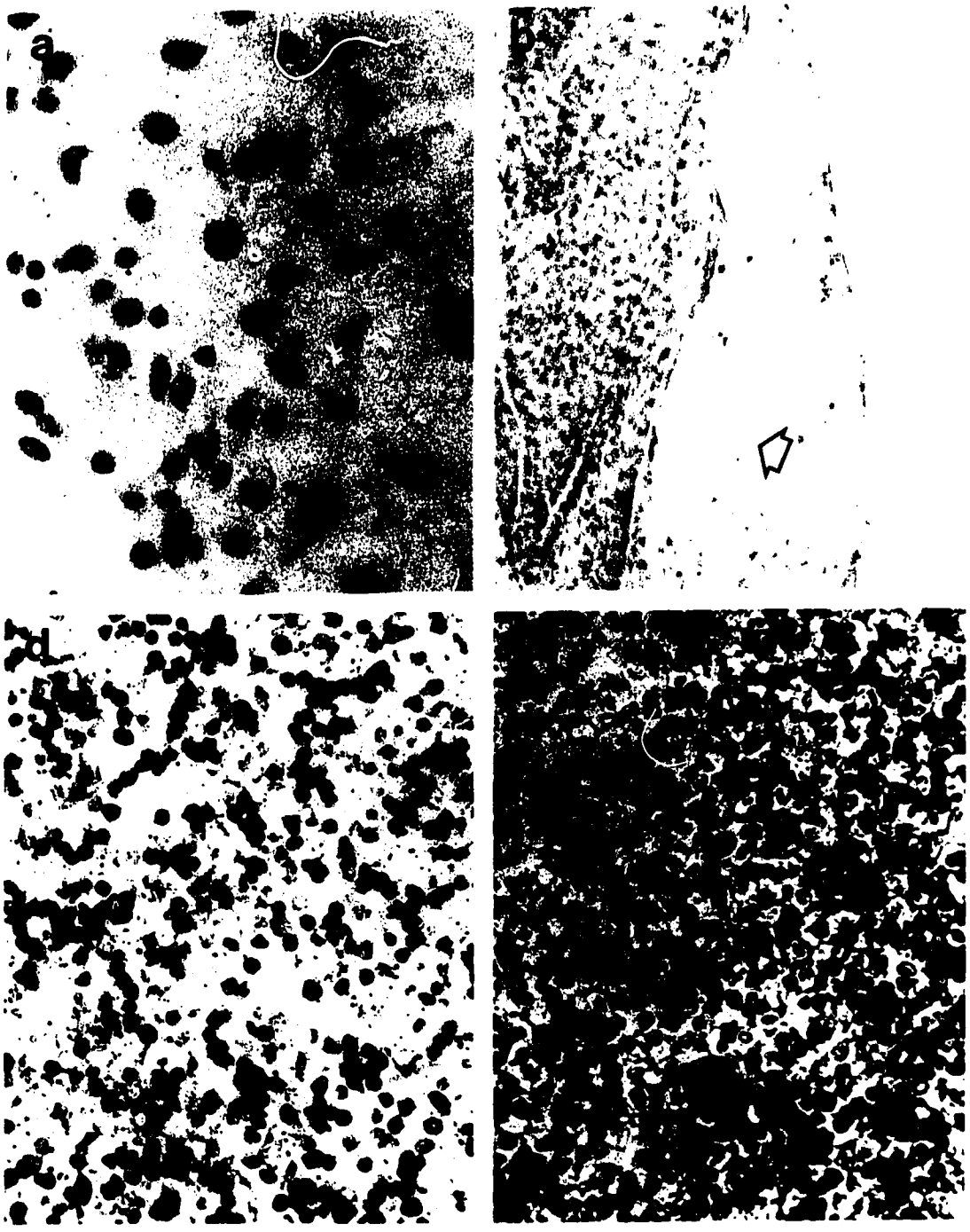
Fig. IV.5. Identification of Class I and Class II MHC antigens.

a) Using a mouse monoclonal anti- Class I primary antibody, Jag-1 cells do not express Class I antigens.

b) Fresh trophectoderm from the tips of day 14 blastocysts also do not express Class I antigens.

c) As the positive control, all the Pig PBLs expressed Class I antigens. Jag-1 cells and fresh trophectoderm also did not show staining with the monoclonal anti-Class II antibody (not shown, but photomicrographs are similar to 5a and 5b respectively).

d) As expected, a portion of the positive control pig PBLs were stained with the anti-Class II antibody.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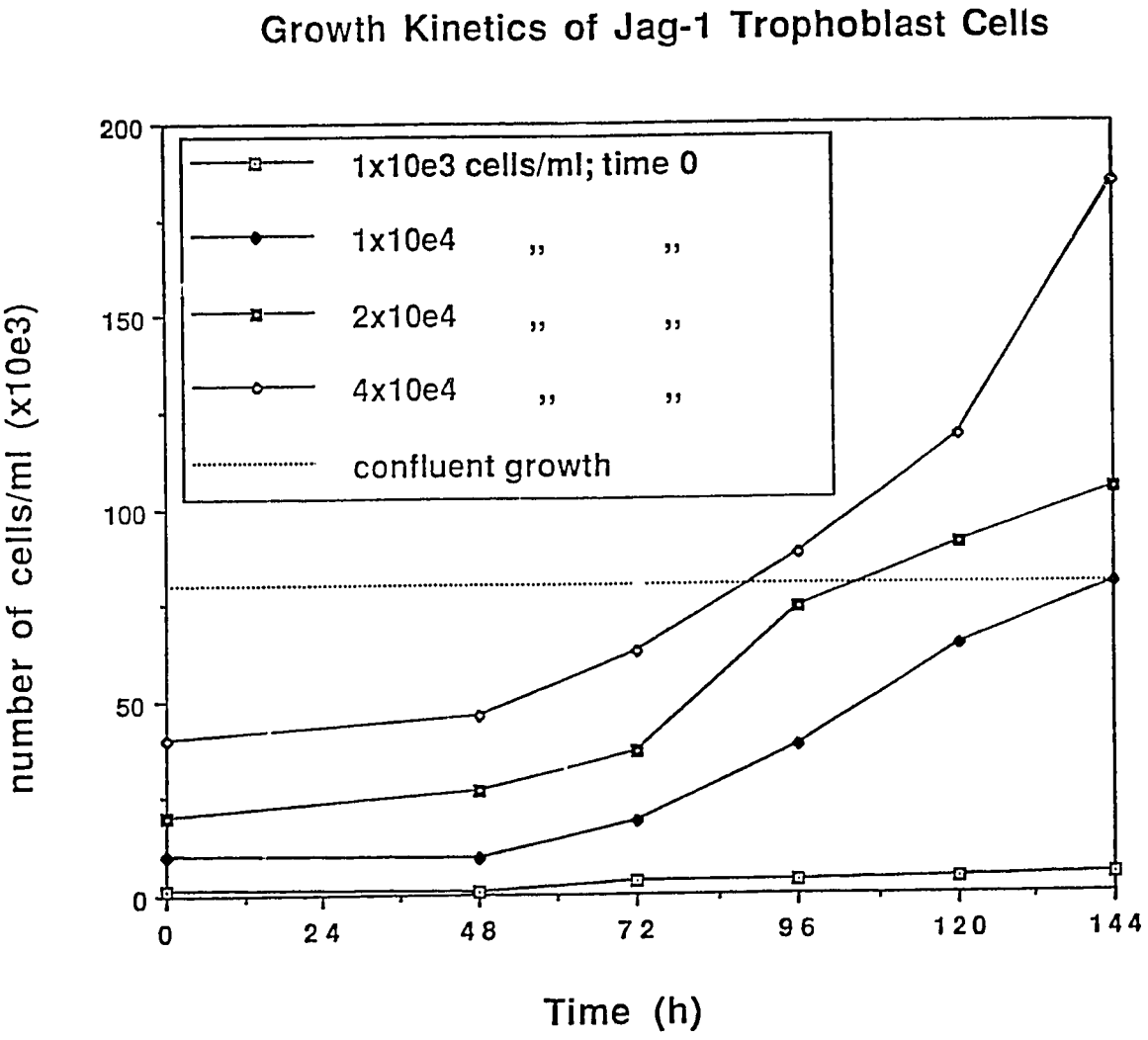


Fig. 6. Kinetics of Jag-1 cell growth. Effect of different cell concentrations on the time taken to reach confluent growth. The cells were grown in 75^2cm flasks and confluent growth was reached at $2x10^6$ cells/flask.

Fig. IV.7. a) Effect of Jag-1 cell conditioned medium, recombinant bovine GM-CSF and Day 14 Blastocyst conditioned medium on the proliferation of monocyte/macrophages from the pig.

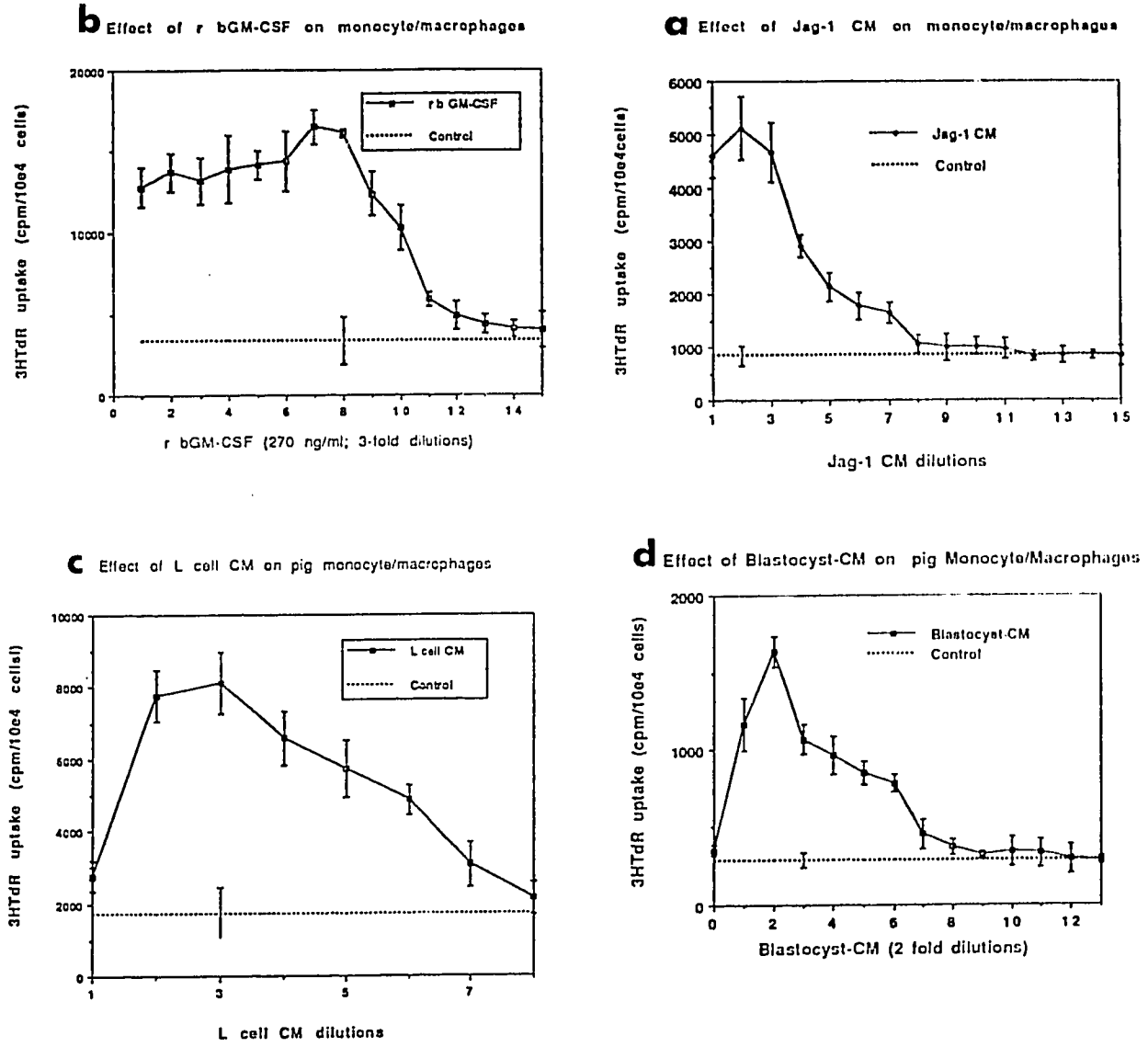
Maximum proliferation occurred at 50% Jag-1 CM. In four independent experiments, this concentration of conditioned medium stimulated at least a five-fold increase in porcine macrophage proliferation.

b) With recombinant bovine GM-CSF peak stimulation of proliferation occurred between 120-370 pg/ml (dilutions 7-8). This experiment was repeated five times and in each, r BoGM-CSF stimulated at least a five-fold increase in proliferation.

c) Effect of mouse L929 cells on the proliferation of porcine monocyte.macrophages. A similar level of stimulation was seen as with r BoGM-CSF

d) A similar level of stimulation of proliferation to that of Jag-1 CM and rBoGM-CSF was seen with day 14 blastocyst conditioned medium. These experiments were repeated each time with monocyte/macrophages from different pigs.

FIGURE 7.


b Effect of r bGM-CSF on monocyte/macrophages
3HTdR uptake (cpm/10e4 cells)
r b GM-CSF
Control
r bGM-CSF (270 ng/ml; 3-fold dilutions)
a Effect of Jag-1 CM on monocyte/macrophages
3HTdR uptake (cpm/10e4cells)
Jag-1 CM
Control
Jag-1 CM dilutions
c Effect of L cell CM on pig monocyte/macrophages
3HTdR uptake (cpm/10e4 cells)
L cell CM
Control
L cell CM dilutions
d Effect of Blastocyst-CM on pig Monocyte/Macrophages
3HTdR uptake (cpm/10e4 cells)
Blastocyst-CM
Control
Blastocyst-CM (2 fold dilutions)

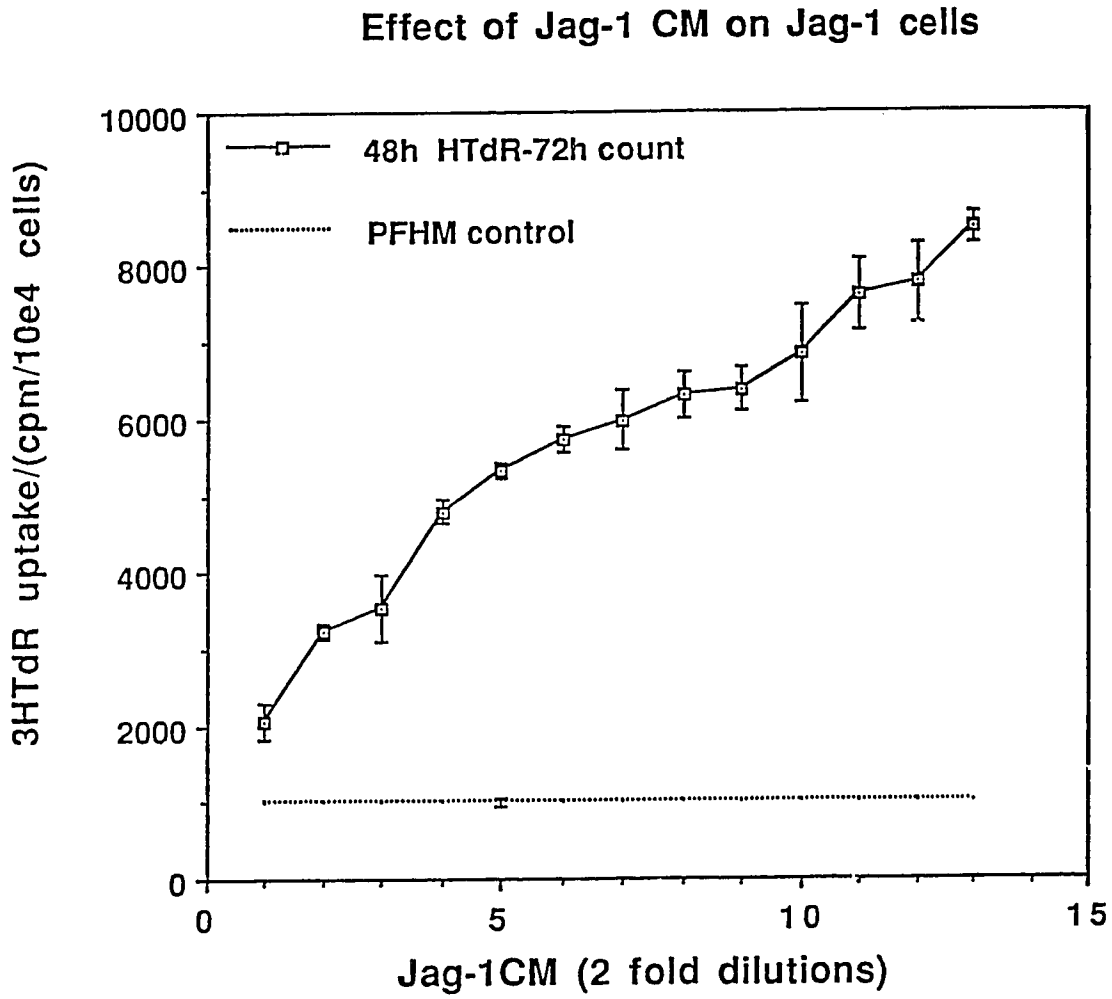


Figure 8. Effect of Jag-1 cell-conditioned medium (48 h) on Jag-1 cell proliferation in a protein-free hybridoma culture medium (PFHM). The PFHM was incubated in the absence of cells and used as a control.

FIGURE 9

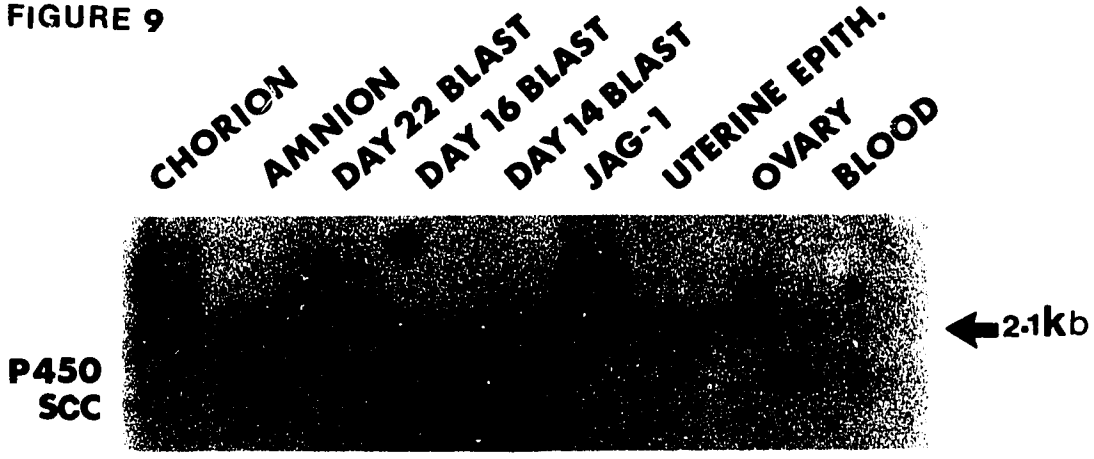


Figure 9. A cDNA probe specific for porcine cytochrome P450 cholesterol side chain cleavage enzyme (P450 SCC) was used in a Northern blot analysis to determine whether the Jag-1 cells expressed mRNA for this enzyme. Total RNA from Days 14, 16 and 22 blastocysts, uterine epithelial cells, the ovary , peripheral white blood cells, and term extrafetal membranes were used for comparison. The mRNA for this enzyme was strongly expressed in all the tissues except blood. It was especially strong in the Jag-1 cells and days 14 and 16 blstocysts. The protocol used is described in chapter IV of this thesis.

CHAPTER V

LACK OF CLASS I SLA ANTIGENS ON TROPHOBLAST OF PERI-IMPLANTATION BLASTOCYSTS AND TERM PLACENTA IN THE PIG

INTRODUCTION

Trophoblast tissue constitutes the major barrier between the maternal and fetal circulations. This cell type has developed unique mechanisms for protecting the fetus from maternal immunologic rejection responses (essentially graft rejection). Genes of the major histocompatibility complex (MHC) encode for highly polymorphic antigens involved in T cell recognition of foreign molecules in the context of self [Kline *et al.*, 1981; Zinkernagel and Doherty, 1979] and as such are central to the graft rejection response. Regulation of MHC antigen expression on trophoblast in the placentas of mammals is therefore central to the understanding of the survival of the semiąllogeneic fetus within the uterus of an immunocompetent mother. Given the economic importance of farm

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A version of this chapter has been submitted for publication. Jagdeece Ramsoondar, Robert J. Christopherson, Aziz Ghahary, Shirley Ellis, Larry J. Guilbert, Thomas G. Wegmann. Biology of Reproduction. 1994

animals, it is noteworthy that the MHC antigens of domestic animals have been implicated in influencing reproductive performance [Mallard *et al.*, 1991] and therefore have been considered in selective breeding programs [Mallard *et al.*, 1991;Van der Zijpp and Egbert, 1989].

Although MHC expression in farm animals such as sheep [Gogolin-Ewens, *et al.*, 1989], cow [Templeton *et al.*, 1987; Low, *et al.*, 1990], horse [Crump *et al.*, 1987; Donaldson *et al.*, 1990] and pig [Meziou, *et al.*, 1983; Warner *et al.*, 1986] resembles that of the better studied mouse and human models [see reviews Head *et al.*, 1987; Loke and King, 1991; Head, 1991; Hunt, 1992], the development and morphology of placentas in the two groups are quite different [Amoroso, 1952]. Especially notable is the epitheliochorial placentation of the pig [Amoroso, 1952]. The pig blastocyst undergoes extensive elongation just prior to attachment, which is achieved by interlocking microvilli on the surface of juxtaposed trophoblast and uterine endometrial epithelium between days 13 to 20 of gestation [Amoroso,1952; Dantzer, 1985].

The outer surfaces of the allanto-chorion and the endometrial epithelium become extensively folded as pregnancy progresses. The chorionic ridges or 'villi' fit into corresponding fossae of the uterine epithelium and *vice versa*. The trophoblast remains a non-invasive single layer. Practically the entire surface of the allantochorion forms the placenta, hence, the name placenta diffusa. In contrast to the placenta haemochorialis of humans and rodents where maternal blood comes into direct contact with fetal tissue, epitheliochorial types place both fetal and maternal layers of tissue between fetal and maternal blood. In pig beginning around midgestation the separation distance begins to decrease. The capillary plexuses at the tips of the chorionic villi penetrate between

the trophoblast cells until only a thin (~ 2μm) layer of trophoblast remains near term [Amoroso, 1952].

Class II MHC antigens have not been detected on the trophoblast of any species [see reviews Head *et al.*, 1987; Loke and King, 1991; Head, 1991; Hunt, 1992; Bulmer and Johnson, 1985]. In contrast class I MHC is expressed, but differently, among species. In the placenta, it is variable in expression among the different trophoblast subpopulations within species [Low, *et al.*, 1990; Donaldson, et al,. 1990; Head *et al.*, 1987; Loke and King, 1991; Head, 1991; Hunt, 1992; Bulmer and Johnson, 1985; Stern, et al., 1987]. Class I MHC is not expressed in the villous trophoblast, the predominant population in the mature human placenta [Sunderland, et al., 1981; Redman, et al., 1984; Hunt, et al., 1988]. However, invasive, extravillous trophoblast subpopulations express HLA-G, a monomorphic form of class I MHC [Ellis, *et al.*, 1986; 1990; Kovats, *et al.*, 1990; Risk, *et al.*, 1990]. A novel form of class I MHC is also expressed in the baboon placenta [Stern, 1987], but in contrast to humans, it is expressed on the syncytiotrophoblast (exchange area) of villous tissue. Classical forms of class I MHC have been detected on the spongiotrophoblast of the mouse but not on the labrynthine trophoblast (exchange area) [Zuckermann and Head, 1986; Redline and Lu, 1989; Headly, *et al.*, 1989]. In rat, MHC class I antigens are also expressed in spongiotrophoblast but not in the labrynthine layer. [Macpherson *et al.*, 1986; Kanbour, *et al.*, 1987]. The classical RT1.A and nonclassical Pa antigens are expressed on the basal-most (giant cell) trophoblast of syngeneic matings [Kanbour, *et al.*, 1987] Interestingly, in allogeneic rat matings, RT1.A is expressed only in the cytoplasm of giant cells while Pa is expressed both in the cytoplasm and at the cell surface [Kanbour, *et al.*, 1987]. It appears that both forms of rat MHC

class I are genomically imprinted in the placenta since only the p
antigens are expressed [Kanbour-Shakir, *et al.*, 1993].

MHC also appears highly regulated in the embryos of farm a
In the horse the invasive trophoblast cells of the transient chorionic
express high levels of class I antigens that are later downmodula
these cells' progeny, the mature eCG-secreting endometrial cu
Crump, *et al.*, 1987; Donaldson, *et al.*, 1990]. The non-in
trophoblasts of the allanto-chorionic membrane do not express
antigens. MHC class I antigens have been detected on day seve
embryos [Templeton, *et al.*, 1987] and on trophoblasts of the a
chorion in areas outside the placentomes but not on the villous trop
within the placentomes [Low, *et al.*, 1990]. In contrast (and with th
monoclonal anti-MHC antibody preparations), no class I MHC ha
found in the sheep placenta at any stage of gestation [Gogolin-Ewen
1989]. Pre-implantation pig blastocysts have been reported to expr
microglobulin between days six and twelve of gestation [Meziou
1983] and class I MHC antigen was serologically detec
preimplantation embryos between days two to six of gestation [Wa
al., 1986]. However, there has been no localization of MHC expre
pig embryos.

The rather high resorption rate in pig pregnancies [Flint
1982; Pope, *et al.*, 1985; Bolet, *et al.*, 1986] and the com
importance of the species argues for a greater understanding of wh
how MHC class I is expressed in the embryo. The present study is
to localize class I MHC expression on peri-implantation blastocy
extrafetal membranes at term. We have characterized pig class
expression both at the pre-(mRNA) and post- (antigen) translatio

the former by Northern and *in situ* analyses with cross-reactive bovine cDNA probes and the latter immunohistochemically with a monoclonal antibody that recognizes a common determinant on all class I MHC antigens. We found that MHC class I antigen could not be detected on the surfaces of extraembryonic/fetal membranes during gestation but that specific mRNA could be detected at low levels.

MATERIALS AND METHODS

Tissue collection and preparation

Outbred pigs of the Yorkshire breed maintained at the University of Alberta Swine Facility were used in this study. Blastocysts were collected at slaughter from second estrus gilts on days 14, 16 and 22 after mating (day of first estrus = day 0). Uterine tracts were exised from the animals within 15 min of slaughter, chilled on ice and taken to the lab. Blood was also collected from the pregnant animals at slaughter and pooled for isolation of total RNA from leucocytes (PBL). In the lab, blastocysts from pigs with the same gestational age (days: 14 n= 5 pigs, 62 blastocysts as indicated by the number of embryonic discs found, an average of 73 CLs were counted on the ovaries; 16 n= 5 pigs, 58 blastocysts counted with an average of 70 CLs,) were flushed from each uterine horn with cold phosphate buffered saline (PBS) and pooled.A total of 32 implanted blastocysts were removed mechanically from 3 pigs on day 22 postmating. After flushing, the uterine horns from days 14 and 16 pregnant animals were ligated at both ends and infused with a 0.2% collagenase solution and incubated for 30 min at 38°C.

The dissociated endometrial epithelial cells were then pelleted, washed and pooled. Cell smears were prepared for immunocytochemistry and the rest of the cells used for isolation of total RNA. Samples of tissues from different regions of the blastocysts were dissected from each of the three blastocyst pools for immunohistochemistry and from the day 16 pool for *in-situ* hybridization studies (described below).

The remainder of the three pools of blastocyst tissues were then aliquoted into 50-ml polypropylene centrifuge tubes (Corning Inc, Corning, NY; ~ 1g of tissue/tube) containing guanidine thiocyanate salt (GTC; Sigma, St. Louis, MO) solution (7 ml) and immediately homogenized with an electronic homogenizer (Ultra Turrax T 25-SI). The homogenates were then flash frozen in liquid nitrogen and stored at -70°C. Placental tissues at term were collected from two multiparous sows at farrowing. The amniotic and allanto-chorionic membranes were separated immediately after extrusion of the placentas. Samples from different conceptuses were pooled, minced and immediately homogenized in 50-ml tubes containing GTC and then frozen in liquid nitrogen to stop the action of placental ribonucleases. Frozen samples were taken to the lab and stored at -70°C for the extraction of total RNA. Also, samples of the amniotic and allanto-chorionic membranes from randomly selected conceptuses were directly spread onto microscope slides with either the fetal- or maternal-face surface exposed for immunohistochemical staining.

Immunohistochemistry

Pieces of extraembryonic tissues and embryos (n=10), randomly selected from each of the three blastocyst pools, were air dried onto chrom-alum gelatin coated microscope slides and then fixed with cold

acetone for 20 min at -18°C. Term amniotic and allanto-chorionic membrane pieces, uterine epithelial cells and PBL were also air dried onto slides and similarly fixed with cold acetone. In addition, pieces of tissues and embryos (n=10) randomly selected from each of the blastocyst pools were embedded in OCT compound (Miles Inc., Elkhart, IN) and frozen in liquid nitrogen. Frozen sections (six mm) were cut and collected onto coated slides, air dried and fixed with cold acetone.

The indirect immunoperoxidase labelling procedure was as previously described [Ramsoondar, *et al.*, 1993]. A mouse monoclonal anti-porcine class-1 antibody (mAb, PT85; VMRD INC., Pullman, WA) was used as the primary antibody in the detection of class I MHC antigen expression. This mAb specifically recognizes a monomorphic determinant on all class I swine leucocyte antigens [SLA; Lunney, *et al.*, 1988]. A mouse monoclonal anti-porcine vimentin antibody (Dakopatts, Glostrup, Denmark) was also used to indirectly label term-placental tissues. Peripheral blood leucocytes and uterine epithelial cells were used as positive controls and normal mouse serum as the reagent controls.

Preparation of Radiolabeled Probes

The bovine class I cDNA probe used for Northern blot analysis was previously described [Bensaid, *et al.*, 1991]. The following procedure to prepare the class I MHC plasmid construct was carried out by Dr. Shirley Ellis (AFRC Institute for Animal Health, Berkshire, England) as part of our collaboration on this study. Briefly, two cDNA clones 5.1 and 2.1, which code for the Aw10 and KN104 class I MHC molecules, respectively, were inserted into the plasmid pUC19. The inserts, 1378 bp for Aw10 and 1396 bp for KN104, were isolated by digestion with EcoRI. A mixture of

the Aw10 and KN104 cDNA inserts were labeled with ^{32}P-alpha-dCTP by nick translation and used for hybridization.

The BL3 - 7 bovine class I MHC cDNA clone [Ennis, et al., 1988] was used to prepare riboprobes for in-situ hybridization. Polymerase chain reaction (PCR) was used to amplify a fragment of BL3- 7 between exons 2 and the junction of exons 5 and 6, approximately 770 bp in length. This fragment was further amplified using oligonucleotide primers modified to generate an Eco RI site at the 5' end and a blunt 3' end. The transcription vector pcDNA 3 (Invitrogen, San Diego, CA) was cut with EcoRI and EcoRV, and the PCR amplified fragment ligated between the T7 and Sp6 promoters. The pcDNA plasmid was linearized by digestion with Bam HI (antisense) or Not I (sense) restriction enzymes. The production of riboprobes was performed with an in vitro transcription kit (Ambion, Austin, TX). Linearized plasmid (1mg) was incubated with 0.5 mCi/ml ^{35}S-CTP (1200 Ci/mmol; NEN, Du Pont Canada Inc., ON) , 0.5 mM each of nucleotides ATP, GTP and UTP, RNasin, 10mM DTT, 0.2 U/ml RNA polymerase Sp6 (anti-sense) and T7 (sense) for 1h at 37 ^{o}C. Approximately 10^7 cpm were incorporated into RNA probes per mg of template DNA.

Northern Blot Analysis

Total RNA was isolated from the following tissues and cells: days 14, 16 and 22 blastocysts, pooled uterine epithelial cells, PBL, amniotic and allanto-chorionic membranes from term-placenta, and the Jag-1 trophoblast cell line [Ramsoondar, *et al.*, 1993]. Samples of cells (~1 x 10^8) and tissues (~ 1g), previously homogenized in GTC and frozen upon collection, were thawed in a 37^{o}C water bath. The DNA in the samples was sheared using 20-ml syringes fitted with 18 gauge needles. The total RNA from the

tissues and cell homogenates was extracted by the GTC/CsCl method of Chirgwin et al. [Chirgwin, *et al.*, 1979]. Approximately 50 µg of total RNA from each sample (except for that of PBL where 20 µg was loaded onto lane 5 of Fig 3a, and 10 µg, 5 µg and 2.5 µg in lanes 8, 9 and 10 of Fig. 3c, respectively) was separated by electrophoresis on 1% Agarose gel containing 2.2 M formaldehyde and blotted onto nitrocellulose filters (NEN Research Products, Boston MA). Filters were baked for 2 h at 80°C under a vacuum and prehybridized for 3 h at 42°C in a solution of 50% formamide, 20 mM Na H_2 PO_4, 4 x SSC (1x = 0.15 M sodium chloride, 0.015 M sodium citrate), 2mM EDTA, 4 x Denhardt's solution (1 x = 0.02% BSA, Ficoll and polyvinylpyrolidine), 1% SDS and 100mg /ml sonicated denatured salmon sperm DNA.

Hybridization was performed in the same solution at 42°C for 16 - 20 h with a probe prepared from a mixture of two bovine class I cDNAs. The filters were initially washed at room temperature with 2 x SSC and 0.1% SDS. Autoradiography was performed by exposing Kodak X-Omat film (Rochester, NY) to the nitrocellulose filters at -70°C in the presence of an enhancing screen. This experiment was repeated at least three times.

In-situ Hybridization

The procedure used for *in-situ* hybridization was previously described [Lin, *et al.*, 1993]. Briefly, tissue samples from the day 16 blastocyst pool were fixed in a 4% paraformaldehyde /PBS solution overnight at 4°C. The tissues were cryoprotected by immersion for 2 h in each of a graded series of sucrose/PBS solutions (12%, 16%, 18%), embedded in OCT compound and frozen in liquid nitrogen. Frozen sections (6mm) were placed on chrom-alum gelatin precoated slides, fixed with 4%

paraformaldehyde/PBS for 20 min and then dehydrated in a graded series of ethanol.

For *in situ* hybridization, 1 ml of prehybridization mixture was added to each slide and the slides incubated for 1 h at 37°C. Hybridization mixture (100µl) containing the labeled RNA probes were added to each slide and hybridization performed at 45 °C for 15 h in a humidified chamber. The slides were then washed and autoradiography performed at -20°C for 10 days. Thereafter, the slides were developed, fixed, washed and counterstained with hematoxylin and photographed under bright field illumination. This experiment was carried out twice on different tissue samples.

RESULTS

Class I MHC antigens are not expressed on pig trophoblast.

Neither class I nor class II MHC antigens were expressed on a trophoblast cell line (Jag-1) grown from the bulbous tips of elongated day 14 pig blastocysts [Ramsoondar, *et al.*, 1993] nor from round, tubular and elongating blastocysts from day 11 of gestation. In the present study, we utilized immunocytochemistry, Northern blotting and *in situ* hybridization techniques to delineate the pattern of class I MHC antigen or mRNA expression in peri-implantation blastocysts and term extrafetal membranes.

Extraembryonic membranes of days 14, 16 and 22 peri-implantation blastocysts, whether prepared by microsection or by spreading and fixing directly onto slides, did not stain immunocytochemically for class I MHC antigens. Neither the outer trophectoderm nor the inner endoderm stained

for class I MHC antigens (Fig. V.1a is a representative sample of day 16 extraembryonic tissue - data for days 14 and 22 were similar and are not shown). Days 14 and 16 embryo proper were not immunohistochemically stained in this study. Exposure of the fetal face of the term allanto-chorionic membranes to the anti-class I mAb did not stain the parietal endoderm covering this surface. On the other hand, the intricate branches of the blood vessels underlying the endoderm were intensely labelled (Fig. V.1b). When the maternal face of the allanto-chorionic membrane of term placentas were exposed to antibody, the chorionic epithelium (trophoblast) did not stain (Fig. V.1c). However, the inter-epithelial capillary plexuses at the tips and sides of the chorionic folds were intensely stained (Fig. V.1c). A similar pattern of labelling was evident with anti-vimentin antibodies (Fig. V.1d) indicating that only the blood vessels (mesodermal in origin) expressed class I antigens. The close proximity of these blood vessels to the surface that directly contacts maternal tissue presumably allows their reaction with the antibody.

The epithelial surface of the amnion that faces the fetus did not express class I MHC (Fig. V.2a). Instead, prominently labelled foci of rounded cells were evident (Fig. V.2a). These class I positive cell clusters were numerous and in some areas were confluent over this surface of the amnion (not shown). Except for the secondary vascular elements found on this side, the abembryonic surface of the amnion also did not stain for class I MHC (Fig. V.2b). Surprisingly, on both surfaces of the amnion, vimentin positive, round and stellate cells were common but did not stain for class I MHC (Fig.V.2c). These cells were not further characterized in this study. Both uterine epithelial cells, enzymatically harvested after blastocyst collection (Fig.V.2d), and PBL, smeared onto slides (data not

shown), intensely stained for class I and served as positive controls. Replacement of the mouse anti-class I antibody with normal mouse serum served as a reagent control and did not stain cells or tissues (data not shown).

mRNA for Class I MHC Antigens is Expressed at Very Low Levels in Periimplantation Blastocysts and Extra-Fetal Tissues at Term

Figure V.3 a, b and c are three independent Northern blot analyses of mRNA hybridization to a class I MHC-specific cDNA probe. The upper panel in each analysis shows the ethidium bromide-stained RNA. The 28s and 18s ribosomal RNA (rRNA) bands indicate the relative amounts and quality of total RNA loaded. Figure V.3d shows that the ethidium bromide-stained rRNA bands from these tissues accurately reflect mRNA load (as measured by hybridization to the cell-cycle independent gene b-actin). The lower panels in Fig. V.3a, b, and c show the hybridization analysis of the blots for class I MHC. Although there was generally more degradation in samples from tissues than from isolated cells, RNA from the allanto-chorionic membranes appears to have been the most degraded, probably due to the retention of the placentas that normally occurs after birth in the pig. However, in no instance did this degradation preclude some degree of hybridization to specific cDNA probes. The class I-specific cDNA probe detected a single band with an approximate size of 1.5 kb, similar in size to that found in cattle with the same probe (Bensaid, *et al.*, 1991).

It was evident from all three analyses that embryo/fetal tissues and cells expressed considerably lower levels of class I MHC mRNA than did maternal tissues. Even though more than twice the amount of Jag-1 (50 µg,

lane 4) than PBL (20 μg, lane 5) RNA was loaded, the intensity of the PBL band was much greater than that of Jag-1. The relative intensity of MHC to rRNA bands confirms this tendency. In addition, eventhough similar amounts of allantochorionic membrane (partially degraded, Fig. V.3a, lane 1), term amnion (lane 2) and day 16 blastocysts (lane 3) RNA were loaded, a clear and reproducible band was only seen for the tissues at term. This was more evident in Fig. V.3b in which RNA from day 14 blastocysts (lane 4) and uterine epithelial cells (lane 6) were included in the analysis. Relative to day 14 and 16 samples, a higher expression of class I mRNA was seen at term (lanes 1 and 2 of Fig. V.3a, b and c) and for day 22 blastocysts, probably due to the vascularization of the latter tissues. Expression of class I MHC mRNA from endometrial epithelial cells (Fig. V.3b, lane 6 {96h autoradiographic exposure} and Fig. V.3c, lane 7 {48 h exposure}) was relatively similar to that of PBL (Fig 3b, lane 7 and Fig 3c, lanes 8). The large discrepancy in class I mRNA expression between embryo-fetal derived tissues and those of maternal origin was emphasized in Fig. V.3c where RNA from PBL was titrated; 10 μg (lane 8), 5 μg (lane 9) and 2.5 μg (lane 10). This titration shows that 2.5 μg of RNA from PBL contained much more class I mRNA than 50 μg of RNA from any embryo/fetal-derived tissue or cell in that experiment (Fig. V.3c, lanes 1-6).

mRNA for Class I MHC Antigens were Ubiquitously and Uniformly Localized at Low Levels in All Tissues of Day 16 Blastocysts.

Specific hybridization with the antisense riboprobe indicated that class I MHC message was ubiquitously expressed in day 16 blastocysts. Fig.

V.4 shows semi-consecutive sections taken from two different embryos proper (a, b and e, f). Hybridization to the antisense probe is depicted on the left (a, c, e and g) and to the sense (control) probe on the right (b, d, f and h). Silver grains were localized in all layers of the embryos at low densities (determined relative to background densities, Fig. V.4d and h).

A similar pattern of silver grains was localized in the extraembryonic tissues. Representative samples are shown in Fig. V.5 (compare a and c to b and d) for antisense and sense hybridization, respectively. The endodermal layer (Fig. V.5c, arrow) shows a similar level of class I mRNA expression as does the trophectoderm.

DISCUSSION

The cell line secreting mAb PT85 was generated by immunizing mice with pig thymocytes [Davis, *et al.*, 1987]. This mAb recognizes a monomorphic determinant on peripheral blood mononuclear cells of all inbred and outbred pigs tested [Lunney, *et al.*, 1988; Davis, *et al.*, 1987]. It also recognizes SLA class I molecules transfected into mouse L-929 cells even though these molecules are expressed on the cell surface with mouse β_2-microglobulin [see Lunney, *et al.*, 1988;]. The immunohistochemical analysis carried out with this antibody shows that class I MHC antigens were expressed at lower than detectable levels on trophoblastic tissues of day 11 round, tubular or elongating pre-implantation blastocysts [Ramsoondar, *et al.*, 1993]. In the present study, class I antigens also could not be detected on the trophoblastic surface of peri-implantation blastocysts from days 14, 16 and 22 of gestation. There was also no staining for class

I MHC on trophectoderm or endoderm of the blastocysts or on the allanto-chorion of term placentas. Placentas from the intermediate stages of pregnancy were not examined in this study. However, it is unlikely that class I MHC antigens would be expressed at midgestation on the trophoblast but not before day 22 of gestation or after at term. The absence of detectable cell surface expression of class I antigens on pig trophoblast at any stage of development is in accord with similar observations of its absence in sheep placentas at any stage of gestation[Gogolin-Ewens, *et al.*, 1989].

Since the PT85 mAb is directed against pig thymocytes, cells that very likely do not express non-classical, pregnancy-associated class I MHC antigens, it may not recognize non-classical forms and the possibility that unique non-classical forms are expressed on pig trophoblast cannot be formally excluded. Human extravillous trophoblast subpopulations express the HLA-G antigen [Ellis, *et al.*, 1986; 1990; Kovats, *et al.*, 1990; Risk, *et al.*, 1990] while the spongiotrophoblast of the rat expresses the Pa antigen [Redline and Lu, 1989; Headley, *et al.*, 1989; Macpherson, *et al.*, 1986], both unique non-classical class I antigens. The W6/32 mAb directed against a monomorphic determinant of class I MHC detects both classical and nonclassical MHC antigens in humans [Barnstable, *et al.*, 1978]. Therefore, it is possible that the PT85 mAb could also detect putative nonclassical forms of class I MHC antigens in the pig.

The lack of at least classical MHC molecules on pig trophoblast follows both that of the sheep [Gogolin-Ewens, *et al.*, 1989] and the horse, where, except for the expression of class I MHC on the invasive trophoblast of the transient endometrial cups, the non-invasive trophoblasts of the allanto-chorion are class I negative [Crump, *et al.*, 1987; Donaldson, *et al.*,

1990]. In contrast, the cow placenta, which is structurally similar to that of the sheep (syndesmochorial) and in which placentomes develop (i.e., areas where fetal trophoblast invades the uterine epithelium but not beyond the underlying basement membrane), the non-invasive trophoblasts of the interplacentomal allanto-chorion have been found to label for class I in some instances [Low, *et al.*, 1990]. This is especially perplexing since the same monoclonal anti-sheep class I antibodies (SUB-1) were used in both cow and sheep studies [Gogolin-Ewens, *et al.*, 1989; Low, *et al.*, 1990].

In contrast to extra-embryonic tissues, the embryos of peri-implantation pig blastocysts appear to express class I MHC antigens. The ontogeny of class I MHC expression detectable by immunoperoxidase staining was not addressed in this study. However, it should be noted that only one out of five day 16 embryos displayed weak staining when the whole embryo was mounted. It is possible that class I MHC antigens were expressed on embryos earlier than day 16, but at levels below detection by our methods. In similar fashion, mouse class I MHC antigens are barely detectable in the embryo proper as late as day 13.5 of gestation [Philpott, *et al.*, 1988]. Although pre-implantation blastocysts express class I MHC mRNA [Sprink, *et. al.*, 1993] and surface antigens [Warner, *et al.*, 1993], these appear to be downmodulated during implantation and are detected again around day 10 post-implantation after which levels increase progressively until term [Ozato, et al., 1985].

The low levels of class I MHC mRNA expressed in extra-embryonic tissues fits with the general down-modulation of class I expression in the pig placenta. In general, the amount of class I message found in tissues and cells of fetal origin was considerably less than that found in maternal tissues. Whether these low mRNA levels are reflected by less than

detectable protein expression or whether the antigen is translationally regulated is not clear. Although the pig trophoblast cell line Jag-1 expressed slightly higher levels of class I message than day 14-22 blastocysts, cell surface antigen still could not be detected on the line [Ramsoondar, *et al.*, 1993] suggesting translational or post-translational regulation. Post translational regulation of class I expression in embryo-derived cell lines of the mouse has been suggested [Birkoff, *et al.*, 1991]. On the other hand, transcriptional regulation of MHC expression has been suggested in mouse [Drezen, *et al.*, 1993] and human [Boucraut., *et al.*, 1993a] trophoblast subpopulations and may involve DNA hypermethylation [Boucraut., *et al.*, 1993b]. Trophoblast subpopulations in rat and man appear to employ multiple levels of control allowing the expression of non-classical class I while simultaneously repressing classical forms (see introduction). Different regulatory mechanisms appear to control MHC expression in extra-embryonic trophoblast and in the embryo during development [Oudejans, *et al.*, 1989]. In addition, special mechanisms appear to have evolved in trophoblast to control MHC expression. For instance, most trophoblast are refractory to cytokines such as IFNγ that are known to upregulate MHC expression on other somatic cell types [Hunt, *et al.*, 1987; Mattsson, *et al.*, 1992]. The expression of MHC class I antigens on trophoblast thus appears regulated in a temporal and spatial manner during development in a complex but highly efficient manner that operates at multiple levels.

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Fig. V.1. Detection of class I SLA antigens in whole-mounts of peri-implantation blastocysts and the extrafetal membranes at term by immunohistochemistry with mAb PT85. Staining appears dark on the background of hematoxylin counterstain.

(a) X270. Day 16 blastocyst tissue opened to expose the endodermal surface for staining (open arrow). No class I MHC antigens were detected on either the outer trophectoderm (closed arrow) or on the inner endodermal surface (open arrow) of the extraembryonic tissues. Similar results were obtained for day 14 and day 22 blastocysts (data not shown).

(b) X125. The inner endodermal surface of the allanto-chorionic placental membrane at term exposed for staining. This surface did not stain for class I MHC antigens (closed arrows). However, the underlying intricate branches of blood vessels were intensely stained (open arrows).

(c) X125. The outer trophectodermal surface of the allanto-chorionic placental membrane at term exposed for staining. This surface also did not stain for class I MHC antigens (closed arrows). However, the inter-epithelial capillary plexuses at the tips and sides of the chorionic "villi" were intensely stained (open arrows).

(d) X125. The pattern of staining for the vimentin intermediate cytoskeletal elements were similar to that for class I MHC indicating that the class I positive tissue in the pig placenta is of mesodermal origin. Normal mouse serum as reagent controls resulted in no staining for class I (not shown).

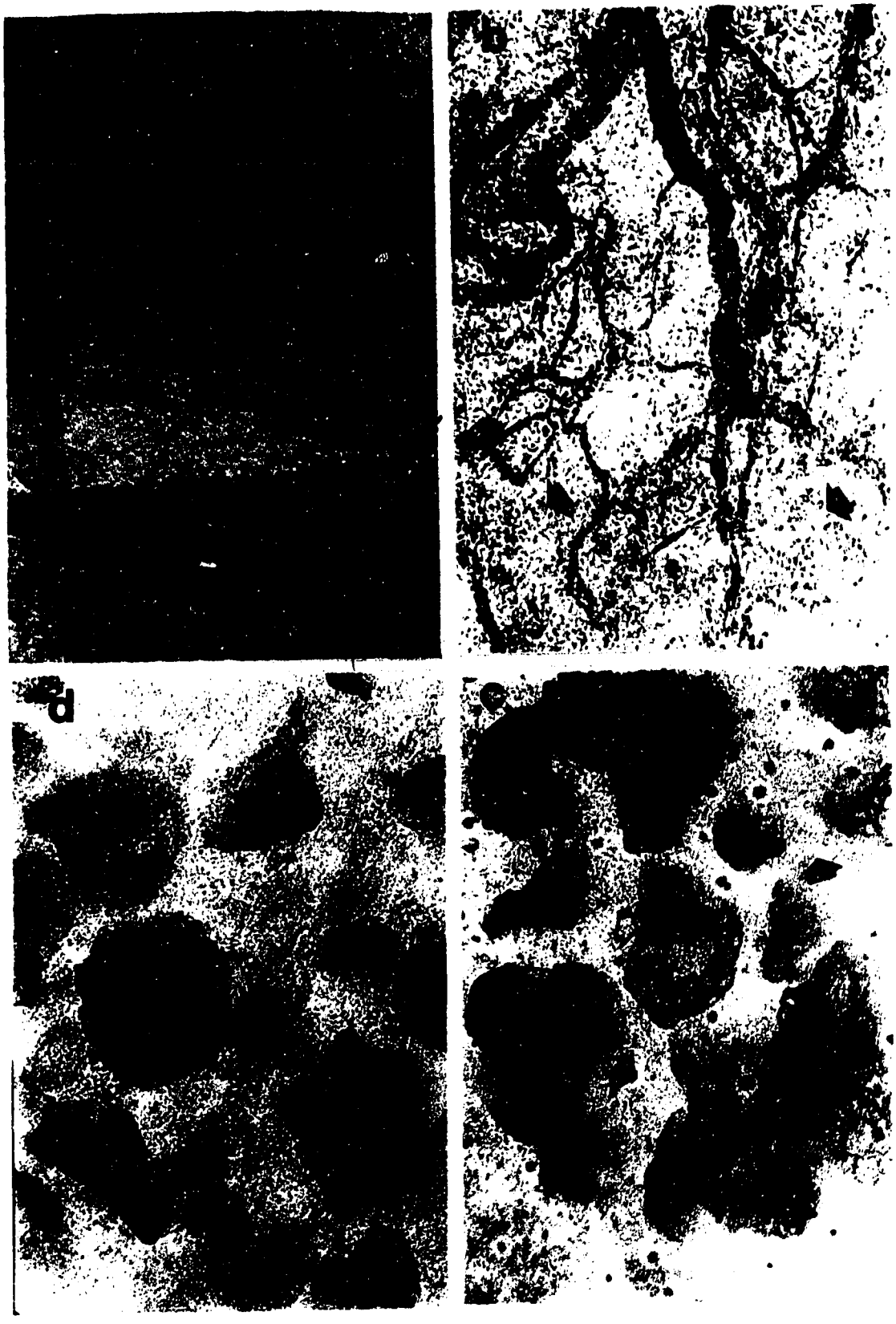

d

Fig. V.2. Detection of class I MHC antigens on whole-mounts of the amnion at term and on pooled endometrial epithelial cells from peri-implantation pig uteri.

(a) X270. The inner epithelial surface of the amnion at term was class I MHC antigen negative (closed arrow). Clusters of class I MHC positive cells were evident on this surface (open arrows).

(b) X125. The outer surface of the amnion away from the fetus was also class I MHC negative (closed arrow) However the secondary vascular element seen at this surface was strongly stained (open arrows).

(c) X125. A representative sample of the round and dendritic cells that stained positive for vimentin and negative for class I MHC antigens were found on both surfaces of the amnion at term.

(d) X125. As positive controls uterine endometrial epithelial cell smears were strongly labelled for class I MHC antigens. So too was PBL (data not shown; see Ramsoondar *et al.*, 1993). There was no staining with nonimmune mouse serum as reagent controls (data not shown).

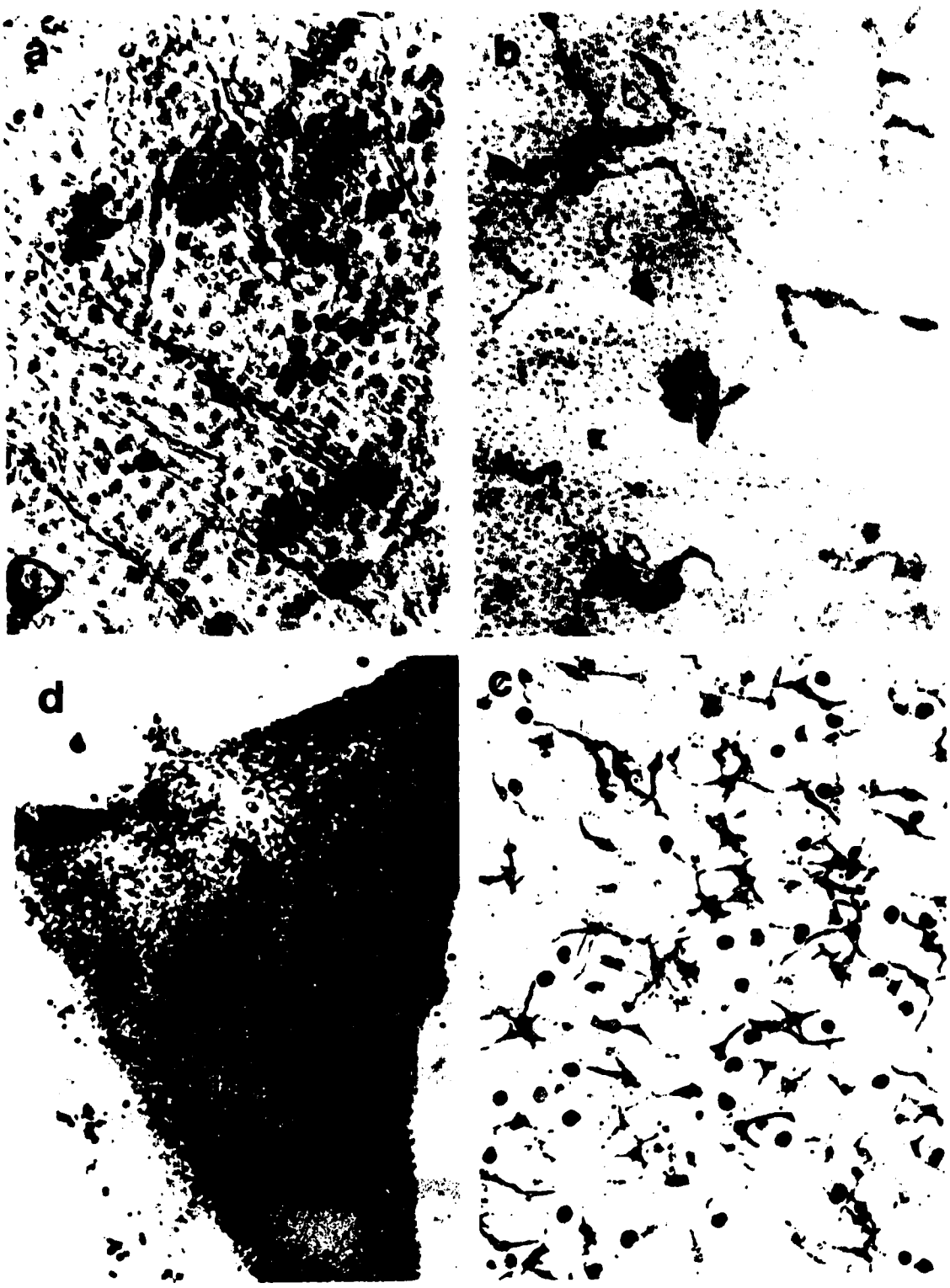

a
b
d
e

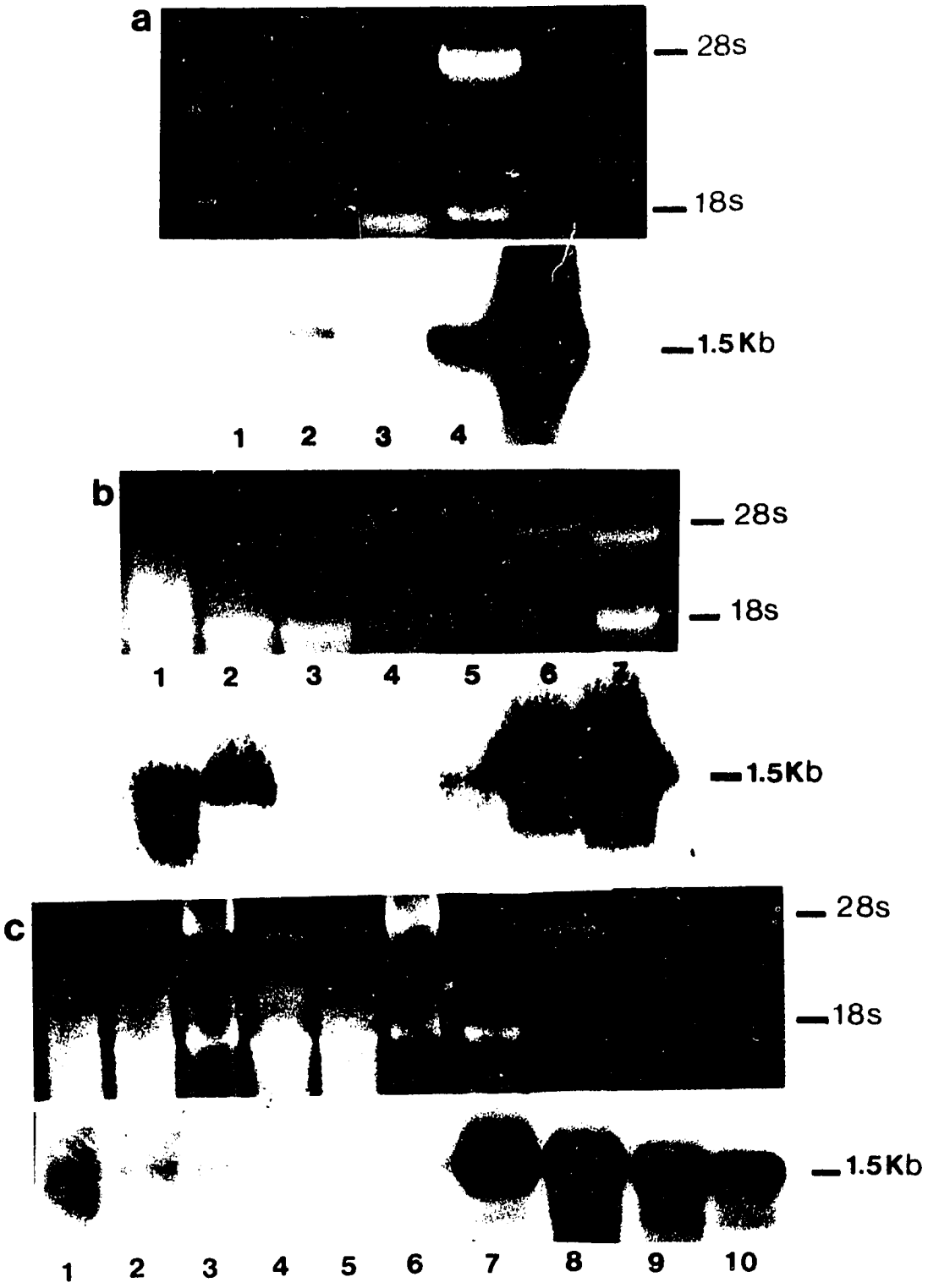
Fig. V.3. Northern blot analyses (a,b, and c) of class I MHC at the mRNA level in peri-implantation blastocysts and extrafetal membranes at term. A cDNA for b-actin was used as controls (d). The upper panel in each analysis represents the ethidium bromide stained RNA prior to transfer onto nitrocellulose membranes. Each panel shows the relative loading of total RNA as indicated by the intensities of the 28s and 18s ribosomal RNA bands. The autoradiograms (for a, c and d were developed after 72 h and b after 96 h) are shown in the lower panels.

(a) Total RNA from the allantochorion (lane 1), and amnion (lane 2) at term, day 16 blastocysts (lane 3), Jag-1 trophoblast cells (lane 4) and PBL (lane 5).

(b) Total RNA from allantochorion (lane 1), amnion (lane 2) at term, days 16 (lane 3), and 14 (lane 4) blastocysts, Jag-1 trophoblast cells (lane 5), endometrial epithelial cells (lane 6) and PBL (lane 7).

(c) Total RNA from allantochorion (lane 1), and amnion (lane 2) at term, days 22 (lane 3), 16 (lane 4) and 14 (lane 5) blastocysts, Jag-1 trophoblast cells (lane 6), endometrial epithelial cells (lane 7) and PBL (lane 8, 9, 10).

(d) Total RNA from allantochorion (lane 1), and amnion (lane 2) at term, day 22 blastocysts (lane 3), day 22 blastocysts polyA$^+$ RNA enriched (lane 4), day 16 (lane 5), and 14 (lane 6) blastocysts, Jag-1 trophoblast cells (50 µg total RNA, lane 7), Jag-1 trophoblast cells (20 µg total RNA, lane 8), endometrial epithelial cells (lane 9) and PBL (10 µg total RNA, lane 10).


a
28s
18s
1.5Kb
1 2 3 4
b
28s
18s
1 2 3 4 5 6 7
1.5Kb
c
28s
18s
1.5Kb
1 2 3 4 5 6 7 8 9 10

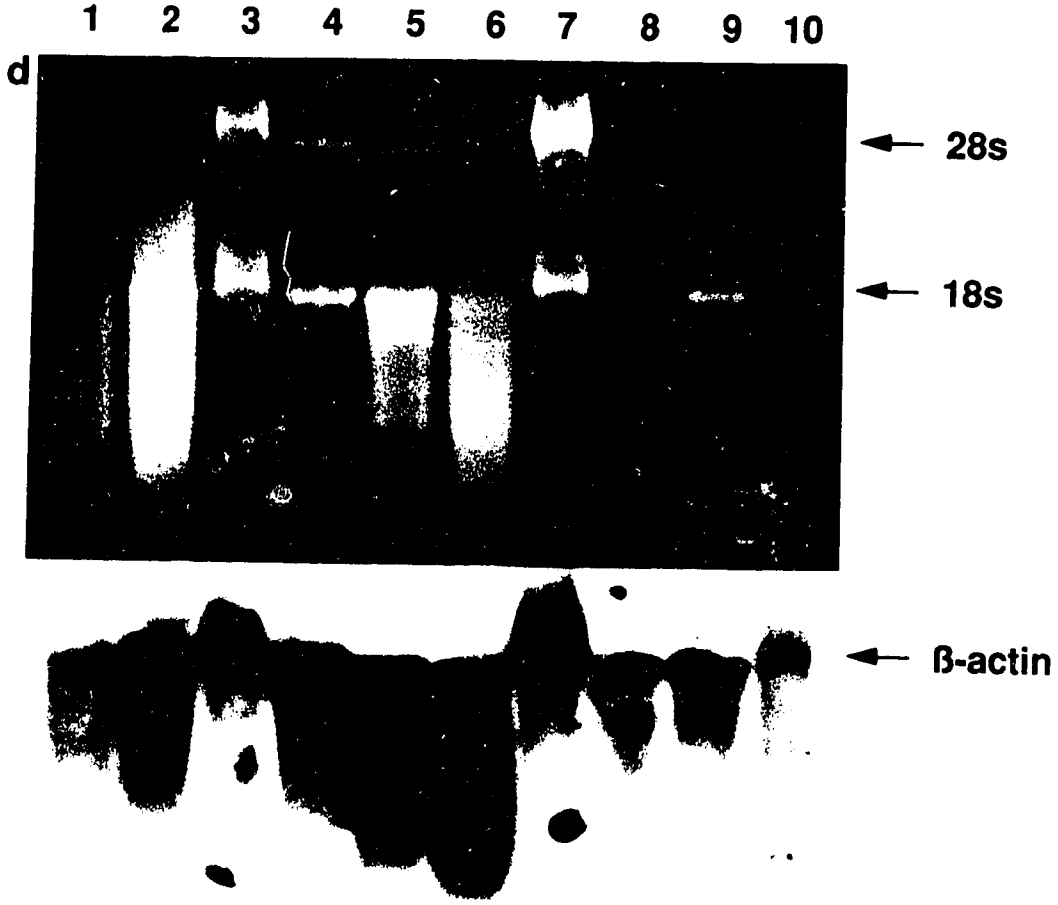

1 2 3 4 5 6 7 8 9 10
d
28s
18s
ß-actin

Fig. V.4 *In-situ* hybridization utilizing the embryo proper from day 16 blastocysts and class I MHC specific riboprobes. Hybridization to the antisense probe is shown on the left and to the sense (control) probe on the right. Sections were counterstained with hematoxylin. **(a)** and **(b)** X 30. Represents semi-serial sections taken from the same embryo. **(c)** and **(d)** (X 270). Enlargement of the fore-limb bud area indicated in a and b by rectangles to show the relative localization of silver grains. **(e)** and **(f)** (X 125). These represent semi-serial sections of another embryo with the areas within the rectangles enlarged in **(g)** and **(h)** (X 270).

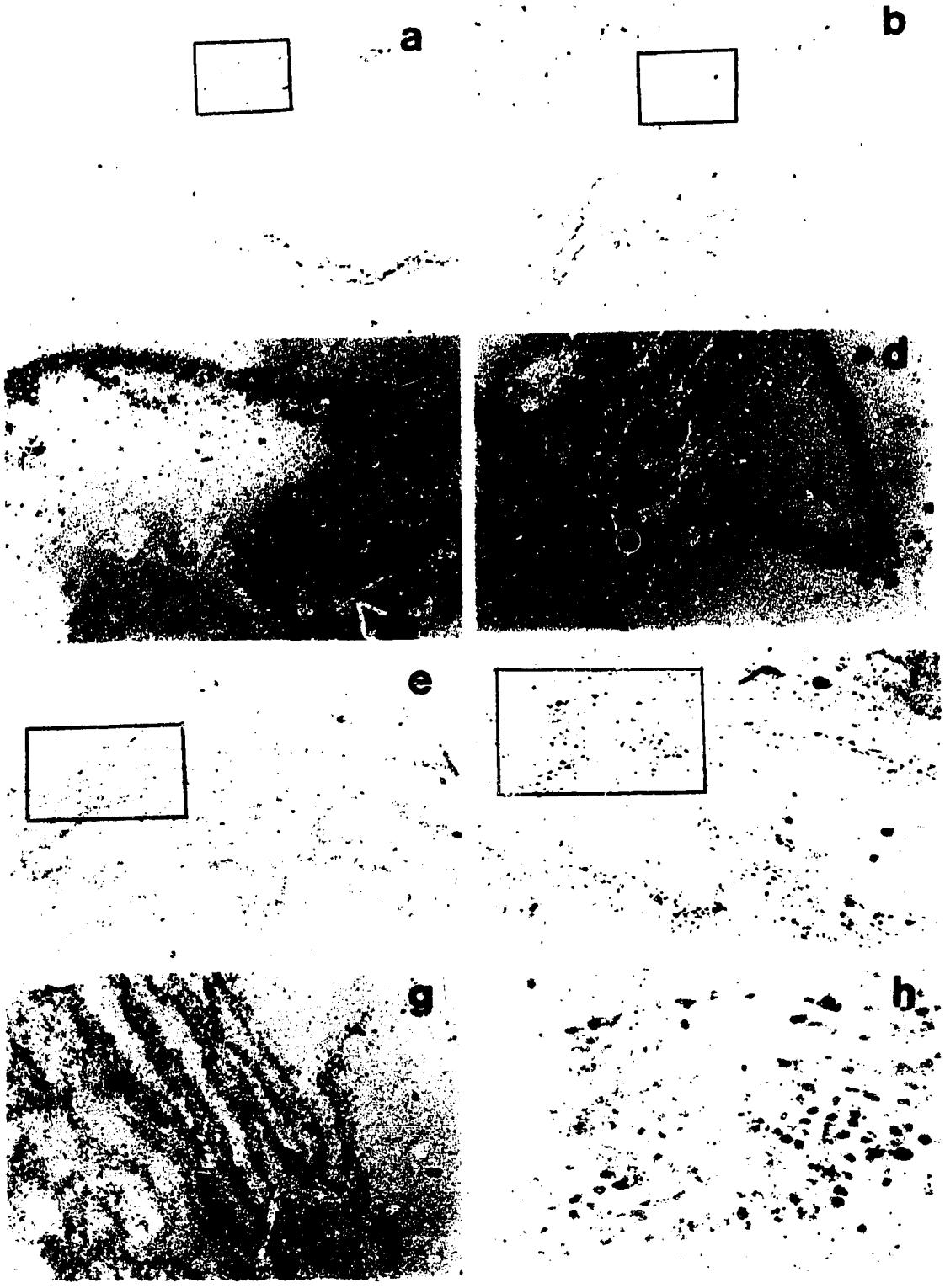
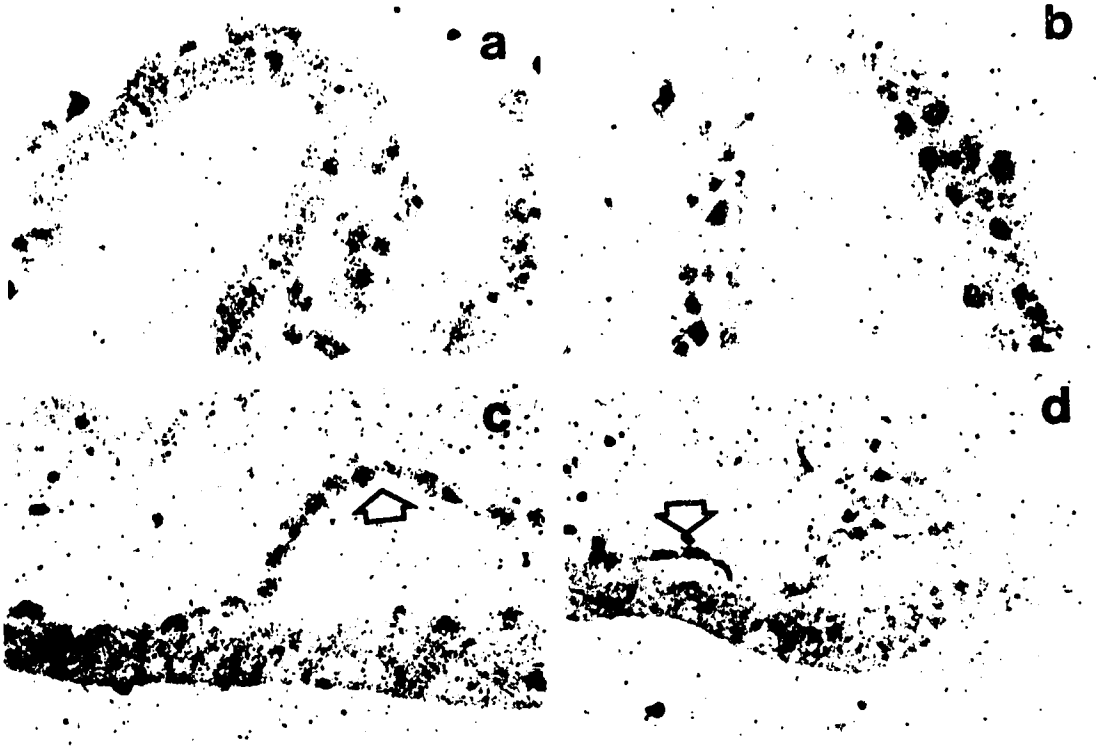

a
b
d
e
g
h

Fig. V.5 Localization of class I SLA mRNA on extraembryonic membranes from day 16 blastocysts by *in-situ* hybridization. Hybridization to the antisense probe is shown to the left and to the sense (control) probe to the right. Sections were counterstained with hematoxylin. **(a)** (X 270) and **b)** (X 420). Representative samples showing the *in-situ* localization of class I mRNA transcripts indicated by the distribution of silver grains. **(c)** and **(d)** (X 270). The same level of grain distribition can be seen for the endodermal cells (arrow) and the trophectoderm.


a
b
c
d

TOP

RAMSOONDAR et. al. 1994

FIGURE 5

CHAPTER VI

CHARACTERIZATION OF CYTOKINES EXPRESSED AT THE MATERNO-FETAL INTERFACE IN THE PIG

INTRODUCTION

Cytokines and growth factors are the most recent members of a growing list of potent regulatory factors identified at the materno-fetal interface during pregnancy. They include lymphokines, the colony stimulating factors CSF-1, GM-CSF, and IL-3, LIF, TGF-α, TGF-β, FGF, EGF and IGF-1 and -II [see reviews by Hunt 1989a; Simmen and Simmen 1990a; Guilbert *et al.*, 1991; 1993; Wegmann *et al.*, 1993; La Bonnardiere 1993]. It is now apparent that these cytokines are organized into a network at the materno-fetal interface, in which most of the diverse cell types of maternal and fetal origin are integrated [Hunt, 1989b; Guilbert, *et al.*, 1993]. Key players in this network are the uterine epithelial cells, macrophages and trophoblast, all highly secretory cell types [Hunt, 1990; Guilbert, *et al.*, 1991; Pollard, *et al.*, 1991; Robertson, *et al.*, 1992].

The CSF family of cytokines, which includes CSF-1 and GM-CSF are not restricted to the lympho-haematopoietic system as they have recently been shown to be involved in the regulation of pregnancy [Hunt, 1989a; Guilbert *et al.*, 1991; 1993; Wegmann *et al.*, 1993]. CSF-1 was proposed to be involved in the regulation of placental development and function throughout pregnancy in the mouse [Pollard *et al.*, 1987; Acerci *et al.*, 1989]. Additional support for this hypothesis was obtained from studies with the CSF-1 deficient osteopetrotic (op/op) mouse model

[Pollard *et al.*, 1991], and also by evidence from human [Daiter *et al.*, 1992] and the cow [Beauchamp and Croy, 1991].

Insulin-like growth factors -I and -II mediate tissue growth and differentiation via autocrine, paracrine and endocrine mechanisms, and are synthesized by many tissues [Daughaday and Rotwein, 1989)]. In the uterus IGFs are implicated in the remodelling processes that preceeds blastocyst implantation [see Simmen *et al.*, 1992] and their production is hormonally regulated [Simmen *et al.*, 1990b]. Moreover, a strong temporal correlation was also observed between the periimplantation changes taking place in the uterus and conceptuses and IGF-I production in the pig [Letcher *et al.*, 1989].

Cross and Roberts [1989] were the first to report that periimplantation pig blastocysts secreted proteins with antiviral activity. IFN-γ and a novel IFN-α were found to be responsible for this activity [Lefevre, *et al.*, 1990]. IFN-γ receptors are not present on implanting trophoblast of the pig nor are they inducible by IFN-γ, but they are found on the uterine epithelium [see La Bonnardiere, 1993]. Therefore, IFN-γ is not autocrine to trophoblast in the pig, but possibly has paracrine effects on the uterine epithelium. Being central to the immune response, IL-2 influences the proliferation, differentation and function of immune cells possessing its receptors, such as B cells, macrophages and other T cells [Smith, 1992]. TGF-β1 and its receptor are widely expressed in embryonic and adult tissues (Moses *et al.*, 1990).

CSF-1, GM-CSF and IGF-I are known to have growth promoting effects [Guilbert *et al.*, 1991; 1993; Wegmann *et al.*, 1993; Simmen and Simmen, 1990a] and IL-2, IFN-γ and TGF-β1, have the potential for generating inhibitory effects [Tezabwala *et al*, 1989; Chaouat *et al.*, 1990;

Massague, 1990] on the development of the placenta and survival of the fetus to term. Very little is known about the cytokines involved in the regulation of development of the conceptus in the pig. Furthermore, most cytokines identified as being important in reproduction in other species [Guilbert, *et al.*, 1993] are not available in purified forms homologous to the pig.

Macrophages are abundant within the uterus during pregnancy [Bulmer and Johnson, 1984; Tachi and Tachi, 1989; Hunt, 1989]. Exactly how this cell type impacts upon the developing conceptus is not fully elucidated. However, they share many morphological and functional characteristics with that of trophoblast including the production of factors to which they are mutually responsive [Guilbert, *et al.*, 1993]. We have recently isolated a trophoblast cell line that secretes growth factor(s) that both appear important for its own proliferation and that stimulates porcine macrophage proliferation [Ramsoondar, *et al.*, 1993]. The characterization of the porcine trophoblast derived macrophage growth factors (MGF) will therefore allow insight into the cytokine regulation of porcine placental development in general and trophoblast development and function in particular.

This report describes the expression of cytokines at the mRNA level in cells and tissues at the maternal-fetal interface and the partial characterization of the MGF produced by the Jag-1 cell line.

MATERIALS AND METHODS

Collection of Cells and Tissues for Total RNA Isolation.

Outbred pigs of the Yorkshire breed maintained at the University of Alberta Swine Facility were used in this study. Blastocysts were collected at slaughter from second estrus gilts on days 14, 16 and 22 after mating (day of first estrus = day 0). Uterine tracts were exised from the animals within 15 min of slaughter, chilled on ice and taken to the lab. Blood was also collected from the pregnant animals at slaughter and pooled for isolation of total RNA from leucocytes (PBL). In the lab, blastocysts from pigs with the same gestational age (days: 14 n= 5 pigs, 62 blastocysts as indicated by the number of embryonic discs found, an average of 73 CLs were counted on the ovaries; 16 n= 5 pigs, 58 blastocysts counted with an average of 70 CLs,) were flushed from each uterine horn with cold phospate buffered saline (PBS) and pooled. A total of 32 implanted blastocysts were removed mechanically from 3 pigs on day 22 postmating. After flushing, the uterine horns were ligated at both ends and infused with a 0.2% collagenase solution and incubated for 30 min at 38°C. The dissociated endometrial epithelial enriched cells (uterine epithelial cells) were then pelleted, washed and pooled.

The three pools of blastocyst tissues were then aliquoted into 50-ml polypropylene centrifuge tubes (Corning Inc, Corning, NY; ~ 1g of tissue/tube) containing guanidine thiocyanate salt (GTC; Sigma, St. Louis MO) solution (7 ml) and immediately homogenized with an electronic homogenizer (Ultra Turrax T 25-S1). The homogenates were then flash frozen in liquid nitrogen and stored at -70°C. Placental tissues at term were collected from two multiparous sows at farrowing. The amniotic and

allanto-chorionic membranes were separated immediately after extrusion of the placentas. Samples from different conceptuses were pooled, minced and immediately homogenized in 50-ml tubes containing GTC and then frozen in liquid nitrogen to stop the action of placental ribonucleases. Frozen samples were taken to the lab and stored at -70°C for the extraction of total RNA.

Jag-1 cells were grown to confluency in 175-cm^2 tissue culture flasks (Falcon, Los Angeles, CA). The supernatant from each flask was decanted and the cell-monolayers washed three times with cold PBS. The cells were harvested directly in GTC. Ten ml of GTC was pipeted into the first flask and allowed to dissolve the monolayer before serial transfer into subsequent flasks for a final concentration of about 10^8 cells/10 ml GTC. The GTC dissolved cells were flash frozen in liquid nitrogen and stored at -70°C.

Preparation of Radiolabeled Probes.

The cDNAs for GM-CSF, IL-2 and INF-γ were bovine in origin and kindly provided by Dr. R.A. Collins (Institute for Animal Health, Berkshire, UK). The cDNA for porcine CSF-1 was a gift from Dr. Fuller Bazer (Texas A&M University, Texas, USA). The porcine TGF-β1, cDNA was obtained from American Type Culture Collection (Rockville Maryland 20852) and the cDNA for porcine IGF-I was a donation from Drs. R.C.M. Simmen and F.A. Simmen (University of Florida, Gainesville, Florida). The GM-CSF cDNA insert cut from the EcoR V site of the pGEM-5Z(+) Vator, was 460 bp in length. IL-2 cDNA insert was cut from the EcoR V site of the Blue-script SK vector and was 550 bp in length. IFN-γ cDNA insert cut from the NcoI site of the pET8c vector was

435 bp long. The pCSF-1 cDNA insert excised from the EcoR I site of the pCR^{TM}II cloning vector was 823 bp in length. The 580 bp pIGF-I insert was excised from the EcoR I site of the pGEM4Z vector, and the 650 bp pTFG-β1 insert was excised by Sac I/PyuII digestion from the pcDVI vector. The cDNA probes were labeled with ^{32}P-alpha-dCTP by using the Nick-translation technique [Rigby *et al.*, 1977].

Northern Blot Analysis

Total RNA was isolated from the following tissues and cells: days 14, 16 and 22 blastocysts, pooled uterine epithelial cells, PBL, amniotic and allanto-chorionic membranes from term-placenta, and the Jag-1 trophoblast cell line [Ramsoondar, *et al.*, 1993]. Samples of cells (~1 x 10^8) and tissues (~ 1g), previously homogenized in GTC and frozen upon collection, were thawed in a 37°C water bath. The DNA in the samples was sheared using 20-ml syringes fitted with 18 guage needles. The total RNA from the tissues and cell homogenates was extracted by the GTC/CsCl method of . Chirgwin, *et al.*, [1979]. Approximately 50 µg of total RNA (20 µg for PBL) from each sample was separated by electrophoresis on 1% Agarose gel containing 2.2 M formaldehyde and blotted onto nitrocellulose filters (NEN Research Products, Boston MA). Filters were baked for 2 h at 80°C under a vacuum and prehybridized for 3 h at 42°C in a solution of 50% formamide, 20 mM Na H_2 PO_4, 4 x SSC (1x = 0.15 M sodium chloride, 0.015 M sodium citrate), 2mM EDTA, 4 x Denhardt's solution (1 x = 0.02% BSA, Ficoll and polyvinylpyrolidine), 1% SDS and 100mg /ml sonicated denatured salmon sperm DNA.

Hybridization was performed in the same solution at 42°C for 16 - 20 h with a probe prepared from a mixture of two bovine class I cDNAs.

The filters were initially washed at room temperature with 2 x SSC and 0.1% SDS. Autoradiography was performed by exposing Kodak X-Omat film (Rochester, NY) to the nitrocellulose filters at -70°C in the presence of an enhancing screen. This experiment was repeated at least twice for each cytokine..

Collection and Processing of Jag-1 Cell Conditioned Medium.

Jag-1 cells were cultured to confluency in 175-cm^2 tissue culture flasks containing RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (RPMI). The culture medium was changed and allowed to be conditioned for 72 h. The conditioned medium (RPMI-CM) was then pooled, filtered through a 0.2 μm filter (Millipore, Bedford, MN), and stored at -20°C for use as the positive controls in the monocyte/macrophage proliferation bioassay. For the isolation of the monocyte/macrophage growth factor(s; MGF) produced by the Jag-1 cells, a protein free tissue culture medium (PFM;Gibco) was used. Firstly, the optimal conditions for collecting Jag-1 cell-conditioned PFM medium was assessed. Jag-1 cells were grown to confluency in RPMI in 15 tissue culture flasks (175-cm^2). For harvesting of PFM-CM, the RPMI medium was decanted and the Jag-1 cell monolayers washed three times with phosphate buffered saline (PBS). PFM (20 ml) was then added to each flask and allowed to incubate for 3 h, 6 h and 12 h. Consequently, three different flasks of cells were used each day over a five day period, beginning on day 3 and ending on day 7 following the onset of culture (day of cell seeding=0). PFM incubated in the absence of cells was used as the negative controls in the bioassay. At the end of each culture period the PFM-CM

was filtered through a 0.2 μm filter and stored at -20°C for testing in the bioassay.

Jag-1 Cell Conditioned Medium Bioassay.

The procedure used for collection and processing of blood monocyte/macrophages was previously described [Ramsoondar, *et al.*, 1993 Chapter IV]. Briefly, blood was collected by jugular veinipuncture into sterile heparinized evucuated tubes. Peripheral blood leucocytes (PBL) were isolated and cultured overnight to obtain adherent monocyte/macrophages. Adherent cells were gently suspended, pelleted and diluted to 10^5 cells/ml in RPMI. Approximately 10^4 cells per well were pipetted into 96-well tissue culture plates. RPMI/10% FCS was incubated with confluent growing Jag-1 cells for 24 h for conditioning. This RPMI-CM was used as the positive controls and was serially diluted (2 fold dilutions) with RPMI and aliquoted into tissue culture plates (100 ml/well; 6 wells/dilution) containing recently harvested monocyte/macrophages. Protein free medium (PFM) conditioned by Jag-1 trophoblast cells was serially diluted (2 fold dilution) with PFM and aliquoted into tissue culture plates (6 wells/dilution) to assay for monocyte/macrophages proliferation stimulating properties. Similarly, the RP-FPLC fractions were each diluted in two fold serial dilutions with PFM and aliquoted into tissue culture plates (2 wells/dilution) and tested in the bioassay.

Culture Conditions.

Incubations were carried out at 37°C in an atmosphere of 5% CO^2 in air and 100% humidity for 48 h. The medium was then decanted from all plates and replenished as described above. Incubations were allowed to

continue for another 24 h and then 1 μCi ^{3}HTdR/well was added and cultured for an additional 18 h. At the end of incubation, wells were harvested and and counted for ^{3}HTdR incorporation.

Concentration and Reverse Phase-Fast Protein Liquid Chromatography (RP-FPLC)

Cell-conditioned medium (PFM-CM) was obtained by incubating confluent growing Jag-1 cells for 12 h in PFM as described above. The PFM-CM was th[illegible], filtered through a 0.2 μm filter (Millipore) and concentrated 10[illegible]ng an Amicon ultrafiltration membrane (YM3> 3000 MW) and system (Amicon Division, W.R. Grace and Co., Beverly, MA) at 4°C. Reverse phase fast protein liquid chromatography (RP-FPLC) was used to separate the proteins of different hydrophobicity present in the concentrated PFM-CM. A ProRPC HR 5/2 column was connected to an LCC liquid chromatography controller (Pharmacia Fine Chemicals AB, Sweden). Acetonitrile and water acidified with 0.1% trifluroacetic acid (TFA) were used as the organic and inorganic solvents, respectively. The concentrated PFM-CM was acidified by dialysis against TFA treated water. This procedure resulted in the precipitation of a mass of proteins from the sample. The sample was centrifuged at 600g for 10 min and the supernatant collected and filtered through a 0.2 μm filter (Millipore). A 1 ml sample of this supernatant was injected into the column and eluted at a flow rate of 0.7 ml/min against an increasing acetonitrile concentration gradient. Fractions were collected at 1 min intervals into sterile microtubes on a fraction collector (FRAC-500; Pharmacia). The fractions were frozen at -70°C overnight and then lyophilized. Each fraction was resuspended in 1 ml

PFM supplemented with 10% FCS and tested in the bioassay. This procedure was replicated twice.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE was carried out to estimate the molecular weight of the substance(s) that displayed high bioactivity in fraction 22. Polyacrylamide gels (15%) were prepared and the tracks loaded with 10 µl samples from each of the fractions collected. Also, whole concentrated PFM-CM before and after TFA treatment was loaded as controls. Gels were stained with silver nitrate to reveal the protein bands. Also, fractions 13-32 were pooled in pairs and proteins radioactively labelled with ^{125}I before separation by SDS-PAGE. The gels were dried and then autoradiographed for 24 h to identify protein bands. Prestained low molecular weight protein standards were used as molecular weigh markers (Biorad).

RESULTS

Expression of Cytokines at the Materno-Fetal Interface is Temporally and Spatially Regulated.

Total RNA from peri-implantation blastocysts, extra fetal membranes at term, pooled day 14-16 uterine epithelial cells, the Jag-1 trophoblast cell line and unstimulated peripheral blood leucocytes, was analysed by Northern blotting for expression of the mRNA of various cytokines in the pig. The CSF-1 cDNA probe hybridized at different

intensities to mRNA present in all the above mentioned total RNA samples (Fig. VI.1A). A single band of approximately 4.6 Kb was observed in all samples. The strongest labelling occured in the days 14 and 16 blastocysts, Jag-1 cells and uterine epithelial cells (Fig. VI.1A).

The level of expression of mRNA for CSF-1 in day 22 blastocysts (lane 3) was much lower than that of days 14 and 16 blastocysts and similar to that of the amnion and allantochorion (taking into consideration RNA degradation in the chorion) at term. Furthermore, enrichment for poly A^+ RNA by one cycle of oligo-dT selection from day 22 total RNA (lane 4, Day 22 $Blast^+$) resulted in a similar hibridization signal as that of days 14 and 16. Hence, it appears that the expression of mRNA for CSF-1 was highest during the early implantation period and reduced thereafter. The intensity of labelling was titratable as 50 ug of total RNA from the Jag-1 cells (Lane 7, Jag-1 50) showed stronger labelling than that of 20 ug of total RNA (Lane 8, Jag-1 20).

IFN-γ mRNA transcripts were presented as bands of approximately 1.4 Kb and 1.0 Kb respectively from days 14 and 16 blastocysts (Fig. VI.1B, Lanes 6 and 5, respectively). Only the 1.4 Kb band was evident in day 22 total RNA (lane 3), day 22 poly A^+ enriched RNA (lane 4) and uterine epithelial cells (lane 9). The 1.4 Kb band was detectable in unstimulated PBL total RNA (lane 10). Neither bands were evident in the other samples tested. The mRNA levels, as indicated by the intensities of labelling for the 1.4 Kb transcript was again highest in days 14 and 16 blastocysts (Fig. VI.1B, lanes 6 and 5 respectively) with a considerable reduction evident in the day 22 blastocysts samples (lanes 3 and 4).

At least four different TGF-β1 transcripts were evident among the samples (Fig. VI.1C). However, the 5.1 Kb and 2.5 Kb bands were the

most pronounced although they were not equally expressed in all samples. Labelling for the 5.1 Kb transcript was relatively low in day 14 blastocysts (lane 6), but increased in samples of days 16 and 22 blastocysts (lanes 5, 3 and 4, respectively). This band was also strong in the Jag-1 trophoblast cells, (lanes 7 and 8), uterine epithelial cells (lane 9) and the amnion at term (lane 2). It was also detectable in the chorion at term (lane 1) and unstimulated blood (lane 10). In contrast, the 2.5 Kb band was strongest in days 14 and 16 blastocysts (lanes 6 and 5, respectively) and decreased by day 22 (lanes 3 and 4). Both bands were of relatively high intensities in the uterine epithelial cells (lane 9) and the amnion at term (lane 2). In addition, the 2.5 Kb band was weak in day 22 blastocysts (lanes 3 and 4) and the Jag-1 trophoblast cells (lanes 7 and 8). It was detectable in the chorion at term (lane 1) and unstimulated PBL (lane 10). The intermediate bands were also expressed at different levels among the samples.

A single GM-CSF transcript of approximately 1 Kb in size was detected in total RNA from day 14 blastocysts and unstimulated PBL (Fig. VI.1D, seen as smears in lanes 6 and 10, respectively due to partial RNA degradation). This 1 Kb band was very faint in the day 14-16 uterine epithelium sample and not detected in the other samples except for the poly A^+ enriched total RNA from day 22 blastocysts (lane 4).

Figure VI.2A shows that a single IL-2 band of approximately 0.8 Kb was strongly expressed in day 14 blastocysts and unstimulated PBL (lanes 5 and 9 respectively, seen as smears due to partial RNA degradation). Fig. VI.2B represents the ethidium bromide stained gels as a reference to the amount of RNA loaded which is indicated by the intensities of the 28s and 18s ribosomal RNA bands. Figure VI.2C is another

representative gel to indicate the amount of RNA loaded for the sample in each of the blots probed in Figure VI.1.

mRNA for IGF-1 was detected as a single species of approximately 1.2 Kb in the uterine epithelial cells (Fig. VI.3A, lane 6) and to a lesser extent in day 16 blastocysts (Fig. VI.3A, lane 3). This band was not detected in any of the other samples tested. Figure VI.3B shows the ethidium bromide stained gel revealing the 28s and 18s bands as a reference to the amount of RNA loaded into each lane.

CSF-1 is a Possible Candidate for the MGF Produced by the Jag-1 Cells

The Jag-1 trophoblast cells adhere but do not form a confluent monolayer when cultured under fetal calf serum free conditions. It appears that some factors(s) present in FCS facilitates spreading of the Jag-1 cells. Therefore the cells were initially cultured in RPMI containing 10% FCS to allow for confluent growth to be attained before incubation in PFM. Days 3-7 after the onset of culture were assessed in order to determine whether PFM would become conditioned if exposed to confluent growing cells during this period. PFM was incubated for 3 h; 6 h and 12 h on each of the days with confluent Jag-1 cells. Results from these preliminary experiments revealed that the monocyte/macrophage growth factor(s) can be secreted by Jag-1 trophoblast cells under protein free condition. In fact, the peak level of stimulation observed with RPMI-CM which contained 10% FCS (Fig.VI.4a) was comparable to that of PFM-CM without FCS (Fig. VI.4b - f).

The half maximal level of stimulation was attained at increasing numbers of dilution from days 3 - 7 (Fig. VI.4b - f). Furthermore, the

maximum level of stimulation for 3 h PFM-CM was about the same as that of 6 h and 12 h (Fig. VI.4b - f). As a result of these observations PFM-CM was collected after 12 h incubations, three flasks each on days 3, 4, 5, 6 and 7 after seeding. The pooled PFM-CM was concentrated 100 fold and subjected to RP-FPLC. Two separate RP-FPLC runs were carried out, one month apart, on two different preparations of PFM-CM. The fractions collected were tested separately on monocyte/macrophages isolated from two different pigs. Pig number 1 was used for both RP-FPLC runs while pig number 2 was used only for RP-FPLC run 2.

Figure VI.5 shows the results of the first RP-FPLC run, the fractions of which were tested on the monocyte/macrophages from pig number 1. The fractions were serially diluted and tested in the bioassay and are represented in Fig. VI.5 by their peak levels of stimulation of ^{3}HTdR uptake. Fraction 22 stimulated a high level of ^{3}HTdR uptake when compared to that of the other fractions which displayed background ^{3}HTdR uptake. This fraction was eluted at 35% acetonitrile concentration.

Fractions from RP-FPLC run 2 were simultaneously tested on cells from pigs number 1 and 2. Again monocyte/macrophages from pig number 1 was highly stimulated to proliferate by fraction 22 (Fig. VI.6). In addition, some stimulation was also observed with fraction 18 (Fig. VI.6). Fraction 22 also stimulated monocyte/macrophages from pig number 2 to proliferate (Fig. VI.7). However, background proliferation of cells from pig number 2 was relatively greater than that of pig number 1. Furthermore, fractions 18 and 30 also showed some stimulatory properties in pig 2 (Fig. VI.7).

SDS-PAGE revealed a common peptide band in all fractions approximately 66 kd in size which was most likely serum albumin (Figs.

VI.8 and VI.9). Fractions 22 and 23 (Fig. VI.8, lanes 22 and 23; fractions 21-22, Fig. VI.9 lane 5 and 23-24; lane 6) also possessed a common band approximately 50 Kd in size. In addition, fraction 22 (Figs. VI.8 and VI.9) contained two unique bands that were not present in any of the other fractions. The molecular weights of these bands were between 38-45 kd (Figs. VI.8 and VI.9) and are possible candidates for possession of the bioactivity. A protein band of similar size was evident among the ^{35}S-labelled proteins secreted by the Jag-1 cells (Fig. VI.10).

DISCUSSION

One of the striking aspects of the results of this study is the temporal expression of cytokine mRNAs to coincide with the period of implantation in the pig, that is, between days 13-18 of pregnancy [King *et al.*, 1982; Dantzer, 1985]. It was no coincidence since this is the period when complex and dynamic changes are taking place within the conceptus and the uterus, [King et al 1982, Dantzer, 1985], which underscores the need for precise well balanced mechanisms of regulation. CSF-1, is constitutively secreted by the uterine epithelium throughout pregnancy in the mouse [Pollard *et al.*, 1987]. It appears that this may also be the case in the pig. The CSF-1 mRNA was strongly expressed in days 14-16 pooled uterine epithelial cells. Moreover, the conceptus may be an additional source of this cytokine throughout pregnancy. The expression of CSF-1 mRNA was strongest between days 14-16 but decreased by day 22 to levels similar to that of the amnion and allantochorion at term.

CSF-1 and its receptor were found to be temporally and spatially variable in their expression within the uterus of the mouse during

pregnancy [Acerci *et al.*, 1989]. Similar observations were made in humans [Jokhi *et al.*, 1993]. CSF-1 was detected in serum, endometrium, placenta, chorion, amnion and amniotic fluid throughout gestation with the highest levels occuring at first trimester [Daiter *et al.*, 1992]. CSF-1 mRNA was found in all three tissues except the amnion and levels of expression matched that of the protein [Daiter *et al.*, 1992], with a steady decline in localization of mRNA and protein in the placenta between first trimester and term. Our results support the hypothesis that CSF-1 is essential for the development of the placenta and survival of the fetus [Pollard *et al.*, 1987; Acerci *et al.*, 1989].

In-situ hybridization has localized GM-CSF mRNA in the decidua and spongiotrophoblast [Kanzaki *et al.*, 1991] and novel transcripts ranging in size between 1 Kb and 5.1 Kb appear to be expressed in the placenta [Crainie *et al.*, 1990] of the mouse. In the sheep, GM-CSF stimulated the production of oTP-1 (mRNA and protein) by day 17 conceptus *in vitro* [Imakawa *et al.*, 1993]. These workers found that GM-CSF mRNA was localized in the luminal and glandular epithelium of the endometrium but not in the conceptus at day 17. The data presented in this study shows that a ~ 1 Kb transcript was expressed in the pig. This band was significantly higher in day 14 blastocysts than in day 14-16 uterine epithelium. The striking thing is that the levels rapidly declined. It was not detected in total RNA from day 16 blastocysts and a sharp band was only seen in poly A^+ enriched RNA at day 22. It was not detectable in the allantochorion or the amnion at term. Therefore it appears to have a narrow window of expression when compared to that of CSF-1. Furthermore, the pattern of GM-CSF mRNA expression in the pig may be different from that of the mouse and sheep during pregnancy.

IGF mRNA and protein have been detected in the uterus of a number of species including the rat [Murphy *et al.*, 1987, Ghahary *et al.*, 1990], pig [Letcher *et al.*, 1989] and the cow [Geisert *et al.*, 1991]. A strong temporal correlation was found between IGF-1 production by the endometrium and synthesis of estrogens by the conceptuses [Geisert *et al.*, 1982]. Letcher *et al.*, [1989] found that endometrial IGF-1 mRNA increased to a peak level from days 8-12, then decreased by day 14 and continued to do so past day 30 of gestation. They also found that uterine luminal IGF-1 paralleled the temporally regulated pattern of expression seen with endometrial IGF-1 mRNA synthesis.

In the present study a 1.2 Kb IGF-I mRNA transcript was expressed in the endometrial epithelial cells at days 14-16 and most likely represented declining levels as peak expression was reported at day 12 of pregnancy [Letcher *et al.*, 1989]. Interestingly conceptus derived IGF-I was not detected in day 14 blastocysts but a faint band was detected in day 16, which was not expressed in the amnion or chorion at term. Since day 16 was the latest periimplantation-stage blastocysts looked at, it was not possible to tell whether this represented the onset of conceptus synthesis of IGF-I mRNA. IGF-I was found to modulate estradiol biosynthesis in perimplantation pig blastocysts *in vitro* [Hofig *et al.*, 1991)].

Until recently IFN-γ was thought to be a lymphokine produced only by activated T lymphocytes and NK cells, however, it has now been shown to be expressed at the mRNA and protein levels by trophectoderm cells of perimplantation pig blastocysts [Lefevre *et al.*, 1990]. These researchers found that two mRNA species 1.4 Kb and 1.3 Kb, respectively, were expressed between days 14-20 of gestation. They also found IFN-γ to be secreted in culture Our data are in agreement with their findings. In

addition, we found that the 1.4 Kb transcript was also expressed in days 14-16 uterine epithelial cells and in day 22 blastocysts. The 1.0 Kb band found in our study was possibly due to partial degradation of the 1.3 Kb band found by Lefevre *et al.*, [1990]. This was indicated by the smear ranging between 0.8 -1.3 Kb seen in days 14 and 16 blastocysts total RNA.

It is not clear what role IFN-γ, secreted by the conceptus and possibly the uterine epithelium, play, during the implantation period. In addition to its antiviral activity and immunoregulatory functions IFN-γ may be involved in the normal physiological processes of cell growth and differentation in the placenta [Loke and King, 1990]. Now that IFN-γ "knock-out" mice and trophoblast cell lines from the pig are available, elucidating the role of IFN-γ in reproduction should be accelerated. The Jag-1 trophoblast cell line may have lost its ability to express mRNA and protein for IFN-γ in culture and this may be due to an inherent programme of down regulation, similar to that seen *in vivo* [present study; Lefevre *et al.*, 1990].

Another unique feature in the pig is the present finding that IL-2 was expressed strongly in day 14 blastocysts (and presumably at earlier stages; that were not examined). The lack of expression in day 16 blastocysts indicates that IL-2 mRNA was down-regulated rapidly. A similar size transcript (0.8 kb) as that observed in the present study was detected in human placenta. It was localized in the syncytiotrophoblast layer from all three trimesters of pregnancy [Boehm *et al.*, 1989]. This correlated well with the observations by Soubiran *et al.*, [1987] that an IL-2 like factor(s) was present in the human placenta, localized on syncytiotrophoblasts, however, an IL-2 receptor was not located. Again, one can only speculate as to the role of IL-2 in periimplantation blastocysts in the pig and human

placentas. Like IFN-γ, IL-2 presence in the placenta is not unique in itself but its production by trophoblast is. Soubrian *et al.*, [1987] theorized that an IL-2 like material in the placenta could be immunosuppressive by competing locally with IL-2 on activated T lymphocytes. Since the placenta is a simiallogeneic organ possessing and attracting cells with receptors for IL-2 and INF-γ, an immune interaction involving the trophoblast cannot be ruled out.

Most eukaryotic cells encode a 2.5 Kb TGF-β1 mRNA, however other mRNA species are also expressed at varying intensities [Kondaiah *et al.*, 1988]. These workers found that porcine tissues expressed two major TGF-β1 RNAs, 3.5 and 2.5 kb in size, respectively, together with other minor species. They presented evidence for multiple mechanisms of transcriptional regulation of porcine TGF-β1 resulting in the generation of different mRNA species by alternative splicing and selection of different poly (A) sites. Up to five different bands were found to be expressed at varying intensities among the tissues in this study. The two major bands seen were 2.5 Kb and about 5.1 Kb in size. Two transcripts of 4.9 Kb and 2.5 Kb were the major bands found in cultured human fibroblast [Ghahary *et al.*, 1993]. TGFs-β are expressed throughout embryonic development with ubiquitous distribution of their receptors [see Massague, 1992].

The conceptus of the pig secretes a vast array of proteins the majority of which are of unknown identity and function [Godkin *et al,*. 1982; Powell-Jones *et al.*, 1984]. The Jag-1 trophoblast cells appear to constitutively produce the MGF in culture. In protein free culture medium secretion levels were similar at 3 h, 6 h, and 12 h, with respect to peak levels of ^{3}HTdR uptake. The presence of bioactivity in the conditioned PFM on each

day, between days 3-7 of culture, made it possible to collect sufficient PFM-CM for concentration of the factor into a small volume for RP-FPLC.

Activity is retained after being subjected to conditions necessary for RP-FPLC. It is thus stable to ultrafiltration, exposure to a pH of 2 and the organic solvent acetonitrile, and after lyophilization and rehydration in RPMI medium. Separation of proteins according to hydrophobicity is a common method used in the isolation of cytokines [Ihle., 1985; Ohara *et al.*, 1985; Takatsu *et al.*, 1987; Nagata *et al,* 1990]. Most cytokines are stable under such conditions, one exception being IL-10 [Lin *et al.*, 1993]. In our lab, this technique has been used successfully to isolate a number of cytokines from mouse placental and decidual cell supernatants [Lin *et al.*, 1993].

The RP-FPLC fractions, however, do not represent the MGF purified to homogeneity. Indeed, silver nitrate staining of the proteins in the RP-FPLC fractions followed by SDS-PAGE indicated the presence of at least four different protein bands in fraction 22. This was confirmed by radioiodination of the proteins in the fractions and SDS-PAGE. On the other hand, fraction 22 contained two proteins that were unique, in that, they were absent from the other fractions. These two peptides were approximately 42 and 45 Kd respectively and are possible candidates for the MGF bioactivity. The MGF present in fraction 22 eluted around 35-40% acetonitrile indicating that the MGF is relatively hydrophobic. The acetonitrile profile of most cytokines indicated similar hydrophobicity, eliciting around 30-50% acetonitrile concentration [Lin *et al.*, 1993].

What is the significance of the finding that the Jag-1 trophoblast cells produce a MGF. One possibility is that this is a trophoblast autocrine factor that coincidentally has a paracrine stimulatory effect on macrophages.

Trophoblast and macrophages share many characteristics including the production and response to certain cytokines [Hunt, 1989b; 1990, Guilbert *et al.*, 1993]. Uterine macrophages have the potential to function in both a nonimmunologic and immunologic capacity [Hunt, 1989b]. Some of these functions such as phagocytosis of invading microorganisms, tissue remodeling especially during early pregnancy, secretion of monokines and immunosuppressive factors such as PGE_2 may be paramount to the success of pregnancy in animals with diffuse placentas.

Cytokines produced by trophoblast cells from other species that have the potential to simulate macrophages to proliferate and differentiate include the colony stimulating factors, GM-CSF and CSF-1 [Athanassakis *et al.*, 1987, Kanzaki *et al.*, 1991, Garcia-Lloret, 1991].

A possible candidate for the MGF secreted by the Jag-1 cells is CSF-1 for the following reasons: The CSF-1 probe hybridized strongly with RNA isolated from the Jag-1 cell line, uterine epithelium and days 14 and 16 blastocysts. The expression of the CSF-1 mRNA appears to decrease after day 16 since it was lower in day 22 total RNA. The mRNA for GM-CSF on the other hand was only detected in day 14 blastocyst and peripheral blood total RNA. Enrichment for poly A^+RNA from day 22 blastocysts revealed a sharp band for GM-CSF indicating that the mRNA for this cytokine decreased after day 14, and as such, may be present at a low copy number in total RNA from Jag-1 cells. CSF-1 is a disulfide-linked homodimer with a molecular weight of 70 Kd in mouse and 70-90 Kd in humans (Metcalf 1986). Under the reducing condition used for SDS-PAGE a band of between 35-45 kd is expected if the activity was attributable to CSF-1. The two peptide bands unique to fraction 22 in this study fall within this

molecular weight range. The molecular weights of other CSF cytokines range between 18-30 under nonreducing conditions [Metcalf 1986].

In conclusion, data are presented as evidence for the mRNA expression of cytokines known to either have beneficial or harmful effects on the development of the feto-placental unit. The expression of these mRNAs were found to be spatially and temporally regulated in periimplantation, conceptus and uterine epithelial tissues. As shown here, the same cells and tissues can simultaneously express mRNA for both putatively "beneficial and harmful" cytokines. However, in light of the changes taking place during the peri-implantation period in both maternal and fetally derived tissues, and the lack of precise knowledge of the interplay among these cytokines in the control of these changes, the classification of these factors as beneficial or harmful represents an over simplification.

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Fig. VI.1. Northern blot analysis for the detection of mRNA for CSF-1, IFN-γ, TGF-β and GM-CSF. Total RNA was isolated and 50 μg electrophoresed from the allantochorionic (lane 1) and amniotic membranes (Lane 2) at term, days 22 (lane 3), 16 (lane 5) and 14 (lane 6) perimplantation blastocysts, the Jag-1 trophoblast cell line (lanes 7, 50 μg, 8, 20 μg), days 14 and 16 pooled uterine epithelial cells (lane 9) and unstimulated peripheral blood leucocytes (lane 10, 20μg). Poly A^+ mRNA from day 22 blastocysts was enriched for by one round of oligo -dT selection. (lane 4, 10 μg).

A). CSF-I mRNA was detected in all samples, It was strongest in the Jag-1 cells , uterine epithelial cells, and days 14 and 16 blastocysts.

B). IFN-γ was strong in days 14 and 16 blastocysts and weak in the uterine epithelium and day 22 blastocysts poly A^+ mRNA.

C). TGF-β mRNA was expressed in all the tissues . Multiple bands were seen at varying intensities in all the samples.

D). GM-CSF was weakly expressed in day 14 blastocyst at a similar intensity to that of blood. It was also seen in the poly A+ enriched day 22 blastocysts.

(see Chapter V for the β-actin control.)

FIGURE I

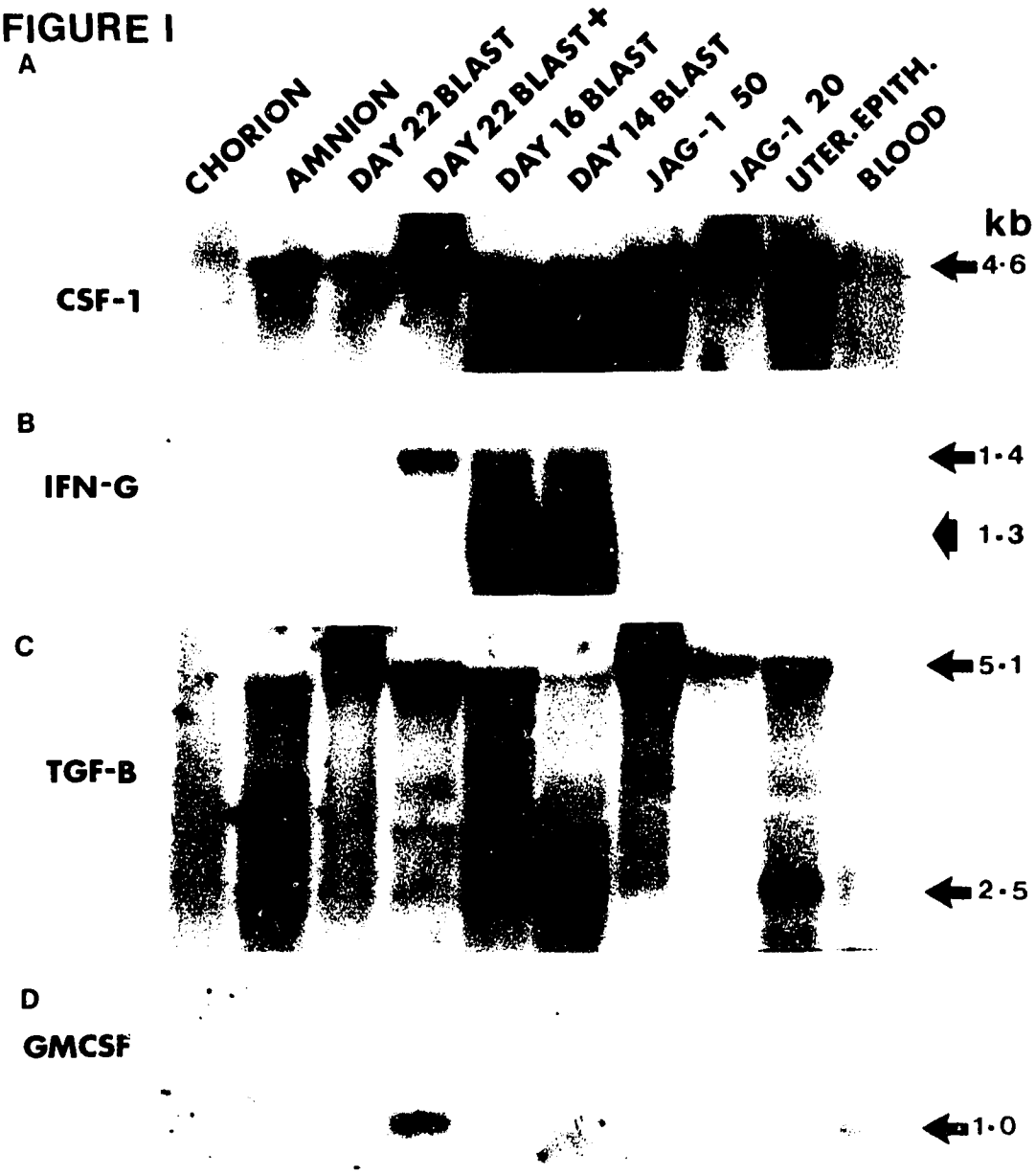

A
CHORION
AMNION
DAY 22 BLAST
DAY 22 BLAST+
DAY 16 BLAST
DAY 14 BLAST
JAG-1 50
JAG-1 20
UTER. EPITH.
BLOOD
kb
CSF-1
4·6
B
IFN-G
1·4
1·3
C
TGF-B
5·1
2·5
D
GMCSF
1·0

Fig. VI.2. Northern blot analysis for the detection of mRNA for IL-2,. Total RNA was isolated and 50 μg electrophoresed from the allantochorionic (lane 1) and amniotic membranes (Lane 2) at term, days 22 (lane 3), 16 (lane 4) and 14 (lane 5) perimplantation blastocysts, the Jag-I trophoblast cell line (lanes 6), days 14 and 16 pooled uterine epithelial cells (lane 7), the ovary (lane 8) and unstimulated peripheral blood leucocytes (lane 9, 20μg).

A). The IL-2 mRNA was strongly expressed in day 14 blastocyst and peripheral blood. It was not detected in any other tissues.

B). The ethidium bromide stained gel showing the 28s and 18s ribosomal bands to give an indication of the amount of total RNA loaded into each lane for the blot probed for IL-2.

C) A representative ethidium bromide stained gel showing the 28s and 18s ribosomal bands to give an indication of the amount of total RNA loaded into each lane for the blots probed for CSF-I, IFN-γ, TGF-β and GM-CSF.

FIGURE 2

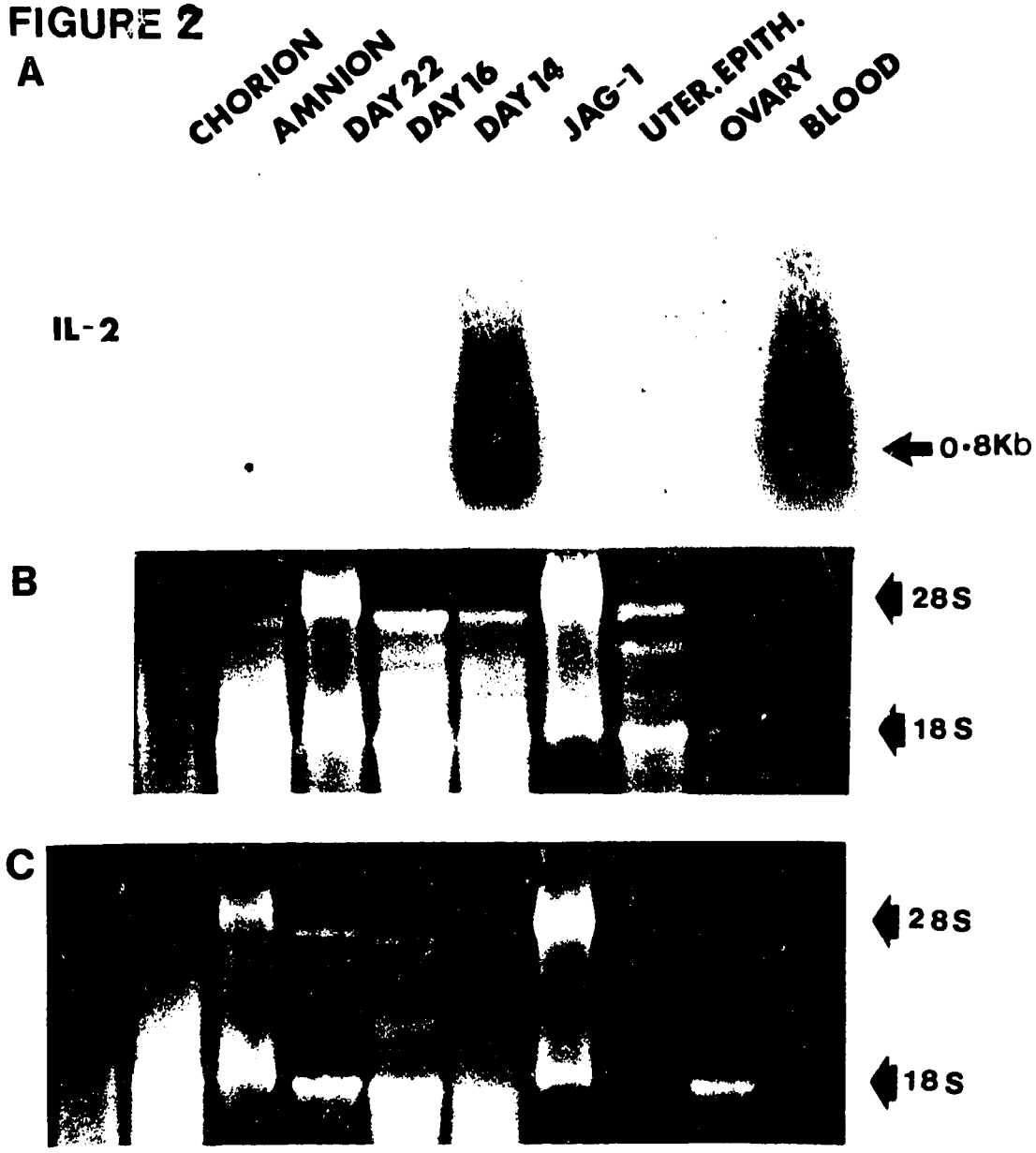

A
CHORION
AMNION
DAY 22
DAY 16
DAY 14
JAG-1
UTER. EPITH.
OVARY
BLOOD
IL-2
0·8Kb
B
28S
18S
C
28S
18S

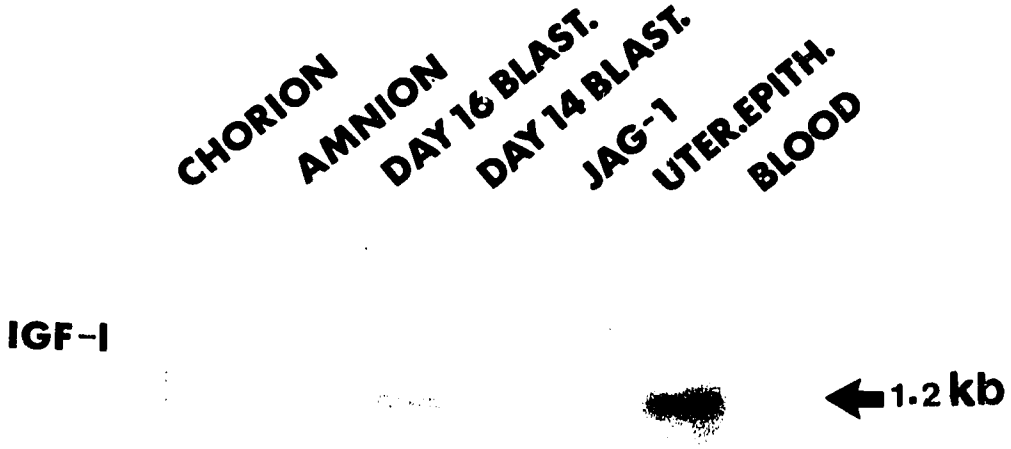
Fig. VI.3. Northern blot analysis for the detection of mRNA for IGF-I,. Total RNA was isolated and 50 μg electrophoresed from the allantochorionic (lane 1) and amniotic membranes (Lane 2) at term, days 16 (lane 3) and 14 (lane 4) perimplantation blastocysts, the Jag-I trophoblast cell line (lanes 5), days 14 and 16 pooled uterine epithelial cells (lane 6), and unstimulated peripheral blood leucocytes (lane 9).

A). The IGF-I mRNA was detected only in day 16 blastocysts and the uterine epithelial cells.

B). A representative ethidium bromide stained gel showing the 28s and 18s ribosomal bands to give an indication of the amount of total RNA loaded into each lane for the blots probed for IGF-I.

FIGURE 3

A


CHORION
AMNION
DAY 16 BLAST.
DAY 14 BLAST.
JAG-1
UTER.EPITH.
BLOOD
IGF-I
1.2 kb

B


28S
18S

Fig. VI.4. The effects of Jag-1 conditioned protein-free medium (PFM-CM) harvested 3 h, 6 h, and 12 h on each of days 3 - 7 postconfluency compared to Jag-1 cell conditioned RMI medium containing 10% fetal calf serum on the proliferation of porcine monocyte/macrophages.

a) The effect of 24h Jag-1 cell conditioned RPMI medium containing 10%FCS. Half-max level of stimulation occured around dilution number 5.

b) The effects of Jag-1 conditioned protein-free medium (PFM-CM) harvested 3 h, 6 h, and 12 h on days 3. The half-max level of stimulation occured at around dilution number 3.

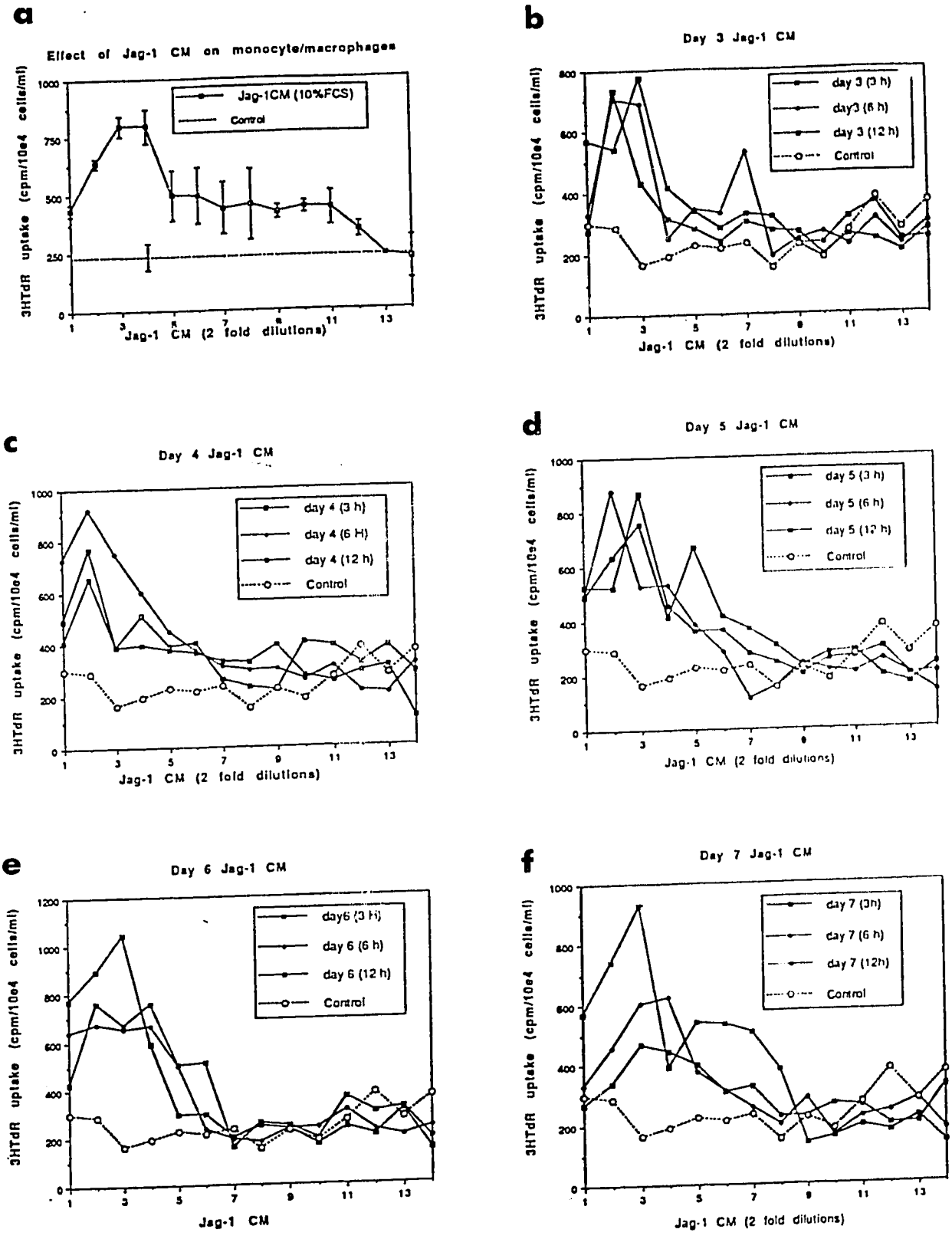
c) The effects of Jag-1 conditioned protein-free medium (PFM-CM) harvested 3 h, 6 h, and 12 h on day 4. The half-max level of stimulation occured at around dilution number 4.

d) The effects of Jag-1 conditioned protein-free medium (PFM-CM) harvested 3 h, 6 h, and 12 h on day 5. The half-max level of stimulation occured at around dilution number 5.

e) The effects of Jag-1 conditioned protein-free medium (PFM-CM) harvested 3 h, 6 h, and 12 h on day 6. The half-max level of stimulation occured between dilutions number 5 and 6.

f) The effects of Jag-1 conditioned protein-free medium (PFM-CM) harvested 3 h, 6 h, and 12 h on day 7. The half-max level of stimulation occured between dilutions number 6 and 7.

Figure 4


a
Effect of Jag-1 CM on monocyte/macrophages
Jag-1CM (10%FCS)
Control
3HTdR uptake (cpm/10e4 cells/ml)
Jag-1 CM (2 fold dilutions)
b
Day 3 Jag-1 CM
day 3 (3 h)
day3 (6 h)
day 3 (12 h)
Control
3HTdR uptake (cpm/10e4 cells/ml)
Jag-1 CM (2 fold dilutions)
c
Day 4 Jag-1 CM
day 4 (3 h)
day 4 (6 H)
day 4 (12 h)
Control
3HTdR uptake (cpm/10e4 cells/ml)
Jag-1 CM (2 fold dilutions)
d
Day 5 Jag-1 CM
day 5 (3 h)
day 5 (6 h)
day 5 (12 h)
Control
3HTdR uptake (cpm/10e4 cells/ml)
Jag-1 CM (2 fold dilutions)
e
Day 6 Jag-1 CM
day6 (3 H)
day 6 (6 h)
day 6 (12 h)
Control
3HTdR uptake (cpm/10e4 cells/ml)
Jag-1 CM
f
Day 7 Jag-1 CM
day 7 (3h)
day 7 (6 h)
day 7 (12h)
Control
3HTdR uptake (cpm/10e4 cells/ml)
Jag-1 CM (2 fold dilutions)

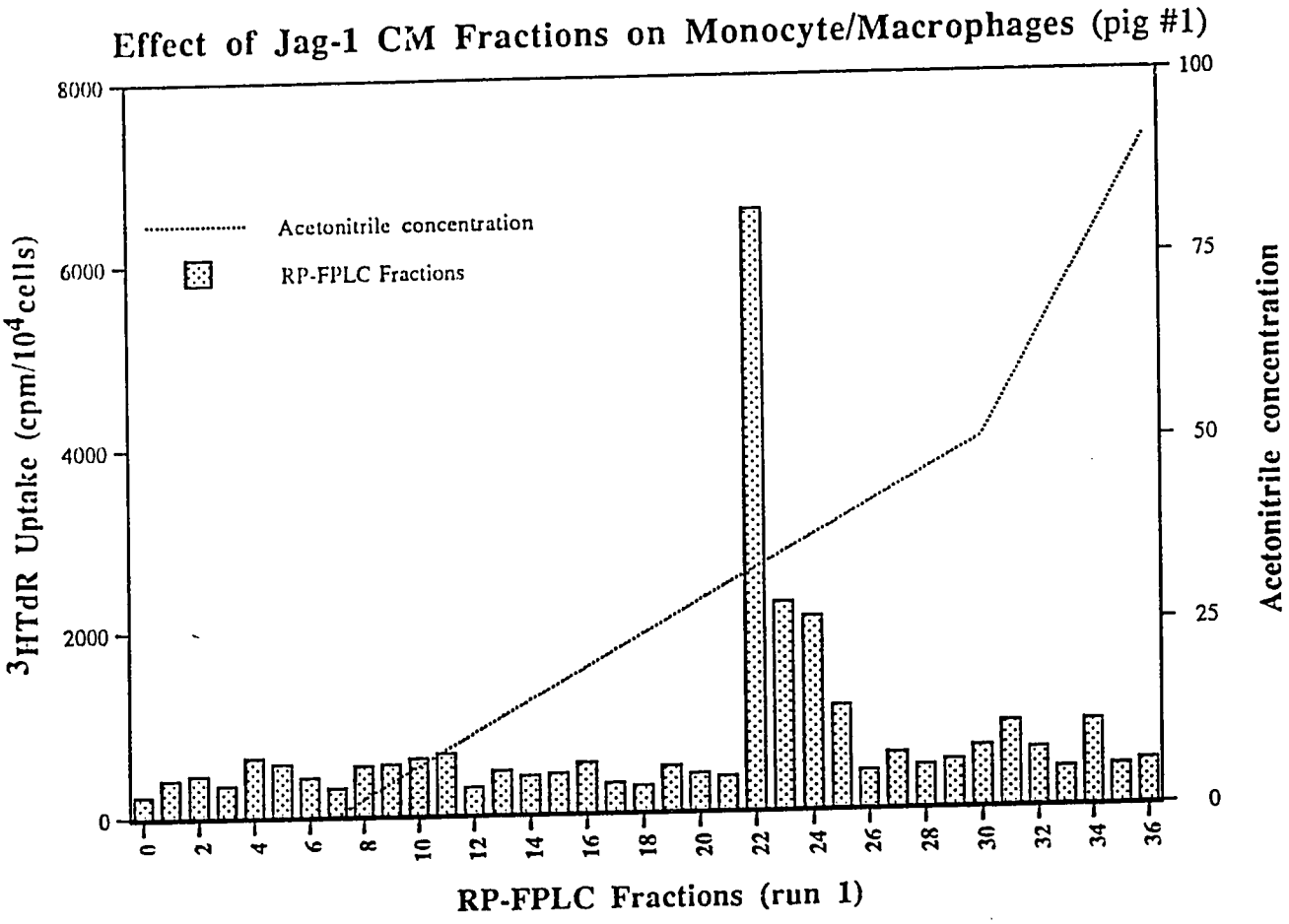


Fig. VI.5. The effect of Jag-1 CM fractions, from the first of two reverse phase-fast protein liquid chromatography (RP-FPLC) runs, on the proliferation of porcine monocyte/macrophages from **pig number 1.** The values represent the peak level of stimulation obtained for each of the 36 fractions collected.

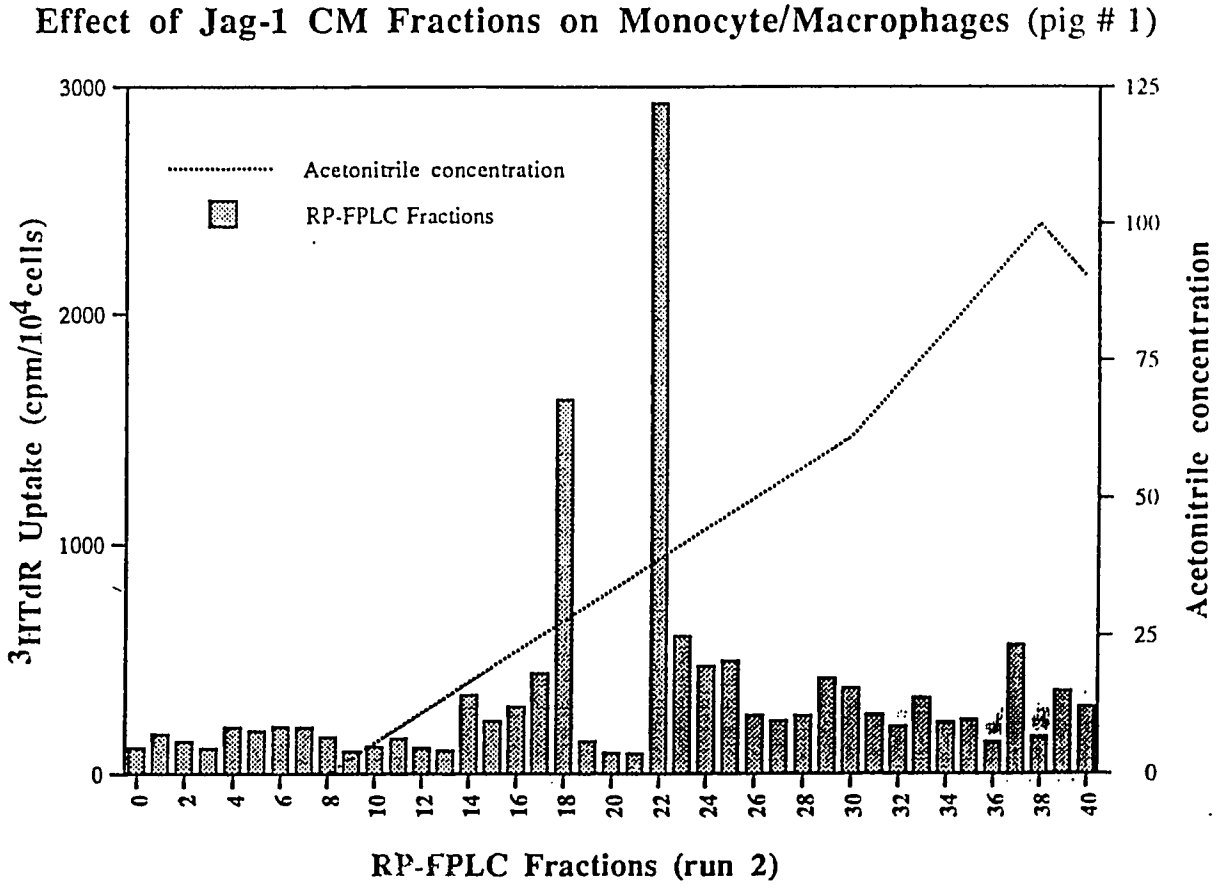


Fig. VI.6. The effect of Jag-1 CM fractions, from the second reverse phase-fast protein liquid chromatography (RP-FPLC) run, on the proliferation of porcine monocyte/macrophages from **pig number 1**. The values represent the peak level of stimulation obtained for each of the 40 fractions collected.

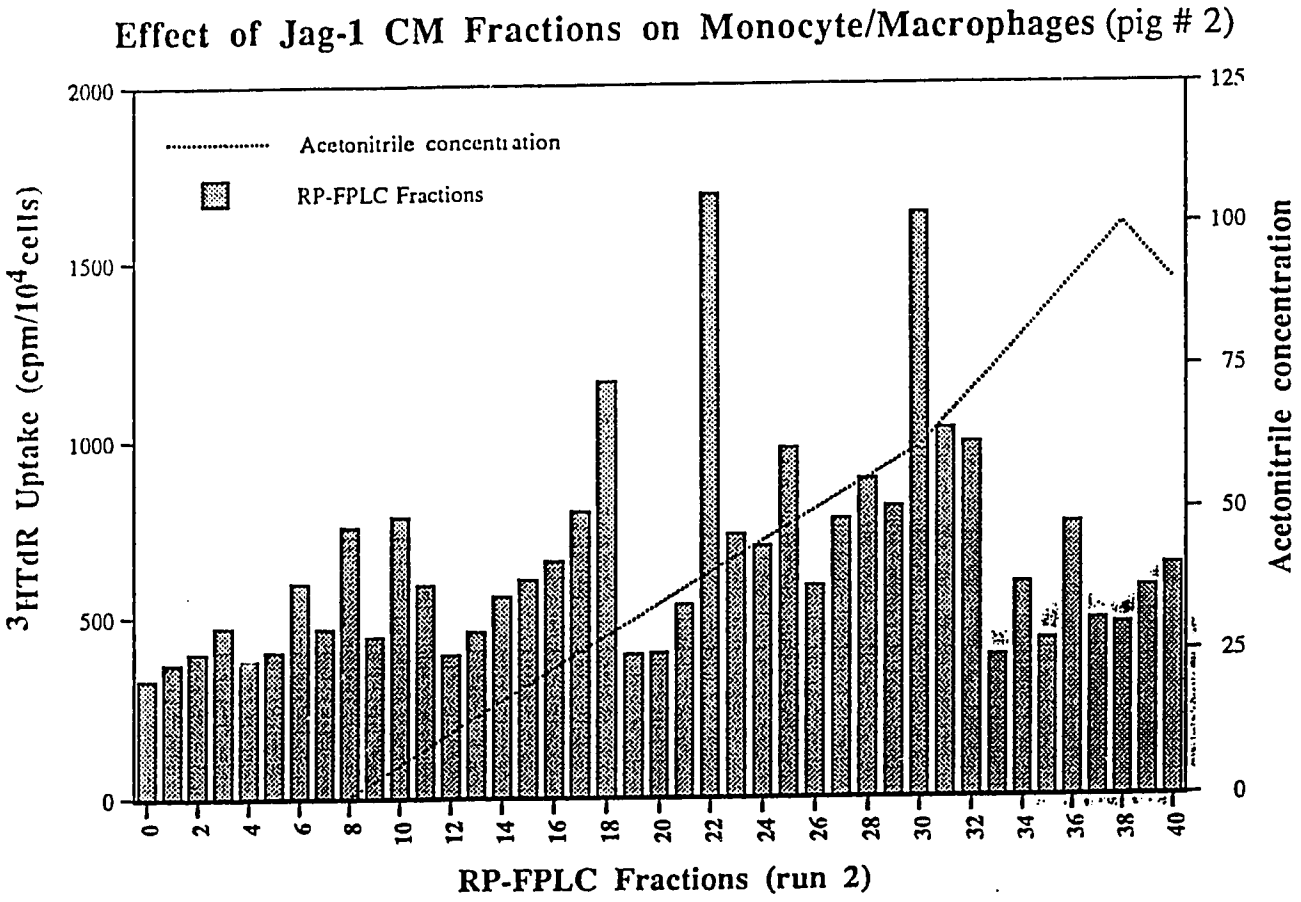


Fig. VI.7. The effect of Jag-1 CM fractions, from the second reverse phase-fast protein liquid chromatography (RP-FPLC) run, on the proliferation of porcine monocyte/macrophages from **pig number 2.** The valucs represent the peak level of stimulation obtained for each of the 40 fractions collected.

Fig. VI.8. Separation of the proteins present in fractions 1 to 39 by SDS-PAGE electrophoresis. Silver nitrate staining of the proteins showed that fraction 22 contained two unique protein bands, one stronger than the other (lane 22). They ranged between 38-45 Kd in size.

Fig. VI.9. Separation of the proteins present in fractions 13 to 31 by SDS-PAGE electrophoresis. The fractions were pooled into paired fractions and labeled with ^{125}I. The same two bands seen in fraction 22 by silver nitrate staining were confirmed to be in the same weight range in the pooled fractions 21and 22 (lane 5).

Fig.VI.10. ^{35}S-methionine labelling of the proteins secreted by the Jag-trophoblast cell line. A number of labelled proteins were secreted into the culture medium within 4 h. the band around 45 Kd corresponds with that seen in fraction 22 (Fig. VI.8, lane 22; Fig. VI. 9, lane 5).

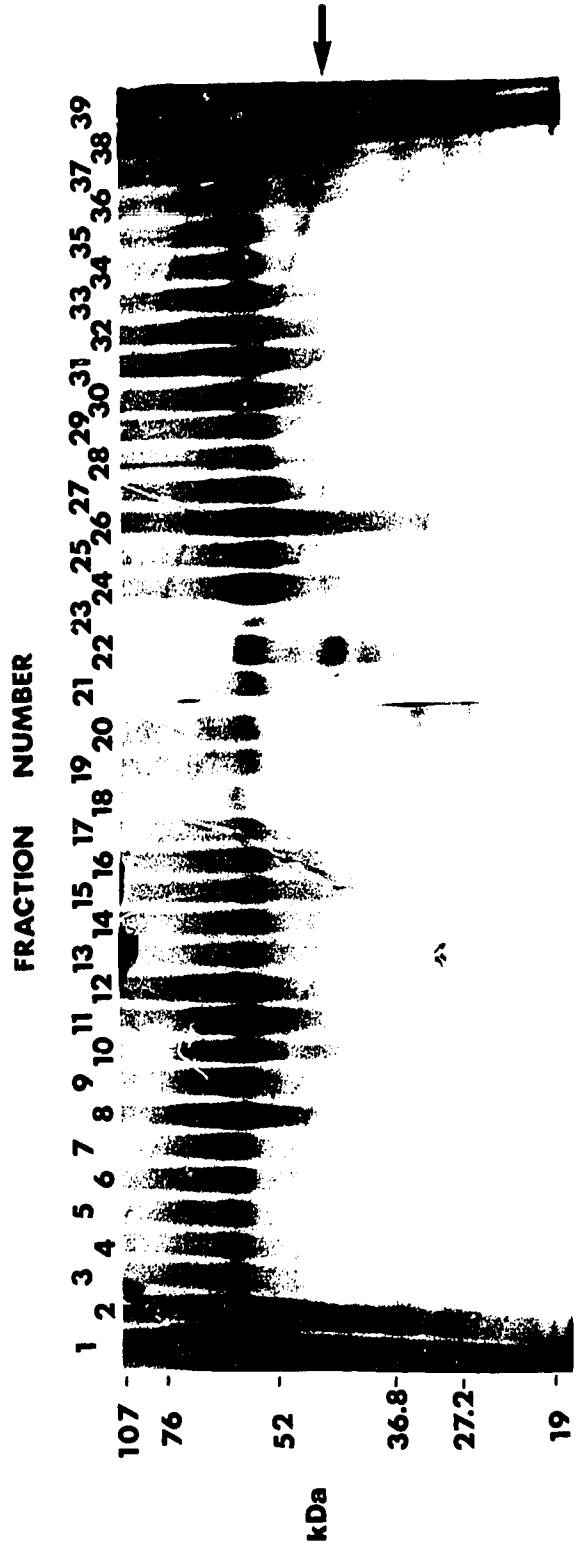

FRACTION NUMBER
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
107
76
52
36.8
27.2
19
kDa

FIGURE 5

FIGURE6

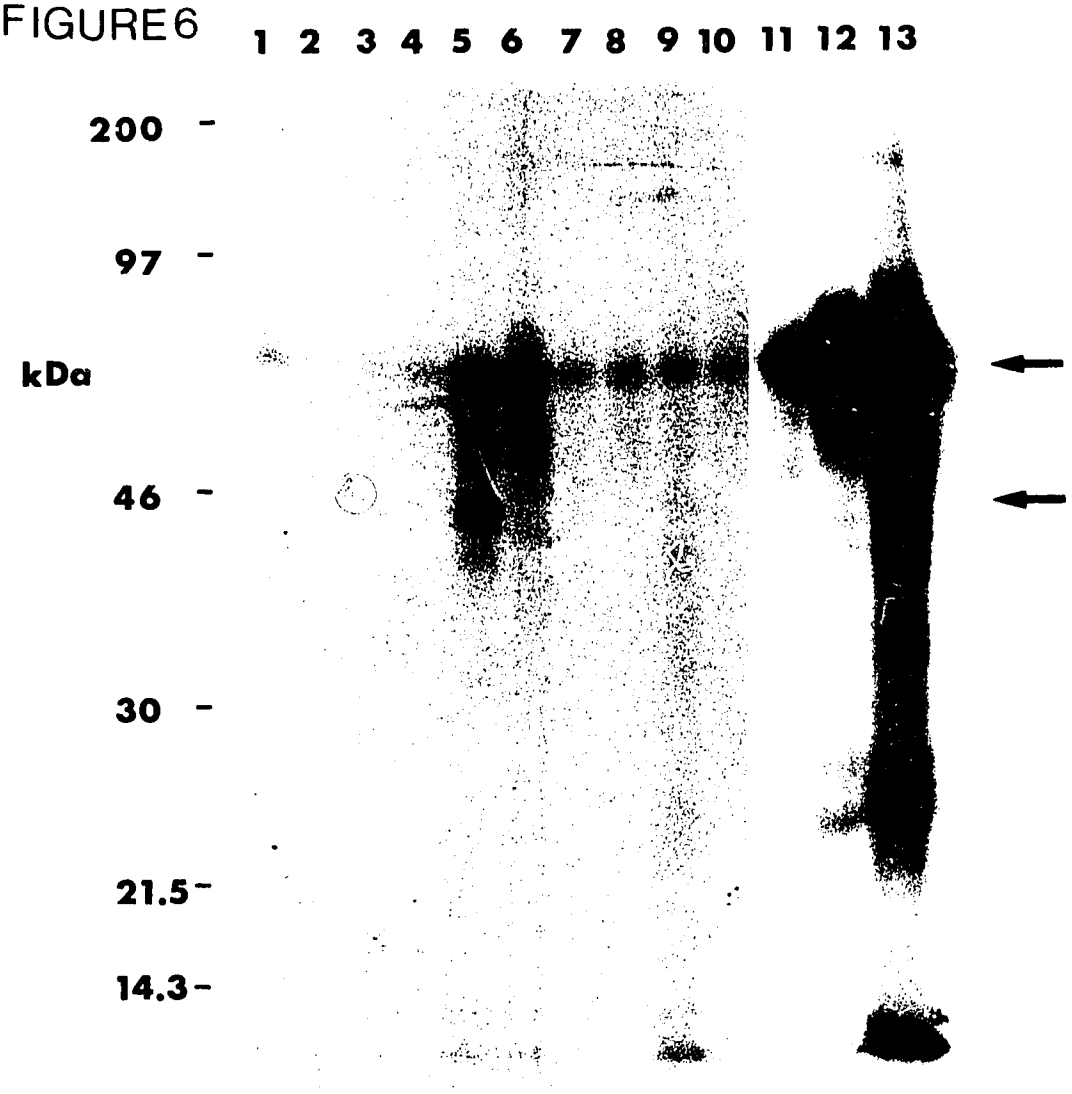
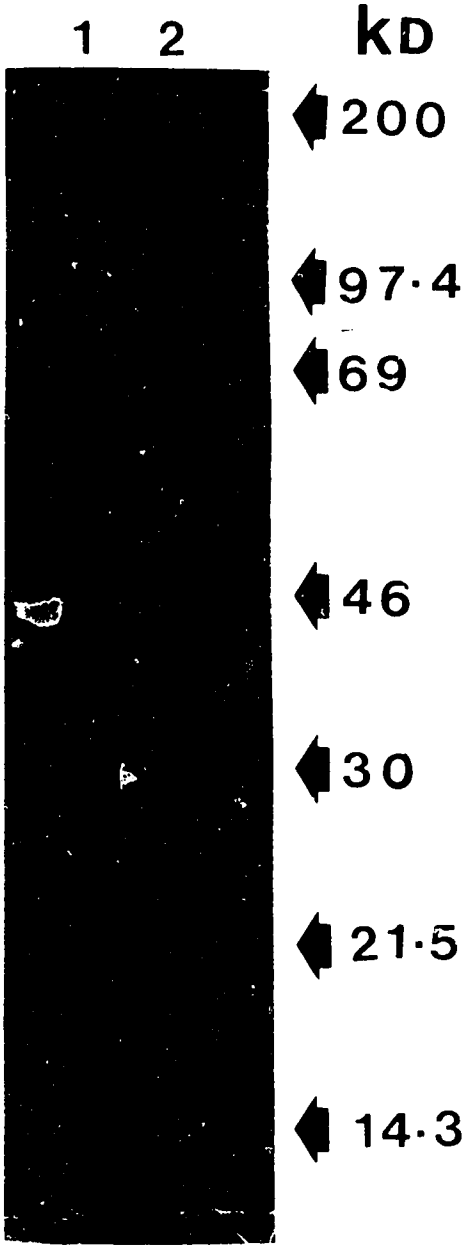


FIGURE 7



CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

The pig is an agriculturally important species that experiences a high percentage of embryonic loss [Pope and First, 1985]. This loss can range between 20-50% [Bolet, 1986], the majority of which is centered around the implantation period [Ford and Young, 1993]. Incidentally, this period is characterized by dynamic morphological, endocrinological, physiological and apparently immunological changes taking place in both the uterus and implanting conceptuses [see, Chapter I]. Few studies have addressed directly how the immune system impacts upon the reproductive process in the pig. Our research represents only the beginning of such studies, and underscores the need for more participants in this fertile area of the study of pig reproduction.

There is evidence for interactions between the reproductive and immune systems during pregnancy in animals with hemochorial placentas such as human and mouse [see Gill and Wegmann, 1987]. Instead of generating harmful rejection responses, the interactions between these two systems appear to facilitate growth and development of the feto-placental unit and survival of the fetus to term [see Beer, 1988; Chapter I]. Boosting maternal immunity against paternal antigens inherited by the fetus have been reported to improve reproductive performance in human, mouse, horse [see, Gill and Wegmann, 1987] and pig [Almlid, 1981; Murray, 1983, 1986; Blichfeldt, 1984; Van der Lende *et al.*, 1986]. The most convincing evidence for the success of this treatment was found in the mouse [see Gill and Wegmann, 1987] since its efficacy is questionable in

human [Clark and Daya, 1991; Coulam and Coulam, 1992] and pig [Blichfeldt, 1984; Van der Lende *et al.*, 1986; the present study, Chapter III].

The potential of this treatment in improving embryo survival and consequently litter size in pigs made it necessary to verify, one way or the other, its effectiveness. The results shown in Chapter III support other studies [see, Blichfeldt, 1984] in confirming that within an outbred population of pigs kept under optimum husbandry conditions, pretreatment of females with leucocytes from the sire prior to insemination offers no enhancement of subsequent reproductive ability. There was no evidence of improved reproductive performance in animals receiving this treatment. Overall, mean embryo survival was within the normal range reported for first parity animals during the fifth week, the stage of pregnancy looked at in this study [Flint *et al.*, 1982; Bolet, 1986]. However, embryo survival for animals treated with leucocytes, whether autologous or sire's, were on average lower than that of the PBS treatment group.

The increase in placental, embryo and chorioallantoic fluid weights observed for the leucocyte treatment group was most likely as a result of fewer numbers of conceptuses within the uterus. However, an immunotrophic effect cannot be ruled out [Wegmann 1990, Wegmann *et al.*, 1989, 1993]. It appears that any putative involvement of the maternal immune system resulting in enhancement of the reproductive process only becomes evident under suboptimal husbandry conditions. The literature supports this assumption since the subjects in human studies are all RSA women, and in the mouse and horse, abortion-prone models were utilized.

Trophoblast tissue constitutes the major barrier between the maternal and fetal circulations. Its strategic position at the maternal fetal interface

and its unique properties facilitate its functions of protection and nurturing of the fetus in normal pregnancies. The trophoblast in the epitheliochorial placenta is simple constituting a single layer of cells (Amoroso, 1952; Dantzer, 1985), however, its functions are no less important than that of the more complex hemochorial placenta. Furthermore, because of the long preimplantation period in the pig, the developing embryos rely solely on the uterine secretions for its nutrition and the absorptive potential of the trophoblast to facilitate this process (Roberts and Bazer, 1988).

The trophoblast from human and rodents have been shown to possess unique features that prevent the semiallogeneic fetus from being rejected (Gill, 1988). Objective two was to develop a method to purify trophoblast in quantities that would facilitate their study, and at the same time attempt to isolate a spontaneously growing cell line. We have satisfied this objective. Knowledge of the morphological development of the pig blastocyst has led to the development of a reproducible technique for the isolation of pure trophoblast from periimplantation blastocysts, in sufficient quantities for study. Moreover, the bulbous tips of day 14 elongated blastocysts consists of pure trophectoderm [Mattson *et al.*, 1987]. Knowing this allowed us to isolate the first pig trophoblast cell line to be reported.

Elongated pig blastocysts, cut into fragments or enzymatically dissociated, form multicellular vesicles and monolayer growth in culture [Whyte *et al.*, 1987; Kuzan and Wright , 1980; Selgrath and Wright, 1988]. Surprisingly, only the dense, free floating or attached type of vesicles have been described in the literature. This study is the first to describe the evanescent, transparent, monolayer vesicles that form within 24 hours of culture and collapse soon after formation to grow as concentric colonies at

the bottom of the dish. The pooling of these colonies and expanding their growth by passaging resulted in the isolation of the cell line.

Efforts to characterize this cell line have revealed that it displays epithelial characteristics common to trophoblast as evident under light and electron microscopy. These cells displayed pronounced cell polarity as described by Griepp and Robins, [1988]. Additional evidence that Jag-1 cells are trophoblasts is that they expressed cytokeratin as their intermediate cytoskeletal elements and not vimentin. Furthermore, they do not express class I or class II MHC antigens on their surface. Also, trophectodermal tips of day 14 blastocysts (the region from which the Jag-1 cell line was isolated) were found to lack MHC antigen expression.Therefore, the lack of MHC antigen expression on Jag-1 cells parallel that seen on day 14 trophoblasts. This finding is not unique to the pig, as trophoblast from several other species lack MHC antigens [Loke and King, 1991; Head, 1991].

The lack of class I MHC antigens on the Jag-1 cells and the primary tissue from which they originated, was supported by the finding that mRNA for class I MHC antigen was also severely down-modulated [Chapter V].

To determine whether the Jag-1 cells secrete cytokines, we first had to develop a bioassay for possible cytokines known to be produced by trophoblast. Macrophages are known to share many characteristics with trophoblast including the types of cytokines they produce and/or to which they respond [Hunt, 1989; Guilbert, 1993]. In order to determine whether porcine trophoblast produce or respond to CSF cytokines, other sources had to be found because there are no available porcine CSF cytokines. A modification of an assay used by Genovesi *et al.*, [1989] to show that mouse CSF-1 can stimilate porcine monocyte/macrophages was used to test the

species cross-reactivity of cytokines. In this bioassay recombinant bovine GM-CSF, L929 cell-conditioned medium (a source of mouse CSF-1) and medium conditioned by the Jag-1 trophoblast cell line, all simulated to a similar extent porcine monocyte/macrophages to proliferate. Jag-1 cells, were also found to proliferate in response to their own conditioned medium indicating that they not only produce a paracrine activity but also one that is autocrine. Whether these two activities can be attributed to the same factor has not yet been determined. Day 14 blastocyst conditioned medium was also mitogenic in this assay confirming the authenticity of the mitogenic properties of the Jag-1 conditioned medium.

The third objective of this study was to characterize the expression of class I MHC antigen expression at the maternal-embryo/fetal interface in the pig. Evidence from the previous experiments [Chapter IV] indicated that pig trophoblast probably regulates the expression of MHC on its surface in a manner similar to that of other species [Low, *et al.*, 1990; Donaldson, et al,. 1990; Head *et al.*, 1987; Loke and King, 1991; Head, 1991; Hunt, 1992; Bulmer and Johnson, 1985; Stern, et al., 1987]. The present findings are important in that the number of species found to have regulated expression of MHC antigens in the placenta now includes the pig. Class II MHC antigens have not been detected on the trophoblast of any species [see reviews Head *et al.*, 1987; Loke and King, 1991; Head, 1991; Hunt, 1992; Bulmer and Johnson, 1985].

We determined the pattern of class I MHC antigen expression at the protein and mRNA levels in periimplantation blastocysts and extra fetal membranes at term. Immunohistochemistry was used to detect Class I MHC antigens on these tissues. Northern blot analysis was used to determine their relative expression of Class I mRNA and *in situ*

hybridization was used to localize the mRNA in day 16 blastocysts. As predicted Class I MHC antigens were not expressed on days 14, 16 and 22 extra embryonic tissues nor on the days 14 and 16 embryos proper. It was surprising though to discover that Class I MHC antigens were absent from both the embryonic and abembryonic surfaces of the amniotic and allantochorionic membranes at term. The finding that Class I MHC antigens were not present on surfaces of periimplantation blastocysts and term placental membranes suggests that MHC antigens were absent throughout gestation from the surface of the placenta. This is a reasonable suggestion since it would be difficult to explain why the appearance of Class I antigens after day 22 of pregnancy (postimplantation) and then complete disappearance from these surfaces at term.

One study in sheep also failed to find Class I antigens at any stage during pregnancy (Low *et al.*, 1990). Further evidence support the hypothesis that Class I MHC antigens are not expressed on the surfaces of the pig extra embryonic/fetal membranes throughout gestation. Compared to maternal class I mRNA levels, class I mRNA expressed in all tissues of fetal origin was barely detectable as determined by Northern blot analysis. In addition, the copy number of class I mRNA in day 16 blastocysts was low, as indicated by the number of silver grains detected, and was found to be ubiquitously distributed. No class I antigens were detected on day 16 embryos which correlates well with the low density of silver grain distribution seen in the embryos. The silver grain distribution in the extraembryonic and embryonic tissues were similar in density.

Collectively, the observations made during this study represent convincing evidence for downregulation of Class I MHC antigens on the placenta of the pig during gestation. The lack of Class I MHC antigens on

the surface of the Jag-1 trophoblast cells and the correspondingly low level of mRNA detected indicates that the regulation of Class I MHC antigens is programmed since they were not upregulated by the long term culture conditions. Moreover, it appears that one mechanism of control is post-transcriptional as mRNA was detected but not protein antigens. Class I MHC antigens were restricted to the vascularized mesenchyme of the extrafetal membranes at term. This is confirmation of the specificity of the PT85 monoclonal anti-porcine Class I MHC antibody, since it recognized class I MHC antigens in one part of the same tissue (Mesenchyme) and not another (the inner and outer surfaces).

The fourth and last objective was to characterize the expression of cytokines at the maternal-embryo/fetal interface and the MGF produced by the Jag-1 trophoblast cell line. Three of the cytokines tested; GM-CSF, CSF-1 and IGF-1, are known to be mitogenic [Guilbert *et al.*, 1991; 1993; Simmen and Simmen, 1990a] and the other three; IFN-γ, IL-2 and TGF-β1, are inhibitory in their characteristics [Tezabwala *et al.*, 1989; Chaouat *et al.*, 1990; Massague, 1990].

Striking evidence for regulated expression of mRNA in a temporal and spatial manner was obtained. This point is well illustrated in days 14-16 blastocysts which expressed mRNAs for all six cytokines. The intensity of labelling was stronger than that of the other tissues and cell samples. CSF-1 and TGF-β1were the only cytokines found to be expressed, albeit at variable densities in all the tissue samples.

The results for IFN-γ are in agreement with that of Lefevre *et al.*, (1990). They reported the presence of two bands (1.4 and 1.3 Kb, respectively) from days 14 to 17 blastocysts which was also present in our days 14 and 16 samples. The 1.0 Kb band in our study most likely

corresponds to their 1.3 Kb band probably due to some RNA degradation. A smear ranging between 0.8 Kb and 1.3 Kb was seen in our days 14 and 16 blastocysts samples. The new information contributed by the present study is that by day 22 only the 1.4 Kb band is expressed, but at a substantially reduced level. In our study, the 1.4 Kb band was also detected in days 14-16 uterine epithelial cells but at a much lower level than that of days 14 and 16 blastocysts. It was comparable to that of day 22 blastocysts. However, it cannot be refuted that this INF-γ mRNA could have originated from intrauterine immune cell contaminations of the enzymatically obtained uterine epithelial cells.

The fact that CSF-1 mRNA was expressed in both periimplantation tissues and extrafetal membrane samples at term indicates that CSF-1 is important for the regulation of placental development and function as proposed by [Pollard *et al.*, 1987; Acerci *et al.*, 1989]. The strong expression of CSF-1 in the uterine epithelium at days 14-16 indicates paracrine mechanisms of action. Moreover, both uterine epithelium and trophoblast tissues are known to express the CSF-1 receptor [Pollard et al., 1987], implicating autocrine actions as well. CSF-1 mRNA was also strongly expressed in the Jag-1 cell line making this cytokine a possible candidate for the MGF produced by these cells.

Multiple TGF-β1 mRNA transcripts have been reported to be present in a wide variety of tissues [Kondaiah *et al.*, 1988]. Our results showed that pig placental tissues are no exception. Temporal expression of the different bands at variable intensities, and the detection of more than one band in all tissues, also implicates TGF-β1 in placental development throughout pregnancy. An immunosuppressive role for this cytokine in the

placenta is attractive; however, its involvement in the developmental processes is also highly likely.

The limited expression of GM-CSF is a surprise. In the pig, an uterine epithelium source of GM-CSF at day 14-16 appears to be secondary to that of day 14 blastocysts when the levels of mRNA expression are compared. It was not detectable in the total RNA after day 14 indicating a sudden decline in expression. The relative importance of the CSF cytokines in pig reproduction remains to be determined.

Our results for IGF-1 expression supports that of Letcher *et al.*, [1989]. The 1.2 Kb IGF-1 mRNA band in the day 14-16 uterine epithelium was weak. This was most likely due to its decline after peak expression on day 12 as indicated by Letcher *et al.*, (1989). A weak band was also detected in day 16 total RNA. Since day 16 was the latest periimplantation stage examined, it was not possible to determine whether this represents the initiation of detectable levels of this cytokine in embryo derived tissues.

Another intriguing observation was the expression of IL-2 in day 14 blastocysts but not at any stages thereafter. The presence of mRNA for this cytokine in day 14 blastocyst coincides with the onset of implantation and indicates an immunological role in activating maternal immune cells that possess the IL-2 receptors in the vicinity of the implantation sites. The 5.1 Kb TGF-β1 band was one weakly expressed in day 14 blastocysts. It is tempting to speculate that down-regulation of TGF-β1 on day 14 represents, a relaxing of immunosuppression sufficiently to allow a limited action of IL-2.

This paper makes an important contribution towards the eventual purification and identification of the MGF produced by the Jag-1 cells. The MGF appeared to be constitutively secreted by the Jag-1 cells in a

protein free culture medium. This permitted the continuation of our attempts to characterize the MGF. The remaining activity is stable after being subjected to the conditions of ultrafiltration and RP-FPLC. This was a fortunate finding because it enabled us to obtain a fraction that contained the activity as determined from the bioassay. Separation of the proteins in the fractions indicated that there were two unique peptide bands in fraction 22 that are possible candidates for the activity. The 42-45 kd bands fall within the size range of some cytokines, including CSF-1.

Taken together, the observations that the Jag-1 trophoblast cell line strongly expressed the mRNA for CSF-1 and that the CSF- 1 protein is a dimer of two approximately 45 Kd bands [Metcalf, 1986] strongly suggests CSF-1 as a candidate. Of more significance though, is that these bands are sufficiently strong to be detected by both silver nitrate staining and ^{125}I-labelling indicating that sufficient quantity can be isolated by electrophoresis for amino terminal sequencing for comparison with known cytokines. One cannot rule out the possibility that the MGF could prove to be a novel cytokine. The isolation and characterization of the autocrine factor was not addressed in this study but it would be interesting to find out whether these activities are represented by one or two factors.

In conclusion I have presented data in this thesis that implicates a strong immune involvement in the processes of development taking place in the uterus and conceptuses during reproduction in the pig. The data in this thesis show that the pig reproductive and immune systems share elements of cytokine networks. Thus the potential for maternal immune involvement in reproduction is present. However, the masking of any improvement in reproductive ability with leucocyte priming in this study could be due to the efficient management of the herd. Probably, beneficial effects of this

treatment only become evident under suboptimal husbandry conditions. The isolation of a trophoblast cell line opens a window to a number of possible studies with the general aim of better understand the functioning of this cell type. We know now that pig trophoblast cells do not express MHC antigens and employ a similar strategy that mammals take to evade any harmful maternal immune effector responses to the presence of the conceptus. I have shown that periimplantation blastocysts of the pig express mRNA for cytokines including lymphokines and that these are regulated in a temporal and spatial manner during gestation. The Jag-1 cell line produces mitogenic factors that appears to be autocrine to trophoblast but also paracrine to macrophages, a cell type that is important in the generation of immune responses. The possibility exists that this MGF is CSF-1.

These studies point to a complex network of cytokines in the utero-placental unit during pregnancy. The profile for cytokine mRNA expression at the maternal-fetal interface will prove important in a number of studies. These studies also now make it possible to purify and identify some mitogenic factors produced by pig trophoblast. The Jag-1 trophoblast cell line can also be used as a tool to study the functions of trophoblast such as adhesion to substrata, steriodogenesis, phagocytosis, and the production of reactive oxygen radicals and to study the regulation of genes such as MHC Class I antigen and CSF-1. Finally, my hope is that the results of this study will add fuel to the eventual goal of fully understanding the mechanisms behind the high embryonic loss inherent in the pig and lead to effective ways of reducing such losses.

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