Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF): A Paracrine Regulator in the Pre-Implantation Mouse Uterus

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Abstract

The period between mating and implantation is characterised by a rapid and dramatic series of remodelling events in the endometrium, which culminate in a transient state permitting attachment and implantation of the embryo. Events during this period are now recognised to be critical to the outcome of pregnancy. The mechanisms underlying these steroid hormone-driven processes are not clearly defined, but recent studies implicate lymphohemopoietic cytokines as molecular mediators of remodelling at the cellular level. In this study, granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-6 (IL-6) are identified as members of a family of cytokines that are synthesised by the epithelial cells in the murine uterus. GM-CSF and IL-6 appear to be important components of a local cytokine network that is initiated as an inflammatory response to seminal factors at mating. This network appears to coordinate the recruitment and behaviour of endometrial leukocytes, and is implicated in establishing an immunological environment that can accommodate the semi-allogeneic conceptus.

These studies were initiated following the recognition that lymphohemopoietic cytokines can act as paracrine regulators of the growth and function of the placenta. The initial aim of this study was to investigate whether cytokines might also influence the development of the embryo prior to implantation. GM-CSF was identified as potentially important in this capacity when it was found to promote the attachment and outgrowth of blastocysts *in vitro*. Blastocysts were found to express GM-CSF receptor in both radiolabelled-ligand binding studies and reverse-transcription polymerase chain reaction (RT-PCR) analysis of GM-CSF receptor mRNA expression.

This activity was postulated to be of physiological relevance when it was found that GM-CSF is synthesised during the pre-implantation period of pregnancy within the endometrium *in vivo*. A dramatic but transient increase in the uterine luminal fluid GM-CSF content was found to occur as a consequence of the interaction between seminal components and the endometrium following mating. Matings with accessory gland deficient mice, together with preliminary chromatographic analyses implicated a specific, high molecular weight factor/s of seminal vesicle origin as the inducing stimulus.

GM-CSF was found to be constitutively synthesised *in vitro* by endometrial cells harvested at oestrus and during early pregnancy. The endometrial-cell derived cytokine was immunologically and physiochemically indistinguishable from GM-CSF synthesised by other tissues. In Northern blot analysis endometrial cells were found to express a 1.2 kb GM-CSF mRNA both *in vitro* and *in vivo*. GM-CSF mRNA was expressed at peak levels in the day 1 uterus.

The IL-6 content of uterine luminal fluid was also found to be markedly elevated after mating, and endometrial cells released IL-6 activity in culture. In contrast, no evidence was found for IL-1, TNFα, IL-2, or IL-3 bioactivities in the luminal fluids or culture supernatants of endometrial cells prepared from mice at oestrus or during early pregnancy.

In view of reports suggesting that T-lymphocytes are the origin of GM-CSF in the mid-gestation mouse uterus, uterine GM-CSF and IL-6 synthesis were examined in genetically lymphocyte-

deficient strains of mice. The GM-CSF and IL-6 contents of luminal fluids and of endometrial cell supernatants from nude, SCID and beige mice were found to be similar to those prepared from immunocompetent mice. To determine the cellular origin of these cytokines, a panning technique employing monoclonal antibodies against a range of leukocyte and other lineage markers was used to isolate uterine cell subsets *in vitro*. These experiments identified glandular and/or luminal epithelial cells as the major source of GM-CSF and IL-6 in oestrous and pregnant uteri. For GM-CSF, this was confirmed by *in situ* hybridisation data indicating that within the intact endometrium GM-CSF mRNA is expressed predominantly by the luminal and to a lesser degree the glandular epithelium.

The role of ovarian steroid hormones in GM-CSF synthesis was examined by measuring the GM-CSF content of supernatants from short-term primary cultures of endometrial cells prepared from mice in which steroid levels were perturbed by ovariectomy and steroid replacement, or by steroid antagonists. GM-CSF synthesis was found to fluctuate through the oestrous cycle, with maximal secretion from endometrial cells harvested at oestrus. Endometrial cells derived from ovariectomised mice secreted 25-fold less GM-CSF than cells from oestrous mice, and production was restored by administration of oestrogen, but not progesterone. GM-CSF output was significantly enhanced in cultures derived from ovariectomised mice as early as 3 hours after administration of oestrogen, suggesting that upregulation of synthesis was a direct consequence of oestrogen action. The oestrogen antagonist ZK 119,010 blocked GM-CSF output whereas the anti-progestin RU486 enhanced its synthesis. These studies demonstrated that GM-CSF synthesis by uterine epithelial cells is stimulated by oestrogen with progesterone having a moderate inhibitory effect. *In vitro*, GM-CSF output was also found to be modulated by factors including bacterial LPS and the T-lymphocyte and NK-cell product IFNγ, but factors that act to promote GM-CSF synthesis from other cell types, including IL-1, TNFα and prostaglandins, did not alter the release of this cytokine from epithelial cells.

GM-CSF is characteristically a regulator of the recruitment, proliferation and functional activation of macrophages and granulocytes in peripheral tissues. There are large populations of these cells in the virgin and particularly in the day 1 pregnant uterus. That the uterus contains GM-CSF responsive cells was suggested by the finding, using RT-PCR analysis, that mRNA for both the α and β chains of the GM-CSF receptor were strongly expressed in uterine tissue at oestrus and during early pregnancy. Binding of radiolabelled ^{125}GM-CSF to uterine tissue sections localised GM-CSF receptor expressing cells to the subepithelial endometrial stroma, suggesting that endometrial leukocytes are targets of GM-CSF action. To investigate the influence of local release of GM-CSF on the recruitment and activation of leukocytes, the densities and distributions of macrophages and granulocytes in the uterus were examined immunohistochemically after instillation of recombinant GM-CSF into the uterine lumen of ovariectomised mice. This treatment resulted in a striking influx into the endometrium of eosinophils, and to a lesser degree macrophages, suggesting that the recruitment of these cells into uterine tissues is a physiological action of this cytokine. Furthermore, leukocyte infiltration was associated with an increase both in the number of endometrial glands and in the overall size of the uterus, implicating GM-CSF responsive leukocytes as mediators of endometrial growth.

Together, these studies identify GM-CSF and IL-6 as potentially important members of a family of cytokines, also including CSF-1, LIF, TNFα, and TGFβ , that are released by epithelial cells in the pregnant uterus. GM-CSF and IL-6 are likely to be of special importance during the preimplantation period, when a striking increase in their secretion is stimulated by specific factors in the ejaculate. It is postulated that the cascade of events initiated by this surge of cytokine activity has repercussions that help to generate a 'receptive' endometrial microenvironment. GM-CSF appears to have a role in coordinating the infiltration and behaviour of macrophages and granulocytes, which have the capacity to synthesise an array of bioactive substances in a microenvironment specific manner. These leukocytes may have critical roles both in tissue remodelling and in establishing a local immune milieu that is tolerant of the semi-allogeneic conceptus. The finding that the embryo is also a target for GM-CSF action, suggesting that this factor may be an important component of a cytokine circuit interlinking the resident and hemopoietic cells in the uterus with the developing conceptus.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. The author consents to this thesis being made available for photocopying and loan, if applicable and if accepted for the award of the degree.

Sarah A Robertson.
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Table of Contents

List of Tables

List of Figures

Abbreviations

A	adenine
amp	ampicillin
bp	base or nucleotide pair
BSA	bovine serum albumin
°C	degrees celsius
C	cytosine
CAM	cell adhesion molecule
CM	conditioned medium
cpm	counts per minute
CSF	colony-stimulating factor
CSF-1	colony-stimulating factor-1 (or M-CSF)
d	day (s)
DAB	diaminobenzidine
dpm	disintegrations per minute
DMEM	Dulbecco's modified minimal essential medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
epo	erythropoietin
EtBr	ethidium bromide
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluorescien isothiocyanate
FMLP	formyl-met-leu-phe
G	guanine
GIFT	gamete intrafallopian tube transfer
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMG	granulated metrial gland
h	hour (s)
HBSS	Hank's buffered salt solution
hCG	human chorionic gonadotrophin
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLA	
HRP	horseradish peroxidase
Ig	immunoglobulin
IGF	insulin-like growth factor
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ip	intraperitoneal
iv	intravenous
IVF	*in vitro* fertilisation
IU	international unit (s)
kb	kilobase pair(s) or 1,000 bp
kD	kilodalton
LCA	leukocyte common antigen
LCCM	lung cell conditioned medium
LFA-1	lymphocyte functional antigen-1
LHRH	leuteinising hormone releasing hormone
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
mAb	monoclonal antibody
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
min	minute (s)
mRNA	messenger RNA
MTT	
NA	nutrient agar
NB	nutrient broth
NK	natural killer
NMS	normal mouse serum
O/N	overnight
PAF	platelet activating factor
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PEC	peritoneal exudate cells
PG	prostaglandin
PHA	phytohaemagglutinin
PMA	4b-phorbol-12b-myristate 13a acetate
PMC	polymorphonuclear cell
PMSG	pregnant mare serum gonadotrophin
RNA	ribonucleic acid

RNAse	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerasse chain reaction
SCF	stem cell factor
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
ssDNA	salmon sperm DNA
SSEA	stage specific embryonic antigen
T	thymine
TCA	trichloroacetic acid
TE	Tris EDTA
tRNA	transfer RNA
TsF	T-lymphocyte suppressor factor
TGF	transforming growth factor
TLX	trophoblast/ lymphocyte cross-reactive antigen
TNF	tumor necrosis factor
U	uracil
UV	ultraviolet
v/v	volume per volume
wk	week
w/v	weight per volume

1 Review of the Literature

1.1 Introduction

Local events within the uterus during very early gestation are of paramount importance in determining the outcome of pregnancy. A less than optimal interaction between the embryo and maternal tissues at implantation compromises placental development. The result may be spontaneous abortion, which occurs in up to 70% of human pregnancies (Chard 1991), or low birth weight and attendant health problems through post-natal and adult life (Barker *et al.* 1989 and Barker 1991).

Successful implantation and placentation are dependant on the 'receptivity' of the endometrium, which is characterised by;

(1) an endometrial surface which will allow the embryo to attach and invade, and;

(2) an immunological environment which will allow the semi-allogeneic embryo to escape rejection.

The uterine environment at implantation is the culmination of a rapid and dramatic reorganisation of uterine tissue during the preceding oestrous cycle and primarily, as argued later in this thesis, during the period between exposure to semen at mating and implantation. This process involves a steroid hormone-orchestrated cascade of communicative events, linking both immunocompetent cells and resident cells within the uterus, and the developing conceptus (Fig 1.1).

Evidence emerging over recent years has revealed that, as in other tissues, polypeptide growth factors comprise the basis for an inter-cellular signalling language in the uterus. A subset of growth factors known as 'lymphohemopoietic cytokines' because of their origins in, or actions on cells of hemopoietic origin are now implicated as pivotal components of this communication network. In this capacity, it is speculated that cytokines may play a central role in protecting the fetal allograft by recruiting leukocytes into the uterus and coordinating their behaviour. In addition, the effects of cytokines on the development and function of the placenta may directly influence embryonic growth.

This discussion will focus on recent advances in our understanding of the molecular and cellular mechanisms at play in the uterus during the oestrous cycle and during early pregnancy, with a special emphasis on the roles of cytokines and the leukocytes with which they interact.

1.2 The pre- and peri-implantation period in murine pregnancy

1.2.1 The mouse as a model for human pregnancy

Rodents, like humans, have interstitial implantation and hemochorial placentas, and so provide a valuable model species in which to study aspects of reproductive physiology that are potentially common to pregnancy in humans. The mouse has a relatively short oestrous cycle and gestation period, and has been the species of choice for many reproductive biologists because of the availability of genetically defined inbred strains, and an extensive range of probes and reagents with

which to identify leukocytes and cytokines. The physiology of the murine oestrous cycle and pregnancy are comparatively well studied and are comprehensively described in Finn and Porter (1975), Hogan *et al.* (1986) and Allen (1921). Data described in this review will be confined to murine studies unless otherwise indicated.

1.2.2 Mating and the ejaculate

The mouse has a 4 day oestrous cycle which is synchronous with a diurnal rhythm, and mating coincides with ovulation just after midnight in the middle of the oestrous period. The ejaculate is composed of sperm delivered from the epididymis, bathed in fluid derived from the accessory glands (which include the seminal vesicle, prostate and coagulating gland). The seminal plasma provides a nutritive and protective medium for sperm transport, and in the rodent it contains enzymes that interact with coagulating factors to form the copulation plug.

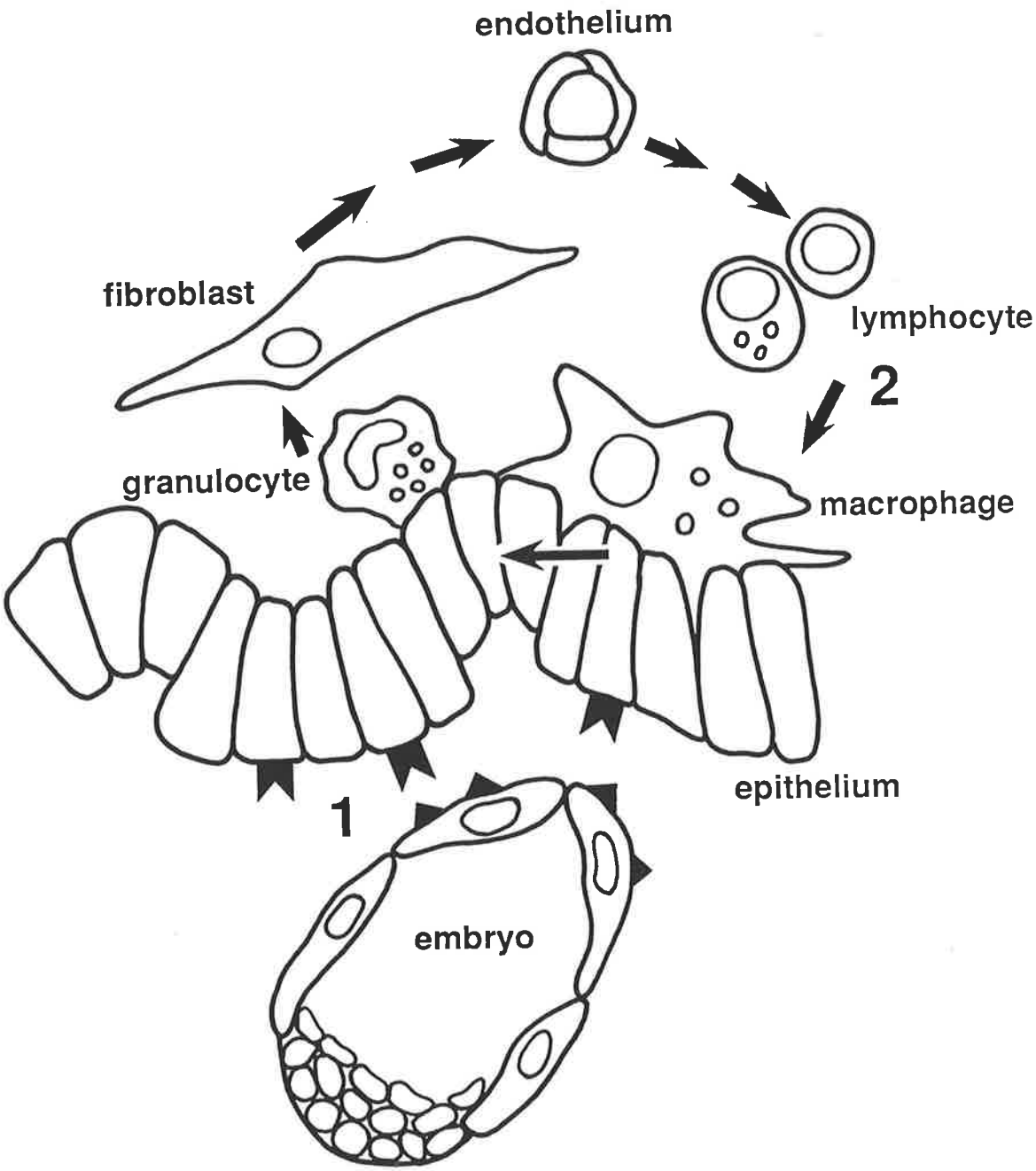
Whilst embryo transfer experiments have shown that seminal factors are not an absolute requirement for successful pregnancy, implantation rates are reduced in the rat in the absence of insemination (Carp *et al.* 1984) and fertility in rodents is reduced when male accessory glands, particularly the seminal vesicle, are removed (Pang *et al.* 1979; Queen *et al.* 1981). The importance of exposure to seminal fluid may be heightened when fertility is otherwise compromised (Pollard *et al.* 1991a). In the human, exposure to semen reduces the risk of pre-eclampsia (Klonoff-Cohen *et al.* 1989), and enhances the implantation rate in gamete intrafallopian tube transfer (GIFT; Marconi *et al.* 1989) and *in vitro* fertilisation (IVF) programs through an effect on the endometrium (Bellinge *et al.* 1986). Furthermore, seminal vesicle deficiency or obstruction is a cause of infertility in men (Dominguez *et al.* 1991, Abbitt *et al.* 1991). In the pig, pre-exposure of gilts to seminal plasma increases litter size through more efficient implantation and enhanced placental function (Murray *et al.* 1986; Stone *et al.* 1987; Bischoff *et al.* 1990).

1.2.3 Implantation

In the mouse, the conceptus develops in the oviduct and is delivered into the uterus as a 16-32 cell morula on day 3, 60-72 hours (h) after fertilisation. Apposition, attachment and implantation take place during the subsequent 24-30 h period on day 4. Implantation is a complex phenomenon encompassing a series of interactive processes in which both the conceptus and maternal tissues participate. The molecular and cellular mechanisms underlying these major tissue remodelling events are poorly understood. For a full discussion of the implantation process the reader is referred to Weitlauf (1988) or Finn (1977). This review will briefly describe the process of implantation, with an emphasis on the factors that appear to be important determinants for a successful outcome.

a. Uterine receptivity

Certainly the single most important determinant in the success or otherwise of implantation is the 'receptivity' of the endometrium. A receptive uterus is, by definition, in an exclusive and



UTERINE RECEPTIVITY

1. Adhesin expression
2. Immuno-receptive state

Figure 1.1 Uterine 'receptivity' at implantation. Receptivity is viewed as the culmination of a series of steroid hormone-driven events, during which communication between immunocompetent cells and resident cells within the uterus, and the developing conceptus, gives rise to (1) an endometrial surface which will allow the embryo to attach and invade, and (2) an immunological environment which will allow the semi-allogeneic embryo to escape rejection.

transient state during which embryo attachment and implantation can occur. The concept of receptivity arose after embryo transfer experiments in the rabbit demonstrated the importance of chronological synchrony between the embryo and the uterus. Preparation of the uterus for implantation begins during the oestrous cycle, when prominent changes occur in the cytology of the endometrium, which is the mucosal lining of the uterine wall. Ovarian steroid hormones induce proliferation and differentiation of resident epithelial and stromal cells (described in detail in Finn and Porter 1975), accompanied by major changes in the density and composition of infiltrating leukocytes. At mating, mechanical stimulation of the cervix promotes central nervous system-regulated release from the pituitary of the anti-luteotrophic hormone prolactin, which prolongs the life of the corpus luteum and ensures the continued secretion of pregnancy hormones.

Studies in ovariectomised mice have defined the precise ovarian steroid hormone parameters necessary to induce the characteristic sequences of proliferation and differentiation in the luminal and glandular epithelium and endometrial stroma that culminate in the receptive state (Finn 1977, Psychoyos 1986). Priming of the uterus with progesterone for about 48 h gives rise to a pre-receptive, 'neutral' state. A short phase of receptivity occurs in response to a minute amount of 'nidatory' oestrogen in the progesterone conditioned uterus, but within 36 h this is followed by a state of refractoriness in which the endometrium becomes hostile to unimplanted embryos. These constraints appear to be specific to the uterus: in extrauterine sites or in culture, embryos can attach and grow regardless of the hormonal environment. *In vitro* co-culture experiments suggest that factors that act to inhibit embryonic development may be secreted by the endometrium during the pre-receptive phase, whilst the refractory action of a post-receptive uterus may be mediated by embryotoxic molecules (O'Neill and Quinn 1983; Glasser *et al.* 1991). In the mouse, the dependence of implantation on the state of the uterus is demonstrated clearly by the 'delayed implantation' phenomenon. The blastocyst can exist in a dormant state within the uterine lumen during a prolonged phase of pre-receptivity, before implanting normally in response to nidatory oestrogen. Delayed implantation can be induced experimentally in progesterone-maintained, ovariectomised mice, and occurs naturally in lactating mice (Weitlauf 1988).

The recent findings that various growth factors, including cytokines, are synthesised in uterine tissues in response to the actions of ovarian steroid hormones (see 1.7) has led to the proposal that these factors mediate many of the actions of steroid hormone at the cellular level (Pollard 1990). Interestingly, perturbation in ovarian steroid levels by stimulation with superovulating agents interferes with embryonic survival and implantation (Beaumont and Smith 1975) and this appears to be mediated through reduced uterine receptivity (Fossum *et al.* 1989).

b. Apposition and attachment

Murine embryos enter the uterus from the oviduct on day 3, and become spaced at regular intervals along the length of the horn, where with time they become apposed more closely to the antimesometrial side of the luminal epithelium. Endocrine-dependant resorption of fluid from the uterine lumen and oedema of the endometrium, together with contractile activity in the

myometrium, contribute to uterine closure, and the clasping and spacing of the embryos. During this period local protease activity dissolves the zona pellucida.

The molecular basis for the initial adhesion of the embryonic trophoblast cells to the luminal epithelium is not yet determined, although there is evidence that binding is mediated by the expression of complimentary surface membrane molecules. Modulation of cell surface glycoconjugates of both the uterine epithelium and the trophectoderm accompany a decrease in both the surface charge and the thickness of the epithelial glycocalyx immediately prior to implantation (Weitlauf 1988). Heparin or heparan sulphate proteoglycans are major cell surface components of embryos and agents that remove or compete with heparin block the attachment and spreading of embryos on laminin, fibronectin, and uterine epithelial cells *in vitro* (Farach *et al.* 1987). The trophectoderm expresses a receptor for a hormone-dependant epithelial glycoconjugate carrying a structure related to the milk oligosaccharide lacto-N-fucopentaose I (LNF-I)(Lindenberg *et al.* 1990; Kimber and Lindenberg 1990), and LNF-I blocks the adhesion of murine blastocysts to epithelial cells *in vitro* (Lindenberg *et al.* 1988). The affinity of this trophectoderm receptor for fucosylated lactosamines suggests it may be a member of the 'selectin' family of cell adhesion molecules (CAMs)(Vestweber 1992). Lactosaminoglycans are cell surface components of uterine epithelial cells in the mouse, and the finding that the blastocyst expresses the complimentary ligand galactosyl transferase just prior to implantation (Dutt *et al.* 1987; Sato *et al.* 1984) suggests that these may also be important cell adhesion molecules during the initial stages of implantation.

c. Decidualisation and invasion

As the apposition phase proceeds there is evidence typically of death and detachment of the underlying epithelial cell layer, which allows trophoblast cells to penetrate the basal lamina and stroma in the first stages of placental formation. 'Decidual transformation' begins prior to death of the overlying epithelial cells. There are conspicuous changes in the morphology of the adjacent endometrial stroma, including proliferation and differentiation of stromal fibroblast cells into the characteristic binucleate and polyploid 'decidual cells', which have a typical epithelioid appearance. These changes, together with tissue oedema resulting from local increases in vascular permeability, soon result in grossly observable increases in the size and weight of the uterus (Finn *et al.* 1989; Weitlauf 1988). The earliest development of fenestrations between endometrial epithelial cells at implantation sites can occur even while blastocysts are still enclosed in the zona pellucida, and so do not require direct epithelial/ trophoblast cell contact (McLaren 1969).

Decidualisation rarely occurs spontaneously in the uteri of psuedopregnant animals, demonstrating that a signal of embryonic origin is needed to induce the local changes within the implantation site. A role for embryo-derived prostaglandins, histamine, and various other proteins and metabolites in this capacity have been postulated (Weitlauf 1988). Rodent embryos have steroid synthesising enzymes and although studies with anti-oestrogens implicate oestrogen of embryonic origin in implantation, embryo-associated steroids and their metabolites have been difficult to detect (Weitlauf 1988). Platelet activating factor (PAF) is a vasoactive and

immunoactive phospholipid (Braquet and Rola-Pleszczynski 1987) secreted by mouse and human embryos, as well as by endometrial cells, during the pre-implantation period (O'Neill 1991). A role for PAF in embryonic development and in the maternal tissues at implantation is suggested by the finding that PAF antagonists inhibit implantation (Spinks *et al.* 1990), and that PAF can induce decidualisation (Acker *et al.* 1989). However these results have not been confirmed by others (Milligan and Finn 1990).

Decidual transformation in the uterine endometrium can be induced by a variety of physical and chemical stimuli during a limited period (corresponding to the period of receptivity to implantation) in psuedopregnant or ovariectomised mice treated with the appropriate steroids (Finn 1977; Finn *et al.* 1989). Interestingly, various inflammatory cytokines (Choudhuri and Wood 1991) and some lectins (Shaw and Murphy 1992) can induce decidual reactions in psuedopregnant mice, presumably by mimicking an embryo-derived signal to the epithelium.

The ability of such a wide range of agents to induce the decidual response suggests that they may all stimulate production by the endometrium of a common factor. Indeed, studies in mice with de-epitheliated uteri (Lejeune *et al.* 1981) show that the epithelium may have a critical role in transmitting the decidualising stimulus. The nature of the epithelial cell-derived factors in this process remain unknown, but various biological mediators have been implicated on the basis that they are released locally in the uterus at implantation as well as in response to various artificial stimuli. One such factor is histamine, which is released from mast cells in response to oestrogen together with a factor of blastocyst origin (Cocchiara *et al.* 1992). Histamine can induce decidualisation when instilled into the uterine lumen of rats, and both histamine antagonists and inhibitors of histamine release from mast cells can block implantation and decidualisation (Shelesnyak 1952; Dey et al 1978).

Several studies implicate prostaglandins, particularly prostaglandin E_2 (PGE_2), in the development of endometrial vascular permeability and decidual transformation during implantation. The most compelling evidence in this regard is the finding that prostaglandin levels are elevated in implantation sites and in artificially induced deciduomata, and that indomethecin blocks implantation and decidualisation in many species. This effect is, at least partly, overcome with exogenous PGE_2. Intraluminal PGE_2 administration induces decidualisation, and stromal cell expression of PGE_2 receptors parallel the decidual response (Weitlauf 1988).

1.3 Immunological Considerations

1.3.1 Pregnancy is an immunological phenomenon

Viviparity implies direct physical contact between genetically disparate maternal and fetal cells, and of necessity has required the evolution of efficient mechanisms for nurturing the young in the face of a functional immune system able to detect and eliminate alien cells. The trophoblast, derived from the conceptus, expresses paternal transplantation antigens and so would be expected to elicit an allo-reaction by the maternal immune system, resulting in rejection. Trophoblast cells of

the placenta and extra-placental membranes are exposed to maternal blood and tissues from the time of zona dissolution and for the duration of pregnancy.

However, far from being detrimental to pregnancy outcome, genetic disparity between parents appears to contribute positively to the health and size of the placenta and hence the fetus, and is the basis for 'hybrid vigour' in both rodents and humans (Gill 1983; McIntyre and Faulk 1983; Beer *et al.* 1975; Beer and Billingham 1974). A selective advantage of placental alloantigenicity has been suggested to be a driving force behind maintenance of major histocompatibility (MHC) locus polymorphism and heterozygotic diversity within a species (Lewis *et al.* 1986). Indeed, some pregnancy disorders have been attributed to inadequate activation of the maternal immune system to paternal alloantigens (Mowbray 1990) when there is sharing of MHC alleles between parents (Lewis *et al.* 1986; Scott *et al.* 1987; Redman and Sargent 1986). How these advantages of placental allo-reactivity can be reconciled with the ability of the semi-alloantigenic conceptus to evade rejection by the maternal immune system remains an area of considerable conjecture.

Together, these data suggest that the maternal immune system has an important role in recognising and promoting development of the products of conception. In normal circumstances, the status of the endometrial immune environment would therefore be a consideration of utmost importance in determining the outcome of implantation and placentation.

1.3.2 Maternal immune responses to semen

During pregnancy, the maternal immune system is interactive with events in the reproductive tract as early as at the time of mating. Sperm and seminal fluid carry various antigens, including MHC antigens and minor histocompatibility antigens including H-Y, blood group antigens, trophoblast-lymphocyte cross-reactive antigen (TLX, or CD56) and sperm specific autoantigens, as well as leukocytes (Clarke 1984; Kajino *et al.* 1988; Alexander and Anderson 1987). Washed allogeneic epididymal spermatozoa inoculated directly into the uterine lumen of rats incite local lymph node enlargement and transplantation immunity (Beer and Billingham 1974). Seminal plasma contains immunosuppressive and immunoactivating substances and may have a role in regulating maternal immune responses to insemination (Alexander and Anderson 1987; Thaler 1989; Hadjasavas 1992).

An inflammatory-like response occurs within the endometrium during the 24 h period following mating. Interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα) synthesis by leukocytes within the endometrium is induced within 12 hour after copulation. Although the nature of the inducing agent is not clear, mating with vasectomised males elicits a similar response implicating factors other than spermatozoa (McMaster *et al.* 1992). Upregulation of messenger ribonucleic acids (mRNAs) encoding TNFα, IL-1α and IL-1β, colony stimulating factor-1 (CSF-1), IL-6 and granulocyte-macrophage CSF (GM-CSF) in the uterus following mating has also been reported (Sanford *et al.* 1992).

These changes in cytokine expression are associated with a dramatic increase in the numbers of mononuclear phagocytes and neutrophils found in both the endometrium (1.5.2; 1.5.3), and the uterine lumen (Austin 1960; Ball and Mitchinson 1977). These cells may act to phagocytose micro-organisms that are introduced at mating, and also the bulk of the sperm, although the basis upon which some sperm escape phagocytosis is not clear (Cohen 1984). It has been suggested that maternal immunisation against paternal antigens may be facilitated by the processing of seminal debris by uterine antigen presenting cells at mating (Kajino *et al.* 1988). Uterine luminal epithelial cells are endocytotic in early pregnancy (Parr 1980) and since they can express class II MHC antigens (Head 1987) these cells may also present antigen in a role analogous to that of other epithelia (Mayrhofer *et al.* 1983; Mayer and Shlein 1987).

1.3.3 Maternal immune responses to the conceptus

The mother appears to recognise the conceptus as an independent immunological entity as early as implantation. An hypertrophy of the lymph nodes draining the uteri was found to occur prior to implantation on day 4 after mating to allogeneic, but not syngeneic males. This reaction was primarily a consequence of exposure to seminal antigens since it also occurred following ligation of the oviduct (Beer *et al.* 1975; Beer and Billingham 1974). Lumbar lymph node hypertrophy was more pronounced in multiparous females (Clarke 1984), and interestingly, was associated with increased litter sizes. Implantation rates were also enhanced in uteri that had been previously sensitised to paternal leukocytes or skin grafts. That this increased reproductive efficacy had a local, immunologic basis was shown by the finding that allo-sensitisation of one horn did not increase implantation sites in the contralateral horn, and had no effect on the pregnancies resulting from syngeneic matings (Beer *et al.* 1975; Beer and Billingham 1974). Dramatic changes in the weight of the thymus associated with allogeneic pregnancy are evident by day 4, and are modified by prior pregnancy or sensitisation against paternal antigen (Clarke 1984).

MHC antigens have been detected on embryos as early as at the 8-cell stage (Searle *et al.* 1976; Goldbard *et al.* 1985; Warner *et al.* 1988), as well as minor histocompatibility antigens and stage specific embryonic antigens (SSEA) (Warner *et al.* 1988; Hamilton 1983). The embryo is susceptible to maternal effector cell attack as soon as the physical protection afforded by the zona pellucida is lost (Warner *et al.* 1988), and is bathed in maternal immunoglobulin from conception (Clarke 1984). Allogeneic blastocysts transferred surgically to beneath the kidney capsule of an immunised host are rejected rapidly, and this attack is directed towards non-MHC histocompatibility antigens (Searle *et al.* 1976).

Local mechanisms preventing the initiation of destructive immune processes must therefore be in place from the moment of primary contact between embryonic and maternal tissues at implantation. However, the midgestation fetal-placental unit has been the focus of studies aimed at identifying these mechanisms, and although substantial progress has been made in this area in recent years, the temporal sequence of their induction during the per-implantation period has been somewhat ignored.

An effective host anti-graft immune response is dependant on (1) expression of appropriate allo-antigens by the graft; (2) generation of specific allo-reactive effector cells; (3) physical contact between graft and effector cells, and (4) susceptibility of the graft to effector-mediated cytolysis. The following sections summarise what are currently regarded as the major strategies employed at the maternal fetal interface to prevent the generation and the execution of maternal rejection responses.

a. Afferent suppressive mechanisms

Several factors may limit maternal recognition of conceptus antigens. After implantation, the fetus is anatomically separated from the mother by the placenta and extra-embryonic membranes and trophoblast is the fetal tissue in direct contact with maternal blood and tissue. Study of the distribution of MHC antigens in placentae of many species has revealed a lack of class II antigens and also a highly regulated expression of class I antigens in the placental trophoblast cells to which the maternal tissues are exposed. There is substantial evidence that class I MHC antigens are not expressed by the trophoblast giant cells adjacent to maternal tissues after implantation (Head *et al.* 1987). In humans and rats the non-polymorphic non-classical class I genes HLA-G and Pa respectively are expressed in place of other class I genes (Hunt and Orr 1992).

Whether a similar situation exists in the mouse is less clear. Expression of class I antigens occurs at least as early as in the (pre-placental) ectoplacental cone (Hedley *et al.* 1989). Immunohistochemical studies have revealed that in the mid-gestation placenta spongiotrophoblast cells express class I antigens (Redline and Lu 1989), but this appears not to be immunostimulatory, since no evidence of an enhanced maternal allo-response was found following upreglation of expression by treatment with interferon–γ (IFNγ) (Mattsson *et al.* 1991). Class I protein is not detected in labyrinthine trophoblast cells, and *in situ* hybridisation studies show that although class I heavy chain transcripts are synthesised in these cells their failure to be processed into cell surface protein is associated with a lack of $\beta_2$microglobulin mRNA expression (Jaffe *et al.* 1991). Non-polymorphic Qa-2 antigen is present on pre-implantation embryos, where its expression appears to be positively correlated with growth and development (Warner *et al.* 1988; 1991). However, classical class I (H-2) antigens have also been reported to be expressed by preimplantation murine embryos (Searle *et al.* 1976; Goldbard *et al.* 1985; Warner *et al.* 1988).

A block in local antigen handling and processing is a potential mechanism whereby maternal sensitisation to conceptus-derived antigens is inhibited. Lymphatic vessels are not found near the uterine lumen after mating, nor in the vicinity of the conceptus during or after implantation (Head and Billingham 1986). There is a paucity in Ia-positive dendritic cells near the implanting blastocyst, and macrophages are excluded from the vicinity of trophoblast tissue by the decidual zone from the initiation of implantation onwards (1.5.2). Together, these conditions probably function to restrict the access of antigenic material to the lymph nodes draining the uterus.

Soluble factors that have immunosuppressive activities *in vitro* are present within the pregnant uterus and have been proposed to influence local T-lymphocytes dynamics. T-

lymphocytes as well as lymphocytes of an unusual non-T phenotype have been implicated as the source of molecules that may suppress the activation and expansion of effector cells in the mouse uterus and draining lymph nodes. Hoversland and Beaman (1990) have described a T-suppressor cell derived, antigen-specific suppressor factor (TsF) that is highly active during implantation. *In vivo* antibody-mediated depletion of either TsF or T-suppressor cells induces abortion (Beaman and Hoversland 1988; Clark *et al.* 1989). Non-T suppressor cells appear in the decidua following implantation and a reduction in their numbers is associated with early pregnancy failure (Clark *et al.* 1987, Chaouat *et al.* 1990). These cells secrete a molecule of the transforming growth factor–β2 (TGFβ2) cytokine family, which suppresses IL-2 mediated activation and proliferation of natural killer (NK) and T-lymphocytes *in vitro* (Clark *et al.* 1988 and 1990; Lea *et al.* 1992). Successful pregnancy in humans as well as mice has also been correlated with systemic induction on lymphocytes of the progesterone receptor, together with the capacity to secrete an NK cell inhibiting activity after exposure to progesterone (Szekeres-Bartho *et al.* 1991).

Other soluble factors within the uterus have immunosuppressive activity. Of particular importance may be PGE_2, since indomethacin is a powerful abortifacient. PGE_2 is produced by macrophages as well as other resident uterine cells and interferes with activation of T-lymphocytes and NK cells (Tawfik *et al.* 1986a and 1986b; Lala *et al.* 1988 and 1990). Cytokines including TGFβ, TNFα and GM-CSF are present within uterine and placental tissues and may have 'immunosuppressive' properties, primarily through inducing suppressive phenotypes in local leukocyte populations (1.7.1; 1.7.6). The activities and potential functions of cytokines in the uterus will be considered fully in later sections of this review (1.7).

b. Efferent suppressive mechanisms

Mechanisms that may act to inhibit sensitisation to conceptus antigens are insufficient to fully account for the survival of the conceptus, since humoral and cellular responses to paternal class I and minor histocompatibility antigens, as well as to oncofetal antigens, can both occur naturally in parous animals without obvious detriment to fetal survival (Bell and Billington 1983; Hamilton 1983; Heyner and Komar 1987). Pregnancy is not compromised in females rendered hyperimmune to paternal tissue antigens by prior immunisation with paternal leukocytes (Beer and Billingham 1974). Similarly, passive transfer of *in vitro* expanded anti-paternal allotype cytotoxic T-lymphocytes (CTLs) has no effect on pregnancy outcome, although these cells readily mediate paternal skin graft rejection (even in pregnant mice) (Kamel and Wood 1991). Thus it appears that mechanisms operating locally in the uterus at the maternal-fetal interface provide a second line of defence, through blocking of the effector arm of immune rejection.

In the event that specific T-effector cells gain access to the placenta, a further level of protection is afforded by the resistance of trophoblast cells to T-effector cell attack. Firstly, the trophoblast cells that are exposed to the maternal tissues do not express the classical MHC molecules to which anti-paternal allo-reactive effector cells are targeted (Hunt and Hsi 1990; Head 1989). Furthermore, there is little definitive evidence that cytolytic cells can cause placental

damage by killing trophoblast cells. Whilst highly activated CTLs can lyse cultured trophoblast cells *in vitro*, fresh trophoblast cells prove remarkably resistant (Drake and Head 1989). CTLs found within resorption sites appear to be present as a consequence rather than a cause of the pregnancy failure (Head 1989). Trophoblast cells also appear to be protected from NK cell lysis, and this has been related to their expression of non-classical MHC (Kovats *et al.* 1990), although $\beta_2$microglobulin gene knockout experiments show that MHC expression is not critical to the success of pregnancy (Koller *et al.* 1990).

Antibodies to trophoblast membrane proteins, including both major and minor histocompatibility antigens, are detectable in the serum and placenta during normal rodent (Head and Billingham 1983; Heyner and Komar 1987) and human (Davies and Browne 1985) pregnancy, but the trophoblast also appears to be resistant to complement mediated cytolysis. This may be due to expression by the embryo and placental trophoblast cells of proteins including membrane co-factor protein (MCP) and decay accelerating factor (DAF) that inhibit activation of the complement cascade (Holmes and Simpson 1992).

There is evidence that in some circumstances trophoblast cells may be susceptible to killing by innate immune cells of the NK cell lineage. Uterine NK cells (or granulated metrial gland [GMG] cells) are abundant in the uterus and decidua during pregnancy. These cells can kill trophoblast cells *in vitro*, and so may provide a mechanism whereby excessive placental growth can be limited (1.5.6).

c. Immunotrophism

In an attempt to address the apparent dichotomy between the benefits and potential dangers of maternal immune recognition of the conceptus, Wegmann *et al.* (1989; Wegmann 1988) have put forward the 'immunotrophism' hypothesis which states that certain types of immune responses may be beneficial to pregnancy and actively prevent rejection of the fetal 'graft'. This concept arose from studies showing that the elevated rate of fetal resorption in the CBA/J x DBA/2 strain combination can be reduced to normal levels by active immunisation with cells bearing paternal MHC antigens (Balb/c spleen cells), and that this leads to a significant increase in fetal and placental size (Chaouat *et al.* 1985). Immunisation correlates with the induction of both humoral and cellular responses to allo-antigen, and with a local recruitment of decidual non-T suppressor cells (Clark *et al.* 1987), and a reduction in the accumulation of presumptive NK cells (or GMG cells) at the implantation site (Gendron and Baines 1988). Protection against abortion can be adoptively transferred with post-partum serum or CD8 positive T-cells from immunised or multiparous (by Balb/c males) CBA/J mice (Chaouat *et al.* 1985).

It was originally postulated that the beneficial effects of anti-paternal immunity were mediated through the local release from allo-reactive T-lymphocytes of cytokines that enhance placental growth and development (Athanassakis *et al.* 1987). Placental cells were shown to proliferate and exhibit enhanced phagocytic activity in response to T-cell derived cytokines (1.8.1). When T-lymphocyte parameters were altered by *in vivo* administration of monoclonal antibodies

(mAbs) against CD4 or CD8 during pregnancy, placental proliferation and phagocytosis were diminished (Athanassakis 1990). However, neither the resorption frequency nor placental or fetal weights were affected by depletion of CD8 positive T-cells in another study (Sulila *et al.* 1988). Further support for a trophic role of activated T-cells came from the observation that depletion of T-cells in the autoimmune MRL *lpr/ lpr* strain corrected placental hypertrophy and placental phagocytosis was then normal (Chaouat *et al.* 1988). These findings have led to the controversial treatment of recurrent spontaneous abortion in women with immunisation against paternal leukocytes, but whether this has a beneficial effect on subsequent pregnancy outcome remains inconclusive (Hill 1991).

However, the validity of the 'immunotrophism' hypothesis was bought into question when it was found that there is no absolute requirement for an intact maternal immune system for pregnancy to proceed successfully. Genetically T-cell deficient mice have normal placental and fetal weights (1.5.1), suggesting that any role of the maternal immune system in promoting successful pregnancy on the basis of classical anti-alloantigenic reactivity is limited to an adjunctive rather than a necessary one.

Thus the capacity of both the specific and innate arms of the maternal immune system to respond *appropriately* to pregnancy may prove to be more important for pregnancy outcome than the status of maternal immunity to paternal allo-antigens. This interpretation of the data is consistent with findings that non-specific immunopotentiating agents such as Freund's complete adjuvant can prevent fetal resorption as effectively as immunisation with paternal antigens (Toder and Strassburger 1990), whereas environmental pathogens can have the opposite effect (Hamilton and Hamilton 1987). A balance between the activities of the two subsets of $CD4^+$ T-lymphocytes (T_H1 and T_H2) has been proposed to be central to the success of pregnancy and may be influenced by these treatments (Guilbert *et al.* 1993).

Furthermore, the findings that systemic administration of small quantities of cytokines during mid-gestation can also enhance fetal survival in abortion-prone mice (Chaouat *et al.* 1990) has led to the extension of this hypothesis to accommodate the concept of cytokine regulatory networks at the fetal-maternal interface (1.8.5). Local cytokines may alter the outcome of pregnancy through maintaining an appropriate balance between the cytotoxic actions of uterine NK cells (1.5.6) and the activity of local immunosuppressive mechanisms. The recruitment, proliferation and activation of T-lymphocytes are sensitive to the effects of various cytokines, and so cytokines are also likely to be important in initiating and maintaining the balance between T_H1 and T_H2 cells. The potential roles of cytokines in regulating the functions of immunocompetent cells are considered in greater detail in later sections of this discussion.

1.4 Leukocyte trafficking

Cycling and pregnant uteri host large and diverse populations of leukocytes. The nature and behaviour of these cells is presumably central to strategies employed at the maternal fetal interface to prevent generation or execution of destructive immune responses. Leukocytes, especially those

of myeloid lineages, also have important roles in the tissue remodelling that accompanies inflammatory and reparative processes, and may act in similar capacities in the tissue remodelling that is characteristic of reproductive events. Recent data pertaining to the occurrence and behaviour of the individual leukocyte populations that are resident within the murine uterus will be discussed in the following sections of this review, following a brief overview of mechanisms that regulate leukocyte trafficking through peripheral tissues.

1.4.1 Inflammation

The nature and degree of the leukocyte infiltrate into the uterus during the oestrous cycle and early pregnancy, together with the accompanying vascular changes, have caused comparisons to be drawn between these events and an inflammatory response.

During inflammation leukocytes and serum molecules are diverted towards areas of damage in peripheral tissues, usually following infection or injury. The principle components of an inflammatory process include local increases in blood flow and capillary permeability, and the infiltration of leukocytes including polymorphonuclear cells, lymphocytes and macrophages. The kinetics of leukocyte recruitment depend on the cell type; neutrophils migrate into tissue within minutes of stimulation but die within 24 h, whereas macrophages and lymphocytes arrive within hours and persist (or are constantly renewed) for days or even weeks.

Recent studies in epithelial tissues highlight the importance of resident sessile cells within tissues in the generation of inflammatory reactions. Non-hemopoietic cells in the skin (including epidermal keratinocytes and dermal fibroblasts) upon appropriate stimulation produce various cytokines that can decisively influence the behaviour and function of lymphocytes, granulocytes and monocyte/macrophages. Many of these cytokines (GM-CSF, IL-1, TNFα) are the same factors that influence CAM expression by endothelial cells and epithelial cells (Kupper 1990; Carlos and Harlan 1990). Kupper (1990) surmises that "Taken to their logical conclusions, these observations suggest that nonhemopoietically derived cells of skin, by virtue of their potential for cytokine production, have the indirect capacity to trap appropriate classes of leukocytes within microvasculature at precise anatomical sites, guide them through vessel walls and through dermal tissue along chemotactic gradients, and then activate them in situ." In the case of the lung, recruitment of granulocytes, particularly eosinophils, follows the upregulated release by pulmonary endothelial and epithelial surfaces of factors which are chemotactic or enhance CAM expression (Ohtoshi *et al.* 1991). This may be important in the pathogenesis of asthma (Leff *et al.* 1991; Corrigan and Kay 1992).

1.4.2 Leukocyte recruitment

The physiological processes by which leukocytes are selectively exported from the circulation and trapped at precise anatomical sites within normal or inflamed peripheral tissues are exquisitely regulated by specific cell-cell and cell-extra cellular matrix (ECM) interactions,

mediated by integrins and other CAMs, and by the local production of chemotactic factors such as IL-5, IL-8 and GM-CSF (Albelda and Buck 1990; Osborn 1990; Vestweber 1992).

The endothelial cell-neutrophil interaction has been studied extensively and a model involving a multi-step recognition process that provides for both specificity and diversity in leukocyte-endothelial cell recognition has emerged from these studies (Butcher 1991). An inflammatory reaction begins when resident cells at the site of insult release biologically active mediators including cytokines (eg. IL-1, TNFα, IFNγ, GM-CSF) or other inflammatory mediators [eg. leukotrienes, histamine and bacterial products including FMLP and lipopolysaccharide (LPS)] which act to upregulate expression of endothelial CAMs (Albelda and Buck 1990; Obsorn 1990). Within minutes after tissue injury, the passage of leukocytes through the local microvasculature is slowed, as they become loosely associated with the endothelial cell via a primary adhesion pathway mediated by molecules of the selectin family of CAMs (Vestweber 1992). This allows local chemoattractant/ activating factors (including cytokines, lipids, complement components and bacterial products) to induce leukocyte expression of 'activation-dependant' CAMs that stabilise the leukocyte-endothelial cell interaction. Finally, leukocytes migrate into the tissue by opening up tight junctions between endothelial cells. Chemotactic factors produced by cells within the inflammatory site induce directional migration of leukocytes through the endothelium and into tissues. The profile of chemoattractant and activating factors disseminating from an inflammatory site presumably regulates local endothelial and leukocyte CAM expression and so determines the composition of the infiltrating leukocyte population (Butcher 1991; Obsorn 1990; Mantovani *et al.* 1992).

Both T and B lymphocytes leaving the blood are destined to traffic through all peripheral tissues, but primarily enter either lymphoid tissues or sites of inflammation. Tissue specific expression of endothelial cell markers or 'vascular addressins' on high endothelial venules in lymphoid tissues, and the preferential expression of their ligands by specific subsets of lymphocytes, accounts for the remarkable tissue specificity of lymphocyte extravasation. Addressins for skin, and for mucosal and peripheral lymphoid tissues have been identified (Albelda and Buck 1990; Siegelman 1991). Whilst expression of many of these molecules is constitutive, in inflammatory sites lymphocyte-endothelial cell interactions appear to be dependant on activation (Osborn 1990, Butcher 1991).

1.4.3 Regulation of leukocyte recruitment in the uterus

There is considerable evidence indicating that the trafficking and behaviour of each of the leukocyte populations resident within the uterus are subject to regulation by ovarian steroid hormones. For example, macrophages and eosinophils are the largest populations in the oestrous uterus and their numbers and distribution within the endometrium appear to be regulated by steroid hormone-induced synthesis of local factors (De and Wood 1991; Lee *et al.* 1989; 1.4.2 and 1.4.4). There is increasing evidence to implicate cytokines, acting as chemotactic agents or as inducers of CAM expression in the local microvasculature, as local mediators of these dramatic cyclic changes in uterine cytology (see 1.7).

1.5 Uterine leukocyte populations

1.5.1 T and B Lymphocytes

T-lymphocytes comprise a small proportion of the lymphocytes found in the oestrous and early pregnant endometrial stroma (Head 1987; Redline and Lu 1989). A CD8 positive T-cell population with suppressive activity that is not MHC restricted accumulates in the oestrous uterus and persist through the implantation period (Clark *et al.* 1989). Following implantation, T-lymphocytes are extremely sparse in the decidua of normal mice and rats, even in the presence of local *Listeria* infection (Redline and Lu 1989; Head 1987; Redline and Lu 1988). Only a small proportion of lymphocytes from murine decidua bear cytotoxic markers (Kearns and Lala 1985). Exclusion of lymphocytes from decidual tissue may be a function of the decidual cell ECM [analogous to the mechanism underlying macrophage exclusion described by Redline *et al.* (1990)], but other factors including production of local inhibitory factors (Clark *et al.* 1985) or lack of adhesion molecules on the decidual microvasculature may also be important. Expression of the CD5 antigen by local endothelial cells has been suggested to be a mechanism which blocks T-lymphocyte extravasation into the pregnant sheep uterus (Gogolin-Ewens *et al.* 1989).

Densities of immunoglobulin (Ig)-secreting plasma cells are similar in the reproductive tract and the small intestinal tract. Ovarian steroid hormones regulate the migration of these cells into the murine reproductive tract; at pro-oestrous plasma cells are 2-3 fold increased in number and the ratio of IgA:IgG secreting cells is relatively greater than at di-oestrous (Rachman *et al.* 1983). IgA-containing plasma cells are more common in the uterine endometrium and Ig transport across the epithelium is enhanced following implantation on day 4. These changes are influenced by the embryo since they do not occur in psuedopregnant mice (Rachman *et al.* 1986). Both progesterone and oestrogen were found to increase the number of plasma cells in the uterus of ovariectomised mice, suggesting that the marked increase in endometrial plasma cells at implantation is a response to progesterone acting on an oestrogen primed uterus (Parr and Parr 1986).

T-lymphocytes in the decidua secrete soluble molecules that have immunosuppressive activity in IL-2 dependant T-cell activation assays *in vitro*, and studies in which these cells were depleted with specific mAb *in vivo* suggest that these cells may have a role in suppressing destructive maternal immune responses (Beaman and Hoversland 1988)(see 1.3.3).

Cytokines liberated by T-lymphocytes have also been implicated as trophic factors for trophoblast cells of the developing placenta (1.3.3; 1.8.1). However, experiments with two strains of genetically T-cell deficient mice have shown that T-cells are not an absolute requirement in pregnancy. T-and B-lymphocyte, NK cell deficient *scid/scid* x *bg/bg* double mutant mice have no reduction in litter size and placentas of normal weights (Croy and Chapeau 1990), whilst $\beta_2$microglobulin deficient mice with defective cytotoxic T-cell function through absence of class I MHC expression are also normally fertile (Koller *et al.* 1990). This finding does not exclude the possibility that products of lymphocytes (some of which, for example IFNγ, are extremely potent) can not impact directly or indirectly through cytokine networks on placental growth.

1.5.2 Macrophages

Tissue macrophages are long-lived cells derived from circulating monocytes. These cells are capable of phagocytosis, antigen processing, antigen presentation, and synthesis of such factors as cytokines, prostaglandins, vasoactive amines and proteolytic enzymes. Macrophages are, therefore, equipped to participate in physiological activities as diverse as defence against invading pathogens or tumor cells, recruitment of leukocytes, suppression or activation of leukocyte activity, angiogenesis and tissue remodelling (Rappolee and Werb 1989 and 1992; Papadimitriou and Ashman 1989; Leibovich and Ross 1975; Nathan 1987). Furthermore, their ability to produce enormous amounts of a specific molecule (eg. TNFα) suggests that macrophages may in some circumstances amplify signals derived from other types of cells.

The morphological and functional characteristics of tissue macrophages, including their importance in innate immunity and profile of bioactive products, are extraordinarily sensitive to local microenvironmental signals. In particular, cytokines can induce and suppress various properties reversibly, to give rise to macrophages with tissue-specific phenotypes (Gordon 1986, Papadimitriou and Ashman 1989; Mazzei *et al.* 1990; Turner at al 1991; Heidenreich *et al.* 1989; Geppert and Lipsky 1989). Extracellular matrix proteins are also potent mediators of tissue-specific influences on the profile of cytokines and other mediators released by resident macrophages (Nathan and Spom 1991).

Macrophages are normally resident in all connective and mucosal tissues, including the myometrial and endometrial stroma of the virgin and pregnant uterus (Hume *et al.* 1983; 1984; 1985; Hunt *et al.* 1985). De and Wood (1990) have used the macrophage specific mAb F4/80 to analyse the numbers of macrophages in suspensions of enzymatically digested uterine tissue, and to demonstrate their distribution in intact tissue from virgin mice. In accord with the findings of Hunt *et al.* (1985), these authors report that approximately 10% of collagenase-digested cells from oestrous uteri are F4/80 positive. However, leukocytes are released preferentially by tissue disaggregation protocols employing collagenase and this figure may be an over-estimate of the proportion in undisrupted tissue (Bulmer 1989). The numbers of leukocyte common antigen (LCA)-positive and F4/80-positive cells were similar, suggesting that macrophages account for all or nearly all of the collagenase-extractable leukocytes in the virgin uterus. Macrophages were evenly distributed through the endometrium and myometrial stroma in di-oestrous uteri, but during pro-oestrus and oestrus were found to accumulate in the stroma lying immediately subjacent to the luminal epithelium and around the endometrial glands. Ovariectomy resulted in a significant decrease in the numbers of uterine macrophages within 6 days, although whether this was the result of their death, or active migration from the tissue is unknown. Administration of oestrogen or progesterone to ovariectomised mice restored macrophage numbers and their distribution to an oestrous pattern, and oestrogen and progesterone in combination further increased macrophage levels to 13% (De and Wood, 1990). The authors concluded that ovarian steroids regulate the quantity and distribution of uterine macrophage populations, and hypothesised that factors which are chemotactic for macrophages are produced by uterine epithelial cells in response to ovarian steroids.

During the 48 h period following natural mating the proportion of F4/80 positive cells in collagenase preparations increased approximately 2.5 fold. This number declined from 27% on day 2, to 17% and 19% on days 3 and 4 respectively. After implantation macrophages increased to 35-40% on day 5 through to day 8. Numbers of F4/80 positive cells detected in sections of uterus were consistent in number with the data derived from cell suspensions for each of the first 8 days of pregnancy. They were found to be localised predominantly to areas immediately below the luminal and glandular epithelium, including within the superficial endometrium adjacent to the implanting embryo (De *et al.* 1991). Following implantation, F4/80 positive cells are found infrequently in the expanding primary decidual zone (Redline and Lu, 1989; De and Wood 1991) even in the presence of local *Listeria* infection (Redline and Lu 1988). The exclusion of macrophages from the decidua appears to be a function of the substratum of decidualised stromal cells, which blocks the adhesion, spreading and lysis of tumor cell by macrophages *in vitro* (Redline *et al.* 1990). F4/80 positive cells were found to accumulate in large numbers in the decidua basalis and the metrial gland area in the mesometrial triangle until day 12 of pregnancy. At this time, disappearance of luminal epithelium in the mesometrial uterus leads to regression of these two structures and the coincident disappearance of F4/80-positive cells. Association of F4/80-positive cells with epithelium was also found to be a feature in inter-implantation sites throughout pregnancy, and during late gestation as new luminal epithelium surrounds the fetus and placenta (De and Wood 1991).

The pattern of macrophage distribution is similar in the cycling and pre-implantation rat uterus. In the virgin rat, cells bearing monocyte/macrophage antigens are most numerous at oestrus, when they are found to accumulate predominantly in the luminal endometrium. A transient 4-fold increase in macrophages occurs within the luminal endometrium during the 12 h period following mating, and this is followed by a progressive decline to oestrous levels over the following 4-5 days under the influence of progesterone (Kachkache *et al.* 1991).

The functional status of uterine macrophages has not been studied extensively. Few cells in suspensions of cells harvested from virgin uterus but approximately 50% of the F4/80 positive cells in suspensions from pregnant uterus express class II MHC (Ia) antigen, a marker of activation (Hunt *et al.* 1985). Similar proportions of class II-positive cells were also observed in sections of pregnant uterus (Redline and Lu 1989). Isolated stromal cells with a subepithelial distribution, presumed to be macrophages, have been reported to have greatly increased IL-1 (α and β) and TNFα mRNA expression on day 1 of pregnancy (McMaster *et al.* 1992).

The versatility of the macrophage as a regulator of both immunological and non-immunological events, coupled with the extraordinary numbers of these cells in the endometrium, suggests that they may be pivotal cells in uterine processes (Hunt 1989 and 1990). The capacity of macrophages to synthesise an array of bioactive substances including cytokines, prostaglandins (PG), vasoactive amines and tissue remodelling enzymes, suggests that these cells may be active participants in the tissue remodelling that is a characteristic of uterine processes. Macrophages are thought to have important roles as accessory cells in lymphocyte activation, and may therefore play a key role in establishing and maintaining an immunological environment that is refractory to rejection of the fetal graft. For example, GM-CSF and TGFβ1 both induce macrophages to assume an 'immuno-inhibitory' phenotype. TGFβ1 and TGFβ2 are potent inhibitors of the respiratory burst

(Tsunawaki *et al.* 1988), and induce secretion of IL-1 inhibitor (Turner *et al.* 1991). GM-CSF is a less potent inhibitor of macrophage activation (Tsunawaki *et al.* 1988), and it also induces secretion of IL-1 inhibitor (Mazzei *et al.* 1990; Roux Lombard *et al.* 1989) and PGE (Heidenreich *et al.* 1989). IL-1 inhibitor and PGE are both important potentially as inhibitors of T-lymphocyte and NK cell activation processes. By contrast, exposure to IFNγ enhances the antigen-presenting capabilities of macrophages (Geppert and Lipsky 1989). Interestingly, secretion of GM-CSF by tumor cells is associated with induction of a local immunosuppressive microenvironment that permits tumor proliferation and metastasis (Tsuchiya *et al.* 1988; Sotomayor *et al.* 1991; Fu *et al.* 1991). A precedent for down-regulation of local immune responses by macrophages exists in the lung, where suppression of the pulmonary immune response to lung microbial flora appears to be a major function of the alveolar macrophage (Thepen *et al.* 1989).

However, the precise significance of the large numbers of macrophages within the uterus is not clear. Tachi (1991) has shown that carrageenan, a pharmacological suppressor of macrophage activation, blocks implantation when administered to rats on day 4 (although a non-specific cytotoxic effect was not ruled out) whilst macrophage activation with yeast glucans had no adverse effect. Studies employing the genetically CSF-1 deficient *op/op* mouse have generated data relevant to the role of macrophages in pregnancy (Pollard *et al.* 1991a). Homozygous mutant crosses (*op/op* x *op/op*) were found to be infertile consistently, whilst *op/op* females mated to heterozygote males bore litters 46% of the size of those of phenotypically normal heterozygote females. Macrophages are not detected in the uteri of virgin *op/op* mice but they were found to accumulate in the uterus during early pregnancy in numbers approaching those in the heterozygote controls on day 7 and 8. However, these macrophages exhibited an uncharacteristically rounded morphology (and hence presumably less differentiated phenotype), failed to persist and were undetectable by day 14. The finding that *op/op* females did maintain pregnancies when mated to heterozygote males can be interpreted as evidence that macrophages are beneficial, but not absolutely critical, to the success of pregnancy. However, interpretation of these data is difficult since the absence of CSF-1 in these mice is also likely to contribute to their infertility (1.6.4).

1.5.3 Dendritic cells

Antigen presentation in peripheral tissues is primarily accomplished by Ia antigen-bearing dendritic cells. Studies on the rat uterus have revealed that the virgin uterus is well endowed with Ia antigen-bearing cells which are distributed throughout the endometrial stroma, particularly in areas adjacent to glands. These cells lack non-specific esterase, are dendritic in shape and have been found to have potent antigen-presenting capacity *in vitro* (Head *et al.* 1987) Administration of oestradiol to ovariectomised animals increased the density of endometrial dendritic cells, suggesting that, like other myeloid lineages in the uterus, these cells are influenced by steroid hormone levels (Head and Gaede 1986). During early pregnancy in the rat, the density of Ia-positive cells in the endometrial stroma was found to decline, particularly in decidualised areas adjacent to the implantation site (Head and Billingham 1986). Macrophage-like cells with a dendritic morphology

and antigen-presenting capacity have also been identified in the murine decidua (Searle and Matthews 1988).

1.5.4 Neutrophils

Polymorphonuclear cells (PMNs), or granulocytes, are classified into neutrophils, eosinophils and basophils. These cells are not antigen specific and they are short-lived (2-3 days) and readily-activated phagocytic cells that comprise the immune system's first line of defence. Neutrophils are the most abundant circulating granulocyte, comprising approximately 50% of the total blood leukocytes under normal circumstances. They are normally only sparsely distributed in peripheral tissues, unless associated with an inflammatory response to a pathogenic agent, foreign body or trauma (Roitt *et al.* 1985; Male *et al.* 1987).

De *et al.* (1991) have quantified PMNs in smears of uterine cells obtained by collagenase digestion. They comprised only 1% of uterine cells in cycling mice but this proportion increased to 36% on the first day of pregnancy. This concurs with earlier reports that a transient accumulation of PMNs occurs in the murine uterus within hours after mating (Austin 1960; Ball and Mitchinson 1977). These cells are recruited from the blood, migrate into the endometrium and pass through the luminal epithelium into the uterine lumen. Here, they appear to have a role in phagocytosis of sperm and seminal debris. Only a fraction of sperm in an ejaculate escape coating with antibody and phagocytosis within the female reproductive tract. This has led to hypotheses that leukocytes can discriminate between sperm and dispose of some selectively, thereby permitting only a fraction to compete for fertilisation of the oocyte (Cohen 1984). Granulocytes may account for a proportion of the endometrial leukocytes which exhibit transient upregulation of TNFα and IL-1 (α and β) mRNA synthesis following mating (McMaster *et al.* 1992).

Neutrophil numbers are reduced to 3% of total uterine cells by day 3 of pregnancy and remain low for the remainder of gestation (De *et al.* 1991; De and Wood 1991). Finn and Pope (1991) have reported histological evidence for a short-lived accumulation of neutrophils in the endometrial stroma at the implantation site (but not areas between implantation sites) early on day 5 of pregnancy, apparently in response to the decidualising stimulus. This is consistent with the suggestion that the evolutionary origin of the uterine reaction to the implanting blastocyst may be the inflammatory reaction to a foreign body (Finn 1986). Activated neutrophils produce several bioactive substances which could affect the progress of decidualisation but at present no direct evidence is available for such a role. Neutrophils are not concentrated selectively in the endometrium underlying implantation sites in the rat (Rogers *et al.* 1992), suggesting that they may not have an important role in implantation in all species.

1.5.5 Eosinophils

Eosinophilic leukocytes comprise less than 5% of total blood leukocytes. During the oestrous cycle in rats, there is an approximately 50-fold increase in the numbers of eosinophils in the uterus at oestrus. Eosinophils are absent from the uterus of immature and ovariectomised rats,

but appear in large numbers after oestrogen administration (Ross and Klebanoff 1966; Lee *et al.* 1989). Tchernitchin *et al.* (1974) found that after exposure to oestrogen for 12-24 h the uterus in immature rats contains as many eosinophils as are found normally in the entire peripheral circulation. In contrast, progesterone decreases the oestrogen-induced accumulation of eosinophils in the uterus of the ovariectomised animal (Ross and Klebanoff 1966). Oestrogen induces (but progesterone inhibits) the synthesis of a 20 kD protein that is chemotactic for eosinophils, and so may regulate their traffic in the rat endometrium (Lee *et al.* 1989, Leiva *et al.* 1991). The dynamics of eosinophils are similar in the mouse uterus (Paul *et al.* 1967), and are not dependant on mast cell activity (Terada *et al.* 1985). Eosinophil numbers during pregnancy have not been characterised, apart from a note in Ross and Klebanoff (1966) that they are not found in uterine tissues during late gestation in the rat.

The striking cyclic variation in eosinophil numbers suggests that these cells participate in the profound changes in uterine tissue mass and morphology that are characteristic of the oestrous cycle. Although eosinophils are phagocytic cells, Ross and Klebanoff remark that they found no evidence for phagocytosis within the uterus; rather the eosinophils themselves undergo lysis late in oestrus and both whole cells and cellular remnants are phagocytosed by resident macrophages (Ross and Klebanoff 1966). Oestrogen can induce eosinophils to degranulate and release potent granule enzymes (Tchernitchin *et al.* 1985). Klebanoff (1965) has shown that one of these, eosinophil-derived peroxidase, can catalyse the inactivation of oestrogen in the presence of hydrogen peroxide (H_2O_2), suggesting that one function of eosinophils may be to regulate oestrogen metabolism. Activated eosinophils are also a major source of PAF (Lee *et al.* 1984), and it is of interest that an increase in the uterine and circulating levels of this mediator is one of the earliest responses to conception (O'Neill 1991). Eosinophil derived PAF has the potential to act in an autocrine manner, and thus may act to amplify signals for eosinophil activation and degranulation (Kroegel *et al.* 1988 and 1989).

1.5.6 Natural killer cells

High levels of natural killer (NK) cell activity are found in early decidua (day 6), but this activity declines rapidly during maturation of the placenta (Gambel *et al.* 1985). At about this time a morphologically distinct, bone marrow-derived population of cells with some NK cell characteristics (GMG cells), appears and within days becomes a major constituent in the decidua basalis (Stewart and Peel 1980). It has been demonstrated recently that GMG cells and NK cells represent distinct stages of differentiation in the same cell lineage. Parr *et al.* (1991) have shown by immunohistochemical staining with mAb against the NK cell specific marker LGL-1 that there are substantial numbers of NK cells in the mouse uterus throughout the oestrous cycle and during early pregnancy. No substantial influx of these cells was found to be associated with either oestrus or mating. NK cells were not found in the primary decidual zone but became concentrated in the mesometrial decidua of the uterus from day 6 of gestation. Cells in this area, but not in inter-

implantation sites, became enlarged and expressed perforin (implicated as a mediator in cellular cytotoxicity) and serine esterases as pregnancy progressed. By day 12-14 of gestation the zone of activated presumptive NK cells was found to have shifted to the metrial gland area in the uterine periphery. Factors produced locally at implantation sites are implicated in the activation process. These authors (Parr *et al.* 1991) excluded the possibility that IL-2 and IFNγ may function in this role, on the basis that these factors were undetectable in the mouse uterus. However, there is a report that human trophoblast cells can express IL-2 mRNA (Boehm *et al.* 1989), which if translated could function to limit placental growth *via* an NK-cell mediated negative feed-back mechanism.

The functions of uterine natural killer cells remain unknown. Granulated metrial gland (GMG) cells cultured out of decidual explants can kill freshly isolated trophoblast cells selectively (Stewart and Mukhtar 1988), and when they have been activated with IL-2 they can become cytolytic to NK target cell lines (Linnemeyer and Pollack 1991). These observations suggest that GMG cells may have a role in surveillance against aberrant or overly aggressive trophoblast proliferation, and in support of this there is a histological correlation between fetal resorption and the appearance in the vicinity of cells bearing NK markers (Gendron and Baines 1988). Activators of NK cells such as poly IC or IL-2 cause fetal resorption when they are administered *in vivo* (Lala *et al.* 1990; Kinsky *et al.* 1990), whereas treatment with anti-asialo-GM-1 (NK cell specific) antibody reduces the rate of fetal resorption (deFougerolles *et al.* 1987). Interestingly, a physiological role for PGE_2 in suppressing decidual NK cell activity has been suggested by the finding that the abortifacient effects of indomethacin are associated with an increase in decidual asialo-GM-1 positive cells with lytic activity towards trophoblast cells (Lala *et al.* 1990). Furthermore, GMG cells have been shown to produce transcripts and/or bioactivity *in vitro* for cytokines, including CSF-1, IL-1 and leukaemia inhibitory factor (LIF) (Croy *et al.* 1991).

1.5.7 Other

Small, granulated cells that do not express either T-cell or myeloid markers appear in the decidua following implantation. Their appearance seems to be in response to hormonal as well as trophoblast-derived signals (Clark *et al.* 1986). The ontogeny and origin of these cells remains unclear, but they appear to have a role in maintaining an immunosuppressive uterine environment through the secretion of a TGFβ2-like molecule (Clark *et al.* 1988). Insufficient activity of these cells is correlated with fetal resorption (1.3.3).

Numbers of uterine mast cells also fluctuate during the oestrous cycle and during early pregnancy. Their density peaks during the attachment phase of implantation before declining, apparently in response to a factor of blastocyst origin. An embryo-derived factor causes the release of histamine from rat uterine mast cells and this release is enhanced by oestrogen (Cocchiara *et al.* 1992). These findings, together with the data reviewed in (1.2.3c), suggest that mast cells may have a role in the process of decidualisation.

1.6 Cytokines

One of the most exciting recent developments in reproductive biology has been the increasing recognition of the role of polypeptide growth factors, including lymphohemopoietic cytokines, in intercellular communication networks in reproductive tissues. Of special relevance has been the finding that cytokines are synthesised by, and can act upon, cells of non-hemopoietic as well as hemopoietic lineages, including trophoblast cells of the conceptus and placenta. Cytokines are implicated both directly and indirectly (through the actions of their target cells) as mediators both of remodelling processes and of immune processes within the pre-implantation uterus. The following overview will highlight the features common to the cytokine family that confer their suitability as molecular mediators of reproductive processes. The individual cytokines that appear to be important in the murine uterus will then be discussed in some detail.

1.6.1 Cytokines: overview

Polypeptide growth and differentiation factors, collectively termed cytokines, have emerged as important regulators of a wide variety of physiologic and pathophysiologic processes. Nathan and Sporn (1991) define a cytokine as "a soluble (glyco)protein ...released by living cells of the host, which acts nonenzymatically in picomolar to nanomolar concentrations to regulate host cell function". Cytokines thus constitute a further class of soluble intercellular signalling molecules, alongside neurotransmitters, endocrine hormones, and autocoids. They have relatively low molecular weights, with a proteinaceous core of about 15 kD to which is linked a similar weight of carbohydrate, often in heterogeneous glycosylated forms. Apart from CSF-1 and TNFα, these proteins exist as monomers and experiments with recombinant material shows that the carbohydrate moieties are not required for activity. However, modification of the carbohydrate may promote degradation of the cytokine.

Although most cytokines were described originally on the basis of their production by and/or their effects on lymphohemopoietic cells, [and indeed almost all of them are implicated as regulators of hemopoiesis (Metcalf 1989)], it is now clear that they mediate communication between cells of diverse lineages. All the cytokines so far described are highly pleiotrophic, acting on multiple, often unrelated cell types to elicit extraordinarily diverse and sometimes even seemingly opposing effects on different targets. Almost all data relating to cytokine actions have been generated *in vitro* and it is a current challenge to determine how closely these findings apply *in vivo*.

Cytokines have a number of properties which help to overcome the problems inherent in a system composed of relatively few factors which share so many targets and functions. Cytokines are made and secreted at very low levels, and with few exceptions (eg. IL-1, IL-6 and TNFα) they are not detected easily in the circulation, unlike endocrine hormones. Cytokines in general act at short range, having either paracrine, autocrine or juxtacrine effects. The production of cytokines is usually under tight regulatory control and they are only secreted by most cells in response to an activation signal. The potency of cytokines is conferred by the ability of only very few molecules to

elicit a response; receptors are often expressed at only a few hundred per cell (Nicola 1987; D'Andrea *et al.* 1990). The finding that combinations of cytokines often act either synergistically or antagonistically has given rise to the concept that they act *in vivo* not as single entities but in a context defined by local levels of other cytokines and environmental factors. Sporn and Roberts (1988) have provided a useful analogy whereby cytokines can be viewed as 'symbols in an alphabet'. Target cells are then seen as responding to 'words', as opposed to individual symbols of the cytokine alphabet. Natural inhibitors including non-signal transducing antagonists [eg the IL-1 inhibitor (Seckinger *et al.* 1987; Roux Lombard *et al.* 1989)], soluble receptors (see 1.6.2) and autoantibodies (Arend *et al.* 1991), are also likely to influence cytokine activities. Cytokines are usually synthesised and secreted as soluble molecules, but differential splicing of mRNA transcript can also give rise to membrane associated CSF-1 (Rettenmier *et al.* 1987). Variants of GM-CSF and LIF that become incorporated into the ECM have also been described (Roberts *et al.* 1988; Rathjen *et al.* 1990). Immobilisation at the site of production may serve to restrict the sphere of influence of these factors. Nathan and Sporn (1991) highlight the importance of the ECM, which is viewed as a repository of information reflecting a cell's metabolic history, in modifying the production of and response to cytokines. Temporal or developmental regulation of the cellular receptors, or of the intracellular signalling molecules mediating cytokine action, may provide controls on the specificity of cytokines.

1.6.2 Cytokine receptors: overview

There is in general little sequence homology between cytokines at either the amino acid or the genomic levels, suggesting that they have evolved independently. In contrast, analysis of their receptors has revealed several receptor superfamilies (D'Andrea *et al.* 1990; Gillis 1991; Miyajima *et al.* 1992). 'Cytokine' receptors are classified into three distinct families (Miyajima *et al.* 1992), all of which are integral membrane proteins that lack tyrosine kinase activity. They include the receptors for all of the known lymphohemopoietic cytokines apart from CSF-1 receptor and c-kit, which are members of the 'growth factor' receptor family. The receptors for interleukins 2 through 7, for GM-CSF and granulocyte-CSF (G-CSF) and for erythropoietin (epo), comprise the type-1 cytokine receptors. For IL-3, IL-5, IL-6, LIF and GM-CSF, the receptor binds ligand specifically but at relatively low affinity and cannot transduce signal. In hemopoietic cells, functional receptors for these cytokines are heterodimeric combinations of the specific (α-) subunits with 'adaptor' or 'affinity converter' (β-) subunits, which bind cytokine with high affinity. Formation of a complex composed of ligand, α–chain and β-subunit facilitates signal transduction (Kitamura *et al.* 1991; Miyajima *et al.* 1992). The β-subunit has a longer cytoplasmic domain than the α-subunit, and it is proposed that it is this molecule that transmits signal, although it is not clear whether this is mediated through its association with additional proteins. The relatively recent finding that β-subunits can associate into complexes with more than one type of α-subunit and different ligands suggests that different cytokines can elicit the same response by triggering a common intracellular

signalling pathway (Nicola and Metcalf 1991; Vairo and Hamilton 1991). Competition for β-subunit would also explain the hierarchal receptor 'down-modulation' that cytokines can exert over each other.

There is evidence that some α-subunits can be elaborated as soluble molecules (Raines *et al.* 1991; Downing *et al.* 1989; Takaki *et al.* 1990). These diffusible receptors can act as cytokine inhibitors by binding factor in the soluble phase (Raines *et al.* 1991), or they can participate in formation of signal-transducing receptor-ligand complexes in the vicinity of cells expressing the β-subunit (Hibi *et al.* 1990). This provides a potential mechanism whereby cytokines can act upon cells that do not express specific (α-subunit) receptors. Indeed, the soluble IL-6 receptor/ IL-6 complex has been shown to bind to the β-subunit common to the high affinity IL-6 and LIF receptors (designated gp 130) on embryonic stem cells (which are IL-6 receptor-negative) and induce their proliferation (Kishimoto *et al.* 1992).

1.6.3 Cytokines and cellular differentiation

It is difficult to conceive of how a single cytokine can exert different or even opposite effects on different target cells, particularly when each target cell expresses receptors for, and can have the same response to, a number of factors. Lineage commitment in hemopoiesis (Nicola and Metcalf 1991; Miyajima *et al.* 1992) presents a model for ligand cross-reactivity and biological redundancy in the action of cytokines. The 'selection' theory proposes that hemopoietic progenitor cells that bear a particular cytokine receptor will proliferate in response to a given cytokine. According to this hypothesis, the commitment of cells to a particular developmental pathway occurs through irreversible shutdown of gene programs that are no longer of relevance through an intrinsic stochastic process that is cytokine independent. In this model, cytokines function merely to activate committed cells to execute their existing programs. In contrast, the 'instructive' theory proposes that cell differentiation follows inductive or instructive signals from cytokines and other external stimuli. There is only limited data available in support of either model. However, the first model allows for greater flexibility in the response to cytokines that associate with the same β-subunit 'adaptor' molecules and share common intracellular signalling pathways (Nicola and Metcalf 1991; Vairo and Hamilton 1991).

Miyajima *et al.* (1992) have proposed that the cytokine receptor family has evolved in relatively recent times, along with the evolution of cellular and humoral immunity. In contrast, growth factor receptors are found in invertebrates. However, it is interesting that embryonic stem cells express both the α- and β-subunits of the LIF receptor (specific receptor and gp 130) and that their commitment to differentiate is extremely sensitive to LIF action (Williams *et al.* 1988). This, together with the finding that LIF deficient animals cannot support early embryogenesis (Stewart *et al.* 1992; 1.7) shows that critical roles for cytokine receptors are not limited to lymphohemopoietic cells, and suggests that the conceptus and its products may have adopted the use of cytokines from cells of the immune system.

1.7 Cytokine synthesis in the pre- and peri-implantation uterus

1.7.1 GM-CSF

a. Biochemistry and Genetics

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a member of the colony-stimulating-factor (CSF) family of cytokines. These were identified initially by their ability to stimulate the proliferation and differentiation of primitive hemopoietic precursor cells into macroscopic colonies in semisolid culture media (Bradley and Metcalf 1966).

The gene encoding murine GM-CSF was cloned from complementary deoxyribonucleic acid (cDNA) prepared from the T-lymphocyte cell line EL-4 by Gough *et al.* (1985), and it has been expressed in both prokaryote and eukaryote systems. The mouse and human genes are 60% homologous at the cDNA level, and are represented as single copies on chromosome 5 and 11 respectively. Both genes are 2.5 kb in length and each contains three introns and four exons (Gough *et al.* 1985, Miyatake *et al.* 1985). Native GM-CSF is synthesised as a 23-29,000 kD heterogeneously glycosylated protein which has a 16,000 kD proteinaceous core and two intrachain disulphide bonds that are important for activity (Burgess *et al.* 1977; Burgess and Nice, 1985; Sparrow *et al.* 1985). The mouse and human proteins are 54% homologous in amino acid sequence and there is no cross-species bioactivity. Interestingly, nonglycosylated recombinant GM-CSF generated in bacteria is equally or more active (at least in hemopoietic cells) than the glycosylated native or yeast-derived recombinant forms (Metcalf *et al.* 1986; Miyajima *et al.* 1986).

Structure/ function studies for murine GM-CSF have been performed by *in vitro* mutagenesis and critical residues on which binding and function of mouse and human proteins are dependant have been pin-pointed (Gough *et al.* 1987; Lopez *et al.* 1992). A human GM-CSF analogue with a single amino acid mutation that confers binding but does not transduce signal has been identified, and will be of use as an experiment antagonist (Lopez *et al.* 1992).

In vitro studies of GM-CSF synthesis by various cell types have led to the view that GM-CSF is released as a diffusible protein into intercellular spaces. However it is not clear whether this is an accurate reflection of the *in vivo* situation. GM-CSF is one of a number of cytokines that is now known to be sequestered and immobilised in the ECM. Although not detectable in the supernatant of bone marrow stromal cell cultures, GM-CSF can be eluted from the solid phase of cultures (Gordon *et al.* 1987) where it is associated, in a biologically active form, with the major ECM component heparan sulphate (Roberts *et al.* 1988).

b. Cellular Sources

Although originally identified as a product of activated T-lymphocytes, GM-CSF is now known to be a product of a diverse variety of normal and transformed cell lineages. Non-transformed rodent sources include macrophages (Thorens *et al.* 1987), T-lymphocytes (Kelso and Metcalf 1985), mast cells (Wodnar-Filipowicz *et al.* 1989), osteoblasts (Horowitz *et al.* 1989), fibroblasts (Koury and Pragnell 1982), endothelial cells (Malone *et al.* 1988), astrocytes (Malipiero

et al. 1990), and renal mesangial cells (Budde *et al.* 1989). In the human, the list of cellular sources includes all of the above, plus NK cells and B-lymphocytes (Gasson 1991; Ruef and Coleman 1990; Baldwin 1992). GM-CSF derived from different cell types appears to have identical physical and biological characteristics apart from variation in the degree to which the molecule is glycosylated (Ruef and Coleman 1990). Until relatively recently, epithelial cells were notable for their absence from the list of cellular sources, but GM-CSF synthesis from keratinocytes (Koury *et al.* 1983; Kupper *et al.* 1988; Chodakewitz *et al.* 1988), thymus epithelium (Denning *et al.* 1988; Galy *et al.* 1990), and rat (Smith *et al.* 1990) and human tracheal epithelium (Ohtoshi *et al.* 1991) are now described.

Uterine tissues have been recognised for some time as potent sources of CSF (Bradley *et al.* 1971; Rosendaal 1975). GM-CSF is a component of CSF bioactivity in supernatants from cultures of human placenta (Burgess *et al.* 1977) and murine (Wegmann *et al.* 1989) and rat decidual cells (Shiverick *et al.* 1990). GM-CSF mRNA transcripts of conventional and unique sizes have been detected in murine placental tissues (Crainie *et al.* 1989). It has been proposed that T-lymphocytes responding to fetal alloantigens are the origin of decidual GM-CSF (Wegmann 1988; Wegmann *et al.* 1989; Athanassakis *et al.* 1987). *In situ* localisation of GM-CSF mRNA transcripts in murine placentae show that GM-CSF is synthesised within small round cells (thought to be leukocytes) and endothelial cells within the decidua, as well as trophoblast-like cells within the spongiotrophoblast zone of the placenta (Kanzaki *et al.* 1991).

c. *Regulation of Synthesis*

Several agents have been identified that alter the synthesis of GM-CSF. Most notable are IL-1, TNFα and bacterial lipopolysaccharide (LPS), all of which induce synthesis of the cytokine in macrophages, T-lymphocytes (IL-1 and TNFα) fibroblasts, and endothelial cells. In T-cells, non-specific mitogens including lectins and phorbol esters stimulate synthesis of GM-CSF and a range of endogenous and exogenous bioactive agents (including prostaglandins, bacteria and viruses and various drugs) can also affect production (Ruef and Coleman 1990; Gasson 1991; Baldwin 1992). IFNγ antagonises the synthesis of GM-CSF induced in fibroblast-like synovial cells by TNFα (Alvaro-Gracia *et al.* 1990). It also enhances synthesis of GM-CSF by T-lymphocytes (Lu *et al.* 1988; Piacibello *et al.* 1986), while in macrophages it can be either stimulatory or inhibitory, depending on the dose (Piacibello 1985; Schlick *et al.* 1985; Thorens *et al.* 1987). Human blood monocytes release GM-CSF after crosslinking of type 1 receptors for $F_C\gamma$ with mouse IgG_{2a} (Herrmann *et al.* 1992).

In murine skin, GM-CSF synthesis is enhanced following topical exposure to inflammatory diterpene esters (Koury *et al.* 1983), and airway epithelial cells cultured from inflamed nasal polyps or from allergic rhinitis tissue synthesise significantly more GM-CSF than cells from normal tissue (Ohtoshi *et al.* 1991). In rats, GM-CSF expression in tracheal epithelial cells is enhanced by LPS (Smith *et al.* 1990).

In vitro culture can lead to GM-CSF expression in macrophages. Fetal calf serum (FCS) is known to have inflammatory properties *in vivo* and it has been found to contain a powerful inducing agent (other than LPS) for GM-CSF synthesis. The potential influence of ECM components in FCS (Nathan and Sporn 1991) was demonstrated by the ability of fibronectin to stimulate GM-CSF synthesis (Thorens *et al.* 1987).

Regulation of GM-CSF gene expression involves transcriptional and post-transcriptional control mechanisms. The GM-CSF gene is transcribed constitutively at low levels in monocytes, endothelial cells and fibroblasts, probably because the mRNA has a short half-life (as do mRNAs for most cytokines) and does not accumulate. Activation of these cell types by a variety of stimuli leads to transient mRNA accumulation, through increased transcription and by enhanced mRNA stabilisation (Thorens *et al.* 1987; Koeffler *et al.* 1988; Seelentag *et al.* 1987). Stability is controlled through AUUU sequences in the 3' untranslated region (Shaw and Kamen 1986; Iwai *et al.* 1991). Transcriptional regulation of the GM-CSF gene is important in T-lymphocytes, which only transcribe the GM-CSF gene after they are activated. In these cells regulation is mediated by both *cis* and *trans*-acting regulatory elements which can have positive or negative effects (Nimer *et al.* 1989; Shannon *et al.* 1990). Transcription factors, including nuclear proteins induced by TNFα and phorbol ester, bind to consensus sequences in the GM-CSF promoter region that are homologous to promoter region sequences in IL-3 and other cytokine genes (Shannon *et al.* 1990).

d. Actions

GM-CSF derives its name from its ability to stimulate the differentiation and proliferation of myeloid progenitors to produce colonies containing granulocytes and macrophages (Metcalf 1985; Metcalf *et al.* 1986; Begley *et al.* 1988). The molecular cloning and expression of the GM-CSF gene in bacteria and yeast has since permitted the extensive study of its actions both *in vitro* and *in vivo*. The biological effects of this molecule can be divided into (1) induction of growth of several different cell lineages, and (2) alterations in the function of relatively differentiated cells. The effects of GM-CSF are concentration dependant: induction of proliferation by target cells requires higher concentrations of the factor than does either differentiation or activation (Metcalf *et al.* 1986).

Mature macrophages appear to be dependant on CSF for survival, since its withdrawal from *in vitro* cultures of hemopoietic cells leads to cell death by apoptosis (Williams *et al.* 1990). The proliferation of monocyte precursors and of elicited peritoneal macrophages is enhanced in the presence of GM-CSF, and GM-CSF is implicated in promoting the differentiation of blood-borne monocytes into mature macrophages in the peripheral tissues (Chen *et al.* 1988a; 1988b; Chodakewitz *et al.* 1988). The maturation of other peripheral antigen presenting cells, including lymph-borne rat dendritic cells (MacPherson 1989; MacPherson *et al.* 1989) and Langerhan's cells in murine skin (Heufler *et al.* 1988) is also mediated by GM-CSF. Markers expressed characteristically by mature macrophages are induced by GM-CSF. These include expression of class II MHC and enhancement of antigen presentation (Chantry *et al.* 1990, Coleman *et al.* 1988,

Falk *et al.* 1988, Fischer *et al.* 1988). GM-CSF activates macrophages to increase release of cytokines including TNFα (Cannistra *et al.* 1988; Hart *et al.* 1988), interferon (Wing *et al.* 1989), CSF-1 (Horiguchi *et al.* 1987), and granulocyte-CSF (G-CSF)(Oster *et al.* 1989), and it also increases synthesis of enzymes and factors (such as plasminogen activator) implicated in tissue remodelling (Hamilton *et al.* 1991). Expression of receptor molecules for CSF-1 by human myeloid precursors is downregulated in response to GM-CSF (Gliniak and Rohrschneider 1990), although in tissue macrophages a transient decrease in CSF-1 receptor numbers was followed by restoration (and later upregulation) of their expression (Chen *et al.* 1988a). Human blood-derived mononuclear phagocytes accumulate IL-1β mRNA and secrete IL-1β after exposure to GM-CSF (Oster *et al.* 1992).

GM-CSF and IFNγ appear to induce opposite phenotypes in macrophages. Whilst IFNγ enhances the antigen-presenting capabilities of macrophages (Geppert and Lipsky 1989), GM-CSF can induce macrophages to synthesise 'immunosuppressive' molecules, which include IL-1 inhibitor (Mazzei *et al.* 1990; Roux Lombard *et al.* 1989) and PGE_2 (Heidenreich *et al.* 1989). These molecules may act to downregulate local T-lymphocyte activation; IL-1 inhibitor by blocking the binding of IL-1 at the receptor level, and PGE_2 by inhibiting cytotoxic T-lymphocyte generation (Hunt *et al.* 1984, Tawfik *et al.* 1986a and 1986b) through interfering with the IL-2 dependant activation sequence (Lala *et al.* 1988 and 1990). GM-CSF-induced PGE expression also mediates an autocrine reduction in IL-2 and IFNγ receptor expression, and this may represent a negative feedback mechanism for control of monocyte activation (Hancock *et al.* 1988b; Fischer *et al.* 1990). Constitutive secretion of GM-CSF by tumors in mice has been associated with a metastatic phenotype, and a mechanism has been suggested whereby this affects local monocyte/macrophages and thus local T-lymphocytes. The effect may be to allow expansion of a T-suppressor population, which inhibits local anti-tumor defences (Tsuchiya *et al.* 1988). GM-CSF secretion by mammary tumors has been found to downregulate the tumoricidal capability of elicited peritoneal macrophages obtained from tumor bearing mice (Sotomayor *et al.* 1991). Administration of rGM-CSF to normal mice caused similar alterations in systemic immune functions, including the induction of an immunosuppressive macrophage phenotype (mediated at least partially by PGE_2 release), suppression of T-lymphocyte responses to mitogens, and reduction of the cytotoxicity of T-lymphocytes and NK cells (Fu *et al.* 1991).

The differentiation and survival of dendritic cells in mucosal tissues may also be regulated by GM-CSF. GM-CSF has been reported to support the outgrowth of mature dendritic cells from progenitor cells in peripheral blood or bone marrow (Inaba *et al.* 1992; Caux *et al.* 1992), to enhance the accessory activity of lymph node dendritic cells (Bowers *et al.* 1990), and to increase the survival of lymph-borne dendritic cells *in vitro* (MacPherson 1989).

The survival and function of neutrophils and eosinophils is also dependant on GM-CSF (Lopez *et al.* 1986). GM-CSF has indirect or 'priming effects' on neutrophils, whereby it enhances the response of neutrophils to secondary stimuli, including the bacterial peptide formyl-met-leu-phe (fMLP), complement component C5a and leukotriene B4 (LTB4). Neutrophils activated by GM-CSF secrete IL-1 (Lindemann *et al.* 1988). GM-CSF also primes neutrophils to synthesise

leukotrienes and PAF (DiPersio *et al.* 1988; DeNichilo *et al.* 1991; Stewart *et al.* 1991), and enhances their oxidative metabolism (Lopez *et al.* 1986; Nathan 1989).

GM-CSF has been reported to stimulate the synthesis of leukotrienes and PAF by human eosinophils (Silberstein *et al.* 1986; Lee *et al.* 1984), and it also upregulates their expression of IL-5 receptors (Chihara et al. 1990) and CD23, CD25 and CD4 (Chihara *et al.* 1992). In the case of basophils, exposure to GM-CSF induces histamine release (Hirai *et al.* 1988; Haak Frendscho *et al.* 1988).

GM-CSF appears to enhance the recruitment of monocytes and granulocytes into inflammatory sites, possibly through actions at the endothelial cell/ leukocyte interface. It may also act as a chemotactic factor within tissues. Expression of CAMs by human neutrophils and monocytes is modulated by GM-CSF. The possible importance of this factor in trapping these cells within inflammatory sites *in vivo* is suggested by findings that exposure to GM-CSF *in vitro* reduces expression of the MEL-14 homologue LAM-1, whereas CD18/ CD11b (Mac-1) expression is enhanced (Arnaout *et al.* 1986; Kishimoto *et al.* 1989; Gamble *et al.* 1990; Griffen *et al.* 1990; Fu *et al.* 1992). GM-CSF has also been found to stimulate a CD11/ CD18-mediated increase in the adhesion of human peripheral blood monocytes to umbilical vein endothelial cells (Elliott *et al.* 1990; Gamble *et al.* 1989) and to mesangial cells (Brady *et al.* 1992). GM-CSF modulates the adhesiveness of human neutrophils and their sensitivity to chemotactic factors by mobilising cytoplasmic granules containing both CD11/ CD18 and formyl-peptide receptors (Weisbart *et al.* 1986 and 1987; English and Graves 1992). GM-CSF itself is chemotactic for both monocytes and polymorphonuclear cells; this is demonstrated in experiments in which these cells migrate towards an increasing concentration of factor (Wang *et al.* 1987), or by the inhibition of neutrophil migration in the presence of a uniform concentration of GM-CSF (Gasson *et al.* 1984).

Several aspects of the responses of polymorphonuclear leukocytes and macrophages to GM-CSF in inflammatory sites are important in relation to host defence against pathogenic agents and tumor cells. GM-CSF upregulates the phagocytic activity (Fleischmann *et al.* 1986; Coleman *et al.* 1988) and microbicidal activity (Villalta and Kierszenbaum 1986; Handman and Burgess 1979) of neutrophils and macrophages. Tumor cells are among the variety of target cells killed more readily by macrophages (Grabstein *et al.* 1986; Cannistra *et al.* 1988), neutrophils (Kushner and Cheung 1989; Lopez *et al.* 1983) and eosinophils (Lopez *et al.* 1986; Silberstein *et al.* 1986) that have been exposed to GM-CSF. Neutrophil phagocytosis and cytotoxicity are enhanced when target cells or particles are coated with immunoglobulin. GM-CSF appears to enhance these processes in neutrophils by upregulating in a period of minutes the number or affinity of receptors expressed by neutrophils for the Fc component of IgA (Weisbart *et al.* 1988) or IgG (Buckle *et al.* 1990).

Non-hemopoietic cells also respond to GM-CSF. GM-CSF stimulates proliferation *in vitro* of human nonhemopoietic tumor cells, including two osteogenic sarcoma cell lines, a breast carcinoma cell line, and an SV40 transformed marrow stromal cell line (Dedhar *et al.* 1988), human colon adenocarcinoma cell lines (Berdel *et al.* 1989; 1990), SCCL (small carcinoma of the lung) cells (Baldwin *et al.* 1989), and JAR choriocarcinoma cells (DiPersio *et al.* 1990). There has been considerable interest in determining whether GM-CSF can exert similar effects on the normal

counterparts of these cells. GM-CSF stimulates the proliferation of normal human osteoblasts but antagonises their synthesis of osteocalcin and alkaline phosphatase (Evans *et al.* 1989). Baldwin *et al.* (1992) have reported that primary rat oligodendrocytes proliferate in response to GM-CSF, as do human keratinocytes (Hancock *et al.* 1988a). Bussolino *et al.* (1989a and 1989b) have shown that exposure of human umbilical vein endothelial cells to GM-CSF induces rapid activation of the Na^+/H^+ exchanger followed by increased directional migration and proliferation. Although Yong *et al.* (1991) report contradictory data, this may be the result of different culture conditions which may induce activation-associated autocrine responsiveness to GM-CSF (Baldwin 1992).

Interpretation of the physiological implications of the results of *in vitro* experiments is difficult, primarily because *in vitro* experiments often fail to accommodate the multifactorial role of the local *in vivo* microenvironment. In particular, the role of the ECM and of other cytokines on the response to a cytokine signal are difficult to reproduce. However, many of the biological activities predicted by *in vitro* experiments have been substantiated *in vivo*. Systemic administration of GM-CSF to mice causes the numbers of circulating macrophages and neutrophils to increase (Metcalf *et al.* 1987) and it also enhances Fc receptor and class II MHC expression, IL-1 release and phagocytic activity by macrophages (Morrissey *et al.* 1988; Elliott *et al.* 1991). Transgenic mice that over-express the murine GM-CSF gene have elevated levels of GM-CSF in the blood and in many other body fluids. Large, activated macrophages accumulate in the eye, peritoneal and pleural cavities and within striated muscle tissue (Lang *et al.* 1987). In humans, administration of GM-CSF leads to a prominent and dose-dependant increase in the number and survival of hemopoietic progenitor cells, in mature circulating neutrophils and eosinophils, and monocytes to a lesser degree. The functional capabilities of cells, in particular the priming of neutrophils, the synthesis of IL-1 by monocytes and the cytotoxicity of monocytes are also somewhat upregulated in GM-CSF treated patients (Baldwin 1992).

e. Receptor and Signal Transduction

The bioactivity of GM-CSF is mediated following binding of the protein to its membrane-bound receptor (Nicola *et al.* 1988). In hemopoietic cells, the human and mouse GM-CSF receptors are both heterodimers composed of subunits belonging to the cytokine receptor superfamily (Gearing *et al.* 1989; Hayashida *et al.* 1990; Park *et al.* 1992; Gorman *et al.* 1990). The α-subunit of the mouse GM-CSF receptor is a 387 amino acid protein which binds GM-CSF with low affinity, and which shares 35% homology with the corresponding subunit of the human GM-CSF receptor (Park *et al.* 1992). The β-subunit, designated AIC2B, does not bind GM-CSF by itself, but together with the α-subunit it forms a high affinity ligand binding complex. Association between the α- and β-receptor molecules is necessary for receptor activation and growth signal transduction, at least in hemopoietic cells (Kitamura *et al.* 1991; Park *et al.* 1992). However studies with mutant GM-CSF molecules show that this activation is not dependant upon high affinity binding but is a consequence of a ligand-induced interaction between the two receptor molecules (Shanafelt and Kastelein 1992). The mechanism of signal transduction remains unclear, since neither the human

nor the mouse α-or β-subunit molecules have obvious signal transducing elements such as tyrosine kinase or G-protein interaction domains. Despite this, biochemical events including activation of microtubule-associated protein 2 (MAP-2) kinase (Raines *et al.* 1991), and phosphorylation of multiple intracellular substrates (Duronio *et al.* 1992), follow GM-CSF binding. Critical residues for inducing phosphorylation or cell proliferation have been identified in the cytoplasmic domain of the human β-subunit (Sakamaki *et al.* 1992).

AIC2B is also the signal transducing subunit for the IL-3 and IL-5 receptors, and a closely related molecule, AIC2A, can also act as a β-subunit of the IL-3 receptor (Park *et al.* 1992; Hara and Miyajima 1992). This explains the ability of IL-3 to compete for GM-CSF binding and to dampen GM-CSF responses (Walker *et al.* 1985; Nicola 1987).

In the human, alternatively spliced mRNAs give rise to at least another two isoforms of the α-subunit of the GM-CSF receptor in addition to the (α1)-subunit described above. The functional significance of the existence of different isoforms is not understood, but it is possible that their differential expression is lineage-dependant and/ or plays a role in determining the nature of the cellular response. One of the additional isoforms, designated α2, is expressed in human bone marrow and placenta and has a longer cytoplasmic domain than the α1 molecule (Crosier *et al.* 1992). This domain is rich in serine residues, a feature that is typical of regions critical for signal transduction for other receptors of the hemopoietin receptor superfamily. The α2 GM-CSF receptor was functionally active in a murine myeloid cell line, but whether signal transduction was dependant upon its association with the murine β-subunit is not clear.

In addition, a soluble form of the GM-CSF receptor α-subunit has been found to be released from the JAR, BeWo and JEG-3 human choriocarcinoma cells but secretion was not detected from GM-CSF responsive hemopoietic cells (Raines *et al.* 1991; Sasaki *et al.* 1992). This receptor is transcribed from an alternatively spliced α-subunit mRNA (Raines *et al.* 1991) that is similar to an mRNA found in human placental tissue (Ashworth and Kraft 1990). Soluble GM-CSF receptor-ligand complexes may transmit signal after association with membrane bound β-subunit, as has been documented for the IL-6 receptor system (Hibi *et al.* 1990). Furthermore, in view of the recent report that GM-CSF has a nucleotide binding site (Doukas *et al.* 1992), it will be of interest to determine whether GM-CSF has an alternative mode of action within the target cell nucleus, as has been described for the structurally-related protein prolactin (Clevenger *et al.* 1991).

Expression of the β-subunit has not been detected in non-hemopoietic cells. The mechanism by which non-hemopoietic cells respond to GM-CSF therefore remains unclear, but may involve the α2 or other alternatively spliced or nuclear forms of the GM-CSF receptor. Whereas SSCL cells are reported to express high affinity receptors (Baldwin *et al.* 1989), other non-hemopoietic cells that proliferate in response to GM-CSF express low affinity receptors (Baldwin *et al.* 1989, 1991 and 1992; Bussolino *et al.* 1989a), in some instances at significantly increased numbers (DiPersio et al. 1990). Free or receptor-bound GM-CSF is internalised in human melanoma cells that express only low affinity receptors (Baldwin *et al.* 1991), and it is clear that GM-CSF can initiate cytoplasmic signalling events in non-hemopoietic cells since activation of the N+/ H+ pump follows low affinity binding of GM-CSF to human endothelial cells (Bussolino *et al.* 1989b). Whether the α-subunit

can initiate certain types of signalling in the absence of the β-subunit, perhaps by associating with other signal transducing proteins, remains to be investigated.

1.7.2 CSF-1

CSF-1 (or macrophage-CSF, M-CSF) is a homodimeric glycoprotein with a molecular weight of 45-90 kD. In humans, alternative promoter usage can give rise to a either a cell membrane bound form or a soluble form, and a significant proportion of CSF-1 remains bound to the surface of the cell (Rettenmier *et al.* 1987). Two mRNA species (2.3 and 4.6 kb) are transcribed from a single gene on chromosome 3. Compared to many of the other cytokines, CSF-1 is relatively restricted in its function as a regulator of the proliferation and differentiation of mononuclear phagocytes. The importance of CSF-1 in the regulation of this lineage is demonstrated in mice homozygous for the *op* mutation, which results in a systemic and specific deficiency in CSF-1. *Op/op* mice suffer from congenital osteopetrosis due to a severe deficiency of osteoclasts and macrophages, despite normal macrophage progenitor activity (Wiktor-Jedrzejczak *et al.* 1990).

The synthesis of CSF-1 and its potential roles in pregnancy have been studied in detail by Pollard and colleagues (reviewed by Daiter and Pollard 1992). During pregnancy serum and tissue concentrations of CSF-1 are elevated approximately 2-fold, and this is associated with increased numbers of circulating monocytes. Uterine CSF-1 immunoactivity is enhanced 5-fold by day 5, and it increases progressively during the course of gestation to reach by day 14 levels that are 1,000-fold higher than are found in non-pregnant uteri (Bartocci *et al.* 1986). CSF-1 mRNA and CSF bioactivity are found to be associated predominantly with the extraembryonic tissues of the conceptus (Azoulay *et al.* 1987). Administration of human chorionic gonadotrophin (hCG) to cycling mice, but not ovariectomised mice, was found to mimic the increase in activity associated with early pregnancy, and this suggests a role for ovarian steroid hormones in regulation of uterine CSF-1 synthesis (Bartocci *et al.* 1986). A small increase in CSF-1 synthesis in ovariectomised mice occurs in response to either oestrogen or progesterone alone, while in combination (in doses mimicking levels found in early pregnancy) these steroids induce CSF-1 production at physiological levels, especially when accompanied by a decidual stimulus (Pollard *et al.* 1987).

In situ hybridisation experiments demonstrate clearly that glandular and luminal epithelial cells are the major cell types responsible for CSF-1 mRNA synthesis in the uterus. In contrast to other tissues that synthesise CSF-1, the 2.3 kb transcript (which may have a longer half-life than the 4.6 kb alternative) is the dominant transcript in the pregnant uterus (Pollard *et al.* 1987). Expression is detected prior to implantation when the uterus first becomes progestational (day 3), and it reaches a peak on days 14-15 of pregnancy (Arceci *et al.* 1989).

The gene encoding the CSF-1 receptor (*c-fms*) is expressed by embryos from the 2-4 cell stage, by trophoblast cells in the blastocyst and by the trophectoderm in the ectoplacental cone following implantation. As the placenta forms, CSF-1 receptor mRNA expression is highest in trophoblast giant cells, and weaker in the underlying spongiotrophoblast and labyrinthine layers.

Maternal decidual cells in the primary decidual zone, probably of stromal fibroblast origin, also express CSF-1 receptor from day 6 until placentation is complete on day 11 (Arceci *et al.* 1989).

CSF-1 expression during human pregnancy follows a similar pattern, where it is localised to maternal epithelial cells, cells lining the blood vessels within the endometrium (presumed to be either endothelial cells macrophages) and to both trophoblast and stromal cells in the placenta (Daiter and Pollard 1992; Kanzaki *et al.* 1991). The production of CSF-1 receptor mRNA in the placenta increases as pregnancy progresses, and CSF-1 receptor protein is localised by immunohistochemical staining to placental trophoblast cells, and to epithelial and decidual cells in the uterus (Pampfer *et al.* 1992).

1.7.3 TNFα

Tumor necrosis factorα (TNFα) is a trimeric molecule composed of 17 kd subunits. It was identified originally as a product of activated macrophages, with potent cytotoxic effects that were responsible for many of the symptoms of endotoxic shock. TNFα is now identified as a product of many different cell lineages, and it is clear that it has important functions as a pleiotrophic regulator of the growth and function of a variety of hemopoietic and non-hemopoietic cells in normal physiological processes (Tovey *et al.* 1988; Vassalli 1992). These include a recently discovered role in the differentiation of monocyte precursors in hemopoiesis (Witsell and Schook 1992; Jacobsen *et al.* 1992). The actions of this factor exhibit a remarkable degree of overlap with those of IL-1 (Rosenblum and Donato 1989; Vassalli 1992). TNFα expression is regulated at both the transcriptional and translational levels by a variety of factors which include cytokines and bacterial products (Beutler and Cerami 1989; Beutler *et al.* 1992).

Various actions are relevant to the role of this factor in the uterus during early pregnancy. This cytokine can influence the expression of a variety of genes in target cells (Vassalli 1992). TNFα is chemotactic for monocytes and neutrophils (Ming *et al.* 1987; Newman and Wilkinson 1989). In addition to the cytotoxic properties after which this factor was originally characterised and named, TNFα has growth stimulating effects for some types of cells (Baglioni 1992). The activation of distinct intracellular signalling pathways, as a result of the differential expression of the two TNFα receptor proteins, is thought to determine the effect of TNFα in a target cell. There is evidence that this factor may have a role in angiogenic processes; TNFα-treated endothelial cells undergo marked but reversible changes in shape and function, associated with intercellular gap formation. This effect appears to be mediated by the autocrine actions of PAF (Camussi *et al.* 1991).

Hunt and colleagues have studied in detail the expression of TNFα mRNA and protein in reproductive tracts of humans and rodents. In the cycling rat and mouse uterus, synthesis was found by *in situ* hybridisation to be associated primarily with epithelial cells lining the lumen and the endometrial glands. The signal was also associated strongly with stromal macrophages [which on a quantitative basis are likely to be very significant sources of this factor (Beutler *et al.* 1992)] and weakly with myometrial fibroblasts (Yelavarthi *et al.* 1991; Hunt 1993). Following implantation,

TNFα expression was found to be increased first in uterine epithelial cells, and later in the primary decidua (Yelavarthi *et al.* 1991). Within the placenta, cells which were positive for mRNA transcripts included trophoblast cells initially and later giant cells and villous stromal cells (probably macrophages). Immunoreactive TNFα was co-localised with mRNA in the uterus and placenta and was also found in placental blood vessels (Yelavarthi *et al.* 1991; Hunt 1993). Expression patterns were found in general to be similar in the human uterus and placenta to those observed in rodents (Chen *et al.* 1991; Hunt 1993).

De *et al.* (1992b) have also reported the synthesis of TNFα mRNA and protein in the uteri of cycling and of pregnant mice, where its expression was found to parallel that of IL-1. Northern blot analysis showed that expression of mRNA transcripts for both factors were highest in the cycling uterus at oestrous. Immunohistochemical analysis of uterine cell suspensions prepared by collagenase digestion showed that the number of cytokine-secreting cells followed a similar pattern. Expression of TNFα was detected by Northern blot in pre- and peri-implantation uteri, with transient elevations in transcripts for both cytokines (particularly IL-1β) on day 1 of pregnancy (Sanford *et al.* 1992; De *et al.* 1992b). These increases following mating are likely to reflect the transient activation of expression of TNFα and IL-1β by cells within the endometrial stroma that are presumed to be macrophages (McMaster *et al.* 1992). Following implantation, TNFα expression was seen to increase progressively to reach a peak immediately before parturition, whereas expression of IL-1 was elevated during decidualisation. It then remained high until day 17, before decreasing to very low levels with the approach of parturition (De *et al.* 1992a).

Expression of IL-1 and TNFα are ablated following ovariectomy, and whilst administration of oestrogen induces expression of IL-1α and β transcripts, both oestrogen and progesterone are required to induce maximal TNFα mRNA synthesis (De *et al.* 1992a). This may reflect differential regulation of TNFα synthesis by different uterine tissue types. In humans, *in situ* hybridisation analysis reveals that cytokine synthesis by epithelial cells is oestrogen-dependant, whilst expression by stromal cells only occurs during the progesterone-dominated half of the menstrual cycle (Hunt *et al.* 1992). CSF-1 and TGFβ are both reported to influence TNFα expression in hemopoietic cells, but neither appears to be related to TNFα expression in the uterus (Hunt 1993).

1.7.4 LIF

Leukaemia inhibitory factor (LIF), encoded on chromosome 11, is a 38-67 kD glycoprotein with a 25 kD proteinaceous core. Use of alternative promoters gives rise to two distinct transcripts (each of 4.2 kb) that encode diffusible and ECM-associated forms respectively (Rathjen *et al.* 1990). Depending on the cellular target, LIF can act to stimulate either the proliferation or differentiation of myeloid leukaemic cells (Gearing *et al.* 1987; Moreau *et al.* 1988). A molecule called differentiation inhibitory activity (DIA), which inhibits the differentiation of embryonic stem cells *in vitro*, is identical to LIF (Williams *et al.* 1988). LIF is notable for the panoply of other properties that are attributed to it. These include actions in bone, the liver and the nervous system, and on the processes of lipid metabolism (Kurzrock *et al.* 1991).

Bhatt *et al.* (1991) have assessed LIF expression in various tissues and they have found a dramatic but transient burst of LIF mRNA expression in the uterus on day 4, coinciding with the initiation of implantation. *In situ* hybridisation analysis shows that LIF mRNA is expressed predominantly in the glandular epithelium and only weakly in the luminal epithelium. Expression also occurs in females that are mated to vasectomised males, and is therefore independent of the presence of an embryo. Progesterone and oestrogen appear to act synergistically to regulate LIF synthesis, since nidatory oestrogen induces its synthesis in progesterone-maintained ovariectomised mice (Bhatt *et al.* 1991). Genetically LIF-deficient mice are fertile, but their blastocysts fail to implant or develop without administration of exogenous LIF (Stewart *et al.* 1992). How LIF induces implantation is not clear but an action in the uterus, as opposed to the blastocyst, is suggested by the failure of wild-type blastocysts to implant after 24-48h culture in LIF prior to transplantation into the uteri of LIF-deficient recipients.

Trophoblast cells of pre-implantation blastocysts (A. Gabriel and L. Williams, unpublished data) and placental trophoblast cells (D. Hilton, D. Metcalf and N. Nicola, unpublished data) each bind radio-labelled LIF with high affinity, suggesting that both the α-subunit and the β-subunits (gp 130) of the LIF receptor are expressed by these cells. A physiologic role for LIF in embryogenesis is suggested by the recent report that this factor can inhibit the formation of primitive ectoderm, while permitting the differentiation of primitive endoderm, in embryoid bodies derived from embryonic stem cells (Shen and Leder 1992).

1.7.5 IL-6

IL-6 is a 26 kD multifunctional cytokine which has diverse biological activities on various hemopoietic and non-hemopoietic cells and tissues. These include regulation of the proliferation and function of B- and T-lymphocytes, the stimulation of hemopoietic progenitor cells, and the expression of antiviral activity (Le and Vilcek 1989; Lee 1992; Kishimoto 1989). IL-6 shares many biological properties with LIF: both factors induce synthesis acute phase proteins and they mediate differentiation of myeloid leukaemic cells and neuronal cells. However, IL-6 has no effects on ES cells (Lee 1992). These similarities in action may be due in part to the sharing of common β-subunits (gp 130) by the high affinity IL-6 and LIF receptors (Kishimoto *et al.* 1992; Gearing *et al.* 1992).

Expression of IL-6 can be induced in a wide variety of cell types in response to stimuli including cytokines [TNFα, IL-1, IL-2, IL-3, colony stimulating factors, IFNβ and IFNγ and platelet derived growth factor (PDGF)], bacteria and their products and viral infection (Le and Vilcek 1989). These include fibroblasts, monocytes/macrophages, T- and B-lymphocytes, endothelial cells, synovial cells and various tumor cells. Mucosal epithelia in the skin, intestine and airways are also reported to synthesise this cytokine. IL-6 immunoactivity and mRNA localise to keratinocytes in normal human skin and both are elevated in psoriasis (Kirnbauer *et al.* 1989; Grossman *et al.* 1989; Neuner *et al.* 1991). Keratinocytes proliferate in culture in response to IL-6, and this cytokine may therefore contribute directly to the epidermal hyperplasia seen in psoriasis, as

well as having an effect on the function of dermal inflammatory cells (Grossman *et al.* 1989). IL-6 and its receptor are expressed in gastric and small intestinal epithelial cells, and enhanced expression is associated with colonic carcinoma cells (Shirota *et al.* 1990). Human bronchial epithelial cells express IL-6 and IL-1 when exposed to inflammatory stimuli (Mattoli *et al.* 1991).

De *et al.* (1992b) have reported steroid-dependant expression of IL-6 in the uteri of cycling and pregnant mice. IL-6 (measured in a bioassay employing the IL-6 dependant B9 lymphoblastoid cell line) was detected in homogenates prepared from uteri of cycling mice, with the highest levels measured at pro-oestrus/ oestrus. Bioactivity and mRNA were not detected in uteri of ovariectomised mice, but were induced following administration of oestrogen and progesterone in combination. A transient elevation in IL-6 mRNA (but not bioactivity) was found to occur in the uterus on day 1 after mating (Sanford *et al.* 1992). Both bioactivity and mRNA were reduced on day 2, but mRNA synthesis increased again on day 3 and it continued at elevated levels through mid and late gestation (De *et al.* 1992a; 1992b).

Various cell types may contribute to IL-6 production in the uterus. Cultures of polarised epithelial cells secreted immunoactive and bioactive IL-6 vectorially, with a 2.5 to 5-fold greater secretion at the apical surface compared with the basal surface (Jacobs *et al.* 1992). Stromal cells were also found to synthesise IL-6 *in vitro*, and levels were increased in response to IL-1 and to factors secreted by epithelial cells. The amount of cytokine released was not dependant on the steroid status of the animal from which the cells were obtained, but was inhibited by physiological levels of oestrogen and progesterone added *in vitro* (Jacobs *et al.* 1992). Regulation by oestrogen of IL-6 mRNA expression in human endometrial stromal cells has been reported (Tabibzadeh *et al.* 1989). During mid-gestation, IL-6 mRNA is expressed by endothelial cells in the developing vascular system of the murine decidua basalis (Motro *et al.* 1990), and synthesis by human placental trophoblast cells has also been reported (Nishino *et al.* 1990).

IL-6 has various actions that may be relevant to tissue remodelling in the uterus and to the numbers and activities of leukocytes. This cytokine has been shown to increase markedly the permeability of endothelial cell monolayers *in vitro*, and so may be important in the development of oedema during inflammation (Maruo *et al.* 1992). Myeloid precursor cells are induced to proliferate and/or differentiate in response to IL-6 (Shabo *et al.* 1988; Chiu and Lee 1989), and a synergistic activity with GM-CSF has been reported (Hoang *et al.* 1988). IL-6 has an autocrine role in the differentiation of macrophages from human peripheral blood monocytes, and it is an exogenous stimulator of the accessory function of macrophages in T-cell activation (Ruppert and Peters 1991).

1.7.6 TGFβ

Transforming growth factor-β (TGFβ) is a multifunctional cytokine which has multiple forms. It belongs to a large gene family which has roles in cell growth, differentiation and migration as well as in the formation of extracellular matrix and in regulation of the expression of cell surface molecules. TGFβ molecules are either homo- or heterodimers composed of two 12.5 kd proteins. The dimeric molecules are released from the cell as latent precursor complexes from

which are released biologically active TGFβ by the actions of exogenous proteases (Wakefield *et al.* 1988). The state of activation and secretory profiles of tissue macrophages are very sensitive to TGFβ1, which has been reported to induce chemotaxis, release of fibroblast growth factors, accumulation of IL-1 mRNA and release of angiogenic factors (Wahl *et al.* 1989; Wiseman *et al.* 1988). TGFβ1 and TGFβ2 can both act to 'deactivate' macrophages through inhibition of the respiratory burst. Expression of class II MHC molecules and release of TNFα are also inhibited, and these effects are blocked by TNFα, IFNγ and TNFβ (Tsunawaki *et al.* 1988). The proliferation of mature murine macrophages induced by GM-CSF and CSF-1 is reported to be enhanced by TGFβ1 (Celada and Maki 1992).

Immunohistochemistry and *in situ* hybridisation have been employed to study the temporal and spatial expression of TGFβ1 in the mouse uterus during the peri-implantation period. TGFβ1 mRNA was found to be associated primarily with the luminal epithelium, and to a lesser degree with the glandular epithelium during days 1-4 of pregnancy. From day 3, message was also found in peri-epithelial stromal cells. After implantation, TGFβ1 expression was observed in a diffuse pattern throughout the deciduum (Tamada *et al.* 1990), possibly associated with the decidual cells (Manova *et al.* 1991). Immunostaining with a mAb directed against intracellular TGFβ1 was found to co-localise with mRNA synthesis, and a mAb against the extracellular form showed that the primary sites of accumulation of secreted factor at implantation (and hence likely sites of action) were in the decidualising stroma, and in the secondary decidual zone and the decidua capsularis from days 5-8.

A molecule termed decidual suppressor factor (DSF) that is related to TGFβ2 in structure and bioactivity (but has a slightly lower molecular weight), is released by small non-T lymphocytes in murine decidua (Clark *et al.* 1990). This factor has potent immunosuppressive properties *in vitro*. It has been shown to inhibit Con A-stimulated T-cell proliferation, activation of NK cells, and generation of cytotoxic T-lymphocytes (CTLs) and lymphokine activated killer cells (LAKs). Lack of DSF production has been associated with spontaneous abortion in mice and humans (Clark *et al.* 1990). *In situ* hybridisation studies have revealed that mRNA transcripts for TGFβ2 are associated with a small population of cells in the decidua basalis and in the metrial gland area from day 8.5 of pregnancy, correlating with the appearance of detectable DSF bioactivity, and with the numbers of DSF-producing cells isolated from the decidua (Lea *et al.* 1992).

Affinity labelling and competition studies show that human placental trophoblast cells express low levels of type I and type II TGFβ receptors, as well as two forms of the type III betaglycan TGFβ receptor. In combination, these receptors permit binding of both TGFβ1 and TGFβ2 (Mitchell *et al.* 1992).

1.7.7 Others

Other cytokines have been detected in the murine uterus. Stem cell factor (SCF) mRNA and protein is expressed in the mouse uterus and decidua (Horie *et al.* 1992) and placenta (Matsui *et al.* 1990), and SCF receptor is expressed strongly in cells of the decidua, placenta and extra-embryonic

membranes (Pollard *et al.* 1991b; Orr Urtreger *et al.* 1990; Motro *et al.* 1991). IL-1α and IL-1β mRNA are expressed in subepithelial cells thought to be macrophages, particularly on day 1 following mating (Takacs *et al.* 1988; McMaster *et al.* 1992), and are also produced by the mouse placenta (Azoulay *et al.* 1987). The granulocyte-CSF (G-CSF) receptor is expressed by human trophoblast (Uzumaki *et al.* 1989), and G-CSF is synthesised in the human decidua (Shorter *et al.* 1992). However G-CSF activity has not been reported in the murine reproductive tract.

Interferons of the Type 1 family, related to IFNα, are secreted in massive amounts by ovine and bovine blastocysts, where they are implicated as anti-luteotrophic agents (Roberts 1991). Although human and rodent blastocysts have been reported to secrete anti-viral activity, type 1 IFNs have not been detected in the rodent embryo (Cross *et al.* 1990) and are not implicated in corpus luteum rescue in these species. IFNs, particularly IFNγ, are potent modulators of both lymphocytes and immuno-accessory cells, and so are important potentially as modulators of immune processes in the endometrium. Interferons α, β and γ have been detected in placental trophoblast and are produced by decidual leukocytes during human pregnancy (Bulmer *et al.* 1990), but they have not been studied in the placenta or uterus of the rodent.

Various well-characterised polypeptide growth factors, including epidermal growth factor (EGF), insulin-like growth factor (IGF)-1 and transforming growth factor-α (TGFα), are implicated in the autocrine/ paracrine regulation of growth and differentiation in other tissues and they are all synthesised in the uterus (Brigstock *et al.* 1989, Pollard 1990). In each instance ovarian steroid hormones regulate synthesis in cell-specific patterns which have a temporal relationship to the oestrous cycle and to the stage of pregnancy. Functional roles for growth factors in uterine growth and/ or conceptus development are suggested by receptor localisation studies, and data derived from studies of their actions in other tissues suggests that they may have important influences on the dynamics of uterine leukocytes. However, these factors will only be discussed superficially, since lymphohemopoietic cytokines are intended to be the focus of this review.

EGF is a strong candidate for a growth factor in the cycling as well as pregnant uterus. The growth factor and its receptor are each induced by oestrogen in the immature mouse uterus (DiAugustine *et al.* 1988; Huet-Hudson *et al.* 1990), and EGF stimulates proliferation of epithelial cells from the immature uterus *in vitro* (Tomooka *et al.* 1986) and *in vivo* (Nelson *et al.* 1991). An autocrine loop, whereby oestrogen causes cleavage of the EGF precursor into its active form and also stimulates synthesis of the EGF receptor, is suggested by the finding that in mature epithelial cells the *de novo* synthesis of EGF precursor is unaffected by oestrogen (DiAugustine *et al.* 1988).

IGF-1 synthesis is regulated by oestrogen in the rodent uterus and it is implicated in autocrine/ paracrine growth regulation in the cycling uterus (Murphy and Ghahary 1990). In the mouse, oestrogen induces synthesis of IGF-1 in the luminal and glandular epithelium and progesterone (alone or together with oestrogen) induces stromal cell synthesis. In pregnancy, the levels of IGF-1 mRNA increase during the pre-implantation period, and epithelial cells are the predominant site of IGF-1 mRNA and protein synthesis on days 1 and 2 after mating. On days 3 and 4 when progesterone dominates, synthesis in the uterus is associated with stromal cells and later with decidual cells in the implantation site (Kapur *et al.* 1992).

TGFα mRNA and protein co-localise primarily in luminal epithelial cells on days 1 and 2 of early pregnancy, and also in stromal cells after day 3, where expression is associated with decidualisation at the implantation site (Tamada *et al.* 1991). During the oestrous cycle, the amount of TGFα mRNA and protein in the endometrium parallels oestrogen levels, and αTGFα antibodies significantly reduce oestrogen-induced proliferation of uterine cells *in vivo* (Nelson *et al.* 1992). These findings implicate epithelial cell TGFα as an autocrine mediator of the growth-promoting actions of oestrogen in the endometrium.

1.8 Cytokine actions in the cycling and pregnant uterus

In situ mapping of cytokine receptor expression has provided information on the potential targets for the various cytokines that are produced in the uterus. Mice with mutations that prevent synthesis of individual cytokines have been used to determine the relative importance of the respective factors in the reproductive process. However, the overlap of function between cytokines remains an important constraint on the interpretation of experiments employing the 'knockout' strategy. Furthermore, since each of the cytokines implicated in reproductive functions also has roles in hemopoiesis and/or immune regulation, it is difficult to apportion the blame for a fertility defect between local actions of a factor in the uterus and the indirect consequences of disturbances in the lymphohemopoietic system.

Mutation in the *op* locus gives rise to a complete absence of CSF-1 and a severe reduction in circulating monocytes (Witkor-Jedrzejczak *et al.* 1990). Homozygous females are infertile when mated to homozygous males. When homozygous females are mated to heterozygous males they produce litters reduced in number by 60%. The placental weights of surviving fetuses are normal, and the fertility defects are thought to occur at both the pre- and post-implantation stages of pregnancy (Pollard *et al.* 1991a). This result shows that while a maternal source of CSF-1 contributes to the success of pregnancy, it is not an absolute requirement for production of offspring. Furthermore, it suggests that either heterozygous semen, or heterozygosity in the fetus, can partially compensate for the deficiency of uterine CSF-1.

In contrast, the transient expression of LIF in the endometrial glands immediately prior to implantation appears to be absolutely essential for successful implantation. Transgenic mice lacking a functional LIF gene are fertile, but blastocyst development is arrested at the implantation stage (Stewart *et al.* 1992). Whether the target for LIF action is the blastocyst, or another cell type within the endometrium, remains to be determined.

Mice with mutations in the *Sl* and *W* loci, leading to deficiency of SCF and SCF receptor respectively, have apparently normal pregnancies, although in their severest form these mutations can lead to embryonic death in mid-gestation through fetal anaemia (Russell 1979). This demonstrates that local SCF activity is not an absolute requirement for pregnancy, perhaps because compensatory mechanisms, probably involving other cytokines, operate at the maternal-placental interface in these mice. Such a mechanism is reminiscent of the redundancy of cytokine actions observed in the hemopoietic system.

1.8.1 Embryonic and placental tissues as cytokine targets

a. Pre-implantation embryo

The development of murine embryos to the blastocyst stage can occur *in vitro* in simple defined medium (Whitten and Biggers 1968). Therefore embryogenesis is (at least partially) autoregulated, perhaps by growth factors including EGF, IGF, PDGF, fibroblast growth factor (FGF), TGFα and TGFβ (Schultz and Heyner 1992). The development of pre-embryos cultured *in vitro* is retarded compared to their *in vivo* counterparts (Harlow and Quinn 1982) or to embryos cultured in the presence of other cells (Lavranos and Seamark 1990). These observations, together with studies of the development of embryos in the reproductive tracts of hormone-depleted mice (Roblero and Garavagno 1979), suggest that additional paracrine regulators may originate from the oviduct and the uterus under the influence of oestrogen and progesterone.

There is evidence that cytokines are among the growth factors synthesised by uterine cells that can influence the growth and development of embryos. Supernatants from activated lymphocytes have been reported to both accelerate and inhibit embryo development at the morula and blastocyst stages (Fukada *et al.* 1989; Hill *et al.* 1987; Haimovici *et al.* 1991).

Purified natural cytokines and their recombinant counterparts appear to have both positive and negative effects on the development of embryos in culture. Their effects may be dependant on the developmental stage of the embryo and the influence of other growth factors. Supplementation of the culture medium with LIF improves the proportion of 8-cell embryos that develop to blastocyst stage (Robertson *et al.* 1991) and CSF-1 has a similar action on 2-cell embryos (Pampfer *et al.* 1991). GM-CSF has been shown to inhibit the development of 2-cell embryos into morulae (Hill *et al.* 1987) and the attachment of blastocysts to fibronectin-coated culture dishes (Haimovici *et al.* 1991), while TNFα at high doses has an inhibitory effect on 2-cell embryo development (Hill *et al.* 1987). IFNγ, produced by activated T-lymphocytes and NK cells, was found to be cytotoxic to both 2-cell embryos and to the trophoblastic outgrowth of implanted blastocysts (Haimovici *et al.* 1991; Hill *et al.* 1987). IL-6 is reported to inhibit the attachment and trophoblast outgrowth of murine blastocysts *in vitro* (Jacobs *et al.* 1992).

Transcripts for CSF-1 and SCF have not been detected in embryos but their receptors are expressed by pre-embryos from the 2-cell stage onwards, and in the blastocyst they are associated with trophoblast cells as opposed to cells of the inner cell mass (ICM) (Arceci *et al.* 1989; Manova and Bachvarova 1991; Pampfer *et al.* 1991). Transcripts for LIF and IL-6 (but not for GM-CSF or IL-3) are detectable in blastocysts (Murray *et al.* 1990), and the binding of radio-labelled LIF to trophoblast cells of blastocysts indicates that they express the LIF receptor (A Gabriel and L Williams, personal communication).

b. Placental trophoblast

Placental trophoblast cells express receptors for many of the cytokines that are released by uterine cells during pregnancy, and have biological responses to them *in vitro*. This has led to the view that the placenta may benefit directly from the local abundance of cytokines.

CSF-1 receptor is expressed at high levels by placental giant cells with lower levels observed in the spongiotrophoblast layer. The temporal pattern of CSF-1 receptor expression is consistent with a role for CSF-1 in regulating placental development. Studies in the *op/op* mutant mouse show that a deficiency in CSF-1 can affect fertility, but the normal placental weights observed in the fetuses of homozygous females mated to heterozygous males suggests that factors other than CSF-1 also contribute to regulation of placental development.

GM-CSF receptor (α-subunit) mRNA is expressed in human placenta (Gearing *et al.* 1989), but expression of the GM-CSF receptor in the rodent placenta has not been examined. Interestingly, human choriocarcinoma cell lines and human placenta have been identified as rich sources of the soluble and alternatively spliced GM-CSF receptors (1.7.1e). The soluble receptor may modulate the local response to GM-CSF by competing with membrane bound receptor for available ligand. Alternatively, it could contribute to an agonist effect by associating with membrane-bound components to form a ligand-receptor complex.

Primary cultures of cells from day 12 mouse placentae, and cytokeratin-positive placental cell lines have been found to proliferate in response to GM-CSF as well as IL-3 and CSF-1 (Athanassakis *et al.* 1987; Wegmann 1988; Wegmann *et al.* 1989). Purified natural GM-CSF also stimulated the proliferation of trophoblast cells harvested as outgrowths from day 8-9 ectoplacental cones, or by enzymatic disruption of day 12 placentae (3- and 4-fold enhanced ^{3}H-thymidine incorporation respectively) (Armstrong and Chaouat 1989). Difficulties in identifying the responding population, and/ or excluding the possibility that increased ^{3}H-thymidine incorporation is the result of endoreduplication as opposed to cell division have made interpretation of these experiments difficult. GM-CSF and CSF-1 also stimulate the release of placental lactogen II from explant cultures of rat trophoblast (Shiverick *et al.* 1990). Human choriocarcinoma cell lines JAR, JEG and BeWo secrete GM-CSF and an autocrine action for this factor is indicated by the finding that an anti-GM-CSF antibody blocks their proliferation (Garcia-Lloret 1991). GM-CSF and CSF-1 have been shown to induce the differentiation of human cytotrophoblast cells into syncytium, and to stimulate release of placental lactogen and chorionic gonadotrophin *in vitro* (Garcia-Lloret 1991). The granulocyte-CSF (G-CSF) receptor is also expressed by human trophoblast (Uzumaki *et al.* 1989), but there is no evidence that G-CSF is synthesised in either the human or rodent uterus.

IL-6 and TNFα may also have roles in regulating the growth and function of placental cells. Human placental trophoblast cells synthesise IL-6 and IL-6 receptors, and an IL-6-mediated autocrine regulation of hCG synthesis has been proposed (Nishino *et al.* 1990). TNFα appears to impact upon this circuit, through stimulating the release both of IL-6 and hCG from trophoblast cells (Li *et al.* 1992). Whether a parallel situation exists in the mouse remains to be determined. The p60 and p80 TNFα receptors are expressed by trophoblast cells in the human placenta (Hunt 1993), suggesting that various autocrine or paracrine pathways for TNFα action in placental development may operate. These may include both growth promoting and inhibitory actions depending on the stage of maturation of the trophoblast cells; TNFα and TGFβ have been reported to inhibit the proliferation of rat trophoblast cell lines (Hunt *et al.* 1989 and 1990), whilst TNFα

was found not to alter the rate of ^{3}H-thymidine incorporation into murine trophoblast cells from late gestation placentae (Drake and Head, 1990).

1.8.2 Stromal fibroblasts as cytokine targets

Stromal fibroblasts may also be targets for CSF-1, SCF and GM-CSF action in the pregnant uterus. During implantation, CSF-1 receptor mRNA is detected in the antimesometrial stroma immediately below the implanting embryo. As decidualisation progresses, expression of the receptor gene becomes limited to the primary decidua and to the decidua basalis (Arceci *et al.* 1989; Pollard 1991). Expression has been localised by *in situ* hybridisation to cells that are not macrophage-like in morphology and these cells are thought to be of stromal fibroblast origin. SCF receptor is also expressed by cells in the secondary decidual zone (Pollard 1991). Cytokines, released in response to the presence of the blastocyst or local trauma, may provide the epithelial cell-derived signal for differentiation of stromal fibroblasts into decidual cells (Lejeune *et al.* 1981). This is suggested by the finding that CSF-1, TNFα and IL-1 can induce decidualisation when injected into the uterine lumen of psuedopregnant mice (Choudhuri and Wood 1991).

The profile of biological mediators released by stromal fibroblasts might also be sensitive to local cytokine activity. Secretion of PGE and PGF_{2a} by stromal fibroblasts from pregnant bovine endometrium is reduced moderately by GM-CSF (Betts and Hansen 1992). However contaminating leukocytes remain to be excluded as mediators of this effect. These cytokines, and perhaps others, may therefore have a role in regulating the function and/ or the differentiation of these non-hemopoietic cells.

1.8.3 Leukocytes as cytokine targets

The data reviewed above show that resident cells within reproductive tissues are potent sources of a variety of pleiotrophic cytokines which have capacities to recruit macrophages and granulocytes and influence their behaviour decisively. The dynamic changes observed in leukocyte composition, distribution and activation during the oestrous cycle and in the pre-implantation uterus parallel and indeed appear to be regulated by ovarian steroid hormone levels. The synthesis of a number of uterine cytokines follows a similar pattern.

Immunohistochemical demonstration that macrophages accumulate beneath the basement membrane of the uterine epithelium at oestrous has led De and Wood (1990) to postulate that epithelial cells secrete macrophage chemotactic factors. At least in part, this activity may be attributable to CSF-1. Data derived from the *op/op* mouse model suggests that CSF-1 is an obligatory component in the recruitment of uterine macrophage populations. However, it is not clear whether the deficiency in uterine macrophages in these mice is caused by the diminished pool of circulating monocyte precursors or by failure of recruitment into the uterus (Pollard *et al.* 1991a). The presence of some macrophages early in gestation in *op/op* mice indicates that other recruitment and activation factors are also operative during pregnancy. Evidence that CSF-1 synthesised by

epithelial cells in the pregnant uterus can act to regulate local macrophage populations has been provided by the demonstration that very large numbers of macrophages are found in the endometrium of ovariectomised mice within hours after an intraluminal injection of recombinant CSF-1 (Wood *et al.* 1992). However the relative lack of CSF-1 activity during the oestrous cycle and prior to day 3 of pregnancy suggests that other factors are responsible for regulating macrophage dynamics at these times. Furthermore, with the exception of TNFα, none of the factors so far described during oestrous or on the first days of pregnancy target granulocytes. This suggests that additional cytokines are involved in the recruitment of uterine neutrophil and eosinophil populations into the uterus. Thus a direct association between cytokines liberated by resident cells in the uterus and the recruitment and behaviour of infiltrating leukocytes has been established, although the relationships between individual factors and their target leukocyte populations remain to be defined.

The transient increases observed in cytokine expression and infiltration of the endometrium with granulocytes and macrophages during the 24 h period after mating are reminiscent of an inflammatory reaction (1.3.2). Although increased synthesis of IL-1 and TNFα by infiltrating macrophages occurs at this time, it is clear that many of the cytokines synthesised within the uterus (including TNFα, CSF-1, LIF and TGFβ) originate from resident tissues, in particular the epithelium. The available evidence does not preclude the possibility that these cells are responsible in part for the elevation in other cytokine activities following mating.

Endometrial cytokines may influence the activities as well as the trafficking of local leukocytes. The cytokine microenvironment is known to be an important determinant of the antigen-presenting capacities and immunoaccessory function of macrophages and dendritic cells. For example, whilst GM-CSF and TGFβ can reduce the immunocompetence of antigen-presenting cells, IFNγ has the converse effect (1.5.2; 1.7.1). Regulating the phenotype of local macrophages is therefore likely to be an important function of cytokines within the uterus, particularly during implantation and placentation when destructive maternal immune responses to fetal alloantigens must be inhibited.

Lymphocytes may also be targets for cytokine action within cycling and pregnant uteri. A balance between the activities of the two subsets of $CD4^+$ T-lymphocytes (T_H1 and T_H2) has been proposed to be central to the success of pregnancy (Guilbert *et al.* 1993). Local cytokines are likely to be important in initiating and maintaining this balance, because the recruitment, proliferation and activation of T-lymphocytes are sensitive to the effects of various cytokines. Furthermore, IL-6 production has been linked with secretion of IgA in the uterus (C. Wira, personal communication) and the breast (Saito et al. 1991), although it is not clear whether this action is mediated through effects on IgA secretion by B-cells or on IgA uptake and secretion into the lumen by epithelial cells.

1.8.4 Endothelial cells as cytokine targets

The periodic growth and regression of uterine tissues is accompanied by rapid proliferation and development of new blood vessels to an extent unparalleled in other normal tissues.

Angiogenesis is a feature of the cycling uterus but it is particularly marked during placental development (Findlay 1986; Reynolds *et al.* 1992). Dramatic increases in vascular permeability and development of oedema occur immediately adjacent to the attachment site during the very early phase of the implantation process (Finn 1986). Changes in endothelial cell junctions leading to increased capillary diameter and fragility occur as a response to embryonic signals, transmitted via the epithelium, even before the blastocyst has hatched from the zona pellucida. (McLaren 1969; Rogers 1992).

Many of the cytokines and other growth factors produced within uterine tissues have effects on endothelial cells. GM-CSF induces proliferation and migration of endothelial cells (Bussolino *et al.* 1989a), and TGFβ is also a possible mediator in control of the local vascular bed (Klagsbrun and D'Amore 1991). TNFα and PAF both induce leakage in endothelial cell junctions (Camussi *et al.* 1991), and IL-6 has a similar action (Maruo *et al.* 1992). Analysis of day 8.5-11.5 pregnant uteri by *in situ* hybridisation revealed that endothelial cells within the developing decidua basalis express IL-6 mRNA. This is consistent with a role for IL-6 in the angiogenic process (Motro *et al.* 1990).

1.8.5 Cytokine circuits in the uterus

The importance to the ongoing success of pregnancy of maintaining an appropriate cytokine milieu is highlighted by the dramatic consequences of administering relatively small amounts of cytokines to pregnant mice. Failure of implantation or fetal resorption occurs in mice bearing a CSF-1 secreting tumor and in animals injected with very small amounts of CSF-1 (Brosh *et al.* 1991; Tartakovsky *et al.* 1991a; 1991b). This effect depends on the combination of parental strains and presumably has an immunological basis. Daily injections of nanogram amounts of GM-CSF or TNFα during the pre-implantation period reverse the effect of CSF-1 administration and also reduce the high rate of early embryo malformation and embryo loss that occurs spontaneously in the CBA/J x DBA/2 mating combination (Tartakovsky and Ben-Yair 1991).

Administration of a single dose of IFNγ, TNFα, IL-2 or LPS on day 7.5 of pregnancy induces fetal resorption in some strain combinations (Chaouat *et al.* 1990). In the abortion prone CBA/J x DBA/2 mating combination, where microbial factors have been implicated in interfering with gestation (possibly through activating non-specific effector cells) (Hamilton and Hamilton 1987), small amounts of GM-CSF or IL-3 can protect against fetal resorption. Treatment with these cytokines enhanced fetal and placental weights and in particular, it expanded the spongiotrophoblast zone in the placenta (Chaouat *et al.* 1990). In the same CBA/J x DBA/2 model, immunisation with non-specific immunopotentiating agents also reduces resorption rates (Toder and Strassburger 1990; Szckeres-Bartho *et al.* 1991).

The doses of cytokines used in all of these studies appear to be far too small to effect placental cell growth directly. The authors suggest that they may act by precipitating a 'cytokine regulatory cascade' that involves cytokine responsive leukocytes within the uterus.

1.9 Summary

The mammalian uterus is a remarkable tissue in view of its capacity to (1) undergo repeated cycles of rapid growth and regression, and (2) accommodate and nourish the fetus, which in most instances can be regarded as a semi-allogeneic allograft.

The outcome of pregnancy is dependant critically on the successful implantation of the conceptus into the uterine endometrium. Implantation will occur only during a very brief period of 'receptivity', which is the culmination of a series of cellular remodelling processes which take place during the preceding oestrous cycle and in the period between mating and implantation. Most notable of these changes is a dramatic infiltration by leukocytes, particularly those of the monocyte/ macrophage and granulocyte lineages. These cells may have roles in endometrial remodelling analogous to their roles in inflammatory and reparative processes. Events in the pre-implantation uterus are driven at a systemic level by ovarian steroid-hormones, but growth factors (including some lymphohemopoietic cytokines), that are produced locally are now implicated as the molecular mediators of these events. Cytokines are ideally suited to provide communication links between the various types of uterine cells, by virtue of their pleiotrophic effects and their production by a range of cell lineages. Their effects may extend, therefore, to the full range of cell lineages that are represented in the uterus.

Intrinsic to the success of implantation and placentation is the ability of the embryo to escape rejection by the maternal immune system. The conceptus expresses paternally encoded, allogeneic antigens that would be expected normally to elicit transplantation immunity and graft rejection. However, the lack of strongly immunogenic molecules on the surfaces of conceptus-derived tissues at the fetal-maternal interface, the resistance of the trophoblast to T-cell mediated lysis, and the physical protection from immunocompetent and accessory cells afforded by the decidual zone help to block the generation and execution of destructive immune responses.

The 'immunotrophism' hypothesis, which suggests that an appropriate maternal immune response to fetal alloantigens can be beneficial to the outcome of pregnancy, has been instrumental in promoting research into the role of cytokines in the growth and development of the placenta. Recent studies suggest that both non-specific activation as well as specific priming of the maternal immune system against paternal antigens, can reduce the rate of fetal resorption in abortion-prone mice. T-cell-derived cytokines may be important in influencing the rate of placental growth, either directly through release of trophic factors or indirectly by influencing the behaviour of leukocytes or other uterine cells. However, studies in genetically lymphocyte-deficient or MHC-deficient mice show that these effects are adjunctive, since an intact maternal immune response is not obligatory for successful pregnancy.

T-lymphocytes are likely to function as a component of a broader leukocyte network in the uterus. It is becoming evident that different leukocyte lineages within the endometrium can both contribute to suppression of maternal rejection mechanisms and guard against uncontrolled invasion of maternal tissues by placental trophoblast. The various leukocyte populations in the endometrium therefore appear to act in a coordinated manner to 'monitor' the extent of the growth of

conceptus tissues and the degree of their invasion into the endometrium. Cytokines are implicated in the recruitment and function of the leukocytes resident within uterine tissues, and are likely to be critical in mediating the interactions between different subpopulations.

Current evidence indicates clearly that locally synthesised cytokines also function directly as paracrine mediators of the growth and development of maternal and fetal tissues within the uterus during pregnancy. Trophoblast cells of the placenta and the pre-implantation embryo express receptors for, and/ or have biological responses to many of the cytokines synthesised within the uterus.

Together, these data suggest that during pregnancy cytokines are important mediators of a three-way communication network interlinking maternal cells (of hemopoietic and non-hemopoietic origin) with the conceptus. This cytokine network is exquisitely sensitive to experimental perturbation, as early as during the pre-implantation period, suggesting that its operation coincides with the establishment of the receptive uterine state. Cytokines may therefore have important roles in the uterus during the pre-implantation period, with potential implications for the outcome of pregnancy.

1.10 Aims of this study

In view of the role of cytokines in inflammation, and the emerging concept that these factors are ubiquitous in tissue remodelling events, it is highly probable that cytokines participate in establishing a uterine state that can accommodate the implanting conceptus. However, at the outset of this study, the synthesis and roles of cytokines in the early stages of pregnancy had received little attention.

These considerations led to formulation of the following hypotheses:

(1) Lymphohemopoietic cytokines have pivotal roles in establishing a uterine state conducive to implantation and development of the conceptus.
(2) Cytokines act through coordinating the recruitment and behaviour of leukocytes, which may be important as mediators of tissue remodelling and in generating a receptive immunological milieu.
(3) Cytokines play an important role in enhancing embryo survival and embryo development as a consequence of their direct action on the trophoblast cells of the developing embryo.

The purpose of this study was examine these hypotheses in the murine model.

2 Materials and Methods

2.1 Mice

Adult (8-12 wk) [Balb/c x C57Bl] F1 female mice and [CBA x C57Bl] F1 male mice were used throughout except where stated. Pre-pubertal (4 wk) [Balb/c x C57Bl] F1 or Balb/c female mice were superovulated and mated with [CBA x C57Bl] F1 or Balb/c male mice respectively as specified. In some experiments , female CBA-*nu/nu*, C57Bl/6J-*bg/bg*, C.B-17-*scid/scid* and Balb/c adult (6-12 wk) females were mated with Swiss adult male studs. C57Bl/6J-*bg/bg* were obtained from ARC, Perth and C.B-17-*scid/scid* from the Walter and Eliza Hall Institute [WEHI], Melbourne. All other mice were obtained from the University of Adelaide Central Animal House and were maintained in minimal security barrier, specific pathogen free (SPF) facilities, on a 12 h light/ 12 h dark cycle with food and water available *ad libitum*. Lymphocyte-deficient *nu/nu*, *scid/scid* and *bg/bg* mice, and Balb/c mice were housed and mated with Swiss males in microisolator cages within sterile laminar flow cabinets, in medium security barrier, SPF facilities .

2.1.1 Natural mating

Adult naturally cycling or synchronised (see 2.1.4) female mice were placed 2 per cage with individual males. The day of sighting of a vaginal plug was day 1 of pregnancy. Pregnant females were separated from males on day 1.

2.1.2 Superovulation

Adult or pre-pubertal (4 wk) females were primed at 1400 h with 7.5 IU PMSG (Folligon, Intervet, Artarmon, NSW) followed 48 h later with 7.5 IU hCG (Chorulon, Intervet) and then placed 1 per cage with males. Females were checked for vaginal plugs the next morning, designated day 1 of pregnancy, and pregnant females were caged separately from males.

In some experiments (6.3.2) blood was obtained from hCG-treated mice by orbital bleeding under light ether anaesthesia immediately prior to sacrifice, and the serum oestrogen and progesterone content was measured in RIA kits from Serono (Italy) (<7% inter- and <5% intra-assay variation) and Amersham (UK) (<5% inter- and <2% intra-assay variation) respectively.

2.1.3 Surgical procedures

a. General procedures

Mice were anaesthetised prior to all other surgical procedures with avertin (1 mg/ ml tribromoethyl alcohol in tertiary amyl alcohol was diluted to 2.5% (v/v) in saline, and approximately 15 µl/ g body weight injected ip). Surgical instruments were sterilised by autoclaving prior to use.

b. Ovariectomy and intraluminal injection

Adult [Balb/c x C57Bl] F1 female mice were anaesthetised with avertin and bilaterally ovariectomised by severing the oviduct close to the uterine-oviduct junction through a single small dorsal incision as described by Hogan *et al.* (1986). The incision was closed with a wound clip and animals were allowed to recover for 2-3 weeks prior to use.

In some experiments solutions of antibody or cytokine, seminal vesicle fluid or peanut oil, were injected into the uterine lumen of intact or ovariectomised adult [Balb/c x C57Bl] F1 mice. Uteri were accessed by the procedure described for ovariectomy and between 10 and 50 µl of fluid was injected directly into the proximal end of the uterine horn using a 30 gauge needle attached to a 1 ml syringe.

c. Vasectomy and seminal vesicle excision

Adult [CBA x C57Bl] F1 male mice were anaesthetised with avertin and vasectomised by bilateral ligation of the vas deferens through a transverse incision in the abdomen as described by Hogan *et al.* (1986). Seminal vesicles were removed from mice of the same age and strain by ligation followed by severing of the proximal tubule at the base of the gland, through a transverse incision in the abdomen. The body wall and skin were sutured and the mice were allowed to recover for at least a week prior to mating.

2.1.4 Synchronisation of oestrous cycle

For studies requiring oestrous mice, Balb/c F1 females were synchronised using a modification of a procedure described previously (Vickery and McRae 1980). Twenty micrograms of LHRH superagonist [des-Gly_{10},D-Ala_6,-$ProNEt_9$] LHRH (Sigma Chemical Co, St Louis, MO) was injected ip at 1200 h on day -4 and mice were placed with males for natural mating on day -1, or checked for oestrous by examination of vaginal smears, then used as oestrous mice on day 0.

In some experiments adult female mice were induced into oestrus by the Whitten effect (Whitten 1956), where they were caged within close proximity to a stud male (a small cage containing the male was placed within a larger cage containing the female mice). On the third day approximately 75% of the female mice were found to have an outward vaginal appearance typical of oestrus and this was confirmed by making vaginal smears.

2.1.5 Cervical stimulation

The cervices of oestrus stage, synchronised mice were stimulated mechanically for 2 min at 2400 h on day -1 (to approximate the time, at the mid-point of the dark cycle, when mating frequencies are naturally highest). The device employed in this procedure consisted of a small plastic pipette tip (cut and rounded over a flame) attached by a piece of plastic tubing to the nozzle of a battery-operated electric toothbrush.

2.2 Growth media

Media used for cell line and primary cell culture included RPMI-1640, DMEM, Hams F-12 or Iscoves modified DMEM (Gibco, Grand Island, NY) supplemented with 20 mM HEPES, 10% Fetal Calf Serum (FCS)(Commonwealth Serum Laboratories, Australia), 5 x 10^{-5} M β-mercaptoethanol and antibiotics (RPMI-FCS, DMEM-FCS, Hams-FCS or IMDM-FCS respectively); RPMI supplemented with 0.5% FCS, 1% Nutridoma-NS (Boehringer Mannheim, Germany), 5 x 10^{-5} M β–mercaptoethanol and antibiotics (RPMI-NS) and serum-free DMEM supplemented with 1% Nutridoma-NS, 5 x 10^{-5} M β–mercaptoethanol and antibiotics (DMEM-NS), as specified. 'E-free' DMEM was phenol red free and contained 0.5% charcoal-stripped FCS, 1% Nutridoma NS (Boehringer Mannheim, Germany), 1% Low Protein Serum Replacement (Sigma), 5 x 10^{-5} M β–mercaptoethanol and antibiotics.

Media used for embryo culture included Human Tubal Fluid (HTF)(Quinn *et al.* 1985) and DMEM supplemented with 10% FCS and antibiotics (DMEM-Em). The osmolarity of each batch of HTF and DMEM-Em was measured in an osmometer (Wescor, Logan, Utah), and adjusted to between 275 and 280 mOsm/ kg with water prior to addition of serum and antibiotics. HTF and DMEM-Em used for embryo collection and handling contained 20 mM HEPES.

2.3 Chemicals and reagents

2.3.1 Recombinant cytokines

Recombinant *E. coli*-derived murine GM-CSF was the kind gift of N. Nicola [The Walter and Eliza Hall Institute of Medical Research (WEHI), Melbourne], recombinant human IL-6 was purchased from Boehringer Mannheim, recombinant murine IL-2 and IL-3 were purchased from Genzyme Immunobiologicals (Cambridge, MA), recombinant murine IL-1, TNFα and TNFβ were purchased from British Biotechnology (Oxford, UK), and recombinant murine LIF was from AMRAD Corp. Ltd. (Kew, Vic.). Recombinant murine interferon gamma (IFNγ) was from Genentech (Palo Alto, CA), and recombinant human interferon alpha-2a (Roferon-A; IFNα) was the gift of F. Hoffman La Roche and Co., Ltd. (Basle, Switzerland). ^{125}I-labelled GM-CSF was prepared by N. Nicola (WEHI), using the Chloramine-T-catalysed two-phase method of Tejedor and Ballasta (1982), which yields high specific activity ^{125}I-GM-CSF (50,000 cpm/ ng) and retains 100% biological activity (Walker and Burgess 1985).

2.3.2 Monoclonal and polyclonal antibodies

Rat/ mouse hybridoma cell lines including TIB 107 (αThy1.2) and TIB 122 (αCD45), TIB 207 (αCD4), TIB 104 (αCD5), TIB 213 (αLFA-1)and TIB 128 (αMac-1) from the sources specified in Table 2.1 were cultured to late log phase when monoclonal antibody (mAb)-containing supernatants were harvested. Other rat mAbs including α mouse thymic stroma #12 and #24 (MTS #12 and MTS #24) (Godfrey *et al.* 1988; 1990) were kindly provided as hybridoma supernatants by R. Boyd, Monash University, Melbourne.

Table 2.1 The antigenic specificities and cell lineage reactivities of rat αmouse monoclonal antibodies (mAbs). These mAbs were used for isolation of cell subpopulations by panning, and in complement-mediated depletion, immunofluorescence and immunohistochemical analyses. The sources of each mAb are given in the text or in Table 2.2. LCA = leukocyte common antigen; LFA = leukocyte functional antigen; ND = not determined

mAb	antigenic specificity	reactive cell lineages
MTS #24	ND	thymus epithelial cells
MTS #12	ND	thymus endothelial cells; T-lymphocytes
TIB 107	Thy 1.2	pan T-lymphocytes; NK cells; some fibroblasts
TIB 122	CD45 (LCA)	pan leukocytes
TIB 128	Mac-1 (CD11b/ CD18)	macrophages; neutrophils
F4/ 80	ND	macrophages
TIB 120	I-A	activated macrophages/ dendritic cells
TIB 207	CD4 (L3T4)	T-lymphocytes (suppressor); some macrophages
TIB 105	CD5 (Lyt 1)	T-lymphocytes, some B-lymphocytes
TIB 213	LFA-1 (CD11a)	pan leukocytes

Sheep αrat-FITC (Silenus, Hawthorn, Vic.) and goat αrat-horseradish peroxidase (HRP)(Dakopatts, Copenhagen) were used to detect rat immunoglobulin in immunofluorescent and immunohistochemical assays respectively. Affinity purified goat αrat (Calbiochem, La Jolla, CA) was used as the capture antibody in panning experiments.

A goat polyclonal antibody to murine GM-CSF (goat αGM-CSF) and a rat monoclonal antibody to murine IL-6 (rat αIL-6) (Shabo and Sachs 1988) were kindly provided by J. Schreurs (DNAX, Palo Alto, CA) and L. Sachs (Weismann Institute of Science, Rehevot) respectively.

2.3.3 Steroids and steroid antagonists

17β-oestradiol (E), progesterone (P) and testosterone were obtained from Sigma, USA. ZK 119,010 (Nishino *et al.* 1991) was the generous gift of E. von Angerer (University of Regensburg, D-8400 Regensburg, Germany), and RU486 (Baulieu 1989) was kindly provided by Roussel UCLAF, Romainville, France. Stocks of 17β-oestradiol, progesterone, RU486 or ZK 119010 were made in ethanol and added to cultures at a final concentration of 0.1 % ethanol. Control cultures contained 0.1 % ethanol alone.

2.3.4 Sundry chemicals and reagents

Salmonella typhimurium lipopolysaccharide (LPS) was the gift of I. Kotlarski (University of Adelaide). Prostaglandin E (PGE) and prostaglandin F_{2a} (PGF_{2a}) were from Upjohn Pty. Ltd. (Rydalmere, NSW). All other chemicals and reagents were purchased from BDH Chemicals (Dorset, UK) or Sigma Chemical Co., unless specified in the text.

2.4 Cell culture

2.4.1 General

All harvesting and culture of tissues and cells was performed under aseptic and pyrogen-free conditions. Solutions and media were prepared from stocks reserved for cell culture using water purified in a Milli-Q Reagent Grade Water System (Millipore, Bedford, MA) (MQ H_2O), and sterilized by filtration through 0.22 µm filters (Millex, Millipore) or by autoclaving. Disposable plastic culture-ware and pipettes were used throughout. Glassware, including glass Pasteur pipettes, was washed in 7X detergent (ICN, Seven Hills, NSW) and rinsed extensively in MQ H_2O, and autoclaved prior to use.

Cell cultures were routinely examined for confluency and health with the aid of a phase contrast inverted microscope (Olympus).

2.4.2 Cell lines

Cell lines were obtained from the sources and maintained in the media specified in Table 2.2. Frozen stocks were maintained in 10% DMSO in liquid nitrogen. All cell lines were cultured in 250 ml culture flasks (Costar, Cambridge, MA) at 37°C, 10% CO_2 in air in a humidified incubator.

Table 2.2 The species, lineage, origins and growth media for cell lines used in these studies. M = mouse; H = human; R = rat; G = gibbon.

Cell line	Species, Lineage	Origin	Growth Media
32Dcl3	M, myeloid	A Kelso, WEHI, Melbourne	RPMI-FCS
7TD1	M, myeloid	J Van Snick, Ludwig Inst. Cancer Res., Brussels	RPMI-NS
A375	H, melanoma	D. Haynes, Pathology, Uni. of Adelaide, Adelaide	RPMI-FCS
CTLL	M, T-cell	L Ashman, Hanson Centre for Cancer Res., Adelaide	RPMI-FCS
eEND.2	M, endothelial	L Williams, Ludwig inst. Cancer Res., Melbourne	DMEM-FCS
EL-4	M, T-cell	Kotlarski, Immunology, Uni. of Adelaide, Adelaide	RPMI-FCS
EL-4 EU	M, T-cell	A Kelso, WEHI, Melbourne	RPMI-FCS
F4/ 80	R/M, hybridoma	P Kenny, Clin. Immunol., Flinders Uni., Adelaide	RPMI-FCS
FD 5/12	M, myeloid	A Kelso, WEHI, Melbourne	RPMI-FCS
J774A.10	M, macrophage	ATCC, Rockville, Maryland	IMDM-FCS
L929	M, fibroblast	D. Haynes, Pathology, Uni. of Adelaide, Adelaide	RPMI-FCS
L cells	M, fibroblast	A Lopez, Hanson Centre for Cancer Res., Adelaide	Hams F12-FCS
MLA144	G, T-cell	L Ashman, Hanson Centre for Cancer Res., Adelaide	RPMI-FCS
TIB 104	R/M, hybridoma	ATCC, Rockville, Maryland	RPMI-FCS
TIB 107	R/M, hybridoma	ATCC, Rockville, Maryland	RPMI-FCS
TIB 120	R/M, hybridoma	ATCC, Rockville, Maryland	RPMI-FCS
TIB 122	R/M, hybridoma	ATCC, Rockville, Maryland	RPMI-FCS
TIB 128	R/M, hybridoma	ATCC, Rockville, Maryland	RPMI-FCS
TIB 207	R/M, hybridoma	ATCC, Rockville, Maryland	RPMI-FCS
TIB 213	R/M, hybridoma	ATCC, Rockville, Maryland	RPMI-FCS
WEHI-3BD-	M, promyeloid	A Lopez, Hanson Centre for Cancer Res., Adelaide	RPMI-FCS

2.4.3 Cell quantitation

The densities of cells in suspensions of cell lines, spleen cells or uterine cells were determined using a Neubauer haemocytometer (Assistent, Germany). Cells with a viable morphology within an area corresponding to 10^{-4} ml were counted using an Olympus BH-2 phase contrast microscope (Olympus, Lake Success, NY). The numbers of adherent cells in uterine cell cultures were determined by uptake of Rose Bengal dye as described in 2.10.1a.

2.5 Embryo harvesting and culture

Fertilised eggs or two-cell embryos were flushed from the oviducts of mated, superovulated mice 22-24 h post hCG or 46-48 h post-hCG respectively with HEPES-HTF containing 300 IU/ ml hyaluronidase (Sigma) according to the procedure described by Hogan *et al.* (1986). Eight-cell stage embryos were flushed from the oviducts and uteri of mated, superovulated mice 70-72 h post hCG with DMEM-Em containing 20 mM HEPES. Blastocysts were flushed from the uteri of mated, superovulated mice 92-94 h post hCG with DMEM-Em containing 20 mM HEPES. Approximately 20-30 healthy embryos or blastocysts were obtained per Balb/c x C57 female, and 10-15 per Balb/c female. In some experiments the zona of 8-cells, morulae or blastocysts were removed by incubation for a few minutes (until zona was no longer visible) in acidic Tyrode solution (Hogan *et al.* 1986) or 0.5% pronase (Boehringer Mannheim) in phosphate buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 pH 7.2).

Embryos were handled and cultured under aseptic and pyrogen-free conditions as described for cell culture in section 2.4.1. A 'mouth pipette', constructed from a sterile Pasteur pipette, drawn over a flame to a fine diameter, and connected to plastic tubing, was used to physically manipulate embryos. Fertilised eggs were cultured in 10 µl microdrops of HTF containing 5 mg/ ml BSA under paraffin oil in 35 mm plastic Petri dishes (Disposable Products, Adelaide, SA) and 8-cell embryos were cultured in DMEM-Em in 1 ml wells of 4-well dishes (Nunc, Roskilde, Denmark). All cultures were incubated at 37°C in a 10% CO_2 in air, humidified atmosphere.

2.6 Measurement of embryonic viability and development

2.6.1 Microscopic assessment of developmental stage and viability of embryos

The developmental stage of embryos in culture was assessed visually with the aid of an inverted or dissecting microscope. Preimplantation embryos were scored as 1, 2, 4, or 8-cell, morulla, blastocyst, hatching blastocyst, or hatched blastocyst according to the criteria described by Hsu (1979). Blastocysts were defined as attached when gentle swirling of the culture dish failed to dislodge them. Blastocysts were defined as 'implanted' when differentiated trophectoderm, morphologically identifiable as adherent cytoplasmic projections extending from the base of the attached blastocyst, were visible.

2.6.2 *Quantitation of nuclei in embryos by PI-Hoescht staining*

The numbers and viability of cells in morulae and blastocysts were determined following nuclear staining with the fluorescent dyes acridine orange, or bisbenzimide (Hoescht 33342; Sigma) together with propidium iodide (PI; Sigma), essentially as described by Papaioannou and Ebert (1988). Stock solutions of 5 mg/ ml acridine orange (Sigma), 10 mg/ ml Hoescht 33342 (Sigma) and 100 mg/ ml propidium iodide (Sigma) were prepared in MQ water. Embryos were incubated in 20 µg/ ml Hoescht together with 100 µg/ ml propidium iodide in DMEM-FCS for 30 min at 37°C. Following a brief wash in DMEM-FCS, embryos were mounted on a microscope slide under a coverslip and viewed with the aid of an Olympus BH-2 fluorescent microscope fitted with a 400 nm excitation filter. Blue nuclei were scored as viable and pink nuclei as dead.

Similarly, embryos were stained in 10 µg/ ml of acridine orange for 5 min at 37°C, washed, mounted and viewed with the aid of a fluorescent microscope fitted with a 500 nm excitation filter. Nuclei exhibited apple green fluorescence.

2.6.3 *Measurement of DNA synthesis in 'implanted' embryos*

Embryos harvested at 72h post hCG (8-cells/ morulae) were cultured individually in 200 µl DMEM-FCS in 96 well microtitre trays (Costar, Cambridge, MA). On day 4 of culture, approximately 24 after 'implantation', embryos were pulsed with 1 µCi/ ml ^{3}H-thymidine (Amersham, Arlington Heights, IL) for 6 h, and harvested onto glass fibre paper (Enzo Diagnostics, NY) using a Titretech automated cell harvester. Scintillant (Ready-Safe, Beckman Instruments Inc., Fullerton, CA), was added to vials containing individual paper discs and radioactivity was measured as disintegrations per minute (dpm) in a liquid scintillation beta counter (Beckman).

2.7 Cytokine-enriched conditioned media

Conditioned media (CM) from L cells, MLA-144, WEHI-3BD^{-} and EL-4 cell lines were prepared in DMEM-Em for use in embryo co-culture experiments. EL-4 cells were grown to 2 x 10^{6} cells/ ml, then phorbol mystic acid (PMA) was added to 10 ng/ ml and supernatants harvested 24 h later. L cells, EL-4 and WEHI-3BD- were each cultured for 72 h from a starting density of 1 x 10^{5} cells/ ml. Lung cell CM (LCCM) was prepared according to the method of Burgess *et al.* (1977; 1985). 10 Balb/c female mice were injected iv with 5 µg LPS in saline. Three hours later lungs were collected, roughly chopped, and incubated for 48 h in 50 ml culture flasks (Nunc) cotaining 5 ml DMEM-Em and 10 mM LiCl. LCCM was then cleared by centrifugation at 1000 g. Both EL-4 and LCCM were dialysed twice against 100 volumes of PBS, then once against 50 volumes of DMEM. All CM were sterilised by filtration through 0.22 µm filters (Millex).

To generate CM for comparison of GM-CSF output on a 'per cell' basis, various normal and transformed cells were each cultured for 24 h with a starting density of 2 x 10^{5} cells/ ml. Spleen cells and peritoneal exudate cells (PEC) were harvested from adult [Balb/c x C57Bl] F1 female mice. Spleen cells were cultured at 1 x 10^{6}/ ml in RPMI-FCS with 4 µg/ ml PHA (Sigma) for 48 h,

washed, and supernatants collected after 24 h further culture at 2 x 10^5/ ml in RPMI-FCS with 4 μg/ ml PHA and 10 ng/ ml PMA (Sigma). PEC were cultured in RPMI-FCS with 5 μg/ ml *S. typhimurium* LPS (gift of I. Kotlarski, University of Adelaide). EL-4.BU cells were cultured with 10 ng/ ml PMA, J774A.1 cells with 5 μg/ ml LPS, and L cells with 5 μg/ ml LPS, in media specified in Table 2.1. Supernatants were stored at -80°C prior to GM-CSF and IL-6 bioassay.

2.8 Cytokine bioassays

All cytokine assays were performed in flat-bottomed 96-well microtitre trays (Costar) and standardised against recombinant cytokines. GM-CSF and IL-6 titres were determined from dose response curves. Minimum detectable amounts were GM-CSF: 1 U/ ml [50 U/ ml defined as the concentration of GM-CSF stimulating half maximal FD5/12 cell proliferation; equivalent in our hands to (1) 1 pg/ ml of yeast-derived recombinant murine GM-CSF, or (2) 50 CFUc U/ ml, where 50 U/ ml stimulates half maximal colony development in a bone marrow assay], IL-2: 1 BRMP (NIH Biological Response Modifiers Program) U/ ml, IL-3: 1 CFUc U/ ml, IL-6: 5 U/ ml (50 U/ ml defined as producing half maximal 7TD1 growth), and IL-1 and TNFα: 10 U/ ml (50 U/ ml defined as 50% cytolytic activity).

2.8.1 GM-CSF bioassay

GM-CSF was assayed using the GM-CSF-dependant cell line FD5/12, essentially as described by Kelso and Owens (1988). Duplicate serial 1:2 dilutions of uterine fluids or culture supernatants were incubated with 2000 FD5/12 cells in 200 μl of RPMI-FCS. After 2 days, cultures were pulsed with 1 μCi of ^{3}H thymidine (Amersham) for 6 h, then harvested and counted as described in 2.6.3.

2.8.2 IL-6 bioassay

IL-6 was assayed using the IL-6-dependant cell line 7TD1 (Van Snick *et al.* 1986). Duplicate serial dilutions of samples were incubated with 2000 7TD1 cells in 200 μl RPMI-NS. After 3 days, the number of cells was estimated colorimetrically by conversion of the tetrazolium salt MTT to formazan in a modification of a method described by Mossman (1983). Briefly, 25 μl of 4 mg/ ml MTT (Sigma) in RPMI was added to each well. After incubation for 4 h at 37°C, media was aspirated carefully and formazan product was solubilised by addition of 100 μl of 95% ethanol, and absorbance was measured at 570 nm using a multiwell ELISA reader.

2.8.3 TNFα and IL-1 bioassays

TNFα and IL-1 were assayed in cytotoxicity assays employing respectively the TNFα sensitive cell line L929 as described by Matthews and Neale (1987) and the IL-1 sensitive human melanoma cell line A375 as described by Nakei *et al.* (1988). Duplicate serial dilutions were incubated with 20,000 L929 cells in 200 μl of RPMI-FCS and 4 μg/ ml cycloheximide overnight, or

with 2000 A375 cells in 200 µl of RPMI-FCS for 4 days. Cell lysis was measured by methyl violet uptake (0.5% in 20% methanol for 10 min at room temperature). Incorporated dye was dissolved in 50% acetic acid and quantitated by measuring absorbence at 570 nm using a multiwell ELISA reader.

2.8.4 IL-2 and IL-3 bioassays

IL-2 and IL-3 were assayed using respectively the IL-2 dependent T-cell line CTLL (Gillis and Watson, 1980) and the IL-3 dependent cell line 32Dcl3 (Greenberger *et al.* 1983) as described by Kelso and Owens (1988). Duplicate serial dilutions of samples were incubated with 500 CTLL cells for 3 days or 2000 32D cells for 2 days in 200 µl of RPMI-FCS. Cultures were pulsed and harvested as described for the GM-CSF assay.

2.8.5 CFUc bioassay

Clonal culture of murine bone marrow cells was performed according to Metcalf (1984). Bone marrow cells were extruded from the femur of adult [Balb/c x C57Bl] F1 female mice. Fifty-thousand cells were cultured in 1 ml of half-strength Iscoves modified DMEM containing 25% FCS and 0.33% Bacto-agar (Difco Laboratories, MI), and colony stimulating factor from various sources (see below) in 35 mm plastic Petri dishes (Disposable Products). Cultures were performed in quadruplicate, and incubated in air-tight boxes gassed with 5% CO_2/ 5% O_2/ 90% N_2. Fourteen days later, the numbers of colonies in cultures were determined by visual inspection (with the aid of a dissecting microscope) and individual colonies were identified morphologically as of macrophage, granulocyte, or mixed lineage composition.

Supernatants from uterine cells or cytokine-enriched CM (derived as described in 2.8) were added to bone marrow cell cultures at final concentrations ranging from 1:10 to 1:2500. The colony stimulating activity (CSA) of each was determined by comparison of dose response curves to recombinant murine GM-CSF and CSF-1 standards included in the same assay at 1 to 1000 U/ ml.

2.9 Uterine cell cultures

2.9.1 Ovariectomised, cycling and pre-implantation pregnant uteri

a. Epithelial cell-enriched endometrial cell cultures

Uterine endometrial cell monolayers were prepared under sterile conditions from whole uteri using a modification of a procedure described by Sherman (1978) for harvesting an epithelial cell-enriched endometrial cell population. Immunofluorescence analysis using lineage-specific mAbs showed that these cultures were composed of approximately 70% epithelial cells, 25% stromal fibroblasts and 5% leukocytes and endothelial cells. Between 1000 h and 1200 h on the day of oestrus or day 1 of pregnancy, uteri from individual mice were excised, placed in cold PBS pH 7.2, trimmed of fat, mesentery and blood vessels and slit lengthwise. After another rinse in PBS, pairs

of uterine horns were incubated in 1 ml of 0.5% trypsin (Sigma) plus 2.5% pancreatin (bovine pancreatic, type III)(Sigma) in PBS for 45 min at 4°C, followed by 45 min at 37°C. In some experiments, collagenase (Sigma type II) was used at 250 U/ ml in the place of trypsin and pancreatin. One millilitre of DMEM-FCS was added and the uteri agitated gently by passage 3-4 times up and down a plastic Pasteur pipette. The supernatant contained epithelial and other liberated cells which were pelleted at 200 g and washed in DMEM-FCS. The cell yield was 7-14 x 10^5 cells per uterus for oestrous and day 1 mice.

Harvested cells were plated in duplicate at 1-3 x 10^5/ ml in 1 ml of DMEM-FCS in 4 well multidishes (Nunc) and incubated at 37°C in 5% CO_2 in air. Culture supernatants were collected 24 h later (unless otherwise stated), centrifuged at 2000 g and the supernatant stored at -80°C prior to cytokine bioassay. Adherent cells were quantified after uptake of Rose Bengal dye (Faulding, Adelaide, SA)(0.25% in PBS) and lysis in 1% SDS by measurement of absorbence at 540 nm, by comparison to a standard curve constructed by counting the number of cells harvested with 2 mM EDTA from duplicate wells in a haemocytometer.

b. Stromal cell cultures

A cell fraction enriched in stromal cells was obtained from the tissue remaining after stripping of epithelial cells. Immunofluorescence analysis showed approximately 80% of the cells in these cultures were stromal fibroblasts, and the remaining 20% were epithelial cells, leukocytes and endothelial cells. Pairs of uterine horns were transferred to 2 ml of 0.5% trypsin, 20 mM EDTA in PBS, finely minced with sterile scissors, and incubated for 30 min at 37°C. FCS was added to a concentration of 10% and the fragments were further dissociated by pipetting and allowed to settle. The supernatant was collected, centrifuged at 200 g and the pellet washed in DMEM-FCS. Cells were cultured and supernatants collected as described in 2.10.1a.

c. Mixed uterine cell cultures

In some experiments cells were harvested from minced whole uteri (including epithelial cell layer) by trypsin / EDTA treatment as described in 2.10.1b, or by incubation of intact uteri in 300 U/ ml collagenase (Sigma, type II) in PBS in place of trypsin/ pancreatin. Immunofluorescence analysis showed that these cultures were composed primarily of epithelial and stromal fibroblast cells, in approximately equal proportions. Cells were cultured and supernatants collected as described in 2.10.1a.

2.9.2 Day 10 uteri

Uteri excised from day 10 pregnant mice were slit lengthwise midway between the mesometrial and the antimesometrial sides and conceptus derived structures were completely removed with the aid of a dissecting microscope. Endometrial cells were prepared by the same trypsin/ pancreatin digestion and subsequent processing described in 2.10.1a. Separate populations

were harvested from uterine tissue dissected from (1) between embryonic units (inter-conceptus); (2) the antimesometrial side of implantation sites (peri-conceptus), and (3) the mesometrial metrial gland region underlying the placenta (MG); individual nodules (2-3 mm in diameter) were dissected free of surrounding uterine tissue, and 4-6 x 10^4 cells were obtained per nodule. Cells were cultured and supernatants harvested as described in 2.10.1a.

2.10 Uterine luminal fluid collection

Uterine intraluminal fluid was harvested essentially as described by Khurana and Wales (1989). Mice were killed by cervical dislocation between 1000 h and 1200 h on the day specified. Individual horns were isolated by cutting near the oviduct and the cervix. A blunt 26-gauge needle attached to a 1 ml syringe containing RPMI-FCS was then inserted into the oviductal end of the uterine horn and uterine washings (25-50 µl in 500 µl RPMI-FCS per horn) obtained via the cervical end. Flushings were collected over a period of 30-40 seconds to minimise tissue damage, then centrifuged at 2000 g and the supernatant stored at -80°C prior to cytokine bioassay.

2.11 Male accessory gland fluid collection

The seminal vesicle, prostate and coagulating gland secretions of adult fertile and vasectomised male mice were collected by excising the appropriate gland and dispersing the contents and tissues in 1.0 ml of RPMI per gland or pair of glands with the aid of fine needles. Epididymal secretions were collected by flushing the ampullary region of the epididymis with 1.0 ml of RPMI using a blunt 26 gauge needle. All preparations were pelleted at 5000 g, then supernatants collected and stored at -80°C until cytokine assay.

In some experiments 50-100 µl of neat seminal vesicle fluid was collected directly into a 1 ml insulin syringe with a 27 gauge needle attached (Terumo, Melbourne, Vic.) by insertion of the needle into the proximal end of the intact gland *in situ*, within 1-2 minutes after killing the animal. This fluid was either diluted in DMEM-FCS and added to uterine cell cultures, or diluted 1:2 into 6 M guanidine HCl and subjected to fractionation protocols described in 2.17.2.

2.12 Antibody plus complement-mediated depletion of uterine T-lymphocytes

A protocol for antibody plus complement-mediated lysis of T-lymphocytes was established using spleen cells. The incorporation of ^{3}H-thymidine by spleen cells incubated with 4 µg/ ml of PHA (Sigma) and normal peritoneal cells (at a ratio of 10:1) was reduced to 8% of the control value after the incubation of spleen cells in αThy1.2 and 5% guinea pig serum (CSL, Australia) for 90 min at 37°C. Incubation of spleen cells in antibody or complement alone did not inhibit their subsequent proliferation.

Pancreatin/ trypsin-dissociated uterine cells were cultured for 16-18 h and adherent cells harvested with 200 µM EDTA. Cells were incubated with αThy1.2 or other mAbs alone or in the presence of 5% guinea pig serum for 90 min at 37°C, then cultured for a further 24 h either in 1 ml

culture wells or in 500 µl Lab-Tek 8-well chamber slides (Nunc), when supernatants were harvested and assessed for GM-CSF content. In some experiments the numbers of mAb-reactive cells present in cultures at the conclusion of the culture period were assessed by immunofluorescence.

2.13 Uterine cell subpopulation isolation

2.13.1 Density gradient centrifugation of uterine cells

Pancreatin/ trypsin-dissociated uterine cells were cultured for 16-18 h and adherent cells harvested with 200 µM EDTA. Clumps of cells were removed by passage through cotton wool. Approximately 2 x 10^6 cells were layered over a Percoll (Pharmacia) density gradient composed of 5 x 2 ml density steps of 50, 40, 30, 20 and 10% Percoll in PBS, and centrifuged for 300 g/ 30 min/ 4°C. Bands of cells were recovered from each density interface, washed, counted and cultured in DMEM-FCS for an additional 24 h. Supernatants were harvested and stored for cytokine assay at -80°C.

2.13.2 Panning of mAb-reactive uterine cells

Pancreatin/ trypsin-dissociated uterine or decidual cells were cultured for 16-18 h and adherent cells harvested with 200 µM EDTA. They were then incubated with individual mAbs (1:2 dilution of hybridoma supernatant in Hanks buffered salt solution with 10% FCS and 10% normal mouse serum [HBSS-NMS]) for 1 h at 4°C. The labelled cell suspensions were washed, then 'panned' by a modification of the procedure described by Wysocki and Sato (1988), using Petri dishes (Disposable Products) coated with affinity purified goat αrat (Calbiochem, La Jolla, CA)(10 µg/ ml in PBS for 24 h at 4°C). Monoclonal antibody-labelled uterine cells were incubated in coated Petri dishes for 2 h at 4°C in HBSS-NMS, (or in uncoated dishes for control) and the non-adherent cells were collected. Adherent cells were detached by incubation in normal rat serum (10% in HBSS-NMS, 20 min at 37°C) followed by forceful pipetting. Both populations were cultured in DMEM-FCS for 24 h and the supernatants assayed for cytokine content. Cells were quantitated by Rose Bengal dye uptake (2.10.1a). GM-CSF or IL-6 synthesis by adherent, non-adherent or control (unpanned) cells is expressed in U per 10^5 cells. Values are normalised to the overall mean cytokine production per 10^5 control cells for the set of panning experiments, thus allowing comparison of cytokine synthesis by the isolated sub-populations from each experiment. In some experiments, the proportion of mAb labelled cells was determined by immunofluorescence analysis (2.14).

2.14 Immunofluorescence analysis

The proportions of cells within suspensions of uterine or other cells were determined by immunofluorescence analysis, using mAbs specific for a variety of cell lineages (Table 2.2). Approximately 5 x 10^5 cells were incubated in mAb for 1 h at 4°C. MAbs were used as neat supernatants containing 10% NMS, except for F4/ 80 which was precipitated in 33% ammonium

sulphate, dissolved in 1/ 10th volume of PBS, dialysed against 3 x 100 volumes of PBS pH 7.2, then concentrated 10-fold to achieve an approximately 100-fold concentrate prior to use. Cells were washed 4 x in PBS containing 10% FCS (PBS-FCS), and incubated for 1 h at 4°C in sheep αrat-FITC (Silenus) diluted 1:20 in PBS-FCS containing 10% NMS, then washed 4 x in PBS-FCS prior to microscopic examination using an Olympus BH-2 fluorescent microscope fitted with a 500 nm excitation filter. Labelled cells were identified as those exhibiting apple green fluorescence.

2.15 Immunohistochemistry

Monoclonal antibodies specific for a variety of cell lineages (Table 2.2) were used to determine the distribution of reactive cells in intact tissue. Uteri from cycling or pregnant mice, or from ovariectomised mice following intraluminal injection of GM-CSF, were placed in OCT compound (Tissue-Tek, Miles Inc., Elkhar, IN) and frozen by immersion in liquid N_2 cooled isopentane (BDH Chemicals). Sections (6 μm thick) were cut on a Bright (Huntingdon, UK) model OTF cryostat and air-dried, then fixed in 96% ethanol (BDH) at 4°C for 10 min. Sections were incubated in mAbs for 18 h at 4°C, washed 3 times in PBS, incubated in goat αrat-HRP (Dakopatts, Copenhagen)(1:20 in PBS containing 1% BSA and 10% normal mouse serum (PBS-NMS) for 2 h at 4°C), and washed again 3 times in PBS. All incubations were carried out in humidified chambers. MAbs were used as neat supernatants containing 10% NMS, except for F4/ 80 which was prepared as described in 2.15 and diluted 1:10 in PBS- NMS. Reactivity was visualised by incubating slides in diaminobenzidine (DAB; Sigma)(5 mg/ ml in 0.05 M Tris-HCl pH 7.2) plus 0.02% hydrogen peroxide for 10 min at room temperature. Tissue was counterstained in Gill's haematoxylin (Sigma), dehydrated in 2 changes of absolute ethanol, cleared in 2 changes of Safsolvent (Ajax Chemicals, Auburn, NSW), mounted in Depex (BDH) and viewed and photographed using an Olympus BH-2 light microscope and black and white Pan F film, or 100 ASA colour film (Ilford Ltd., Cheshire, UK).

In some experiments, sections were stained in Gill's haematoxylin and washed in tap water, prior to staining for eosinophils in 1% Congo Red (Gurr, BDH) for 20 min, and destaining in 3 x 30 sec washes of 50% ethanol. Sections were air-dried, then cleared in 2 changes of Safsolvent prior to mounting in Depex.

2.16 Chromatography

2.16.1 Endometrial cell conditioned media

Conditioned medium was prepared from pooled endometrial cells harvested from 10 oestrous mice. Cells were cultured at 2 x 10^5 cells/ ml for 24 h in DMEM-FCS and then 48 h in serum-free DMEM-NS. Eighty millilitres of conditioned medium was filtered (0.22 μm Millex-GS; Millipore, Bedford, MA), concentrated to 5 ml over a PM10 membrane (Amicon, Danvers, MA), dialysed against PBS pH 7.0, concentrated to 0.5 ml over a YM2 membrane (Amicon), and chromatographed on a Sephacryl S-200 column (1.0 x 30 cm)(Pharmacia, Uppsala) equilibrated in

6 M guanidine HCl at a flow rate of 18 ml/ h. Fractions of 0.45 ml were collected into 5 mg/ ml BSA, and desalted on calibrated 1 ml columns of Sephadex G-25 superfine (Pharmacia) by spin-gel filtration. Aliquots from fractions were diluted 1:10 in RPMI, filtered (0.22 μm Millex-GV; Millipore) and assayed for GM-CSF and IL-6. Molecular weights were estimated from a linear plot of log molecular weight *versus* V_e/ V_o (elution volume/ void volume), using bovine serum albumin, ovalbumin, α-chymotrypsinogen and cytochrome-c as standards.

2.16.2 Seminal vesicle fluid

Neat seminal vesicle fluid was collected as described in 2.12, and diluted 1:5 into 6 M guanidine HCl. In some experiments this material was desalted over 5 ml of Sepharose Superfine G-25 (5,000 Mr cut-off) (Pharmacia) equilibrated in DMEM. The 3 ml eluate was assessed for GM-CSF-stimulating activity by culture with oestrus endometrial cells. Endometrial cells were harvested from oestrus mice, and 3 h after the initiation of the culture eluate was added at a 1:2 dilution (= 1:30 dilution of seminal vesicle fluid) to duplicate wells and incubated for 16 h, when the supernatants were removed and replaced with fresh media, and the morphology of the cultured cells was examined microscopically. Following an additional 24 h culture period, supernatants were harvested and assayed for GM-CSF content.

In other experiments seminal vesicle fluid dissolved in guanidine HCl was chromatographed on a Sepharose CL4B column (1.0 x 30 cm)(Pharmacia) equilibrated in phenol red-free HBSS at a flow rate of 20 ml/ h. Fractions of 1.5 ml were collected, and 250 μl aliquots were assessed for GM-CSF-stimulating activity by addition to 250 μl cultures of endometrial cells in DMEM-(20%) FCS containing 100 μg/ ml gentamycin sulphate in addition to the usual supplements.

2.16.3 Protein-G sepharose

Hybridoma supernatants containing rat IgG2b immunoglobulin (Ig) were filtered (0.22 μm; Millex), and loaded onto a 10 ml Protein-G Sepharose 4 Fast Flow column (Pharmacia) equilibrated in 20 mM Na phosphate, pH 7.0, at a flow rate of 0.8 ml/ min. After extensive washing with 20 mM phosphate pH 7.0, pure immunoglobulin was eluted in 0.1 M glycine HCl, pH 2.7, and dialysed against two 50 x volumes of PBS pH 7.2. The immunoglobulin content of eluted fractions was estimated by measuring absorbence at 280 nm.

2.17 GM-CSF receptor analysis

2.17.1 FD 5/12 cells

Iodinated *E.coli*-derived recombinant murine GM-CSF (^{125}I-GM-CSF; specific activity 50,000 cpm/ ng) was generously provided by N. Nicola (WEHI). A protocol for the use of this material in autoradiographic analysis of GM-CSF receptor expression was established using the GM-CSF responsive myeloid cell line FD 5/12. Suspensions of approximately 5 x 10^6 cells in 100

µl PBS-FCS were incubated for 60 min/ 20°C with either 250,000 or 50,000 cpm of ^{125}I-GM-CSF (50 or 10 ng ^{125}I-GM-CSF/ ml). Control suspensions were incubated in PBS-FCS alone, and in the presence of a 10x excess (500 ng/ ml) of cold recombinant yeast-derived GM-CSF as well as 50 ng/ ml ^{125}I-GM-CSF. After 3 washes in PBS, smears of cells from each treatment were made onto gelatin-coated microscope slides. Smears were air-dried and post-fixed for 10 min in 2.5% gluteraldehyde in PBS.

Slides were dipped in NTB-2 (Eastman Kodak Co., Rochester, NY) nuclear emulsion at 42-45°C under a # 2 (Kodak) dark red safelight. To allow emulsion to gel, slides were placed flat on an aluminium cold bench for 15 min, then emulsion was allowed to dry completely O/N at room temperature. Following incubation in light-tight boxes at 4°C for 14 days, slides were developed for 4 min in D19 (Kodak) , and fixed in Hypam Rapid-fix (Ilford) diluted 1:4 in H_20. Cell smears were counterstained in hematoxylin as described in 2.15, and examined and photographed with a Nikon Microphot-FXA (Nippon Kogaku K.K., Japan) under (a) brightfield and (b) darkfield illumination.

2.17.2 Blastocysts

Blastocysts harvested from the uterus of superovulated mice early on the morning of day 4 of pregnancy were stripped of their zona pellucidae in 0.5% pronase in PBS, then co-cultured for 8 h on confluent eEND.2 monolayers in Em-DMEM in 1 ml wells (Nunc). Hatched blastocysts continue to expand but fail to attach and 'implant' onto eEND.2 cells (S. Robertson, unpublished data). Blastocysts were incubated with 50 ng/ ml ^{125}I-GM-CSF (as in 2.18.1) for 60 min at 20°C in 25 µl drops of DMEM-FCS under paraffin oil in 35 mm plastic Petri dishes. After rapid washing in 5 x 1 ml DMEM-FCS, blastocysts were fixed in 2.5% gluteraldehyde in PBS for 10 min, and dried onto gelatin coated microscope slides.

Controls included blastocysts incubated in the absence of ^{125}I-GM-CSF, and blastocysts incubated in the presence of a 10x excess of cold recombinant yeast-derived GM-CSF as well as ^{125}I-GM-CSF. Slides were coated in emulsion, incubated for 14 days, developed, and photographed as described in 2.17.1, except that Giemsa (Gurr, BDH) was used as the counterstain in place of hematoxylin.

2.17.3 Uterine tissue

Ethanol-fixed fresh frozen sections of day 1 uterus were incubated with 50 ng/ ml ^{125}I-GM-CSF (as in 2.17.1) in PBS-FCS for 60 min at 20°C, washed and post-fixed in 2.5% gluteraldehyde for 10 min. Control sections were incubated in the absence of ^{125}I-GM-CSF, or the presence of a 10x molar excess of cold recombinant yeast-derived GM-CSF in addition to ^{125}I-GM-CSF. Sections were coated in emulsion, incubated for 14 days, developed, counterstained and photographed as described in 2.17.1.

2.18 General procedures for RNAse-free conditions

Ribonuclease (RNAse)-free conditions were maintained for materials, chemicals and solutions used in the generation and handling of riboprobes, preparation and processing of tissue and cell culture-derived RNA used in Northern blot analysis, and preparation and processing of tissue used in *in situ* hybridisation. Precaution against RNAse contamination included use of disposable plastic pipettes and tubes where possible, baking of glassware, stainless steel-ware and fleas at 160°C O/N prior to use, DEPC (Sigma)-treatment of H_2O and solutions (as described by Sambrook *et al.* 1989), and soaking of gel-boxes in 10% H_2O_2 prior to use. In addition, disposable plastic gloves were worn throughout these experiments.

2.19 Riboprobe preparation

2.19.1 Growth and maintenance of bacterial strains

The *Escherichia coli* strain E381 was used throughout and cultured in either nutrient broth (NB)(Difco), or on nutrient agar (NA)(Difco). Ampicillin (amp; Sigma) was added to broth and solid media at 50 µg/ ml for culture of plasmid-containing derivatives of E381. Incubations were at 37°C.

All strains were maintained as frozen stocks in glycerol (32% v/v) and peptone (0.6% w/v) stored at -80°C. Fresh cultures from glycerols were prepared by streaking a loopful of glycerol suspension onto a NA plate.

2.19.2 Plasmids and DNA

Complementary DNA (cDNA) for murine GM-CSF (398 bp *Bam*H1-*Eco*RV fragment from pGM5'D17)(Gough et 1985) and β_2-microglobulin (β_2-MG; 300 bp *Pst*1 fragment)(Parnes *et al.* 1983) in the amp-resistant (amp^R) gene-containing plasmid SP65 were kindly provided by N Gough (WEHI). A1 *Giardia lamblia* cDNA (450 bp *Eco*R1-*Sac*1 fragment) in the amp^R gene-containing plasmid pGEM7 was kindly provided by P Ey (Department of Microbiology and Immunology, University of Adelaide).

2.19.3 Transformation

cDNA containing plasmids were amplified by transformation into E381. E381 bacterial cells were made competent by treatment with $CaCl_2$ and $MgCl_2$ (Brown *et al.* 1979). For transformation, approximately 1 µg of each DNA was mixed with approximately 10^9 competent bacterial cells in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and incubated at 4°C for 45 min followed by 42°C for 2 min. Following a further 10 min at 4°C, 200 µl of NB was added and bacteria were grown for 45 min at 37°C, then plated on amp^+ NA plates and cultured O/N.

2.19.3 Plasmid purification

a. Small scale plasmid preparation

For qualitative analysis, plasmid was extracted from approximately 10^9 transformed bacterial cells using a modification of the three-step alkali lysis method (Garger *et al.* 1983). Cells were resuspended in 100 µl solution 1 (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0), and 200 µl of solution 2 [0.2 N NaOH, 1% (w/v sodium dodecyl sulphate (SDS)] was added to induce cell lysis during a 5 min/ 4°C incubation. Protein, chromosomal DNA and high molecular weight RNA were precipitated by the addition of 150 µl of solution 3 (3 M potassium acetate, 2 M acetic acid, pH 4.8) for 5 min/ 4°C, and pelleted (5 min, Eppendorf 5414). Plasmid DNA was extracted from the supernatant in an equal volume of TE saturated phenol (BDH), chloroform and isoamyl alcohol mixture (25:24:1) and precipitated in 70% (v/v) ethanol and dried *in vacuo*.

b. Large scale plasmid preparation and purification by CsCl gradient centrifugation

Large amounts of plasmid DNA were prepared from approximately 10^{12} bacterial cells using a scaled-up version of the three step protocol described in 2.19.3a. Bacterial cells were incubated in 30 ml freshly prepared lysozyme (Sigma; 600 µg/ ml) in solution 1 for 10 min/ room temperature prior to addition of 55 ml of solution 2, and 5 min/ 4°C incubation. Twenty-eight millilitres of solution 3 was added, and after an additional 15 min/ 4°C, the precipitated material was pelleted at 5,000 g/ 15 min. Plasmid DNA was extracted from the supernatant in an equal volume of TE saturated phenol (BDH), chloroform and isoamyl alcohol mixture (25:24:1) and precipitated in a 0.6 volume of isopropyl alcohol (30 min/ 4°C). Precipitated plasmid DNA was washed in 70% (v/v) ethanol and dried (1h/ room temperature).

Plasmid DNA was purified from contaminating protein and RNA by centrifugation through a two step CsCl ethidium bromide (EtBr) gradient according to Garger *et al.* (1983). DNA was dissolved in 1.7 ml of 5.7 M CsCl in TE (refractive index = 1.4080), and layered beneath 3.4 ml of 4.3 M CsCl in TE (refractive index = 1.3780) in a 5.1 ml Beckman Quick-Seal polyallomer tube. Gradients were centrifuged at 65,000 rpm/ 5 h in a TLN-100 rotor (Beckman). The DNA band was collected using a 21 gauge needle and syringe, and the EtBr extracted 5 x into equal volumes of isopropanol. CsCl was removed by dialysis against 3 changes of a 50 x volume of TE at 4°C. DNA was quantitated by measurement of absorption at 260 nm and assuming an A_{260} of 1.0 is equal to 50 µg DNA/ ml (Miller 1972), and stored at -20°C.

2.19.4 Restriction endonuclease digestion and DNA electrophoresis

Plasmid DNA was analysed for appropriately sized cDNA inserts by restriction enzyme (RE) cleavage and electrophoresis. Excision of insert from plasmid was achieved by cleavage of 0.5-1 µg plasmid DNA with the restriction enzymes *Bam*H1 and *Eco*R1 (for GM-CSF), *Pst*1 (for β_2-MG) or *Sac*1 and *Eco*R1 (for A1)(all Boehringer Mannheim). Digestion was performed with 2-

10 U of enzyme per µg of DNA at 37°C/ 2 h in restriction enzyme buffer (Boehringer Mannheim) as recommended by the manufacturer.

Electrophoresis of digested DNA was carried out in a horizontal 1% agarose (Seakem HGT) 'minigel' in TBE buffer (67 mM Tris base, 22 mM boric acid, 2 mM EDTA, pH 8.8). Digested DNA (0.5-1 µg) containing tracking dye [0.01% (w/v) bromophenol blue in 15% (v/v) Ficoll] was electrophoresed at 10 V/ cm at room temperature, and DNA bands were visualised by trans-illumination with UV light following staining of the gel in 2 µg/ ml EtBr in water. Gels were photographed with Polaroid 667 positive film and the size of DNA bands determined by comparison of their relative mobility to *Eco*RI digested *Bacillus subtilis* bacteriophage SPP1 DNA.

2.19.5 Riboprobe synthesis by reverse transcription

a. Linearisation of template

'Antisense' ^{32}P-labelled RNA probes (riboprobes) were synthesised by reverse transcription *in vitro* from the SP65 or pGEM7 cDNA template. To prepare template, 5 µg of CsCl purified plasmid DNA was linearised with 40 U of *Hind*III (for GM-CSF or β_2-MG) or *Eco*R1 (for A1) for 4 h / 37°C. One microgram of linearised DNA was electrophoresed on a 'minigel' (2.19.4) to check that digestion was complete (ie. that no circular plasmid remained). Linearised DNA was then extracted in an equal volume of TE saturated phenol (BDH), chloroform and isoamyl alcohol mixture (25:24:1), precipitated in 70% ethanol and 100 mM Na acetate, dried *in vacuo* and resuspended to 200 µg/ ml in TE.

b. In vitro transcription

One microgram of linearised template was incubated with 2 U SP6 RNA polymerase (Bresatec) and 50-100 µCi (approx 3000 Ci/ mmol) ^{32}P-UTP (Bresatec) in a final volume of 20 µl reaction mixture containing 1 U/ ml placental RNAse inhibitor (Promega, Madison, WI), 40 mM Tris-HCl, 6 mM $MgCl_2$, 10mM dithiothreitol (DTT) and 0.5 mM ATP, CTP and GTP pH 7.6. In some experiments cold transcript was generated by including 0.5 mUTP in the place of ^{32}P-UTP. The reaction was allowed to proceed for 1 h at 37°C, then the DNA template was removed by adding 10 U of RNAse-free for a further 15 min at 37°C. The volume was made up to 200 µl with H_2O, and the percent incorporation of radiolabel was determined by comparing the TCA precipitable to total CPM in duplicate 1 µl aliquots. Eighty to ninety percent incorporation was routinely achieved. Radio-labelled riboprobe was extracted in an equal volume of TE saturated phenol, chloroform and isoamyl alcohol mixture (25:24:1), and precipitated in the presence of 20 µg carrier tRNA (Boehringer Mannheim) in 70% ethanol and 100 mM Na acetate (O/N, -20°C).

On the day of use, riboprobe was pelleted, dried *in vacuo*, resuspended in H_2O and the specific activity determined by counting a small aliquot in a beta-scintillation counter. Two to five hundred nanograms of riboprobe with a specific activity of 1-2 x 10^9 cpm/ µg was considered an average yield.

2.20 RNA analysis by Northern blotting

2.20.1 RNA extraction from tissue and cells

RNA was extracted from cultured cells or uterine tissue according to the method described by Chomczynski and Sacchi (1987). Briefly, 1-5 x 10^8 cells were harvested (with 2 mM EDTA for adherent uterine cells), pelleted and then rapidly denatured by the addition of 1 ml of 'solution D' (4 M guanidine thiocyanate, 0.5% sarkosyl, 0.1 M β–mercaptoethanol, 25 mM Na citrate pH 7.0). Sequentially, 0.1 ml of 2 M Na acetate pH 4.0, 1 ml of phenol (water saturated), and 0.2 ml of chloroform-isoamyl alcohol mixture (49:1) were added, and the mixture was vortexed and incubated at 4°C for 10 min. Uterine tissue was treated similarly, except that upon removal from the mouse a single uterus was added to 2 ml of solution D and disrupted using an Ultra-Turrax high speed homogeniser (Janke and Kunkel, Staufen, Germany). Samples were centrifuged at 10,000 g for 20 min at 4°C, and the RNA precipitated from the aqueous phase by addition of an equal volume of isopropanol (-20°C, 1-2 h). The RNA was then pelleted (10,000 g/ 20 min/ 4°C), redissolved in 500 µl solution D (by heating to 70°C), and reprecipitated then sedimented in an equal volume of isopropanol as above. The pellet was washed in 75% ethanol, dried *in vacuo*, and dissolved in H_2O for northern blot analysis. RNA was quantitated by measurement of absorption at 260 nm and assuming an A_{260} of 1.0 is equal to 40 µg RNA/ ml. RNA was judged acceptably free of contaminating protein if the A_{260}:A_{280} ratio was greater than 1.8. RNA was stored precipitated in 70% ethanol/ 30 mM Na acetate at -20°C.

In some experiments RNA from cell lines or tissues were further purified by centrifugation through a CsCl two-step gradient according to the method described by Davies *et al.* (1986). Following precipitation in isopropanol or ethanol, RNA was dissolved in 4 M guanidine thiocyanate, 0.1 M β–mercaptoethanol, 25 mM Na citrate pH 7.0 and 3.3 ml was layered over 1.7 ml of 5.7 M CsCl in a Beckman Quick-Seal 5.1 ml polyallomer tube. Gradients were centrifuged at 80,000 rpm/ 6 h in a TLN-100 rotor. RNA pellets were recovered after aspiration of the supernatant, and stored precipitated in 70% ethanol/ 30 mM Na acetate at -20°C.

Residual chromosomal DNA was removed from RNA prior to RT-PCR analysis by treatment with DNAse. RNA (1-50 µg) was incubated with 5 U of RNAse-free DNAse I (Bresatec) and 5 U of placental RNAse inhibitor (Promega) in 100 µl of 100 mM Tris pH 7.8, 2 mM EDTA, 20 mM $MgCl_2$, 0.2 mM DTT for 60 min at 37°C.

2.20.2 RNA extraction from oocytes and embryos

Total RNA was isolated from mouse oocytes and one cell, two cell, 8-cell and blastocyst stage embryos using an adaption of the phenol-chloroform extraction method originally described by Braude and Pelham (1979), as modified by Arcellana-Panlilio and Schultz (1993). Essentially, pools of 100-300 eggs or embryos were collected in FCS-free DMEM-Em in a 500 µl microcentrifuge tube and all but 5-10 µl of the supernatant aspirated. Pellets were stored at -80°C prior to RNA extraction. Three hundred microlitres of a 1:1:1 mixture of extraction buffer (0.2 M

NaCl, 25 mM Tris-HCl pH 7.4, 1 mM EDTA), TE (pH 7.4 Tris-HCl, 1 mM EDTA) equilibrated phenol and Sevag's solution (24:1 chloroform: isoamyl alcohol) containing 10 µg of tRNA (Sigma) was added to the frozen pellet at 4°C and vortexed for 3 x 10 sec bursts. After centrifugation (10 min/ 13,000 rpm/ room temperature) the aqueous phase was re-extracted with 200 µl Sevag's solution., then precipitated in 75% ethanol O/N at -20°C. RNA was treated with DNAse prior to use in RT-PCR as described in 2.20.1.

2.20.3 RNA electrophoresis

RNA was electrophoresed through 1% agarose gels (dimensions 15 x 15 x 0.4 cm) in 2.2 M formaldehyde, and 1 x running buffer [20 mM 3-(N-morpholino)propanesulphonic acid, 8 mM Na acetate and 1 mM EDTA (pH 7.0)] as described by Sambrook *et al.* (1989). RNA was denatured (by heating to 15 min at 65°C) in 0.5 x running buffer, 2 M formaldehyde, and 50% formamide. Loading buffer was added to samples to give final concentrations of 5% glycerol, and 0.025% bromophenol blue and xylene cyanol FF respectively. Ten to thirty micrograms of RNA was loaded per track, and the gel was run in 1 x running buffer at 3-4 V/ cm until the bromophenol blue had migrated two thirds of the total distance. Duplicate RNA samples and RNA molecular weight markers, containing 50 µg/ ml EtBr, were run in adjacent tracks and after electrophoresis these tracks were cut off and ribosomal RNA or molecular weight marker bands were visualised by trans-illumination with UV light. Gels were photographed with Polaroid 667 positive film to facilitate the sizing of RNA bands by comparison of relative mobilities after hybridisation and autoradiography.

2.20.4 Northern transfer and hybridisation

Transfer of RNA from agarose gels to nylon membrane (Hybond; Amersham) was performed by capillary elution in 20 x SSPE as described by Southern (1975) and modified by Sambrook *et al.* (1989).

Prior to hybridisation with radio-labelled riboprobe, filters were baked at 80°C for 2 h then incubated for 8-16 h at 65°C in a pre-hybridisation solution containing 50% formamide (v/v), 10% dextran sulphate (w/v), 1% SDS, 0.1% polyvinyl pyrrolidone (PVP), 0.1% Ficoll, 40 mM Tris-HCL pH 7.5, 5 x SSPE (750 mM NaCl, 44 mM NaH_2PO_4, 20 mM EDTA, pH 7.4) and 50 µg/ ml denatured salmon sperm DNA (ssDNA). Approximately 1 x 10^8 cpm of denatured riboprobe were then added and hybridisation was allowed to proceed O/N at 65°C. Filters were then washed 3 times in 1 x SSPE/ 0.1% SDS for 20 min at 65°C, once in 0.1 x SSPE/ 0.1% SDS for 20 min at 65°C. After a rinse in 2 x SSC (300 mM NaCl, 30 mM Na citrate pH 7.0), filters were incubated in 5 µg/ ml RNAse A (Sigma) in 2 x SSC for 30 min at 37°C to remove non-specifically bound probe, then washed twice in 1 x SSPE/ 0.1% SDS. Filters were then sealed within plastic bags and exposed to X-Omat K film (Kodak) for 24 h to 6 d at -70°C with intensifying screens. Film was developed for 3 min in D19 (Kodak) and fixed for 3 min in Hypam Rapid Fixer (Ilford) diluted 1:5 in H_2O.

2.21 RNA analysis by *in situ* hybridisation

Fresh frozen (6 µm thick) sections of uterine tissue on APES (Sigma) coated slides were dried for 20-30 min and then fixed in freshly prepared 4% paraformaldehyde/ 5mM $MgCl_2$ (in PBS, pH 7.2; 5 min/ room temperature). Following 2 x 5 min washes in PBS, tissue was acetylated in fresh 0.25% (v/v) acetic anhydride in 0.2 M triethanolamine pH 8.0 (10 min/ room temperature), washed 2 x 5 min in PBS, then dehydrated through a sequence of 40, 70, 90, and 2 x 100% ethanol (5 min each) and dried.

Denatured ^{32}P-labelled riboprobe was prepared in 1 x FDST (50% formamide, 10% dextran sulphate, 500 µg/ ml tRNA, 500 µg/ ml ssDNA), 1 x hybridisation mix (2 x SSC, 100 mM Tris-HCL pH 7.6, 10mM NaH_2PO_4, 10mM Na_2HPO_4, 0.02% Ficoll and 0.02% PVP), 0.5 mg/ ml BSA and 1 U/ ml placental RNAse inhibitor (Promega). Two and a half microlitres (containing 1-2 x 10^5 cpm) were applied to each section, and covered with a 12 mm diameter coverslip siliconised in Prosil (PCR Incorporated, Gainsville, FL). Slides were immersed in a paraffin oil bath and hybridisation was allowed to proceed for 16 h at 55°C. The oil was then removed from slides in 2 x chloroform washes (10 min/ room temperature), and following a rinse in 2 x SSC/ room temperature, coverslips were dislodged during two sequential 1 h washes in 2 x SSC/ room temperature . Non-specifically bound riboprobe was removed in two 10 min washes in 0.1 x SSC at 65°C, followed by two 1 h washes in 0.1 x SSC/ room temperature. Tissue was then dehydrated through a sequence of 40, 70, 90, and 2 x 100% ethanol (5 min each) and dried.

Slides were dipped in Ilford K5 nuclear emulsion diluted 1:2 in H_2O and 2% glycerol at 42-45°C, under a dark orange (Ilford 904) safelight. To allow emulsion to gel, slides were placed flat on an aluminium cold bench for 15 min, then emulsion was allowed to dry completely O/N at room temperature. Following incubation in light-tight boxes at 4°C for 4-8 days, slides were developed for 4 min in D19 (Kodak), fixed for 4 min in Hypam Rapid Fixer (Ilford) diluted 1:4 in H_20, and then counterstained in hematoxylin as described in 2.15, and examined and photographed with a Nikon Microphot-FXA (Nippon Kogaku K.K., Japan) under (a) brightfield and (b) darkfield illumination.

2.22 RNA analysis by reverse transcriptase-polymerase chain reaction (RT-PCR)

2.22.1 cDNA generation from RNA by reverse transcription

RNA was harvested from tissues, cell lines or embryos and treated with RNAse-free DNAse as described in 2.20.1 and 2.20.2. First strand cDNA synthesis was achieved by reverse transcription (RT) of RNA primed with oligo dT, employing a Superscript RNase H- reverse transcriptase kit (Gibco BRL) essentially according to the manufacturer's instructions. Approximately 1 µg of ethanol precipitated RNA from cell lines or tissue, or the RNA extracted from 100-300 mouse oocytes or embryos, was pelleted (15 min/ 13,000 rpm/ 4°C), washed once in 75% ethanol, and dried under vacuum. RNA was dissolved in 10 µl or RNAse free water, and

incubated with 1 µl of oligo dT (500 µg/ ml; Gibco BRL) at 70°C for 10 min, then chilled on ice for 5 min. Following addition of 4 µl of 5 x RT buffer (250 mM Tris-HCl pH 8.3, 300 mM KCl, 15 mM $MgCl_2$), 2 µl 0.1 M DTT, 2 µl 10 mM dNTP's (10 mM each dATP, dTTP, dCTP and dGTP), and 1 µl of RT enzyme the reaction was allowed to proceed for 90 min at 43°C, prior to RNA-cDNA denaturation and enzyme inactivation at 94°C for 5 min, and quenching on ice for 5 min. The cDNA (enough for 10 PCR reactions) was made up to 50 µl with water and stored at -20°C.

2.22.2 Construction of GM-CSF-R and AIC2B oligonucleotide primers

A primer pair specific for GM-CSF-R cDNA was designed with the aid of Primer Designer software (Scientific and Educational Software). Primer GMRa corresponds to the cDNA sequence from nucleotide 717 to 738, and primer GMRb is anti-sense to the cDNA sequence from nucleotide 930 to 951 (Park *et al.* 1992). The primer sequences are given in Table 2.3. Each primer is a 21-mer, with 57% GC content, a melting temperature of 73°C, and low primer-dimer potential either individually or in combination. The predicted amplified product is 235 bp in length with a melting temperature of 84°C and an annealing temperature of 59°C.

A primer pair specific for AIC2B cDNA, that does not amplify the closely related AIC2A cDNA has been designed and published by Fung *et al.* (1992). The primer MF44 is specific for AIC2B cDNA sequence from 595-618, and primer MF45 is antisense to the cDNA sequence from 898-920 of AIC2B cDNA (Gorman *et al.* 1990). The primer sequences are given in Fig 2.3. MF44 is a 24-mer and MF45 is a 23-mer. MF44 and MF45 have 37% and 43% GC content, and melting temperatures of 74 and 69°C respectively. The amplified product is a 326 bp fragment.

Table 2.3. Sequences of the primer pairs for GM-CSF-R (GMRa and GMRb), and for AIC2B (MF44 and MF45).

Primer	Length	Sequence
GMRa	21	5'-CCAGTGCTTCATCCTCGTGTC-3'
GMRb (complimentary strand)	21	5'-CACCGCGTCCTGTAACTCTTC-3'
MF44	24	5'-ATACACGATTTTCCATCACAAACG-3'
MF45 (complimentary strand)	23	5'-TAGATGCTGTTGGGTAGGAATAG-3'

Oligonucleotide primers were synthesised in a DNA synthesiser (Applied Biosystems, Foster City, CA) (operated by C. Cursaro in the Department of Microbiology and Immunology, University of Adelaide). Approximately 500 µg of each was recovered after purification by passage through an OPC cartridge (Applied Biosystems). A primer pair specific for murine actin cDNA, which generate a 381 bp product, was kindly provided by A. Watson, University of Western Ontario. Primers were stored at 100 µM in TE at -80°C.

2.22.3 Establishment of optimal PCR protocol for GM-CSF-R and AIC 2B primers

The PCR amplification employed reagents supplied in a Taq DNA polymerase kit (Bresatec) according to a protocol based on that described by Arcellana-Panlilio and Schultz (1993). For each 50 µl reaction volume the following were combined at 4°C; 5 µl of 10x PCR reaction buffer (670 mM Tris-HCl pH 8.8, 166 mM $(NH_4)_2SO_4$, 2 mg/ ml gelatin, 4.5% Triton X-100), 5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP's, 0.2 µl of 10 U/ µl Taq DNA polymerase, 1 µl of 100 µM 3' primer, 1 µl of 100 µM 5' primer, 5 µl of cDNA and 32 µl of water. 'Master mixes' were prepared and aliquoted across reaction tubes where possible to minimise inter-tube variation. Mixed contents were overlayed with 50 µl of paraffin oil. The tubes were placed in the thermocycler (Corbett Research, Australia) programmed to give the following temperature profile: (cycle 1) 5 min at 94°C; (cycle 2-n, where n = the number of amplification cycles), denaturation for 1 min at 94°C, annealing for 2 min at x°C (where x = 56-62°C), and extension for 2 min at 72°C; (cycle n+1) 7 min at 72°C, then soak at 20°C prior to short-term storage at 4°C or long-term storage at -20°C. Twenty microlitres of each reaction product was analysed by 'minigel' electrophoresis (2.19.4) through a 2% agarose (electrophoresis grade, Gibco BRL) gel containing 0.5 µg / ml EtBr in TAE buffer (40 mM Tris, 20 mM Na acetate, 1 mM EDTA, pH 7.2), and visualised by trans-illumination with UV light. Gels were photographed with Polaroid 665 negative film and the size of the PCR products were determined by comparison of their relative mobility to molecular weight markers (1 kb DNA ladder; Gibco).

In some experiments 1 µCi of ^{32}P dATP was included in the reaction mix. Following electrophoresis through agarose gels, reaction product was transferred to nylon membrane (Hybond) by rapid capillary elution in 1 M NaCl, 0.5 M NaOH buffer. Filters were neutralised, dried and autoradiographed for up to 10 days.

The number of amplification cycles (n) and the optimal annealing temperature (x) were determined empirically for each primer pair, employing FD 5/12 cDNA as a positive control and L cell and MLA 144 cDNA as murine and heterologous-species negative controls respectively. Optimal signal to noise ratios (ie a single band of amplification product in the absence of bands in negative controls) were obtained for GM-CSF-R and actin primers at 39 cycles at 62°C, and for AIC2B primers at 40 cycles at 56°C. PCR analysis of embryo cDNA was performed under these conditions. Positive (FD 5/12) cDNA and negative (no cDNA) controls were included in each PCR amplification.

2.22.4 Diagnostic analysis of PCR products by restriction enzyme analysis

The amplified GM-CSF-R and AIC2B reaction products each contain a number of restriction enzyme sites, and these were employed to verify the identity of the reaction product amplified by the GM-CSF-R and AIC2B primers. Reaction product was precipitated in 75% ethanol, washed in 75% ethanol and vacuum dried prior to dissolution in water for restriction enzyme analysis.

3 Cytokines and embryonic development

3.1 Introduction

Until the second half of the 1980's, lymphohemopoietic cytokines were commonly perceived as having roles exclusive to hemopoiesis or to regulation of the immune system. The physiological reason for the wealth of cytokine activity in the reproductive tract remained an enigma until recent years, when it became apparent that these factors have an extended role as mediators of communication between cells of non-hemopoietic as well as hemopoietic lineages. The recognition that cytokines could act to promote the formation and maintenance of the placenta followed the discovery that murine placental trophoblast cells expressed CSF-1 receptor (Pollard *et al.* 1987), together with the finding that cytokines of the CSF family could enhance ^{3}H-thymidine incorporation and phagocytosis by placental cells (Athanassakis *et al.* 1987; Wegmann 1988; Wegmann *et al.* 1989), and alter their secretory profile (Shiverick *et al.* 1990).

These developments occurred during a period when there was growing interest in the role of tubal and uterine factors as paracrine regulators in pre-embryonic development. In the mouse, the rate of development of embryos *in vitro* was found to be retarded markedly in comparison with development *in vivo* (Harlow and Quinn 1982). The development of murine embryos could be enhanced by co-culture with either endometrial cells, or a number of other normal cells or transformed cell lines (Lavranos and Seamark 1990; Robertson *et al.* 1991). The spectrum of growth factors and other hormones, and the balance of growth-promoting and inhibitory activities produced by different cell types, presumably accounted for the quantitative differences in the effects observed in various experimental systems. The exciting finding of enhanced development when embryos were co-cultured with the murine fibroblast L-cell line (a potent source of the cytokines CSF-1 and LIF) was highly relevant to the studies implicating cytokines in placental growth and development (Robertson *et al.* 1991).

The central hypothesis of the studies described within this chapter is that cytokines can play an important role as paracrine regulators of growth and development of the pre-implantation murine embryo. This hypothesis was tested initially *in vitro,* where embryos will develop from the fertilised oocyte through to stages correlating with attachment and implantation *in vivo*, in simple culture medium. To determine whether cytokines can influence embryonic development, the extent and rate of embryo development in culture was examined in the presence of cytokine-rich 'conditioned media' (CM) generated from cultures of various hemopoietic and non-hemopoietic cells.

Conditioned media rich in GM-CSF were found to be effective particularly in promoting the attachment of hatched blastocysts to plastic culture dishes. Recombinant GM-CSF and CSF-1 were then assessed for their ability to replicate this effect and also for their influences on the development of zygotes into blastocysts and on DNA synthesis in post-implantation embryos. Expression of GM-CSF receptors by implantation-stage blastocysts was assessed by (1) binding of radio-labelled

ligand, and (2) analysis of GM-CSF receptor α and β chain (GM-CSF-R and AIC2B respectively) mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR).

3.2 Effect of cytokine-enriched conditioned media on embryonic growth and development *in vitro*

Cytokine-rich conditioned media were prepared from LPS-activated mouse lung tissue (LCCM), and from transformed cells which included the murine T-lymphocyte cell line EL-4, the murine myeloid cell line WEHI 3B, and the murine L-cell fibroblast cell line. These cells are reported to be potent sources of specific colony stimulating factors, including GM-CSF (LCCM and T-lymphocyte cell lines), IL-3 (WEHI 3B) and CSF-1 (L-cells). All conditioned media were prepared in DMEM-Em and diluted 1:2 in fresh DMEM-Em prior to use in embryo culture. The gibbon T-cell line MLA-144 was also included to determine the effect of IL-2, which unlike many of the other cytokines produced by T-cells (such as GM-CSF and IL-3), can act across the mouse/ human species barrier.

3.2.1 Cytokine content of conditioned media

The IL-2, IL-3 and GM-CSF content of each of the conditioned media were measured in specific bioassays employing the murine factor-dependant cell lines CTLL, 32Dcl3 and FD 5/12 respectively, and total CSF activity was determined in a bioassay employing clonal culture of bone marrow-derived myeloid progenitor cells. The cytokine contents of each of the conditioned media are shown in Table 3.1. In each instance, the cytokine content approximated that reported by other investigators.

Table 3.1 The cytokine content of conditioned media (CM) as determined in specific bioassays. Data is given as U/ ml.

CM	IL-2	IL-3	GM-CSF	Total CSF
L-cell	< 1	< 1	2	
LCCM	< 1	< 1	2130	1500
WEHI-3B	< 1	14	2	6
EL-4	8	1	50	80
MLA-144	33	<1	<1	<5

3.2.2 Effect of conditioned media on the development of eight cell embryos

a. Effect on the proportion of eight-cell embryos developing to 'implantation'

Eight-cell (C57BL x Balb/c) embryos were cultured in DMEM-Em or conditioned media diluted 1:2 in DMEM-Em (15-20 embryos/ 1 ml culture well). Observations on embryo

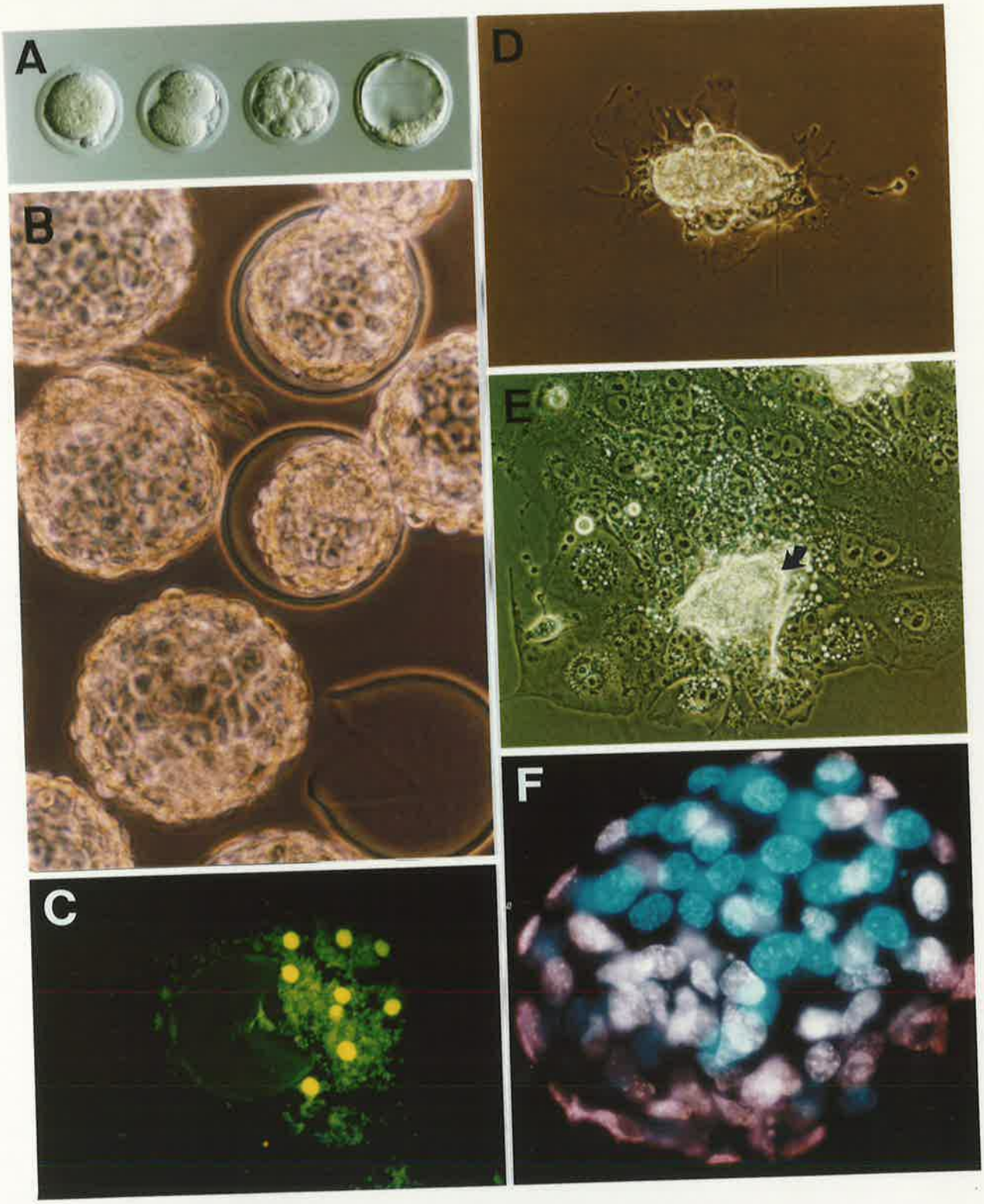

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Figure 3.1 Development of murine embryos *in vitro*. (A) Embryos at 1-cell, 2-cell, 8-cell and blastocyst stages of development. (B) Expanded blastocysts during and after hatching from the zona pellucida. (D) A recently attached blastocyst with adherent trophoblastic projections marking the early stages of 'implantation'. (E) An 'implanted' embryo with substantial trophoectodermal outgrowth and inner cell mass (arrowed). (C) An eight cell embryo stained with acridine orange to enable quantification of cell numbers, and (F) a hatched blastocyst stained with Hoescht 33342 and propidium iodide (PI) to enable quantification of viable (blue) and dead (pink) cells.

development were made daily (at 1800 h), and records were kept of the developmental stages of embryos (according to the criteria specified in 2.6.1). The numbers of embryos that developed to blastocyst and 'implantation' stage by day 5 of culture (172 h post-hCG) were not found to differ significantly between treatment and control groups (Table 3.2).

b. Effect on the rate of embryo development from eight-cell to 'implantation'

An additional experiment was undertaken to determine whether the rate of development of embryos is altered in the presence of conditioned media. Eight cell embryos (C57BL x Balb/c) were cultured in DMEM-Em with or without conditioned media (15-20 embryos/ 1 ml culture well), and observations on embryo development were made twice daily (at 1000 h and 1800 h). The numbers of morulae, blastocysts, hatched blastocysts, attached blastocysts and 'implantation' stage embryos were recorded at each time point. Whilst the rate at which eight-cell embryos developed into blastocysts and then hatched from the zona was not affected by culture in conditioned media (data not shown), there was a significant increase in the rate at which embryos cultured in LCCM-conditioned media attached to the culture dish and developed to 'implantation' stage (Table 3.3). This effect was most pronounced at 124 h post-hCG, when there was an 11-fold increase in the proportion of hatched embryos that had already attached. Other conditioned media had either no effect, or inhibited the rate of embryo attachment to the culture dish moderately. In addition, embryos cultured in LCCM, and to a lesser extent those cultures in EL-4 conditioned media, appeared to have an altered pattern of trophoblast outgrowth and maintained a more organised morphology. Embryos grown in these conditioned media also appeared to have larger numbers of trophoblast cells surrounding each inner cell mass, after the same period of time in culture. LCCM and EL-4 conditioned media were notable for their content of GM-CSF (2100 and 50 U/ ml respectively), and it was concluded that GM-CSF might be one of the factors within these supernatants that had acted to promote peri-implantation stages of embryonic development.

Table 3.2 The effect of cytokine-conditioned media (CM) on development of eight-cell embryos during culture *in vitro*. The numbers of embryos that developed to the blastocyst stage after 5 days in culture (n_1) are expressed as the percentage of the initial number of eight-cell embryos (N). The numbers of blastocysts that proceeded to hatch and 'implant' (n_2) are expressed as a percentage of the total number of blastocysts (n_1). No effect of CM on development to either blastocyst or 'implantation' stages was apparent when data were subjected to Chi square analysis with Yates' correction ($^a X^2 = 1.60$, $p = 0.520$; $^b X^2 = 5.42$, $p = 0.368$)

CM	N	% Blastocyst (n_1)[a]	% 'Implanted' (n_2)[b]
DMEM-Em	87	97 (84)	70 (59)
L-cell	259	97 (251)	79 (199)
LCCM	83	95 (79)	68 (54)
WEHI-3B	63	100 (63)	67 (42)
EL-4	56	98 (55)	78 (43)
MLA-144	62	98 (61)	74 (45)

Table 3.3 The effect of cytokine-conditioned media (CM) on the rate of attachment of hatched blastocysts to the culture dish. The number of attached blastocysts (n) are expressed as a percentage of the initial number of eight-cell embryos. Embryos were designated attached when they were not dislodged upon gentle swirling of the culture dish. N = the number of embryos within each group. Data were subjected to Chi square analysis with Yates' correction ([a] $X^2 = 21.03$, $p < 0.001$; [b] $X^2 = 12.76$, $p = 0.026$; [c] $X^2 = 13.12$, $p = 0.023$). *$p < 0.001$.

		% Attached (n)		
CM	N	124 h[a]	140 h[b]	148 h[c]
DMEM-Em	122	3 (7)	94 (115)	100 (122)
L-cell	37	7 (6)	89 (33)	100 (37)
LCCM	54	33 (18)*	96 (52)	100 (54)
WEHI-3B	29	23 (7)	72 (21)*	100 (29)
EL-4	43	17 (7)	90 (39)	91 (39)*
MLA-144	31	13 (4)	90 (28)	97 (30)

3.3 Effect of recombinant cytokines on embryonic growth and development *in vitro*

The preceding experiments identified the effectiveness of GM-CSF-rich conditioned media in promoting the post-hatching development of embryos. However these conditioned media presumably contained other growth factors and cytokines apart from GM-CSF, any of which could have contributed to their embryotrophic activity. To determine whether GM-CSF could have been the active component in these supernatants, an examination was made of the effects of recombinant GM-CSF on embryonic development *in vitro*. Recombinant CSF-1 was also tested for possible embryotrophic activity in view of the effectiveness of co-culture with L-cells on embryo growth (Robertson *et al.* 1991). A role has also been implied for this factor in regulating the growth of post-implantation trophoblast cells (Pollard *et al.* 1987; Athanassakis *et al.* 1987).

3.3.1 Effect of cytokines on the development of one-cell embryos

To examine whether embryos are responsive to these cytokines during the early cleavage stages, one cell (C57BL x CBA) embryos were cultured in HTF with added recombinant GM-CSF or CSF-1 at 40, 200, 1000 or 5000 U/ ml (15-20 embryos/ 20 µl drop). Observations on embryo development were made daily (at 1800 h), and a record was made of the numbers of embryos achieving 8-cell, morula and blastocyst stages by day 4 of culture (100 h post-hCG). Development to the eight-cell stage was inhibited in the presence of 200 U/ ml of either GM-CSF or CSF-1 (Table 3.4), where growth was arrested either at the first or second division. Neither cytokine influenced development from eight-cell to the blastocyst stage.

Table 3.4 The effect of recombinant GM-CSF and CSF-1 on development of one-cell embryos during culture *in vitro*. The numbers of embryos that developed to eight-cell stage after 4 days of culture (n_1) are expressed as a percentage of the initial number of one-cell embryos (N), and the number of eight-cell embryos that proceeded to blastocyst stage (n_2) are expressed as a percentage of the number of eight cell embryos (n_1). Data were subjected to Chi square analysis with Yates' correction ($^a X^2 = 51.56$, $p < 0.001$; $^b X^2 = 14.22$, $p = 0.077$). $*p < 0.001$.

	N	% Eight-cell (n_1)[a]	% Blastocyst (n_2)[b]
DMEM-Em	117	74 (87)	100 (87)
GM-CSF (U/ ml)			
40	94	62 (58)	100 (58)
200	91	40 (36)*	100 (36)
1000	100	73 (73)	100 (73)
5000	86	63 (54)	100 (54)
CSF-1 (U/ ml)			
40	126	64 (81)	100 (81)
200	116	43 (50)*	98 (49)
1000	140	64 (90)	100 (90)
5000	101	72 (73)	100 (73)

3.3.2 Effect of cytokines on the development of eight-cell embryos

a. Effect on the proportion of eight-cell embryos developing to 'implantation'

Eight cell embryos (C57BL x Balb/c) were cultured in 1 ml DMEM-Em supplemented with recombinant GM-CSF or CSF-1 (15-20 embryos/ 1 ml culture well), and assessed for development as described in 3.2.2. The numbers of embryos that developed to blastocysts and 'implantation' stage were not significantly different between treatment and control groups (Table 3.5).

b. Effect on the rate of embryo development from eight-cell to 'implantation'

An additional experiment was undertaken to determine whether culture with recombinant GM-CSF could alter the rate of development of embryos. Eight cell embryos (C57BL x Balb/c) were cultured in DMEM-Em with or without cytokine (15-20 embryos/ 1 ml culture well), and observations were made on embryo development as described in 3.2.2. Although eight-cell embryos developed to blastocysts and hatched from the zona at similar rates, regardless of the presence of cytokine (Fig 3.2), there was a significant increase in the rate at which hatched blastocysts cultured in recombinant GM-CSF attached to the culture dish and developed to 'implantation' stage (Table 3.6). The increase in attachment rates was greatest at 200 U/ml and slightly less at 1000 U/ml of recombinant GM-CSF. Forty U/ ml of recombinant GM-CSF had no significant effect on attachment rates, suggesting that the effect of this factor is dose-dependant. This effect was most pronounced at 124 h post-hCG, when there was a 9-fold increase in the proportion of hatched embryos that had already attached. Comparison of the difference between

Table 3.5 The effect of recombinant GM-CSF and CSF-1 on the proportion of mouse embryos developing to blastocyst and implantation stages during culture *in vitro*. The number of embryos that developed to blastocyst after 5 days of culture (n_1) are expressed as a percentage of the initial number of eight-cell embryos (N), and the number of blastocysts proceeded to hatch and 'implant' (n_2) are expressed as a percentage of the number of blastocysts (n_1). No effect of cytokines on development to either stage was revealed when data were subjected to Chi square analysis with Yates' correction ([a] $X^2 = 4.61$, $p = 0.592$; [b] $X^2 = 6.42$, $p = 0.378$).

	N	% Blastocyst (n_1)[a]	% 'Implanted' (n_2)[b]
DMEM-Em	325	95 (308)	69 (224)
GM-CSF (U/ ml)			
40	94	96 (90)	77 (73)
200	91	95 (86)	78 (71)
1000	100	99 (99)	77 (77)
CSF-1 (U/ ml)			
40	126	98 (124)	75 (95)
200	116	95 (110)	73 (85)
1000	140	97 (136)	78 (109)

Table 3.6 The effect of recombinant GM-CSF and CSF-1 on the rate of attachment of hatched blastocysts to the culture dish. The numbers of attached blastocysts (n) are expressed as percentages of the initial numbers of eight-cell embryos. Embryos were designated attached when they were not dislodged upon gentle swirling of the culture dish. Data were subjected to Chi square analysis with Yates' correction ([a] $X^2 = 45.01$, $p < 0.001$; [b] $X^2 = 14.07$, $p = 0.029$; [c] $X^2 = 8.57$, $p = 0.200$). $^*p < 0.001$ $^{**}p = 0.002$

		% Attached (n)		
CM	N	124 h[a]	140 h[b]	148 h[c]
DMEM-Em	162	3 (5)	83 (134)**	100 (162)
GM-CSF (U/ ml)				
40	69	11 (8)	95 (66)	96 (66)
200	81	26 (21)*	89 (72)	100 (81)
1000	95	22 (21)*	93 (88)	97 (92)
CSF-1 (U/ ml)				
40	87	6 (5)	94 (82)	100 (87)
200	82	5 (4)	89 (73)	100 (82)
1000	102	7 (7)	94 (96)	100 (102)

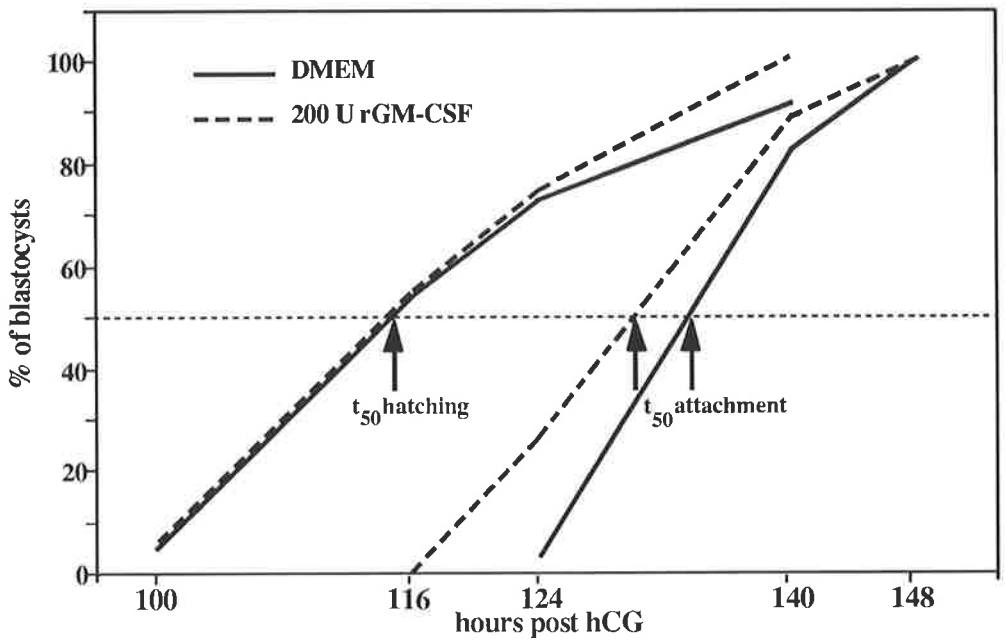

Figure 3.2 Graphical representation of the effect of recombinant GM-CSF on the rate of hatching and attachment of embryos *in vitro*. Eight-cell embryos were cultured in 200 U/ ml of recombinant GM-CSF or DMEM-Em alone. Embryos were designated hatched when they were entirely extruded from the zona pellucida, and attached when they were not dislodged upon gentle swirling of the culture dish. The percentage of embryos hatched or attached is plotted against the number of hours post hCG (data from Table 3.6). The horizontal dotted line and arrows delineates the times at which 50% of embryos are hatched or attached ['t_{50} (hatching)' and 't_{50} (attached)' respectively]. The t_{50} (hatching) was calculated to be 115.0 h post-hCG for both control and GM-CSF groups, whereas the t_{50} (attached) was 134.0 h and 130.3 h respectively for the control and GM-CSF groups.

't_{50} (hatching)' and the 't_{50} (attached)' (when 50% of blastocysts are hatched or attached respectively) for control and cytokine-treated groups (Fig 3.2) reveals that culture in the presence of 200 U/ ml GM-CSF reduced the delay between hatching and attachment to the culture dish from 19 h to 15 h (a 27 % decrease).

In some experiments, the effect of GM-CSF on the viability and rate of growth of blastocysts was assessed by comparing the numbers of cells in blastocysts grown in the presence or absence of GM-CSF. Eight cell embryos were grown for 48 h in 200 U/ ml of GM-CSF, or in DMEM-Em alone and then stained with PI-Hoescht or acridine orange. There was no difference between the numbers or viability of cells in blastocysts from either group (Fig 3.1, data not shown).

3.3.3 Effect of cytokines on DNA synthesis in post-implantation embryos

To determine whether GM-CSF or CSF-1 can influence the rate of DNA synthesis in post-'implantation' embryos, eight-cell embryos were cultured individually in microtitre trays in the presence or absence of recombinant cytokine. Cytokine was added (1000 U/ ml) at the initiation of 'implantation' on the third day of culture (at approx 120 h post-hCG). Forty-eight hours later, embryos were pulse-labelled with 1 µCi / well of ^{3}H-thymidine for 6 h. The amount of ^{3}H-thymidine incorporated was increased moderately in the presence of GM-CSF in two out of three experiments (9% and 18%) and in one out of three experiments in the presence of CSF-1 (26%) (Table 3.7). GM-CSF and CSF-1 in combination did not alter the rate of ^{3}H-thymidine incorporation in any of the three experiments.

Table 3.7 The effect of recombinant GM-CSF and CSF-1 on the rate of ^{3}H-thymidine incorporation into 'implanted' embryos. 'Implanted' embryos were cultured in 1000 U/ ml cytokine for 48 h, then pulsed in ^{3}H-thymidine for 6 h prior to harvest. Mean dpm ± SEM (x 10^{-3})/ embryo are shown. n = the number of embryos in each experimental group. Data comparison is by Bonferoni *t*-test for unpaired samples. $*p < 0.001$; $**p < 0.05$.

	Expt 1 (n)	Expt 2 (n)	Expt 3 (n)
DMEM-Em	9.7 ± 0.6 (31)	7.2 ± 0.4 (51)	4.7 ± 0.5 (52)
GM-CSF	10.6 ± 0.8 (27)**	8.5 ± 0.5 (51)*	4.1 ± 0.5 (26)
CSF-1	10.1 ± 0.8 (27)	9.1 ± 0.7 (48)*	5.2 ± 0.5 (51)
GM-CSF + CSF-1	9.1 ± 0.9 (27)	7.9 ± 0.6 (54)	4.6 ± 0.4 (47)

3.4 Expression of GM-CSF receptors by blastocysts

The preceding studies suggest that implantation stage embryos are responsive to the cytokine GM-CSF. Responsiveness to GM-CSF in hemopoietic cells is dependant on expression of functional GM-CSF receptor at the plasma membrane. In hemopoietic cells, functional GM-CSF receptors are comprised of the monomeric GM-CSF receptor protein (GM-CSF-R or α-subunit),

which on its own confers low affinity ligand binding, together with the AIC2B protein (β-subunit) which is required for high affinity ligand binding and for transduction of the proliferative signal. In the studies that follow, synthesis of GM-CSF receptors by blastocyst stage embryos was assessed in ^{125}I-GM-CSF binding experiments. Reverse transcription-polymerase chain reaction (RT-PCR) was used to analyse the expression of GM-CSF-R and AIC2B mRNA during development in embryos from the 1-cell through to the blastocyst stage.

3.4.1 ^{125}I-GM-CSF binding studies

a. Protocol work-up with FD 5/12 cells

The murine GM-CSF-dependant myeloid cell line FD 5/12 was used to evaluate the ligand binding and detection protocol. In preliminary experiments FD 5/12 cells were incubated in either 10 or 50 ng/ ml of ^{125}I-GM-CSF (specific activity = 50,000 cpm/ ng), in the presence or absence of 500 ng/ ml of cold recombinant GM-CSF, or in media alone. Cell smears were coated in autoradiographic emulsion and exposed for 8 or 14 days. A satisfactory signal to noise ratio was achieved when cells labelled with 50 ng/ ml of ^{125}I-GM-CSF were developed after 14 days exposure. Labelled cells were overlaid with between 5 and 10 silver grains per cell, and specificity of binding was demonstrated by the reduction to background levels (1-2 silver grains per cell) of specimens incubated in the presence of a ten-fold excess of cold GM-CSF (Fig 3.3)

b. Blastocyst ^{125}I-GM-CSF binding study

Blastocysts harvested from the uteri of superovulated mice on the morning of the fourth day of pregnancy were stripped of their zona pellucidae in 0.5% pronase. They were cultured for eight hours and then incubated in 50 ng/ ml of ^{125}I-GM-CSF. The washing steps were completed within 3-4 min, to minimise the loss of ligand in the event that it was bound to low affinity receptor. The labelled blastocysts were deposited onto slides and exposed for 14 days to autoradiographic emulsion. Silver grains were found to localise over blastocysts (> 50 grains per blastocyst) in the absence but not in the presence of a 10-fold concentration of cold ligand (Fig. 3.3). This demonstrates that ^{125}I-GM-CSF binds specifically to blastocysts, suggesting that they express at least the low affinity component of the GM-CSF receptor complex. There was no preferential distribution of silver grains over the inner cell mass or over either embryonic pole, suggesting that GM-CSF receptors are distributed evenly over the trophoblast cells of the blastocyst.

3.4.2 Reverse transcription-polymerase chain reaction studies

a. Protocol work-up with FD 5/12 cells

Primers for GM-CSF-R cDNA were designed with the aid of 'Primer Designer' software and were constructed as described in 2.22, together with primers that discriminate between AIC2B and the closely related AIC2A cDNAs (Fung *et al.* 1992). Primers for actin cDNA were also used as a

'quality control' for RNA extraction and first-strand synthesis by reverse transcription. Since the actin primers spanned an intron-containing region of the actin gene, amplification of actin mRNA also served as a control for contamination of the RNA extracts with DNA.

RNA was harvested from the murine GM-CSF responsive cell line FD 5/12, the murine L-cell fibroblast cell line, and the gibbon T-cell line MLA 144 by guanidine thiocyanate extraction and caesium chloride density gradient centrifugation, followed by treatment with RNAse-free DNAse to remove residual DNA. RNAs were reverse transcribed into cDNA using oligo dT primers. These cDNAs were used to determine empirically the optimal PCR amplification protocols for each primer pair. Complementary DNA from FD 5/12 cells was used as a positive control and L cell and MLA 144 cDNAs as murine and heterologous-species negative controls respectively. Strong signal to noise ratios (ie. a single band of amplification product in the absence of bands in negative controls) were obtained for GM-CSF-R and actin primers (39 cycles at 62°C), and for AIC2B primers (40 cycles at 56°C). The actin primers amplified a 381 bp band from each of the cell line cDNAs, including the gibbon cell line MLA 144, as well as from murine liver and murine ovary (Fig 3.4). The GM-CSF-R and AIC2B primers respectively amplified a 235 bp and a 326 bp fragment from FD 5/12, liver and ovary cDNAs, but failed to amplify any material from the L-cell and MLA 144 cDNAs, or from the negative control which did not contain any cDNA (Fig 3.4). Expression of mRNA for both subunits of the GM-CSF complex in ovarian and liver tissue was expected in view of the large populations of GM-CSF-responsive macrophage and/or granulocytes normally resident within these tissues (Hume *et al.* 1983; 1984).

The identities of the GM-CSF-R and AIC2B amplicons was confirmed by restriction enzyme analysis. GM-CSF-R cDNA contains a single Sma-1 site, and AIC2B cDNA contains two Sau-3A sites within the regions spanned by the primers. Sma-1 digestion of the GM-CSF-R amplicon yielded bands approximating the predicted sizes of 164 bp and 71 bp, and Sau-3A digestion of the AIC2B amplicon yielded bands approximating the predicted sizes of 188 bp, 84 bp and 54 bp. The size of each amplicon was not altered after incubation with the converse enzymes (Fig 3.5).

b. Embryo GM-CSF-R and AIC2B expression

To determine whether pre-implantation stage embryos express mRNA for either subunit of the GM-CSF receptor, mRNA was harvested from 1-cell, 2-cell, 8 cell and blastocyst stage embryos and subjected to RT-PCR analysis with primers for the GM-CSF-R, AIC2B and actin cDNAs. Ten to fifteen embryos were sufficient to yield a substantial band of the expected size (381 bp) using actin primers under the reaction conditions described in 3.4.2a (Fig 3.5).

One cell, 2-cell, 8-cell and blastocyst stage embryos were found to express GM-CSF-R mRNA. cDNAs from each embryonic stage generated a GM-CSF-R amplicon of the expected size (235 bp) under the same reaction conditions as those described in 3.4.2a (Fig 3.6). The reaction product from 2-cell stage cDNA yielded an additional, minor band of approximately 450 bp. The identity of this band is unknown, but it is the same size as a band that is a more abundant product of

Figure 3.3 GM-CSF receptor expression by blastocyst stage embryos.
Blastocysts were incubated in ^{125}I-GM-CSF and autoradiographed, then counterstained in Giemsa. Preparations were photographed under (A) darkfield and (B) brightfield illumination. Binding of label was blocked when cells were incubated in ^{125}I-GM-CSF in the presence of a 10-fold excess of cold GM-CSF; photographed in (C) with darkfield and (D) brightfield illumination. To establish the efficacy of the labelling protocol, FD 5/12 cells were incubated in (E)^{125}I-GM-CSF alone or (F) ^{125}I-GM-CSF in the presence of a 10-fold excess of cold GM-CSF. Smears of labelled cells were autoradiographed, then counterstained in haematoxylin, and photographed under brightfield illumination.

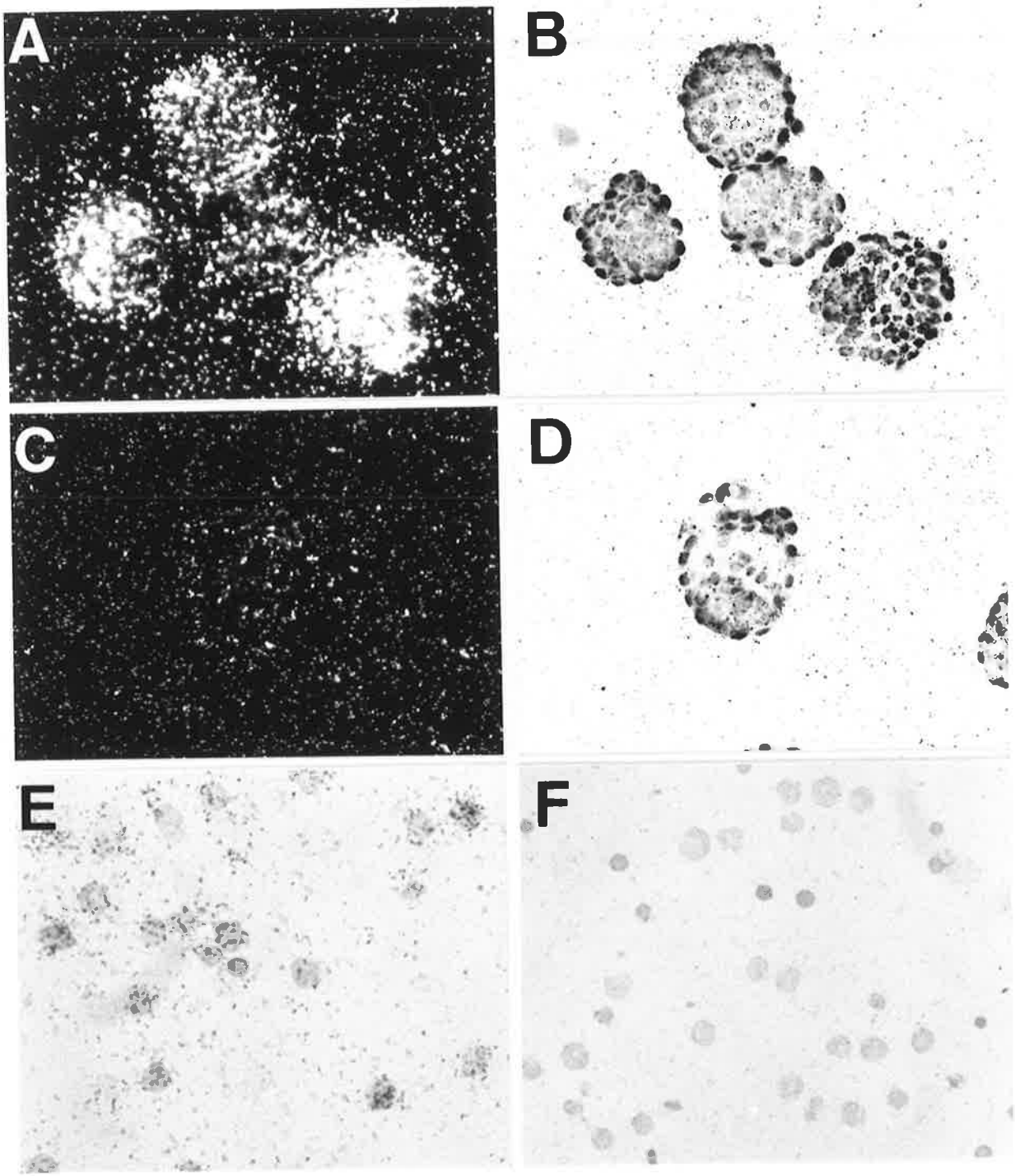
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Figure 3.4 RT-PCR analysis of GM-CSF-R and AIC2B mRNA expression by FD 5/12 myeloid cells. RNA was isolated from murine myeloid cells (FD 5/12) and fibroblasts (L-cells), gibbon T-cells (MLA144), murine ovary (ov) and murine liver (liv). First strand cDNA was reverse-transcribed from mRNA using oligo-dT primers and then amplified using primers for (A) actin, (B) GM-CSF-R and (C) AIC2B. 20 µl aliquots of reaction products were analysed by electrophoresis through a 1% agarose gel containing 50 pg/ ml of ethidium bromide, and photographed with UV illumination. (D) The identities of GM-CSF-R and AIC2B amplicons were confirmed by diagnostic Sau 3A and Sma1 restriction enzyme analysis. The 235 bp GM-CSF-R amplicon yielded fragments of the predicted sizes of 164 and 71 bp after digestion with Sma-1, but remained intact with Sau-3A. The 326 bp AIC2B amplicon yielded fragments approximating the predicted sizes of 188, 84 and 54 bp after digestion with Sau-3A, but remained intact with Sma-1.

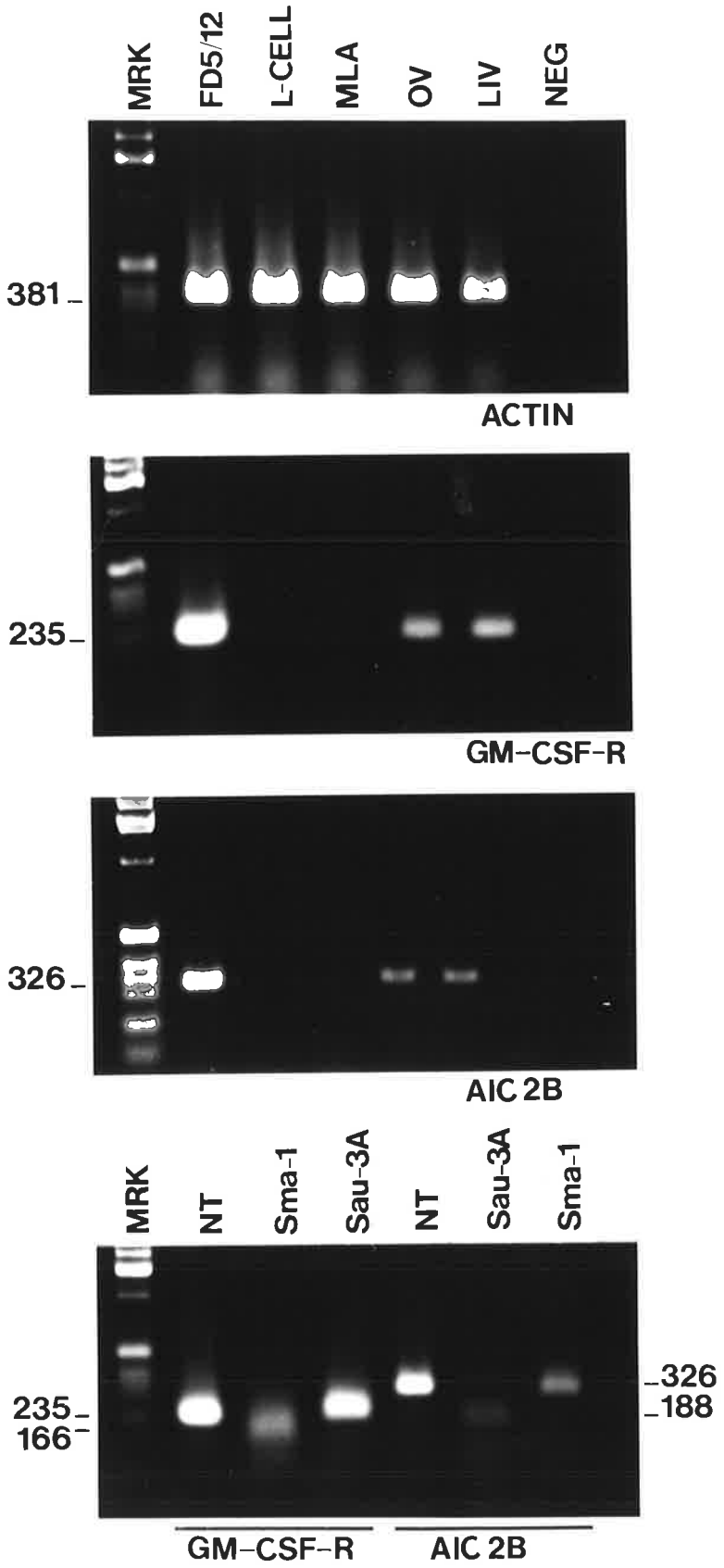
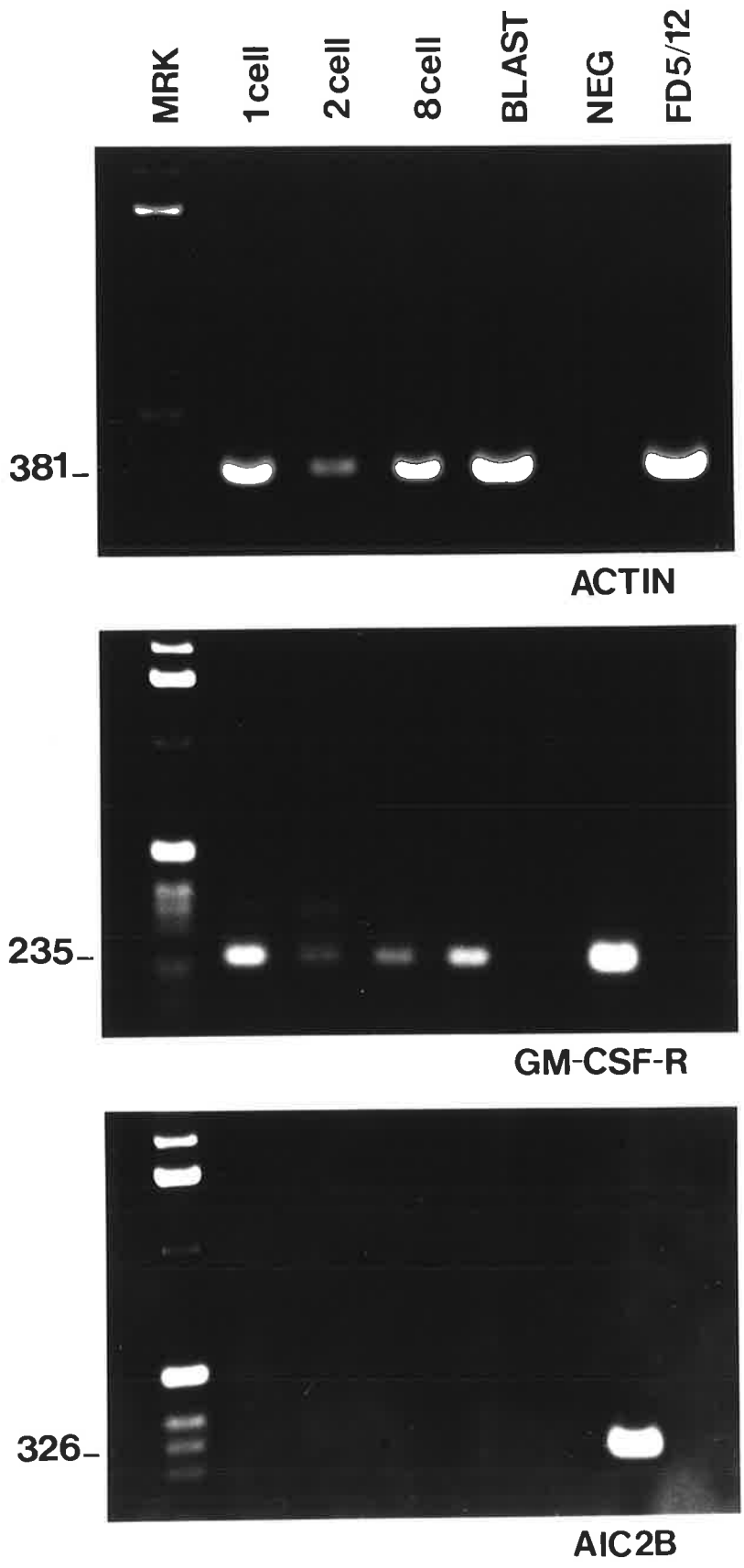

MRK
FD5/12
L-CELL
MLA
OV
LIV
NEG
381
ACTIN
235
GM-CSF-R
326
AIC 2B
MRK
NT
Sma-1
Sau-3A
NT
Sau-3A
Sma-1
235
166
326
188
GM-CSF-R
AIC 2B

Figure 3.5 RT-PCR analysis of GM-CSF-R and AIC2B mRNA expression by 1-cell to blastocyst stage embryos. RNA was isolated from 1-cell , 2-cell, 8-cell and blastocyst (Bl) stage embryos, and from murine myeloid cells (FD 5/12). First strand cDNA was reverse-transcribed from mRNA using oligo-dT primers and then amplified with primers for (A) actin, (B) GM-CSF-R and (C) AIC2B. 20 µl aliquots of reaction products were analysed by electrophoresis through a 1% agarose gel containing 50 pg/ ml of ethidium bromide, and photographed with UV illumination.


MRK
1cell
2cell
8cell
BLAST
NEG
FD5/12
381
ACTIN
235
GM-CSF-R
326
AIC2B

uterine cDNA (7.1.4). The potential origin of this band is fully discussed in chapter 7.

mRNA for the β-subunit of the GM-CSF receptor was not detected in embryos. AIC2B primers failed to amplify product from any of the four embryo cDNAs under reaction conditions that yielded substantial product from FD 5/12 cDNA (Fig 3.5). To increase the sensitivity of the analysis, ^{32}P-dATP was included in the reaction mix, and gels were autoradiographed after capillary transfer to nylon filters. No reaction product was detected when filters were exposed for up to two weeks (data not shown).

3.5 Conclusions and discussion

The studies described within this chapter have identified GM-CSF as a potential 'embryotrophic' factor, that may act to regulate the development of peri-implantation blastocysts during early pregnancy in the mouse. GM-CSF was found to promote the 'implantation' of blastocysts in an *in vitro* model of murine embryonic development. Radiolabelled-ligand binding studies demonstrate that blastocysts express the GM-CSF receptor.

Initially, media conditioned by lung tissue from LPS-injected mice, shown by specific bioassay to be rich in native GM-CSF, was found to enhance the rate at which hatched blastocysts attached to the culture dish in an *in vitro* culture system. Recombinant GM-CSF was found to mimic this effect, further implicating GM-CSF as the attachment-promoting agent in the conditioned medium. Enhanced development appeared to be dependant on the concentration of the recombinant cytokine, with maximal activity obtained at 200 U/ ml. This is in the same order of magnitude as the concentrations at which other biological activities of GM-CSF are exhibited *in vitro* (Gasson 1991; Ruef and Coleman 1990).

In addition, ^{3}H-thymidine incorporation studies suggested that GM-CSF, and to a lesser degree CSF-1, may have a modest enhancing effect on the rate of DNA synthesis in post-implantation embryos (^{3}H-thymidine incorporation was increased up to 18% and 26% respectively for GM-CSF and CSF-1). However these results were inconclusive, since significant effects of cytokine were achieved in only two out of three experiments.

The development of fertilised oocytes into eight-cell embryos was inhibited by both recombinant GM-CSF and CSF-1. This effect was concentration dependant, and maximal for both cytokines at 200 U/ ml. The reasons for their lack of activity at 1000 U/ ml or higher concentrations are unclear, although refractoriness to high concentrations of cytokines is also observed using other target cells and it may result from receptor down-modulation or other negative feed-back mechanisms (Lopez *et al.* 1992).

Other groups have reported both positive and negative effects of GM-CSF on various stages of early embryo development. Hill *et al.* (1987) have found that GM-CSF at high doses (> 1000 U/ ml) inhibited the development of 2-cell embryos into morulae. In two studies, ectoplacental cone trophoblast has been found to proliferate in response to GM-CSF (Armstrong and Chaouat 1989; Lea and Clark 1993), but in the second instance an effect was obtained with native but not recombinant cytokine. The results presented in this chapter support these observations. However,

in contrast to the findings reported here, Haimovoci *et al.* 1991 found that 250 U/ ml or more of GM-CSF inhibited the attachment of blastocysts to fibronectin-coated culture dishes in the absence of serum. Lea and Clark (1993) have reported that recombinant GM-CSF (at between 10 and 100 U/ ml) inhibited the incorporation of ^{3}H-thymidine into outgrowing, implanted blastocysts, in a dose dependant manner. Tartakovsky and Ben-Yair (1991) found that systemic GM-CSF administration markedly enhanced early embryonic development *in vivo*, but did not note any effect of GM-CSF on embryonic development *in vitro*. These results are difficult to reconcile. However, the differences are likely to be related to the developmental stages examined, the methods for embryo culture, the strains of mice, and the sources and concentrations of cytokine used. For example, some cytokine preparations may contain potentially embryotoxic contaminants such as endotoxin. In addition, there is emerging evidence that there may be more than one mechanism by which GM-CSF is able to exert its effects in target cells (1.7.1e), and it is possible that the glycosylation state of the cytokine (which would also be dependant upon its source) may be important for binding to unconventional receptors.

Studies of radio-labelled ligand binding show clearly that blastocysts bind ^{125}I-GM-CSF specifically, indicating that they express at least the low affinity form of the GM-CSF receptor. This conclusion was supported by RT-PCR analysis, which showed that blastocysts express mRNA for the α-subunit of the GM-CSF receptor complex. GM-CSF-R was expressed at similar levels through the first four days of embryo development, from fertilisation to blastocyst stage. However mRNA for the β-subunit of the GM-CSF receptor complex was not detected in embryos by the RT-PCR technique. Together, these data suggest that embryos express GM-CSF receptor from at least as early as fertilisation, but that it may be of the low affinity form. The murine embryo therefore falls into the same category as endothelial cells and other non-hemopoietic cells which exhibit a biological response to GM-CSF (1.7.1d and 1.7.1e) although expressing only low affinity receptors. Although it seems clear in hemopoietic cells that the α-subunit of the GM-CSF receptor cannot on its own transduce proliferative signal (1.7.1e), it is not known whether the α-subunit can in some circumstances initiate responses in cells in the absence of the β-subunit. The recent discovery of unconventional forms of the GM-CSF receptor in the human suggests that this may be possible (1.7.1e)

There are a number of possible mechanisms through which GM-CSF may act to enhance the rate of 'implantation' of blastocysts *in vitro*. Embryos appear to employ multiple adhesion systems *in vitro* in the presence of serum: attachment factors that include fibronectin, collagen and laminin support embryo attachment and outgrowth (Jenkinson and Wilson 1973; Armant *et al.* 1986). GM-CSF can enhance the adhesion of myeloid cells by upregulating expression of CD11/ CD18b (Mac-1). However, a primary function for Mac-1 in implantation is unlikely, because pre-implantation embryos were found not to express this integrin (Weitlauf and Knisley 1992). A selectin-like molecule has been implicated in the attachment of the blastocyst to uterine epithelial cells (1.2.3b), and GM-CSF can enhance the expression of selectins in myeloid cells (1.7.1d). It would be of interest to investigate the effect of GM-CSF on expression of these and other myeloid attachment factors by blastocysts, particularly in view of the other similarities that trophoblast cells share with

cells of the myeloid lineages (Guilbert *et al.* 1993). For example, Mac-2 is the major non-integrin laminin-binding protein of macrophages and it is expressed in late-blastocyst stage embryos immediately prior to implantation (Weitlauf and Knisley 1992).

It seems plausible that binding of GM-CSF to blastocysts could alter their charge or adhesive properties perhaps without a requirement for signal transduction to the cell cytoplasm. Solid phase GM-CSF bound to extracellular matrix could provide an anchor for the receptor-bearing blastocyst, and heparan sulphate could provide such a matrix (Roberts *et al.* 1988), particularly since heparan sulphate is a potentially important mediator of blastocyst binding to fibronectin and laminin (1.2.3b). Experiments aimed at detecting evidence of GM-CSF signal transduction (for example protein phosphorylation or MAP-2 kinase induction) are required to resolve this. Furthermore, the molecules in serum that mediate the attachment of trophectoderm cells to serum-coated tissue culture plastic *in vitro* and to the endometrium *in vivo* may or may not be related. Whether GM-CSF has a physiological role in attachment of the blastocyst to the epithelium *in vivo* will remain unclear until the specific factors mediating this attachment are characterised more fully.

4 GM-CSF synthesis in the cycling and pregnant uterus

4.1 Introduction

The studies described in Chapter 3 provided evidence that GM-CSF could act as a regulator of early embryonic development *in vitro*. In this chapter, experiments were undertaken to investigate whether GM-CSF is present within the pre- and peri-implantation mouse uterus, as a first step towards determining whether GM-CSF may have a physiological role in early pregnancy. Initially, this was achieved by measuring the bioactivity of (1) fluid collected from the uterine lumen, and (2) the supernatants from cultures of uterine cells harvested by various enzymatic techniques.

Luminal fluid collected from the uteri of mice 8-12 h after mating was found to be a potent source of GM-CSF-like activity, and experiments were performed aimed at determining whether this material was of uterine origin or whether it was contained within the ejaculate. These experiments were followed by a preliminary characterisation of the nature and source of a factor within the ejaculate that acts to stimulate synthesis of GM-CSF within the uterus after mating.

Cells harvested from the endometrium of mated and cycling mice were also found to be a potent source of GM-CSF-like bioactivity. The immunochemical and physiochemical characteristics of the bioactive protein, and the size of the GM-CSF mRNA synthesised by endometrial cells (both *in vitro*, and within the endometrium *in vivo*), were compared with GM-CSF mRNA and protein synthesised by T-lymphocytes.

Uterine luminal fluid and supernatants of endometrial cell cultures were also assessed for IL-1, IL-2, IL-3, IL-6, and TNFα bioactivity. The immunochemical and physiochemical characteristics of an activity detected in the IL-6 bioassay were also investigated.

4.2 GM-CSF-like bioactivity in uterine luminal fluid

4.2.1 Effect of day of pregnancy

To determine whether GM-CSF is secreted into the uterine cavity, fluids harvested by lavage from within the uterine lumen of oestrous mice and on days 1 to 4 after natural mating were assessed for content of GM-CSF-like bioactivity in the FD 5/12 bioassay. GM-CSF activity was undetectable in fluids collected at oestrous and on days 3 or 4 of pregnancy. Activity was present in the uterine luminal fluid from 10 of 12 mice (Fig. 4.1) on day 1, with median levels in mated mice at least 20-fold higher than in oestrus mice [median (95% confidence range) = <1 (<1) U/ uterus at oestrous, *versus* 17 (<1-50) U/ uterus on day 1, p <0.05]. However, it was detected in only 1 of 6 day 2 mated mice (6.3 U/ uterus).

4.2.2 Effect of superovulation

To determine whether superovulation has an effect on the GM-CSF content of mice, uterine luminal fluids were harvested by lavage 20-22 h after hCG injection from the uteri of superovulated

mice, and assessed for content of GM-CSF-like bioactivity in the FD 5/12 bioassay. The GM-CSF contents of unmated adult mice, mated 4 week-old mice and mated adult mice were compared. GM-CSF activity was detected in only 9 of 33 mice mated at 4 weeks, and in only 2 of 21 mice mated as adults (Table 4.1). In contrast to naturally-mated mice, the uterine GM-CSF contents of superovulated mice were not found to be altered significantly after mating.

Table 4.1 GM-CSF in uterine luminal fluid of mice following mating of superovulated mice. Values are median (95% confidence range) with number of observations in parentheses. No significant differences between groups were found when data were compared by Kruskal-Wallis one-way ANOVA ($p = 0.269$)

Uterine Status			GM-CSF (U/ uterus)
Unmated:	Superovulated adult	(11)	<1 (<1)
Mated :	Superovulated adult	(21)	<1 (<1-10)
	Superovulated 4 wk	(33)	<1 (<1-12.4)

Additional experiments investigated the effects of synchronising mice into oestrus prior to natural mating. Oestrous mice were synchronised by administration of LHRH superagonist, or by the Whitten's effect, to minimise adverse endocrine effects during the ensuing cycle. They were mated naturally, together with randomly cycling mice. The GM-CSF content of day 1 mated mice was not altered by either synchronisation method (data not shown). Since LHRH administration was found to be the less cumbersome and more efficient of the two methods, it was employed in subsequent experiments requiring oestrous mice.

4.2.3 GM-CSF content of male accessory gland secretions

Because GM-CSF was detected in the uterine luminal fluid of naturally-mated mice, the GM-CSF contents of male accessory organs (including the prostate, seminal vesicle, coagulating gland and epididymis) were measured to determine whether the ejaculate could be the source of this activity. GM-CSF was not detected in any accessory gland secretion from 5 fertile and 5 vasectomised males.

4.2.4 Effect of cervical stimulation on post-mating GM-CSF response

Cervical stimulation can cause prolactin release and result in psuedopregnancy in rodents (Freeman 1988). To determine whether the neuroendocrine response to the physical act of mating caused the post-mating increase in uterine GM-CSF content, the cervices of oestrous mice were stimulated mechanically at 2400 h (mid-dark cycle). Control oestrous mice were left untreated, or placed with males for natural mating. Uterine luminal fluids were harvested from each group 10-12 h after cervical stimulation or mating and assayed for GM-CSF-like bioactivity. The GM-CSF contents

of fluids harvested from unmated mice were not altered by cervical stimulation, and the contents of both untreated and cervically stimulated oestrous mice were significantly less than in fluids collected from naturally mated mice (Table 4.2).

Table 4.2 GM-CSF in uterine luminal fluid of mice following cervical stimulation or natural mating. Values are median (95% confidence range) with number of observations in parentheses. Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed)-test. $^{*}p$ = 0.002; $^{**}p$ = 0.006

Uterine Status			GM-CSF (U/ uterus)
Unmated:	Oestrus, untreated	(10)	<1 (<1-14.3)*
	Cervically stimulated	(9)	<1 (<1-7.9)**
Mated :	Natural	(11)	38.1 (<1-137.8)

4.3 Synthesis of GM-CSF-like bioactivity by endometrial cells *in vitro*

4.3.1 Effect of method of harvesting uterine cells

Since male accessory glands were not the origin of the GM-CSF in luminal fluid following mating, experiments were carried out to determine whether uterine cells can synthesise GM-CSF.

Initially, the GM-CSF outputs (in U/ 10^5 cells/ 24 h) of cultures of uterine cells harvested by different enzymatic techniques were compared. Cell suspensions enriched for either uterine endometrial cells (predominantly epithelial cells; 2.9.1) or stromal cells were prepared according to the methods of Sherman (1978) from groups of day 1 pregnant mice and cultured for 24 h, when supernatants were harvested and the GM-CSF content of each measured.

Both preparations of uterine cells were found to release GM-CSF during the 24 h period following initiation of the culture. Endometrial cells produced more GM-CSF per 10^5 cells (mean $\pm$ SEM; 47.1 $\pm$ 12.2 U) than stromal cells (15.3 $\pm$ 4.2 U) (p <0.05, n = 9), indicating that endometrial cell cultures were enriched comparatively for GM-CSF-producing cells compared to stromal cell preparations. In additional experiments the GM-CSF output of mixed uterine cell cultures, which contain both endometrial and stromal cells, was found to be less than that of endometrial cell cultures but greater than that of stromal cell cultures (data not shown). Cells harvested by digestion of intact uteri with type II collagenase in place of trypsin/ pancreatin produced approximately 5-fold less GM-CSF (208 U/ 10^5 cells *versus* 41 U/ 10^5 cells respectively).

Microscopic examination of endometrial cell cultures revealed that 50-70% of cells formed an adherent monolayer within the first 24 h of culture, whilst the remaining non-adherent population contained primarily dead or large, vacuolated cells. To determine whether the major source of GM-CSF was the adherent or the non-adherent cells in the endometrial cell cultures, cultures were separated into adherent and non-adherent fractions 24 h after initiation of the culture, and then

cultured separately for a further 24 h. Adherent cells were identified as the source of approximately 75% of the GM-CSF activity.

4.3.2 Effect of day of pregnancy

Uterine endometrial cells were harvested from individual oestrous, day 1 and day 10 pregnant mice and cultured separately. Supernatants from cells prepared from both oestrous and day 1 mice contained GM-CSF [55 (12-137) U/ 10^5 cells and 51 (16-149) U/ 10^5 cells respectively] (Fig. 4.2). There was no significant difference between levels in supernatants obtained from cells of oestrous and day 1 mice ($p > 0.1$). Cells harvested from day 10 pregnant uteri, [whether from between implantation sites (inter-conceptus) or adjacent to embryonic membranes (peri-conceptus)], secreted GM-CSF at levels comparable to cells from oestrous and day 1 uteri. In comparison, cells harvested from metrial gland tissue synthesised significantly less GM-CSF ($p < 0.05$) (Fig. 4.2), and when individual nodules were trimmed of surrounding tissue more thoroughly, less GM-CSF was produced in culture (data not shown).

A broad range of values was obtained for the GM-CSF content of culture supernatants. This was attributed to *in vivo* factors rather than to error introduced during tissue preparation or assay, because GM-CSF activities of supernatants derived from either of the two uterine horns within individual animals did not differ significantly ($p > 0.1$; n = 8 oestrous mice). The activities of supernatants derived from the two horns of an individual animal differed from the mean of the two values by an average of 8.2%.

4.3.3 The kinetics of endometrial cell proliferation and GM-CSF synthesis during culture

To examine the relationship between GM-CSF output, cell proliferation and the duration of the culture period, endometrial cells were prepared from pools of uteri harvested from oestrous mice. The cumulative and daily GM-CSF output was determined after culture for periods of up to 7 days. Cells were plated at an initial concentration of 1 x 10^5 cells per 1 ml culture well, which was observed in preliminary experiments to yield approximately 25% confluent monolayers at 24 h and fully confluent monolayers at 72 h. GM-CSF output was found to be substantial during the 0-24 h period, and to peak during the 24-48 h period (Fig. 4.4A). Output declined thereafter, but remained easily detectable until at least 120 h.

Adherent cells were quantified by the uptake of Rose-Bengal dye at the end of each 24 h period. Figure 4.4C shows that the number of adherent cells increased continually over the first 120 h of the culture period, plateaued and then subsequently declined. Measurement of incorporation of ^{3}H-thymidine into cells cultured from a similar plating density showed that cell proliferation was paralleled by a continuously increasing rate of DNA synthesis over the initial 120 h, and a decline thereafter (Fig. 4.4C).

Calculation of the GM-CSF output as a function of the number of adherent cells revealed that the GM-CSF output was substantial during the first 24 h, peaked during the 24-48 h period, and declined thereafter (Fig 4.4D). The GM-CSF outputs (per 10^5 cells) for each of four replicate culture

wells differed from the mean by an average of 1.5% at 24 h, and increased as the culture period lengthened to 7.6% at 96 h.

4.3.4 Potency of endometrial cell GM-CSF bioactivity, compared with other cellular sources

The output of GM-CSF from endometrial cells was compared with that from other normal cells and immortal cell lines (Fig. 4.5). The amount of GM-CSF produced per 10^5 cells in 24 h of culture was two to thirty-fold greater for endometrial cells than for polyclonally activated spleen cells, immortalised T-lymphocytes, LPS-activated peritoneal exudate macrophages, macrophage cell lines or for fibroblasts.

4.4 Identity of GM-CSF-like bioactivity in endometrial cell-conditioned media

4.4.1 Neutralisation of bioactivity with polyvalent anti-GM-CSF antiserum

The FD 5/12 cell lines used in the GM-CSF bioassay is reported to be responsive specifically to GM-CSF (Duhrsen 1988). However, since the endometrium is not a conventional source of this cytokine, it was of interest to further characterise the endometrial cell-derived molecules. Goat αGM-CSF polyclonal antibody, a polyvalent antiserum raised against recombinant murine yeast-derived GM-CSF (Miyajima *et al.* 1986), reduced the FD 5/12 cell proliferative response to 260 U/ ml of uterine cell-derived GM-CSF activity by approximately 7-fold at a dilution of 1:250,000, whilst 2.5 µg/ ml of a rat αIL-6 mAb had no effect (Fig. 4.6). Furthermore, titration of endometrial cell supernatant in the presence of antibody and additional recombinant GM-CSF reinstated the FD 5/12 proliferative response, and so eliminated the possibility that cytotoxic activity associated with the antisera interfered with the assay.

4.4.2 Determination of the molecular weight of endometrial cell GM-CSF by Sephacryl S-200 chromatography

To further investigate the nature of the endometrial cell-derived cytokine, an approximate molecular weight of the GM-CSF bioactivity was determined by Sephacryl S-200 size exclusion chromatography. Peak proliferation in the FD 5/12 bioassay was obtained in fractions eluted at Mr 23,000 (Fig. 4.7).

4.4.3 Northern blot analysis of endometrial cell GM-CSF mRNA

Total RNA was harvested both from PHA/ PMA activated EL-4 (T-lymphocyte) cells, and from endometrial cells derived from oestrous mice following 48 h in culture. Agarose-formaldehyde gel electrophoresis followed by Northern hybridisation with a ^{32}P-labelled riboprobe specific for murine GM-CSF revealed that endometrial cells express a GM-CSF mRNA of 1.2 kb, which was indistinguishable in size from GM-CSF mRNA expressed by activated EL-4 cells (Fig. 4.8).

4.5 Stimulation of GM-CSF synthesis by seminal factors

The experiments described in section 4.2 showed that there is a dramatic but transient increase in the luminal fluid GM-CSF content after mating. Since GM-CSF was absent from the accessory gland fluids that comprise the ejaculate, and endometrial cells were found to be a potent source of GM-CSF *in vitro*, it was speculated that the increase in luminal fluid activity at mating was a consequence of an interaction between semen and cells within the endometrium. The experiments described in this section were aimed at describing this interaction further, with particular reference to defining the nature of the seminal stimulus.

4.5.1 Effect of allogeneic versus *syngeneic mating*

GM-CSF is one of a number of cytokines released upon lymphocyte activation (Woods *et al.* 1987; Lu *et al.* 1988). To determine whether the post-mating increase in luminal GM-CSF content occurred as a consequence of alloantigenic stimulation of endometrial lymphocytes by MHC antigens (associated with sperm or other cells contained within the semen), a comparison was made of the effect of mating females to syngeneic or to allogeneic males. The content of GM-CSF in the uterine luminal fluids from both CBA x C57BL (H-2 b/d) and Balb/c x C57BL (H-2 k/d) females after mating to CBA x C57BL males was similar ($p = 0.215$), and in each instance was significantly greater than the content in fluid from oestrous Balb/c x C57BL mice ($p < 0.001$) (Table 4.3).

Table 4.3 GM-CSF in uterine luminal fluid of mice following allogeneic *versus* syngeneic natural mating. Females are Balb/c x C57BL unless otherwise specified. Values are medians (95% confidence range) with numbers of observations in parentheses. Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed)-test. $^{*}p < 0.001$

Uterine Status			GM-CSF (U/ uterus)
Unmated:	Oestrous	(22)	<1 (<1-9.4)
Mated:	Allogeneic	(18)	8.8 (<1-40.0)*
	Syngeneic[a]	(9)	11.4 (2.8-38.4)*

[a]CBA x C57BL

4.5.2 Effect of male accessory gland deficiency on post-mating GM-CSF response

To determine whether a deficiency in either sperm or the seminal vesicle-derived component of the semen influences the post-mating elevation in uterine GM-CSF content, females were mated naturally with either intact, vasectomised, or seminal vesicle-deficient males. Vaginal smears were

made from females caged with seminal vesicle-deficient males each morning and checked for the presence of sperm, since absence of the seminal vesicle fluids from the ejaculate prevented copulatory plug formation. Luminal fluids were collected on day 1, and the pellets obtained after centrifugal clearing of the fluid were examined microscopically for sperm content. Fluids were not included in the analysis unless the sperm content was comparable to the content in luminal fluids from mice mated with intact males.

Luminal fluids from mice mated with seminal vesicle-deficient males (SV-) were found to contain significantly less GM-CSF than mice mated to intact studs [median (95% confidence range) = <1 (<1-6.4) U/ uterus for SV- *versus* 8.1 (<1-36.8) U/ uterus for intact, $p = 0.001$], and levels are not significantly increased over those of fluids from unmated, oestrous females [<1 (<1-4.7) U/ uterus; $p = 0.123$] (Fig. 4.9). In contrast, GM-CSF levels in mice mated with vasectomised males were not significantly different from those of mice mated to intact studs [11.6 (<1-32.6) U/ uterus].

4.5.3 Effect of male accessory gland fluids on GM-CSF synthesis in vitro

To determine whether factors derived from the seminal vesicle gland interact with endometrial cells to increase GM-CSF release, endometrial cells harvested from the pooled uteri of oestrous mice were cultured in the presence of fluids collected from the seminal vesicle, prostate, coagulating gland and epididymis of 4-6 month old stud males. Accessory gland fluids were harvested from a 6 month old male mouse by dispersion of seminal vesicles, prostate or coagulating glands with the aid of fine needles. Epididymal fluid was obtained by flushing of the epididymis. Extracts were incubated with endometrial cells for 16 h, following which the media was replaced and supernatants were harvested after a further 24 h culture period.

GM-CSF output (per 10^5 adherent cells) from endometrial cells was significantly greater in the presence of seminal vesicle fluid (7.6 $\pm$ 0.2 fold increase) when compared with the output from endometrial cells cultured in the absence of accessory gland fluid. Culture of endometrial cells with coagulating gland, prostate or epididymal fluids did not alter their GM-CSF output (Fig 4.10A).

In additional experiments, an examination was made of various protocols for obtaining seminal vesicle gland fluid and of its effects on the assay system. GM-CSF-stimulating activity was measured in the seminal vesicle fluids of 4-6 month old male mice. Cytotoxic activity was evident to varying degrees in all seminal vesicle preparations, causing a reduction in the number of adherent cells in the cultures (data not shown). It was found that even very slight contamination with coagulating gland fluid during excision of the seminal vesicle, delay of more than a minute or so between death of the animal and maceration, or aeration during maceration and clearing by centrifugation, caused seminal vesicle fluids to precipitate. This resulted in loss of GM-CSF stimulating activity. Maximal stimulation of GM-CSF secretion was achieved after 16 h (compared to 4 or 8 h) co-incubation of seminal vesicle extract with endometrial cells. In a total of six experiments, the median (95% confidence range) increase in endometrial cell GM-CSF output (following incubation with precipitate-free seminal vesicle extracts) was 320 (150-760) per cent ($p < 0.05$).

4.5.4 *Effect of age, sexual experience and testosterone status on seminal factor activity*

To determine whether age, sexual experience and testosterone status influence the GM-CSF-stimulating capacity of male mice (and so to identify an appropriate population to use as the source of material for characterisation of the nature of the stimulating factor/s), seminal vesicle fluid was harvested from 3 month old male mice that had been caged either in groups or individually for 3 weeks, and from 4-6 month old males that had been caged individually but had been in use as stud males on a regular basis for at least two months. In addition, seminal vesicle fluid was harvested from unseparated 3 month old male mice following injection with 200 µg of testosterone 48 h prior to sacrifice. Excised glands were weighed immediately prior to maceration. Fluids were incubated with endometrial cells as described in 4.5.3 and measurements were made of the amount of GM-CSF released per 10^5 adherent endometrial cells.

The capacity of seminal vesicle fluids to enhance GM-CSF release from endometrial cells differed between treatment groups. Stud males yielded significantly larger seminal vesicles (mean ± SD = 254 ± 35 mg; $p = 0.001$) than unseparated or inexperienced males, and yielded significantly more GM-CSF-stimulating activity than all other groups [median (± 95% confidence range) GM-CSF output = 233 (119-439)% control; $p = 0.001$] (Fig. 4.10). Caging inexperienced male mice individually significantly increased both seminal vesicle weights (unseparated; 127 ± 31 mg *versus* separated; 183 ± 18 mg; $p = 0.001$), and GM-CSF-stimulating activity (unseparated; 77 (27-156)% *versus* separated; 127 (86-318)%; $p = 0.021$). Despite increasing the mean ± SD weight of seminal vesicles in unseparated males (236 ± 16 mg; $p = 0.001$), testosterone treatment caused many of the seminal vesicle fluids to inhibit, rather than stimulate, endometrial cell GM-CSF output. Endometrial cells cultured with seminal vesicle fluids from testosterone-treated males released significantly less GM-CSF than all other groups, including those exposed to fluids from untreated, unseparated males (33 (13-176)%; $p = 0.010$).

4.5.5 *Molecular weight of seminal factor*

To determine whether the GM-CSF stimulating activity was excluded by Sepharose G-25 (which excludes molecules of greater than 5,000 Mr), a 26-gauge needle and syringe was employed to withdraw neat fluid from seminal vesicles *in situ*. Approximately 100 µl of fluid was recovered from individual 6 month old stud mice, dissolved in five-times its volume of guanidine HCl, and passed through Sepharose G-25 equilibrated in DMEM. When fractions were added at a 1:2 dilution to endometrial cell cultures, GM-CSF-stimulating activity was found consistently within the first 1 ml fraction eluted from the column. Preparations from three individual 6 month old male mice were found to increase the GM-CSF outputs of endometrial cells by 374%, 186% and 120% respectively. In one of these experiments, the activity was shown to be concentration dependant, with maximal activity at a 1:2 dilution, titrating to undetectable levels at dilutions beyond 1:16 (corresponding respectively to an approximately 1:20 and 1:160 dilution of neat seminal vesicle fluid).

A preliminary attempt was made to characterise the molecular weight of the endometrial cell-

stimulating activity within seminal vesicle fluid, by the use of chromatography through a broad resolution (10,000 - 4,000,000 Mr) Sepharose CL4B column. Seminal vesicle fluid harvested from four 4-6 month old stud mice was pooled in 5 M guanidine HCl and passed through an HBSS-equilibrated column. Eluted fractions were assessed for their capacity to stimulate GM-CSF release from oestrous endometrial cells *in vitro*. GM-CSF-stimulating activity was eluted over a broad range of high molecular weight fractions, with two peaks in activity eluted within the first one-third of fractions collected (Fig. 4.11). The second half of fractions collected were found to be contaminated with guanidine HCl which interfered with the bioassay.

4.5.6 Northern blot analysis of GM-CSF mRNA expression in vivo

To determine whether the increase in the luminal fluid content of GM-CSF bioactivity occurred as a consequence of an increase in transcription of the GM-CSF gene, GM-CSF mRNA levels were assessed in the uteri of oestrus, and day 1 through day 4 pregnant mice. Total RNA was isolated from homogenised whole uteri, and 30 μg of each preparation was electrophoresed through an agarose-formaldehyde gel and transferred to a nylon filter. Northern hybridisation with a ^{32}P-labelled riboprobe specific for murine GM-CSF revealed that uteri from day 1 mated mice express a GM-CSF mRNA of 1.2 kb, whilst no GM-CSF transcripts could be detected in RNA prepared from mice at oestrus or later than day 1 of pregnancy. The filter was also probed with a ^{32}P-labelled riboprobe specific for murine $\beta_2$microglobulin, to act as an internal control for comparison of the amounts of RNA loaded onto each track. This probe labelled a pair of bands of the expected sizes of 930 and 715 bp (Parnes *et al.* 1983).

4.6 IL-6 synthesis in the cycling and pregnant uterus

4.6.1 Assay of uterine luminal fluid and endometrial cell culture supernatants for other cytokines

The concentrations of IL-1, IL-2, IL-3 and TNFα in the uterine luminal fluids collected in the experiments described in 4.2.1, and in the supernatants from cultures of uterine endometrial cells from oestrus, day 1 and day 10 pregnant mice (from the experiments described in 4.3.2), were measured using specific bioassays. No cytokine bioactivity was detected in any of the supernatants or luminal fluids tested.

4.6.2 IL-6 activity in uterine luminal fluid

The IL-6 content of the luminal fluids collected in the experiments described in 4.2.1 were measured in an IL-6 specific bioassay employing the 7TD1 cell line. IL-6 activity was detected in uterine luminal fluid (Fig. 4.1) from both oestrous and day 1 mated mice, with median levels approximately 250-fold greater in mated mice than in mice at oestrus [median (95% confidence range) = 9 (<2-56) U /uterus at oestrous *versus* 2318 (34-12,800) U /uterus on day 1, p <0.05].

4.6.3 Synthesis of IL-6 by endometrial cells in vitro

The IL-6 content of the supernatants from cultures of uterine endometrial cells obtained from oestrous, day 1 and day 10 pregnant mice (from the experiments described in 4.3.2), were also measured. IL-6 was detected in supernatants harvested from at oestrus [3900 (2420-16,990) U/ 10^5 cells] and day 1 mice [5440 (2020-14,580) U/ 10^5 cells] (Fig. 4.3). There was no difference between levels in supernatants obtained from cells harvested at either time ($p > 0.1$). Cells harvested from day 10 pregnant uteri, from either between implantation sites (inter-conceptus), adjacent to embryonic membranes (peri-conceptus), or from the metrial gland secreted GM-CSF at levels comparable to cells from oestrous and day 1 uteri.

A broad range of values was obtained for IL-6 levels in intraluminal fluids and culture supernatants. This is attributed to *in vivo* factors rather than to error introduced during tissue preparation or assay, because IL-6 activities of supernatants derived from each of the two uterine horns within individual animals were not significantly different ($p > 0.1$; n = 8 oestrous mice). The activities of supernatants derived from each horn differed from the mean in individual animals by an average of 8.0%.

4.6.3 Kinetics of IL-6 synthesis over time in culture

The supernatants generated in the experiment described in 4.3.3 were assayed for IL-6 content to determine the kinetics of IL-6 output during endometrial cell culture. Figure 4.4B shows that the output of IL-6 increases continuously over the first 96 h of endometrial cell culture. This is primarily a function of the increase in cell numbers during this period however, as the output on a cellular basis remains relatively constant over 120 h (Fig. 4.4D). The IL-6 outputs (per 10^5 cells) for each of four replicate culture wells differed from the mean by an average of 8.0% at 24 h, and increased as the culture period lengthened to 22.4% at 120 h.

4.6.4 Potency of endometrial cell IL-6 bioactivity, compared with other cellular sources

Production of IL-6 by uterine epithelial cells was compared with that from other normal and immortal cellular sources (Fig. 4.5). The amount of bioactive cytokine produced per 10^5 cells in 24 h of culture was two to five hundred-fold greater for uterine cells than for either polyclonally activated spleen cells, immortalised T-lymphocytes, LPS-activated peritoneal exudate macrophages, macrophage cell lines or for fibroblasts.

4.6.5 Neutralisation of IL-6 bioactivity with an anti-IL-6 monoclonal antibody

The 7TD1 cell line used in the IL-6 bioassay is reported to be specifically responsive to IL-6 (Van Snick *et al.* 1986). To further characterise the endometrial cell-derived molecule, antibody against murine Krebs II ascites carcinoma cell-derived IL-6 (Shabo and Sachs 1988) was assessed for its capacity to neutralise the endometrial cell bioactivity. Rat αIL-6 (2.5 μg/ ml) reduced the 7TD1 proliferative response to uterine cell-derived IL-6 activity (660 U/ ml) approximately 8-fold, while

goat αGM-CSF had no effect (Fig. 4.6). Furthermore, titration of endometrial cell supernatant in the presence of antibody and additional recombinant IL-6 reinstated the 7TD1 proliferative response, thus discounting the possibility of cytotoxic activity associated with the antisera.

4.6.6 Determination of the molecular weight of endometrial cell IL-6 by Sephacryl S-200 chromatography

To further investigate the nature of the endometrial cell-derived cytokines, the approximate molecular weight of the IL-6 bioactivity was determined by Sephacryl S-200 size exclusion chromatography. Peak proliferative capacities in the 7TD1 bioassay were eluted in fractions corresponding to Mr 23,000-26,000 (Fig. 4.7).

4.7 Conclusion and discussion

The experiments reported within this chapter identify GM-CSF within the early pregnant mouse uterus, where seminal components interact with the endometrium to invoke a dramatic but transient increase in the luminal fluid GM-CSF content after mating. The findings are consistent with the hypothesis that GM-CSF has an embryotrophic role.

The volume of uterine fluid on the day after mating is about 90 µl (Wales and Edirisinghe 1989) so the median luminal GM-CSF concentration at this time can therefore be calculated to be about 200 U/ ml. This is well within the bioactive range, and local concentrations at sites of production are likely to be much higher. GM-CSF contents of individual uteri covered a broad range, and this may reflect variable efficiency of recovery of fluids, but could also be the result of heterogeneity in uterine responses or in stimulating activity of individual males. The volume of fluid in the uterine lumen at oestrus is similar to that in mated animals, but the volume after day 1 decreases rapidly to 4 µl on day 3 (Wales and Edirisinghe 1989). The dilution factor involved in collecting fluids after day 1 would therefore have prevented the detection of all but extremely high concentrations of cytokine. Therefore, while the total output into luminal fluids is markedly reduced after day 1, the concentration of GM-CSF could not be determined in fluids collected after day 1. Moreover, there appeared to be no simple way of quantitating the total uterine content of GM-CSF, since activity could not be detected within homogenised uterine tissue (from any stage of early pregnancy) either by bioassay or by specific ELISA (in the laboratory of Dr Tom Wegmann, University of Alberta, Edmonton, Alberta). This may have been due to proteolytic digestion of the cytokine or to interference by cytotoxic material (data not shown).

The post-mating elevation in GM-CSF levels was inhibited in superovulated animals, where the normal endocrine parameters are grossly perturbed. This was not simply a result of immaturity, because GM-CSF was not detected in either pre-pubertal or adult superovulated mice. Mice synchronised into oestrous using LHRH superagonist, which does not alter steroid hormone levels during the ensuing oestrous cycle, had GM-CSF contents on the day after mating similar to those in naturally mated, randomly cycling mice.

GM-CSF was not detected in any of the accessory gland fluids that comprise the ejaculate, and cervical stimulation failed to elevate the luminal fluid GM-CSF content. These findings suggested that elevated GM-CSF content was a consequence of the interaction of seminal factors with a cellular source of the cytokine within the female reproductive tract. Further evidence for this possibility was the finding of GM-CSF activity in the supernatants of cultured uterine cells. Endometrial cells were a richer source of GM-CSF than uterine stromal cells, and secreted constitutively greater amounts of GM-CSF on a per cell basis than conventional sources of this cytokine (including activated T-lymphocyte or macrophage cell lines). GM-CSF output from endometrial cells remained high for the first 48 h *in vitro*, and although it declined subsequently, it remained significant for at least another four days of culture. Endometrial cell-derived bioactivity was blocked by an αGM-CSF antiserum, and the estimated molecular weight of Mr 23,000 is similar to the size reported for GM-CSF synthesised by other tissues (Nicola *et al.* 1979). These findings, together with Northern blot analysis showing that endometrial cells expressed a 1.2 kb GM-CSF mRNA both *in vivo* and *in vitro*, confirmed the identity of the cytokine.

In experiments designed to investigate the nature of the seminal stimulus for GM-CSF release, females were mated with both syngeneic and vasectomised males. These mice were found to have a normal post-mating GM-CSF response. However, females mated with males from which the seminal vesicles had been removed surgically failed to exhibit the post-mating increase in GM-CSF activity. These results excluded the possibilities that either sperm or histocompatibility antigens provided the stimulus for release, and implicated factors provided by the seminal vesicle. *In vitro*, fluids derived from the seminal vesicle but not from other male accessory glands stimulated enhanced GM-CSF release from endometrial cells. These findings further implicate a specific interaction between factor/s of seminal vesicle origin and endometrial cells as the underlying basis for the post-mating GM-CSF response. Northern blot analysis of RNA extracted from uterine tissues revealed that an increase in GM-CSF mRNA transcription accompanies this interaction.

In preliminary attempts to further characterise the seminal vesicle-derived factor/s, activity stimulating enhanced GM-CSF release from endometrial cells was found to elute at high molecular weights (estimated to be greater than 100,000) from molecular sieving columns. However, the heterogeneity of the peak suggested that the fractionation was complicated by a substantial degree of intermolecular association. It therefore remains possible that the active factor is related to a 15, 000 Mr boar seminal plasma protein called Adhesion Inducing Factor-1. This factor is synthesised in the seminal vesicle epithelium and induces integrin-mediated cell adhesion between, and activation of, peripheral blood mononuclear cells (Hadjasavas 1992).

Interestingly, there was variation in the potency of seminal vesicle fluid activity, depending on the sexual status of the male. Active stud males of four to six months of age had a superior capacity to enhance GM-CSF release from endometrial cells compared with younger males caged either individually or in groups. This appeared consistent with a role for testosterone in regulating seminal vesicle factor activity. However, the potency of activity from males caged in groups was found to decline rather than improve following testosterone administration, suggesting that other factors may also be important.

The luminal fluids and endometrial supernatants were also assessed for their content of other cytokines. Interleukin-1, IL-2, IL-3 and TNFα activities were not be detected in specific bioassays of either uterine fluids or endometrial cell conditioned media. In contrast, the luminal fluids of mated mice were a potent source of IL-6, and comparison with fluids from oestrous mice revealed that there was an approximately 200-fold increase in luminal fluid content after mating. Culture supernatants from endometrial cells released IL-6 constitutively for up to six days in culture, and this activity was neutralised with a monoclonal antibody reactive specifically with murine IL-6. Chromatography of endometrial cell-derived activity revealed that it was comparable in size to IL-6 synthesised by other cell lineages, which is generally estimated to be between Mr 22-29,000 (Van Snick *et al.* 1986; Van Snick 1990).

These findings are in agreement with a recent report that mRNA transcripts for GM-CSF and IL-6 are elevated after mating (Sanford *et al.* 1992). The expression of mRNAs for IL-1α and IL-1β, CSF-1 and TNFα, as well as the content of IL-1, IL-6 and TNFα bioactivities in uterine homogenates, were found in the same study to be greater on day 1 of pregnancy than in oestrous uteri. In another recent study, factors apart from sperm in seminal plasma were implicated in the functional activation of endometrial macrophages which were found to express IL-1α, IL-1β and TNFα mRNAs on day 1 of pregnancy (McMaster *et al.* 1992). These post-mating changes in cytokine expression are accompanied by a dramatic infiltration into the endometrium of mononuclear phagocytes and neutrophils (1.5.2; 1.5.3), and together are evidence that seminal fluid initiates a local inflammatory response.

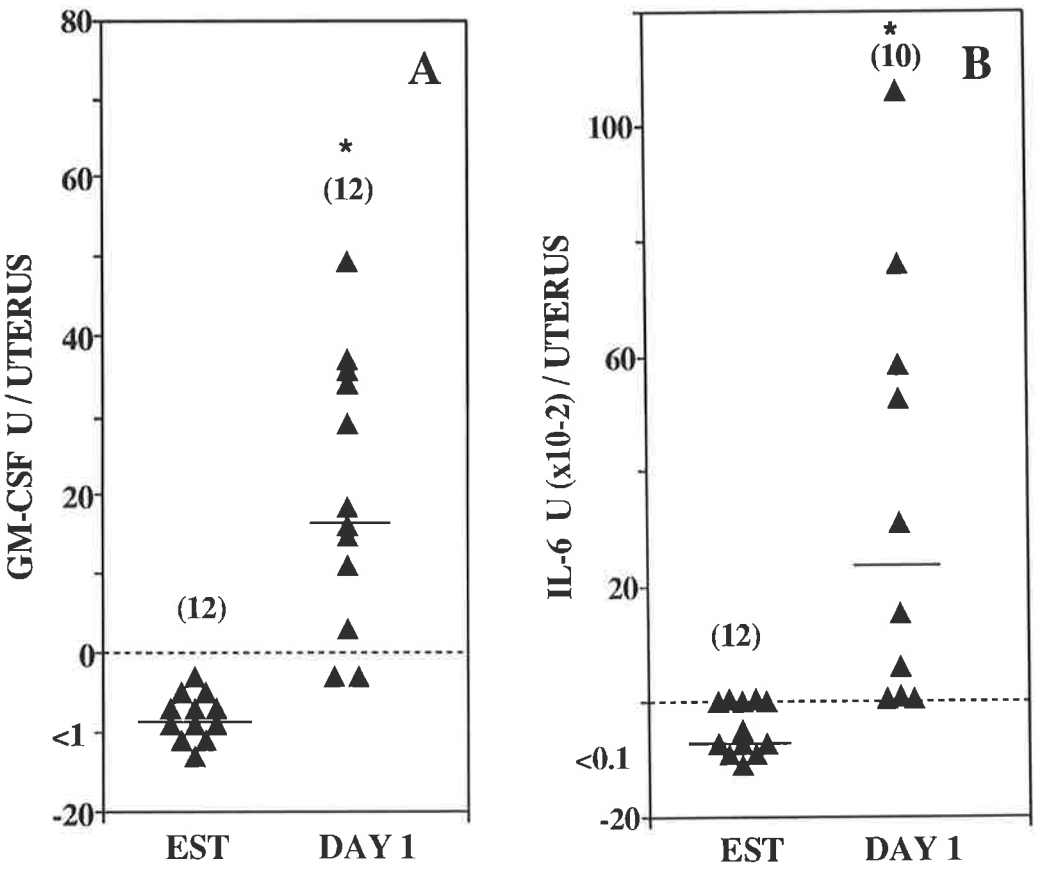


Figure 4.1 GM-CSF and IL-6 bioactivity in uterine intraluminal fluid of oestrous and day 1 pregnant mice. Luminal fluid was collected by lavage from oestrous (EST) and day 1 pregnant (DAY 1) mice and assessed for GM-CSF and IL-6 content respectively in the FD 5/12 and 7TD1 bioassays. Symbols represent individual animals and median values are scored, with numbers of observations in parentheses. Symbols below the dotted line have a cytokine content below the limit of detection for the bioassay. Data were compared by Mann-Whitney Rank Sum Test (two-tailed)-test. $*p < 0.05$

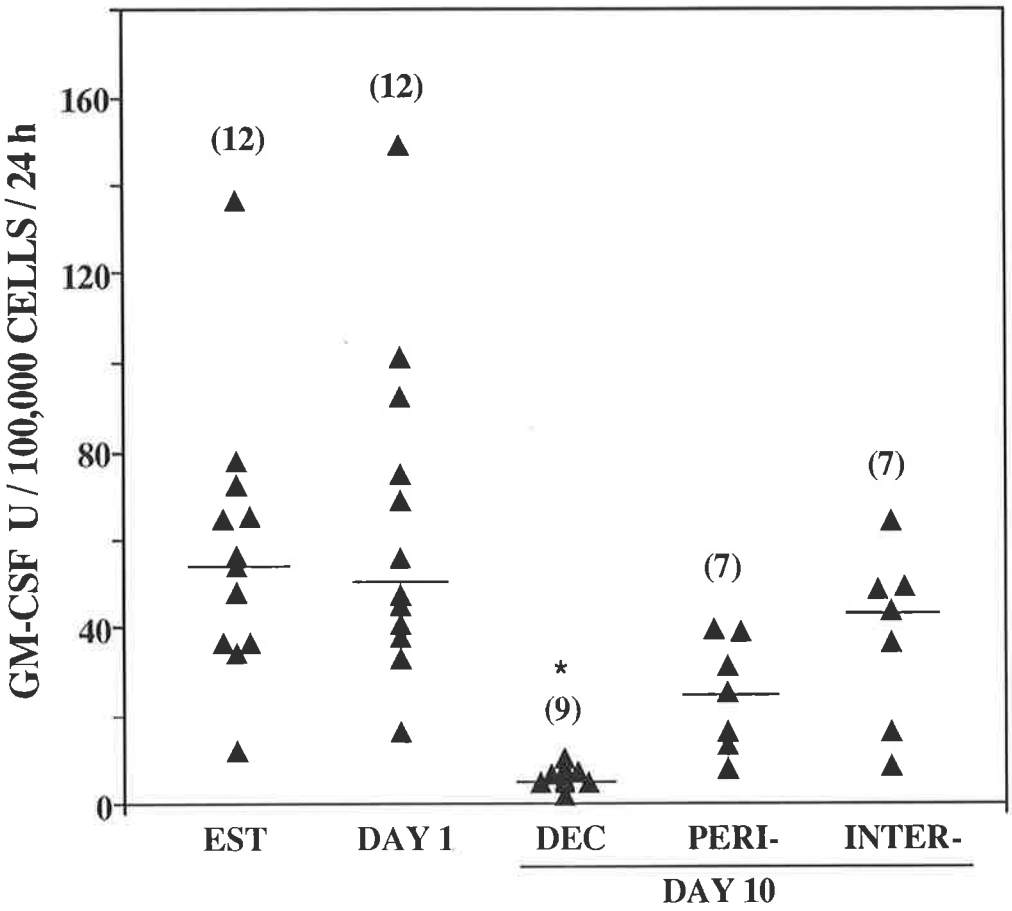


Figure 4.2 GM-CSF bioactivity in the supernatants of endometrial cell cultures prepared from oestrous, day 1 and day 10 pregnant mice. Endometrial cells were harvested from oestrous (EST) and day 1 pregnant (DAY 1) uteri, and from deciduae (MG), and non-decidualised peri-conceptus (PERI-) and inter-conceptus (INTER) uteri of day 10 pregnant mice. Supernatants were collected at 24 h and assessed for GM-CSF content in the FD 5/12 bioassay. Symbols represent individual animals and median values are scored, with numbers of observations in parentheses. Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed)-test. *$p < 0.05$

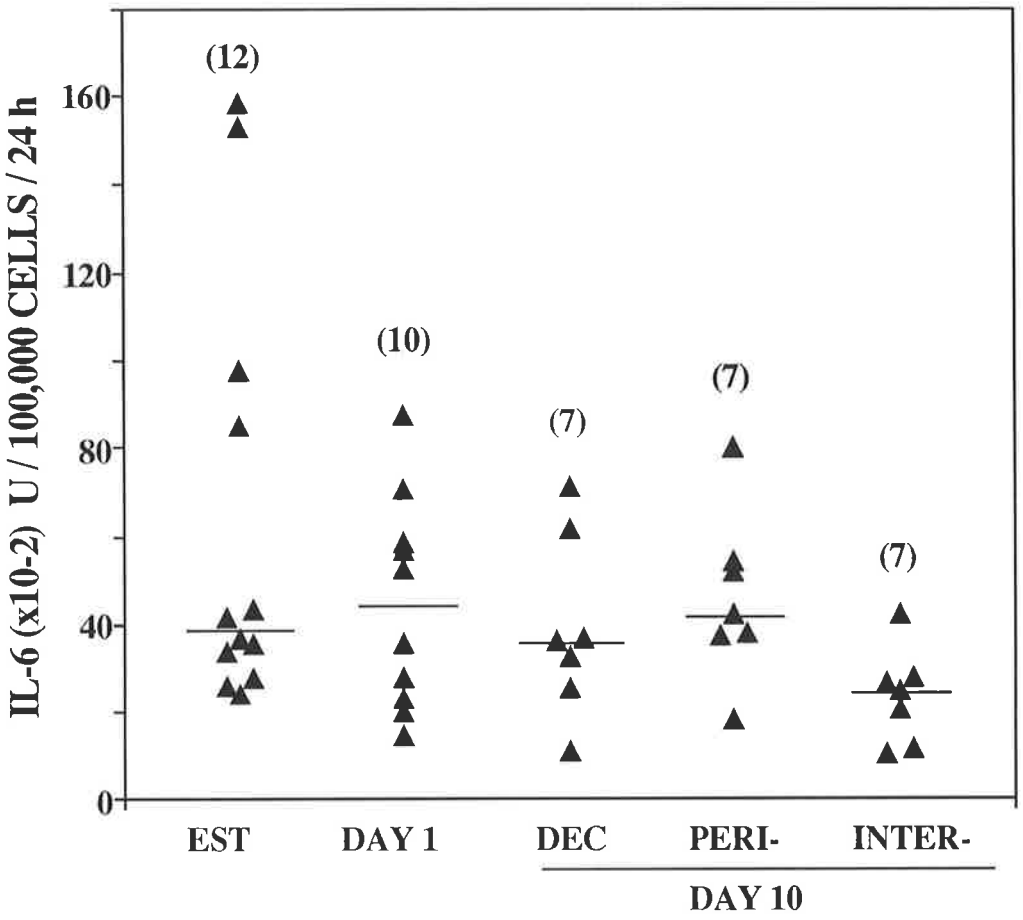


Figure 4.3 IL-6 bioactivity in the supernatants of endometrial cell cultures prepared from oestrous, day 1 and day 10 pregnant mice. Endometrial cells were harvested from oestrous (EST) and day 1 pregnant (DAY 1) uteri, and from deciduae (MG), and non-decidualised peri-conceptus (PERI-) and inter-conceptus (INTER) uteri of day 10 pregnant mice. Supernatants harvested at 24 h were assessed for IL-6 content in the 7TD1 bioassay. Symbols represent individual animals and median values are scored, with numbers of observations in parentheses. There were no significant differences between groups when data were compared by Kruskal-Wallis one-way ANOVA.

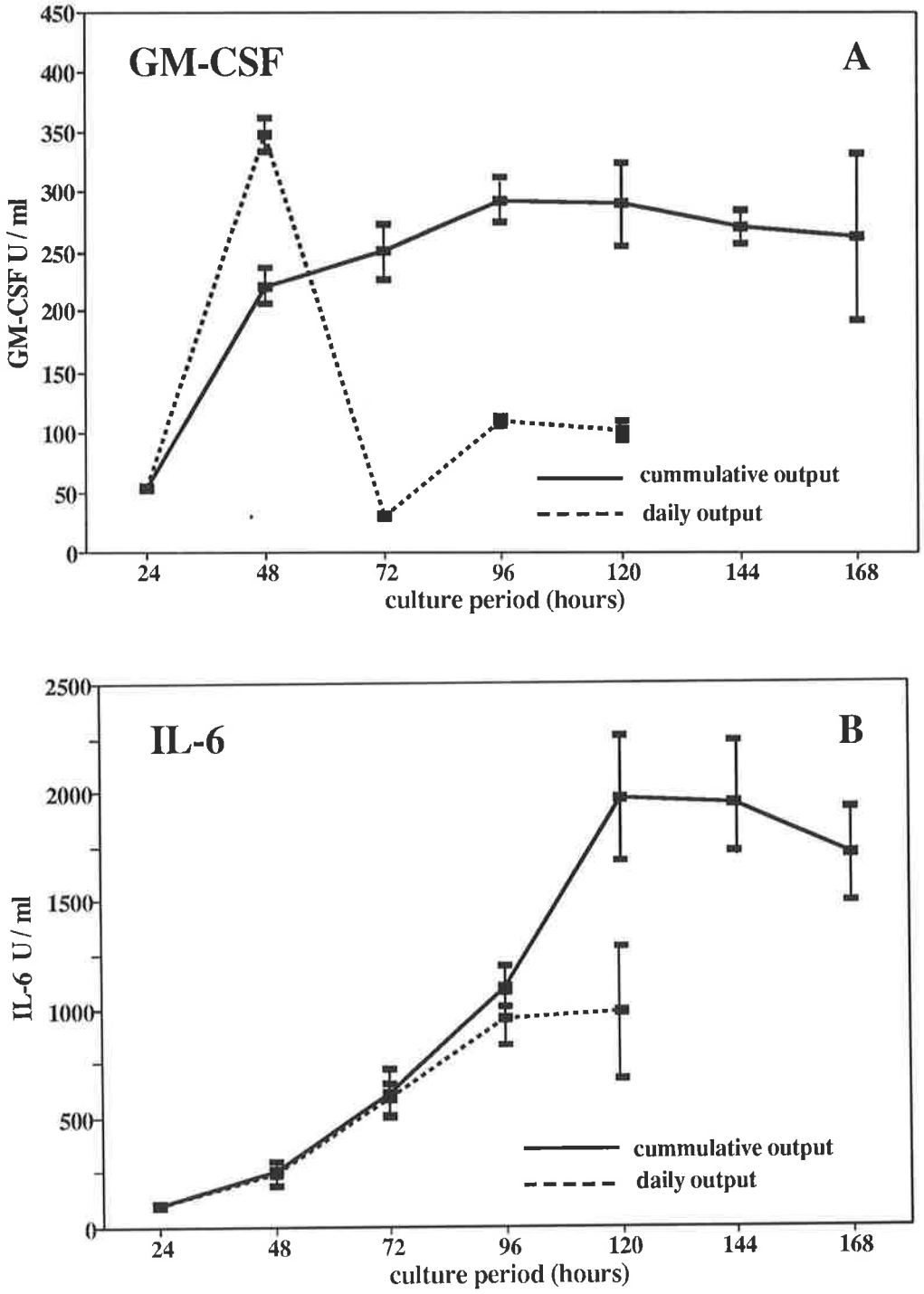
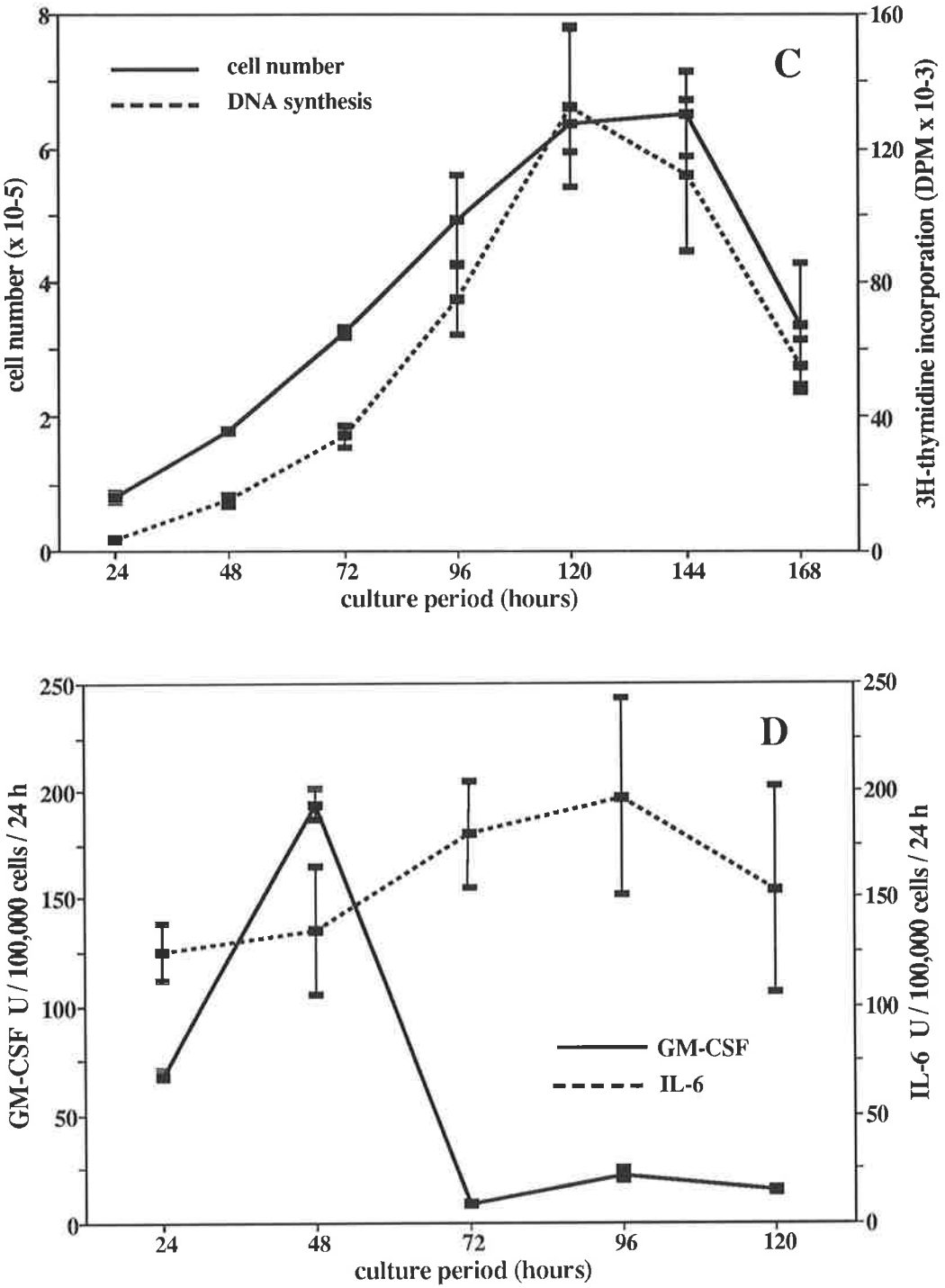


Figure 4.4 The kinetics of endometrial cell proliferation and cytokine synthesis *in vitro*. Endometrial cells prepared from pools of uteri harvested from oestrous mice were seeded at 1 x 10^5 cells/ 1 ml well and cultured for periods of up to 7 days. To examine cumulative cytokine output, supernatants harvested from quadruplicate wells at 24 h intervals were assayed for GM-CSF and IL-6 content. In a separate set of four wells, the media was changed every 24 h over 7 days and the cytokine output during each 24 h period was determined. (A) Cumulative and daily GM-CSF output, and (B) cumulative and daily IL-6 output from endometrial cells during 7 days in culture. (Fig. 4.4 continued overleaf)



(Figure 4.4 cont.) At the end of each 24 h period, the numbers of adherent cells in each well were determined on the basis of their uptake of Rose-Bengal dye, and used to calculate the cytokine output per 10^5 cells during each 24 h period. In addition, the rate of DNA synthesis in endometrial cell cultures was measured by ^{3}H-thymidine incorporation. Wells of a 96-well plate were seeded with 2 x 10^3 cells and individual plates were pulsed with 1 μCi of ^{3}H-thymidine for 7 h prior to harvesting at 24 h intervals. (C) Endometrial cell number and DNA synthesis during 7 days in culture. (D) Cytokine output per 10^5 adherent endometrial cells during each 24 h period.

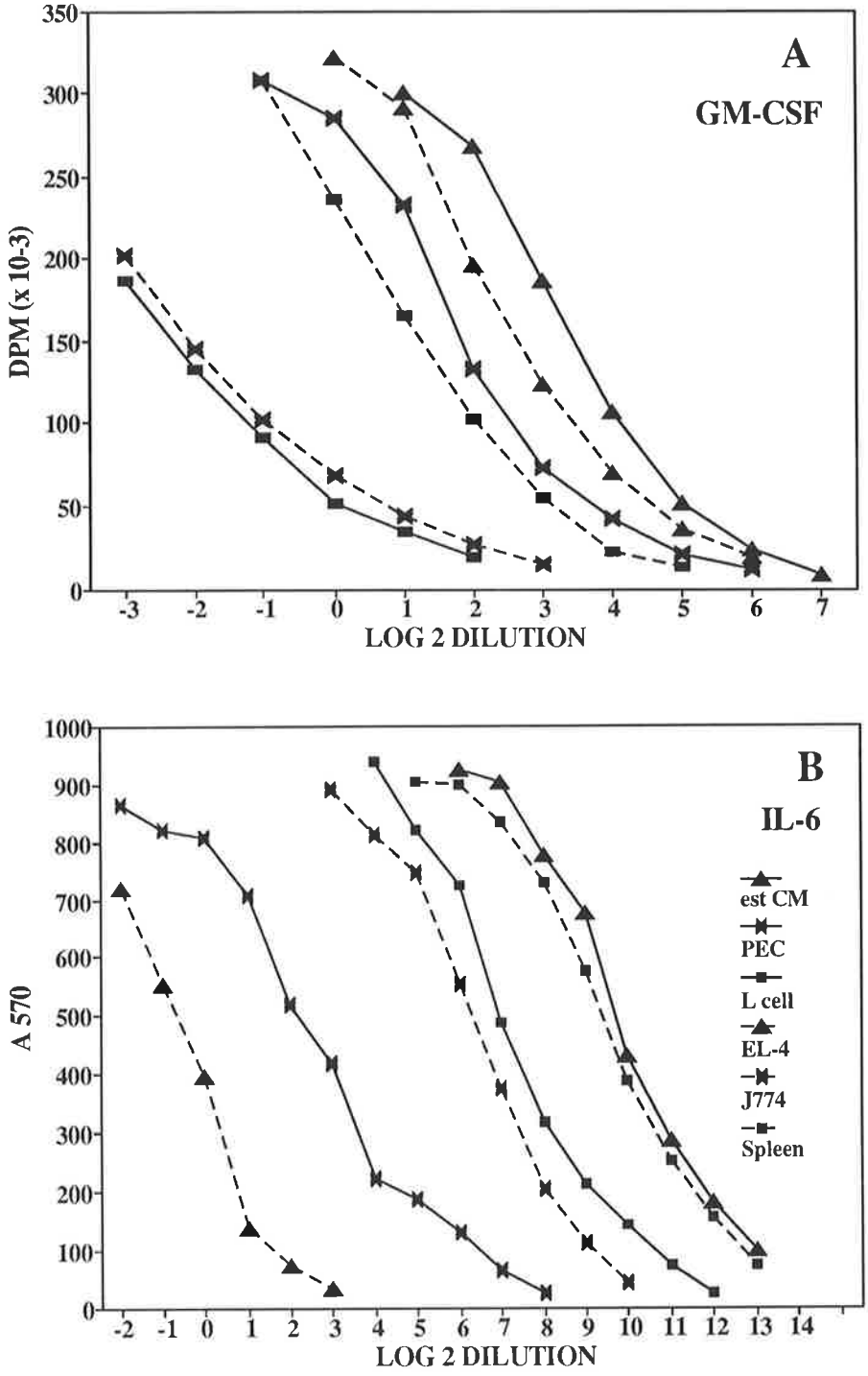


Figure 4.5 Potency of endometrial cells as a source of GM-CSF and IL-6. Supernatants from 24-h cultures of 2 x 10^5 cells/ml of oestrous endometrial cells (est S/N); PMA/ PHA-activated spleen cells (spleen); PMA-activated EL-4 T-lymphocytes (EL-4); LPS-activated peritoneal cells (PEC); J774 macrophages (J774); and L-cell fibroblasts (L-cell) were assessed for GM-CSF and IL-6 content in the FD 5/12 and 7TD1 bioassays respectively. The number of indicator cells at the end of the assay period [as assessed by ^{3}H-thymidine incorporation (FD 5/12) or by formazan production (7TD1)] is plotted against the dilution of supernatant.

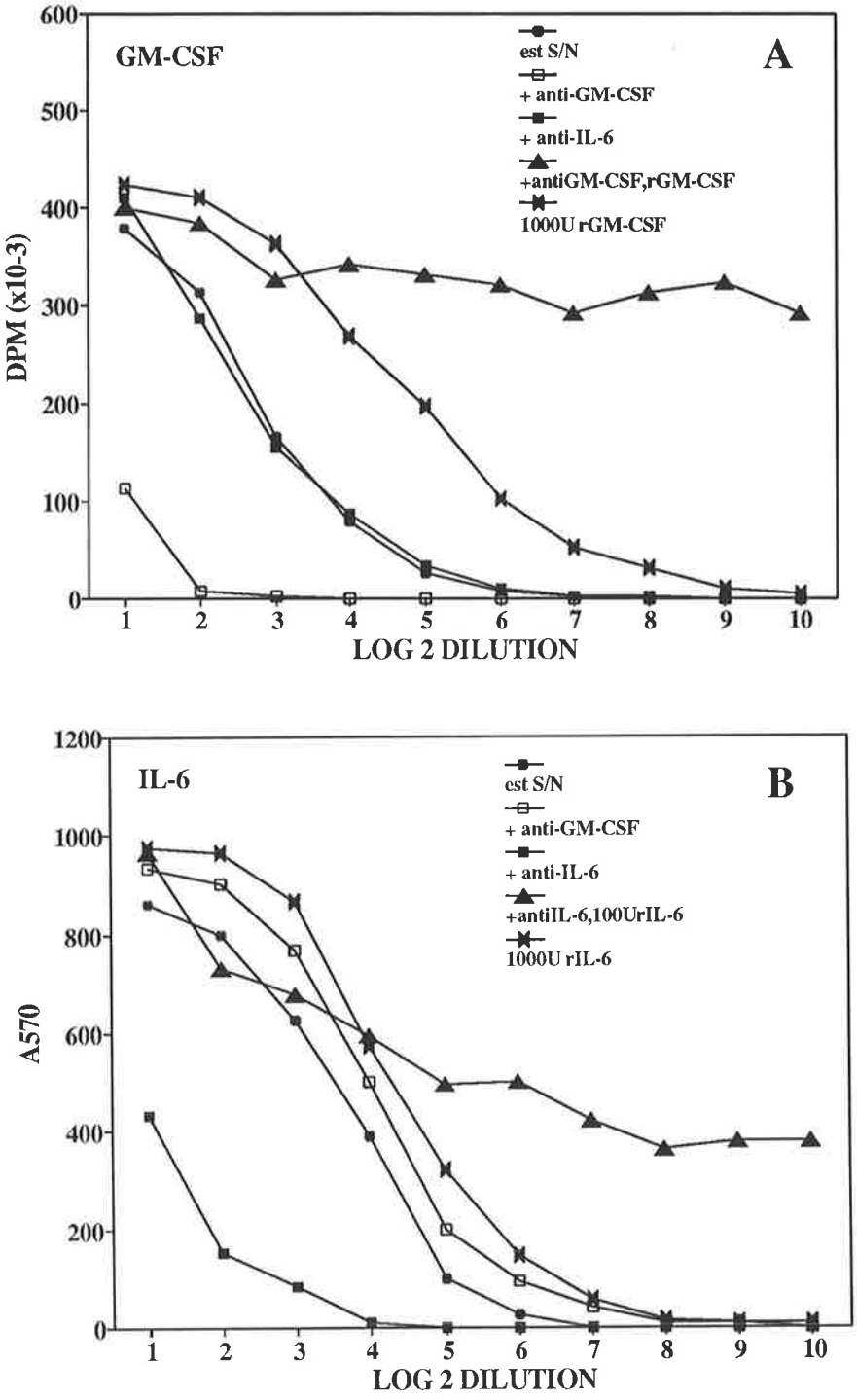


Figure 4.6 Neutralisation of GM-CSF and IL-6 bioactivity with specific antibodies. The proliferative responses of (A) FD 5/12 and (B) 7TD1 to GM-CSF and IL-6 bioactivity in supernatant from 24 h culture of endometrial cells pooled from 6 oestrus mice (est CM) were assessed in the presence or absence of goat αGM-CSF (1:250,000) or rat αIL-6 (2.5 μg/ml). To determine whether the antibody preparations interfered with the proliferative response of the FD 5/12 of 7TD1 cells, est CM was incubated in the presence of (A) goat αGM-CSF (1:250,000) and 300 U/ ml rGM-CSF, or (B) rat αIL-6 (2.5 μg/ml) and 100 U/ ml rIL-6. The proliferative responses of FD 5/12 cells to 1000 U recombinant murine GM-CSF (A) or 7TD1 cells to recombinant human IL-6 (B) are also shown. The number of indicator cells at the end of the assay period [as assessed by ^{3}H-thymidine incorporation (FD 5/12) or by formazan production (7TD1)] is plotted against the dilution of supernatant.

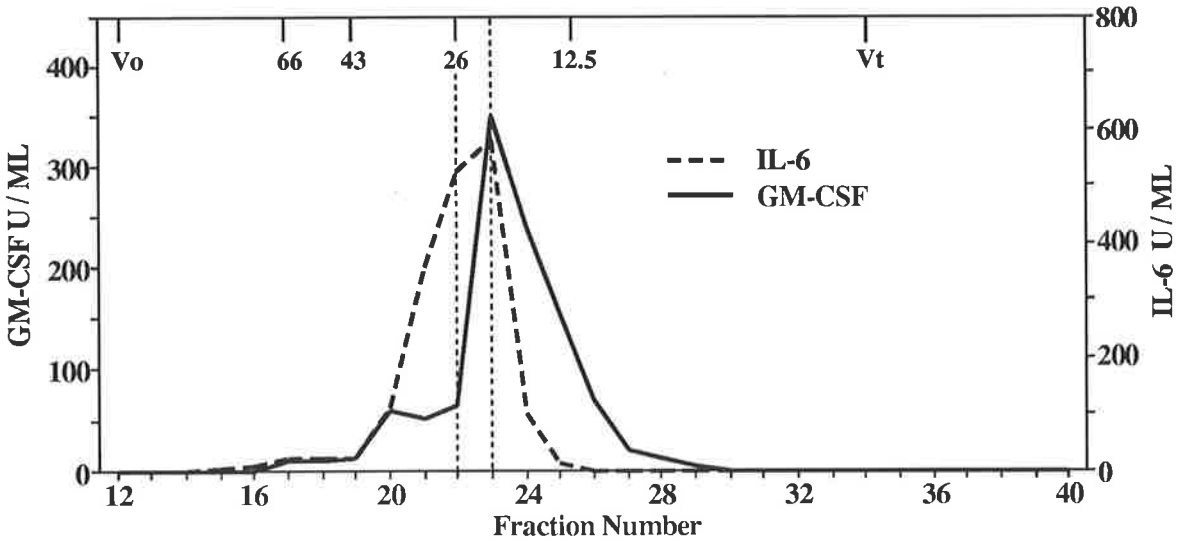
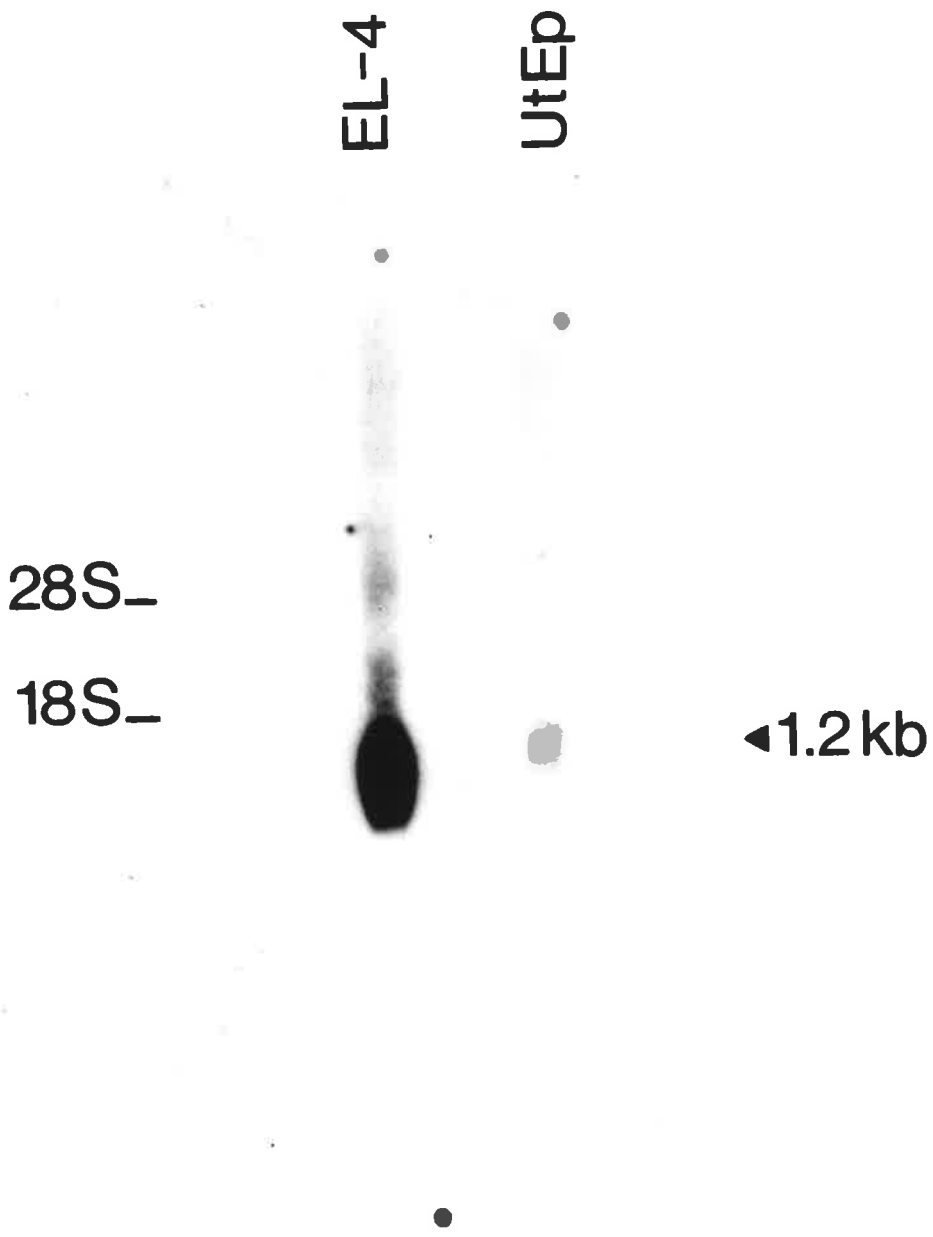


Figure 4.7 Sephacryl S-200 chromatography of endometrial cell-derived GM-CSF and IL-6. Endometrial cell conditioned media was prepared from oestrous uteri, concentrated approximately 200-fold over Amicon PM10 and YM2 membranes, and chromatographed on a Sephacryl S-200 column equilibrated in 6 M guanidine HCl. Fractions of the eluate were desalted over Sepharose G-25 by spin-gel filtration, and assayed for GM-CSF and IL-6 content in the FD 5/12 and 7TD1 bioassays respectively. Dotted vertical lines indicate the expected position of elution of proteins with Mr 23,000 and 26,000, which are the reported Mr for GM-CSF and IL-6.

Figure 4.8 Northern blot analysis of GM-CSF mRNA expression by endometrial cells *in vitro*. Total RNA was harvested from oestrous endometrial cells (End) after 40 h of culture, and from EL-4 (T-lymphocyte) cells 8 h after activation with PMA and PHA. 30 μg of RNA was electrophoresed through a 1% agarose-formaldehyde gel, transferred to Hybond nylon membrane, and hybridised with a ^{32}P-labelled riboprobe for murine GM-CSF. Both endometrial cells and EL-4 cells expressed a 1.2 kb GM-CSF mRNA.


EL-4
UtEp
28S
18S
1.2kb

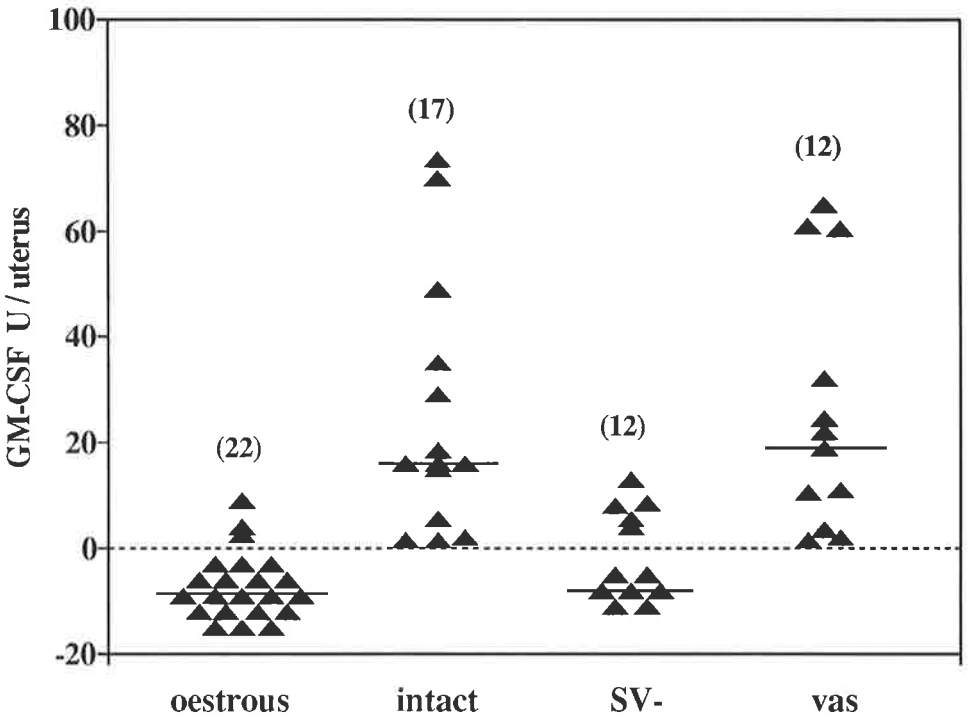


Figure 4.9. GM-CSF in uterine luminal fluid of mice mated with accessory gland deficient males. The GM-CSF contents of luminal fluids collected from oestrous mice, or mice mated with intact males, vasectomised (vas) males, or males from which the seminal vesicle glands had been surgically removed (SV-) were assessed in the FD 5/12 bioassay. Symbols represent individual animals and median values are scored, with numbers of observations in parentheses. Symbols below the dotted line have a cytokine content below the limit of detection for the bioassay. Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed)-test. The *p* values for differences between groups are shown below. NS = not significant.

	vas	SV-	est
intact	NS	0.001	0.001
vas		0.003	0.001
SV-			NS

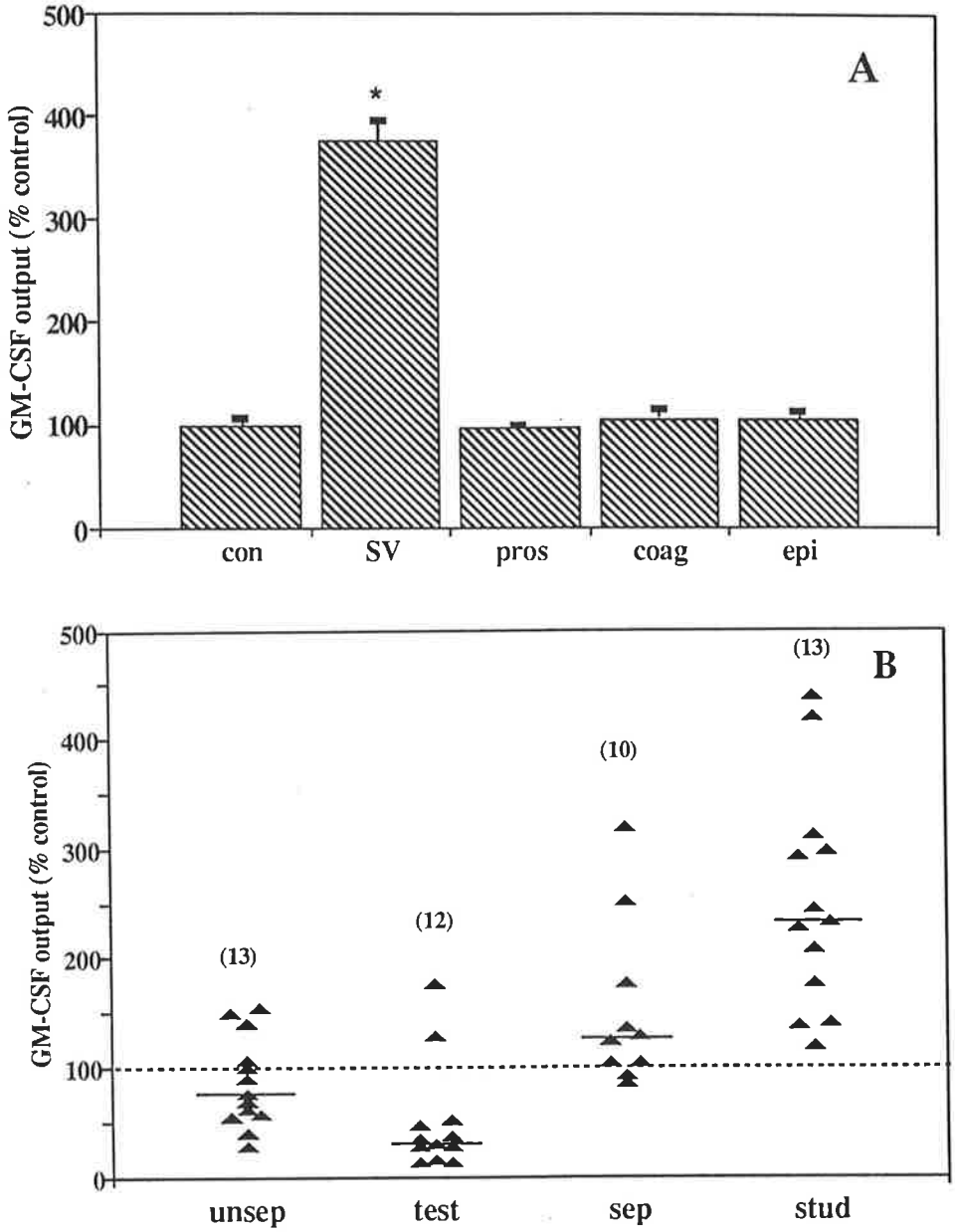


Figure 4.10 GM-CSF-stimulating activity of seminal vesicle fluid *in vitro*. (A) Accessory gland fluids were prepared from a 6 month old stud mouse and cultured with oestrous endometrial cells for 16 h, when they were replaced with fresh medium. The GM-CSF content of supernatants harvested 24 h later are graphed as a percentage of the content of supernatants harvested from endometrial cells cultured in DMEM-FCS alone. Cells cultured with seminal vesicle fluid (SV) secreted approximately 7.6 times the amount of GM-CSF as the control (con). The GM-CSF output of endometrial cells was not altered by culture with coagulating gland (coag), prostate (pros) or epididymal (epi) fluids. $^{*}p < 0.001$ (B) Effect of testosterone status on GM-CSF -stimulating activity of seminal vesicle fluid. The GM-CSF outputs of oestrus endometrial cells following incubation with seminal vesicle fluids from testosterone treated (test) and untreated unseparated (unsep) 2-3 month old males, 2-3 month old male mice separated for 3 weeks (sep) and 4-6 month old male stud (stud) mice are plotted as the percent output from endometrial cells cultured in DMEM alone. Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed)-test. Each group was significantly different to all other groups, and the p values for differences between groups are:

	test	sep	stud
unsep	0.010	0.021	0.001
test		0.002	0.001
sep			0.015

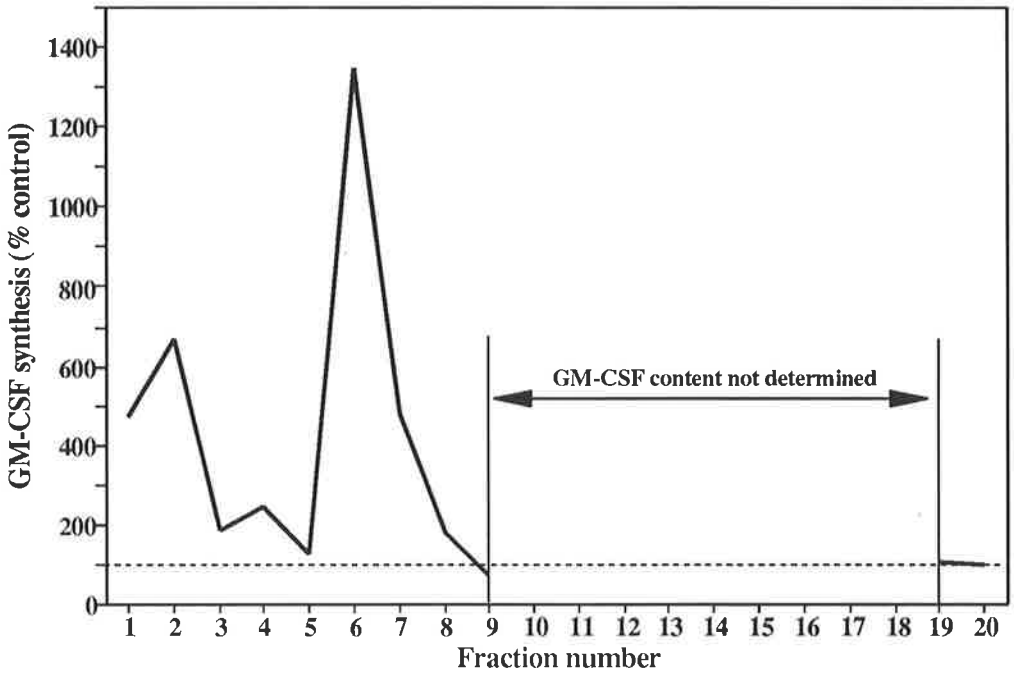
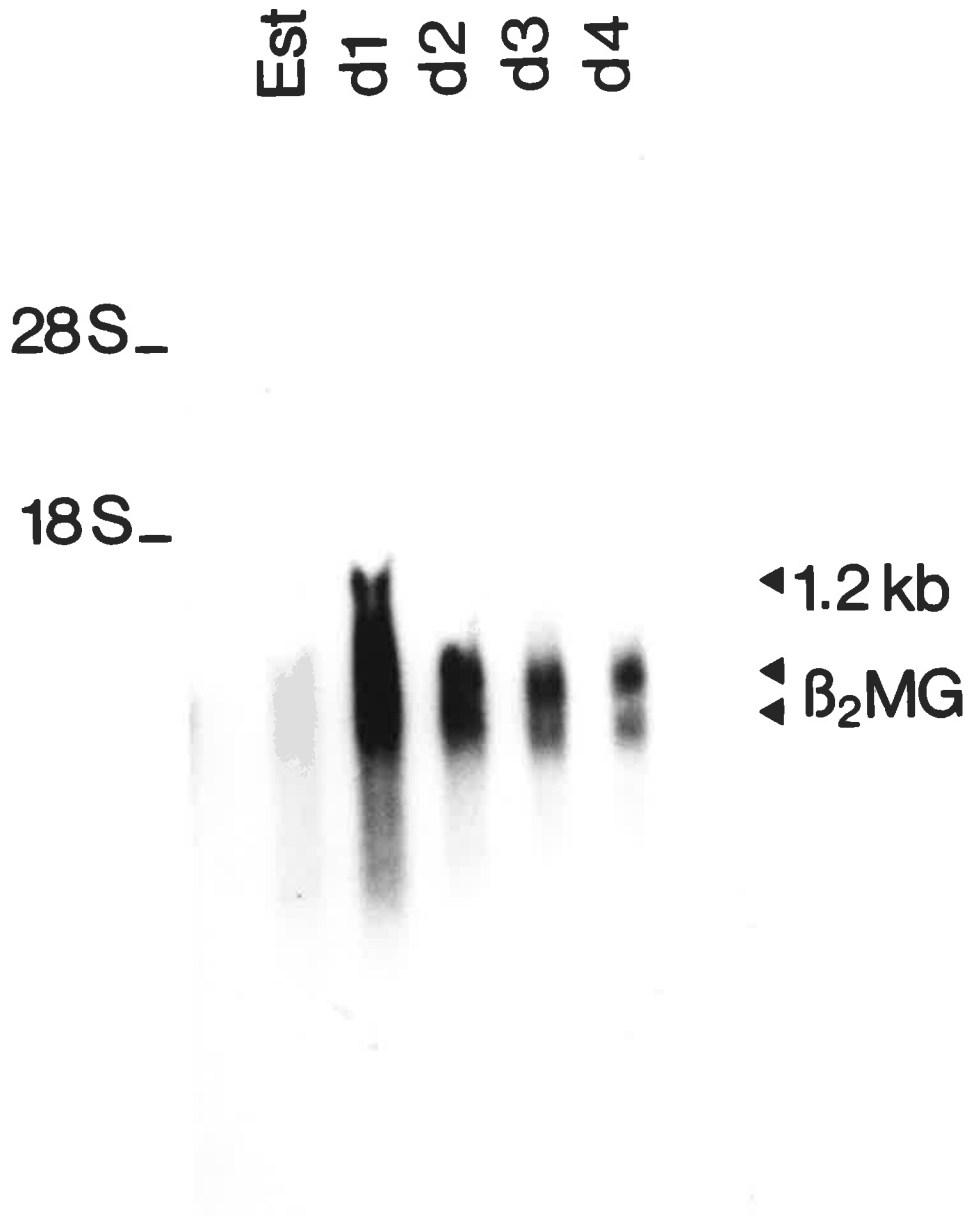


Figure 4.11 Sepharose CL4B chromatography of seminal vesicle gland-derived GM-CSF-stimulating activity. Fluid was collected from the seminal vesicle glands of 6 month old stud mice and solubilised in guanidine HCl, then chromatographed through Sepharose CL4B. Eluted fractions were cultured with oestrous endometrial cells for 16 h, then replaced with fresh medium. The GM-CSF content of supernatants harvested 24 h later are graphed as a percentage of the content of supernatants harvested from endometrial cells cultured in DMEM-FCS alone. GM-CSF stimulating activity was eluted in high Mr fractions, with two peaks in activity found in fractions 2 and 6. Guanidine HCl contamination of fractions 10 to 18 interfered with the bioassay, so that the stimulating activity within these fractions was not determined.

Figure 4.12 Northern blot analysis of GM-CSF expression *in vivo* during early pregnancy. Total RNA was harvested from whole uteri excised from oestrus, and day 1 through day 4 pregnant mice. 30 μg of RNA was electrophoresed through a 1% agarose-formaldehyde gel, transferred to Hybond nylon membrane, and hybridised with ^{32}P-labelled riboprobes for murine GM-CSF and for murine $\beta_2$microglobulin (β_2MG). A 1.2 kb GM-CSF mRNA is evident in the track containing day 1 RNA. The β_2MG mRNA doublet at 930 bp and 715 bp in all tracks confirms that similar amounts of each RNA were loaded.


Est
d1
d2
d3
d4
28S
18S
1.2 kb
β2MG

5 Cellular origin of endometrial GM-CSF

5.1 Introduction

The studies described in the preceding chapters show that the uterine endometrium of cycling and pregnant mice is a potent source of GM-CSF. The cellular origin of GM-CSF within the endometrium is identified in this chapter.

A variety of cell lineages present within the endometrium are known sources of GM-CSF, including T-lymphocytes, macrophages, fibroblasts, and endothelial cells [Gasson 1991; Ruef and Coleman 1990]. Athanassakis *et al.* (1987 and 1990) have reported that *in vivo* depletion of cells bearing the T-lymphocyte markers Thy1.2, CD8 and CD4 reduced the subsequent output of GM-CSF from murine decidual cell cultures, suggesting either that T-lymphocytes may be the origin of GM-CSF within the decidua, or that their presence otherwise impacts upon GM-CSF synthesis from another decidual cell population. In *in situ* hybridisation studies, GM-CSF mRNA was found to be expressed by isolated cells within the mid-gestation decidua that were presumed to be leukocytes (Kanzaki *et al.* 1991).

In the studies described in this chapter, similar levels of GM-CSF were found in the uterine luminal fluid and in supernatants from cultures of endometrial cells from genetically lymphocyte-deficient nude, SCID and beige mice on day 1 of pregnancy. These findings indicate that cells other than lymphocytes were a major source of GM-CSF in the endometrium. To identify the cellular source of GM-CSF, different lineages of cells were isolated from the endometrium by physical fractionation, followed by selective purification using lineage-specific mAbs. Endometrial cell cultures were examined from oestrous uteri, from the uteri of day 1 pregnant and from mid-gestation mice. Finally, GM-CSF mRNA expression within the intact uterus was localised by *in situ* hybridisation.

IL-6 has also been identified as a product of the cycling and early pregnant endometrium. The supernatants generated in these experiments were assessed for their IL-6 content to determine which of the endometrial cell lineages contribute to the synthesis of this cytokine.

5.2 GM-CSF synthesis in the uteri of genetically lymphocyte-deficient nude, SCID and beige mice

5.2.1 Content of luminal fluid on day 1 of pregnancy

The GM-CSF contents of luminal fluids from uteri of lymphocyte-deficient mice were compared with those from immunocompetent mice in order to evaluate the contributions made by T-lymphocytes and NK cells to the post-mating GM-CSF response. Female CBA *nu/nu* (α/β T lymphocyte-deficient), C57 Blk/6J-*bg/bg* (natural-killer (NK) cell activity-deficient), C.B-17-*scid/scid* (T and B lymphocyte-deficient) and Balb/c mice were mated with Swiss outbred males. Uterine luminal flushings were prepared on the day of detection of a vaginal plug. The GM-CSF

contents of luminal fluids were found to be similar in Balb/c mice mated with Swiss males and in Balb/c F1 females mated with CBA F1 males (data not shown). The GM-CSF and IL-6 contents of luminal fluids from immunodeficient strains were not significantly different from those of Balb/c mice (Fig. 5.1A), suggesting strongly that neither T lymphocytes, B lymphocytes, nor NK cells are major sources of post-mating intra-luminal GM-CSF *in vivo*.

5.2.2 *Content of conditioned media from endometrial cell cultures*

After collection of luminal fluid, endometrial cells were harvested from the uteri of day 1 mated lymphocyte-deficient and Balb/c mice (5.2.1). Endometrial cells from Balb/c mice mated with Swiss males and from Balb/c F1 females mated with CBA F1 males were found to secrete similar amounts of GM-CSF (data not shown). The GM-CSF contents of supernatants of cultured cells prepared from lymphocyte-deficient mice were not significantly less than those of Balb/c mice (Fig. 5.2). Neither T lymphocytes, B lymphocytes, nor NK cells were therefore major sources of GM-CSF in cultures of endometrial cells. Cells obtained from *bg/bg* mice synthesised significantly higher levels of GM-CSF (p <0.001) than cells from Balb/c, *nu/nu* or *scid/scid* mice (Fig. 5.2).

5.3 Antibody and complement-mediated depletion of endometrial lymphocytes

Endometrial cells from day 1 mated mice were incubated with mAbs specific for various leukocyte lineages, then treated with complement to deplete leukocytes from endometrial cell monolayers. However, protocols and reagents that depleted T-lymphocytes from spleen cell suspensions failed to deplete mAb-reactive cells from endometrial cell suspensions, since residual cells could be detected by indirect immunofluorescence immediately following treatment, and 24h after treatment (data not shown). In additional experiments, it was also found that epithelial cells could not be depleted from endometrial cell cultures with specific antibody (MTS #24; see 5.5.1) and complement (data not shown).

The GM-CSF output by endometrial cell monolayers was also not reduced by treatment by any of the antibodies in the presence of complement. In contrast, it became evident during the course of these experiments that incubation of endometrial cells with some leukocyte-specific mAbs significantly and consistently enhanced GM-CSF output (Table 5.1). Hybridoma supernatants that promoted release of GM-CSF release included αThy1.2, αCD4, and αI-A (MHC class II). These mAbs are all of the IgG2b isotype. Protein-G affinity purified αThy1.2 and αCD4 immunoglobulin were also found to promote GM-CSF release, but supernatant from the non Ig-secreting X-63 mouse myeloma cell line, and hybridoma supernatants containing comparable concentrations (determined by specific ELISA; data not shown) of other mAbs of the IgG2b isotype but different specificities (eg. αLFA-1) had no effect. These findings suggest that these mAbs, reactive with T-lymphocytes (αThy1.2 and αCD4), macrophages (αCD4 and αI-A), and fibroblasts (αThy1.2)(see 5.5.1), can act directly or indirectly to specifically enhance GM-CSF output from endometrial cells.

Table 5.1. Effect of incubation of endometrial cells from mice on day 1 of pregnancy with rat αleukocyte mAbs. Endometrial cells were harvested from day pregnant mice and cultured for 24 h in the presence or absence of mAb-containing supernatants or protein-G purified Ig from rat αleukocyte hybridomas, normal rat serum (NRS) or supernatant from the X-63 myeloma cell line. The GM-CSF contents of supernatants were determined by bioassay, and expressed as U/ 10^5 cells and percent of the output in control cells cultured in DMEM only.

Treatment		Ig isotype	U/ 10^5 cells (% control)
Experiment 1:			
control			52 (100)
NRS (0.2%)		mixed	45 (87)
mAbs (S/Ns):			
αThy1.2		IgG2b	204 (391)
αCD5		IgG2a	46 (88)
αLFA-1		IgG2b	39 (75)
αCD4		IgG2b	208 (398)
αMac-1		IgG2b	122 (234)
αI-A		IgG2b	271 (520)
Experiment 2:			
control			55 (100)
NRS (2%)		mixed	80 (146)
mAbs (S/NS):			
αThy1.2		IgG2b	195 (308)
αCD4		IgG2b	117 (215)
mAbs (affinity purified)			
αThy1.2	(5 μg/ ml)		83 (182)
	(25 μg/ ml)		150 (274)
αCD4	(5 μg/ ml)		86 (157)
	(25 μg/ ml)		136 (249)

5.4 Isolation of GM-CSF-producing endometrial cell subsets by density gradient centrifugation

In an effort to further characterise the GM-CSF-secreting cell population, endometrial cells from day 1 pregnant uteri were subfractionated on the basis of their densities by centrifugation through Percoll gradients composed of 10, 20, 30, 40, and 50% Percoll steps. Cells were recovered from each interface and cultured for 24 h. The cellular morphology of each culture was then assessed microscopically and supernatants were harvested and assayed for GM-CSF content. Cells which formed a band at the interface between the 30% and 40% Percoll steps were found to be enriched for GM-CSF secreting cells (Table 5.2). Cultures from this fraction contained large numbers of cells with an unusual 'balloon-like' morphology resulting from a single large cytoplasmic vacuole which often comprised as much as 80% of the total cell volume (Fig. 5.3). Staining of smears of these cells with acridine orange or with Giemsa revealed that they were intact cells with eccentric nuclei, however the cytoplasmic vacuole failed to react with either Schiff's reagent for carbohydrate or Toluidine blue.

Table 5.2. Subfractionation of endometrial cells by density gradient centrifugation. Endometrial cells were harvested from day pregnant mice and cultured for 24 h. Cells were detached from culture wells with EDTA, and centrifuged through a Percoll density step-gradient (10-50%), prior to recovery of fractions and culture for a further 24 h. The GM-CSF contents of supernatants were determined by bioassay. Similar data were obtained in two additional experiments.

Fraction	cell number (x 10^{-5})	U GM-CSF / 10^5 cells
control (mixed)	7.5	24.5
10 / 20	0.4	13.3
20 / 30	0.9	56.7
30 / 40	3.6	107.2
40 / 50	12.0	20.6
pellet	6.2	5.5

5.5 Isolation of endometrial cell subpopulations by panning

5.5.1 Immunohistochemical screening and demonstration of the specificities of rat mAbs

In order to determine the origin of GM-CSF, it was planned to use mAbs to isolate individual cell lineages from cultures of endometrial cells. Firstly, the specificities of the antibodies used were examined by indirect immunoperoxidase staining of fresh frozen sections of uterus from a day 1 pregnant mouse. CD45 and Mac-1 are pan leukocyte and neutrophil/macrophage markers respectively, and were each found to react with large numbers of cells within the endometrial stroma, particularly localised to areas adjacent to the luminal epithelium and to the endometrial glands. The majority of these cells were large and had a macrophage-like morphology. This finding is consistent with reports of large numbers of macrophages and neutrophils in the day 1 endometrium (Hunt *et al.* 1985; De *et al.* 1991).

Thy1.2 is expressed by all T-lymphocyte and NK cells and it is also expressed by some fibroblast cells (Golub 1988). Anti-Thy1.2 was found to react with fibroblasts in endometrial stroma (Fig. 5.4). The degree of reactivity of αThy1.2 mAb with stromal fibroblasts varied in relation to their distance from the epithelium; myometrial cells were only marginally positive, and a progressive increase in the intensity of labelling of endometrial cells was evident, with the most strongly labelled cells closest to the epithelium.

Immunohistochemical screening of a panel of mAbs raised against stromal elements of murine thymus by R. Boyd and colleagues (Monash University, Melbourne) revealed some which reacted with epithelial cells and endothelial cells in the mouse uterus. MTS #24 reacts with epithelial cells in the thymus stroma (Godfrey *et al.* 1988) and in the uterus it was found to detect an epitope with a subcellular distribution in glandular and luminal epithelial cells that is predominantly apical and cytoplasmic (Fig. 5.4). This mAb does not react with either spleen, lymph node or thymic leukocytes

(R. Boyd, personal communication). MTS #12 reacts with endothelial cells and with T-lymphocytes in the thymus (Godfrey *et al.* 1990) and a staining pattern consistent with reactivity with these cells was obtained in the uterus (Fig. 5.4).

5.5.2 *Identification of the cellular origin of GM-CSF* in vitro

The proportions of endometrial cells reactive with each mAb were determined in 24 h cultures by immunofluorescence analysis (Table 5.3). No more than 5% of cells were labelled with mAb against the leukocyte markers (CD45 and Mac-1) and less than 1% were labelled with the endothelial cell marker (MTS #12). Larger proportions of cells reacted with αThy1.2 (between 20% and 40%) and MTS #24 (between 60% and 80%).

Depletion by panning of endometrial cells reactive with mAb against leukocytes (αCD45 and αMac-1), endothelium (MTS #12) or stromal fibroblasts (αThy1.2) did not reduce the GM-CSF synthetic capacity of preparations from oestrous mice (expressed as units of GM-CSF per 10^5 cells in Table 5.3). Furthermore, populations selected positively by these mAb did not produce any GM-CSF. Other mAbs against leukocyte markers including CD3, CD4, CD5, CD8, CD11a, I-A, sIg and NK1.1 also failed to select cells that released GM-CSF (data not shown).

In contrast, MTS #24$^+$ epithelial cells produced 95% of the total GM-CSF (secreted by adherent plus non-adherent cells), while MTS #24-depleted cultures produced only 5% of the activity secreted by control cultures (Table 5.3). Results obtained for cultures prepared from day 1 uteri were similar, with the MTS #24$^+$ cells secreting 65% of the total GM-CSF (Table 5.3). Cultures of MTS #24 positive cells contained large numbers of adherent cells with a 'cobblestone' morphology typical of epithelial cells, as well as large numbers (up to 30%) of non-adherent or semi-adherent cells containing a single large vacuole and having the 'balloon cell' morphology described in 5.4 (Fig. 5.3). In some experiments suspensions of these cells were assessed by indirect immunofluorescence for their reactivity with MTS #24, and all were found to be positive.

Variability in the efficiency of positive selection was attributed to the tendency of cells to form small aggregates when harvested by detachment with EDTA. These aggregates were easily dislodged from the panning dish during washing steps, and this may be the reason for the consistent retention of significant GM-CSF synthetic capacity in the antigen-negative populations of day 1 cells after panning with MTS #24. Nevertheless, the possibility cannot be excluded that an MTS #24$^-$, Thy1.2$^-$, CD45$^-$, Mac-1$^-$, MTS #12$^-$ population of epithelial or other lineage contributed to GM-CSF secretion in these cultures.

5.6 Synthesis of GM-CSF by uterine epithelial cells during mid-gestation pregnancy

Cells harvested from between implantation sites (inter-conceptus) or adjacent to embryonic membranes (peri-conceptus) in day 10 pregnant uteri, secreted GM-CSF at levels comparable with cultures prepared from oestrous and day 1 uteri (see 4.3.2). Specificity of MTS #24 for glandular and luminal epithelium was confirmed by immunohistochemistry on frozen sections of day 10 pregnant uterus (Fig. 5.4). MTS #24$^+$ cells isolated from either inter- and peri-conceptus

Table 5.3. GM-CSF and IL-6 production by subpopulations of endometrial cells isolated by panning with mAbs.

		GM-CSF[b]			IL-6[b]		
mAb	% cells[a]	adherent	non-adherent	% total output[c]	adherent	non-adherent	% total output[c]
oestrus							
none	0		57	100		41	100
MTS #24	60-80	113	5	95	42	36	74
αThy1.2	20-40	2	95	<1	5	67	5
αCD45	5	ND	57	<1	ND	38	<1
αMac-1	5	ND	58	<1	ND	41	<1
MTS #12	<1	ND	57	<1	ND	40	<1
day 1							
none	0		67	100		36	100
MTS #24	60-80	98	57	65	28	63	55
αThy1.2	20-40	3	202	<1	27	55	48
αCD45	3	ND	67	<1	ND	34	5
αMac-1	<1	ND	68	<1	ND	35	4
MTS #12	<1	ND	67	<1	ND	35	2
day 10 (peri-conceptus)							
none	0		46	100		45	100
MTS #24	60-80	77	21	74	50	41	50
αThy1.2	20-40	2	113	3	57	38	49
αCD45	5	ND	46	<1	ND	42	9
day 10 (inter-conceptus)							
none	0		48	100		20	100
MTS #24	40-60	81	25	69	32	11	65
αThy1.2	40-60	1	183	1	8	53	30
αCD45	5	ND	50	<1	ND	17	16

[a] The percentage of cells in endometrial cultures reactive with each mAb was determined by immunofluorescence analysis (see Materials and Methods for details).
[b] Subpopulations of endometrial cells were isolated from oestrous, day 1 and day 10 pregnant mice by panning with mAb (see Materials and Methods for details). Results of representative panning experiments are shown (similar results were obtained in at least two other experiments for each mAb). Specificities of mAb used were MTS #24; epithelial cells, αThy1.2; stromal fibroblasts, NK cells and T lymphocytes, αCD45; all leukocytes, αMac-1; macrophages and neutrophils, and MTS #12; endothelial cells and T-lymphocytes. The amount of cytokine in supernatants harvested by separated adherent (mAb^+) and non-adherent (mAb^-) populations is expressed in $U/10^5$ cells/24 h (GM-CSF) and $U(x10^{-2})/10^5$ cells/24 h (IL-6). ND signifies populations for which accurate determination of synthesis was prevented by low cell numbers.
[c] The amount of cytokine synthesised by the adherent (mAb^+) subpopulation expressed as a percentage of the total amount synthesised, calculated according to the formula;

$$\% \text{ total output} = \frac{\text{U made by adherent cells}}{\text{U made by adherent + non-adherent cells}}$$

endometrium both synthesised major proportions of the total GM-CSF produced by the cultures. Cells with a fibroblast morphology (panned with αThy1.2), and leukocytes panned with αCD45 or αMac-1 mAb, did not produce any detectable GM-CSF. This indicates that epithelial cells remain a major source of GM-CSF in mid-gestation pregnancy, regardless of their proximity to embryonic tissue.

5.7 Identification of the cellular origin of GM-CSF *in vivo* during early pregnancy by *in situ* hybridisation

To determine whether epithelial cells synthesise GM-CSF mRNA *in vivo*, sections of uteri from oestrous and day 1 pregnant mice were probed with an antisense ^{32}P-labelled riboprobe specific for murine GM-CSF. In both tissues, GM-CSF mRNA expression was associated predominantly with the epithelial cells lining the uterine lumen, and to a lesser and variable degree with epithelial cells comprising many of the endometrial glands, particularly those close to the luminal surface. Myometrial cells, particularly those adjacent to the serosal surface of the uterus, were also positive in both tissues. The intensity of the hybridisation signal was similar in both oestrous and day 1 uterus. In contrast, a riboprobe complementary with the anti-sense strand of GM-CSF cDNA, or with an irrelevant mRNA species (*G. lamblia* A1) failed to hybridise to uterine tissue. The specificity of the GM-CSF riboprobe hybridisation reaction was examined further by competition with a 10-fold excess of cold homologous riboprobe, or by treating the tissue with RNAse prior to hybridisation. The pattern of labelling in the presence of cold inhibitor, or after RNAse treatment, was indistinguishable from the binding of the irrelevant A1 probe.

5.8 The origin of IL-6 in the endometrium

5.8.1 Studies with genetically lymphocyte-deficient mice

The content of IL-6 in luminal fluid collected from day 1 mated lymphocyte-deficient and Balb/c mice (5.2.1) were measured. No significant differences in IL-6 content were found between lymphocyte deficient and immunocompetent Balb/c mice (Fig 5.1). IL-6 levels were also measured in the supernatants of endometrial cell cultures prepared from the same uteri (5.2.2). Cells from lymphocyte-deficient and from Balb/c mice produced similar amounts of IL-6 (Fig 5.2). These findings suggest strongly that T lymphocytes, B-lymphocytes and NK cells are not major sources of IL-6 after mating either *in vivo*, or in endometrial cell cultures *in vitro*.

5.8.2 Synthesis of IL-6 by isolated subsets of endometrial cells from cycling, and early and mid-gestation pregnant uteri

Epithelial cells purified by panning with MTS #24 mAb were found to be a major source of IL-6 in cultures of endometrial cells prepared from oestrous and day 1 pregnant mice (74% and 55% of the total output respectively)(Table 5.3). Thy1.2$^+$ stromal cells and leukocytes (CD45$^+$ cells) were also found to contribute significantly to IL-6 output (48% and 5% respectively in day 1 cultures), but

interestingly, this was not the case in cultures of cells prepared from unmated animals. The finding of IL-6 bioactivity in the supernatants of cultured Mac-1^+ cells suggests that macrophages are the leukocyte population responsible for IL-6 synthesis. MTS $\#24^+$ epithelial cells isolated from both inter- and peri-conceptus sites on day 10 of pregnancy synthesised a large proportion of the total IL-6 (66 % and 50%). Leukocytes purified by panning with αCD45 or αMac-1 mAbs, and cells with a fibroblast morphology panned with αThy1.2 mAb, also contributed significantly to IL-6 output at this time (Table 5.3).

5.9 Conclusions and discussion

The experiments described in this chapter provide compelling evidence that glandular and/or luminal epithelial cells harvested from the murine uterus during the oestrus cycle and pregnancy are a potent source of GM-CSF *in vitro*. Together with *in situ* hybridisation data indicating that GM-CSF mRNA is associated predominantly with luminal and glandular epithelial cells in the intact cycling uterus and in the endometrium on the first day of pregnancy, these studies show clearly that GM-CSF synthesis is a physiological function of uterine epithelial cells.

Isolation of individual endometrial cell subpopulations by the use of mAbs against lineage-specific markers provided an opportunity to study pure populations of these cells, and thus identify conclusively the cells which synthesise the bulk of the GM-CSF in endometrial cell cultures. Panning proved to be the most effective technique to achieve this objective. Studies on positively selected epithelial cell populations identified these cells as the main source of GM-CSF in endometrial cell cultures. A curious feature of the epithelial cells in culture was the differentiation of a proportion of the cells into unusual, large 'balloon-like' cells containing a single large vacuole. Whether this was an artefact of the *in vitro* culture conditions or whether it is an indication of the secretory function of these cells remains to be determined.

Earlier attempts aimed at depleting endometrial cell subpopulations by treatment with mAb and complement failed, even when protocols and reagents were used that successfully depleted T-lymphocytes from spleen cell suspensions. It is tempting to speculate that expression of the C3b-binding complement regulatory protein CD46, which is strongly expressed by uterine glandular epithelium in humans (Johnson and Bulmer 1984), may protect endometrial cells from complement-mediated lysis. A second interesting observation to arise out of these experiments was the specific enhancement (by up to 500%) of GM-CSF release from endometrial cell cultures caused by their incubation with mAbs against Thy1.2, CD4 or MHC class II antigen (in the absence of complement). The underlying mechanism remains to be investigated, but an indirect action of the mAbs mediated by mimicking binding of a cognate ligand to non-epithelial cells is possible. This sort of mechanism is suggested by the finding that the relevant mAbs failed to influence the secretion of GM-CSF from pure epithelial cell cultures (data not shown).

Athanassakis *et al.* (1987) and Wegmann *et al.* (1989) have proposed that T-lymphocytes responding to fetal alloantigen are the source of decidual GM-CSF. The findings in this study do not support this hypothesis. Normal levels of GM-CSF were found in the uterine luminal fluids of

genetically lymphocyte-deficient mice. Furthermore, endometrial cells from lymphocyte-deficient mice produced normal levels of GM-CSF *in vitro*. These findings indicate that endometrial T cells are not a major source of GM-CSF at least during the oestrous cycle and post-mating period. This conclusion is supported by the finding that leukocytes make a negligible contribution to the GM-CSF secreted by endometrial cells from oestrous, day 1 or day 10 pregnant mice in culture. However GM-CSF synthesis by decidual cells and the proliferative or secretory responses of endometrial T-lymphocytes to seminal or fetal alloantigen have not been examined in this study. The production of GM-CSF activity by cultures that are likely to be rich in metrial gland cells (from day 10 pregnant mice) is in accordance with the exclusion of the epithelium from these structures. It has been described previously that GM-CSF bioactivity and mRNA levels are low in metrial gland cell preparations (Croy *et al.* 1991). The finding that GM-CSF synthesis was significantly elevated in cultures of endometrial cells from *bg/bg* mice may suggest that NK cells or their products can act to inhibit epithelial cell GM-CSF release. However, it is not known whether this is a result of the *bg/bg* mutation or simply due to inter-strain variation. Studies with congenic C57Blk mice are necessary to investigate this phenomenon.

GM-CSF synthesis has been described in various epithelial cells, including keratinocytes (Koury *et al.* 1983; Kupper *et al.* 1988; Chodakewitz *et al.* 1988) and thymus epithelium (Denning *et al.* 1988; Le *et al.* 1990; Galy *et al.* 1990). However, reports of its synthesis by mucosal epithelia are limited to tracheal epithelium in the rat (Smith *et al.* 1990) and in humans (Ohtoshi *et al.* 1991). It is notable that in each instance GM-CSF is found not to be produced constitutively, but as a consequence of activation by an inflammatory or cytokine stimulus.

Epithelial cells also appear to be a predominant source of IL-6 in the endometrium, but they are not the sole source of this cytokine. The panning studies showed that leukocytes and stromal fibroblasts also contribute to IL-6 synthesis, particularly following mating and later in pregnancy. This is in accordance with data published recently by Jacobs *et al.* (1992), who found that IL-6 is synthesised *in vitro* both by epithelial cells and stromal cells harvested from the murine endometrium. Croy *et al.* (1991) were unable to detect IL-6 mRNA in uterine NK cells, suggesting that these cells are unlikely to be the origin of the leukocyte-derived IL-6 activity in cells prepared from uteri of mated and mid-gestation pregnant mice. However, the endometrium is rich in macrophages, which are known to be a potent source of IL-6 and a number of other cytokines after activation. A report suggesting that murine decidual endothelial cells may also synthesise IL-6 (Motro *et al.* 1990) is not inconsistent with the results presented here, since the cultures used in this study are unlikely to have contained many cells of decidua basalis origin. The apparent failure of these authors to detect hybridisation signal associated with the uterine epithelium suggests that these cells express less IL-6 mRNA than decidual endothelial cells.

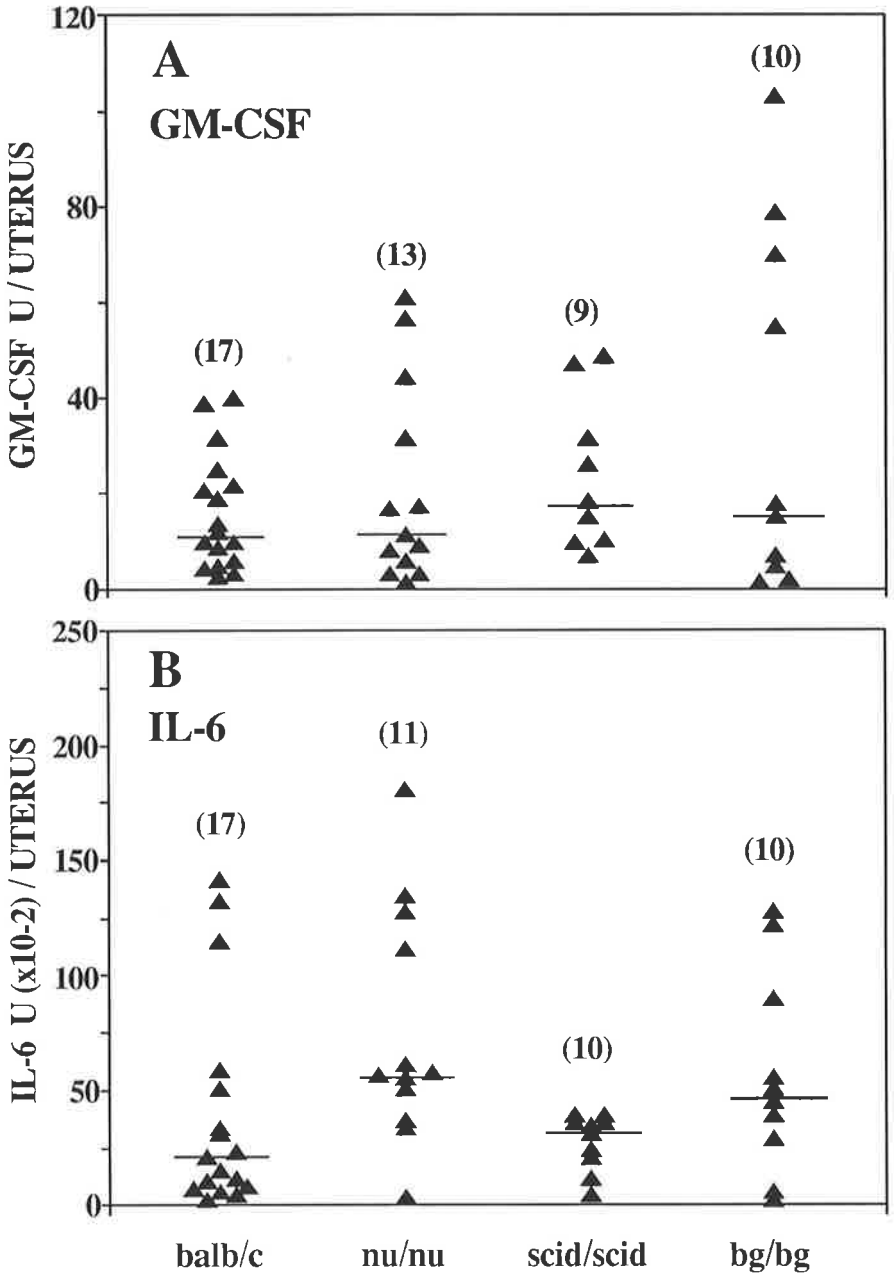


Figure 5.1 GM-CSF and IL-6 bioactivity in uterine intraluminal fluid of day 1 pregnant lymphocyte-deficient (nu/nu, scid/scid and beige/beige) mice. Luminal fluid collected by lavage from day 1 pregnant lymphocyte-deficient and immunocompetent (Balb/c) mice was assessed for GM-CSF and IL-6 content respectively in the FD 5/12 and 7TD1 bioassays. Symbols represent individual animals and median values are scored, with numbers of observations in parentheses. No significant differences within groups were found when data were compared by Kruskal-Wallis one-way ANOVA (A: $p = 0.125$, B: $p = 0.046$).

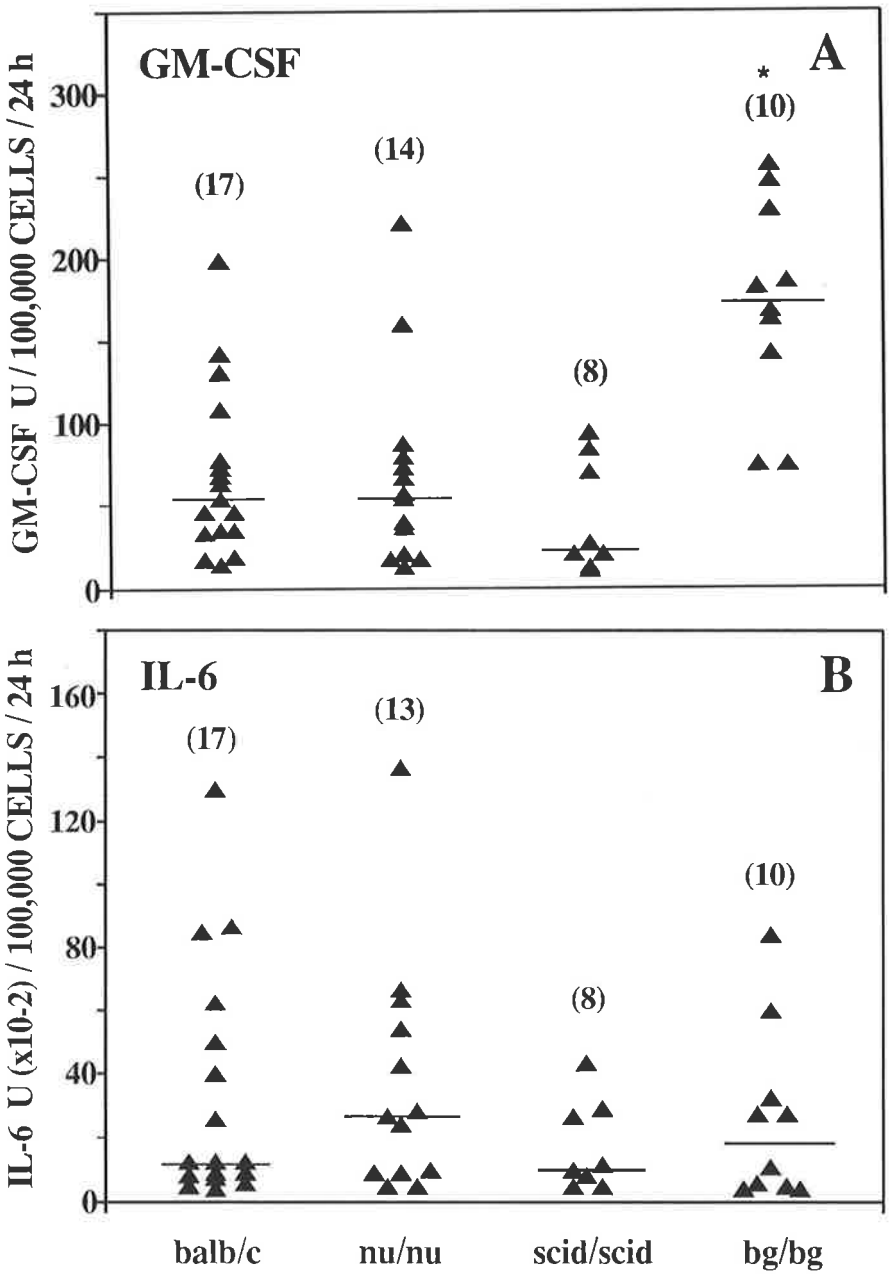


Figure 5.2 GM-CSF and IL-6 bioactivity in the supernatants of endometrial cell cultures prepared from day 1 pregnant lymphocyte-deficient (nu/nu, scid/scid and beige/beige) mice. Endometrial cells were harvested from day 1 pregnant uteri of lymphocyte-deficient and immunocompetent (Balb/c) mice, and supernatants were collected after 24 h. Culture supernatants were assessed for GM-CSF and IL-6 content respectively in the FD 5/12 and 7TD1 bioassays. Symbols represent individual animals and median values are scored, with numbers of observations in parentheses. No significant differences were found when data in B were compared by Kruskal-Wallis one-way ANOVA. Data in A were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed)-test. $*p < 0.001$

Figure 5.3 Morphology of subpopulations of endometrial cells isolated by density gradient centrifugation and panning. (A) Endometrial cells in culture after harvesting by trypsin/ pancreatin digestion. (B) Epithelial cells in culture after purification by panning with MTS #24 mAb from endometrial cells. (C) Stromal fibroblasts in culture after purification by panning with aThy1.2 mAb from endometrial cells. (D) Endometrial cells cultured in the presence of 100 U/ ml of IFNγ. Non-adherent cells with a 'balloon-like' morphology, purified by Percoll density gradient centrifugation, have eccentric nuclei (identified by staining with acridine orange) (G) and (H), and are MTS #24 positive by indirect immunofluorescence (E) and (F).

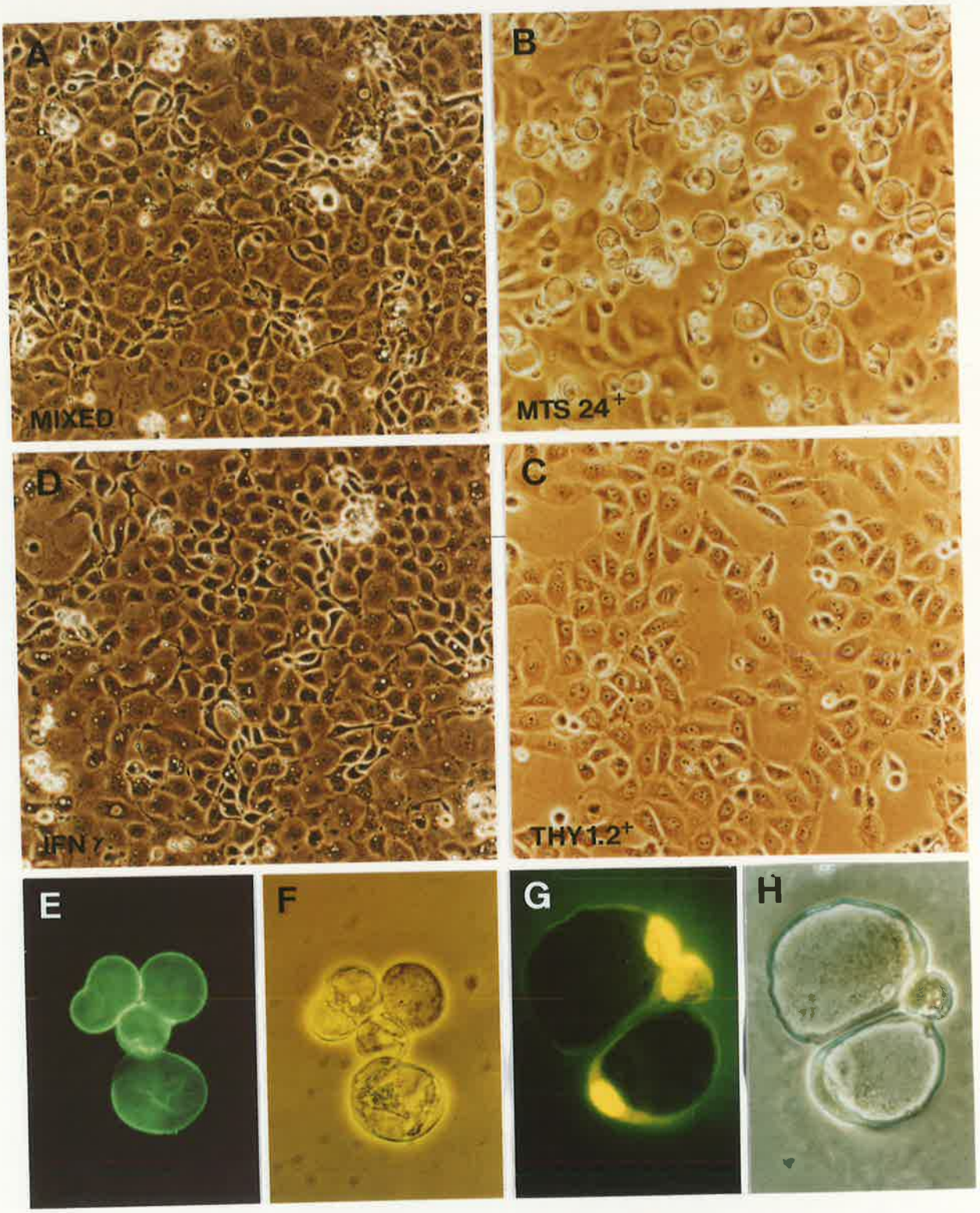

A
MIXED
B
MTS 24+
D
IFN γ-
C
THY 1.2+
E
F
G
H

Figure 5.4 Immunohistochemical staining of uterine tissue by mAbs used to isolate endometrial cell subpopulations. (A) MTS #24 labels luminal and glandular epithelial cells; (B) αThy1.2 reacts with stromal fibroblasts; (C) αLCA/ CD45 labels stromal and intra-epithelial leukocytes; (D) MTS #12 labels stromal endothelium; (E) αMac-1 labels macrophages and neutrophils in the stroma (all in day 1 uterus), and (F) MTS#24 labels glandular epithelium in day 10 pregnant uterus. Eosinophils stain non-specifically as a result of endogenous peroxidase activity and are evident as scattered cells in the uterine stroma in most sections.

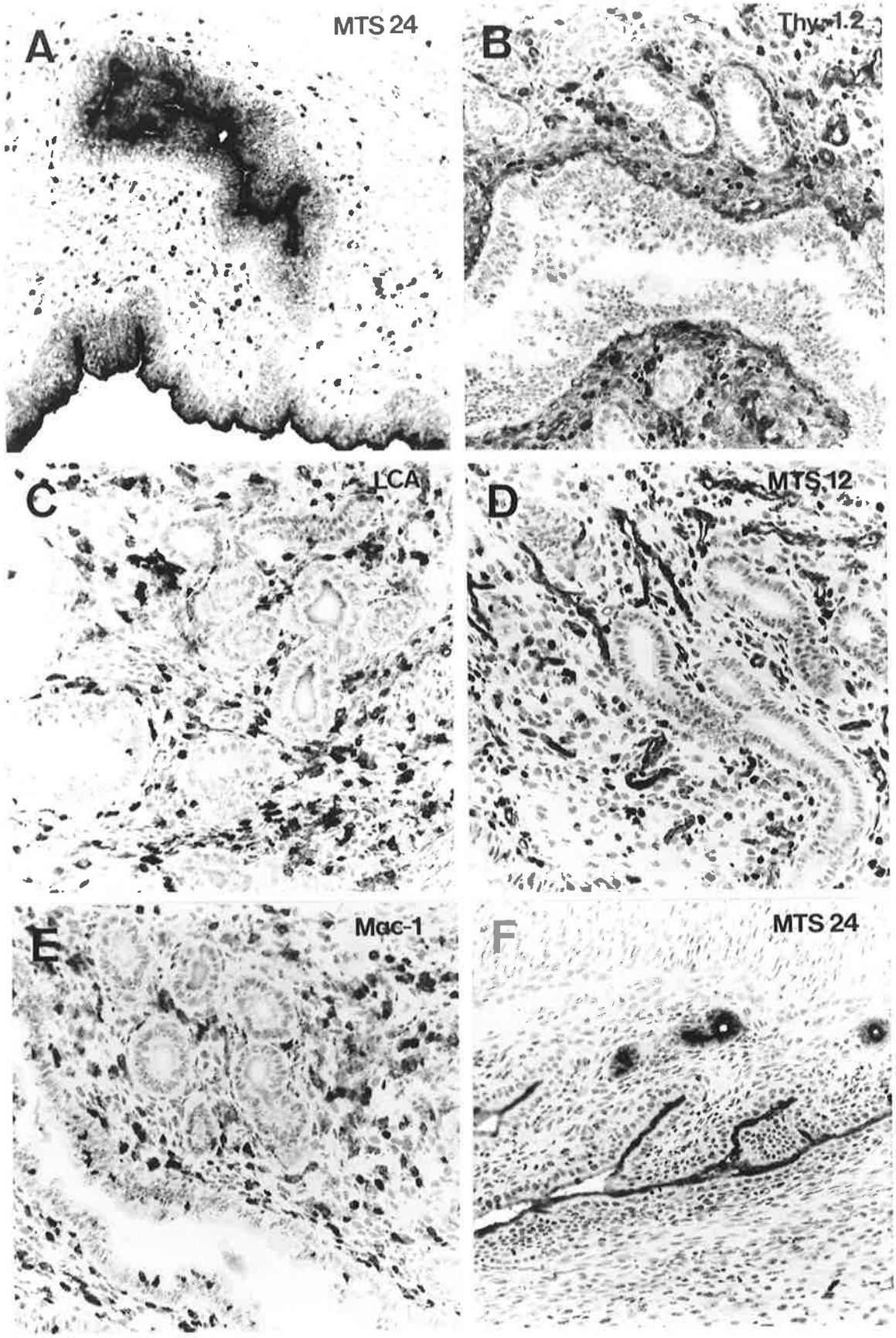
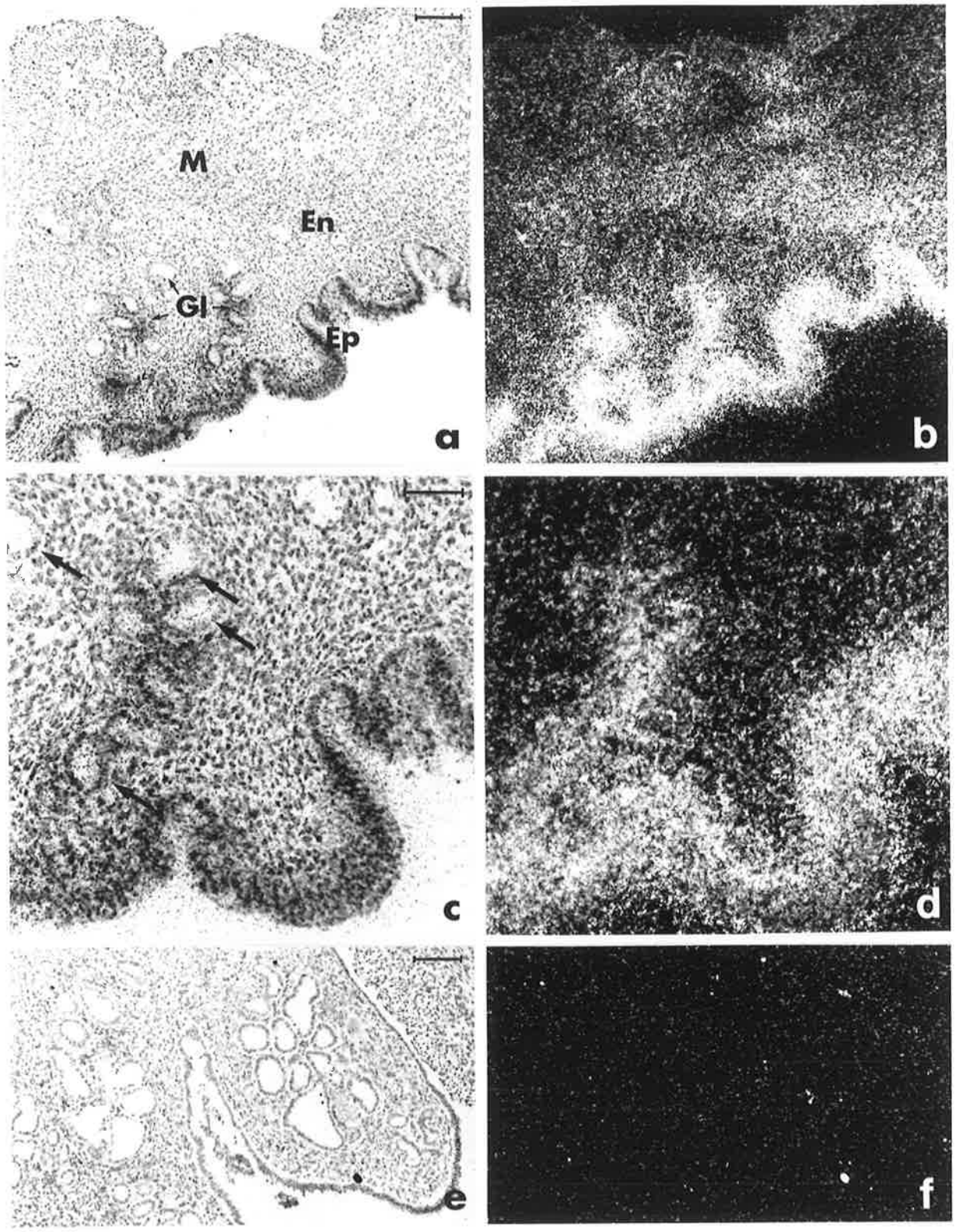

A
MTS 24
B
Thy-1.2
C
LCA
D
MTS 12
E
Mac-1
F
MTS 24

Figure 5.5 Localisation of GM-CSF mRNA in the intact uterus by *in situ* hybridisation. Sections of uteri from oestrous mice were hybridised *in situ* to a ^{32}P-labelled GM-CSF riboprobe. Low power brightfield and corresponding darkfield photomicrographs (x 20, a and b) illustrate that within the uterus GM-CSF mRNA is localised predominantly to epithelial cells (Ep) lining the uterine lumen and endometrial glands (Gl). (En = endometrium; M = myometrium; bar = 100 μm). Higher power photomicrographs (x 100, c and d) show that GM-CSF mRNA expression is strongest in luminal epithelial cells, weaker in the epithelial cells of some endometrial glands, and absent from the epithelial cells of other glands (endometrial glands are arrowed; bar = 25 μm). Low power photomicrographs (x 20, e and f) illustrate an irrelevant probe (matched for %GC, size and specific activity) failed to bind to uterine tissue (bar = 100 μm).


M
En
Gl
Ep
a
b
c
d
e
f

6 Regulation of GM-CSF release from uterine epithelial cells by steroid hormones and other biological mediators

6.1 Introduction

6.1.1 Ovarian steroid hormone regulation of epithelial cell cytokine synthesis

The proliferation and secretory function of endometrial epithelial cells are controlled primarily by ovarian steroid hormones (Martin and Finn 1968, and Martin *et al.* 1973), and there is evidence that synthesis of at least some of the cytokines made by these cells is also steroid-dependant. Pollard *et al.* (1987) have demonstrated that epithelial CSF-1 synthesis is regulated at the transcriptional level by the synergistic action of oestrogen and progesterone, using *in vivo* steroid replacement experiments in mice. This accords with the finding that uterine CSF-1 mRNA expression and bioactivity is first detectable on day 4 and is elevated progressively during placentation (Arceci *et al.* 1989), an event mimicked by ovarian-hyperstimulation with hCG in non-pregnant mice (Bartocci *et al.* 1986). Regulation of LIF synthesis appears to be regulated in a similar manner, as evidenced by the finding of a transient peak in LIF mRNA expression on day 4 of pregnancy, although this is followed by a decline thereafter (Bhatt *et al.* 1991). The expression of TNFα and TGFβ mRNAs during the oestrous cycle and early pregnancy follow different spatial and temporal patterns (Yelavarthi *et al.* 1991; De *et al.* 1992a; Tamada *et al.* 1990), thus implicating steroid hormones in mechanisms which specifically regulate the synthesis of these factors.

Although ovarian steroid hormones are not known to influence GM-CSF synthesis by other cells, their potential influence on its synthesis in the endometrium was suggested by the finding that the post-mating elevation in luminal GM-CSF activity fails to occur in superovulated mice (4.2.2). This chapter reports data from several experiments which examine the effects of steroid hormones on GM-CSF synthesis by uterine epithelial cells. Cytokine production has been measured from endometrial cells that were harvested after manipulation of the steroid hormone status of intact and ovariectomised mice. This experimental approach was chosen because preliminary experiments showed that measurement of *in vivo* activity was not feasible using available methodologies. No GM-CSF bioactivity could be detected in homogenates of uterine tissue from oestrous mice, even after procedures to minimise exposure to proteases, or after concentrating GM-CSF protein by absorption onto and elution from ConA-sepharose.

6.1.2 Regulation of epithelial cell cytokine synthesis by other biological mediators

A considerable array of mediators of GM-CSF production have been described. These include inflammatory cytokines, prostaglandins and bacterial LPS (see 1.6.1c, and Ruef and Coleman 1990 for review). Many of these agents are present in reproductive tissues and hence are potential candidates for regulation *in vivo* of GM-CSF release in the uterus. Bacterial LPS, other inflammatory agents and various chemical compounds promote GM-CSF release from keratinocytes and airway

epithelia. The influence of these and other biological mediators on release of GM-CSF by uterine epithelial cells *in vitro* is reported in this chapter.

6.2 Effect of stage of the oestrous cycle on GM-CSF release

In order to determine whether the oestrous cycle-dependant variation in steroid hormone levels has an impact on GM-CSF synthesis by epithelial cells, endometrial cells were harvested from mice synchronised with LH-RH superagonist (2.1.4). Animals were sacrificed on each of four consecutive days during the ensuing, normal oestrous cycle, beginning 70 h after LHRH administration (pro-oestrus), and with mice at oestrus, di-oestrus and met-oestrus stages of the cycle sacrificed at 94 h, 118, and 142 h respectively. In all cases, the stage of the cycle was confirmed prior to sacrifice by examining vaginal smears. Endometrial cells were harvested and cultured for 24 h, and the output of GM-CSF per 10^5 cells during this period was determined for each animal (2.9.1a). The GM-CSF output was highest from endometrial cells harvested at oestrus, when levels were approximately 8-fold greater than at other stages of the cycle ($p < 0.001$) (Fig. 6.1). This suggests that there may be a direct relationship between GM-CSF output and circulating oestrogen levels, which are highest during the 24 h period immediately prior to ovulation (Finn and Porter 1975).

6.3 Cytokine release following ovariectomy or hCG treatment

6.3.1 Effect of ovariectomy on GM-CSF release

To investigate the effect of absence of ovarian steroids on GM-CSF synthesis, endometrial cells were collected from ovariectomised and oestrous mice. The GM-CSF output was found to be approximately 25-fold less from cells harvested from ovariectomised mice than from cells prepared from oestrous mice [2 (<2-16) and 53 (13-138) U/ 10^5 cells respectively, $p < 0.001$] (Fig. 6.2). Therefore, ovarian-derived factors appeared to have a positive influence on GM-CSF release by epithelial cells.

6.3.2 Effect of hCG treatment on GM-CSF release

To investigate the effect of elevated circulating ovarian steroid hormone levels on GM-CSF synthesis, intact mice were injected ip daily with either 50 IU hCG or saline for 4 days, and then sacrificed on day 5. All mice received LH-RH superagonist on day 1 to induce oestrus on day 5. In one experiment, serum was obtained by orbital bleeding of 12 hCG-treated and 12 control mice, and the oestrogen and progesterone contents were determined. Circulating levels of both progesterone and oestrogen were substantially elevated in intact hCG-treated mice compared to oestrous mice (mean $\pm$ SD serum progesterone concentrations were 39 $\pm$ 20 and 243 $\pm$ 36, and serum oestrogen concentrations were 57 $\pm$ 16 and 84 $\pm$ 33 for oestrous and hCG treated mice respectively). After sacrifice, endometrial cells were harvested and cultured for 24 h, and the output of GM-CSF per 10^5 cells was determined for each animal. GM-CSF outputs were significantly less from cells harvested

from mice hyperstimulated with hCG, than from cells from oestrous mice [28 (7-66) U/ 10^5 cells respectively, $p < 0.001$] (Fig. 6.2).

6.3.3 Effects of ovariectomy and hCG on release of other cytokines

Endometrial cells from cycling and pregnant mice also synthesise IL-6 *in vitro* (4.6.1). The supernatants generated in these experiments were assessed for their IL-6 content to determine whether gross manipulation of steroid hormone levels by ovariectomy or hCG treatment influenced the release of this cytokine. In contrast to the effects on GM-CSF production, there was no significant difference in the IL-6 output from cells from ovariectomised and oestrous mice [2230 (310-5330) and 2970 (740-5050) U/ 10^5 cells respectively]. Furthermore, IL-6 release was not altered significantly by treatment with hCG [1950 (240-4670) U/ 10^5 cells], suggesting that synthesis of IL-6 by epithelial cells may be independent of factors derived from the ovary. Interleukin-1, IL-2, IL-3 and TNFα were not secreted in detectable quantities by endometrial cells from oestrous mice (4.6.10), and bioassay of supernatants of cells from hCG-treated mice also failed to reveal any of these activities (data not shown).

6.4 Effects of exogenous ovarian steroid hormones and induction of a decidual response on GM-CSF release following ovariectomy

6.4.1 Effects of 17β-oestradiol and progesterone

To determine whether the steroid hormones oestrogen and progesterone are the ovarian mediators active in inducing production of GM-CSF by endometrial cells, a study was made of the effects of administering exogenous 17β-oestradiol and progesterone on cytokine release in ovariectomised mice. A hormone replacement protocol (detailed in Table 6.1) was utilised that mimics the pre-and peri-implantation period of pregnancy. This treatment has been shown previously to allow implantation and decidualisation to occur (Finn and Martin 1969; Finn and Martin 1972), and to induce CSF-1 synthesis at physiological levels (Pollard *et al.* 1987). Endometrial cells were harvested from steroid-treated mice and cultured for 24 h, when supernatants were assayed for GM-CSF content. The experiment was repeated 4 times, each time with 6 mice per treatment group. The data obtained for each experiment were combined after normalisation to take account of differences in control (group A) values, and expressed as the cytokine output in U/ 10^5 cells/ 24 h (Fig 6.3).

Comparison of median values shows that GM-CSF release by cells harvested from ovariectomised mice treated with 25 ng of 17β-oestradiol (group C) was approximately 30-fold greater than release from cells from control (group A) mice ($p < 0.001$) (Fig. 6.3) and within the same range as the output from oestrus endometrial cells (6.2). These data indicate that 17β-oestradiol administration was sufficient to induce GM-CSF release from epithelial cells, and also suggest that release *in vivo* may be dependant upon the action of ovarian oestrogen.

In contrast, cells harvested from mice treated with progesterone alone (group B) released quantities of GM-CSF similar to cells from control (group A). Mice treated with progesterone in

Table 6.1 Protocol for administration of 17β-oestradiol and progesterone to ovariectomised mice. Nine groups of ovariectomised mice (A to I) received 17β-oestradiol and/ or progesterone (sc, in 100 µl peanut oil) according to the following protocol. All mice were primed with 100 ng of 17β-oestradiol on days 1 and 2, then after 2 days rest were maintained on daily doses of carrier, progesterone and/ or 17β-oestradiol for 6 days. Groups E and F, and H and I, received the same steroids as group D and G respectively but were given an intraluminal stimulus of 10 µl of peanut oil (oil; F and I) or saline (sal; E and H) as a control on day 7 of the schedule. On day 11 all mice were killed and the uteri were excised, weighed, and the endometrial cells were harvested and cultured as described in 2.9.1a. P = 500 µg progesterone; 10 E, 25 E and 100 E = 10, 25, and 100 ng of 17β-oestradiol respectively; c = carrier.

Day	A	B	C	D	E	F	G	H	I
1	all mice (groups A-I) received 100 E								
2	all mice (groups A-I) received 100 E								
5	c	P	25 E	10 E P			25 E,P		
6	c	P	25 E	10 E P			25 E,P		
7	c	P	25 E	10 E P	sal	oil	25 E,P	sal	oil
8	c	P	25 E	10 E P			25 E,P		
9	c	P	25 E	10 E P			25 E,P		
10	c	P	25 E	10 E P			25 E,P		

combination with either 10 ng or 25 ng of 17β-oestradiol (group D and G respectively) yielded cells that released GM-CSF at approximately 8-fold and 23-fold higher median levels respectively than the control (group A) value (both $p < 0.001$) (Fig. 6.3). There was a decrease in GM-CSF output by cells harvested from mice receiving progesterone in addition to 25 ng of 17β-oestradiol compared with those harvested from mice receiving 17β-oestradiol alone ($p = 0.049$) (Fig. 6.3). These data show that progesterone alone did not induce GM-CSF production by epithelial cells, and had a moderate inhibitory effect when combined with 17β-oestradiol.

6.4.2 *Effects of administration of a decidual stimulus and induction of a decidual response*

Decidual transformation of the uterine stroma, mimicking that which occurs during blastocyst implantation, can be achieved by instillation of oil or other irritants into the uterine lumen in ovariectomised mice maintained on an appropriate regimen of exogenous steroids or in psuedopregnant mice (Finn 1977; Finn and Martin 1972; Finn *et al.* 1989), and is associated with enhanced CSF-1 synthesis by epithelial cells (Pollard *et al.* 1987). To assess the effect of decidualisation on GM-CSF synthesis, groups of progesterone and 17β-oestradiol-treated mice were given intraluminal peanut oil 4 days prior to sacrifice. Extensive decidualisation was induced in 18 of 24 mice receiving 10 ng 17β-oestradiol, but a decidual reaction was either absent or confined to an area surrounding the injection site in mice that received 25 ng 17β-oestradiol. Similar results were observed in mice given either dose of 17β-oestradiol and intraluminal saline, in accord with the findings of others (Finn and Martin 1972; Pollard *et al.* 1987). In mice maintained on 10 ng 17β-

oestradiol, cytokine secretion was reduced significantly in cultures of endometrial cells derived from oil injected-uteri compared to saline-injected uteri ($p = 0.029$) (Fig. 6.3), but there was no difference between saline- and oil-injected groups in mice treated with 25 ng of 17β-oestradiol.

The effect of decidualisation became more evident when data were regrouped according to whether or not they were obtained from uteri in which decidual reactions were visible. In mice maintained on 10 ng 17β-oestradiol plus progesterone, GM-CSF output by cells harvested from decidualised uteri was approximately 2.5-fold less than output from non-decidualised uteri [8.2 (2.4-46.7) U/ 10^5 cells (n = 26) and 20.6 (2.1-59.3) U/ 10^5 cells (n = 21) respectively, $p = 0.003$]. In mice maintained on 10 ng 17β-oestradiol plus progesterone, GM-CSF secretion by cells harvested from decidualised uteri was approximately 1.8-fold less than secretion from cells prepared from non-decidualised uteri [21.5 (2.8-57.6) U/ 10^5 cells (n = 14) and 39.0 (0.5-149.3) U/ 10^5 cells (n = 28) respectively, $p = 0.015$]. The extent of decidualisation was inversely proportional to GM-CSF output ($r = -0.44$, $p = 0.005$) (Fig. 6.4). Immunofluorescence analysis revealed that there was a negative correlation between the degree of decidualisation and the proportion of epithelial cells in the endometrial cells harvested from individual uteri [control (undecidualsed) uteri, and medium (400-800 mg) and large (800-1200 mg) deciduomas yielded cultures containing approximately 75 %, 65 % and 30 % MTS #24 positive epithelial cells respectively]. This would have contributed significantly to the diminished GM-CSF output by endometrial cells prepared from decidualised uteri, but did not account for it fully. In summary, these data show that administration of a decidual stimulus itself had little effect on GM-CSF output, whilst decidual transformation of the uterine stroma caused a moderate reduction in the GM-CSF output of endometrial cells.

6.4.3 Time course of response following 17β-oestradiol administration

To determine the rate of onset of GM-CSF synthesis after administration of 17β-oestradiol, endometrial cells were harvested from ovariectomised mice between 3 and 24 h after a single injection of 50 ng of 17β-oestradiol. To mimic oestrus, an additional group of mice received a second injection of 50 ng of 17β-oestradiol 24 h after the first, and endometrial cells were harvested 24 h later. Endometrial cells were cultured for 24 h, and the output of GM-CSF per 10^5 cells was determined for each animal. Cells harvested as early as 3 h after 17β-oestradiol administration were found to have an approximately 4-fold greater GM-CSF output than those from control (ovariectomised) mice ($p = 0.020$). Maximal GM-CSF output (approximately 24-fold greater than control) was achieved after 9 h of exposure to 17β-oestradiol *in vivo* ($p = 0.014$) (Fig 6.5). The 17β-oestradiol-induced response was short-lived, since output declined to approximately 10-fold greater than control after 15 h exposure ($p = 0.014$). These data demonstrate that a single injection of 17β-oestradiol is sufficient to induce GM-CSF release from epithelial cells, and suggest that induction may be a relatively direct response to the action of this steroid hormone. Comparison of the GM-CSF output and the weight of uteri indicates that the onset and peak of the former occurred more rapidly than the uterine hypertrophy and hyperplasia induced by 17β-oestradiol, which increased continually over the 48 h period studied.

6.5 Effects of steroid antagonists on GM-CSF release

6.5.1 Steroid antagonists in cycling mice

The effects of the progesterone antagonist RU486 (Baulieu 1989) and of the oestrogen antagonist ZK 119010 (Nishino *et al.* 1991) on GM-CSF release were investigated in oestrous mice. Endometrial cells from antagonist-treated and control mice were harvested and cultured for 24 h, and the output of GM-CSF per 10^5 cells was determined. After administration of ZK 119010 to oestrous mice, endometrial cells produced 4-fold less GM-CSF than cells from the control mice ($p < 0.001$) (Fig. 6.6). Treatment with RU486 did not affect the capacity of uterine cells to produce GM-CSF *in vitro*.

6.5.2 Steroid antagonists in steroid-maintained ovariectomised mice

The effects of RU486 and ZK 119010 on GM-CSF release by endometrial cells were also investigated in ovariectomised mice maintained on progesterone and 17β-oestradiol. Cells harvested from ovariectomised mice that received ZK 119010 in addition to 17β-oestradiol plus progesterone produced 8-fold less GM-CSF than cells from mice that did not receive ZK 119010 ($p < 0.001$) (Fig. 6.7). In contrast, RU486 treatment of ovariectomised mice maintained on both steroids stimulated a small but significant increase in the GM-CSF output of endometrial cells ($p = 0.021$) (Fig. 6.7). Together with the results in 6.5.1, this finding provides further evidence that GM-CSF production is oestrogen-dependant.

6.5.3 RU486 in hCG-hyperstimulated mice

Intact mice given RU486 in addition to hCG (500 mg and 50 IU respectively injected daily over 4 days) yielded cells that synthesised 1.4-fold more GM-CSF than mice that received hCG plus carrier [51 (36-169) U/ 10^5 cells/ 24 h (n = 14) and 37 (7-101) U/ 10^5 cells/ 24 h (n = 15) respectively, $p = 0.013$]. This finding suggests that the cause of the inhibition of endometrial cell GM-CSF release by hCG treatment (6.3.2) is elevated progesterone levels. Together with the findings in 6.5.2 these data implicate further an inhibitory action of progesterone.

6.6 Effects of steroids and steroid antagonists on GM-CSF release in vitro

To determine whether steroid hormones can act to regulate epithelial cell GM-CSF synthesis *in vitro*, endometrial cells were harvested from ovariectomised mice were cultured in 'oestrogen-free' DMEM for periods of up to 72 h, either in the presence of 17β-oestradiol, progesterone, or with a combination of 17β-oestradiol plus progesterone. Supernatants from endometrial cells cultured in 'oestrogen-free' DMEM or in DMEM-FCS contained similar amounts of GM-CSF. There was no significant alteration in the GM-CSF output of endometrial cells cultured for 24, 48 or 72 h with

steroids at concentrations of 17β-oestradiol between 10^{-7} M and 10^{-12} M and of progesterone between 10^{-4} M and 10^{-7} M. However, progesterone (on its own or in combination with 17β-oestradiol) significantly inhibited epithelial cell proliferation (as judged by number of adherent cells at the end of 72 h culture compared to controls) at concentrations of greater than 10^{-6} M (data not shown).

Endometrial cells harvested from oestrous mice were cultured in DMEM-FCS for periods of up to 72 h in the presence of ZK 119010 or RU486 (the oestrogen and progesterone antagonists respectively) to examine whether inhibition of steroid hormone action *in vitro* affects GM-CSF release. Steroid antagonists had no obvious effect on the morphology or proliferation of endometrial cells, and there was no significant alteration in the GM-CSF content of supernatants harvested at 24, 48 or 72 h from cell cultures containing these agents at concentrations of between 10^{-6} M and 10^{-8} M.

6.7 Effect of other biological mediators on GM-CSF release *in vitro*

6.7.1 Effect of cytokines, prostaglandins, mitogens and Ca^{++} ionophore

The effect of a variety of cytokines and other mediators on GM-CSF release by oestrous endometrial cells was assessed *in vitro* after co-incubation with the factors for 6 or 16 h.

LPS and IFNγ were the only substances found to have a substantial effect on GM-CSF output by endometrial cells (Table 6.2). Incubation with LPS (20 μg/ ml) for 6 h or 16 h stimulated a 4.7 fold and 10.5 fold increase in GM-CSF release respectively. Conversely, 6 and 16 h exposure to 1000 U/ ml of recombinant murine IFNγ resulted respectively in 98 % and 96% inhibition of GM-CSF synthesis. Apart from IL-2 (which at 1000 U/ ml inhibited GM-CSF output by 49%), and Ca ionophore and PMA (which were also inhibitory at high concentrations), the GM-CSF output of endometrial cells was not altered (ie. was within ± 40% of control values) in cultures incubated with each of the other cytokines or with prostaglandins (Table 6.2).

Neither LPS nor IFNγ influenced the viability or the rate of proliferation of endometrial cells. Cell numbers (as assessed by Rose-Bengal uptake) after incubation for 6 or 16 h in these substances were similar to control cultures (data not shown). However, on microscopic examination, epithelial cells in endometrial cell cultures grown in the presence of IFNγ were found to have altered morphology, characterised principally by the presence of up to 10-12 small cytoplasmic vacuoles per cell (Fig 5.3).

6.7.2 Effect of the duration of exposure and concentration of IFNγ and LPS on GM-CSF release

To determine whether there was a relationship between GM-CSF production and the duration of exposure to either LPS or IFNγ, endometrial cells from oestrous mice were incubated for 4, 8 or 16 h with the respective mediators. Incubation with IFNγ for 4 h, 8 h or 16 h inhibited GM-CSF output to the same extent (95-96%), but the effect of LPS was found to be somewhat dependant upon the duration of exposure. Inhibition at 4 h and 8 h was 67% and 52% respectively of the effect observed at 16 h (Fig. 6.8).

Table 6.2 Effect of incubation of endometrial cells with cytokines and other potential regulators of GM-CSF release. Substances were added to duplicate wells at the initiation of the culture period and the media was replaced at either 6 h or 16 h later. Culture then continued for a further 16 or 24 h respectively. Supernatants were assessed for GM-CSF and IL-6 content by specific bioassay, and the mean ± SD GM-CSF or IL-6 concentrations of supernatants are expressed as a percentage of the concentrations of supernatants from control cultures (DMEM alone). M = murine; Hu = human.

Treatment	Species, Supplier[a]	Concentration	GM-CSF		IL-6
			6 h	16 h	16 h
Control (DMEM)			100 ± 10	100 ± 6	100 ± 15
IL-1	M, British Biotechnology	1000 U/ ml	88 ± 10	97 ± 6	96 ± 7
		100 "	77 ± 12	97 ± 11	356 ± 115
IL-2	M, Genzyme	1000 "	95 ± 28	51 ± 17	
		100 "	76 ± 8	101 ± 20	
IL-3	M, Genzyme	1000 "	68 ± 19	126 ± 19	
		100 "	70 ± 1	113 ± 1	
IL-6	Hu, Boehringer Mannheim	1000 "	79 ± 2	91 ± 2	409 ± 93
		100 "	84 ± 5	90 ± 1	330 ± 99
TNFα	M, British Biotechnology	1000 "	83 ± 8	92 ± 13	67 ± 9
		100 "	83 ± 11	79 ± 0	206 ± 18
TNFβ	M, British Biotechnology	1000 "		92 ± 0	
		100 "		86 ± 0	
LIF	M, AMRAD	1000 "	67 ± 7	85 ± 9	179 ± 93
		100 "	68 ± 6	91 ± 6	79 ± 4
CSF-1	M, N. Nicola	1000 "	71 ± 6	82 ± 12	
		100 "	60 ± 3	80 ± 7	
GM-CSF	M, N. Nicola	1000 "	115 ± 2	105 ± 7	
		100 "	82 ± 5	114 ± 12	
IFNγ	M, Genentech	1000 "	2 ± 0	4 ± 2	39 ± 1
		100 "	2 ± 1	8 ± 4	89 ± 8
$IFN\alpha_{2a}$	Hu, Roche	1000 "	124 ± 4		
		100 "	110 ± 2		
PGE	UpJohn	5×10^{-7} M	67 ± 2	98 ± 13	108 ± 18
		5×10^{-8} M	62 ± 2	95 ± 6	137 ± 19
$PGF_{2\alpha}$	UpJohn	5×10^{-7} M	89 ± 8	94 ± 0	190 ± 12
		5×10^{-8} M	86 ± 5	99 ± 12	160 ± 1
LPS	*S. typhimurium*, I. Kotlarski	20 µg/ ml	470 ± 40	1052 ± 42	184 ± 23
		2 µg/ ml	407 ± 4	577 ± 56	166 ± 67
PMA	Sigma	100 ng/ ml	29 ± 6	107 ± 15	54 ± 2
		10 ng/ ml	26 ± 2	96 ± 3	101 ± 40
Ca^{++} ionophore	Sigma	10^{-6} M	61 ± 15	48 ± 4	118 ± 6
		10^{-7} M	57 ± 3	66 ± 2	117 ± 25

[a]See 2.3.1 and 2.3.3 for details

To determine whether the effects of either LPS or IFNγ are concentration-dependant, endometrial cells from oestrous mice were incubated with LPS at concentrations ranging from 1 ng/ ml to 20 μg/ ml, and with IFNγ at concentrations ranging from 0.08 U/ ml to 1000 U/ ml. The effects of both substances were found to be concentration-dependant, with half-maximal activities at approximately 100 ng/ ml of LPS, and at approximately 0.2 U/ ml of IFNγ (Fig. 6.8).

The hierarchy of the effects of LPS and IFNγ on GM-CSF release were examined in cultures of endometrial cells from oestrous mice. When incubated together, each substance was found to antagonise the effect of the other (Fig 6.9). Titration of IFNγ in the presence of 20 μg/ ml of LPS caused a 10-20 fold increase in GM-CSF output across the 0.08 to 1000 U/ ml concentration range. The stimulating activity of 20 μg/ ml of LPS was inhibited by IFNγ in a dose-dependant manner, with half-maximal activity at approximately 1 U/ ml. Conversely, titration of LPS in the presence of 500 U/ ml of IFNγ resulted in a 10-20 fold decrease in GM-CSF output in cultures containing from 1 ng/ ml to 20 μg/ ml of LPS, with a half-maximal reversal of the effect of 500 U/ ml of IFNγ at 20 ng/ ml of LPS.

6.7.3 *Effect of IFNγ and LPS on GM-CSF release by endometrial cells from uteri of ovariectomised mice, and mice on day 1 and day 10 of pregnancy*

Endometrial cells harvested from the uteri of mice on day 1 or day 10 of pregnancy. In the latter case, tissue was obtained from both between implantation sites and adjacent to implantation sites. The cells were incubated for 16 h with either 100 or 1000 U/ ml of IFNγ, or alternatively with 2 or 20 μg/ ml of LPS. Supernatants from the cultures were assessed for GM-CSF content following an additional 24 h culture in media alone. GM-CSF release from these cells was found to be as sensitive to LPS and IFNγ as release from their oestrous counterparts. GM-CSF output from endometrial cells harvested from day 1 and day 10 pregnant mice was enhanced 8.7-fold and 12.1-fold respectively in the presence of 20 μg/ ml of LPS, and 4.2-fold and 4.5-fold in the presence of 2 μg/ ml of LPS. Following exposure to 1000 U/ ml of IFNγ for 6 h or 16 h, endometrial cells from day 1 and day 10 pregnant mice released 96% and 94% less GM-CSF respectively than cells cultured in the absence of IFNγ, and 88% and 82% less following exposure to 100 U/ ml of IFNγ.

To determine whether epithelial cells are sensitive to LPS in the absence of oestrogen, endometrial cells from ovariectomised mice were cultured in 'oestrogen-free' DMEM in the presence or absence of 2 or 20 μg/ ml of LPS. Supernatants were collected at 24 h and in each instance were found to contain < 1 U/ 10^5 cells of GM-CSF.

6.7.4 *Specificity for epithelial cells of the action of IFNγ and LPS*

To determine whether LPS and IFNγ acted directly on epithelial cells in endometrial cell monolayers (as opposed to contaminating fibroblasts or leukocytes), epithelial cells and stromal cells were purified from oestrous endometrial cells by panning with MTS #24 and Thy1.2 mAbs. The purified cells were then incubated with either LPS or IFNγ. GM-CSF release from purified epithelial cells (MTS #24$^+$ or Thy1.2$^-$ cells) was enhanced by LPS and inhibited by IFNγ. Purified stromal

fibroblasts (Thy1.2$^+$ cells) did not synthesise any detectable GM-CSF after incubation with LPS (Fig 6.10). These findings show that LPS and IFNγ each influence GM-CSF output through a direct interaction with epithelial cells.

6.7.5 Effect of biological mediators on IL-6 release

To examine the effects of various cytokines and other biological mediators on IL-6 production by epithelial cells, the IL-6 concentrations were measured in many of the supernatants generated in 6.7.1. IFNγ and LPS were found to influence only marginally the release of IL-6. IL-6 output was inhibited at 1000 U/ ml of IFNγ, but not at 100 U/ ml of IFNγ, and 20 ng/ ml LPS enhanced IL-6 output less than 2-fold. High concentrations of cytokines (including IL-2, TNFα, and IL-6 itself), as well as $PGF_{2\alpha}$, promoted IL-6 output 2-3 fold.

6.8 Conclusions and discussion

These studies indicate that GM-CSF production by epithelial cells can be modulated by the ovarian steroids oestrogen and progesterone, as well as by bacterial LPS and IFNγ (Fig 6.11). These agents are present within the endometrium and thus are potentially paracrine or endocrine regulators of epithelial cell GM-CSF release *in vivo*. Whilst oestrogen and LPS each enhance GM-CSF output, IFNγ and progesterone have an inhibitory effect.

GM-CSF output in short term *in vitro* cultures of endometrial cells was found to be dependant on the ovarian steroid status of the mice from which the cells were derived. In normal mice, production fluctuated according to the stage of the oestrous cycle of the donors, being maximal at oestrous. Cells derived from ovariectomised mice produced 25-fold less GM-CSF than cells from oestrous mice, and production was restored to oestrous levels by treatment of mice with 17β-oestradiol but not progesterone. That oestrogen is a principle regulator of GM-CSF release was confirmed by the finding that the oestrogen antagonist ZK 119010 blocked synthesis of GM-CSF both in oestrous mice and in ovariectomised mice maintained on exogenous steroids. Progesterone was found to reduce the ability of 17β-oestradiol to induce GM-CSF synthesis in ovariectomised mice. Furthermore, the progesterone antagonist RU486 reversed the inhibitory effect of hyperstimulation with hCG, and enhanced GM-CSF synthesis in ovariectomised mice maintained on exogenous oestrogen and progesterone. In contrast to the effect of a decidual stimulus on CSF-1 synthesis (Pollard *et al.* 1987), administration of a decidual stimulus to the uterine lumen in mice maintained on both steroids had little effect. However, a moderate reduction in the output of GM-CSF by endometrial cells occurred in mice where extensive decidualisation had taken place.

The nature of the GM-CSF response to ovarian steroids resembles the production of a chemotactic factor for eosinophils by rat uterine epithelial cells. This factor is induced by oestrogen and antagonised by progesterone (Lee *et al.* 1989; Howe *et al.* 1990). However, the GM-CSF response contrasts with that of other epithelial cell-derived cytokines including CSF-1, LIF and TNFα, because the synthesis of these factors (as assessed by mRNA levels) is not maximal until

progesterone synthesis has commenced on day 3 of murine pregnancy (Yelavarthi *et al.* 1991; Bhatt *et al.* 1991; Arceci *et al.* 1989).

The mechanism by which steroid hormones regulate cytokine synthesis by epithelial cells is unknown, although the time course of GM-CSF induction shows that within hours, oestrogen acts to induce GM-CSF release. Oestrogen has not to our knowledge been reported to influence GM-CSF synthesis in other cells. At which point oestrogen acts in the transcriptional and translational events leading to the synthesis of GM-CSF mRNA and protein remains to be determined. However, it is relevant that in leukocytes and fibroblasts an increase in both the rate of synthesis and half-life of GM-CSF mRNA mediate the increase in GM-CSF release in response to various inducing agents (Gasson 1991). Furthermore, steroids appear to increase the synthesis of CSF-1, LIF and TNFα at the mRNA level (Pollard *et al.* 1987; Yelavarthi *et al.* 1991; Bhatt *et al.* 1991) (1.7.2, 1.7.3, 1.7.4). There are no precedents for the mobilisation of a cytoplasmic store of pre-synthesised cytokine as a mechanism for enhanced secretion of GM-CSF, but since epithelial cells are secretory cells this cannot be ruled out. Additional experiments, using sensitive techniques for the quantification of GM-CSF mRNA (ie. solution hybridisation/ RNAse protection), are necessary to investigate whether steroids alter the GM-CSF output of epithelial cells at the mRNA level.

The failure of the oestrogen antagonist ZK 119010 to inhibit synthesis of GM-CSF, and the inability of 17β-oestradiol to induce GM-CSF production in epithelial cells from ovariectomised animals *in vitro*, suggests that factors derived from other endometrial cells may have a role in mediating the effects of oestrogen. There is evidence that TGFβ, EGF, or other growth factors may act in this capacity (Nelson *et al.* 1991; Ignar Trowbridge *et al.* 1992; Beck and Garner 1992). Alternatively, epithelial cells may not respond physiologically to steroids *in vitro*, despite having a functionally intact receptor system (Uchima *et al.* 1991). If epithelial cells are able to respond to

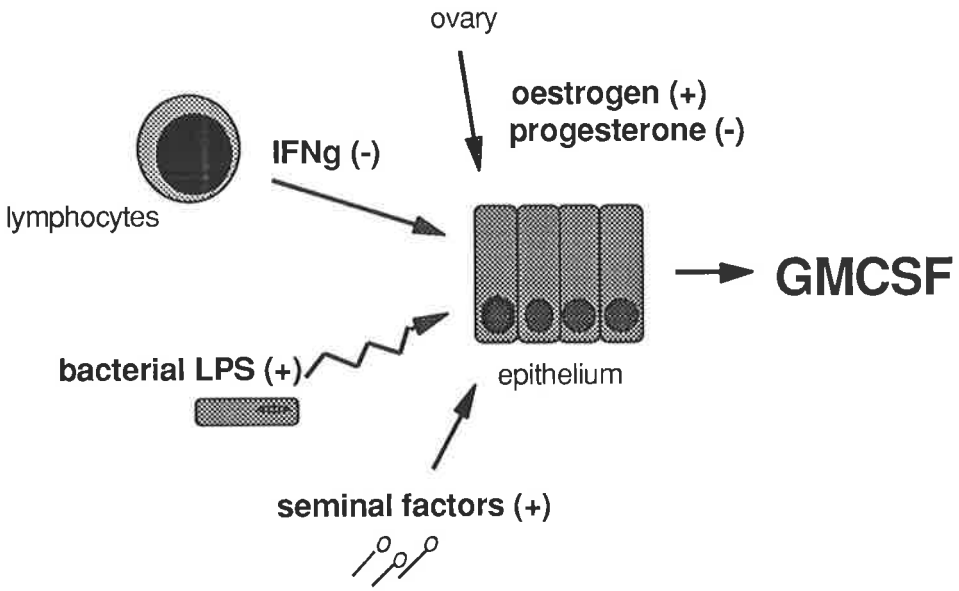


Figure 6.11 Biological regulators of GM-CSF release from uterine epithelial cells

steroid hormones directly, it is possible that embryo-derived oestrogen can act on the local epithelium at implantation to enhance cytokine release and thereby provide the signal for the decidual response (1.2.3c).

The inhibition of GM-CSF synthesis by progesterone may explain the failure of super-ovulated mice to exhibit an elevation in luminal fluid GM-CSF levels observed in response to seminal plasma after natural mating (4.2.2), and this may contribute to the infertility of superovulated mice (Fossum *et al.* 1989).

The failure of oestrogen or progesterone to affect IL-6 production by endometrial cells suggests that IL-6 synthesis by these cells is steroid independent. This is in accord with the finding that secretion of IL-6 by polarised epithelial cells *in vitro* is unaffected by the hormonal status of mice (Jacobs *et al.* 1992).

The gram negative bacterial cell wall component LPS was found to be potent in enhancing the release of GM-CSF by epithelial cells from the uteri of oestrous, day 1 or day 10 pregnant mice. LPS is known to increase GM-CSF mRNA transcription and stability in fibroblasts, endothelial cells and macrophages (Gasson 1991), but whether the same mechanism is responsible for LPS-induced GM-CSF secretion in epithelial cells remains to be determined. As in other tissues, the epithelial cell response to this agent may be relevant in the event of local infection. Furthermore, bacteria introduced into the uterus at mating may strengthen the GM-CSF response to seminal plasma at mating. It is of interest that LPS did not influence the GM-CSF output of epithelial cells from ovariectomised mice. This suggests that oestrogen is an absolute requirement for GM-CSF synthesis by epithelial cells.

The lymphocyte-derived cytokine IFNγ was found to have a potent inhibitory effect on GM-CSF release by uterine epithelial cells harvested from oestrous mice, or from day 1 or mid-gestation pregnant mice. Whilst the physiological significance of IFNγ in the regulation of epithelial GM-CSF synthesis remains unclear. However, it is of interest that immunoreactive IFNγ is found within the human placenta (Bulmer *et al.* 1990), and receptors for IFNγ are expressed by human endometrial epithelial cells (Tabibzadeh, 1990). The synthesis of GM-CSF by a variety of other cells has been reported to be either enhanced or suppressed by IFNγ (1.6.1c).

These data suggest that T-lymphocytes and NK cells have the potential to regulate GM-CSF synthesis and perhaps other functions of epithelial cells. This mechanism may explain both the relationship reported between the degree of maternal recognition of fetal alloantigenicity and production of GM-CSF by decidua (Wegmann 1988; Wegmann *et al.* 1989) and the modulation of GM-CSF synthesis following *in vivo* administration of mAbs against Thy1.2 and CD8 (Athanassakis *et al.* 1987). Chaouat *et al.* (1990) have reported that *in vivo* administration of IFNγ leads to increased fetal resorption in the CBA/J x DBA/2 mouse model, and they cited evidence that IFNγ has direct cytostatic or cytotoxic effects on placental cells. In the light of the results presented in this chapter, it may be speculated that IFNγ-mediated inhibition of GM-CSF synthesis by epithelial cells contributes to the abortifacient effects of IFNγ and other factors which activate T-lymphocytes or NK cells (including IL-2 and Poly(I).Poly(C12)U) (Chaouat *et al.* 1990).

Whether LPS and IFNγ influence the synthesis of other cytokines by epithelial cells remains to be determined. However, the finding that they have little influence on release of IL-6 from these cells is evidence that their effect is not universal and it further supports the contention that there is differential regulation of synthesis of the individual cytokines produced by uterine epithelial cells. Other cytokines which promote GM-CSF release from macrophages, T-lymphocytes, fibroblasts and endothelial cells (including IL-1 and TNFα) (Lu *et al.* 1988, Munker *et al.* 1986; Broudy *et al.* 1986), did not alter release of GM-CSF from uterine epithelial cells. Taken together, these data suggest that the molecular regulation of the release of GM-CSF from the uterine epithelium may be unique when compared with other sources of this cytokine.

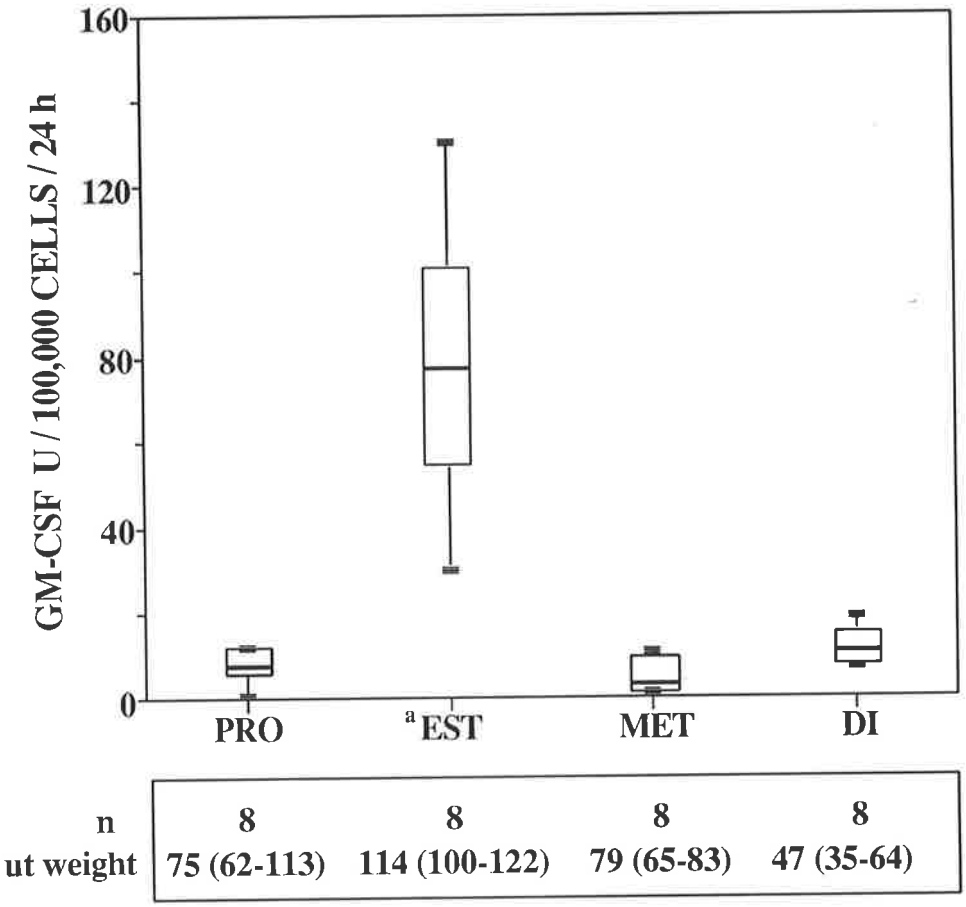


Figure 6.1 Effect of stage of oestrous cycle on GM-CSF release. LHRH superagonist was administered to intact mice on day 1 to induce oestrus on day 5. Mice were sacrificed on consecutive days during the ensuing, normal oestrous cycle, beginning on day 4 (pro-oestrus: PRO), with days 5, 6 and 7 yielding mice in oestrus (EST), di-oestrus (DI) and met-oestrus (MET) respectively. The stage of the oestrus cycle was confirmed by microscopic examination of vaginal smears. Endometrial cells were harvested and cultured for 24 h, and supernatants were assayed for cytokine bioactivity. Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed). Results are graphed as box-and-whisker plots, where the box delineates the 25th to the 75th percentile, and median values are scored. Whiskers define data falling within 1.5 x the inter-quartile-range (IQR), and circles outside the whiskers are outliers and were excluded from statistical analysis. Median (range) uterine weights are in milligrams, and the number of observations (individual mice) are tabulated.
[a] Significantly different to all other groups, $p < 0.001$.

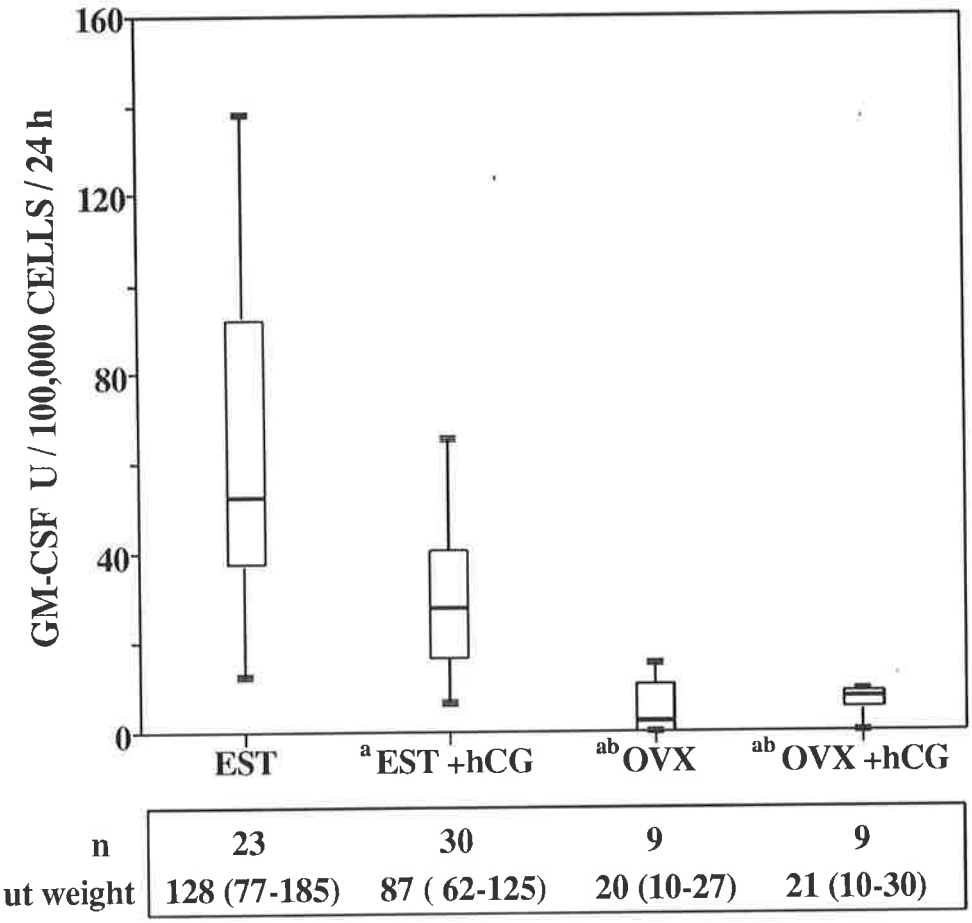


Figure 6.2 Effect of ovariectomy and hCG treatment on GM-CSF release.
Intact (EST) or ovariectomised (OVX) mice were injected daily with 50 IU hCG or saline ip for 4 days, then sacrificed on day 5. All mice received LHRH superagonist on day 1 to induce oestrus on day 5. Endometrial cells were harvested and cultured for 24 h, and supernatants assayed for cytokine bioactivity. Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed). Results are graphed as box-and-whisker plots as described in Fig. 6.1.
[a] Significantly different to EST, $p < 0.001$.
[b] Significantly different to EST + hCG, $p < 0.001$.

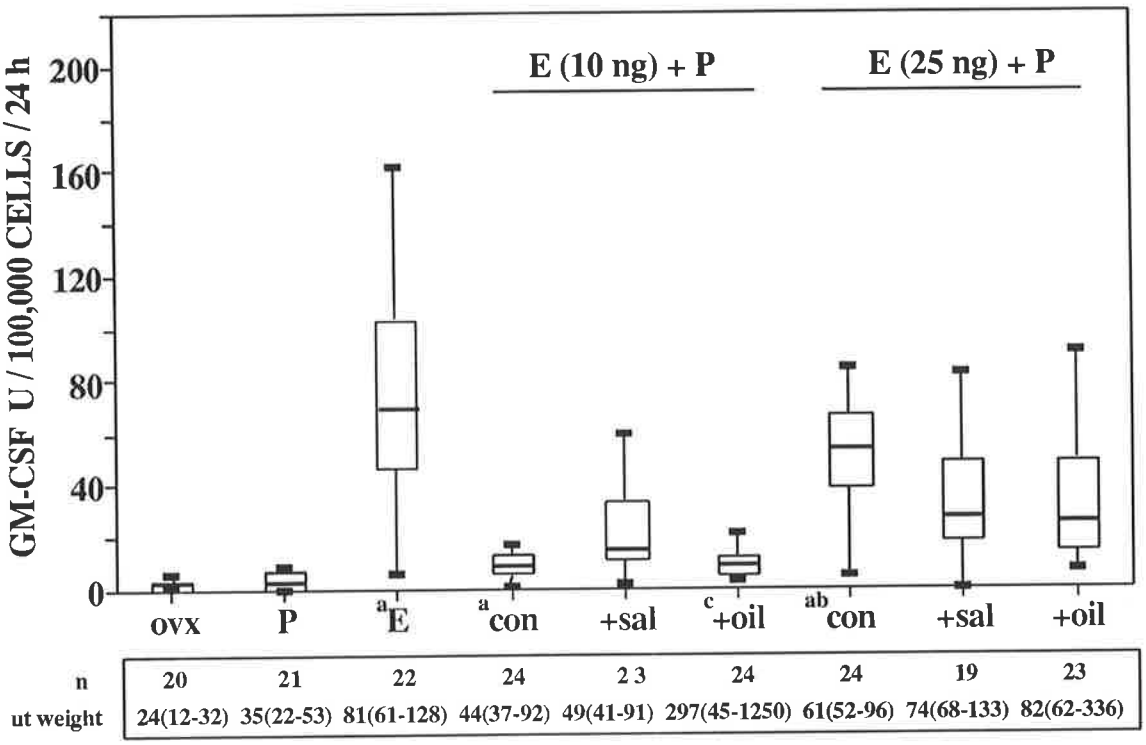


Figure 6.3 Effect of exogenous ovarian steroids on GM-CSF release following ovariectomy. Ovariectomised mice were primed on two consecutive days with 17β-oestradiol (100 ng). Beginning three days later, they were maintained on daily injections for 6 days of either 17β-oestradiol alone (25 ng)(E), progesterone alone (500 μg)(P), or both steroids in combination [E (10 ng) + P and E (25 ng) + P]. Control mice received carrier alone (ovx). Additional groups of mice maintained on both 17β-oestradiol and progesterone [E (10 ng) + P and E (25 ng) + P] received an intraluminal decidual stimulus of peanut oil (+ oil) or saline (+ sal) on day 3 of the 6 day treatment. Mice were sacrificed on day 7 and endometrial cells were harvested and cultured for 24 h. Supernatants were then assayed for cytokine bioactivity. Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed). Results are graphed as box-and-whisker plots as described in Fig. 6.1.

[a] Significantly different to ovx, $p < 0.001$.

[b] Significantly different to E, $p = 0.05$.

[c] Significantly different to E (10 ng) + P (+ sal), $p = 0.002$.

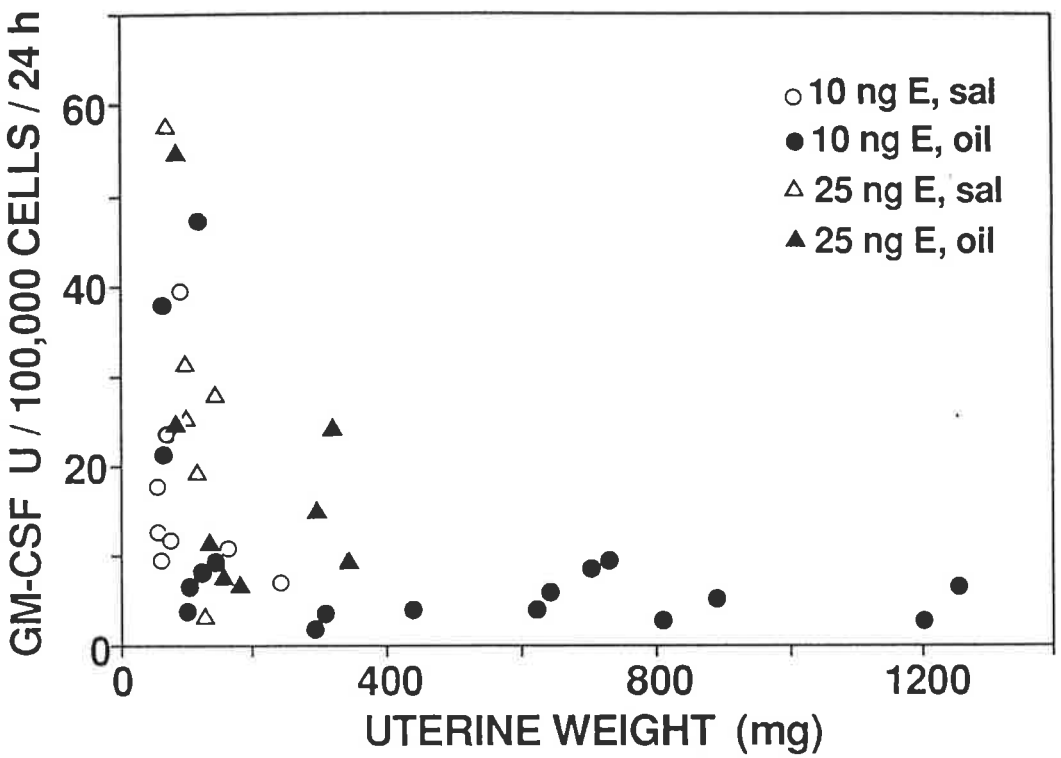


Figure 6.4 Relationship between the degree of uterine decidualisation and GM-CSF release. Data from treatment groups E, F, H and I in Fig 6.3, not including data points corresponding to uteri without a visible decidual reaction, are plotted as a function of decidual weight. Symbols represent individual animals. (Pearson's correlation coefficient $r = -0.44$, $p = 0.005$).

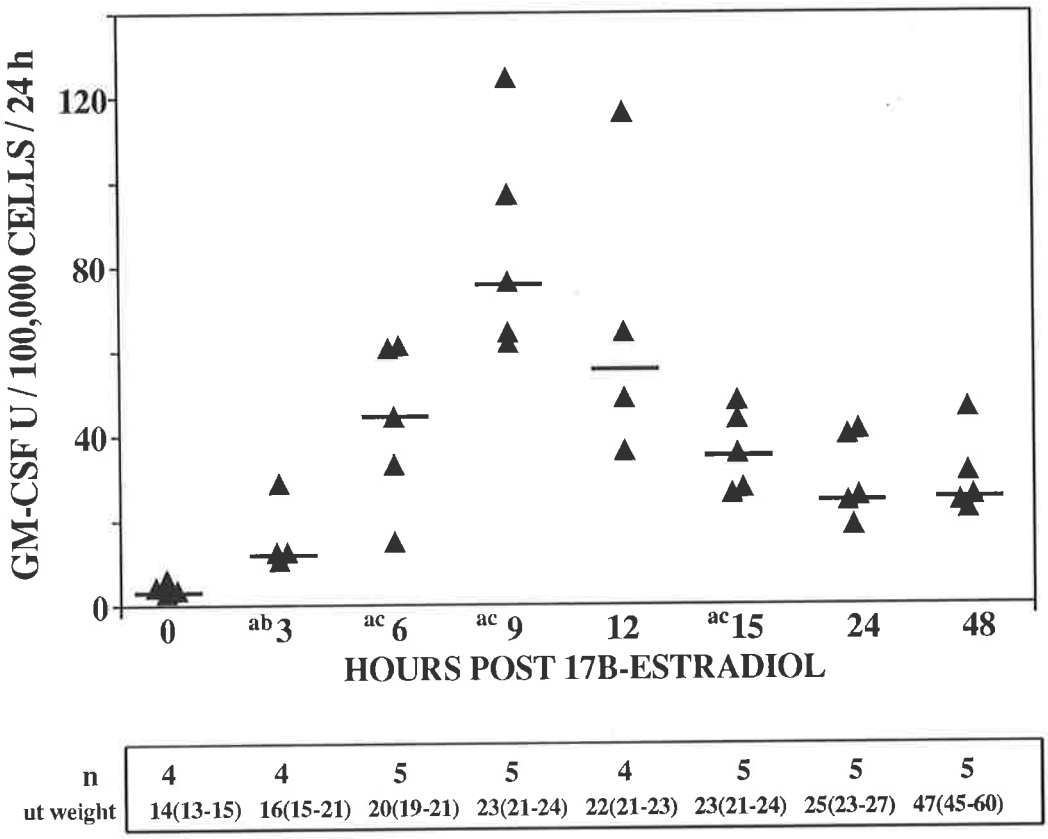


Figure 6.5 Time course of induction of GM-CSF release after administration of 17β-oestradiol. Ovariectomised animals were injected with 50 ng of 17β-oestradiol and sacrificed at 3, 6, 9, 12, 15, and 24 h later. The 48 h group of mice received a second injection of 50 ng of 17β-oestradiol 24 h after the first and were sacrificed 24 h later. Endometrial cells were harvested and cultured for 24 h, and supernatants assayed for cytokine bioactivity. Symbols represent individual animals, and median values are scored. Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed).

[a] Significantly different to preceding time point

[b] Significantly different to t = 0 (ovariectomised control), $p = 0.020$

[c] Significantly different to t = 0 (ovariectomised control), $p = 0.014$

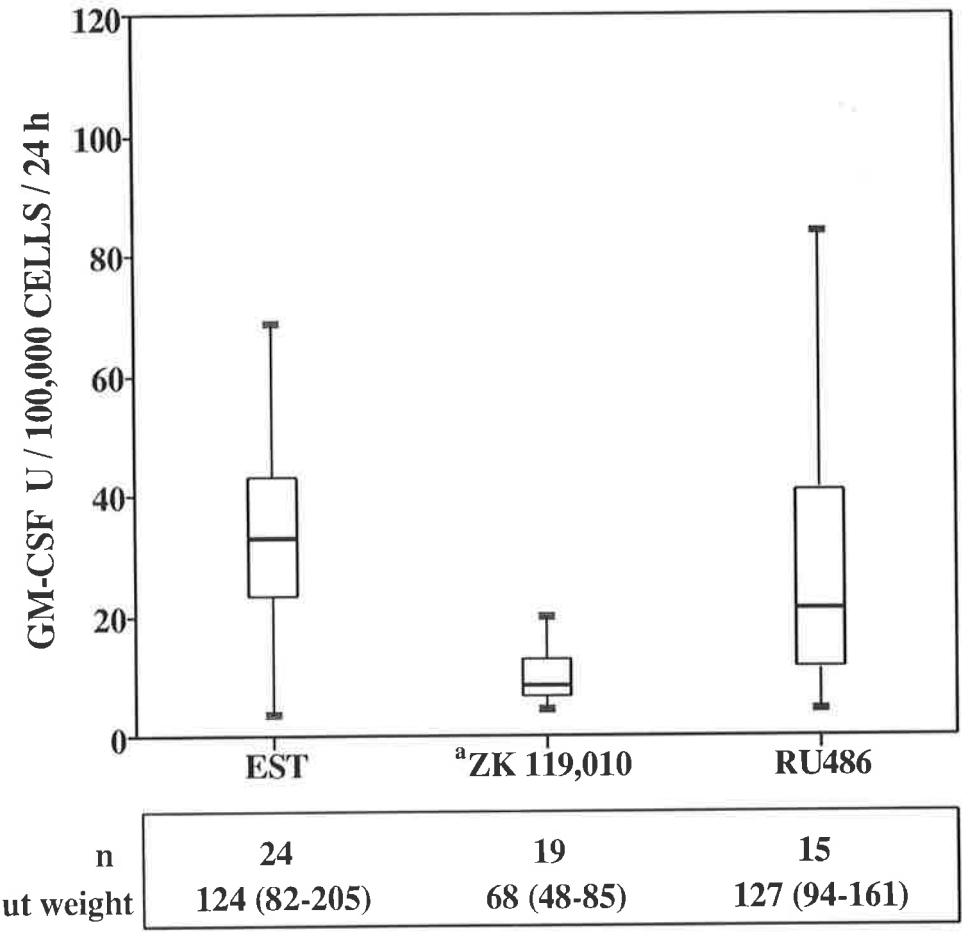


Figure 6.6 Effects of the oestrogen antagonist ZK 119010 and the progesterone antagonist RU486 on GM-CSF release in oestrous mice. Intact mice were administered LHRH superagonist on day 1 to induce oestrus on day 5. They were injected daily, beginning on the same day, with either ZK 119010 (600 μg), RU486 (500 μg) or carrier (EST) for 4 days prior to sacrifice on day 5. Endometrial cells were harvested and cultured for 24 h, and supernatants assayed for cytokine bioactivity. Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed). Results are graphed as box-and-whisker plots as described in Fig. 6.1.
[a] Significantly different to EST, $p < 0.001$.

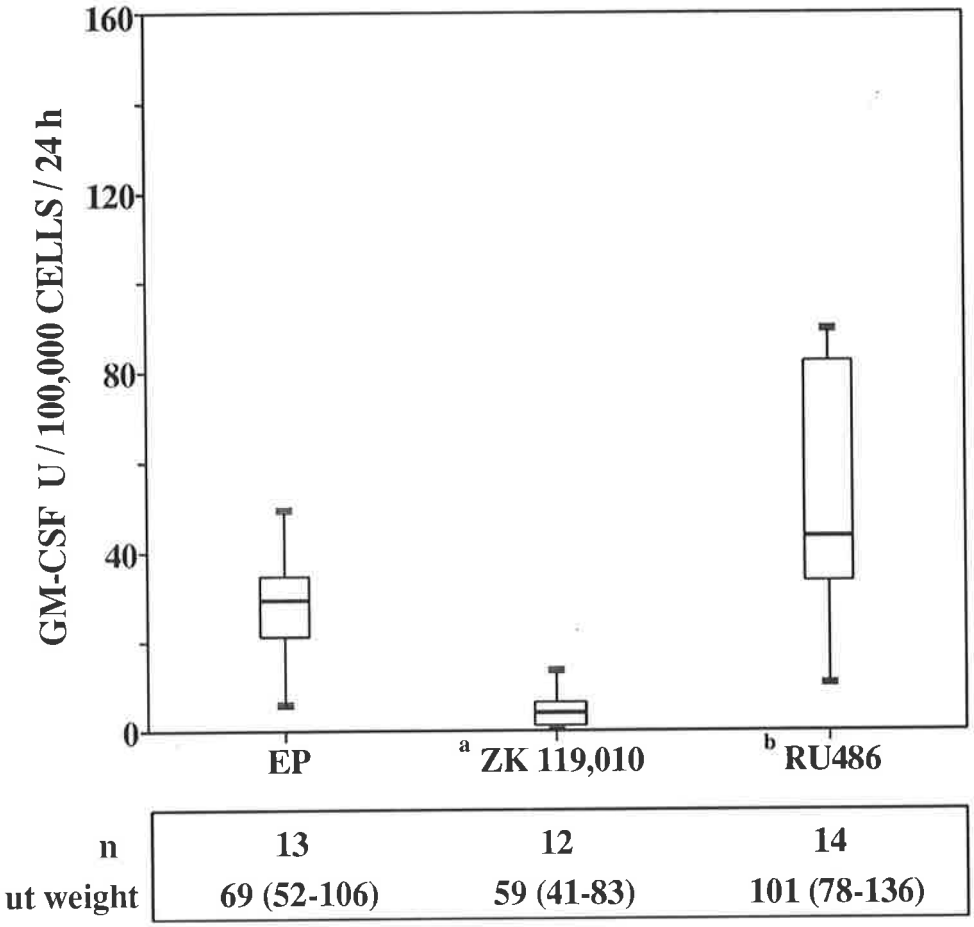


Figure 6.7 Effects of the oestrogen antagonist ZK 119010 and the progesterone antagonist RU486 on GM-CSF release in ovariectomised mice. Ovariectomised mice were primed on two consecutive days with 17β-oestradiol (100 ng). Beginning three days later they were maintained on 17β-oestradiol (25 ng) and progesterone (500 μg) daily for 6 days, in combination with either ZK 119010 (600 μg), RU486 (500 μg) or carrier (EP). Animals were sacrificed on day 7, and endometrial cells were cultured for 24 h then supernatants were assayed for cytokine bioactivity. Data were compared by Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney Rank Sum Test (two-tailed). Results are graphed as box-and-whisker plots as described in Fig. 6.1.
[a] Significantly different to EP, $p < 0.001$.
[b] Significantly different to EP, $p = 0.021$.

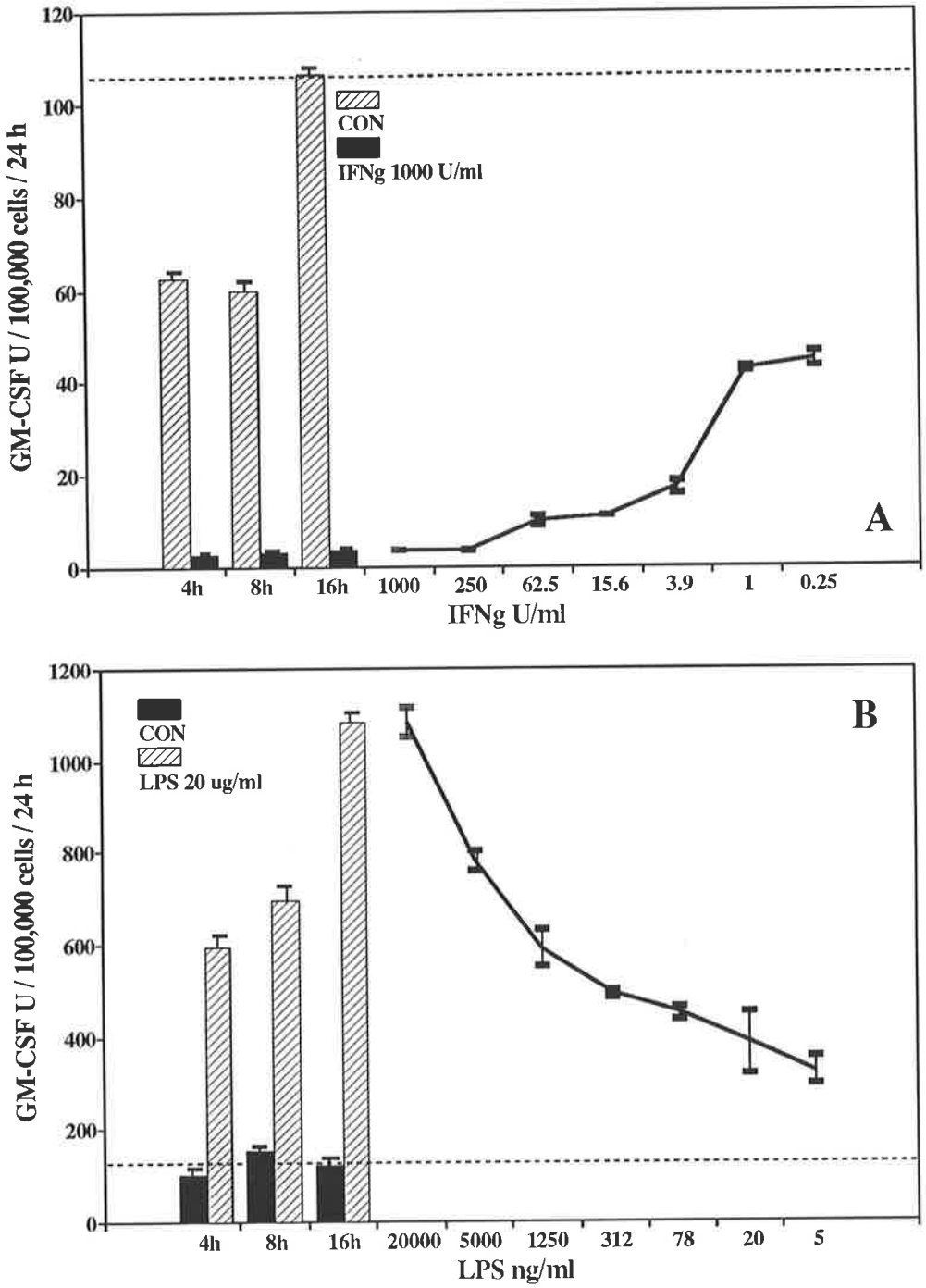


Figure 6.8 Effect of duration of exposure and concentration of IFNγ and LPS on GM-CSF release. Endometrial cells harvested from oestrous mice were incubated with (A) 1000 U/ ml of IFNγ for 4 or 8 h, or concentrations ranging from 0.25 to 1000 U/ ml for 16 h, or (B) 20 μg/ ml of LPS for 4 or 8 h, or concentrations ranging from 5 ng/ ml to 20 μg/ ml for 16 h. In each instance the media containing IFNγ or LPS was then replaced with DMEM-FCS, and supernatants were collected 24 h later and supernatants assayed for cytokine bioactivity. The dotted horizontal line represents the GM-CSF output of endometrial cells cultured in DMEM-FCS alone.

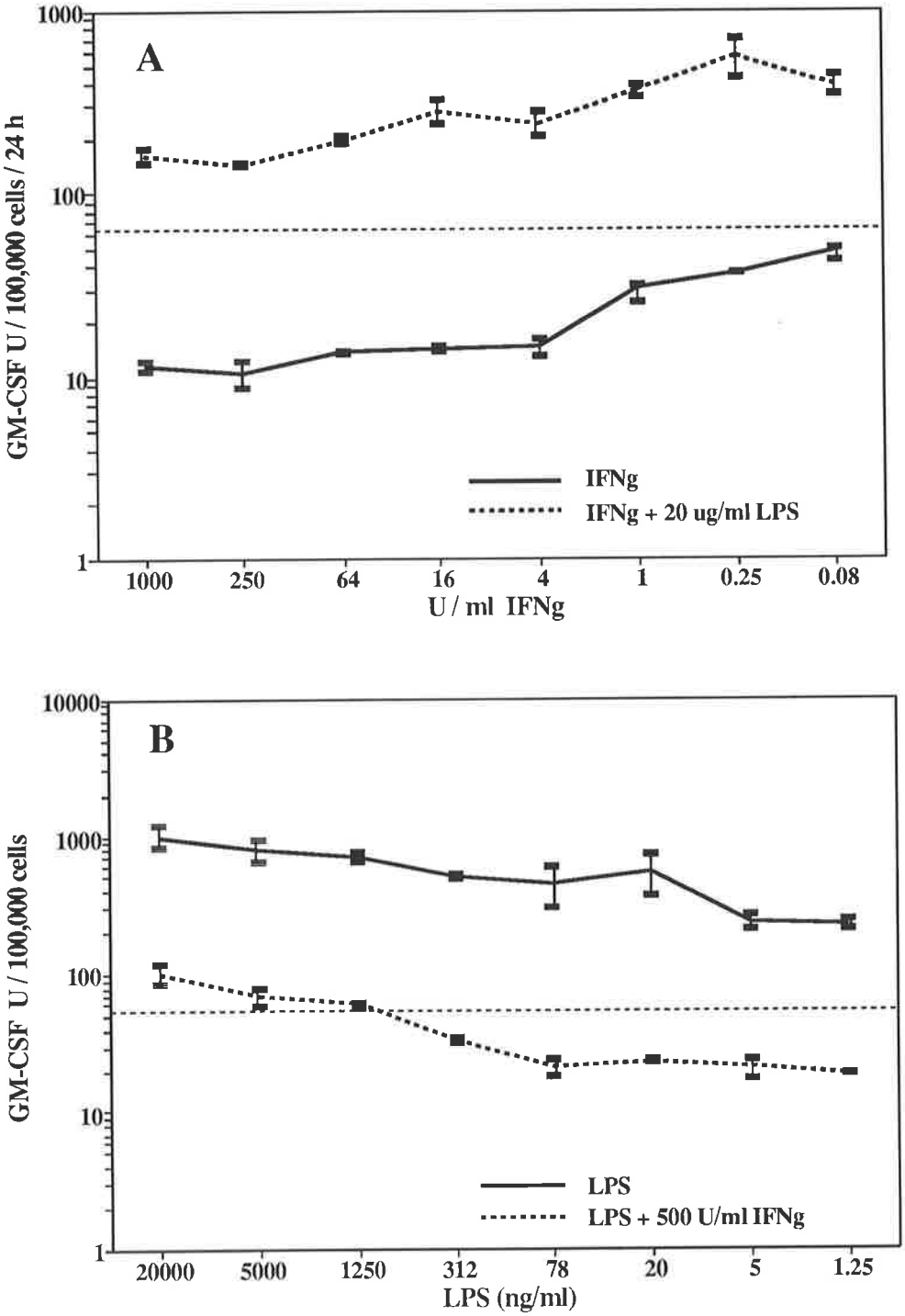


Figure 6.9 Hierarchy of effects of IFNγ and LPS on GM-CSF release.
Endometrial cells harvested from oestrous mice were incubated for 16 h with concentrations of (A) IFNγ ranging from 0.08 to 1000 U/ ml in the presence or absence of 20 μg/ ml of LPS, or (B) LPS ranging from 5 ng/ ml to 20 μg/ ml in the presence or absence of 500 U/ ml of IFNγ. The media containing IFNγ or LPS was then replaced with DMEM-FCS, and supernatants were collected 24 h later and supernatants assayed for cytokine bioactivity. The dotted horizontal line represents the GM-CSF output of endometrial cells cultured in DMEM-FCS alone.

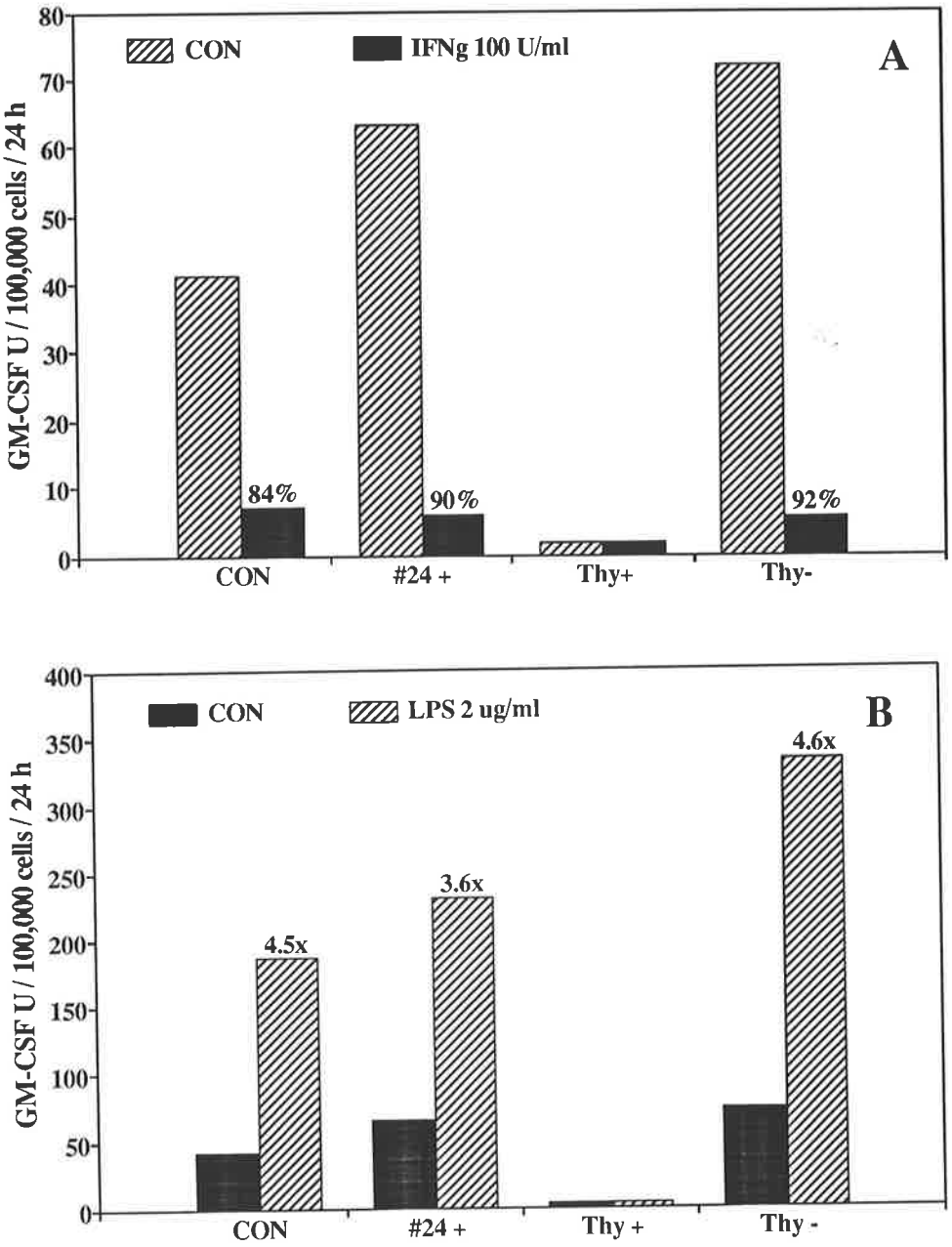


Figure 6.10 Epithelial cell specificity of IFNγ and LPS. Epithelial cells and stromal cells were purified by panning respectively with specific mAbs MTS #24 and Thy1.2 from endometrial cells harvested from oestrous uteri. Positively selected (+) and negatively selected (-) populations were incubated for 16 h with (A) 100 U/ ml IFNγ, or (B) 2 μg/ ml of LPS. The media containing IFNγ or LPS was then replaced with DMEM-FCS, and supernatants were collected 24 h later and supernatants assayed for cytokine bioactivity.

7 The role of GM-CSF as a regulator of uterine leukocyte populations

7.1 Introduction

Immunohistochemical studies which describe in detail the temporal changes observed in the distribution of leukocyte populations during the oestrous cycle and early pregnancy (1.5.2, 1.5.4, 1.5.5), together with the studies presented in earlier chapters, suggest that there is a concurrence between the levels of GM-CSF in the uterus and the traffic and functional status of both macrophages and granulocytes. GM-CSF is known to be a specific chemoattractant for macrophages and neutrophils, and it may also enhance the recruitment of leukocytes into tissues by influencing the adhesive properties of endothelial cells (1.7.1d). The proliferation and functional activities of mature monocyte/ macrophages and granulocytes are also regulated by GM-CSF (1.7.1d).

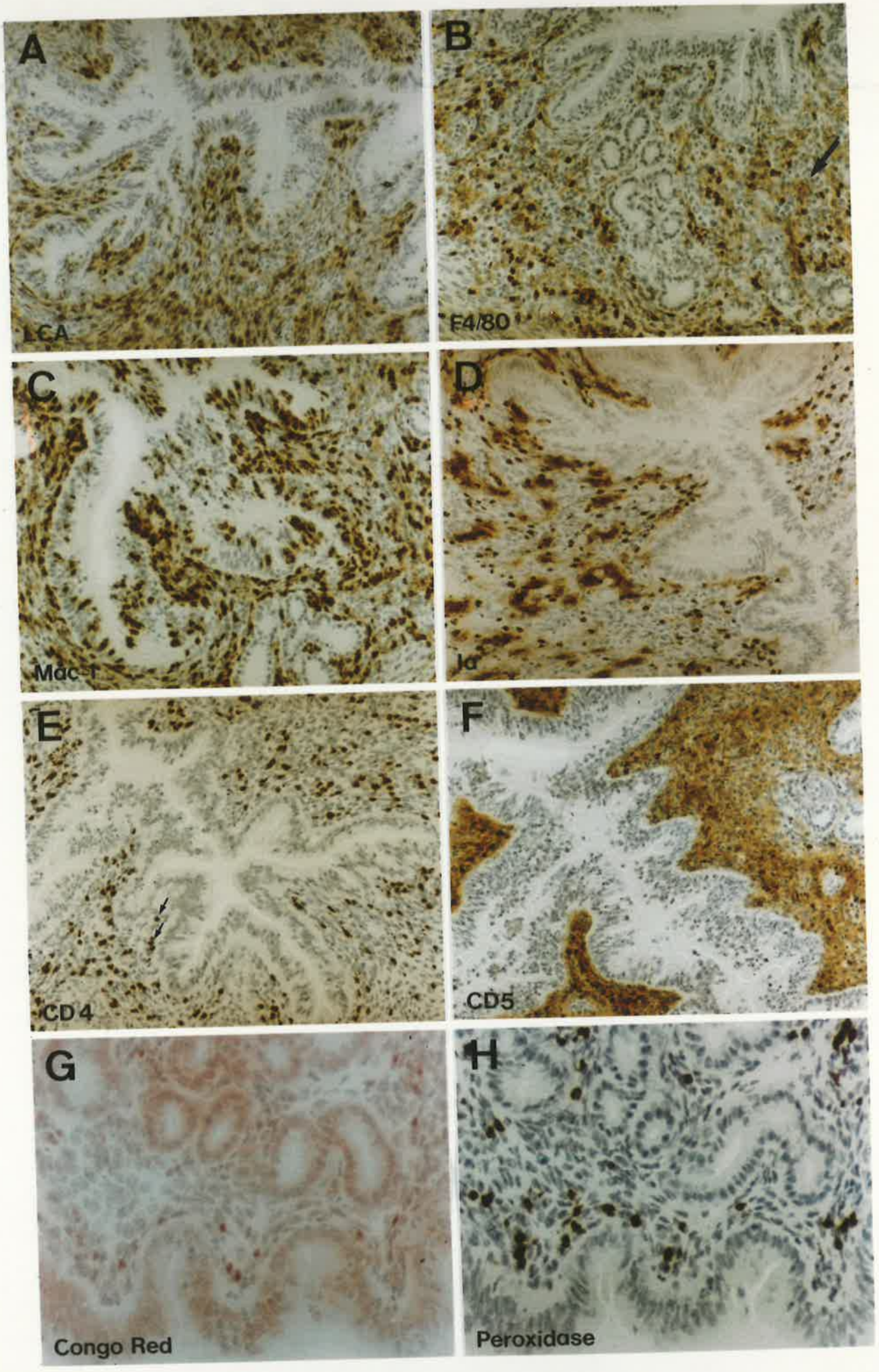
The large populations of macrophages, eosinophils and neutrophils present in the murine endometrium during the oestrous cycle and early pregnancy are therefore potential targets for the actions of GM-CSF and other cytokines that originate in the epithelium. In the experiments described in this chapter, expression of receptor for GM-CSF in the uterus at oestrus and during early pregnancy has been investigated by RT-PCR analysis. ^{125}I-GM-CSF was found to bind to sections of uterine tissue in a pattern consistent with the expression of GM-CSF receptors by endometrial leukocytes. To investigate the influence of local release of GM-CSF on recruitment and activation of leukocytes, the densities and distribution of macrophages and granulocytes in the uterus were examined immunohistochemically after introduction recombinant GM-CSF into the uterine lumen in ovariectomised mice.

7.2 The density and distribution of macrophages and granulocytes in the endometrium on day 1 of pregnancy

To assess the density and distribution of GM-CSF-responsive leukocyte lineages in the uterus, macrophages and granulocytes were identified in sections of uterine tissue from day 1 pregnant mice by immunohistochemistry, using an indirect immunoperoxidase technique. The day 1 uterus was chosen for these experiments because others have reported the presence of large numbers of leukocytes in the uterus at this time. In part, this may be a consequence of the peak in uterine GM-CSF activity that follows exposure to seminal factors at mating (Chapter 4). The mAbs listed in Table 2.1 were used to label individual leukocyte subpopulations in the uteri of three naturally mated mice. Sections of murine small intestine, which contains leukocytes of each of the lineages of interest, were used as positive controls for each mAb. Eosinophils were detected by staining parallel sections with Congo Red, or by the strong endogenous peroxidase activity after reacting with DAB and H_2O_2.

The densities and distribution of leukocytes were very similar in the uteri of all of three mice used in this experiment. Large numbers of cells in the endometrial stroma reacted were detected with mAbs against CD45 (LCA) or LFA-1. Sections labelled with mAbs against myeloid antigens showed

Figure 7.1 Immunohistochemical localisation of leukocytes in the day 1 uterus. Sections of day 1 uterus were incubated with mAbs specific for different leukocyte lineages. Endometrial leukocytes are stained with mAbs against the pan leukocyte marker LCA/ CD45 (A). This population is comprised predominantly of cells reactive with mAbs against macrophage markers F4/80 (B) and Mac-1 (C), or the dendritic cell/ macrophage/ B cell marker Ia (D). Very few cells are stained with mAbs against CD4 or CD8 (E). Stromal fibroblasts are stained with mAb against CD5/ Lyt1 (F). Endogenous peroxidase positive cells are evident as small, granular, dark brown stained cells in sections (A) to (F) (small arrows, E) in addition to lighter brown coloured, mAb-reactive cells (large arrows, B). Serial sections stained with (G) Congo Red and (H) DAB and H_2O_2 show that the majority of the endogenous peroxidase-positive cells in the endometrium are eosinophils.


A
LCA
B
F4/80
C
Mac-1
D
Ia
E
CD4
F
CD5
G
Congo Red
H
Peroxidase

that these leukocytes were predominantly of macrophage, granulocyte and dendritic lineages (Fig 7.1). The macrophage-specific mAb F4/80 reacted with a plentiful population of comparatively large cells with membranous processes that were localised predominantly to stromal areas subjacent to the luminal and glandular epithelia. Similar numbers of cells with a macrophage morphology were found to be positive for Mac-1, which detects the CD18/ CD11b cell adhesion molecule expressed by macrophages and neutrophils in other tissues. In addition, this mAb labelled smaller cells with granular nuclei that were therefore presumed to be neutrophils. Many neutrophils were interspersed between luminal epithelial cells, and so appeared to be migrating into the uterine lumen. Cells with a dendritic morphology and a subepithelial distribution were stained heavily with mAb against Ia antigen. These cells were presumed to be dendritic cells, although even in serial sections it was difficult to discriminate between these cells and those bearing macrophage markers. In addition, the mAb against Ia antigen stained small round cells scattered throughout the endometrial stroma. These were lesser in number than the dendritic cells and endogenous peroxidase-positive cells, and were presumed to be B cells. Large numbers of eosinophils were detected in sections stained with Congo Red. Comparison of serial sections stained with DAB and H_2O_2 revealed that eosinophils accounted for most if not all of the endogenous peroxidase-positive cells in the endometrium (Fig 7.1).

In contrast, T-lymphocytes were distributed very sparsely in the endometrium. Very few positive cells were found in sections stained with mAbs against either CD4 or CD8 antigens. Interestingly, mAb against CD5, an antigen described in the literature as restricted to T- and B-lymphocyte lineages, was found to react strongly and specifically with stromal fibroblasts in the endometrium, in a pattern similar to that found earlier for Thy1.2 (5.5.1).

These data show that on the day after mating, when levels of GM-CSF in the uterus are particularly high, the endometrium is populated richly with leukocytes known to be responsive to GM-CSF, including macrophages, eosinophils, neutrophils and dendritic cells. These findings are in general agreement with reports from other laboratories describing the temporal fluctuations in leukocyte populations during early pregnancy (1.4)

7.3 GM-CSF receptor expression in the endometrium during the pre-implantation period

7.3.1 RT-PCR analysis of uterine GM-CSF receptor mRNA expression

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis was used to determine whether mRNAs encoding the α-subunit (GM-CSF-R) and β-subunit (AIC2B) of the GM-CSF receptor are expressed in the uterus at oestrous and during early pregnancy. RNA was prepared by guanidine thiocyanate extraction, followed by DNAse treatment, from pools of four uteri from oestrus, day 1, day 2, day 3 and day 4 pregnant mice. Complementary DNA (cDNA) was prepared by reverse transcription using oligo-dT primers.

Uterine tissue was found to contain GM-CSF-R mRNA at oestrus and on each of the first four days of pregnancy. cDNAs prepared from uteri at each stage yielded a GM-CSF-R amplicon of the expected size of 235 bp (Fig 7.2). This band was indistinguishable from the product obtained from RT-PCR using murine myeloid cell (FD 5/12) cDNA under the same reaction conditions (described in 3.4.2a).

Figure 7.2 RT-PCR analysis of GM-CSF-R and AIC2B mRNA expression in the oestrous and early pregnant uterus. RNA was isolated by guanidine HCl extraction from oestrous, and day 1, day 2, day 3, and day 4 pregnant uteri and from murine myeloid cells (FD 5/12). First strand cDNA was reverse-transcribed from RNA using oligo-dT primers and amplified with primers for (A) actin, (B) GM-CSF-R and (C) AIC2B. 20 µl aliquots of reaction products were analysed by electrophoresis through a 1% agarose gel containing 50 pg/ ml of ethidium bromide, and photographed with UV illumination.

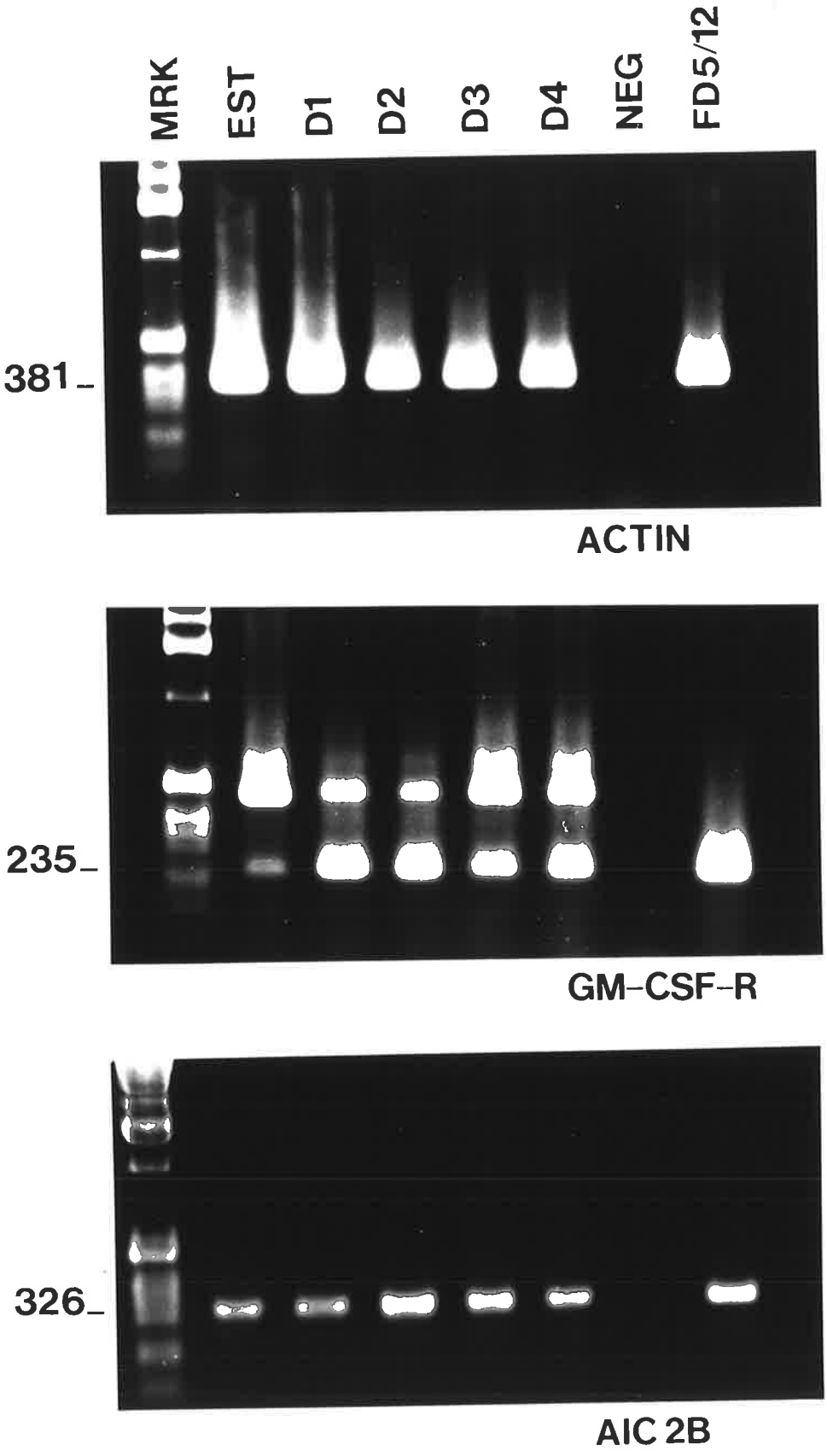
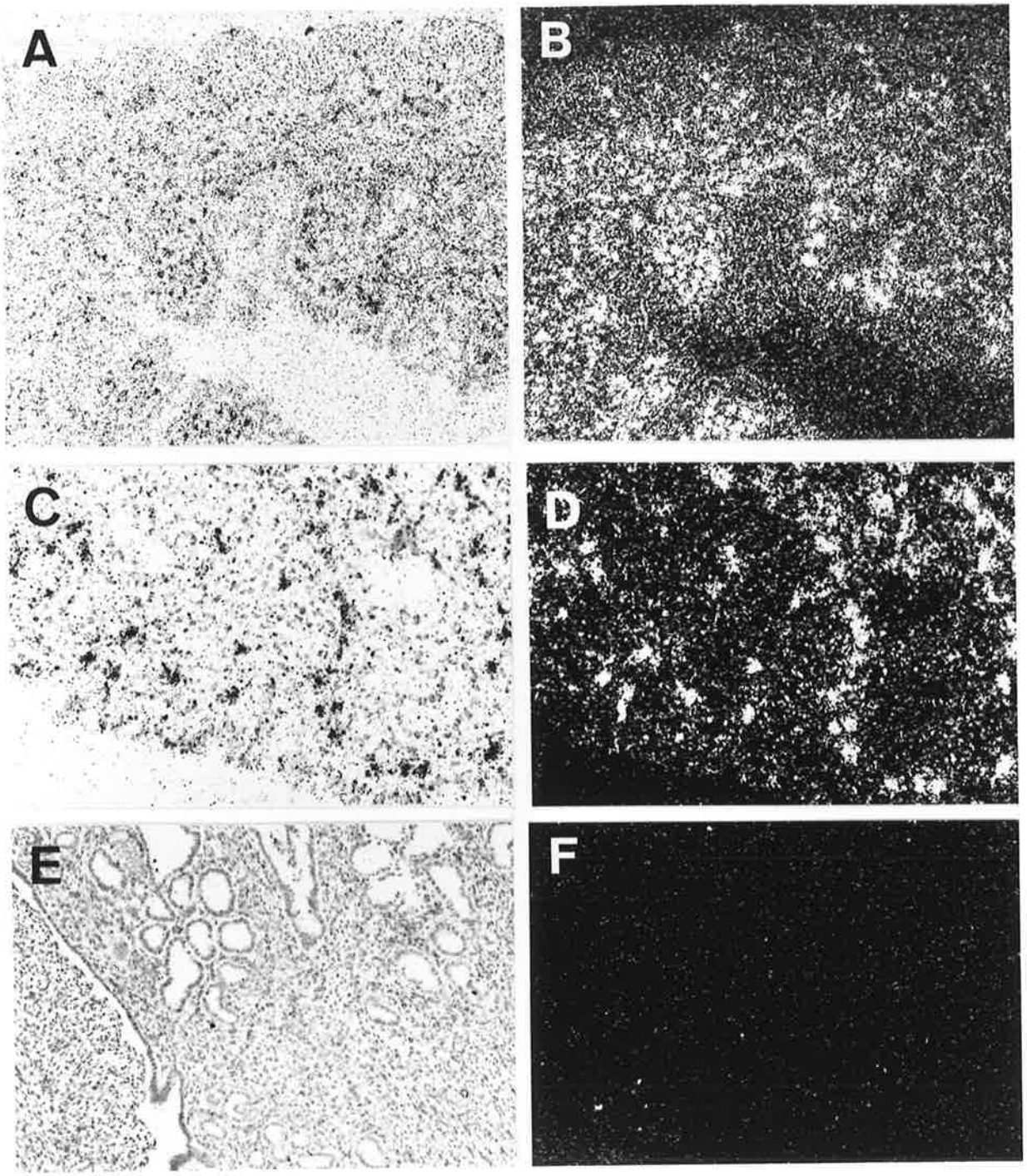

MRK
EST
D1
D2
D3
D4
NEG
FD5/12
381
ACTIN
235
GM-CSF-R
326
AIC 2B

Figure 7.3 Localisation of GM-CSF receptor bearing cells with ^{125}I-GM-CSF on day 1 of pregnancy. Ethanol-fixed fresh frozen sections of day 1 uterus were incubated with ^{125}I-labelled recombinant murine GM-CSF (specific activity 50,000 cpm/ ng) (50 ng/ml; 60 min/ 20°C), washed and post-fixed in 2.5% gluteraldehyde. Sections were autoradiographed for 14 d using Kodak NTB-2 emulsion, developed and counterstained in haematoxylin. They were then photographed under low power magnification (A and B) and high power magnification (C and D), u nder brightfield (A and C) and darkfield (B and D) illumination. (E) and (F) are low power photomicrographs of sections incubated with **^{125}I-GM-CSF in the presence of a** 10-fold excess of cold rGM-CSF.


A
B
C
D
E
F

A second reaction product of 450 bp was also generated by RT-PCR from each of the uterine cDNAs. The identity of this band is not clear, but a band of similar size was sometimes generated from cDNAs prepared from liver and ovary (in addition to the 235 bp product), and also from L cell cDNA (in the absence of the 235 bp product) (data not shown). There appeared to be an inverse relationship between the density of the 235 and 450 bp bands, with the 235 bp band being the major product on day 1 and 2 of pregnancy, and the 450 bp band being of greater intensity at oestrus and on days 3 and 4.

AIC2B mRNA was also found to be present in the uterus on each of the days examined. These results show that cells in the oestrous and early pregnant uterus express mRNAs for both the α-subunit (GM-CSF-R) and β-subunit (AIC2B) of the GM-CSF receptor.

7.3.2 Localisation of GM-CSF receptor-bearing cells with ^{125}I-GM-CSF on day 1

To investigate the identity of the uterine cells that express GM-CSF receptor, sections of day 1 uterus were incubated with ^{125}I-GM-CSF and subjected to autoradiography, according to the protocol developed with the GM-CSF responsive FD 5/12 cell line in 3.4.1a. GM-CSF receptor-positive cells were scattered throughout the endometrial stroma and to a lesser degree in the myometrial stroma. The distribution of receptor-positive cells was similar to that of the leukocytes (7.2), and they were particularly dense in areas immediately subjacent to epithelial surfaces (Fig. 7.2). It was not possible from these experiments to identify precisely which leukocytes within the uterine stroma were positive, or to discount the possibility that non-hemopoietic cells (for example endothelial cells) might also express GM-CSF receptors. However, the predominance of myeloid cells among uterine leukocytes, and the characteristic responsiveness of these cells to GM-CSF, suggests that some or all of the cells from the macrophage, eosinophil, neutrophil and/ or dendritic cell populations express GM-CSF receptor, and hence may be responsive to GM-CSF *in vivo*.

7.4 Effect of GM-CSF on leukocyte traffic in the uterus.

7.4.1 Immunohistochemical evaluation of the GM-CSF-induced leukocyte infiltrate

To investigate whether GM-CSF can alter the distribution or the numbers of leukocytes in the endometrium, a model system was employed in which the leukocyte populations were examined in the uteri of ovariectomised mice, following local exposure to recombinant GM-CSF. The uteri in these mice are relatively 'GM-CSF-deficient' (6.3.1), and are reported to contain less macrophages and granulocytes than the uteri of intact mice (1.5.2, 1.5.4). Groups of four ovariectomised mice were anaesthetised and through a dorsal incision were given a unilateral intraluminal injection of either 40, 200, or 1000 U of recombinant GM-CSF in 50 µl of 1% BSA, or carrier alone. Sixteen hours later, the mice were sacrificed and their uteri were removed. The right and left uterine horns were processed and analysed separately. The numbers and the distribution of uterine leukocytes were assessed immunohistochemically with mAbs against CD45/ LCA, F4/80 antigen, Mac-1, and Ia antigen. Eosinophils were assessed on the basis of their endogenous peroxidase activity.

a. Effect on eosinophils

In each of two experiments, the numbers of leukocytes in endometrial tissues were altered dramatically by intraluminal administration of GM-CSF. Eosinophils appeared to be the subpopulation influenced to the greatest degree. However, not all mice responded to GM-CSF and treated mice fell into two categories; those with uteri that contained very dense populations of eosinophils ('responders') and those with uteri that were indistinguishable from control uteri ('non-responders'). In total, 1 of 4 mice that received 40 U, 6 of 8 mice that received 200 U, and 3 of 8 mice that received 1000 U of GM-CSF were found to have between 4-fold and 200-fold increases in the number of eosinophils within the endometrial stroma, and were classified as 'responders' (Table 7.1).

The data from each experiment were combined and analysed statistically by ANOVA. There were no significant differences between the numbers of eosinophils found in the endometrium of the left and right horns of the uterus ($p = 0.57$). Therefore, the mean number of eosinophils were calculated from counts within areas of 0.27 mm^2 on each of two sections from both the left and the right horns. The ANOVA revealed that the results from each of the two experiments were similar ($p = 0.43$), and overall there was a significant effect of dose of GM-CSF on the numbers of eosinophils ($p = 0.005$). However, since there was a significant interaction between dose and experiment ($p = 0.006$), which was due probably to the difference in the number of eosinophils in the two control groups (mean $\pm$ SD = 25 ± 8 and 0.7 ± 0.4 for experiments 1 and 2 respectively), the two experiments were analysed individually.

In the first experiment, the dose of GM-CSF had a significant effect on the numbers of eosinophils found within the endometrium. Mice that received 200 U of GM-CSF were found to have significantly greater numbers of endometrial eosinophils than mice that received carrier alone, or mice that received 1000 U of GM-CSF (both $p = 0.0001$). In contrast, treatment of mice with 1000 U of GM-CSF did not alter the density of eosinophils in the endometrium. In the second experiment, increased endometrial eosinophils numbers were associated with administration of cytokine in 5 of 12 mice, but the large number (7 of 12) of 'non-responders' in cytokine treated groups reduced the significance of this effect ($p = 0.167$). Eosinophils were distributed throughout the endometrial stroma in GM-CSF treated mice, and in some uteri were concentrated in areas adjacent to the luminal surface (Fig 7.4).

The numbers of eosinophils in the myometrium were also increased in most 'responder' mice. There was no significant difference between the number of eosinophils found in the endometrium or the myometrium of the left and right horns ($p = 0.98$). The effect of GM-CSF was dose dependant in the first experiment, where 200 U, but not 1000 U of GM-CSF, increased the numbers of eosinophils in the myometrium ($p = 0.02$). GM-CSF administration also caused an increase in the density of eosinophils in 5 of 12 mice in the second experiment, but overall the effect was not significant ($p = 0.169$). Eosinophils were predominantly localised to areas adjacent to blood vessels or between the circular and longitudinal muscle layers, and in some mice they were found to congregate in the mesometrial triangle.

Table 7.1 Effect of intraluminal GM-CSF on the number of eosinophils in endometrium and myometrium. Recombinant GM-CSF (20, 200, or 1000 U) in 50 µl of 1% BSA, or 1% BSA alone was injected into the left uterine horn of each of the four mice in each experimental group. Two sections from each uterine horn were stained with DAB and H_2O_2. The numbers of endogenous peroxidase-positive cells within three grid areas of 0.09 mm^2 were counted and pooled. There was no significant difference between the numbers of eosinophils in tissue from the left and right uterine horns. Data are expressed as the mean ± SD number of eosinophils (per 0.27 mm^2) in each of four (two each from the left and right horn) sections per uterus. Values from mice considered to have responded to the treatment are shown in bold.

	Treatment:			
Mouse #	carrier	20 U	200 U	1000 U
ENDOMETRIUM				
Expt 1				
1	14 ± 5		**344 ± 80**	< 1
2	34 ± 4		**214 ± 11**	**90 ± 7**
3	28 ± 13		**249 ± 62**	3 ± 1
4			**222 ± 56**	4 ± 1
Expt 2				
1	< 1	2 ± 1	< 1	3 ± 2
2	< 1	**105 ± 16**	**170 ± 82**	**292 ± 14**
3	< 1	4 ± 3	< 1	< 1
4	< 1	< 1	**170 ± 16**	**257 ± 9**
MYOMETRIUM				
Expt 1				
1	9 ± 2		**80 ± 28**	0.7 ± 0.6
2	57 ± 11		**152 ± 6**	**23 ± 14**
3	28 ± 9		46 ± 14	4 ± 1
4			**119 ± 34**	4 ± 1
Expt 2				
1	< 1	5 ± 2	7 ± 3	7 ± 3
2	3 ± 1	**24 ± 5**	**190 ± 59**	**102 ± 32**
3	3 ± 1	4 ± 2	6 ± 4	2 ± 1
4	< 1	4 ± 1	**119 ± 27**	**47 ± 25**

Figure 7.4 The effect of exogenous GM-CSF on the numbers of leukocytes and endometrial glands, and size of the uterus in ovariectomised mice (low power). sections of uterus are from mice that received intraluminal injections of either 200 U of GM-CSF (right hand panel; B, D, F, H and J), or carrier (left hand panel; A, C, E, G and I). Sections were stained with DAB and H_2O_2 to detect eosinophils (A and B), or incubated with mAbs specific for different leukocyte lineages. All leukocytes are detected with mAbs against the pan leukocyte markers CD45/ LCA (C and D). These cells are predominantly reactive with mAbs against macrophage markers F4/80 (E and F), and Mac-1 (G and H), or the dendritic cell/ macrophage marker Ia (I and J).

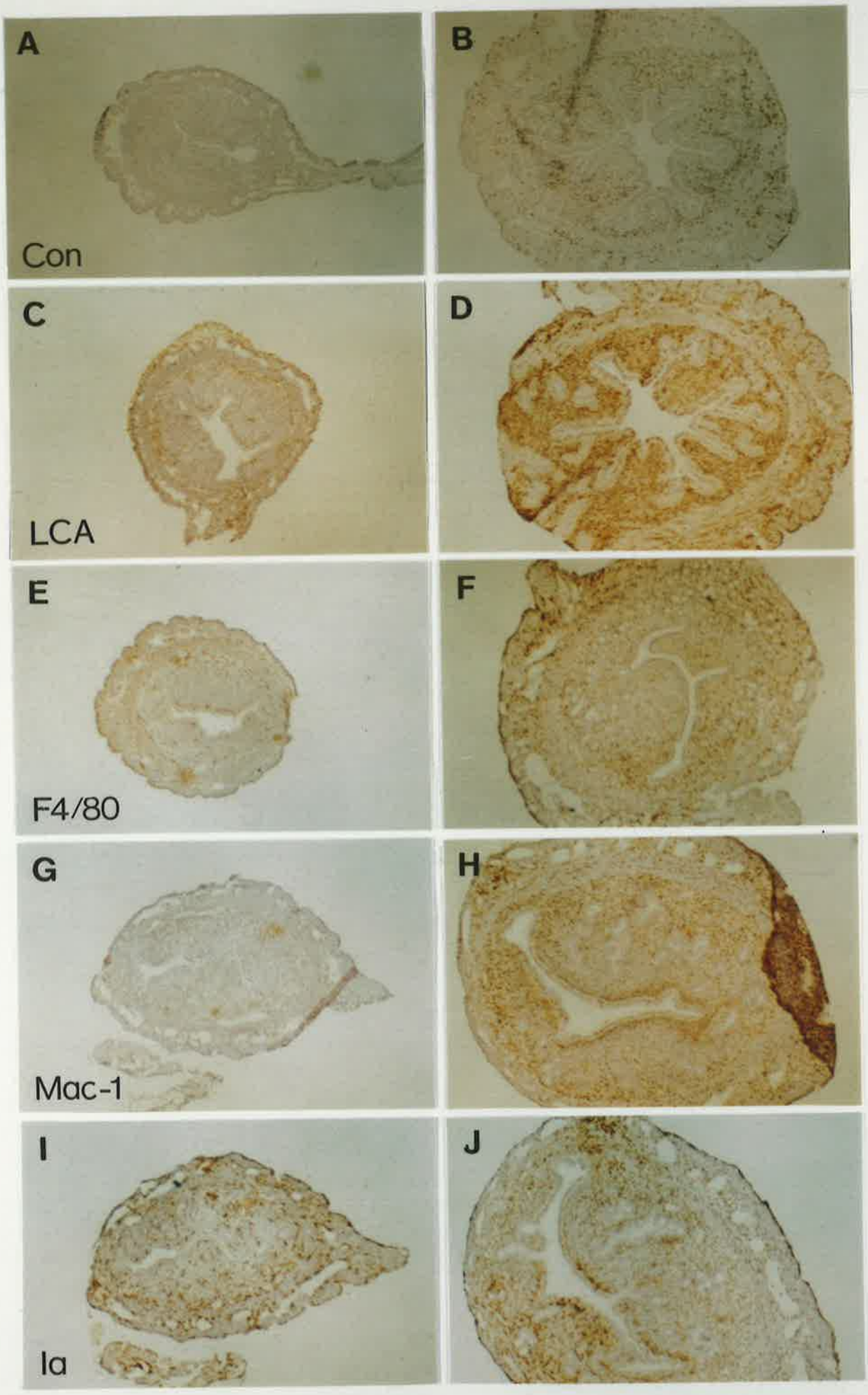
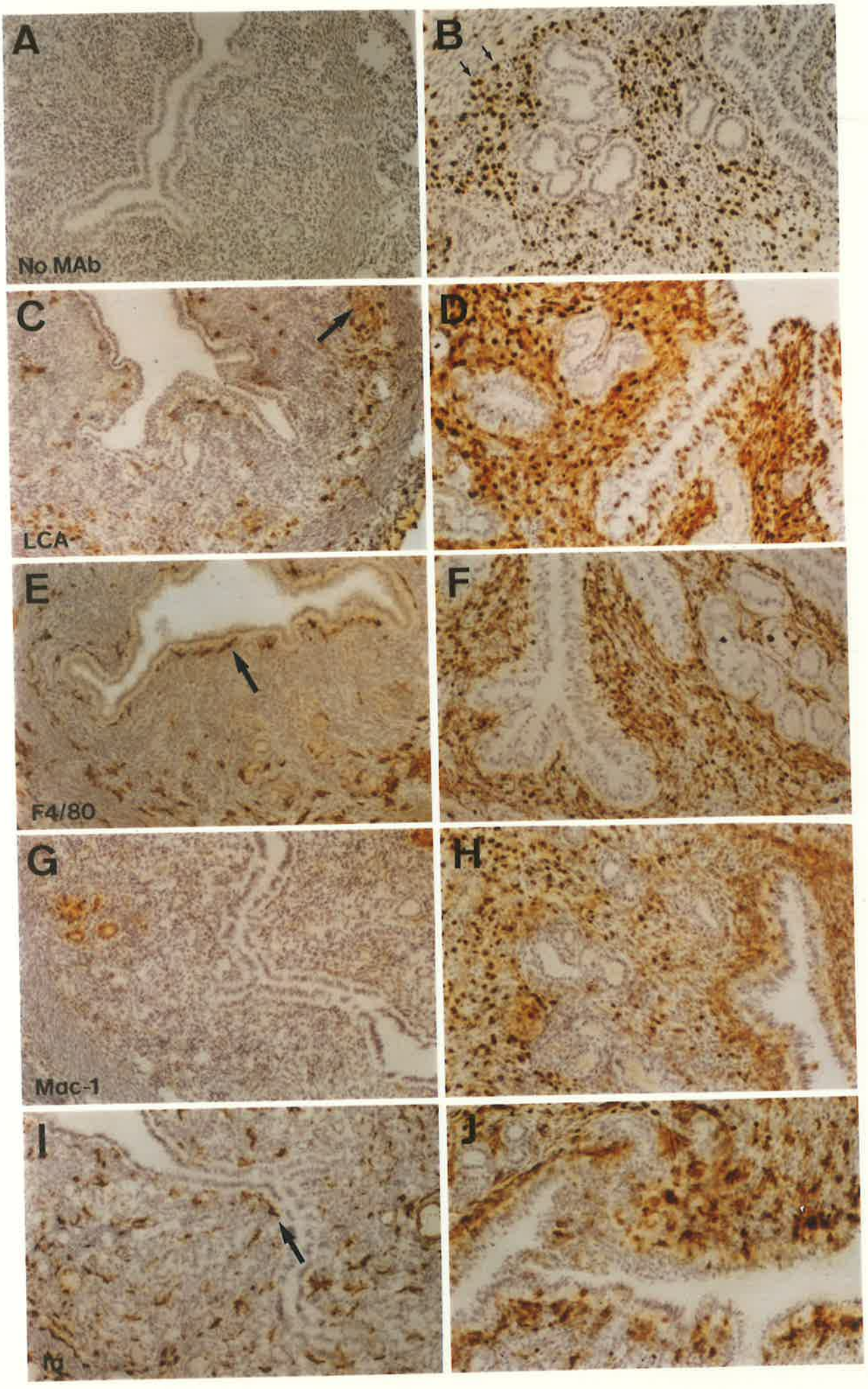

A
Con
B
C
LCA
D
E
F4/80
F
G
Mac-1
H
I
Ia
J

Figure 7.5 The effect of exogenous GM-CSF on the numbers of leukocytes and endometrial glands in the ovariectomised uterus (high power). Sections of uterus are from mice that had received intraluminal injections of 200 U of GM-CSF (right hand panel; B, D, F, H and J), or carrier (left hand panel; A, C, E, G and I). The sections were stained with DAB and H_2O_2 to detect eosinophils (A and B), or incubated with mAbs specific for different leukocyte lineages. All leukocytes are detected with mAbs against the pan leukocyte markers CD45/ LCA (C and D). These cells are reactive predominantly with mAbs against macrophage markers [F4/80 (E and F), and Mac-1 (G and H)], or the dendritic cell/ macrophage/ B cell marker Ia (I and J). Endogenous peroxidase positive cells are evident as small, granular, dark brown stained cells (small arrows, B) in all sections, in addition to lighter brown coloured, mAb-reactive cells (large arrows in sections C to H).


A
No MAb
B
C
LCA
D
E
F4/80
F
G
Mac-1
H
I
ng
J

b. Effect on other leukocytes

GM-CSF was also found to influence the numbers of other leukocytes in the uteri of ovariectomised mice, although the effect of GM-CSF on the difference in numbers of mAb-labelled cells between GM-CSF and carrier-treated mice was not as dramatic as the effect on eosinophils. Cells that reacted with leukocyte-specific mAbs were very difficult to quantitate accurately, both because of their density and often dendritic morphology. However, estimates based on the relative densities of mAb staining were made of the numbers of LCA/ CD45$^+$, F4/80$^+$, Mac-1$^+$, and Ia$^+$ cells in the endometrium and myometrium of uteri from mice treated with GM-CSF, or control mice (Table 7.2).

Table 7.2 Effect of intraluminal GM-CSF on the numbers of uterine cells bearing leukocyte, macrophage and dendritic cell-specific markers. Sections were prepared from GM-CSF and carrier-treated mice as described in the legend to Table 7.1, and incubated with mAbs against CD45/ LCA, F4/80, Mac-1 and Ia. The numbers of cells reactive with each antibody were estimated, and qualitative scores are given for sections of uteri from mice in experiment 1.

Treatment	CD45/ LCA	F4/80	Mac-1	Ia
ENDOMETRIUM				
carrier	++	+	+	++
200 U	++++	+++	+++	+++
1000U	++	+	+	++
MYOMETRIUM				
carrier	+	+ / -	+	+
200 U	++	++ / +	++	++
1000U	+	+	+	+

Uteri from carrier-treated ovariectomised mice contained moderate numbers of cells that reacted with mAbs against F4/80, Mac-1 and Ia (Figs 7.4 and 7.5). These cells were comprised primarily of cells with a dendritic morphology and since approximately equal numbers of cells were positive for LCA/ CD45 and for each of these three mAbs, they were presumed to be predominantly dendritic cells and/ or macrophages. Intraluminal instillation of GM-CSF clearly increased the numbers of F4/80$^+$ and Mac-1$^+$ cells, and whilst the numbers of Ia$^+$ cells increased, the difference was less striking than for the other mAbs (Figs 7.4 and 7.5). Uteri from mice classified as 'responder' mice for eosinophils were found generally to also have greater numbers of cells reactive with F4/80, Mac-1 and Ia than control mice. F4/80$^+$ and Ia$^+$ cells were distributed throughout the stroma but were most concentrated in areas underlying the epithelium in both treated and untreated mice. In some but not all treated mice, the density of cells stained with these mAbs was very much greater in the endometrium than in the myometrium. Small round cells that were Mac-1$^+$ but F4/80$^-$ (and so

presumed to be neutrophils) were found between epithelial cells and appeared to be in the process of trafficking into the luminal cavity in GM-CSF-treated mice. Intraluminal GM-CSF appeared, therefore, to induce an infiltration into the endometrium of macrophages and/ or dendritic cells and neutrophils, in addition to eosinophils.

7.4.2 Effect of GM-CSF on endometrial growth

An unexpected but interesting finding was that the uteri from mice that had received GM-CSF were larger and contained a greater number of endometrial glands (Table 7.3) (Figs 7.4 and 7.5).

Statistical analysis by ANOVA showed that there was a dose-dependant effect of GM-CSF on both the number of endometrial glands and the diameter of the uterus in experiment 1 ($p = 0.0001$). There was a four-fold increase in the mean number of glands ($p = 0.0001$), and a 70% increase in the mean uterine diameter ($p = 0.0001$) after intraluminal injection of 200 U of GM-CSF. However, 1000 U of GM-CSF did not alter the number of glands significantly, and the mean diameter of uteri in this group was less than in control mice ($p = 0.007$). Administration of GM-CSF was also accompanied by an increase in both the number of glands and the diameter of the uterus in experiment 2. However, because only 5 of 12 mice responded to the treatment the effect was not significant ($p = 0.106$ and $p = 0.108$ respectively). Endometrial glands also tended to be up to 10-fold larger in diameter in GM-CSF treated mice (Fig 7.5).

Table 7.3 Effect of intraluminal GM-CSF on the number of endometrial glands and uterine diameter. Sections were prepared from GM-CSF and carrier-treated mice as described in the legend to Fig. 7.1. The total number of endometrial glands were counted in two sections from each horn within three grid areas of 0.36 mm^2. There was no significant difference between the numbers of endometrial glands in tissue from the left and right uterine horns. Data are expressed as the mean $\pm$ SD number of endometrial glands (per 4.3 mm^2) in each treatment group. The diameter of each uterus was calculated by using the microscope graticule to measure the diameter of transverse sections. $^{*}p = 0.106$; $^{**}p = 0.108$; $^{***}p = 0.0001$;

Treatment	n	Glands	Diameter
Expt 1			
carrier	3	39 ± 5	1.2 ± 0.05
200 U	4	$168 \pm 29^{***}$	$2.0 \pm 0.08^{***}$
1000 U	4	24 ± 17	0.9 ± 0.08
Expt 2			
carrier	4	$12 \pm 5^{*}$	$0.8 \pm 0.1^{**}$
40 U	4	38 ± 39	1.0 ± 0.3
200 U	4	77 ± 53	1.0 ± 0.2
1000 U	4	66 ± 54	1.1 ± 0.3

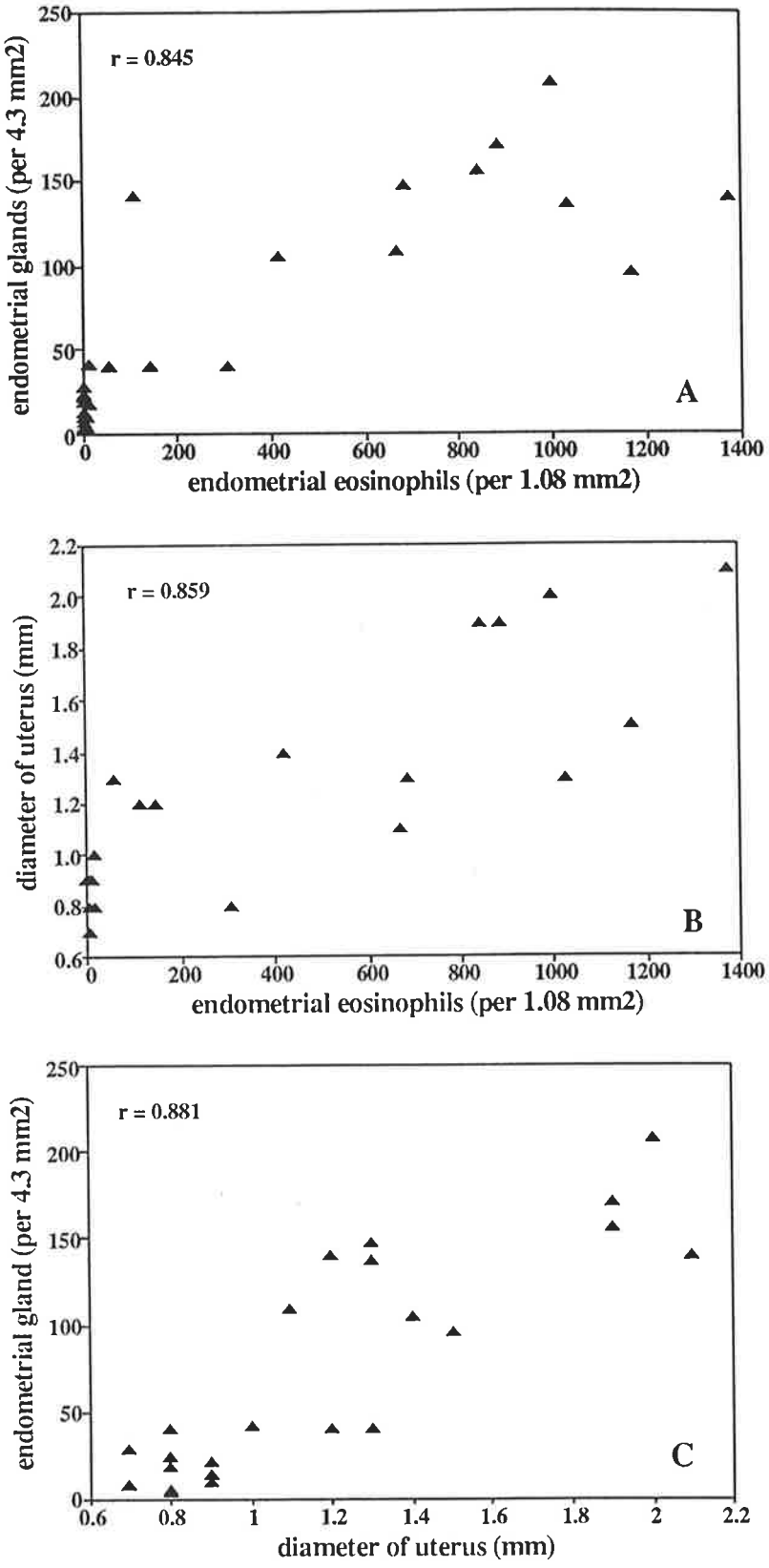


Figure 7.6 The correlation between the number of eosinophils and the number of glands in the endometrium, and the diameter of the uterus. The data from each of two experiments are pooled. (A) The correlation between the number of eosinophils and the number of glands in the endometrium (Pearson Correlation Coefficient r = 0.845, p = 0.0001). (B) The correlation between the number of eosinophils and the diameter of the uterus (r = 0.859, p = 0.0001). (C) The correlation between the diameter of the uterus and the number of endometrial glands (r = 0.881, p = 0.0001).

There was a significant correlation between the number of eosinophils in the endometrium, and both the number of glands (Pearson Correlation Coefficient $r = 0.845$, $p = 0.0001$) and the diameter of the uterus ($r = 0.859$, $p = 0.0001$) (Fig 7.5). The highest values for both measures of uterine growth were obtained in mice that were classified as 'responders' on the basis of their endometrial eosinophil numbers. These data suggest that GM-CSF can influence the proliferation or activity of epithelial cells and possibly other non-hemopoietic cells in the uterus, although whether this effect is direct or mediated by GM-CSF-responsive leukocytes is not clear.

7.4.3 Passage of fluid between uterine horns in ovariectomised mice

That there were no significant differences between the left and right uterine horns in the numbers of leukocytes or endometrial glands was a surprising aspect of the experiments described in 7.4.1 and 7.4.2. One interpretation of this finding is that GM-CSF injected into the uterine horn has systemic as well as local effects. Alternatively, GM-CSF may have flowed across the cervix and into the contralateral horn. To investigate whether fluid can pass between uterine horns, six ovariectomised mice were given intraluminal injections of ^{3}H-inulin (10 μCi) in 50 μl of 1% BSA. Pairs of mice were sacrificed 1 h, 3 h and 6 h later. The left and right horns of the uteri were each cut into three sections (of approximately equal weights) and solubilised, and the ^{3}H-inulin content of each segment was determined by liquid scintillation counting. This result indicates that ^{3}H-inulin had become evenly distributed across the entire uterus within 1 h after injection. These data indicate that, after unilateral injection, the rGM-CSF contents of the contralateral and ipsilateral uterine horns would have been similar in the experiments described above, and suggests that the actions of GM-CSF observed in both horns were due to local spread of the mediator.

7.5 Conclusions and discussion

The experiments described in this chapter identify specific populations of cells within the uterus that are targets for the action of GM-CSF. The results suggest that GM-CSF may have a significant physiological role in the remodelling processes that occur in this tissue. Exogenous intraluminal GM-CSF in the luminal cavity of mice that are functionally GM-CSF-deficient has been shown to induce an infiltration of myeloid leukocytes, and an increase in the both number of endometrial glands and the overall size of the uterus.

In inflammatory sites, GM-CSF acts characteristically as a regulator of the recruitment and activation of leukocytes of myeloid lineages, including macrophages, neutrophils and eosinophils. It has also been implicated as a regulator of the differentiation and survival of dendritic cells in epithelial tissues. A number of researchers have shown that the cycling and early pregnant uterus is heavily populated with cells of these lineages. These findings were confirmed in this study, where mAbs against various leukocyte lineages identified large numbers of macrophages, dendritic cells, eosinophils and neutrophils, but very few T-lymphocytes, in the uterus one day after mating.

Transcripts for both the α and β components of the GM-CSF receptor were detected by RT-PCR in uterine tissue at oestrus and during the pre-implantation period. GM-CSF-R expression

appeared to be greater following mating than in the oestrous uterus, and this might reflect the infiltration of GM-CSF receptor-bearing cells that occurs at this time. An unidentified 450 bp product amplified from uterine cDNAs by the GM-CSF-R primers may be the product of an alternatively spliced GM-CSF-R transcript. There is a precedent for this in the human, where alternatively spliced mRNAs can give rise to two membrane-bound forms (α1 and α2) and the soluble form of the receptor (1.7.1e). However, the primers used in these experiments span a membrane-proximal 235 bp region of the extracellular cDNA that is 5' of the position that is equivalent to the splice point for the soluble and α2 forms of the human receptor, and so would not be expected to span regions that would be different in murine counterparts of the known human isoforms. The aberrant band was unlikely to be the product of contamination of the uterine RNA extracts with genomic DNA, since the RNAs were treated with DNAse prior to reverse transcription. Whether the GM-CSF-R primers span an intron is not known, since the sequence of the murine GM-CSF-R gene has not yet been reported (Genebank and EMBL databases; May 1993).

Cells expressing GM-CSF receptor in the day 1 uterus were localised using ^{125}I-GM-CSF. These cells were judged to be leukocytes on the basis of their distribution throughout the endometrium and myometrium, and predominant localisation to sub-epithelial areas of the endometrial stroma. The precise identification of GM-CSF receptor-bearing cells awaits double-labelling experiments, in which uterine sections would be labelled with leukocyte-specific mAbs prior to ^{125}I-GM-CSF binding and autoradiography.

Evidence that GM-CSF can influence the behaviour of uterine leukocytes was obtained in experiments where rGM-CSF was injected into the uterine lumen of ovariectomised mice. Doses of GM-CSF of 40 U, 200 U or 1000 U per uterus were injected unilaterally. These amounts were considered to cover a broad physiological range, since although 20 U/ uterus is an average value for the GM-CSF content of a uterus on the day after mating, it is likely that a concentration gradient radiates from the epithelium in the physiological state, and that concentrations in the vicinity of production reach much higher levels.

The most striking outcome of administering GM-CSF was an infiltration of leukocytes, predominantly of the eosinophilic lineage but including macrophage. This occurred in both the ipsilateral and contralateral uterine tissues. The subsequent finding that radiolabelled material injected into one uterine horn became evenly distributed across both horns within hours of injection suggested that the action of GM-CSF in the contralateral horn was local, rather than systemic. However, the response of mice to GM-CSF was variable, with some mice responding clearly and others not. The basis for this variability was difficult to determine given the relatively small size of the experimental groups, but may have been influenced by factors including the dose and the technical efficacy of delivery of cytokine into the uterine lumen. In one of the two experiments, the effect of GM-CSF on the numbers of uterine eosinophils was found to be dose dependant, with 200 U of GM-CSF clearly having a greater effect than 1000 U. The reason for the inhibitory effect of the 1000 U dose is not clear, but may be related to receptor down modulation in responsive cells. Alternatively, it is possible that the high GM-CSF concentration caused recruited eosinophils to degranulate and thereby avoid detection. In the second experiment a 40 U dose of GM-CSF was seen to influence

leukocyte numbers in only one of four mice, suggesting that 40 U may be bordering on the lower threshold for an effective dose. It is also possible that some mice failed to receive an effective treatment as a result of a less than 100% rate of success in delivering cytokine into the extremely small diameter luminal space. In future experiments a small amount of radioactive marker or dye stuff will be included with the carrier, and will aid in following the fate of the injected material.

An approximately 10-fold difference between the mean eosinophil content of the control groups for the two experiments also complicated the interpretation of these experiments. This was potentially the result of different lag times between ovariectomy and the initiation of the two experiments. Although this period was at least two weeks, which is sufficient for circulating oestrogen to be removed, some residual eosinophils may have still been present in the uterine tissue. In view of these complications, it can be said in summary that whilst these data provide interesting pilot information, conclusive findings await further studies. Future experiments would include a careful assessment of the rate of decline in endometrial eosinophil numbers following ovariectomy.

The mechanism by which GM-CSF controls the extravasation of leukocytes and their subsequent migration through the endometrial stroma remains to be elucidated. GM-CSF is known to have chemotactic properties for macrophages and granulocytes, and it is possible that this factor may also alter CAM expression by local endothelial cells directly. Alternatively, the few macrophages that are resident within the ovariectomised uterus may act to amplify the recruiting activities of GM-CSF, possibly by releasing other endothelial cell-active factors such as TNFα.

These preliminary experiments suggest that GM-CSF may have a physiological role in regulating the trafficking of uterine leukocytes. The peak in the uterine content of GM-CSF following exposure to seminal factors at mating is accompanied by a large influx of macrophages and neutrophils. However since epithelial cells from the non-pregnant and midgestation uterus also synthesise GM-CSF, this factor may also influence leukocyte numbers and behaviour at these times. Indeed, these data together with the finding that oestrogen controls GM-CSF release, suggest that GM-CSF could have a role in mediating the oestrous cycle-related changes in eosinophil numbers (which are reported to increase 50-fold throughout the cycle), and/ or the effects of steroid hormones on macrophage number and distribution in the uterus. Epithelial cells in the rat uterus synthesise a 20, 000 Mr protein with chemotactic activity for eosinophils (Lee *et al.* 1989). Like GM-CSF, this factor is synthesised in response to oestrogen and inhibited by progesterone , but whether it is related to GM-CSF is unknown.

It is unlikely that GM-CSF is the only factor that promotes leukocyte trafficking into the uterus, and a role for CSF-1 in recruiting macrophages has been demonstrated in ovariectomised mice (Wood *et al.* 1992). Further evidence that CSF-1 regulates uterine macrophage populations is provided by studies in the CSF-1 deficient *op/op* mouse. Macrophages are not detected in the uteri of virgin *op/op* mice but do accumulate in the uterus during early pregnancy when numbers approach those found in the heterozygote controls on day 7 and 8. However, these macrophages exhibit an uncharacteristically rounded morphology (and hence presumably less differentiated phenotype), fail to persist and are undetectable by day 14. That uterine macrophages are present at all in early *op/op* gestation indicates that other recruitment and activation factors (potentially GM-CSF and/ or TNF)

also operate during pregnancy. Similarly, IL-5 and IL-8 are likely to have important roles in eosinophil and neutrophil trafficking respectively, but these factors have not yet been studied in the murine uterus.

An unexpected but intriguing outcome of local administration of GM-CSF was an increase in the number and size of endometrial glands, as well as an increase in the overall size of the uterus. Although additional experiments are needed to investigate the mitotic activity of epithelial cells after exposure to GM-CSF, the data suggest that GM-CSF may act to stimulate their proliferation, thereby promoting gland formation. Since there was no evidence for GM-CSF receptor expression by epithelial cells, it is likely that GM-CSF acts indirectly in this respect, and the GM-CSF responsive endometrial leukocytes are thereby implicated as mediators. Macrophages can produce a variety of epithelial cell-active growth factors (Rappolee and Werb 1992), but since macrophage-deficient *op/op* mice are not reported to have abnormal endometrial architecture, it may be speculated that eosinophils are the source of factors that are active in the endometrium. This contention is supported both by the finding in this study that eosinophil and uterine gland numbers are strongly correlated in GM-CSF-treated ovariectomised mice, and the temporal relationship between eosinophilia and the extent of the glandular network during the normal oestrous cycle. Eosinophils are a potent source of TGFα (Wong *et al.* 1990), and this factor is implicated as a regulator of oestrogen-induced uterine growth. TGFα mRNA expression in the mouse uterus is induced by oestrogen, and anti-TGFα antibody blocks uterine growth *in vivo* (Nelson *et al.* 1992). A model in which the levels of eosinophils are perturbed [for example the IL-5 transgenic mouse (Dent *et al.* 1990)] would be of value in the further investigation of this hypothesis. However, other cellular sources of epithelial growth factors also require investigation. Future experiments aimed at detailing the temporal sequence of the consequences of GM-CSF administration, including measuring the levels and cellular origins of factors that have been implicated in epithelial cell growth (such as TGFβ, EGF, IGF, and PDGF), should help clarify these issues.

8 General discussion and conclusions

Granulocyte-macrophage colony stimulating factor (GM-CSF) has been identified as a major product of luminal and glandular epithelial cells in the murine uterus. Steroid hormones, and other factors including a component of seminal plasma regulate the release of GM-CSF during the oestrous cycle and during pregnancy.

These studies have helped to establish cytokine secretion as an unanticipated, but potentially very important function of the uterine epithelium in reproductive physiology. The family of uterine cytokines now known to be primarily derived from epithelial cells includes GM-CSF, IL-6, CSF-1, TNFα, LIF, and TGFβ1. Epithelial cells are ideally suited to have a coordinating role, through the selective secretion of this armoury of mediators, in the remodelling events that are characteristic of reproductive processes. This activity would be facilitated by their position at the boundary between the uterine cavity and the maternal milieu. Rapid and dramatic changes in uterine cytology are particularly evident during the period between mating and implantation, as the endometrium prepares to accommodate the blastocyst. GM-CSF and IL-6 are likely to be of special importance during this period, when a striking increase in their secretion is initiated within hours after mating by specific factors in the ejaculate. Both the embryo and uterine leukocytes have been shown to be targets for GM-CSF action, suggesting that this factor may be an important component of a cytokine circuit interlinking the resident and hemopoietic cells in the uterus with the developing conceptus.

8.1 Epithelial cell cytokines as mediators of steroid actions in the uterus

Individual cytokines are released by epithelial cells in different temporal patterns, indicating that there are specific mechanisms for the control of their synthesis. Ovarian steroid hormones are known to be principal regulators of the proliferation and function of uterine cells, and the secretion of cytokines and growth factors by epithelial cells has also been found to be steroid-dependant. The current study identifies the importance of oestrogen in priming epithelial cells for GM-CSF release. GM-CSF together with other epithelial cytokines may therefore be amongst the local mediators of steroid hormone action in uterine tissue. The precise mechanism through which steroids influence cytokine synthesis is not clear. Whether steroid responsive elements are directly involved in transcriptional control of cytokine genes, or whether regulation is effected through the paracrine or juxtacrine activities of neighbouring steroid-responsive cells, remains to be determined.

8.2 GM-CSF as a component of an inflammatory cascade in the preimplantation uterus

The GM-CSF and IL-6 contents of uterine luminal fluid increase dramatically within hours after mating, apparently as a consequence of the action on the epithelium of a specific, seminal vesicle-derived component of seminal plasma. For GM-CSF, this increase is (at least in part) the result of enhanced expression of the gene. Seminal plasma is already identified as having an

immunomodulatory role in reproductive processes, and these observations indicate a further dimension in the action of this complex fluid in initiating a local inflammatory response in the preimplantation uterus. The ensuing cascade of events is characterised by a dramatic infiltration of the underlying uterine stroma and the uterine lumen with inflammatory leukocytes, followed by their activation for enhanced cytokine release.

The full physiological significance of the post-mating inflammatory response remains to be elucidated. Infiltrating leukocytes would certainly have a 'housekeeping' role in clearing the uterus of sperm and micro-organisms introduced at mating, and LPS associated with bacterial contaminants of the ejaculate would provide an additional stimulus to enhance cytokine release. Phagocytosis of sperm and seminal debris by macrophages in the luminal cavity, and endocytosis of luminal fluid by epithelial cells and presumably dendritic cells in the endometrium, would facilitate maternal immunisation against paternal antigens.

However a more subtle outcome of the post-mating inflammatory response is potentially of greater importance for pregnancy outcome. It is speculated that the cascade of events initiated by the ejaculate has repercussions that help to generate a 'receptive' endometrial microenvironment. These influences are likely to be mediated directly or indirectly through alterations in the numbers or behaviour of local leukocyte populations, increased local angiogenic activity, or even changes in the composition or growth factor content of the local extracellular matrix. The latter may be of particular importance for the subsequent attachment and outgrowth of the embryo.

Support for a physiologically important role for seminal plasma at mating is obtained from studies indicative of reduced success rates in pregnancies initiated in the absence of exposure to seminal factors, and the finding that 'pre-sensitisation' of the uterus to seminal factors can contribute to pregnancy success in rodents and humans (1.2.2). Although it is abundantly clear that pregnancy can proceed in the absence of seminal factors, this does not exclude a permissive role, as opposed to an obligatory requirement, for seminal factors in optimising fecundity. The importance of exposure to seminal fluid may be heightened where the capacity of the uterus to mount an inflammatory response is undeveloped or otherwise compromised: for example, deficiency in a CSF-1 regulated seminal factor has been suggested as a potential explanation for the difference in the degree of infertility of CSF-1 deficient *op/op* females mated with homozygote compared to heterozygote males. The superovulated mouse, in which mating fails to enhance GM-CSF release (presumably as a result of perturbed endocrine parameters; 4.2.2) and implantation failure is common (1.2.3a), may provide a useful model for the further exploration of this phenomenon.

8.3 Epithelial cytokines as coordinators of endometrial leukocyte activity

This study provides evidence that endometrial leukocytes including macrophages, eosinophils and neutrophils may be responsive to local GM-CSF gradients originating at the luminal surface. Recombinant GM-CSF instilled into the uterine lumen of ovariectomised mice was found to cause a striking infiltration of these cells into the subepithelial stroma (7.4.1). These findings suggest that GM-CSF produced by the epithelium may have a physiological role in regulating the recruitment of

leukocytes into the endometrium during the reproductive cycle. Such a role would explain the spatial relationship that is observed between macrophages and epithelial cells in the cycling uterus and throughout gestation (1.5.2).

A common feature of each of the cytokines secreted by uterine epithelial cells is their ability to influence the differentiation, activation or trafficking of myeloid leukocytes, in various and in some instances opposing manners (1.7). The effects of GM-CSF on its target cells would therefore be in the context of the other cytokines liberated by the epithelium. To use the analogy of Sporn and Roberts (1988), GM-CSF would be only one of many 'symbols' in the cytokine 'alphabet' employed by epithelial cells. GM-CSF could therefore be a component of a diverse array of 'messages' generated by epithelial cells for communicating with adjacent endometrial cells, depending upon the other 'symbols', or cytokines, with which it is released. The profile of cytokines that epithelial cells secrete at a given time would be expected to be determined principally by steroid hormones, but could presumably also be influenced by many other factors such as seminal fluid, LPS and IFNγ. This would provide a remarkably adaptable system for the co-ordination by epithelial cells of endometrial leukocyte activity.

The activities that the epithelium might elicit in its targets cells are many and varied. Macrophages are extraordinarily flexible cells with a broad spectrum of activities including secretion of a vast array of cytokines and other mediators, in a microenvironment-specific manner. Neutrophils and eosinophils are also potential sources of a range of factors including plasminogen activator, collagenase, histamine, PAF and prostaglandins which have all been implicated in implantation and induction of the decidual response. In addition, an exciting preliminary finding in this study was that growth factors derived from macrophages and eosinophils might also be important in endometrial growth during the oestrous cycle. GM-CSF-responsive leukocytes are therefore prime candidates for major effector cells in the pre-implantation uterus, with potentially critical roles in uterine remodelling processes.

8.4 The effect of endometrial cytokines on local immune activity

The survival of the conceptus from the time of its exposure to maternal tissues at implantation is dependant upon its ability to evade rejection by the maternal immune system. However, the suppression of T-lymphocyte and NK cell activities at the maternal-fetal interface normally prevents the generation and execution of destructive immune responses. The activities of these immunocompetent cells would be expected to be linked into cytokine circuits within the uterus. Epithelial cell cytokines might influence the function of lymphocytes indirectly through their regulation of the activities of local macrophages (Fig 8.1). For example, GM-CSF and TGFβ can act to 'dampen' the immuno-accessory functions of macrophages, through stimulating IL-1 inhibitor and PGE release. These factors may be important as inhibitors of the activities of T-lymphocyte and NK cells, and so may help to maintain a local immunological environment that is favourable to accommodation of the semi-alloantigenic conceptus.

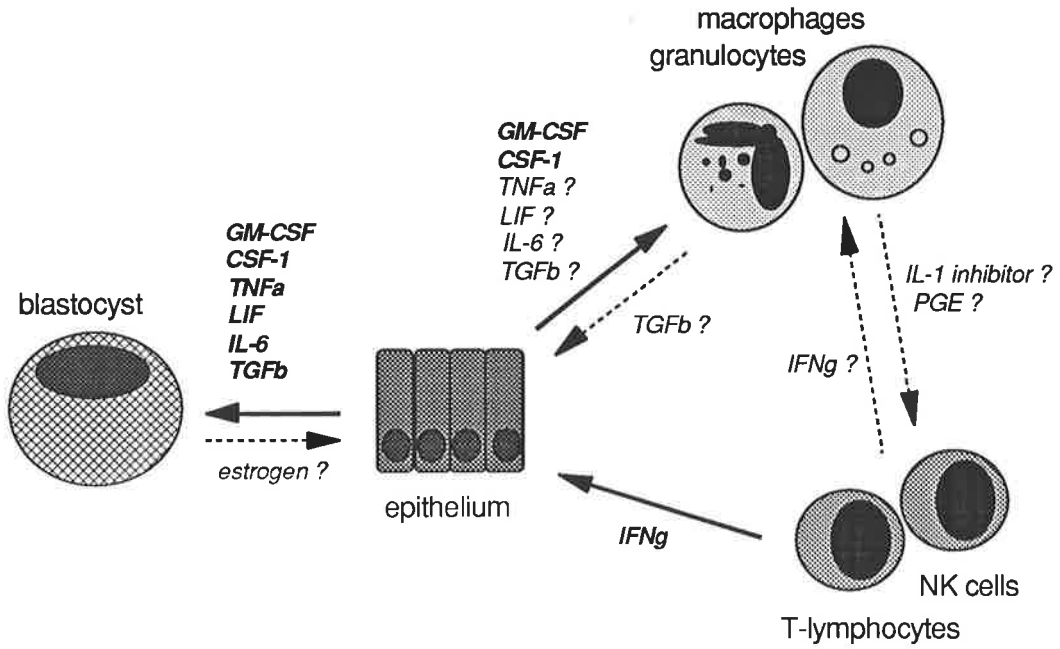


Figure 8.1 Cytokine-mediated interaction between epithelial cells, endometrial leukocytes, and the embryo. Endometrial macrophage and granulocyte populations, together with the embryo, appear to be the predominant targets for the action of cytokines liberated by the epithelium in the pre- and peri-implantation uterus. However, the phenotypes and activation states of local T-lymphocytes and NK-cells may impact indirectly upon the growth and development of the embryo, through cytokine-mediated interactions between these cells, macrophages and the epithelium. Evidence from this study and from other reports suggest that the pathways identified with solid arrows and bold print operate in the endometrium. Dotted arrows and question marks identify pathways for which there is supporting evidence in other tissues, and/ or circumstantial evidence in the endometrium.

However, immunocompetent cells can also be viewed as 'monitors' of the extent or rate of invasion of maternal tissue by the embryonic 'graft', and appear to be poised to swing into action when circumstances warrant their intercession. A mechanism based on a balance between passive and active states of immunocompetent cells might explain the dramatic impact of perturbing T-lymphocyte immune parameters in pregnant mice, including the deleterious effects of *in vivo* treatment with anti-'ı' cell antibodies during pregnancy, and the beneficial effects of non-specific immunopotentiating agents in abortion-prone mice. Conversely, this study shows that factors released by activated T-lymphocytes and NK cells (such as IFNγ) can influence the cytokine-secreting profile of epithelial cells. This suggests that cytokine networks might also provide a mechanism whereby an 'inappropriate' immune response in pregnancy could have detrimental effects on embryo development or placentation through blocking the release of trophic factors from the epithelium (Fig 8.1). Like macrophages, lymphocytes release cytokines and other mediators in patterns that are dependant upon their phenotype and activation state. Therefore, it would be of interest to search for other products of

these cells that could have positive effects on conceptus development through an epithelial cell cytokine-mediated pathway.

8.5 The embryotrophic actions of GM-CSF

The embryo expresses receptor for and/or exhibits an *in vitro* biological response to many of the cytokines liberated by the uterine epithelium (1.8.1). GM-CSF has been shown to have effects on the growth and function of placental trophoblast cells (1.8.1), and the current studies indicate that it may also influence development of the embryo during pre-implantation stages. There is evidence that cytokines released by the epithelium can have both positive and negative effects on embryonic survival and development, and so these factors may contribute to generating both receptive and hostile uterine environments. The different temporal patterns of epithelial cytokine synthesis are likely to target different stages of early conceptus growth, and gradients in soluble and/ or cell-associated forms may be determinants in the spatial orientation of blastocyst implantation and trophoblast invasion.

It will be of great interest to determine whether factors released by the conceptus can influence local epithelial cell cytokine release. Altered cytokine release from the epithelium would be a mechanism well suited to initiating the cascade of local and systemic changes in maternal tissues that is triggered by signals released from the blastocyst at implantation (see below).

8.6 Other potential roles for epithelial cell cytokines in the preimplantation uterus

There is a growing recognition that cytokines may also influence uterine function through their actions on non-hemopoietic target cells. *In vitro*, GM-CSF, TNFα, TGFβ and IL-6 all alter endothelial cell activities in a manner consistent with their having a role in the neovascularisation that occurs in the endometrium during the earliest stages of blastocyst implantation (1.8.4).

Cytokines might also be involved in the role of the epithelium in transmitting the embryo-derived signal for differentiation of local stromal fibroblasts into decidual cells. Support for this view comes from findings that an intact epithelium is necessary for decidualisation, and that a decidual reaction is evoked in psuedopregnant mice when inflammatory cytokines are injected into the uterine lumen (1.8.2). It is possible that cytokine release provides the point of convergence in the sequence of events, leading to decidualisation, that can be initiated by the embryo and by a range of artificial stimulants. Such a mechanism might be analogous to the manner in which inflammatory responses in the skin, which can be elicited by a variety of physical and chemical insults, are all initiated with IL-1 production by keratinocytes (Kupper 1990). CSF-1 is implicated in this capacity, since its release from the epithelium has been found to increase in response to decidual stimuli, and decidualised fibroblast cells in the uterine stroma express receptors for CSF-1. It would be of interest to examine the effect of decidual stimuli on synthesis of other cytokines known to be released by the epithelium. These considerations do not exclude the possibility that endometrial leukocytes also have a role in decidualisation, potentially by amplifying epithelial signals through the release of factors such as PAF, PGE_2 and/ or histamine (1.2.3c).

8.7 GM-CSF as a component of a cytokine network during early pregnancy

These findings and speculations can be integrated into a model for the role of GM-CSF in early pregnancy (Fig 8.2). In this model, GM-CSF (acting in the context of other epithelial cell cytokines) is identified as a key component in a sequence of cytokine and leukocyte-mediated events that are necessary for the establishment and viability of a pregnancy. Preparation of the uterus for implantation begins during the oestrous cycle, when oestrogen-regulated release of chemotactic factors (including GM-CSF) from the epithelium causes macrophages and eosinophils to be recruited into the endometrium. Factors released by these cells may help to promote the proliferation and differentiation of resident epithelial and stromal cells. At mating, an inflammatory response is initiated by the interaction of seminal factors with oestrogen-primed epithelial cells to promote the release of GM-CSF and IL-6. This surge of cytokine activity acts to orchestrate the further recruitment and activation of macrophages, neutrophils and eosinophils in the endometrium. The behaviour of these leukocytes would continue to be responsive to the shifting profile of cytokines released from the epithelium during the first few days of pregnancy. It is proposed that a complex cytokine network interlinking each of the hemopoietic and resident uterine lineages is established, and that this forms the basis for generating an environment that is receptive to implantation of the embryo. This network expands to include the blastocyst, as apposition and attachment phases of implantation proceed. In particular, macrophages are proposed to liberate proteolytic enzymes and arachadonic acid metabolites that would be important in the angiogenesis and tissue remodelling that accompanies decidualisation. As implantation proceeds, other factors released by macrophages would act to help prevent the generation of destructive T-lymphocyte and NK-cell mediated immune responses to placental alloantigens. However, in the event of excessive trophoblast cell proliferation or invasion into maternal tissues, the network might be tipped toward permitting local NK cells, and possibly T-lymphocytes, to limit their further growth. Cytotoxic and cytostatic cytokines such as TNFα or IFNγ could also participate directly or indirectly in this process. The delicate balance in this equilibrium would be expected to be sensitive to natural or experimentally-induced perturbations in the levels of individual network components, and this may lead in some circumstances to compromised implantation and placental development.

8.8 Implications for the human

The extent to which the findings of this study will prove to be common to other species is difficult to predict. The uterine epithelium has been identified as a source of many of the same cytokines in several species including in humans, but the significance of specific cytokines may be quite different. There are also likely to be major differences in the physiology of interactions between seminal factors and maternal tissues. For example, in humans and many other mammals the bulk of the seminal material does not pass the cervix, in contrast to rodents where the ejaculate fills the uterine cavity. In intravaginal ejaculators, components of seminal plasma can gain entry to the uterine cavity through association with sperm, and a cervical leukocytosis that may parallel the post-mating inflammatory response of the mouse has been described for many species. However, if seminal

plasma has an impact through cytokine circuits on implantation in humans, as is postulated in the mouse, the connecting pathways would act over a further physical distance and a longer period of time. It is clear that the further investigation of cytokine networks during the preimplantation period in humans, or other species of interest, is required.

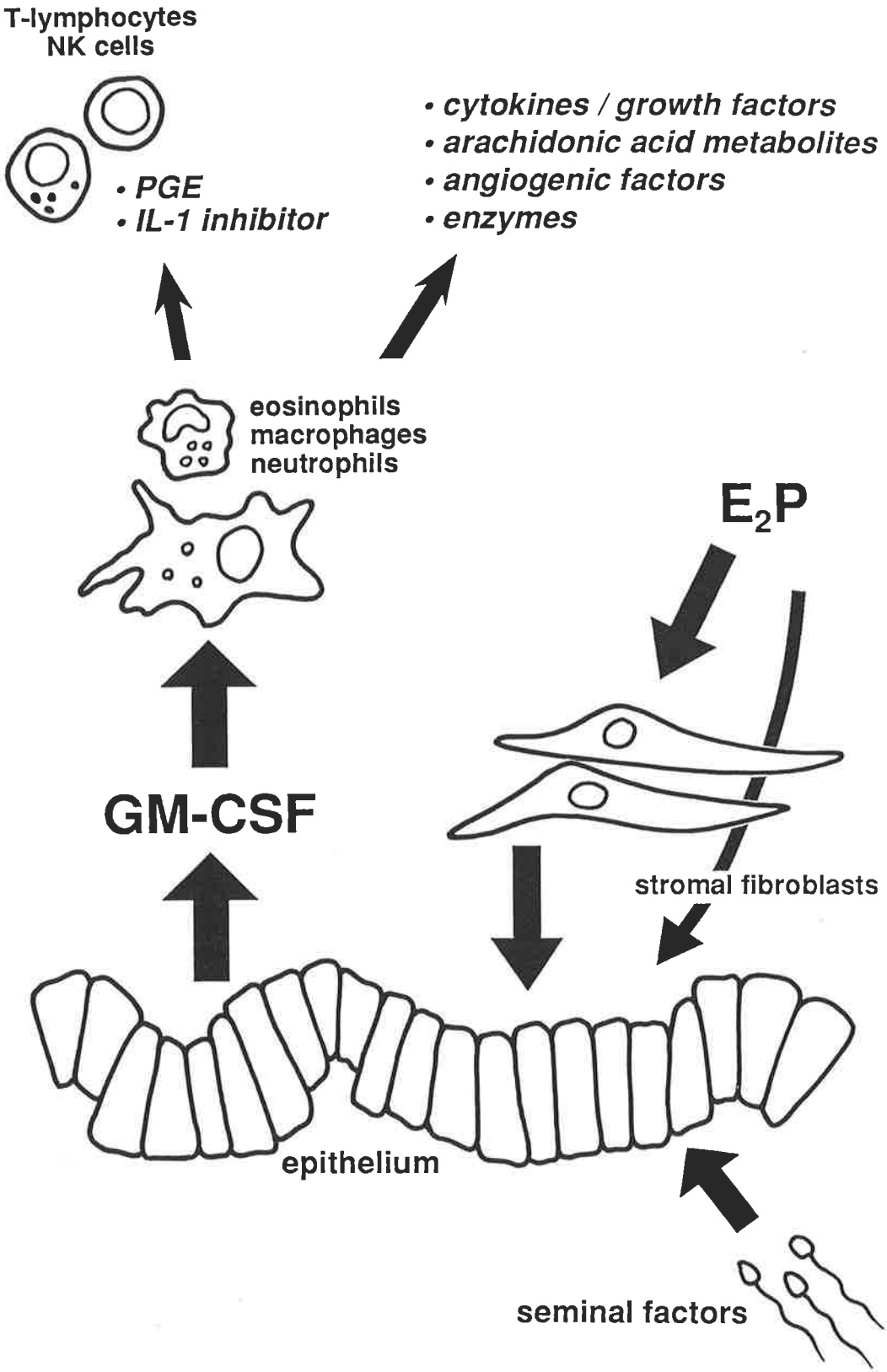
8.9 Future perspectives

The studies described in this thesis identify GM-CSF as a potentially key component of a cytokine circuit operating in the uterus during early pregnancy to facilitate implantation and placentation. Additional studies are obviously required to test the hypothesis that GM-CSF responsive leukocytes have important roles in the uterus. Animal models where uterine macrophage or eosinophil levels are genetically or experimentally perturbed may be enlightening in this regard (Dent *et al.* 1990 , Pollard *et al.* 1991).

In addition, future experiments will be designed to determine the precise physiological significance of GM-CSF in the uterus, and to identify clearly its position in the complex cytokine circuits that operate in the endometrium during early pregnancy. Detailed investigations of the expression and release of the individual cytokines and their receptors during the early stages of pregnancy will help to unravel the causal sequences in individual cytokine-target cell interactions. However, the discovery of soluble cytokine receptors and other natural antagonists, particularly the reported secretion of GM-CSF receptor α-chain by choriocarcinoma cells, promises to add a further dimension to the complexity of these networks.

Evidence that CSF-1 and LIF are critical components during implantation and placentation has been provided by mutant mouse models, and the further investigation of the role of GM-CSF will benefit from the recently generated GM-CSF deficient mouse (A. Dunn, personal communication). Murine counterparts of synthetic GM-CSF receptor agonists (A. Lopez, personal communication) or anti-cytokine antibodies which can be used to block cytokine action *in vivo*, will also facilitate future studies. However, the capacity for interchange and substitution in cytokine function will remain an important constraint in the interpretation of data generated in these models.

Figure 8.2 A model for the role of GM-CSF in establishing receptivity in the pre-implantation uterus. Seminal factors stimulate enhanced release of GM-CSF from oestrogen-primed epithelial cells. GM-CSF initiates an inflammatory cascade during which large numbers of macrophages and granulocytes infiltrate the endometrium and become activated. GM-CSF and other epithelial cell cytokines subsequently coordinate the secretory profile of these cells, during the days leading up to and during implantation of the blastocyst. Growth factors, enzymes and bioactive lipids are macrophage products that are potentially important both in tissue remodelling, and in interacting with local lymphocyte populations to help establish an immunologically receptive uterine environment.


T-lymphocytes
NK cells
• PGE
• IL-1 inhibitor
• cytokines / growth factors
• arachidonic acid metabolites
• angiogenic factors
• enzymes
eosinophils
macrophages
neutrophils
E2P
GM-CSF
stromal fibroblasts
epithelium
seminal factors

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