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The immunoregulatory role of seminal plasma in early murine and human pregnancy

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The desire of knowledge, like the thirst of riches,
increases ever with the acquisition of it.

Laurence Sterne
1713-1768

Abstract

In mice, as in other mammals, deposition of semen in the female reproductive tract provokes local molecular and cellular changes that resemble an inflammatory response. The studies described in this thesis were initiated to investigate two aspects of the post-mating inflammatory cascade. Firstly, the molecular nature of the interaction between semen and cells within the female reproductive tract was characterised. Secondly, the physiological role that the post-mating inflammatory response plays in establishment of an immunological environment conducive to the accommodation of the semi-allogenic conceptus was evaluated.

Previous studies have implicated cytokines produced by the murine uterine epithelium in regulating changes in trafficking and phenotype of endometrial leukocytes after mating. Of particular importance is the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), the synthesis of which is up-regulated at least 20-fold in response to proteinaceous factors in semen originating from the seminal vesicle gland. Fractionation of seminal vesicle gland secretions by size exclusion chromatography identified two moieties which could trigger the release of GM-CSF; an unidentified 650 kDa protein and the cytokine transforming growth factor beta-one ($TGF\beta_1$). Since $TGF\beta_1$ had not previously been identified within murine seminal plasma, the $TGF\beta_1$ content of various male accessory sex glands (seminal vesicle, prostate, coagulating gland) was assessed. This revealed that seminal vesicle secretions contributed more than 90% to the $TGF\beta_1$ content of seminal plasma, consistent with the observation that secretions from this gland, but not other accessory sex glands, were able to initiate an increase in uterine epithelial cell GM-CSF production *in vitro*. Seminal vesicle-derived $TGF\beta_1$ was found to be secreted predominantly in the latent form. However, the murine uterus does appear to have the capacity to release bioactive $TGF\beta_1$ from seminal plasma, since within an hour of mating, the majority of uterine luminal $TGF\beta_1$ is present in the bioactive form.

$TGF\beta_1$, $TGF\beta_2$ and activin, all members of the $TGF\beta$ superfamily, were noted to increase uterine epithelial cell GM-CSF output *in vitro*. $TGF\beta_1$ and $TGF\beta_2$ exhibited similar potency, with activin showing only weak GM-CSF stimulating activity. Neutralisation of $TGF\beta_1$ bioactivity resulted in a 75% decline in seminal vesicle GM-CSF stimulating activity, thereby implicating $TGF\beta_1$ as the dominant

agent responsible for triggering the post-mating inflammatory response in the mouse. Furthermore, intrauterine injection of rTGFβ_1 was observed to increase uterine epithelial cell GM-CSF production, and result in cellular changes that were comparable to those seen following natural mating.

At the onset of these studies, little was known of the nature, location and molecular biology of the post-coital inflammatory response in women. In an attempt to determine if mechanistic parallels existed between mice and women, the effect on GM-CSF output of addition of rTGFβ_1 and semen to human endometrial and cervical keratinocyte cultures was determined. Both whole semen and rTGFβ_1 significantly increased the release of GM-CSF and chemotactic activity (assessed by an *in vitro* leukocyte migration assay) from human cervical keratinocyte cultures. Conversely, only a small increase in output of GM-CSF from endometrial cells was observed following rTGFβ_1 treatment. This response may be less physiologically important than the cervical response, since in the human, ejaculation occurs at the level of the cervix, with seminal plasma not coming into direct contact with the endometrium.

The second main aim of these studies was to explore interactions between the post-mating inflammatory response and maternal immune reactions to paternal antigens in semen. This was prompted by reports that semen exposure could induce maternal hypo-responsiveness in cell-mediated immunity to paternal antigens. Delayed type hypersensitivity and complement-fixing anti-sperm IgG2b responses were significantly reduced in mice that received intrauterine inoculations of epididymal sperm in the presence of rTGFβ_1, compared with mice that received sperm alone, and were comparable to values obtained from mice inoculated naturally by mating. This data suggests that TGFβ and /or other factors within seminal plasma may act to inhibit the Th1 compartment of the female immune response to seminal antigens. In addition, female mice were immunised with sperm or sperm plus rTGFβ_1 prior to mating, to determine if deviation of the immune response to paternal antigens could alter pregnancy outcome. Immunisation of females resulted in a small reduction in litter size, but fetal and placental weights were increased in the group immunised with sperm plus rTGFβ_1. Taken together, these observations suggest that seminal TGFβ_1 may have a role in promoting successful pregnancy through initiating maternal immune "tolerance" to paternal antigens prior to implantation of the conceptus.

Finally, the role of semen exposure in human reproduction was investigated by measuring the effect of intercourse on pregnancy outcome in women undergoing *in vitro* fertilisation (IVF) with thawed embryo transfer. No significant difference in pregnancy success was observed between women who were exposed to semen around the time of embryo transfer compared to those women who abstained.

Together, these studies identify seminal $TGF\beta_1$ as an important triggering agent in both the human and murine post-mating inflammatory response. Furthermore, the cytokine environment provided by seminal $TGF\beta_1$, perhaps in conjunction with other seminal factors such as prostaglandin E, may promote successful pregnancy by initiating maternal "tolerance" to paternal antigens present within the ejaculate. The generation of a permissive immunological environment, prior to the trophoblast making contact with the maternal immune system at implantation, may favour the growth and development of the semi-allogenic conceptus.

Declaration

This thesis contains no material which has been accepted for the award of any 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.

I further grant my consent to the University of Adelaide to make this thesis available for loan and photocopying once accepted for the degree.

Kelton Paul Tremellen

December 1998

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Publications arising from these studies

1. Robertson SA, Mau VJ, Tremellen KP, Seamark RF. Role of high molecular weight seminal vesicle proteins in eliciting the uterine inflammatory response to semen in mice. J. Reprod. Fertl. 1996; 107:265-277.

2. Robertson SA, Mau VJ, Hudson SA, Tremellen KP. Cytokine-leukocyte networks and the establishment of pregnancy. Am. J. Reprod. Immunol. 1997; 37:438-442.

3. Tremellen KP, Seamark RF, Robertson SA. Seminal transforming growth factor β_1 stimulates granulocyte-macrophage colony-stimulating factor production and inflammatory cell recruitment in the murine uterus. Biol. Reprod. 1998; 58:1217-1225.

4. Tremellen KP, Robertson SA. Seminal priming in successful mammalian pregnancy. Proceedings of the VII International Conference of Reproductive Immunology. New Delhi, India, 27th-30th October 1998.

Patent

1. Treatment and diagnosis of infertility using TGFβ or activin.
   International publication number WO 98/39021. Published 11th September 1998.

Short communications

1. Tremellen KP, Robertson SA. Isolation of seminal vesicle proteins responsible for the initiation of the post-mating inflammatory response. Proceedings of the South Australian branch of the Australian Society for Medical Research. 31st May 1996. Awarded the Ansett Australia award for best oral presentation by a new investigator.

2. Tremellen KP, Robertson SA. Potential role for transforming growth factor beta in modulating the maternal immune response during early murine pregnancy. 35th National Scientific Conference, The Australian Society for Medical Research. Surfers Paradise, Queensland. 24th November 1996. Awarded the ASMR Young Investigator award.

3. Tremellen KP, Robertson SA. Potential role of seminal plasma TGFβ in the initiation of the post-coital inflammatory response in humans. First International Conference on Experimental and Clinical Reproductive Immunology. Charlottesville, USA, 2nd October 1997.

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Abbreviations

A	adenine
Ab	antibody
ACAID	anterior chamber associated immune deviation
bp	base pair
BSA	bovine serum albumin
^{0}C	degrees Celsius
C	cytosine
cDNA	complementary DNA
CFA	complete Freund's adjuvant
CSF	colony stimulating factor
CSF-1	colony stimulating factor-1
d	day
DAB	diaminobenzidine
DMEM	Dulbecco's modified minimal essential medium
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dpm	disintegrations per minute
ECM	extra cellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FCS	fetal calf serum
FGF	fibroblast growth factor
FMLP	N-formyl-metionyl-leucyl-phenylalanine
G	guanine
GIFT	gamete intra-fallopian tube transfer
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMG	granulated metrial gland
hr	hour(s)
HBSS	Hank's balanced salt solution
hCG	human chorionic gonadotrophin
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLA	human leukocyte antigen
HRP	horse radish peroxidase
ICSI	intra-cytoplasmic sperm injection
IGF	insulin-like growth factor
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IUI	intrauterine insemination
ip	intra-peritoneal
IUGR	intra uterine growth restriction
iv	intra venous
IVF	*in vitro* fertilisation
IU	international unit(s)
kb	kilobase pairs or 1000 bp
kDa	kilo-dalton
LCA	leukocyte common antigen
LHRH	leuteinising hormone releasing hormone
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatability complex
min	minute(s)
mRNA	messenger RNA
NK	natural killer
NH&MRC	National Health and Medical Research Council of Australia
NMS	normal mouse serum
PAF	platelet activating factor
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
pc	post-coital
PG	prostaglandin
PMIR	post-mating inflammatory response
RNA	ribonucleic acid

RNAse	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
SGSF	seminal GM-CSF stimulating factor
SDS	sodium dodecyl sulphate
T	thymine
TGF	transforming growth factor
TLX	trophoblast/lymphocyte cross-reactive antigen
TMB	tetra methylbenzidine
TNF	tumour necrosis factor
U	uracil
UV	ultra-violet
v/v	volume per volume
w/v	weight per volume

Chapter 1
Literature Review

1. The peri-implantation period of mammalian pregnancy

1.1 Introduction

Central to the success of mammalian reproduction is the process of placentation. The placental organ enables efficient exchange of nutrients and waste, while allowing the mother to nurture her progeny for extended periods of time in the relative safety of her uterus. Giving birth to well developed live young (viviparity) has considerable reproductive advantages compared to reproductive strategies such as laying eggs (oviparity) in a potentially hostile environment accessible to predators and environmental fluctuations. Placental reproduction is one of the key forces behind the evolutionary dominance of mammals in recent time.

Implantation and placental development occur in a stepwise fashion dependent upon precise interactions between the embryo and the uterus. Perturbations in placental development can produce shallow placentation, which in turn may result in spontaneous abortion, intrauterine growth restriction and pre-eclampsia in the human (Khong *et al.* 1986), as well as long-term complications such as diabetes and hypertension in adult life (Barker 1995).

A group of polypeptide growth factors known as cytokines are believed to provide the basis for intercellular communication between the various cell lineages comprising the endometrium and implanting blastocyst. This communication facilitates the coordination of tissue remodelling within the endometrium in readiness for implantation, the initiation of developmental changes in the embryo, and the induction of a permissive immune response to the semi-allogenic embryo, as well as influencing future placental growth and function.

The purpose of the studies described in this thesis is to investigate alterations in the cytokine profiles of the female reproductive tract in response to seminal plasma exposure at the time of mating. It is hypothesised that mating-induced alterations in cytokine production may facilitate implantation and help prevent immunological rejection of the semi-allogenic conceptus. The manner by which seminal constituents modify murine and

human female reproductive tract cytokine profiles, leukocyte recruitment and function, as well as reproductive performance, will be explored.

1.2 Early events in mammalian pregnancy

1.2.1 The mouse as a model for human pregnancy

Like humans, mice exhibit interstitial implantation and haemochorial placentation, and therefore murine pregnancy represents a useful "experimental model" for human pregnancy. An extensive understanding of murine reproductive physiology, combined with economical housing and maintenance, high fertility, short gestational period, and wide availability of immunological reagents for the assessment of cytokine and leukocyte activity, make the mouse an ideal animal for studying mammalian reproductive immunology.

1.2.2 Pre-implantation events

The mouse has a 4-6 day oestrus cycle culminating in the ovulation of between 8-12 oocytes just after midnight at the mid-point of the oestrous period (Hogan *et al.* 1986). Mating coincides with ovulation and results in the introduction of millions of sperm into the uterine lumen, bathed in secretions from the male accessory sex glands (seminal plasma). In addition, the physical thrusting that occurs during mating triggers a neuro-endocrine response which initiates the hormonal changes necessary for the continuation of pregnancy. Within the female reproductive tract, sperm undergo a series of maturational steps called capacitation, which enable them to fertilise oocytes within the oviduct. Each murine embryo then undergoes a series of symmetrical cell divisions to create a solid ball of totipotent cells (morula), which is enclosed within a protective coat of glycoprotein (zona pellucida). By day 3 of pregnancy, the early embryo (32-64 cells) reaches the uterine cavity and undergoes the first round of cell differentiation, with a morphological transformation into a blastocyst, consisting of a hollow ball of cells lined by trophectoderm (future placenta) and an inner cell mass (future embryo).

It has been suggested that interactions between the pre-implantation embryo and the oviduct / uterine environment may assist embryonic development, since co-culturing of murine or human embryos in the presence of oviductal or uterine epithelial cells is

reported to improve pre-implantation embryo development *in vitro* (Bongso *et al.* 1992, Piekos *et al.* 1995, Feng *et al.* 1996). Cytokines such as CSF-1, LIF, GM-CSF, EGF and TGFβ , all produced by the female reproductive tract epithelium, are believed to be likely candidates responsible for this embryotrophic effect (reviewed 1.3).

The human menstrual cycle varies considerably in length, ranging from 24-32 days in the majority of fertile women, yet the point of ovulation is relatively fixed at 14 days prior to the onset of the next menstrual period (Shaw *et al.* 1992). Sexual intercourse occurring within a six day period, ending on the day of ovulation, is reported to be capable of producing conception in the human female (Wilcox *et al.* 1995). Research into the *in vivo* development of the human pre-implantation embryo has been restricted by ethical considerations, however the culturing of human pre-implantation embryos in preparation for assisted reproductive treatment has suggested that these embryos develop in a similar manner to murine embryos, with oviduct / uterine-derived cytokines positively influencing growth and development (Bongso *et al.* 1992, Giudice 1994, Chard 1995).

1.2.3 Implantation

Implantation of murine and human blastocysts occurs soon after the blastocyst hatches from the zona pellucida - day 4.5 in the mouse and day 6 in the human (Johnson and Everitt 1993). The process of implantation can be broken down into three phases: apposition, adhesion and decidualisation / invasion (Weitlauf 1988). Whereas the pre-implantation embryo can develop in the absence of maternal cues, implantation requires an active dialogue between maternal cells and the embryo. Cytokines produced by the embryo and the female reproductive tract play a vital role in cellular communication required for the precisely-timed events of implantation.

The term "apposition" denotes the process by which a blastocyst becomes juxtaposed to the uterine cavity epithelium. In murine pregnancy, a pre-implantation surge in production of oestrogen initiates absorption of uterine luminal fluid and oedema of the endometrium, which in combination with an increase in uterine smooth muscle contraction results in the "clasping" of the uterus around the pre-implantation blastocyst (Enders *et al.* 1967). These three processes lead to apposition of the blastocyst to the anti-mesometrial aspect of the uterine luminal epithelium.

The second phase in mammalian implantation, attachment, is characterised by the formation of carbohydrate-lectin and integrin bonds between the blastocyst and the

endometrial extra cellular matrix (ECM). Mouse blastocysts express carbohydrate molecules such as perlecan, a heparin sulphate proteoglycan, which are able to bind the blastocyst to the uterine epithelial surface via heparin sulphate-binding proteins expressed on the uterine epithelial surface (Wilson *et al.* 1990, Carson *et al.* 1993). Hyaluronic acid (HA) is another candidate-mediating adhesion between the blastocyst and uterine epithelium (Carson *et al.* 1987). During decidualisation, HA is enzymatically removed from the ECM of the uterine wall opposite the future implantation site, while being upregulated at the implantation site (Fenderson *et al.*1993), thereby restricting potential sites for embryo attachment. Glycoprotein binding may also facilitate the attachment of human pre-implantation embryos, since a group of women experiencing recurrent implantation failure have been reported to exhibit altered endometrial expression of glycoprotein lectins compared to fertile women (Klentzeris *et al.* 1991).

Integrins, a group of adhesion molecules mediating cell-cell interactions (Hynes 1992), may also play a role in implantation. Integrins consist of a heterodimeric combination of one of fifteen α and eight β sub-units, with each heterodimer having a different affinity for extra cellular matrix proteins such as fibronectin, laminin and collagen. Speculation that integrins may be involved in murine and human implantation is supported by the observation that there are menstrual / oestrus cycle and pregnancy-related changes in endometrial expression of integrins (Sutherland *et al.* 1988, Lessey *et al.* 1996). With maturation of the mouse blastocyst comes expression of the $\alpha 7\beta 1$ integrin, an integrin that binds laminin (Sutherland *et al.* 1993). Coincidental with this change is an up-regulation of laminin expression in the endometrial ECM, in turn facilitating blastocyst-endometrial binding (Glasser *et al.* 1987). In humans, the $\alpha V\beta 3$ integrin is only expressed by the uterine epithelium between days 19-24 of the menstrual cycle, the period of maximal uterine receptivity to implantation, with this switch in uterine integrin expression not occurring in infertile women experiencing recurrent implantation failure (Lessey *et al.* 1992).

Cytokines are believed to play a major role in the regulation of endometrial integrin expression in women (Sillem *et al.* 1997). IL-1α and IL-1β, both produced by the pre-implantation embryo, have been reported to initiate an increase in human endometrial expression of the $\beta 3$ integrin (Simon *et al.* 1997), a marker of uterine receptivity for implantation. This raises the possibility that the pre-implantation embryo may communicate with its neighbouring endometrium to initiate changes in integrin expression necessary for implantation.

The third and final stage of mammalian implantation is decidualisation of the endometrium and its associated invasion by trophoblast. Decidualisation resembles an inflammatory response with recruitment of macrophages, T and NK cells into the endometrium (Hunt *et al.* 1985, Bulmer *et al.* 1988), together with an increase in angiogenesis and vascular permeability resulting in oedema of the endometrium. The decidual reaction is believed to be mediated by agents such as histamine, prostaglandin and cytokines. Histamine is released from local mast cells in response to the pre-implantation surge in oestrogen production and an unknown blastocyst-derived factor (Cocchiara *et al.* 1992), with histamine antagonists being capable of blocking decidualisation and implantation in the rat (Shelesnyak 1952). The pre-implantation surge in uterine epithelial production of cytokines and growth factors such as IL-1, TGFα, EGF, LIF, CSF-1, GM-CSF and IGF (Robertson *et al.* 1990 and 1992b, Bhatt *et al.* 1991, McMaster *et al.* 1992) may promote angiogenesis and stromal cell proliferation / differentiation within the decidua, as well as initiate the recruitment of leukocytes important to the control of trophoblast invasion. Female mice exhibiting a null mutation of the IL-11 receptor alpha chain are infertile because of defective decidualisation (Robb *et al.* 1998), highlighting the importance of cytokines in decidualisation.

Development of a placenta is the next important step in mammalian reproduction. Trophoblast invasion into the decidua serves to anchor the embryo to the uterine wall, while providing a functional connection between the embryonic and maternal circulation (haemochorial placentation), which enables the efficient exchange of nutrients, gases and waste. The depth of trophoblast invasion differs between mice and humans, with murine trophoblast invasion being limited to the decidua, whereas human trophoblast invades through the entire decidua, the inner third of the myometrium and into the uterine spiral arteries (Pijnenborg *et al.* 1980 and 1981). A delicate balance between pro-invasive and inhibitory processes controls the depth of trophoblast invasion.

Murine and human trophoblast cells release enzymes such as gelatinase B, collagenase and urokinase which facilitate trophoblast invasion by breaking down the decidual ECM (Queennan *et al.* 1987, Librach *et al.* 1991, Behrendtsen *et al.* 1992), while proteinase inhibitors such as α2-macroglobulin and tissue inhibitors of metalloproteases (TIMP-1,TIMP-2 and TIMP-3) inhibit such invasion. Cytokines released by decidual leukocytes and stromal cells are able to modify the depth of trophoblast invasion by regulating the balance of production between proteases and their inhibitors (Graham and Lala 1992, Librach *et al.* 1994).

In addition to modifying trophoblast invasion by controlling protease activity, cytokines control trophoblast invasive behaviour by initiating changes in integrin expression (Damsky *et al.* 1992). The invasive behaviour of human cytotrophoblast is modified by its integrin expression and resulting binding preference for decidual ECM proteins. Trophoblast attachment to the laminin / collagen ($\alpha1\beta1$ integrin) promotes invasion, whereas binding to fibronectin ($\alpha5\beta1$ integrin) promotes anchorage (Damsky *et al.* 1994). In pre-eclampsia, cytotrophoblast invasion is shallow, with uterine arteriole invasion being almost absent (Khong *et al.* 1986). This decrease in invasiveness is accompanied by the failure of cytotrophoblast cells to up-regulate their $\alpha1\beta1$ integrin expression required for the final phase of invasion into maternal arterioles (Zhou *et al.* 1993). There is now increasing evidence that cytokines such as CSF-1 and TGFβ may play a role in the regulation of trophoblast integrin expression and invasion (Irving and Lala 1995, Omigbodun *et al.* 1998).

Decidual natural killer (NK) cells, concentrated at the trophoblast-decidual interface in mice and women, have been implicated in the control of trophoblast invasion since they are capable of destroying trophoblast following stimulation with IFNγ or IL-2 (King *et al.* 1992 and 1993, Croy 1994). Conversely, release of cytokines such as GM-CSF from decidual NK cells enhances trophoblast growth (Saito *et al.* 1993b, Jokhi *et al.* 1994b). The balance between destructive and growth-promoting cytokines at the decidual-trophoblast interface may help decidual NK cells control the depth of trophoblast invasion. Pregnancies from NK and T cell-deficient tgε26 mice have less than 1% of the normal uterine NK cell frequency, and produce small placentas exhibiting atheromatous vascular pathology similar to that seen in human pre-eclamptic placentas (Guimond *et al.* 1997). This placental pathology is associated with a high rate of mid-term fetal loss and low birthweight. Restoration of uterine NK cell numbers in tgε26 mice by bone marrow transfusion from T and B cell-deficient *scid/scid* donors reverses the placental pathology, while increasing fetal viability and birthweight (Guimond *et al.* 1998). Thus, uterine NK cells appear to play a critical role in the development of the murine placenta.

1.3 Cytokines in reproduction

1.3.1 Introduction

Cytokines are known to play an extensive role in reproduction ranging from ovulation, blastocyst maturation, implantation, placental growth and parturition (Robertson *et al.* 1994, Chard 1995). The principal cytokines involved in early pregnancy include IL-1α and β, IL-6, IL-11, CSF-1, GM-CSF, TGFβ, LIF, TNFα, IFNγ and the polypeptide growth factors EGF and IGF. This section of the literature review will focus on the role that cytokines play in early murine and human pregnancy, with particular emphasis on GM-CSF and TGFβ, since these two cytokines are the focus of the experimental work described in this thesis.

Cytokines are low molecular weight (<100 kDa) polypeptides or glycoproteins that are secreted by virtually all cells, but in particular leukocytes. Cytokines act as soluble intercellular signalling molecules capable of effecting cell proliferation, differentiation, adhesion, migration and chemotaxis. The binding of a cytokine to its corresponding membrane-bound receptor typically activates an associated protein kinase, and the resultant phosphorylated proteins stimulate the generation of nuclear transcription factors that activate cellular proto-oncogenes with a resultant cellular response (reviewed by Taga and Kishimoyo 1995). Cytokine receptors are generally heterodimeric combinations of an α and β subunit, with the α subunit providing cytokine specificity while the β subunits, sometimes referred to as "affinity converters", increase the binding affinity of the cytokine to the α subunit, and facilitate signal transduction through their long intra-cytoplasmic domains (Miyajima *et al.* 1992). The interleukins 2, 4 and 7 share a common receptor, while IL-3, IL-5 and GM-CSF also share a receptor (Howard *et al.* 1993). Competition between these cytokines α subunits for affinity converting β subunits explains how the presence of one cytokine can down-modulate the effect of another, thereby modifying the net effect of the cytokine environment.

Cytokines provide the ideal intercellular signalling system because of their capacity to act at very low concentrations (nano-pico molar), short half-life, localised site of action and tightly regulated production at both the transcriptional and translational level. Cytokines do not act in isolation, since most cells will be exposed to several different cytokines at any one time. The same two cytokines can act synergistically or antagonistically on a particular cell depending upon the cell type, its location and the

surrounding milieu (other cytokines, prostaglandins etc). The effect of a cytokine may also be modified by the presence of soluble receptors and binding proteins which may enhance or prevent binding of the cytokine to its receptor.

The concept of cytokine redundancy is an important factor governing the function of the cytokine signalling system in nature (Kelso 1994). Cytokine redundancy describes the situation in which many different cytokines can act on the same cell to produce the same or similar effect. Removal of any one cytokine may have no observable effect on cell phenotypes, since it is functionally backed up by other cytokines. The molecular basis underlying redundancy is now becoming better understood. For example, the cytokines IL-6, LIF, oncostatin M, CNTF and IL-11 are recognised by distinct receptors, yet all use a common gp130 sub-unit signalling pathway. Because of this shared signalling pathway, LIF may substitute for IL-6 and produce the same effect, provided that the LIF receptor is also present on that cell (Gearing *et al.* 1992). Cytokine redundancy is an important limitation to consider when analysing the role that a particular cytokine plays in gene "knock-out" mice.

1.3.2 Granulocyte Macrophage-Colony Stimulating Factor

a. *Biochemistry and genetics*

Granulocyte macrophage-colony stimulating factor (GM-CSF) is a glycoprotein that belongs to the colony stimulating factor family of cytokines, a group discovered due to their capacity to control the proliferation, differentiation and function of granulocytes and macrophages / monocytes.

GM-CSF is secreted as a glycoprotein monomer of 124 amino acids in the mouse and 127 amino acids in the human, giving rise to a 15 kDa protein core, which in turn is variably glycosylated to produce a stable protein of 23 kDa in the mouse and 22 kDa in the human (Burgess *et al.* 1977, Gasson *et al.* 1984, Moonen *et al.* 1987). X-ray crystallography has determined that the GM-CSF molecule consists of four antiparallel α-helical bundles with two interleaving antiparallel β-strands present between the first and last pair of helices (Diederichs *et al.* 1991). A variety of structure-function studies have suggested that the first GM-CSF helix binds to the β-chain GM-CSF receptor and the third and fourth helices bind to the α-chain (Lopez *et al.* 1992).

Murine and human GM-CSF are encoded by genes that are highly homologous in both coding and non-coding regions (Miyatake *et al.* 1985), with both genes being approximately 2.5 kilobase pairs in length, consisting of four exons separated by three intervening sequences (Miyatake *et al.* 1985). The nucleotide sequences of GM-CSF cDNAs cloned from mouse and human (GeneBank accession numbers X03020 and X03021 respectively) predict a protein of 144 (human) and 141 (mouse) amino acids, each containing a leader sequence of 17 amino acids. These proteins are 54% identical at the amino acid level but exhibit no cross-species biological activity.

b. Source of GM-CSF production

A large number of cell types can synthesise GM-CSF in response to a variety of stimuli. T cells and macrophages produce GM-CSF in response to inflammatory stimuli such as IFNγ, TNFα, IL-2, LPS or viral proteins (Chan *et al.* 1986, Quill *et al.* 1989). Macrophages produce GM-CSF when they undergo phagocytosis or adhere to ECM proteins such as fibronectin (Thorens *et al.* 1987). NK cells and B lymphocytes have also been confirmed as sources of GM-CSF production (Baldwin *et al.* 1992). Non-haemopoietic cells such as endothelial cells, fibroblasts and bone marrow stromal cells are known to produce GM-CSF in response to pro-inflammatory mediators such as IL-1 and TNFα (Zsebo *et al.* 1988, Munker *et al.* 1986). Non-haemopoietic cells outside the bone marrow such as osteoblasts (Horowitz *et al.* 1989), astrocytes (Malipiero *et al.* 1990), renal mesangial cells (Budde *et al.* 1989), keratinocytes (Koury *et al.* 1983) and epithelial cells of the trachea (Ohtoshi *et al.* 1991), and thymus (Galy *et al.* 1990) have all been identified as sources of GM-CSF.

The female reproductive tract is a potent source of GM-CSF in both mice and humans. In the mouse, epithelial cells lining the uterine lumen have been identified as a site of production for GM-CSF (Robertson *et al.* 1992a, 1992b), with the release of GM-CSF in cycling animals being maximal at the time of oestrous. Steroid replacement experiments in ovariectomised mice have demonstrated that GM-CSF production is increased by oestrogen, but this increase is antagonised by progesterone (Robertson *et al.* 1996). Within hours of mating, the expression of GM-CSF mRNA within uterine epithelial cells is upregulated (Sanford *et al.* 1992), and GM-CSF protein content within uterine luminal fluid increases more than 20-fold, as a consequence of exposure of the

uterine epithelium to an unidentified high molecular weight seminal vesicle derived factor (Robertson *et al.* 1990, 1992a).

In the human female reproductive tract GM-CSF production is primarily localised to the uterine cavity and fallopian tube epithelium. Uterine GM-CSF mRNA and protein expression is reported to be highest in the late proliferative to mid-secretory phase of the menstrual cycle, suggesting a possible involvement of ovarian steroids in GM-CSF production (Sharpe-Timms *et al.* 1994, Giacomini *et al.* 1995, Zhao and Chegini 1994). Polarised cultures of human endometrial cells reveal vectorial secretion of GM-CSF, with 78% of GM-CSF being released from the apical surface (Matthews *et al.* 1997).

During pregnancy, decidual lymphocytes have been identified as the main source of GM-CSF production at both the human and murine fetal-maternal interface. The production of GM-CSF by decidual large granular lymphocytes (CD56+ NK cells) in human first trimester pregnancies is increased by IL-1, IL-2, IL-4, IL-6, and TNFα, with TGFβ and IFNγ having an indirect effect by blocking the IL-2-induced increase in GM-CSF production (Jokhi *et al.* 1994a). Human trophoblast is also thought to be a site of GM-CSF production (Jokhi *et al.* 1994b). During murine pregnancy, decidual lymphocytes and large trophoblast-like cells in the outer layer of the murine placenta (spongiotrophoblast) have been identified as the cellular origin of placental GM-CSF (Kanzaki *et al.* 1991).

c. Biological activity

The human and murine GM-CSF receptor is a heterodimer consisting of a unique α-chain (GMRα) and a common β-chain (GMRβ) shared with IL-3 and IL-5. GMRα binds GM-CSF with low affinity but can not transmit an activation signal by itself. Conversely, GMRβ cannot bind ligand but is required for high affinity binding of GM-CSF to GMRα, and is responsible for signal transduction. The signal transduction pathways for GM-CSF have not been fully elucidated, however it appears that binding of GM-CSF to its $\alpha\beta$ heterodimer receptor unit results in the activation of JAK2 tyrosine kinase associated with the GMRβ subunit (Quelle *et al.* 1994, Zhao *et al.* 1995). This results in the rapid phosphorylation of the GMRβ cytoplasmic chain, as well as several other cytoplasmic proteins. Signalling proteins such as STAT, SHC, SHP2, MAP kinase and p21ras, as well as the proto-oncogenes c-fos and c-jun, have all been implicated in GM-CSF signal transduction (Sato *et al.* 1993, Itoh *et al.* 1996).

The classic biological effect of GM-CSF is to stimulate proliferation and differentiation of precursors of the myelomonocytic lineages. GM-CSF stimulates the generation of neutrophils, macrophages, eosinophils (Bradley and Metcalf 1966, Metcalf 1985), the proliferation of T cells (Santoli *et al.* 1988), as well as erythrocytes and megakaryocytes in the presence of erythropoietin (Donahue *et al.* 1985), and is implicated in the stimulation of angiogenesis in humans by initiating endothelial cell proliferation and migration (Bussolino *et al.* 1989). GM-CSF enhances neutrophil oxidative metabolism and phagocytic capacity (Fleischmann *et al.* 1986, Baldwin *et al.* 1989). Macrophages exposed to GM-CSF release pro-inflammatory cytokines such as IL-1 and TNFα (Morrissey *et al.* 1987, Heidenreich *et al.* 1989, Wing *et al.* 1989), enzymes such as plasminogen-activator (Hamilton *et al.* 1991), and exhibit increased microbial and tumorocidal activity (Grabstein *et al.* 1986). The antigen-presenting capacity of macrophages and dendritic cells is enhanced by GM-CSF through an increase in their membrane expression of MHC Class II antigen (Fischer *et al.* 1988, Paglia *et al.* 1993), and co-stimulatory molecules such as B7 and CTLA4 (Larsen *et al.* 1994).

d. Role of GM-CSF in reproduction

GM-CSF is a potentially important intercellular communication molecule in the female reproductive tract, with major influences over the development of the pre-implantation embryo, placental trophoblast, and the large population of myeloid leukocytes present within the cycling and pregnant uterus. Both the murine and human uterine epithelium are sites of GM-CSF production, with maximal levels being observed in the mouse during oestrous, under the positive influence of oestrogen and following mating (Robertson and Seamark 1990, Robertson *et al.* 1996). This post-mating increase in GM-CSF production, the result of an interaction between an unknown seminal vesicle protein and the uterine epithelium (Robertson and Seamark 1990), is implicated in mediating the post-mating influx of neutrophils, macrophages, dendritic cells, and eosinophils into the murine endometrium within hours of copulation.

Ovarian production of GM-CSF in the mouse is reported to be associated with recruitment of leukocytes into the corpus luteum, and has been postulated to be involved in the involution of this organ since GM-CSF "knockout" (GM-/-) females have slightly longer oestrus cycles compared to wild type mice (Jasper *et al.* 1998). However, GM-CSF is not critical to murine ovarian function, since GM-/- females have ovulation rates that are

comparable to wild type animals (Robertson *et al.* 1998). A possible role for GM-CSF in human ovarian processes is suggested by the finding that both follicular fluid and plasma GM-CSF concentrations are higher in women undergoing hyper stimulation compared to naturally cycling controls (Jasper *et al.* 1996).

The effect of GM-CSF on pre-implantation embryonic development is not well understood, and the existing studies are some what contradictory. Firstly, while pre-implantation embryos do express mRNA for the α-subunit of the GM-CSF receptor and can bind ^{125}I labelled GM-CSF, it is unclear how GM-CSF could mediate any biological effect, since these embryos do not express mRNA for the β-subunit required for signal transduction (Robertson *et al.* 1994). However, murine pre-implantation embryos appear to respond to GM-CSF, with *in vitro* development (particularly hatching and attachment) being accelerated by the addition of rGM-CSF to culture medium (Robertson *et al.* 1992a). Other investigators have reported that addition of GM-CSF to culture medium inhibits embryo development and attachment to fibronectin matrix (Haimovici *et al.* 1991). These conflicting results may reflect individual *in vitro* culture artefacts or strain differences, since *in vivo*-generated mouse blastocysts from GM-/- females are reduced in cell number by 18% compared to wild type controls (Robertson *et al.* 1998a). Furthermore, the proportion of human embryos developing to blastocysts is doubled in the presence of rhGM-CSF (Sjoblom *et al.* 1998).

Several lines of investigation have indicated that GM-CSF plays an important role in fetal / placental development. The production of GM-CSF by the murine uterine epithelium decreases considerably by day 3 of pregnancy under the negative influence of rising progesterone levels (Robertson *et al.* 1996). From this stage on, decidual cells such as stromal fibroblasts, endothelial cells, and leukocytes such as NK cells are principally responsible for GM-CSF production at the maternal-placental interface in both mice and humans (Crainie *et al.* 1990, Kanzaki *et al.* 1991, Jokhi *et al.* 1994b). Human trophoblast tissue is also a source of GM-CSF production (Jokhi *et al.* 1994b). A lack of GM-CSF at the fetal-maternal interface, as seen in GM-/- females, results in normal implantation rates, yet these litters are 25% smaller by the time of weaning compared to wild type controls because of an increased rate of death in the late fetal and early post-natal period (Robertson *et al.* 1998a). Pups born to GM-/- mothers are significantly growth restricted, presumably due to abnormalities in placental development (Robertson *et al.* 1998a). Specifically, the area of spongiotrophoblast in GM-/- placentae is significantly increased, while the labyrinth component is decreased in comparison to wild type placentae. These

observations are in keeping with previous reports that GM-CSF promotes proliferation, differentiation and enhances secretory activity (hCG, placental lactogen) within rodent and human cytotrophoblast *in vitro* (Garcia-Lloret *et al.* 1991 and 1994, Athanassakis *et al.* 1987, Armstrong and Chaouat 1989) and *in vivo* (Chaouat *et al.* 1990).

A third manner in which GM-CSF may benefit pregnancy outcome is through modification of uterine leukocyte trafficking and behaviour. While GM-CSF has been implicated as the principal initiator of the post mating inflammatory infiltrate (Robertson 1994), an absence of GM-CSF in GM-/- females does not have any significant effects on the numbers or spatial distribution of leukocytes within the uterine endometrium following mating or in the mid-term decidua (Robertson *et al.* 1998a). Factors other than GM-CSF, principally the chemokine family of molecules, have now been identified as being involved in this compensatory cytokine redundancy (Robertson *et al.* 1998b, Pollard *et al.* 1998). Preliminary observations (Robertson *unpublished findings*) have identified subtle defects in GM-/- uterine macrophages and dendritic cells, including a reduction in membrane expression of MHC class II antigen and the co-stimulatory molecule B7-2, both critical to antigen presenting activity. The diminished functional capacity of these cells has been postulated to impede the development of an immuno-permissive environment within the decidua towards the semi-allogenic placenta (Robertson *et al.* 1997). Supporting this concept of GM-CSF as an agent involved in the initiation of immune responses beneficial to reproductive performance is the observation that a single dose of GM-CSF can reverse the high rate of immune-mediated fetal loss that occurs spontaneously in the CBA/J x DBA/2 mating combination (Tartakovsky *et al.* 1991), and is capable of protecting against LPS or IFNγ induced reabsorption (Chaouat *et al.* 1990). This beneficial effect of GM-CSF is not mediated by a direct enhancement of trophoblast growth, but rather through changes in the maternal immune axis, since GM-CSF therapy is without effect if CD8+ T cells are depleted prior to administration of the cytokine (Clark *et al.* 1994b).

1.3.3 Transforming growth factor beta

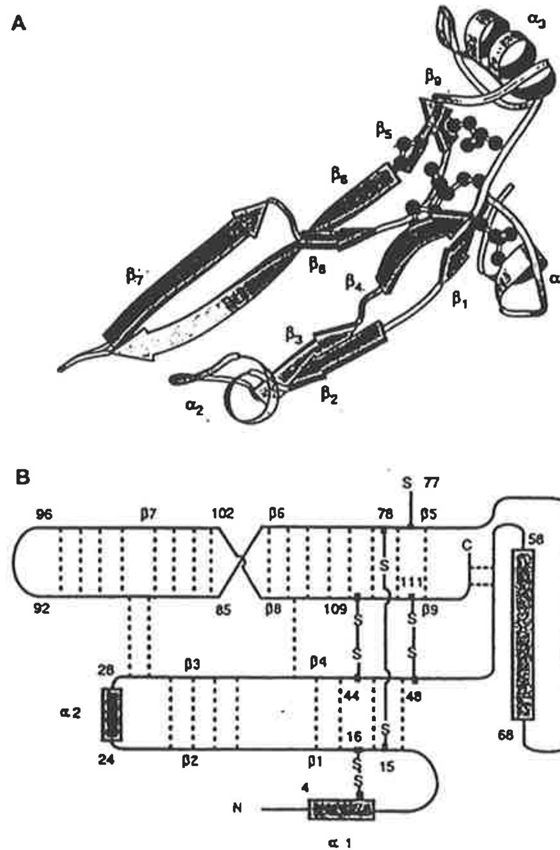
a. *Genetics and biochemistry*

The transforming growth factor beta (TGFβ) family of proteins affect the growth and differentiation of a wide variety of cells. The prototype of the family, $TGF\beta_1$, was first isolated in 1981 and named in accordance with its ability to initiate a transformation of phenotype in rat fibroblasts if administered in conjunction with transforming growth factor α (Roberts *et al.* 1981). The TGFβ family of proteins exhibit 30-80% amino acid sequence homology and share a similar protein structure consisting of a ~ 25 kDa disulphide linked homodimer with several conserved cysteine residues (reviewed in Massague 1990) . Members of this superfamily include $TGF\beta_1$, β_2, β_3 β_4 and β_5, activin, inhibin, mullerian inhibitory substance and bone morphogenic proteins, decapentaplegic, 60A, VG-1, dorsalin, nodal and the growth differentiating factors. The focus of this review will be on the TGFβ isoforms.

TGFβ exist as five different isotypes, with TGF β_1, β_2 and β_3 being present in mammals , $TGF\beta_4$ in birds and $TGF\beta_5$ in Xenopus laevis (Ohta *et al.* 1987, Derynck *et al.* 1988, Jakowlew *et al.* 1988, Roberts *et al.* 1990). The three mammalian isotypes share in excess of 70% amino acid sequence homology at their N terminal ends and in excess of 97% structural identity (Daopin *et al.* 1992), thereby suggesting that they arose as a duplication from a common ancestral gene. In addition, the $TGF\beta_1$ cDNAs from different mammalian species are highly conserved, with virtual amino acid identity over the entire C-terminal domain (GeneBank accession number for murine $TGF\beta_1$ cDNA is M13177; human X02812, J05114), resulting in cross-species bioactivity.

The genes for $TGF\beta_1$,β_2 and β_3 are respectively located on chromosomes 7,1 and 2 in the mouse and chromosomes 19q13, 1q41 and 14q24 in the human. These TGFβ genes encode for pre-proproteins of 390-412 amino acids, consisting of a N-terminal secretory signal sequence, a long precursor segment, and the C-terminal 112 amino acid sequence which corresponds to the mature bioactive TGFβ monomer (Derynck *et al.* 1985). This C terminal region is proteolytically cleaved from its precursor to give a 12.5kDa monomer, which in turn is paired with an identical monomer (although heterodimers are rarely encountered) via disulphide bonds to produce the 25 kDa biologically active form of TGFβ

(A) Topology diagram of a TGF-β2 subunit. The α helices are labeled as α1, α2, and α3 and peptide strands in β sheets are labeled from β1 through β9. The residues involved in the regular secondary structure are: α1, residues 4 to 8; α2, 24 to 28; α3, 58 to 68; β1, 15 to 18; β2, 20 to 23; β3, 37 to 40; β4, 42 to 46; β5, 77 to 80; β6, 82 to 91; β7, 96 to 102; β8, 104 to 106; and β9, 109 to 112. (B) Schematic drawing of the primary and secondary structure of a TGF-β2 subunit. Hydrogen bonds in the β strands and loops are indicated by dashed lines. The analogy to a left hand can be seen. The heel (helix α3) is to the right and the fingers (β strands) are to the left with the third and fourth fingers twisted.



C Sequence comparisons and 3-D-1-D profile scores for the TGF-β superfamily. The sequences were first aligned by the GCG program package . A penalty of −0.1 was applied if a gap or an insertion was in a loop region and −1.0 if it was in an α helix or a β sheet. Included as controls are the variable domains of two mouse immunoglobulin light chains, J539 and McPC603, which are known to have similar structures by x-ray diffraction . The *Z* scores indicate similarity among TGF-β2 and other TGF-β family members.

Sequence	Source	Length	*Z*	Sequence identity (%)
TGF-β 1 to 5*				
TGF-β2	Human	112	50.5	100
TGF-β1	Human	112	48.5	72
TGF-β3	Human	112	47.6	80
TGF-β4	Chicken	114	51.9	66
TGF-β5	*X. laevis*	112	49.8	66
TGF-β superfamily*				
Inhibin βA	Human	112	21.2	39
Inhibin βB	Human	111	16.7	35
BMP 2	Human	100	21.0	34
BMP 4	Human	104	19.6	35
Vg1	*X. laevis*	109	23.7	36
DPP-C	*Drosophila*	102	20.8	36
MIS	Human	107	12.2	23
Inhibin α	Human	113	6.5	26
Immunoglobulin VL†				
J539	Mouse	107	44.6	100
McPC603	Mouse	114	27.9	55

Figure 1.1 Structure of the Transforming Growth Factor beta family of proteins. Family members of the TGFβ group exhibit 66 to 80% sequence identity (C), and nine strictly conserved cysteines. The crystal structure of TGFβ_2 determined by X-ray crystallography (Daopin *et al.* 1992) is summarised in figures A and B. The TGFβ_2 monomer lacks a well defined hydrophobic core and displays an unusual elongated non-globular fold with dimensions of approximately 60 Å by 20 Å by 15Å. Eight cysteines form intra-chain disulphide bonds, which are clustered in a core region forming a network complementary to the network of hydrogen bonds. The TGFβ dimer is stabilised by the ninth cysteine, which forms an inter-chain disulphide bond, and by two identical hydrophobic interfaces.
All figures are copied from Daopin *et al.* Science 257, 369-72.

(Assoian *et al.* 1983). The majority of TGFβ is released as an inactive latent precursor in which the TGFβ dimer (25 kDa) is non-covalently associated with a 75 kDa glycosylated latency protein (LAP), which in turn is covalently linked to a 125-160 kDa binding protein (LTBP) to produce a 225-260 kDa conglomerate (Wakefield *et al.* 1988, Miyazono *et al.* 1993). The release of biologically active TGFβ from its latent precursor can be achieved by extremes of pH, heat, urea, SDS, and through the action of enzymes such as plasmin, thrombospondin, and glycosidases (Lawrence *et al.* 1985, Lyons *et al.* 1988, Miyazono and Heldin 1989, Schultz-Cherry and Murphy 1993).

b. *Cellular origin of TGFβ production*

Most, if not all tissue, express one or more isoforms of TGFβ at different stages in development, usually coinciding with tissue differentiation and morphogenesis. In the mouse, intracellular TGFβ is predominantly localised to platelets / megakaryocytes, adrenal cortex, bone marrow leukocytes, cardiac myocytes, chondrocytes, ovarian glandular cells and placental cells, with extracellular TGFβ being concentrated in the skin, cartilage, pancreas, uterus and placenta (Thompson *et al.* 1989). This pattern of distribution suggests that TGFβ has an important role in controlling interactions between epithelial and surrounding mesenchyme tissue. TGFβ is present at high concentrations within a wide variety of biological fluids including serum, amniotic fluid, breast milk, aqueous humour from the anterior chamber of the eye, and seminal plasma (Childs *et al.* 1982, Wilbanks *et al.* 1992b, Nocera and Chu 1993, Grainger *et al.* 1995b).

c. *TGFβ receptor physiology*

Receptors for TGFβ are widely distributed, reflecting the broad target of cells upon which TGFβ exerts bioactivity. Five different types of membrane-bound TGFβ receptor have been identified, with essentially no cross reactivity in binding between these receptors and other growth factors (Massague *et al.* 1987, Lin and Moustakas 1994). The type I (53kdA) and II receptors (80 kDa) are glycoprotein signal transducers, with all cells expressing between 200 and 10,000 of these receptors. The type III receptor (250 kDa), also referred to as betaglycan, does not have the capacity to induce a response but is

believed to sequester TGFβ to the cell surface (Andres *et al.* 1989). The functions of type IV (60 kDa) and V (400 kDa) receptors are presently unclear.

Tβ-RI and Tβ-RII, the type I and II receptors, are transmembrane proteins with cytosolic domains containing serine-threonine kinase (Wrana *et al.* 1992). $TGF\beta_1$ is capable of binding to Tβ-RII with high affinity in the absence of Tβ -RI, however the binding of $TGF\beta_1$ to Tβ-RI requires the presence of Tβ-RII (Laiho *et al.*1991). In contrast, $TGF\beta_2$ does not directly bind to Tβ -RII but instead requires co-expression of Tβ-RI or Tβ-RIII to bind to Tβ-RII (Rodriguez *et al.* 1995). Heterodimeric complexes of type I and II receptors in the presence of dimeric $TGF\beta_1$ allows the cytosolic domains of Tβ-RI and Tβ-RII to interact so that the cytoplasmic domain of Tβ-RI is transphosphorylated by the Tβ-RII kinase, which in turn activates downstream signalling molecules. The type III receptor has only a short cytoplasmic tail with no apparent signalling motif (Wang *et al.* 1991), and is believed to function by presenting TGFβ ligand to Tβ-RI and Tβ-RII (Lopez-Casillas *et al.* 1994). A soluble non-membrane bound form of the type III receptor is also secreted by some cell types, and may act as a reservoir for ligand retention.

Serine/threonine kinases such as MAD (Chen *et al.* 1996, Liu *et al.* 1997) and TAK1 (Wang *et al.* 1997), which phosphorylate tumour-suppressor SMAD proteins (Kretzschmar and Massague 1998), are responsible for TGFβ signal transduction. Phosphorylated SMAD2 and SMAD3 form hetero-oligomeric complexes with SMAD4 before being translocated to the nucleus, where they then regulate transcriptional responses (Zhang *et al.* 1997). SMAD6 and 7, also produced following receptor activation, complicate the TGFβ signalling pathway by stopping the phosphorylation of SMAD2 and 3, thereby blocking signal transduction (Nakao *et al.* 1997, Imamura *et al.* 1997).

d. *General actions of TGFβ*

TGFβ has the capacity to modify cell proliferation, differentiation and function in a diverse range of cell types. All three mammalian isoforms of TGFβ tend to have similar actions and potency, with isotype specific activity being seen rarely (Jennings *et al.* 1988). Generally speaking, TGFβ is one of the most potent growth inhibitors known. It mediates this effect directly through lengthening or halting of the G_1 phase of the cell cycle (Shipley *et al.* 1986), or indirectly by opposing the action of specific mitogens such as EGF in

keratinocytes (Coffey *et al.* 1988), IL-1 and IL-2 in lymphocytes (Wahl *et al.* 1988), and IL-3, GM-CSF and CSF-1 in haemopoietic cells (Kasid *et al.* 1988, Fan *et al.* 1992, Strassmann *et al.* 1988). Evidence suggests that the growth inhibitory effect of TGFβ on epithelial cells may help prevent unchecked cellular division since mutations in the type II TGFβ receptor have been reported to result in cancer (Markowitz *et al.* 1995). TGFβ is also able to stimulate the proliferation of cells such as fibroblasts (Moses *et al.* 1987) and osteoblasts (Centrella *et al.* 1987) by inducing autocrine secretion of cytokines such as platelet-derived growth factor and induction of c-fos (Moses *et al.* 1987, Machwate *et al.* 1995).

Examples of the capacity of TGFβ to modify cell phenotype include the generation of chondrocytes and osteoblasts from their progenitor cells (Seyedin *et al.* 1985, Pfeilschifter *et al.* 1987), and the inhibition of pre-adipocyte and rat skeletal myoblast development into mature cells (Ignotz and Massague 1985, Florini *et al.* 1986). In addition, TGFβ has been observed to modify the activity of mature cells. Examples of this include blocking steroid hormone production by Leydig cells (Lin *et al.* 1987), and the stimulation of FSH production by pituitary cells (Ying *et al.* 1986). Furthermore, TGFβ is capable of altering interactions between cells by stimulating an increase in the production of ECM proteins such as fibronectin, collagen, osteopontin, and chondroitin / dermatan sulphate proteoglycan (Varga *et al.* 1987, Dean *et al.* 1988, Massague 1990), while altering these cells association with the ECM proteins by initiating cell membrane integrin switching (Ignotz *et al.* 1989, Arrick *et al.* 1992). These properties, in addition to the ability of TGFβ to initiate remodelling of the ECM by stimulating the release of proteases and protease inhibitors (Edwards *et al.* 1987, Arrick *et al.* 1992), places TGFβ in a central role in the control of tissue remodelling.

e. Immune functions of TGFβ

All leukocytes express receptors for TGFβ, with the majority of leukocytes also producing TGFβ, thereby making TGFβ a key regulator of the immune system. TGFβ has the unusual capacity to act as both an immuno suppressive and pro-inflammatory trigger (Wahl 1992). During the first stage of an inflammatory response, TGFβ is directly chemotactic for neutrophils, monocytes and T lymphocytes (Wahl *et al.* 1987, Brandes *et al.* 1991, Adams *et al.* 1991), while indirectly augmenting the inflammatory response by

up regulating the production of pro-inflammatory cytokines such as IL-1α, IL-1β, TNFα, PDGF, as well as its own synthesis, from leukocytes and non-lymphohaemopoietic cells such as fibroblasts (Wahl *et al.* 1987, Chantry *et al.* 1989, Elias *et al.* 1991). Conversely, TGFβ inhibits the proliferation of lymphoid cells such as B cells, thymocytes, T cells and large granular lymphocytes (Kehrl *et al.* 1986 and 1989, Ristow 1986, Lee *et al.* 1987, Ellingsworth *et al.* 1988, Wahl *et al.* 1988, Ortaldo *et al.* 1991). In addition to altering leukocyte proliferation and chemotaxis, TGFβ modifies the phenotype of leukocytes dramatically. TGFβ inhibits immunoglobulin secretion by activated B cells (Kehrl *et al.* 1989 and 1991), promotes isotype switching to IgA production (Coffman *et al.* 1989, Defrance *et al.* 1992), and induces the expression of FcγRIII receptors on monocytes (Welch *et al.* 1990). Alterations in *in vitro* cellular immune responses include inhibiting the generation of cytotoxic lymphocytes (Wallick *et al.* 1990), and suppression of natural and lymphokine-activated killing by large granular lymphocytes (Espevik *et al.* 1988, Ortaldo *et al.* 1991). Furthermore, the capacity of macrophages to kill pathogens and "foreign"cells through the production of oxygen and nitrogen intermediates is diminished following exposure to TGFβ (Tsunawaki *et al.* 1988, Ding *et al.* 1990). The *in vivo* effectiveness of TGFβ as an inhibitor of cellular immune responses is highlighted by the ability of highly immunogenic tumour cells to avoid destruction by cytotoxic T lymphocytes and natural killer cells by secretion of large amounts of TGFβ into their local environment (Tada *et al.* 1991).

TGFβ augments antigen-presenting activity by increasing the differentiation of monocytes into macrophages (Kamijo *et al.* 1990), enhancing the GM-CSF-stimulated proliferation of these macrophages (Celada and Maki 1992), while also increasing dendritic cell numbers by inhibiting their loss from apoptosis (Riedl *et al.* 1997). Important in the context of the current studies is the recognition that antigen-presenting cells exposed to TGFβ generally initiate a shift in the phenotype of naive CD4+ T cells away from Th1 cell-mediated immunity, towards Th2 humoral immunity (reviewed by Letterio and Roberts, 1998). Macrophages exposed to the high TGFβ content within the anterior chamber of the eye are reported to trigger a Th2 phenotype in T lymphocytes, a response characterised by the inhibition of cellular immune responses such as immediate and delayed-type hypersensitivity (Meade *et al.* 1992,Wilbanks *et al.* 1992a). Transforming growth factor beta is believed to polarise the immune response towards the

Th2 axis by increasing macrophage production of IL-10 (Maeda *et al.* 1995), Prostaglandin E_2 (Alleva *et al.* 1995), and TGFβ itself (Takeuchi *et al.* 1997).

The generation of $TGF\beta_1$ "knock-out" mice has confirmed the central role that TGFβ plays in immunological homeostasis. Animals homozygous for the disrupted $TGF\beta_1$ gene [$TGF\beta_1$ (-/-)] exhibit no developmental abnormalities, yet within 3-4 weeks of birth exhibit severe inflammation of the skin and mucosal surfaces and die rapidly. Autopsy of these animals reveals grossly enlarged lymph nodes and widespread inflammation (principally lymphocytes and macrophages) in the heart, liver, gastro-intestinal tract, lung, skin and salivary glands (Shull *et al.* 1992, Kulkarni *et al.* 1993). This phenotype may be the result of failure to establish or maintain tolerance to self-antigens. TGFβ has been reported to inhibit the production of CD4+CD8+ T lymphocytes from their CD4-$CD8^{lo}$ precursors (Takahama *et al.* 1994). It is postulated that in the absence of this negative control, CD4+CD8+ cells are generated too rapidly to allow sufficient time for the complete removal of all auto-reactive clones within the thymus. The resulting auto-reactive T cells that have escaped the normal process of clonal deletion are then capable of eliciting autoimmune disease (Letterio and Roberts 1996). Rodents exhibiting the autoimmune diseases relapsing experimental allergic encephalomyelitis and collagen-induced arthritis, animal models for multiple sclerosis and rheumatoid arthritis respectively, have highlighted the importance of TGFβ for the induction of self tolerance. Both of these experimentally induced autoimmune diseases can be prevented, or their severity reduced, by administration of $TGF\beta_1$ (Johns *et al.* 1991, Kuruvilla *et al.* 1991, Racke *et al.* 1991).

f. TGFβ in reproduction

TGFβ is involved in reproductive processes ranging from spermatogenesis to ovulation and embryogenesis (reviewed by Shull and Doetschman 1994).

All three mammalian isoforms of TGFβ are produced by both the somatic and germinal compartments of the testis (Watrin *et al.* 1991). Serotoli cell-derived TGFβ inhibits the production of testosterone by Leydig cells *in vitro* in response to hCG and cAMP (Avallet *et al.* 1987, Morera *et al.* 1988), while increasing the contractility of peri-tubular cells (Ailenberg *et al.* 1990). It appears that TGFβ does not have any significant effect on mature sperm since the addition of TGFβ to human sperm in culture does not

effect their motility, nor their ability to penetrate zone-free hamster oocytes (Naz and Kumar 1991). Furthermore, TGFβ_1(-/-) males that survive to a reproductive age are fertile (although not as fertile as wild type animals; Doeschtmann, *personal communication*), thereby suggesting that TGFβ_1 is not an absolute requirement for normal testicular function.

The ovary is a major site of TGFβ production and action (Benahmed *et al.* 1993). TGFβ has been identified in the culture medium of murine, porcine and human granulosa cells and thecal / interstitial cells, as well as porcine corpora lutea (Skinner *et al.* 1987, Gangrade and May 1990, Chegini *et al.* 1992, Eramaa and Ritvos 1996). TGFβ mRNA expression in rat granulosa and theca / interstitial cells is regulated *in vitro* by FSH and hCG (Mulheron *et al.* 1991), with TGFβ potentiating the mitogenic effect of oestradiol and FSH on rat granulosa cells (Bendell and Dirrington 1988), in addition to increasing their release of oestrogen and progesterone in response to FSH (Adashi and Reshick 1986). The production of progesterone by thecal / interstitial cells is enhanced by TGFβ, whereas hCG stimulation of androsterone production is inhibited (Magoffin *et al.* 1989, Hernandez *et al.* 1990). TGFβ participates in ovulation by accelerating the growth of early follicle-enclosed oocytes (Feng *et al.* 1988), while suppressing the LH-induced maturation of pre-ovulatory oocytes both *in vitro* and *in vivo* (Tsafriri *et al.* 1989, Juneja *et al.* 1996).

The expression of TGFβ in the peri-implantation uterus, in conjunction with its established roles in the control of cell proliferation, differentiation, migration, tissue remodelling, ECM and angiogenesis, are all consistent with it playing a major role in the implantation process. Immunohistochemical and mRNA techniques have localised all three mammalian isoforms of TGFβ to the uterine and vaginal epithelium in the mouse (Tamada *et al.* 1990, Takahashi *et al.* 1994) and endometrial glandular epithelium in the human (Tang *et al.* 1994). TGFβ production by the murine uterine epithelium is up regulated by oestrogen (Das *et al.* 1992, Takahashi *et al.* 1994), while human endometrial TGFβ mRNA and protein expression peaks in the late proliferate and early secretory phases of the menstrual cycle (Chegini *et al.* 1994), with corresponding uterine luminal fluid TGFβ levels rising after ovulation and remaining elevated until just before menstruation (Bulletti *et al.* 1994). This temporal pattern of TGFβ production, combined with the observation that TGFβ is capable of reducing endometrial stromal cell metabolic activity (Tang *et al.* 1994), suggests that TGFβ may be involved in the initiation of endometrial maturation during the transition from proliferative to secretory endometrium.

Using *in situ* hybridisation, it has been reported that $TGF\beta_1$ mRNA is localised primarily to the luminal and glandular epithelium during early murine pregnancy (Tamada *et al.* 1990, Das *et al.* 1992). By the time of implantation (day 4.5 p.c.), $TGF\beta_1$ mRNA becomes diffusely distributed throughout the decidua, and by day 9 of pregnancy its expression within the decidua capsularis is reduced while still being maintained in the decidua basalis. The pre-implantation embryo is also known to be a source of TGFβ production, since murine embryos express TGFβ mRNA from the four cell stage onwards (Paria *et al.* 1992), while culture medium containing early human embryos has been shown to contain bioactive TGFβ (Austgulen *et al.* 1995).

Receptors for TGFβ have been identified on murine pre-implantation embryos from the eight cell stage, with receptor density increasing with further development (Paria *et al.* 1992). Several studies have indicated that TGFβ has a mitogenic action on early pre-implantation murine and bovine embryos *in vitro*, with the addition of TGFβ to culture medium promoting the development of embryos into blastocysts (Paria and Dey 1990, Larson *et al.* 1992, Lim *et al.* 1993). In addition, TGFβ increases trophectoderm production of oncofetal fibronectin, a protein that is postulated to act as a "molecular glue" promoting the attachment of pre-implantation embryos to the uterine epithelium (Feinberg *et al.* 1991 and 1994). Evidence supporting a role for TGFβ in implantation *in vivo* is provided by the observation that administration of neutralising TGFβ antibody to murine day 3.5 pre-implantation blastocysts results in a halving of implantation rates compared to blastocysts exposed to control antibody (Slager *et al.* 1993).

TGFβ is involved in controlling the depth of trophoblast invasion through its anti-proliferative effect on trophoblast, and its ability to initiate remodelling of the decidual ECM, while enhancing the differentiation of invasive trophoblast into non-invasive multi-nucleate giant cells (Dungy *et al.* 1991, Graham and Lala 1991). TGFβ has been localised to the cytoplasm of villous syncytiotrophoblast and extravillous trophoblast throughout pregnancy, with a shift in decidual TGFβ from the ECM to decidual cells with increasing gestation (Lysiak *et al.* 1995). Endoglin, a component of the TGFβ receptor system (Cheifetz *et al.* 1992), and decorin, a natural inhibitor of TGFβ activity (Yamaguchi *et al.* 1990), have been identified within human placental tissue, and are now recognised to play a key role in TGFβ–mediated control of trophoblast invasion (St-Jacques *et al.* 1994). Endoglin levels are low in the vicinity of cytotrophoblast, transiently increased on invading intermediate cytotrophoblast, and present at high concentrations on

syncytiotrophoblast (St-Jacques *et al.* 1994). Decorin is present throughout pregnancy within the ECM of chorionic villi but is absent from decidual tissue (Lysiak *et al.* 1995). The interplay between localisation of TGFβ, endoglin and decorin results in the facilitation of trophoblast invasion early in pregnancy, while inhibiting excessive invasion at later stages. Apart from controlling trophoblast invasion, TGFβ is reported to upregulate hCG and placental lactogen production by murine and human trophoblast *in vitro* (Aoki *et al.* 1991, Yamaguchi *et al.* 1994).

A further role for TGFβ in pregnancy may be the establishment of maternal tolerance to the allogenic conceptus. A group of $CD56^+$ lymphoid cells in human decidua (Clark *et al.* 1994 a), and small granular lymphocytes in murine decidua (Clark *et al.* 1990, Lea *et al.* 1992) are known to produce a suppressor factor similar to $TGF\beta_2$ (with the same biological action but a slightly smaller molecular weight). This $TGF\beta_2$-related molecule is believed to contribute to an immunosuppressive cytokine milieu at the decidual-trophoblast interface, which may prevent cytotoxic immune responses against the semi-allogenic trophoblast. Diminished numbers of $TGF\beta_2$-producing CD56+ decidual lymphocytes have been linked to recurrent pregnancy loss in the human (Lea *et al.* 1995).

1.3.4 Colony stimulating factor

CSF-1, also known as macrophage-CSF (M-CSF), is a 45 kDa homodimeric glycoprotein initially identified as a haemopoietic growth factor required for the proliferation, differentiation, and viability of cells of the mononuclear phagocytic lineages (Sherr 1990). CSF-1 is constitutively produced by fibroblasts, bone marrow stromal cells, thymic epithelial cells, osteoblasts and keratinocytes, but its production can also be induced in monocytes, endothelial cells and lymphocytes (Stanley and Heard 1977, Le *et al.* 1988, Oster *et al.* 1989, Chodakewitz *et al.* 1990).

A role for CSF-1 during pregnancy was suggested by the detection of high concentrations of CSF-1 in the murine uterus early in pregnancy (Bartocci *et al.* 1986), and the presence of the CSF-1 receptor on murine and human trophoblast (Muller *et al.* 1983, Hoshina *et al.* 1985). CSF-1 mRNA and protein are detectable in the mouse reproductive tract throughout pregnancy, with CSF-1 mRNA being localised to the glandular and luminal uterine epithelium (Arceci *et al.* 1989). Production of CSF-1 by the murine uterine epithelium is increased by both oestrogen and progesterone (Pollard *et al.*

1987), as well as being transiently increased for 24 hours following mating, before again rising on day 4 p.c. (Sanford *et al.* 1992, De *et al.* 1993). Uterine CSF-1 levels continue to increase until day 14-15 of pregnancy, when their level is approximately 1000-fold greater than that seen in non-pregnant animals (Rosendaal 1975, Bartocci *et al.* 1986). In the human, patterns of CSF-1 production are similar to the mouse, with the uterine epithelium being the major site of production (Daiter *et al.* 1992).

The CSF-1 receptor is expressed in both murine and human pre-implantation embryos and trophoblast (Arceci *et al.* 1989, Pollard *et al.* 1991, Sharkey *et al.* 1995, Pampfer *et al.* 1992). CSF-1 stimulates the proliferation of murine (Wegmann *et al.* 1989) and human (Jokhi *et al.* 1994 b) trophoblast, induces the differentiation of cytotrophoblast into syncytotrophoblast, and increases hCG and hPL production (Saito *et al.* 1993b). The expression of fibronectin and its associated receptor, the $\alpha5\beta1$ integrin, is upregulated following exposure of human trophoblast to CSF-1 in culture (Omigbodun *et al.* 1998), a process which is hypothesised to be involved in controlling the depth of trophoblast invasion.

Homozygous mutants for the osteopetrotic mutation ($csfm^{op}/csfm^{op}$) are completely devoid of biologically active CSF-1, and provide a useful model for studying the role of this cytokine in reproduction. Female $csfm^{op}/csfm^{op}$ mice have extended oestrus cycles, with no pre-ovulatory surge in oestradiol, and resulting lower ovulation rates compared to wild type controls (Cohen *et al.* 1997). Of those mice that do become pregnant, implantation rates are lower and embryonic wastage is higher than in wild type females (Pollard *et al.* 1991). CSF-1 has been shown to enhance the development of pre-implantation cultured embryos (Pampfer *et al.* 1992), which may help to explain the observed reduction in implantation rates. An alternative explanation is that the depletion and phenotypic alteration in uterine macrophages observed in the absence of CSF-1 (De *et al.* 1993) may have a negative influence on pregnancy outcome. Male $csfm^{op}/csfm^{op}$ mice have reduced fertility due to low testosterone levels, which in turn impedes spermatogenesis and sexual behaviour (Cohen *et al.* 1996). This defect has been attributed to abnormalities in macrophage-Leydig cell interactions.

1.3.5 Leukaemia Inhibitory Factor (LIF)

Leukemia inhibitory factor (LIF) is a 58 kDa heavily glycosylated single chain polypeptide from the IL-6 family of cytokines. Although named for its ability to inhibit the proliferation of a mouse myeloid leukaemic cell line M1 (Hilton *et al.* 1988), LIF is an extremely pleiotrophic cytokine capable of promoting proliferation and phenotypic changes in a wide array of cell types (reviewed by Metcalf 1992).

LIF production by the murine endometrium is largely confined to an oestrogen-mediated surge on day 4 of pregnancy, just before blastocyst implantation takes place (Bhatt *et al.* 1991). In the human, production of LIF by the endometrium peaks during the luteal phase (Cullinan *et al.* 1996). Female mice lacking a functional LIF gene are fertile, yet their blastocysts fail to implant and do not develop to term, despite the fact that these blastocysts are viable and will develop to term if transferred to pseudo-pregnant recipients that do express a functional LIF gene (Stewart *et al.* 1992). Endometrial explants taken from women with recurrent implantation failure have been reported to secrete lower levels of LIF compared to fertile controls (Chaouat *et al.* 1995, Cullinan *et al.* 1996). Both these observations, and the temporal pattern of endometrial LIF expression, suggest that LIF plays an important role in implantation (Stewart 1994). Receptors for LIF are known to be present on pre-implantation embryos (Fry 1992, Charnock-Jones *et al.* 1994, Sharkey *et al.* 1995). Addition of LIF to embryo culture medium enhances blastocyst hatching and trophoblast outgrowth *in vitro* (Robertson *et al.* 1992a, Lavranos *et al.* 1995, Dunglison *et al.* 1996), and results in a superior implantation rate following transfer to pseudo-pregnant females (Lavranos *et al.* 1995).

1.3.6 Interleukin-6 (IL-6)

IL-6 is a 26 kDa cytokine synthesised by monocytes, endothelial cells, fibroblasts and epithelial cells in response to various stimuli such as IL-1, TNFα, IL-2, colony stimulating factors, lipopolysaccaride and products of viral infection (Lee 1992). The immunological roles of IL-6 include the initiation of antibody production by triggering maturation of B lymphocytes into plasma cells, initiating the differentiation of monocytes into macrophages, and increasing the ability of macrophages to activate T lymphocytes (Ruppert and Peters 1991).

Murine and human uterine epithelial and stromal cells have been identified as sites of IL-6 production (Jacobs *et al.* 1992, Tabibzadeh and Sun 1992). Murine endometrial production of IL-6 is increased by oestrogen (Jacobs *et al.* 1992), and following exposure to semen (Robertson *et al.* 1992b). Uterine IL-6 mRNA dramatically increases following mating, falls on day 2 of pregnancy, before rising on day 3 and continuing to remain elevated for the rest of the pregnancy (Sanford *et al.* 1992). IL-6 may be involved in the timing of blastocyst implantation since its addition to murine blastocysts in culture inhibits their attachment and outgrowth on laminin-coated culture dishes (Jacobs *et al.* 1992). The surge in IL-6 production following mating may serve to augment maternal immune response to paternal antigens / pathogens introduced at the time of mating, since intrauterine administration of IL-6 to ovariectomised rats has been reported to increase antigen presentation and antibody production by the uterine mucosa (Prabhala and Wira 1995). IL-6 has been reported to initiate capacitation of human sperm, which raises the possibility that IL-6 contained within cervical mucous may enhance the fertilising capacity of ejaculated sperm (Naz and Kaplan 1994, Naz and Butler 1996).

1.3.7 Interleukin 1 (IL-1)

Interleukin 1 exists as two 17 kDa ligands, IL-1α and IL-1β. This pair of cytokines share 26% sequence homology, act through a common receptor (IL-1Rt1), and usually produce similar biological actions (diGiovine and Duff 1990). Binding of IL-1 to its receptor is blocked by a natural antagonist, IL-1Ra. IL-1 is principally produced by cells of the monocyte lineage in response to pro-inflammatory signals (TNFα, LPS or IL-1 itself), with its principal activity being to promote T cell activation following antigen exposure.

In the murine uterus, the production of both IL-1α and IL-1β has been localised to endometrial macrophages and endothelial cells, with production transiently peaking 24 hours after mating (Sanford *et al.* 1992), before again increasing on days 4-5 of pregnancy (De *et al.* 1993). Receptors for IL-1 are upregulated on the uterine epithelium on day 4-5 of pregnancy, especially in the vicinity of the implanting blastocyst. In the human, IL-1α and IL-1β production is localised to the uterine epithelium, as well as endometrial lymphoid, endothelial and stromal cells, with the epithelial IL-1 receptor density reaching maximal levels during the luteal phase (Simon *et al.* 1993a, 1993b). Both murine and human pre-implantation embryos are known to produce IL-1 (Austgulen *et al.* 1995), with

embryo-derived IL-1 being capable of increasing IL-1 receptor density in the adjacent human endometrium (Simon *et al.* 1994b).

A critical role for IL-1 in implantation is suggested by the observation that intra-peritoneal injection of an IL-1 antagonist between days 3-9 of pregnancy is able to almost completely block implantation in the mouse (Simon *et al.* 1994a). This observation is not due to a direct toxic effect, since pre-implantation embryos cultured in the presence of the IL-1 antagonist developed normally and are able to attach to fibronectin-coated culture dishes. Furthermore, a positive correlation has been observed between high levels of IL-1 production by human pre-implantation embryos and their successful implantation following embryo transfer (Sheth *et al.* 1991). The precise manner by which IL-1 mediates an effect on implantation is unknown, but may be related to its ability to increase cytotrophoblast invasion of decidual tissue (Librach *et al.* 1994).

1.3.8 Tumour necrosis factor alpha (TNFα)

Tumour necrosis factor alpha (TNFα) is a 51 kDa homotrimer protein principally produced by activated macrophages; although antigen-stimulated T cells, activated NK cells and mast cells may also secrete this cytokine. It is a pleiotrophic cytokine that exerts a variety of effects including pro-inflammatory activity, growth promotion, growth inhibition, angiogenesis and cellular toxicity (Beutler and Cerami 1989).

In the cycling rodent uterus, synthesis of TNFα is localised primarily to the uterine luminal and endometrial gland epithelium, with stromal macrophages and myometrial fibroblasts also being a source of production (Yelavarthi *et al.* 1991, De *et al.* 1992). TNFα production is transiently increased following mating (Sanford *et al.* 1992), and is under the control of sex steroids, since uteri from ovariectomised mice do not produce TNFα without the addition of oestrogen or progesterone (Roby and Hunt 1994). Human uterine epithelium produces TNFα predominantly during the mid-proliferative and late luteal phase of the menstrual cycle (Hunt *et al.* 1992), with endometrial expression of TNFα receptors peaking during the late luteal phase (Terranova *et al.* 1995). This temporal pattern of TNFα expression is proposed to result in the initiation of endometrial cytolysis resulting in menstruation (Tabibzadeh *et al.* 1995).

Production of TNFα by the murine pregnant uterus is elevated compared to the non-pregnant state, with uterine epithelial TNFα mRNA being increased immediately following implantation (Hunt *et al.* 1993), then falling until day 14 of pregnancy, when it again rises. It has been suggested that decidual macrophages at the fetal-maternal interface may play an important role in controlling trophoblast invasion through the release of TNFα, which then activates cytolytic NK cells against trophoblast, thereby preventing overwhelming invasion of maternal tissue (Parr *et al.* 1995). Stress-induced abortion in mice (Arck *et al.* 1995) and endometriosis (Syrop *et al.* 1987), a condition associated with human infertility, have both been associated with elevated levels of TNFα. These observations raise the possibility that high levels of TNFα may initiate NK cell aggression against trophoblast, ultimately resulting in pregnancy failure.

1.3.9 Other growth factors implicated in early pregnancy

Transforming growth factor alpha (TGFα) and epidermal growth factor (EGF) belong to the same growth factor family, share 30% sequence homology, and in general produce similar biological actions via a shared EGF receptor (Massague 1983). TGFα production has been localised to the uterine epithelium of the mouse, with a transient peak in production occurring on days 1 and 4 of pregnancy (Paria *et al.* 1994). No TGFα mRNA is present in the cycling human endometrium, but significant amounts of TGFα transcript are expressed in the pregnant decidua under the positive influence of progesterone (Taga *et al.* 1997). A potential role for TGFα in the control of implantation is suggested by the observation that injection of TGFα-neutralising antibodies into the lumen of a pregnant rat just prior to blastocyst implantation can significantly decrease implantation, whereas injection of rTGFα into the uterus of a pregnant, ovariectomised, progesterone-treated female can initiate decidualisation and implantation in 50% of cases (Tamada *et al.* 1997). Furthermore, the inappropriate expression of a human TGFα transgene in the mouse uterus can down regulate the expression of TGFβ receptors in the endometrium, and delay the initiation of implantation (Das *et al.* 1997).

The production of EGF by the murine uterine epithelium mirrors that of TGFα, with peak levels occurring on day 1 and 4 of pregnancy due to a pre-implantation surge in plasma oestrogen (DiAugustine *et al.* 1988, Huet-Hudson *et al.* 1990). The EGF receptor

has been identified on the murine blastocyst (Wiley *et al.* 1992), with both EGF and TGFα being reported to facilitate pre-implantation embryonic development *in vitro*. When added to culture medium, these growth factors accelerate expansion of blastocyst cell numbers, enhance hatching from the zona pellucida, and stimulate amino acid uptake and incorporation by the blastocyst (Wood and Kaye 1989, Paria and Dey 1990).

Platelet activating factor (PAF), a glycerophospholipid responsible for triggering histamine release from platelets (Harper 1989), is produced by human and rodent uteri (Yasuda *et al.* 1986, Alecozay *et al.* 1989) and embryos (O'Neill 1985, Collier *et al.* 1988). PAF is believed to play a role in murine implantation, since administration of PAF antagonists over the first four days of pregnancy can reduce the proportion of embryos implanting by day 8 (Spinks and O'Neill 1988). PAF may mediate this beneficial effect on implantation by initiating decidualisation, since injection of PAF into the uterine horns of pseudopregnant rats can initiate a decidual response, possibly by releasing prostaglandin and histamine (Acker *et al.* 1989). PAF may also have a direct embryotrophic effect, since human and murine embryos cultured in the presence of PAF exhibit a higher rate of implantation following embryo transfer (O'Neill *et al.* 1989, Ryan *et al.* 1989).

Insulin-like growth factors (IGFs) I and II, and their binding proteins, are produced by both the murine and human endometrium (Heyner *et al.* 1989, Liu *et al.* 1995). Control of IGF production by the murine uterus is under the control of sex steroids, with oestrogen stimulating uterine epithelial cell synthesis, while progesterone increases stromal cell production (Murphy and Ghahary 1990). IGF production increases during murine pregnancy (Kapur *et al.* 1992) and is believed to stimulate uterine stromal growth (Brigstock *et al.* 1989), as well as accelerate blastocyst protein synthesis (Harvey and Kaye 1991). The IGF system may play a role in controlling trophoblast growth, since the migration of human first trimester invasive trophoblast is stimulated by IGF-II and IGFBP-1 *in vitro* (Irving and Lala 1995).

Interferon gamma (IFNγ), produced by NK cells and T lymphocytes, can modulate the antigenicity of tissue by regulating MHC class I and II antigen expression. MHC class I antigen expression on human trophoblast is upregulated by IFNγ (Grabowska *et al.* 1990), with IFNγ also increasing NK cell cytotoxic activity against trophoblast (Haimovici *et al.* 1991). It appears that IFNγ may be involved in the control of trophoblast outgrowth by inhibiting excessive trophoblast invasion.

1.4 The female reproductive tract as an immunological organ

1.4.1 Introduction

The murine and human female reproductive tracts contain a large and diverse population of leukocytes. Interactions between the female reproductive tract and semen, as well as fluctuations in sex steroid levels, result in changes in cytokine production, which in turn alter the composition and phenotype of leukocytes contained within the female reproductive tract (De *et al.* 1990 and 1991, Thompson *et al.* 1992, Robertson *et al.* 1996). The female reproductive tract is thus a competent site for the initiation of local and systemic immune responses against microbial pathogens and seminal antigens introduced at the time of mating (Parr and Parr 1985), as well as antigens expressed on the semi-allogenic conceptus. It is now recognised that the nature of an immune response towards paternal antigens shared by semen and the semi-allogenic conceptus may have an impact on the growth and survival of the embryo (Thaler 1989, Robertson *et al.* 1997). This section of the literature review will focus on the manner that reproductive tract leukocytes influence reproductive function in mice and humans.

1.4.2 Macrophages

Macrophages are multi-functional cells which differentiate from blood-borne monocytes. Macrophages principally serve four main functions: they phagocytose and destroy invading organisms, process and present foreign antigens to T lymphocytes, produce inflammatory substances such as cytokines, prostaglandins, and histamine and release enzymes capable of ECM digestion and tissue remodelling.

Macrophages are distributed throughout the endometrium and myometrial connective tissue in naturally cycling mice and women. The density and distribution of macrophages change according to fluctuations in ovarian sex steroid hormone profiles, with maximal numbers of macrophages accumulating subjacent to the murine uterine epithelium during oestrous and dioestrous-I (oestrogen-dominated), with lower endometrial densities being observed during the progesterone-dominated phase of dioestrous-II (De *et al.* 1990, Huang *et al.* 1995). The influx of uterine macrophages is believed to be mediated by steroid-induced changes in uterine epithelial cell production of cytokines such as CSF-1, GM-CSF, TNFα and IL-6 (Hunt and Robertson 1996), as well as

chemokines such as RANTES and MIP-1α (Pollard *et al.* 1998, Robertson *et al.* 1998). In the human, endometrial macrophage numbers are relatively low during the proliferative phase, but increase in the secretory phase, and peak just before the onset of menstruation (Bulmer 1995). The tissue remodelling activities of these macrophages may contribute to the initiation of menstruation.

During murine and human pregnancy, macrophages increase in density relative to other types of uterine cells (Hunt *et al.* 1985, Bulmer 1995). Shortly following mating in the mouse, interaction between the uterine epithelium and an unidentified seminal protein results in an upregulation in epithelial production of a number of cytokines (IL-1, TNFα, IL-6, GM-CSF, and CSF-1), as well as chemokines (RANTES, MIP-1α, MIP-1β), which in turn attract large numbers of macrophages into the endometrium, principally localised subjacent to the luminal epithelium (Robertson *et al.* 1990, Sanford *et al.* 1992, Brandon 1993, Robertson *et al.* 1998b). By day 3 of pregnancy, macrophage numbers decline, with the remaining macrophages being evenly distributed throughout the entire endometrium. By day 4.5 of pregnancy, decidual macrophages become excluded from the primary decidual sites directly beneath the implanting embryo, instead being relegated to the endometrial tissue between implantation sites and the myometrium (Hunt 1994, Brandon 1995). Increasing progesterone levels after ovulation have been implicated in terminating the post-mating influx of macrophages, since the administration of RU486, a progesterone antagonist, is capable of prolonging the post-mating inflammatory response in rats (Kachkache *et al.* 1991). The pattern of macrophage distribution at human implantation sites has not been studied due to ethical restrictions, however macrophages are known to be spatially associated with first trimester placental villi and the fetal membranes throughout pregnancy (Lessin *et al.* 1988, Hu *et al.* 1992).

Murine studies indicate that macrophages pass through two distinct phenotypic phases during pregnancy. Shortly after mating (day 1-2 of pregnancy), macrophages are in an activated state, characterised by increased membrane expression of MHC Class II antigen and enhanced antigen-presenting capacity (Hunt and Robertson 1996). The post-mating surge in uterine epithelial cell production of GM-CSF and TNFα is likely to be responsible for the change in macrophage phenotype. The purpose of this activation is unclear, but it is hypothesised that it may serve to augment removal of pathogenic bacteria from the uterus introduced at the time of mating (Parr and Parr 1985), as well as facilitate the processing and presentation of paternal antigens contained within the ejaculate (Shaya *et al.* 1981). By day 3 of pregnancy, uterine epithelial cell production of GM-CSF

declines, which is associated with a switch in macrophages to an immuno-suppressive phenotype. It is postulated that by day 3 of pregnancy the relative increase in uterine CSF-1 compared to GM-CSF results in the initiation of a immunosuppressive macrophage phenotype, as has been reported to occur in the lung (Bilyk and Holt 1993 and 1995). Macrophages taken from peri-implantation or later stage murine uteri are capable of inhibiting lymphocyte proliferation (Hunt *et al.* 1984), possibly by their release of PGE_2 (Tawfik *et al.* 1986), TGFβ (Chen *et al.* 1993), or a decline in the production of immunostimulatory molecules such as TNFα (Yelavarthi *et al.* 1991). Progesterone is reported to increase human placental macrophage production of PGE_2 (Yagel *et al.* 1987), while murine placenta releases IL-4, which blocks macrophage TNFα production (Delassus *et al.* 1994).

The existence of immunosuppressive macrophages at the maternal-placental interface may prevent induction of adverse immune reactions against the semi-allogenic conceptus. Failure of uterine macrophages to switch to an immunosuppressive phenotype, as measured by their persistent production of TNFα and nitric oxide, is associated with an increased rate of early fetal loss in the abortion-prone CBA/J x DBA/2 mating combination (Duclos *et al.* 1995).

1.4.3 Neutrophils

Neutrophils are the immune system's first line of defence, being drawn into a site of infection or trauma within hours under the influence of cytokines such as GM-CSF, IL-1, TNFα, TGFβ and IL-8, as well as inflammatory mediators such as leukotrienes and chemokines. Exposure of the murine endometrium and the human cervix to semen has been reported to result in a rapid, yet short-lived, influx of neutrophils (Pandya and Cohen 1985, De *et al.* 1991, Thompson *et al.* 1992, Brandon 1993). It is proposed that the prime function of this neutrophil influx is to remove bacteria and dead or immobile sperm introduced at the time of mating, since murine uterine luminal neutrophils have been observed to contain phagocytosed bacteria and sperm following mating (Parr and Parr 1985). The murine post-mating endometrial neutrophilia declines within 24-48 hours, with neutrophils then accounting for less than 3 % of all uterine cells for the remainder of the pregnancy (De *et al.* 1991). It is presently uncertain if neutrophils play any further role in pregnancy in the absence of intrauterine infection. A sub-epithelial accumulation of neutrophils has been observed beneath implantation sites in the mouse (Smith and Wilson

1974, Finn and Pope 1991), raising the possibility that neutrophils may play some role in murine implantation. However, the depletion of neutrophil numbers with neutrophil specific antibodies or methotrexate during early rat pregnancy has been reported to have no effect on implantation (Orlando and Kennedy 1993), thereby indicating that any role that neutrophils play in rat implantation is not essential. However, implantation in rats does differ from implantation in mice, since there is no accumulation of neutrophils underlying the implantation site (Rogers *et al.* 1992). Therefore, the role of neutrophils in murine implantation remains speculative.

1.4.4 Eosinophils

Eosinophils phagocytose and kill ingested micro-organisms, while dampening inflammation by releasing histaminase (Zeiger *et al.* 1976), which inactivates histamine released by basophils and mast cells during inflammation.

Eosinophils are found in abundance in the cycling and pregnant rodent endometrium (Paul *et al.* 1967), peaking around oestrous (Ross and Klebanoff 1966), yet they are sparsely distributed in both the pregnant and non-pregnant human endometrium (Kamat and Isaacson 1987, Bulmer *et al.* 1988, Starkey *et al.* 1988). Like other uterine leukocytes, eosinophil numbers are exquisitely sensitive to fluctuations in sex steroid hormone levels. Ovariectomy of rats results in a complete depletion of endometrial eosinophils, with administration of oestrogen causing their reappearance, and progesterone antagonising this oestrogen-initiated influx (Tchernitchin *et al.* 1974). Cytokines and chemokines such as IL-5, GM-CSF and eosinophil chemotactic factor (ECF) are likely to control endometrial eosinophil numbers. The production of ECF is upregulated by oestrogen and down-regulated by progesterone (Lee *et al.* 1989). Furthermore, injection of rGM-CSF into the uterine lumen of ovariectomised mice evokes a massive influx of eosinophils (Robertson *et al.* 1994).

Very little information is available concerning the movement of eosinophils during pregnancy apart from the observation that endometrial eosinophil numbers are increased following mating in the mouse (Robertson *et al.* 1994), yet are not present late in gestation in the rat (Ross and Klebanoff 1966). The release of PAF by eosinophils may benefit pre-implantation embryo development (Ryan *et al.* 1989), or be involved in decidualisation (Spinks and O'Neill 1988). However, an important role for eosinophils in murine

reproduction is unlikely, since eosinophil deficient IL-5 "knock-out" mice have normal pregnancy outcomes (Robertson, *unpublished findings*).

1.4.5 T lymphocytes

The T cell population is heterogenous with respect to both functional capabilities and cell-surface phenotype. Broadly speaking, T cells are divided into helper cells (Th cells) which promote cell-mediated (Th1) or antibody-mediated (Th2) responses through the release of appropriate cytokines, and cytotoxic cells (Tc cells), which lyse antigen-bearing target cells.

In the cycling or pregnant murine uterus, T cells comprise only a small proportion of the total lymphocyte population, even in the presence of a strong T cell stimulus such as a local Listeria infection (Head 1987, Redline and Lu 1988 and 1989). Cytotoxic T cells are almost completely absent from the murine endometrium. Small numbers of CD8+ suppressor T cells accumulate within the murine endometrium just prior to implantation and persist throughout pregnancy (Clark *et al.* 1989, Beaman and Hoversland 1988). It is proposed that the Th2 immune bias of pregnancy (as discussed in section 1.5) may be responsible for the predominance of T suppressor cells within the decidua. However, the presence of T suppressor cells is not vital to the success of murine pregnancy since T cell-deficient *nude* mice exhibit normal fertility (Croy 1994).

T cells account for approximately 10% of human endometrial leukocyte numbers (Starkey *et al.* 1991), with their density and distribution remaining relatively constant throughout the menstrual cycle and pregnancy. While no pregnancy-related changes in T cell numbers have been observed, various phenotypic changes have been recorded. These include: down regulation of the CD3 T cell receptor (Chernyshov *et al.* 1993, Morii *et al.* 1993), a marked skewing in the T suppressor: T helper cell numbers, with almost all uterine intra-epithelial T cells being of the T suppressor phenotype (Pace *et al.* 1991), and negligible expression of IL-2 receptors on decidual T cells, thereby limiting their clonal expansion in response to antigenic challenge (Saito *et al.* 1992). Decidual T cells obtained from women experiencing recurrent spontaneous abortion exhibit reduced production of LIF and Th2 cytokines (IL-4 and IL-10) compared to decidual T cells derived from healthy women undergoing elective pregnancy termination (Piccinni *et al.* 1998), suggesting a role for T cells in the maintenance of human pregnancy.

Recently a subset of T lymphocytes, the γδ T cell, have received attention in the field of reproductive immunology (Clark *et al.* 1997). The γδ T cell is located primarily within epithelial tissue, and is believed to play a special role in the generation of immunological tolerance, since these cells have reduced cytotoxic activity compared to systemic αβ T cells (Morita *et al.* 1991). This reduction in cytotoxicity is due to the limited ability of γδT to recognise allogenic MHC antigens and mount a destructive immune response (Hunig *et al.* 1989, Carbone *et al.* 1991, Vaessen *et al.* 1991).

Both the human and murine reproductive tract contain γδ T cells. The number of γδT cells increases 100-fold during murine pregnancy (Heyborne *et al.* 1992), with the majority of these cells being localised adjacent to the trophoblast at the fetal-maternal interface (Heyborne *et al.* 1994). A similar increase in decidual γδ T cells has been observed in human pregnancy (Mincheva-Nilsson *et al.* 1992). It has been postulated that a major role for these γδ T cells is to block the activation of αβ T cells at the fetal-maternal interface, since decidual γδ T cells have been shown to suppress the proliferation of uterine αβ T cells in a one-way mixed allogenic lymphocyte reaction (Suzuki *et al.* 1995). This inhibition is principally mediated by the release of $TGF\beta_1$ and $TGF\beta_2$ from the decidual γδ T cells. Furthermore, it has been reported that the cell-surface antigen required for activation of γδ T cells is not MHC restricted, but rather a non-polymorphic unidentified molecule conserved across murine and human trophoblast populations (Heyborne *et al.* 1994). The interaction between decidual γδ T cells and trophoblast may therefore result in the release of immunosuppressive molecules such as TGFβ, which in turn may help prevent destructive αβT cell responses at the fetal-maternal interface.

1.4.6 B lymphocytes

B lymphocytes, the precursor to antibody-producing plasma cells, are a relatively minor population within the murine endometrium. Endometrial plasma cell numbers appear to be under the control of ovarian steroids, since peak numbers occur at pro-oestrous, with a subsequent fall at dioestrous (Rachman *et al.* 1983). The dominant antibody isotype produced within the murine female reproductive tract is IgA, with IgG also being produced in considerable amounts (Brandtzaeg 1989). The secretion of immunoglobulin by plasma cells within the rat uterus is under ovarian steroid control, with polymeric IgA and its receptor, as well as IgG, being actively secreted into uterine

secretions under the influence of oestrogen (Wira and Sandoe 1977). In contrast, oestradiol has an inhibitory effect on the secretion of IgA and IgG into cervical-vaginal secretions (Wira and Sullivan 1985). Immunoglobulin transport across the uterine epithelium also appears to be controlled by an unknown embryo-derived factor, since an efflux of antibody is seen on day 4 of pregnancy in the presence of an embryo, but not during pseudo-pregnancy (Rachman *et al.* 1986).

The female reproductive tract of the mouse is capable of functioning as an inductive site for the generation of antibody responses. Delivery of replicating micro-organisms to the vagina or the uterine lumen results in the secretion of IgA and IgG throughout the reproductive tract, at concentrations capable of providing a good local protective immune response (Wira and Sandoe 1989, Staats *et al.* 1994). The delivery of non-viable micro-organisms or soluble protein antigens to the female reproductive tract without an adjuvant generally fails to elicit an antibody response (Parr 1988, Staats *et al.* 1994). Antibody responses initiated by the introduction of sperm at the time of mating, or against placental antigens, are generally weak or non-existent in the mouse, and predominantly of the non-complement fixing IgG_1 isotype (Krupen-Brown and Wachtel 1979, Roe and Bell 1982, Bell 1984). This weak antibody response to sperm challenge at the time of mating is probably due to the presence of immunosuppressive components within seminal plasma.

In the human endometrium, a small population of B cells are present in the basal layer of the endometrium throughout the menstrual cycle and during pregnancy (Loke and King 1995). As B lymphocytes are relatively rare, and plasma cells are absent without active infection, local humoral immune responses towards the trophoblast are very uncommon (Kabawat *et al.* 1985). The human cervix is more readily capable of eliciting humoral immune responses since it contains a relatively high number of plasma cells beneath the endocervical epithelium (Bulmer and Fox 1995).

1.4.7 Natural Killer cells

Natural Killer cells (NK cells) are a lymphocyte lineage distinct from T and B lymphocytes. NK cells do not express either the TCR or CD3 molecules, and therefore their activation is not limited by a requirement for antigen expression in the presence of MHC-bearing antigen-presenting cells, nor by prior sensitisation to the eliciting antigen. The primary function of NK cells is to act as a first line of defence against microbial

pathogens (particularly viruses), eliminate tumour cells, and release cytokines which modify haemopoiesis and immune functions (Trinchieri 1989). NK cells differentiate self from non-self, lysing any cells that have diminished MHC class I surface expression due to malignant transformation or viral infection, or which express MHC that is different from that expressed by the host (Ljunggren and Karre 1990).

Substantial numbers of NK cells are found in the murine cycling endometrium, with no influx being noted with change in oestrus cycle status or following mating (Parr *et al.* 1991). During early pregnancy, a population of NK cells, referred to as granulated metrial gland cells, congregate at implantation sites between the placenta and the uterine wall (mesometrial triangle). NK cells are known to be capable of killing trophoblast *in vitro* (Stewart and Mukhtar 1988), as well as to secrete placental growth-promoting cytokines such as GM-CSF and CSF-1 upon stimulation with IL-2 (Trinchieri 1989). The presence of high levels of TGFβ, IL-10 and PGE_2 at the fetal-maternal interface may prevent lysis of the trophoblast by uterine NK cells, while stimulating the production of placental growth-promoting cytokines (Lala *et al.* 1990, Hsu *et al.* 1992, Reiter 1993). Transgenic *tgε26* mice, which have greatly reduced numbers of granulated metrial gland cells in their uterus, have been reported to have smaller litter sizes, lighter fetal and placental weights, and a higher rate of perinatal mortality compared to their wild type control (Guimond *et al.* 1996). Restoration of uterine NK cell numbers by bone marrow transplantation from donor *scid/scid* mice normalises placental function in tg*e*26 females, while also improving fetal weight and viability (Guimond *et al.* 1998), thereby highlighting the importance of uterine NK cells in murine pregnancy.

Endometrial NK cells, often called large granular lymphocytes, are present within the human endometrium in low numbers during the proliferative phase, but their numbers increase dramatically during the secretory phase under the influence of increasing serum progesterone levels (Hamperl and Hellwey 1958, King and Loke 1991). During early pregnancy, endometrial NK cells are concentrated in areas adjacent to trophoblast (Bulmer *et al.* 1991). Human decidual NK cells, like their murine counterparts, may assist in controlling placental development, since they can also secrete large amounts of GM-CSF and CSF-1 (Saito *et al.* 1993c, Jokhi *et al.* 1994b), as well as lyse trophoblast following stimulation with IL-2 (King *et al.* 1989, King and Loke 1990).

1.4.8 Dendritic cells

Dendritic cells are bone marrow-derived cells of the monocyte lineage that play an important role in priming T cell responses. Dendritic cells have limited phagocytic capacity, but are highly efficient at capturing and presenting antigens to T lymphocytes because of their abundant MHC class II antigen expression, as well as their ability to store antigen on their membrane surface for prolonged periods of time (reviewed by Hart 1997).

The rodent endometrium is well-endowed with dendritic cells (Head and Gaede 1986, Searle and Matthews 1988), with a influx being observed during oestrous under the influence of rising oestrogen levels (Zheng *et al.* 1988) and following mating (Robertson, *unpublished findings*). Uterine dendritic cells in cycling mice are present as an immature phenotype, with limited antigen-presenting capacity (Hudson and Robertson 1996). The increase in uterine epithelial cell GM-CSF production following mating has been implicated in drawing dendritic cells into the murine uterus (Robertson *et al.* 1994), as well as switching their phenotype to a mature antigen-presenting state. GM-CSF upregulates dendritic cell expression of MHC class II antigen and the co-stimulatory molecule B7-2, thereby initiating antigen-presenting capacity in these cells (Robertson *et al. unpublished findings*). The density of dendritic cells in the endometrial stroma of the rat declines shortly after mating, particularly in decidualised areas adjacent to the implantation site (Head 1987). At present it is uncertain what role dendritic cells play in murine pregnancy, however it would appear that they are ideally suited to process ejaculate antigens introduced into the female reproductive tract at mating.

Dendritic cells are relatively abundant in the lower reproductive tract of the human female. They are present at their highest density within the ecto-cervical epithelium, where they are believed to play an important role in the eradication of sexually transmitted diseases (Morris *et al.* 1983, Edwards and Morris 1985, White *et al.* 1997). The vaginal mucosa also contains significant numbers of dendritic cells, estimated to account for as many as 5% of the total number of cells in the vaginal epithelium (Bjercke *et al.* 1983). Endometrial dendritic cells are present within both the pregnant and non-pregnant human endometrium, however little is known of their function in reproductive events (Kamat and Isaacson 1987, Dorman and Searle 1988).

1.4.9 Non-professional antigen presenting cells

Keratinocytes have the capacity to act as non-professional antigen-presenting cells (Nickoloff and Turka 1994), a role that is likely to be important in women since the majority of the ejaculate is deposited in the vicinity of cervical / vaginal keratinocytes.

Human uterine epithelium has been reported to express CD1d antigen (Canchis *et al.* 1993), a non-classical MHC class I protein involved in the presentation of antigens to T cells (reviewed by Blumberg *et al.* 1995). It is therefore possible that the uterine epithelium may also be directly involved in antigen presentation.

1.5 The fetal allograft - strategies for avoidance of immune rejection

1.5.1 Introduction

In order to enable efficient transfer of nutrients, gases and waste between a mother and her fetus, mammalian placentation requires the intimate juxtaposition of trophoblast with maternal tissues. This close association exposes trophoblast to recognition by the maternal immune system, a situation that is potentially damaging given that the majority of antigens on trophoblast are paternally derived (Surani *et al.* 1987). Furthermore, the fetus inherits major histocompatibility antigens (MHC) from both its father and mother, a situation that places the fetus in danger of being recognised as foreign, and therefore destroyed by the maternal immune system.

Peter Medwar, a pioneer in the field of reproductive immunology, was first to recognise that successful mammalian pregnancy defies the rules of transplantation immunology (Medwar 1953). Medwar hypothesised three ways by which the semi-allogenic conceptus could avoid maternal immune rejection. His first hypothesis was that the fetus was antigenically immature and therefore incapable of stimulating a maternal immune response; his second hypothesis was that the fetus was anatomically separated from its mother; and his third hypothesis suggested that the maternal immune system was relatively inert during pregnancy.

Over the years each of Medwar's hypotheses have been challenged, as new insights have emerged concerning the immunological relationship between mother and fetus. The following discussion will use the framework of Medwar's hypotheses to outline the

modern understanding of the mechanisms preventing immunological rejection of the mammalian semi-allogenic conceptus.

1.5.2 Hypothesis one: Is the fetus antigenically immature and therefore incapable of stimulating an immune response?

The major histocompatibility complex (MHC) is a group of extremely polymorphic genes encoding for cell surface antigens that play a central role in distinguishing self from non-self, with these antigens being principally responsible for mediating immune rejection of tissue transplants between unrelated donors and recipients. At conception, the future individual receives a copy of both parent's MHC genes, with both sets of genes being expressed in the developing fetus (co-dominant expression). The presence of mRNA encoding for MHC class I antigens has been identified in both the trophectoderm and inner cell mass of mouse blastocysts (Sprinks *et al.* 1993, Arcellana-Panlilio and Schultz 1994), and the translation of this mRNA into protein has been confirmed using immunocytochemical and ELISA techniques (Searle *et al.* 1976, Goldbard *et al.* 1985, Warner and Gollnick 1993), as well as the observation of MHC-dependent cell-mediated cytotoxicity (Ewoldsem *et al.* 1987). Classical paternal-derived class I MHC antigens disappear from the trophectoderm around the time of implantation (Leclipteux and Remacle 1983), but reappear on placental trophoblast by the tenth day of gestation (Raghupathy *et al.* 1981, Head and Billingham 1984). It is currently not understood what controls the temporal expression of class I antigens on the murine trophoblast, nor the role this plays in pregnancy outcome.

In addition to classical polymorphic class I antigens, murine pre-implantation embryos express the non-polymorphic class Ib gene termed Ped, a gene located in the Q region of the H-2 complex in the mouse (Warner *et al.* 1987). This gene encodes for the Qa-2 antigen, which is expressed on the surface of murine pre-implantation embryos. Qa-2 is believed to influence the growth and development of embryos, since those embryos that express this antigen cleave at a faster rate than those that lack expression. Furthermore, the presence of the Ped allele results in larger litter sizes and heavier pups (Warner *et al.* 1991). It is not presently understood how the expression of this non-polymorphic class I antigen can effect pre-implantation embryo development, but it has been suggested that a glycosylphosphosphatidylinositol linkage within the Qa-2 antigen may be responsible for

transmitting a mitogenic signal to embryos in a similar fashion to that reported for transmission of mitogenic signals to T cells (Xu *et al.* 1993).

Immunohistochemical studies of the human placenta have revealed that syncytiotrophoblast, that portion of the placenta in direct contact with the maternal circulation, and the underlying villous cytotrophoblast, are negative for classical polymorphic class I antigens (HLA-A, HLA-B). Interestingly, *in situ* hybridisation studies have revealed that villous cytotrophoblast does contain mRNA for classical class I antigens, but this mRNA is not translated into protein, and therefore is not expressed as a membrane antigen (Hunt *et al.* 1990). Invasive extravillious cytotrophoblast, also in direct contact with the maternal circulation, has been shown to express non-classical MHC class I antigens such as HLA-G and HLA-E (Ellis 1990, Geraghty *et al.* 1993, King *et al.* 1996), and the classical class I antigen HLA-C. The expression of HLA-G is reported to be restricted to "immunologically privileged" sites such as the placenta and the anterior chamber of the eye (Shukla *et al.* 1990, Ishitani and Geraghty 1992). Non-classical MHC class I antigens are peculiar since they exhibit very little polymorphism within a population, and therefore are unlikely to initiate a conventional graft rejection response (Geraghty *et al.* 1993, Morales *et al.* 1993). Since the absence of MHC class I antigen on a cell normally results in its destruction by NK cells, the presence of non-polymorphic HLA-G on trophoblast may serve to protect it from destruction by decidual NK cells, without initiating a conventional destructive $\alpha\beta$ T cell response (Chumbley *et al.* 1994, Pazmany *et al.* 1996). Recently it has been reported that transfecting HLA-G into allogenic stimulator cells promotes a Th2 bias in cytokine production in respondent peripheral blood monocytes (Maejima *et al.* 1997). This process may also favour the growth and survival of the fetal allograft (Clark 1997).

Human blastocysts have been reported to express HLA-G antigens (Jurisicova *et al.* 1996). These investigators noted that only 40% of human blastocysts expressed HLA-G, yet those embryos that were HLA-G positive developed more rapidly in culture than their HLA-G negative counterparts. This finding could be interpreted to suggest that HLA-G may be the human homologue of the murine Qa-2 antigen.

Class II MHC antigens are not expressed on murine or human trophoblast in direct contact with the maternal immune system (Raghupathy *et al.* 1981, Bulmer and Johnson 1985). The importance of this lack of expression is highlighted by the observation that upregulation of trophoblast MHC class II antigen expression by treatment with IFNγ results in immune-mediated abortion in mice (Vassiliadis *et al.* 1994). Furthermore,

human placental tissue obtained from spontaneous miscarriages is MHC class II positive, whereas placental tissue taken from viable pregnancies can be MHC class II antigen negative (Athanassakis *et al.* 1995).

Medwar's first hypothesis is not sustainable, since contemporary research has confirmed that the fetus does express MHC antigens capable of being recognised by the maternal immune system. Contrary to Medwar's original paradigm, maternal awareness of the fetal-placental unit may actually enhance reproductive outcome, rather than be destructive. It has been reported that MHC-incompatible murine (Billington 1964, James 1965) and human (Jazwinska *et al.* 1987, Ober *et al.* 1987) pregnancies result in heavier fetal-placental units than their MHC compatible counterparts. Female mice are able to sense the MHC antigen "make-up" of a prospective mate through smell (Yamazaki *et al.* 1983), and subsequently favour mating with MHC disparate males so as to maximise their reproductive potential (Potts *et al.* 1991). Furthermore, a weak maternal immune response towards fetal antigens, due to a high degree of sharing of HLA antigens between parents, has been suggested to result in recurrent miscarriage in humans (Gill 1983), although this later observation is currently under debate.

1.5.3 Hypothesis two: Is the fetus anatomically separated from its mother?

From an anatomical viewpoint, Medwar's second hypotheses is incorrect since paternal antigen bearing trophoblast does come into contact with maternal tissue. However, several functional barriers do exist. The zona pellucida, an acellular glycoprotein coat encapsulating the pre-implantation embryo, does act as an immunological barrier, helping to prevent cytotoxic lymphocytes from killing the pre-implantation embryo (Ewoldsen *et al.* 1987). At implantation this zona is lost, and a protective shell of extra-villous cytotrophoblast expressing non-classical MHC class I antigens then protects the embryo from immune attack. Compartmentalisation of decidual leukocytes provides an additional functional barrier, since leukocytes with potent antigen-presenting capacity or cytolytic activity are excluded from the decidual tissue adjacent to the fetal-maternal interface.

1.5.4 Hypothesis three: Is the maternal immune system relatively inert during pregnancy?

Pregnancy is not a state of generalised immunosuppression, as is often quoted, but may rather be viewed as a state of trophoblast antigen-specific tolerance. Generalised immunosuppression would be dangerous to a pregnant female, since it would increase her susceptibility to attack by infectious agents, while decreasing her immuno-surveillance against tumour cells. Pregnant females are capable of rejecting allogenic tissue transplants, and mounting an immune response to vaccination, thereby confirming their immunological integrity (Hart 1988).

Tolerance is a state of immunological unresponsiveness to a specific antigen resulting from the elimination or down-regulation of T cells which recognise that antigen. The concept that pregnancy results in a transient state of hypo-responsiveness to paternal antigens on trophoblast cells is supported by the work of Arnold and co-workers (Tafuri *et al.* 1995). These investigators showed that during pregnancy T lymphocytes reactive with paternal class I MHC become "anergic," or unable to recognise antigen, because of internalisation of T-cell receptors. This anergic state is functionally operative from as early as implantation and lasts until shortly after parturition, when lymphocytes regain their reactivity. The initiation of paternal antigen anergy is now believed to occur during mating, since maternal hypo-responsiveness to paternal antigens occurs even in mated tubal-ligated females (Robertson *et al.* 1997).

A shift in systemic immunity away from cell-mediated (Th1) responses towards humoral (Th2) immunity has been suggested to occur during both murine and human pregnancy (Wegmann *et al.* 1993). Th1 immune responses, characterised by the production of cytokines such as IL-2 and IFNγ, result in the generation of complement-fixing antibodies and cytotoxic T cells, both potentially harmful to the semi-allogenic conceptus (Tezabwala *et al.* 1989). Th2 immune responses are characterised by the production of cytokines such as IL-4, IL-5 , IL-6 and IL-10, and result in the generation of predominantly non-complement fixing IgG_1 antibody, while suppressing cytotoxic cell-mediated immune responses (Carter and Dutton 1996). During murine pregnancy, decidual and placental cells mainly produce Th2 cytokines (Lin *et al.* 1993, Chaouat *et al.* 1997), which in turn results in non-complement fixing IgG_1 being generated against placental antigens (Bell and Billington 1980), and an inhibition of cytotoxic cellular responses against placental antigens. This pregnancy-related bias away from Th1 immune responses

occurs systemically, since pregnant mice are unable to mount a curative Th1 immune response against the skin parasite Leishmania (Krishnan *et al.* 1996).

Human pregnancy is also characterised by a Th2 shift in immunity, with cell-mediated diseases such as arthritis being ameliorated during pregnancy, while humoral-mediated diseases such as systemic lupus erythematosus are exacerbated (Varner 1991, Da Silva and Spector 1992). In addition, human placental tissue contains high levels of Th2 cytokines (Cadet *et al.* 1995). An inability to produce these Th2 cytokines, or a shift towards the production of Th1 cytokines, is reported to be associated with repeated implantation failure (Hill *et al.* 1995, Stewart-Akers *et al.* 1998).

A Th2-mediated increase in antibody production does not result in placental damage because the majority of immunoglobulin produced against paternal antigens is non-complement fixing IgG_1 (Bell and Billington 1980). In addition, complement regulatory proteins such as membrane cofactor protein (MCP) and decay acceleration factor (DAF), are deposited on the trophoblast membrane surface (Hsi *et al.* 1991), where they act to block the complement cascade, thereby preventing trophoblast from being lysed by complement-fixing antibodies generated during pregnancy.

1.6 The role of seminal plasma in mammalian reproduction

1.6.1 General physiology

Semen is the collective term describing the cellular and fluid components of the male ejaculate. Semen consists of spermatozoa, non-germ cells such as leukocytes and genitourinary tract epithelial cells, all suspended in a viscous fluid known as seminal plasma. Seminal plasma is derived from the secretions of male accessory sex glands which include the prostate, seminal vesicle, bulbourethral and coagulating glands. The seminal vesicle gland is responsible for the bulk of seminal plasma secretions in both mouse and man (~ 70% total volume), with prostatic secretions making up the majority of the remaining volume (Mann 1981, Aumuller and Riva 1992). In addition, seminal plasma contains plasma transudate, as well as secretions derived from the epididymal and vas deferens epithelium. The typical ejaculate produced by a fertile human male is between 1.5 and 6 ml in volume, containing between 50 - 200 million sperm (Rowe *et al.* 1993). On average, a male mouse deposits approximately six million sperm into the uterine lumen following mating (Tucker 1980).

Seminal plasma is a complex mixture of ions, proteins, peptides, sugars and hormones (reviewed by Mann 1981, Blank 1988, Aumuller and Riva 1992). Seminal plasma contains all the ions present within blood plasma, but its levels of potassium and zinc are many times higher than that seen in blood. Potassium is believed to increase the cationic charge on the sperm membrane, which results in a decrease in sperm motility. The zinc content within seminal plasma is reported to be the highest in the entire body and may serve an antibacterial function (Fair *et al.* 1973). Twenty-five major protein species have been identified in murine seminal plasma using SDS gel electrophoresis (Bradshaw and Wolfe 1977), with up to 19 different proteins being identified in human seminal plasma (Verma *et al.* 1993). Many seminal plasma proteins are derived from serum (albumin, transferin, immunoglobulin), however a large proportion are specialised proteins involved in the processes of semen coagulation and subsequent liquefaction. Fibronectin and semenogelin are the principal structural proteins involved in semen coagulation, while prostatic enzymes such as Prostate Specific Antigen (PSA), a kallikrein-like serine protease, are involved in coagulum liquefaction (Lilja *et al.* 1987). Lactoferrin, an iron-binding protein similar to that found in milk, is present within seminal plasma at relatively high concentrations, and is reported to possess antibacterial activity (Tauber *et al.* 1975). The seminal vesicle gland secretions contain large amounts of the sugar fructose, together with lesser amounts of glucose, fructose and ribose, which act as energy substrates for sperm (Phadke *et al.* 1973). Spermine and spermidine, basic polyamines secreted by the prostate, are believed to accelerate sperm metabolism and trigger sperm capacitation (Shah *et al.* 1975, Parrish and Polakoski 1977).

1.6.2 The role of seminal plasma in reproduction

Seminal plasma augments reproductive potential in rodents and humans by increasing the proportion of ejaculated sperm that reach the oocyte. Human seminal plasma achieves this by increasing sperm penetration through the cervical mucous, possibly by enzymatically modifying the integrity of the cervical mucous barrier (Overstreet *et al.* 1980). Murine seminal plasma increases the proportion of ejaculated sperm that reach the oviduct by increasing their forward motility (Peitz and Olds1986). The vaginal plug, a coagulum of seminal plasma proteins formed within the rodent vagina / cervix shortly after mating, is reported to facilitate the trans-cervical passage of sperm into the rat uterine lumen (Carballada and Esponda 1992 and 1993).

Seminal plasma is not an absolute requirement for fertilisation, since epididymal sperm are capable of fertilising oocytes *in vitro*, as well as following artificial insemination. However, seminal plasma does improve the capacity of sperm to achieve fertilisation by initiating sperm capacitation (Parrish and Polakoski 1977), a transition required before fertilisation can take place. Secondly, seminal plasma enhances the ability of capacitated sperm to penetrate bovine zona-free oocytes *in vitro* (Henault *et al.* 1995). The significance of this second mechanism is presently uncertain, since very little, if any, seminal plasma reaches the oocyte contained within the oviduct at the time of fertilisation.

The presence of seminal plasma within the uterus has been reported to trigger ovulation in the pig (Waberski *et al.* 1997). The mechanism behind this response is not currently understood, but may involve a local reaction to seminal oestrogens. However, seminal plasma has not been reported to induce ovulation in mice or humans.

Several studies have now investigated the regulatory effect of seminal plasma on reproductive outcome. Indirect evidence supporting a role for seminal plasma in human reproduction comes from studies in which exposure of women to semen during IVF or GIFT treatment resulted in a significant improvement in implantation rates, as well as an increase in live birth rates (Bellinge *et al.* 1986, Marconi *et al.* 1989). Furthermore, treatment of women experiencing recurrent miscarriage with seminal plasma pessaries has been reported to enhance implantation rates (Coulam and Stern 1995). Intricate studies involving the selective removal of seminal vesicle, prostate or coagulating glands from stud mice, rats and hamsters prior to mating has revealed that seminal vesicle gland secretions are the most important component of seminal plasma necessary for the establishment of pregnancy (Pang *et al.* 1979, Queen *et al.* 1981, O *et al.* 1988). An absence of exposure of a female hamster to seminal vesicle secretions during mating results in a slower rate of pre-implantation embryonic development, and a higher rate of embryonic loss (O *et al.* 1988). Furthermore, experiments in which sows were exposed to additional volumes of seminal plasma by artificial insemination with heat-killed semen, mating with vasectomised boars, or multiple fertile matings during the one oestrous period, have all reported an improvement in the farrowing rate and litter size following artificial insemination, embryo transfer or intact mating (Murray *et al.* 1983, Mah *et al.* 1985, Gooneratne and Thacker 1989, Flowers and Esbenshade 1993, Soede *et al.* 1993).

1.6.3 Immune modulating effect of seminal plasma

Semen is rich in allo-antigens and powerful immunoregulatory molecules such as prostaglandins and cytokines. Evidence is now emerging that deposition of semen in the female reproductive tract may do more than just deliver sperm to the ovum. Rather, it may induce changes in the maternal tract, perhaps resulting in changes in the maternal immune response which may benefit the growth and survival of the future semi-allogenic conceptus (Thaler 1989, Seamark *et al.* 1992, Robertson *et al.* 1997).

a. Antigens contained within semen

The presence of MHC class I and II antigens on the sperm membrane has been hotly debated for many years, with several investigators being unable to identify these antigens on human (Anderson *et al.* 1982, Haas and Nahhas 1986, Schaller *et al.* 1993) or murine sperm (Harding and Wellhausen 1992), while others have been able to demonstrate MHC class I and II antigens on human sperm (Bishara *et al.* 1987, Kurpisz *et al.* 1987, Ohashi *et al.* 1990). Protagonists of the MHC-negative sperm paradigm believe that the identification of MHC antigens on sperm is an artefact created by a lack of antibody specificity, or somatic cell contamination in the test samples. Only recently has it been conclusively proven that human spermatozoa do express MHC class I and II antigens (Martin-Villa *et al.* 1996). These investigators identified HLA class I and II antigens on human sperm using a set of HLA-specific monoclonal antibodies in combination with fluorocytometry. Importantly, they also identified MHC class I and II mRNA within sperm, and were able to confirm the translation of this mRNA into protein using ^{35}S-methionine labelling. Until similar experiments are performed in the mouse, the debate of whether murine sperm express MHC antigens will remain unsettled.

Leukocytes and genitourinary tract epithelial cells contained within semen provide an additional paternal antigenic stimulus to the female (Collins *et al.* 1994, Wolff 1995). Phagocytosis and uptake of foreign lymphocytes across the cervical-vaginal mucosa has been confirmed to occur in the mouse (Ibata *et al.* 1997). These somatic cells express a high density of MHC antigens on their membrane surface and therefore are likely to be a strong antigenic stimulus, despite their numerical inferiority compared to sperm. Non-germ cells within the human ejaculate have been reported to be more potent than sperm in initiating the activation of maternal lymphocytes (Rodriguez-Cordoba *et al.* 1982).

Somatic cells may therefore be an important antigenic stimulus to the female, since between 10 and 20% of men have in excess of 10^6 leukocytes per ml within their semen (Wolff 1995).

Soluble antigens, not associated with sperm or somatic cells, have also been identified within semen. These include soluble MHC class I antigens (Schaller *et al.* 1993, Dekker *personal communication*), and a group of antigens referred to as Trophoblast-Lymphocyte Cross-Reactive (TLX) antigens. These TLX antigens were discovered when polyclonal rabbit antisera was found to cross react with trophoblast and lymphocytes in an allotypic manner (McIntyre *et al.* 1983). This discovery lead to the proposal that exposure of women to seminal plasma TLX antigens at the time of intercourse may serve as a source of allogenic recognition, which in turn may favour the immunological acceptance of the semi-allogenic conceptus (Kajino *et al.* 1988, Thaler *et al.* 1989). This theory was later invalidated when TLX was found to be identical to CD46, a complement receptor that is theorised to protect the placenta from complement-mediated attack (Purcell *et al.* 1990).

Antigens present within semen are capable of triggering adverse immunological events in some individuals. A small proportion of women are allergic to semen, with the severity of this allergy ranging from post-coital oedema and pruritus, through to an anaphylactic reaction (Jones 1991, Ebo *et al.* 1995, Drouet *et al.* 1997). Interestingly, the onset of semen allergy is not always following first intercourse, but may occur following prostatectomy or vasectomy in the male partner, or pregnancy and reproductive tract surgery in the female (Jones 1991). Provocation of this type of allergy is generally not partner-specific, however a high degree of HLA compatibility between sexual partners has been noted in a small series of couples reporting semen allergy (Bernstein *et al.* 1981)

b. Immunosuppressive capacity of seminal plasma

Immunosuppressive activity within seminal plasma has been extensively studied over the last 20 years (reviewed by James and Hargreave 1984, Alexander and Anderson 1987, Kelly 1995). The search for immunosuppressive molecules within semen was initiated to explain why it is uncommon for adverse anti-paternal immune responses to develop within the female genital tract, despite repeated inoculation with foreign antigens at the time of intercourse (McShane *et al.* 1985, Isojima 1988). The presence of immunosuppressive activity was first reported within human seminal plasma by Stites and Erickson (1975). Since this initial observation, seminal plasma has been reported to

suppress T cell responses to mitogen (Lord *et al.* 1977, Marcus *et al.* 1978, Majumdar *et al.* 1982, Vallely and Rees 1986), inhibit primary and secondary antibody responses to vaginally administered antigens (Anderson and Tarter 1982), decrease natural killer cell activity (James and Szymaniec 1985, Rees *et al.* 1986), inhibit the ability of macrophages to phagocytose and present antigens to T cells (Stankova *et al.* 1976, James *et al.* 1983), decrease neutrophil phagocytic and killing capacity (Peterson *et al.* 1980), and interfere with complement-mediated immunity (Peterson *et al.* 1980, Witkin *et al.* 1983).

A wide variety of seminal plasma compounds such as zinc, transglutaminase, polyamines, prostaglandins and cytokines have been suggested to be responsible for the immunosuppressive effect of seminal plasma, but evidence is now accumulating to suggest that PGE and TGFβ may be the two main immunosuppressive components within seminal plasma (Kelly *et al.* 1976, Bendvold *et al.* 1987, Nocera and Chu 1993, Kelly 1995). Prostaglandin E is a potent immunosuppressive agent capable of inhibiting T cell responses and NK cell activity (Rappaport and Dodge 1982, Bankhurst 1982), as well as skewing the immune system towards a Th2 immune response (Betz and Fox 1991, Kelly *et al.* 1997a). Human seminal plasma contains large amounts of TGFβ (Nocera and Chu 1993, Chu *et al.* 1996). It is currently speculative whether seminal plasma TGFβ has the ability to alter maternal immune responses against ejaculate antigens, yet its abundance within seminal plasma, together with its reported Th2 immune-deviating activity within the anterior chamber of the eye (Streilein 1993), makes TGFβ a likely candidate.

1.6.4 Post-mating inflammatory response

The post-mating inflammatory response describes an influx of leukocytes into the female reproductive tract following exposure to semen. This inflammatory response has been observed to occur in mice, rats, pigs, rabbits and the human female (Lobel *et al.* 1967, Lovell and Getty 1968, Phillips and Mahler 1977, Pandya and Cohen 1985). Intrauterine ejaculators such as the mouse and pig deposit the majority of their ejaculate within the uterine cavity during mating, and therefore exhibit a post-mating inflammatory response that is principally located within the uterine endometrium. In intra-vaginal ejaculators such as humans and rabbits, where the majority of ejaculate is deposited within the vagina / cervix (Asch *et al.* 1977), a cervical inflammatory response that does not extend to the uterine endometrium occurs following mating (Taylor 1982, Pandya and Cohen 1985, Thompson *et al.* 1992).

The post-mating inflammatory response in the mouse is reported to consist of an infiltration of neutrophils, macrophages and eosinophils into the endometrial stroma and lumen within hours of mating (De *et al.* 1991, Kachkache *et al.* 1991, McMaster *et al.* 1992). The influx of neutrophils lasts for less than 24 hours, with macrophage numbers remaining high until day 2-3 of pregnancy, at which stage their numbers begin to decline under the influence of increasing progesterone concentrations (Kachkache *et al.* 1991). This post-mating inflammatory infiltrate is still observed following mating with a vasectomised male (Choudhuri and Wood 1992), yet absent following mating with seminal vesicle-deficient males (Robertson *et al.* 1996b), thereby indicating that the trigger for the post-mating inflammatory response (PMIR) is a seminal vesicle-derived component of seminal plasma.

Fluctuations in the production of pro-inflammatory cytokines by the murine uterus closely parallel leukocyte movements during the first three days of pregnancy. This observation is consistent with the concept that seminal vesicle secretions mediate an influx of leukocytes into the uterus by initiating the production of pro-inflammatory cytokines by the uterine epithelium, rather than the seminal vesicle secretions being directly chemotactic. Analysis of cytokine transcripts from murine uteri during the first few days of pregnancy has revealed that there is a large increase in the production of mRNA for the cytokines IL-1α, IL-1β, IL-6, TNFα, CSF-1 and GM-CSF (Sanford *et al.* 1992, Robertson *et al.* 1990 and 1992b), as well as the chemokines RANTES , Eotaxin, monocyte chemotactic protein (MCP-1), and the macrophage inflammatory proteins (MIP-1α and MIP-1β)(Pollard *et al.* 1998, Robertson *et al.* 1998b). This increase in cytokine / chemokine mRNA only lasts 1-2 days before returning to baseline levels by day 4 of pregnancy, at which time the post-mating inflammatory response has also resolved.

GM-CSF is one of the key cytokines responsible for the production of the murine post-mating inflammatory response. On day 1 of pregnancy there is a large increase in transcription of GM-CSF mRNA, localised to the uterine epithelium by *in situ* hybridisation, with a twenty-fold increase in luminal fluid GM-CSF bio-activity (Robertson *et al.* 1990 and 1992b). This increase in uterine epithelial GM-CSF production is likely to play a key role in the initiation of the post-mating inflammatory response, since administration of recombinant GM-CSF to the uterine lumen of ovariectomised mice is capable of producing an inflammatory reaction indistinguishable from that seen following mating (Robertson *et al.* 1994). Furthermore, seminal vesicle secretions are reported to

precipitate an increase in uterine epithelial GM-CSF production both *in vivo* and *in vitro* (Robertson *et al.* 1996b).

The post-coital inflammatory response has been less extensively investigated in the human than in the mouse. Superficial sampling of cervical cells using an Ayre's spatula, as is performed during a Papanicolaou test screening for cervical cancer, has revealed that within four hours of exposure to semen, there is a massive infiltration of neutrophils into the cervix, in combination with a smaller influx of macrophages and lymphocytes (Pandya and Cohen 1985, Thompson *et al.* 1992). This cervical leukocytosis persists for approximately 24 hours before subsiding. Very little is known about the molecular events responsible for generating this response, however it has been reported that sperm, not seminal plasma, is responsible for triggering the post-coital cervical leukocytosis (Thompson *et al.* 1992).

The post-mating inflammatory response may serve several functions. Firstly, it has been reported to facilitate the removal of infectious pathogens introduced at the time of mating (Parr and Parr 1985). Secondly, the release of inflammatory mediators such as histamine and cytokines from leukocytes involved in the post-mating inflammatory response may assist in remodelling the endometrium in preparation for implantation. Macrophages and granulocytes are well equipped to mediate these pre-implantation tissue remodelling events through the release of proteases and extracellular matrix proteins, and are a potent source of cytokines and immunoregulatory molecules that are capable of controlling the differentiation and function of a wide array of cell types. Since the murine post-mating inflammatory response does not extend to the oviducts (Dalton *et al.* 1994) and has resolved once the blastocyst arrives in the uterine cavity (day 3.5 p.c.), it is unlikely that leukocyte-derived inflammatory mediators have any direct effect on the murine pre-implantation embryo. Furthermore, localisation of the human post-coital inflammatory response to the cervix is likely to preclude any direct effect of leukocyte inflammatory mediators on human pre-implantation embryos.

A third mechanism by which the post-mating inflammatory response may benefit mammalian pregnancy is through its effect on maternal immune awareness of paternal antigens in the ejaculate (Thaler 1989). Antigen-presenting cells such as macrophages and dendritic cells are abundant within the murine endometrium shortly following mating, and are therefore ideally positioned to sample paternal antigens contained within the ejaculate. Although currently speculative, it is possible that immunoregulatory components within seminal plasma may effect the phenotype of antigen-presenting cells so that they present

ejaculate antigens to maternal T cells in a manner that results in paternal antigen-specific tolerance. Experimental evidence supporting this concept is strong. Following mating, tubal-ligated sterilised mice are not capable of rejecting allogenic skin (Lengerova *et al.* 1963) or tumour grafts (Robertson *et al.* 1997) that express the same antigens as expressed by the original mate, yet these females are capable of rejecting third party allografts. These two experiments clearly indicate that exposure of a female to ejaculate antigens can produce paternal antigen-specific immune deviation, even in the absence of ensuing pregnancy.

Paternal-antigen specific immune deviation may benefit the growth and survival of the semi-allogenic conceptus, since preventing maternal immune awareness of paternal antigens by removing the lymph nodes draining the uterus results in a decrease in the litter size and fetal-placental weight (Beer *et al.* 1975a, Tofoski and Gill 1977). It is presently unclear if the human post-coital inflammatory response has any role in maternal immune priming, although this is an attractive proposition that has some circumstantial support (Clark 1994).

1.7 Conclusions and study aims

The precise mechanisms responsible for the paradoxical growth and survival of the semi-allogenic fetal-placental unit are relatively unknown. Cytokines appear to be potent regulators of embryonic and placental growth, as well as mediators of the functional and spatial organisation of leukocytes within the female reproductive tract. In mice, exposure of the uterine epithelium to seminal plasma at the time of mating is associated with heightened production of pro-inflammatory cytokines, which results in an influx of activated leukocytes into the uterus. Many of these leukocytes drawn into the uterus following mating have been identified as playing a significant role in controlling placental growth and function. While the mechanisms and extent to which seminal plasma cytokine-leukocyte networks are necessary for successful pregnancy is presently unknown, there is mounting evidence that seminal plasma plays a role in mammalian pregnancy other than just acting as a transport medium for spermatozoa.

The studies described in this thesis investigate the following hypotheses:

1. Murine semen contains specific factors derived from the seminal vesicle gland that interact with uterine epithelial cells following mating and induce / stimulate production of GM-CSF, which in turn initiates an inflammatory response.
2. The murine cytokine-leukocyte environment afforded by mating facilitates the induction of a protective immune response to paternal antigens.
3. Comparable factors and interactions, as described in hypotheses 1 and 2, occur in the human.

The aim of the studies described in this thesis was to investigate:

1. The identity and nature of factors within seminal plasma which are responsible for triggering the post-mating inflammatory reaction in mice and women.
2. The mechanism by which priming with paternal antigens at the time of mating may influence the maternal immune response towards these antigens.
3. The importance of exposure to seminal plasma in murine and human reproduction.

Chapter 2
Materials and Methods

2.1 Mice and surgical procedures

2.1.1 Animals

Adult (8 - 16 week) Balb/c F1 (Balb/c x C57B1) female mice and CBA or CBA F1 (CBA x C57B1) males were used throughout these experiments. All mice were obtained from the University of Adelaide Central Animal House and were maintained in a minimal security barrier, specific pathogen-free facility, on a 12 hour light / 12 hour dark cycle (0700/1900 hrs), with food and water available *ad libitum*. Ethical approval for experimentation on these animals had been granted by the University of Adelaide animal ethics committee (approval M/30/93 and M/104/95).

2.1.2 Assessment and manipulation of the murine oestrus cycle

Assessment of oestrus cycle stage was performed by taking vaginal smears, as described by Bronson *et al.* (1966). Briefly this consisted of instilling 40 μl of PBS into the vagina using a Gilson pipette (Gilson, France), followed by recovery of the PBS containing desquamated vaginal epithelial cells. The morphology of these vaginal epithelial cells was then examined by phase contrast microscopy (BH-2, Olympus) in order to identify the oestrus cycle stage as oestrous, dioestrous, metoestrous or pro-oestrous.

For studies requiring large numbers of oestrous mice on a particular day, females were synchronised using either the Whitten effect or by pharmacological means. The Whitten method (Whitten 1956) is based on the observation that the majority of females caged in close proximity to a stud male are in oestrous by the third day. The close approximation of females near a stud male, without allowing mating to occur, was achieved by confining the male to a small cage within a larger cage that contained up to 20 females. Using this technique, approximately 75% of females were confirmed to be in oestrous by day 3, as assessed by vaginal smear cytology.

Pharmacological synchronisation of the oestrus cycle was achieved by administering a long acting LHRH analogue (des-Gly_{10}, D-Ala_6, -$ProNEt_9$- LHRH)(Sigma, St Louis, MO) to randomly cycling females. Twenty μg of LHRH super-agonist contained in 200 μl of PBS was injected ip at 1200 hrs, with all females then reaching oestrus 3.5 days later.

2.1.3 Natural mating

Adult naturally cycling females, or those synchronised to pro-oestrous using the techniques described in 2.1.2, were placed two per cage with individual stud males of proven fertility. Mating with intact studs or vasectomised males was confirmed by the identification of a vaginal plug present on the morning after oestrous, with this day being defined as day 1 of pregnancy. Mating with seminal vesicle-deficient males did not result in the deposition of a vaginal plug, so mating was confirmed to have occurred by identifying sperm within a vaginal smear taken on the morning after oestrous. All pregnant females were separated from males from day one of pregnancy.

2.1.4 Surgical procedures

a. General procedures

All surgical procedures were performed under aseptic conditions using protocols designed in accordance with NH & MRC guidelines for the care and use of experimental animals. Mice were anaesthetised prior to all surgical procedures with avertin (1 mg / ml tribromoethyl alcohol in tertiary amyl alcohol - Aldrich Chemical Company, Milwaukee, Wis.) diluted to 2.5% (v/v) in water. A bolus induction dose of 15 μl per gm of body weight was injected ip, with further doses being titrated to produce sufficient anaesthesia for the length of the surgical procedure. All surgical instruments were sterilised by autoclaving or 70% ethanol before use. Wound closure was performed using either 5/0 silk suture on a cutting needle (Ethicon, Sydney, NSW) or stainless steel 9mm wound clips (Becton Dickinson, Sparks, MD). Animals recovering from anaesthetic were kept warm using a warming tray set at 32^0 C, with no animal being returned to its cage until consciousness and ambulance were regained.

b. *Intrauterine delivery of sperm and / or recombinant TGFβ₁*

ascertained for all females in this study, but by standardising the time of sacrifice to 0200hrs it was anticipated that the majority of uterine samples would be collected within 30 to 90 minutes of mating.

d. Collection of male accessory sex gland secretions

Identification of the accessory sex glands of the male mouse was performed with the aid of an anatomical atlas. Following the sacrifice of CBA F1 studs, a large ventral abdominal incision was made to expose the pelvic organs. Through this incision, the seminal vesicle glands were easily identified as large, white, thin-walled bladders. Prior to their removal, the adherent coagulating glands were detached, and then the seminal vesicle glands were severed at their base and quickly transferred to a plastic petri dish to avoid spillage of secretions. The glands and secretions were weighed to the nearest mg. Solubilisation of seminal vesicle secretions was achieved by the addition of 500 μl of guanidine hydrochloride per pair of glands. This solution was then desalted, as described in 2.7.4, before assessment of $TGF\beta_1$ content.

It was found to be impossible to express secretions from the prostate or coagulating glands, so an estimate of the $TGF\beta_1$ content of these glands was made by homogenising each prostate or pair of coagulating glands in 500μl of PBS / 0.1% BSA for 60 seconds with the aid of a tissue homogeniser (Ultra-Turrax, IKA Labortechnick, Germany). Cellular debris was pelleted before collection of the supernatant for determination of $TGF\beta_1$ content.

e. Vasectomy, seminal vesicle removal and castration of stud males

Vasectomy of CBA F1 studs was performed through a ventral abdominal wall incision, and achieved by bilateral cauterisation of the *vas deferens*, followed by ligation of the testicular segment of the vas with a 5/0 silk tie. All vasectomised males were mated at least three times to remove residual sperm from the genital tract, prior to their use in experiments.

Surgical preparation of seminal vesicle-deficient males was achieved by removal of the gland as described in 2.1.4 d, except that the coagulating glands were left intact within the animal.

Castration was achieved by removal of both testicles through an abdominal incision, with meticulous attention being paid to haemostasis.

f. Serum collection

Collection of serum from mice for determination of antibody content was performed by cardiac puncture. A mid-line thoracotomy incision was made under deep anaesthesia to allow for adequate exposure of the heart, and a 23 G needle was then introduced into the left ventricle. Strong suction usually resulted in the collection of between 0.5 - 1 ml of blood per mouse, although on a few occasions it was impossible to collect any blood because of clotting. At the completion of the procedure, all mice were sacrificed by cervical dislocation. Blood was stored overnight at 4 ^{0}C to allow clot formation / retraction, then the clot material was compacted by centrifugation (500g , 10 min), and the serum supernatant collected before being frozen at -80 ^{0}C until assayed.

2.2 Chemical reagents

2.2.1 Recombinant cytokines and cytokine neutralising antibodies

Recombinant yeast-derived murine GM-CSF (rmGM-CSF) was provided by N. Nicola (Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria). Recombinant Chinese Hamster Ovary (CHO) cell-derived human GM-CSF (rhGM-CSF) was provided by A. Lopez (Hanson Center for Cancer Research, Adelaide, Australia). Recombinant CHO-expressed human TGFβ_1 (rhTGFβ_1) was purchased from R and D Systems (Minneapolis, MA). Porcine platelet derived TGFβ_2 was provided by J. Gamble (Hanson Center for Cancer Research, Adelaide, Australia). Recombinant human activin and inhibin were both provided by J. Findlay (Prince Henry's Institute of Medical Research, Melbourne, Australia). All cytokines were suspended in PBS / 0.1 % BSA, and stored at -80 ^{0}C in aliquots.

A goat polyclonal antibody to murine GM-CSF, used for the neutralisation of murine GM-CSF bioactivity, was provided by J. Schreurs (DNAX, Palo Alto, CA), and a mouse α human GM-CSF antibody was purchased from Genzyme (Cambridge, MA) for neutralisation of human GM-CSF bioactivity. Neutralising antibody against TGFβ_1 and a

pan-specific ($TGF\beta_1$,$TGF\beta_2$,$TGF\beta_3$) neutralising antibody were purchased from R and D Systems (Minneapolis, MA).

2.2.2 Sundry chemicals

A 6 M solution of guanidine hydrochloride (Sigma) was made by dissolving 1.2 kg of guanidine salt in 2 *l* of MQ water. The resulting solution contained considerable amounts of insoluble material that was removed by standing the solution for one hour at unit gravity, followed by further clarification of the supernatant by filtration through a No.1 Whatman filter paper and a 0.22 μm cartridge filter (Sterivex-GP, Millipore, Bedford MA). The resulting 6 M guanidine hydrochloride was neutralised to a pH of 7.4 by the addition of 10 M sodium hydroxide, and buffered by the addition of 20 mM Tris-HCl. This neutral guanidine hydrochloride was stored in a dark bottle at room temperature until use.

Salmonella typhimurium lipopolysaccharide (LPS) was a gift from I. Kotlarski (Department of Microbiology and Immunology, University of Adelaide). All other chemicals and reagents were purchased from Sigma or BDH Chemicals, unless otherwise specified in the text.

2.2.3 Sundry procedures

a. Release of active TGFβ from its latent precursor

Active TGFβ is defined as the proportion of TGFβ within a biological fluid that exhibits bioactivity prior to acid activation, whereas total TGFβ refers to the amount of TGFβ measured following acid activation (a process that is reported to result in ~ 100% release of bioactive TGFβ from its latent precursor). Acid-mediated release of bioactive TGFβ from its latent precursor was achieved by the addition of 2 μl of 5 M HC*l* to 0.5 ml of each chromatography fraction for 20 mins at room temperature, followed by neutralisation with 2 μl of 5 M NaOH. The "acid activated" fraction was then desalted into culture medium, as described in 2.7.2. Biological fluids and culture supernatants requiring acid release of TGFβ were treated with 200 μl of 1 M HC*l* per ml of sample for 10

minutes at room temperature, followed by neutralisation with 200 µl of 1.2 M NaOH/0.05 M HEPES.

Two "physiological" techniques for the release of TGFβ from its latent precursor were used. Firstly, murine oestrous uterine fluid and prostate gland homogenate, both reported to contain enzymes capable of cleaving active TGFβ from its latent precursor, were added to dilute seminal vesicle secretions and incubated for 1 hr at 37 ^{0}C, prior to the measurement of active and total $TGF\beta_1$ levels by ELISA. Secondly, human plasmin (Sigma) was incubated (37 ^{0}C) for various lengths of time with murine copulatory plug material (0.1U/ml plasmin), or human seminal plasma (0.5 U/ml plasmin), before assessing the release of bioactive $TGF\beta_1$.

b. Testosterone supplementation

Adult CBA F1 males, previously used as breeding studs, were surgically castrated and allowed to recover for 14 days, before commencing testosterone supplementation. Testosterone propionate (Sigma) was dissolved in absolute ethanol (10 mg/ml), followed by dilution in peanut oil to give the desired working concentration. Two groups of animals received either 100 µg or 500 µg of testosterone in oil s/c, with a third control group receiving peanut oil only. Each animal was treated once a day for seven days before the removal of the seminal vesicle glands, which were then weighed and assayed for total $TGF\beta_1$ and protein content. Seminal vesicle secretions were too viscous to enable measurement of their volume, so estimates were made by weighing the secretions, then calculating their volume using the assumption that seminal vesicle fluid has a density of 1.068 gm / ml . This density was calculated by determining the weight of 200 µl of seminal vesicle fluid harvested from six male CBA F1 studs.

c. Protein quantification

The protein content of murine seminal vesicle secretions was measured using a modification of the bicinchoninic acid protein assay, as described by Smith *et al.* (1985). All reagents for this assay were purchased from Pierce (Rockford, Ill), and the assay was performed according to the manufacturer's instructions, with the exception that all reagent volumes were proportionally scaled down by a factor of five. All test samples were assayed in duplicate and standardised against a calibration curve containing bovine serum

albumin (BSA) fraction V (Pierce). Assays were conducted in 96 well plates, with protein concentration measured by absorbance of the reaction product at 560 nm (reference 410 nm) on a Bio Tek Microplate EL 31 reader (Winooski, VT).

2.3 Cell culture

2.3.1 General

Harvesting, processing and manipulation of primary tissue cultures and cell lines were performed under aseptic conditions using strict sterile working practises in a laminar flow hood whenever possible. Cell cultures were regularly examined to assess viability and microbial contamination with the aid of a inverted microscope fitted with phase contrast optics (Nikon TMS, Japan).

Solutions and media were prepared using water purified in a Milli-Q Reagent Grade Water System (Millipore, Bedford, MA) in culture-grade glassware.

2.3.2 Culture medium

Cell lines and primary cultures were grown in media including RPMI-1640, DMEM (low glucose) and Hams F-12 (Gibco, Grand Island, NY) supplemented with 15 mM HEPES, 5 x 10^{-5} M β-mercaptoethanol (BDH, Poole, UK), 2 mM L-Glutamine (ICN, Costa Mesa,CA) and antibiotics (benzylpenicillin, streptomycin - Sigma). All media contained protein in the form of either heat-inactivated fetal calf serum (FCS) (CSL, Australia) or Neutridoma-SP (Boehringer Mannheim, Germany). PBS or HBSS (Sigma) containing antibiotics and 20 mM HEPES were used as media for the transportation of tissue samples to the laboratory. The osmolarity and pH of all media were adjusted to 280 mOsm and 7.4 respectively, prior to 0.22 μm filter sterilisation (Millex, Millipore) and stored at 4 ^{0}C.

2.3.3 *Cell lines*

Cell lines were obtained from the sources indicated and maintained in media specified in Table 2.1.

Table 2.1 The species, lineage, origins and growth media for cell lines used in this thesis

cell line	species	lineage	origin	growth media
FD 5/12	mouse	myeloid	Anne Kelso, WEHI, Melbourne, Australia	RPMI-10% FCS + murine GM-CSF (0.5 ng/ml)
TF-1	human	myeloid	ATCC CRL- 2003, Rockville, MD	RPMI-10% FCS + human GM-CSF (0.5 ng/ml)
CCL-64	mink	lung fibroblast	ATCC CCL-64, Rockville, MD	DMEM-5% FCS
L cells	mouse	fibroblast	A Lopez, Hanson Centre, Adelaide	DMEM-5% FCS
F4/80	rat/mouse	hybridoma	P Kenny, Immunology, Flinders Uni, Adelaide, Australia	RPMI-10% FCS
TIB 120	rat/mouse	hybridoma	ATCC, Rockville, MD	RPMI-10% FCS
TIB 122	rat/mouse	hybridoma	ATCC, Rockville, MD	RPMI-10% FCS
TIB 128	rat/mouse	hybridoma	ATCC, Rockville, MD	RPMI-10% FCS

2.3.4 *Embryo harvesting and culture*

Pre-implantation embryos were flushed from the reproductive tract of naturally cycling Balb/c F1 females after mating with CBA males (intact or seminal vesicle-deficient) using HEPES- HTF medium, according to the procedure described by Hogan *et al* (1986). In experiments assessing the *in vitro* development of pre-implantation embryos, oviducts were flushed at 1400 hrs on day 2 of pregnancy and the resulting embryos placed into 25 μl droplets of HTF-0.5% BSA (up to 12 embryos per droplet), covered by culture grade liquid paraffin, and cultured for the next four days at 37 ^{0}C, 5% CO_2, in a humidified atmosphere. Pre-implantation embryo development was scored at 0900 hrs as 1, 2, 4, or 8 cell, morula, blastocyst, hatching blastocyst, or hatched blastocyst according to the criteria described by Hsu (1979). *In vivo* development of murine embryos was assessed by flushing embryos from the uterine cavity at 1600 hrs on day 3 of pregnancy, followed by

an assessment of their morphology. Post-implantation embryonic development was assessed by counting implantation sites on day 7 of pregnancy.

2.3.5 Cell quantification

The density of cell lines, primary cell cultures and sperm in uterine luminal flushings were determined using a Neubauer haemocytometer (Assistent, Germany) and an Olympus BH-2 phase contrast microscope.

2.4 Cytokine bio-assays

2.4.1 General conditions

All bio-assays were performed in flat-bottomed 96-well culture plates (Costar) incubated at 37 ^{0}C , 5% CO_2 in a humidified atmosphere. The cytokine content of biological fluids and culture supernatants was determined by linear regression of cell numbers plotted against sample dilution, followed by comparison of these plots to standard curves generated using known concentrations of recombinant cytokine. The specificity of each assay was confirmed by demonstrating the ability of cytokine-specific neutralising antibodies to abrogate the effect of recombinant cytokine on cellular proliferation.

2.4.2 Murine GM-CSF bio-assay

GM-CSF was assayed using the GM-CSF-dependent cell line FD5/12, as described by Kelso and Owens (1988). Duplicate serial 1:2 dilutions of either culture supernatant or uterine luminal fluid were incubated with 2000 FD5/12 cells in 200 μl of RPMI-FCS. Sixteen hours after commencing the assay, 25 μl of Alamar Blue (Alamar Bioscience, Sacramento, CA) was added to the culture wells for a further 36-48 hrs. Alamar Blue is a non-toxic dye which upon reduction by cellular metabolism changes colour from blue to red. Colour changes were monitored by measurement of absorbance at 570 nm (reference filter 600nm) on a Dynatech MR 5000 ELISA plate reader. As a measure of cellular numbers, it was found that Alamar Blue achieved comparable results to those achieved with [^{3}H]- thymidine, at GM-CSF concentrations exceeding 15 U/ml. At concentrations

below 15 U/ml, which were sometimes present within uterine luminal fluid, Alamar Blue was insufficiently sensitive to measure changes in FD5/12 proliferation, and therefore these bio-assays were pulsed with 1 Ci of ^{3}H thymidine for 8 hrs at the end of the culture period. The amount of ^{3}H thymidine incorporated into nucleic acid was determined by harvesting onto glass fibre paper using a Cambridge PhD cell harvester, followed by measurement of radioactivity (DPM) in a liquid scintillation beta counter (Beckman, CA) using Ready Safe liquid scintillant (Beckman).

Every assay was standardised using rmGM-CSF. The minimum detectable amount of GM-CSF was 1 U / ml, where 50 U/ml was defined as the concentration producing half maximal FD5/12 cell proliferation; equivalent to 1 pg / ml of rGM-CSF. The specificity of this assay was confirmed using GM-CSF neutralising antibody (full neutralisation of 2 pg rGM-CSF at an antibody concentration of 1 μg/ml).

2.4.3 Human GM-CSF bio-assay

Human GM-CSF was assayed using the TF-1 cell line, as described by Kitamura *et al.* (1989). Duplicate serial 1:2 dilutions of culture supernatant were incubated with 2000 TF-1 cells in 200 μl of RPMI-FCS. Quantification of cell numbers was performed using Alamar Blue, as described in 2.4.1. Recombinant hGM-CSF was used to generate a standard curve for cytokine quantification. The specificity of this bioassay was confirmed using human GM-CSF neutralising antibodies (full neutralisation of 50pg rGM-CSF at an antibody concentration of 1 μg/ml).

2.4.4 TGFβ bioassay

The mink lung cell bio-assay, described by Like and Massague (1986), was used to quantify murine and human TGFβ content within biological fluids, since TGFβ specifically inhibits the proliferation of these cells. Mink lung fibroblast cells (CCL-64) were incubated for 3 hours at 37 ^{0}C, 5% CO_2, in a humidified atmosphere at a concentration of 5 x 10^3 cells suspended in 100 μl of RPMI-5% FCS to allow attachment of cells to the culture well floor. Test samples or medium containing a known concentration of rTGFβ (serially diluted in 100 μl of RPMI- 5% FCS) were then added. After incubation for a further 48 hrs, 25 μl of Alamar Blue was added to each culture well and the resulting colour change quantified 12 hrs later by measurement of absorbance at

570 nm. The use of Alamar Blue for quantifying CCL-64 cell numbers was found to be far superior to MTT tetrazolium salt, traditionally employed for this purpose. This protocol enabled accurate measurements of TGFβ content above 15 pg / ml. The specificity of the assay for TGFβ was confirmed for each biological fluid by the addition of pan-specific TGFβ neutralising antibody, combined with the demonstration of a lack of non-specific inhibition of cellular growth by adding test samples to TGFβ–insensitive murine fibroblast cells (L cells).

2.5 Cytokine ELISAs

2.5.1 TGFβ_1 ELISA

A TGFβ_1-specific ELISA (R and D systems) was used to measure murine and human TGFβ_1, according to the manufacturer's instructions. Briefly, soluble type II TGFβ receptor molecules were bound to 96 well microtitre plates to capture TGFβ (recombinant standard or assay samples). These type II receptors were able to bind all mammalian forms of TGFβ, however the addition of polyclonal αTGFβ_1 antibody (< 1% cross reactivity with other TGFβ isoforms), conjugated to HRP, was able to confer TGFβ_1 specificity. Bound antibody was then quantified by the addition of a HRP chromogen substrate. After incubation for 20 min at room temperature, the substrate product was acidified by the addition of 50 μl of 1 M H_2SO_4, and absorbance at 450 nm (reference 570 nm) was measured. The concentration of TGFβ_1 within biological fluids was calculated from a standard curve generated using known concentrations of rTGFβ_1. This assay was reported by the manufacturer to have a minimum detection limit of 5 pg/ml, with intra and inter-assay precision of approximately 5%.

2.5.2 Human GM-CSF ELISA

The GM-CSF content of human culture supernatants was determined using a GM-CSF-specific ELISA (R and D Systems). All assays were performed according to the manufactures' instructions. This assay is a quantitative sandwich immunoassay, similar to that described in 2.5.1, with the exception that a mouse monoclonal αhGM-CSF antibody is used as the capture antibody, and a polyclonal HRP-conjugated GM-CSF specific

antibody is used as the detection antibody. This assay was performed essentially as outlined in 2.5.1. The manufacture's assay specifications stated that the minimal detectable level of GM-CSF was 2.8 pg / ml, with an intra-assay precision of 2.5% and an inter-assay precision of 5%. In pilot experiments the GM-CSF content of human reproductive tract cell culture supernatants was found to be at the lower end of the assay detection limits, so all subsequent samples were concentrated four-fold using 10 kDa cut-off low-binding cellulose concentrators (Ultrafree-MC, Millipore) prior to assay.

2.6 Female reproductive tract cultures

2.6.1 Murine uterine epithelial cells

Uterine epithelial cells were harvested from whole uteri under sterile conditions using a modification of the procedure described by Salomon and Sherman (1975). Previous investigations had shown that these cultures comprise of approximately 70% epithelial cells, 25% stromal fibroblasts and 5% leukocytes and endothelial cells (Robertson *et al.* 1992b). At 1000 to 1200 hrs on the day of oestrous or dioestrous, uteri from individual mice were excised, placed in cold PBS (pH 7.2), trimmed of fat, mesentery and blood vessels and then slit lengthwise with the aid of a dissecting microscope. The uteri were rinsed in PBS to remove excess red blood cells, then an enzyme solution consisting of 0.25 % trypsin (Cytosystems, Castle Hill, NSW), and 1 % pancreatin (bovine pancreatic type III, Sigma) dissolved in PBS was added (1 ml per pair of horns) for 45 minutes at 4 ^{o}C, followed by 45 minutes at 37 ^{o}C. At the completion of this incubation step, enzymatic activity was quenched by the addition of FCS to 10% vol / vol, and the uterine horns were then repeatedly pipetted up and down with a wide-bore sterile plastic pipette in order to shear epithelial cells from the underlying stromal tissue. The remaining uterine tissue was removed and the epithelial cells washed by the addition of a 10-fold volume of PBS and then collected by centrifugation at 200 g for 10 minutes. A typical epithelial cell yield was 7- 14 x 10^5 cells / uterus.

Harvested cells were suspended in 500 µl of DMEM-10% FCS at a concentration of approximately 1 x 10^5 cells / ml, then plated in duplicate in 4 x 1.5 ml cell culture multi-dishes (Nunc) before incubation at 37 ^{o}C , 5% CO_2 in a humidified atmosphere. After allowing four hours for cell adherence, a further 500 µl of DMEM-FCS (control), DMEM-FCS plus rTGFβ_1, or DMEM-FCS containing seminal vesicle secretions was added to the

culture wells. Sixteen hours later this medium was harvested (16-hour supernatant) and replaced with fresh medium (DMEM-FCS only). Twenty-four hours later this medium was also harvested (40-hour supernatant). Both the 16 and 40 hour supernatants were centrifuged to remove cellular debris prior to storage at -80 ^{0}C until assay. Adherent uterine epithelial cells were quantified by their uptake of Rose Bengal dye (Faulding, Adelaide, SA, 0.25% in PBS), followed by lysis with 1% SDS and measurement of absorbance at 540 nm. Comparison of dye absorbance between a standard curve, constructed by counting the number of cells harvested with 2mM EDTA from duplicate wells using a haemocytometer, enabled an estimate of epithelial cell numbers.

2.6.2 Human endometrial cultures

Human endometrial cell cultures were prepared under sterile conditions using a modification of the procedure described by Bentin *et al.* (1994). Briefly, this technique involved constructing a three-layered culture system to replicate the structure of an intact endometrium. Stromal cells were embedded in a collagen matrix, covered by a thin layer of Matrigel (Collaborative Biomedical Products, Bedford, MA) acting as an artificial basement membrane, which in turn were overlaid with uterine epithelial cells. The Matrigel artificial basement membrane is reported to ensure correct polarisation of epithelial cells, while the inclusion of stromal cells mounted in collagen matrix is believed to enable cytokine communication between stromal cells and the surface epithelium, thereby creating an approximate model of the human endometrium.

Endometrial tissue was harvested from consenting women using cervical dilatation and curettage performed by a consultant gynaecologist at Ashford Hospital, Adelaide, Australia. All samples were obtained from women who were in the late follicular to early luteal phase of the menstrual cycle, where there was no suspicion of endometrial pathology. Women taking steroidal contraceptives or medication for the treatment of endometriosis were not included in the study.

Curettings were immediately placed in HBSS at 4 °C for transport to the laboratory, where they were cut into approximately 2mm square blocks, and then repeatedly washed in PBS to remove excess red blood cells. The endometrial tissue was then incubated in a trypsin / pancreatin enzyme solution, identical to that described in 2.6.1, for a period of 30 mins at 4 ^{0}C followed by 30 mins at 37 ^{0}C. The enzymatically digested fragments were further disaggregated by repeated vigorous pipetting, with the

resulting cell suspension being passed through a 70 μm nylon cell strainer (Falcon, Franklin Lakes, New Jersey) to enable the separation of large epithelial cell aggregates (10-100 cells) from the single cell stromal / red blood cell fraction. Contaminating red blood cells within the stromal cell fraction were osmotically lysed by the addition of one tenth strength (70 mOsm) Hams F-12 for 30 seconds, followed by osmotic neutralisation by the addition of an equal volume of 5X Hams F-12. The remaining stromal cells were re-suspended in a large volume of physiological (280 mOsm) Hams F-12 prior to collection by centrifugation at 200 g, then suspended in a rat tail collagen solution (pH 7.4) at a concentration of 1-2 x 10^6 cells / ml. One hundred and fifty microlitres of the stromal cell / collagen solution was added to each Millicell-CM culture plate insert (Millipore, Bedford, MA) and allowed to solidify for 30 minutes at 37 °C, 5% CO_2, before 150μl of Matrigel / Hams F-12 (1:1 mix) was layered over the collagen base. After solidification of the Matrigel, 400 μl of uterine epithelial cell suspension was added to each well. Single epithelial cells were obtained by washing the epithelial cell aggregates from the surface of the 70 μm filter, enzymatically disassociating them with 0.1% collagenase (Sigma) for 15 mins at 37 °C, followed by a final incubation in 0.05% EDTA / PBS for 10 mins. These epithelial cells were then pelleted by centrifugation, resuspended in culture medium at a density of 1-2 x 10^5 cells / ml, and applied to culture wells. All wells were cultured for two days before commencing experiments.

Quantification of epithelial cell numbers was not possible using Rose Bengal staining because of the more complex structure of these cultures. An assumption was therefore made that basal GM-CSF production levels would be proportional to the number of epithelial cells in each culture well, so that the GM-CSF output prior to the addition of rTGFβ_1 or semen would provide a good estimate of the relative number of epithelial cells in each well. Uterine epithelial cell supernatants were collected at 12 hrs (basal supernatant), replaced with 400 μl of medium containing either rTGFβ_1, semen, or fresh culture medium (control), and then 12 hrs later the 24 hr supernatants were collected and frozen at -80 °C until assay. The GM-CSF content of the 12 hr supernatant was used to normalise the GM-CSF content of the corresponding 24 hr supernatant, to control for differences in epithelial cell numbers between culture wells. All treatment and control groups were conducted in duplicate or triplicate, depending on the number of cells available for culture.

2.6.3 *Human ectocervical keratinocyte cultures.*

Human cervical keratinocytes were cultured using a modification of the technique described by Rheinwald and Green (1975). Cervical biopsies were obtained from consenting women undergoing hysterectomy (abdominal or laparoscopic-assisted vaginal hysterectomy) for various non-malignant gynaecological indications. All women were premenopausal, but no distinction was made regarding the stage of menstrual cycle at the time of surgery. Cervical biopsies were placed in ice-cold HBSS for transport to the laboratory, then washed twice in medium containing antibiotics, before being incubated overnight at 4 ^{0}C in 5 ml of DMEM medium containing five units of dispase (Boehringer Mannheim). The next morning the cervical biopsy / dispase solution was incubated at room temperature for one hour, by which time large sheets of keratinocytes could be mechanically stripped from the biopsy specimen using sterile forceps. These sheets were then incubated in DMEM / 0.25% trypsin / 0.05 % collagenase for 30 minutes at 37 °C to facilitate the disaggregation of the cervical keratinocyte sheets into smaller clumps of cells. This process was assisted by aspirating the keratinocyte sheets into a 10 ml hypodermic syringe, then extruding the cell suspension through hypodermic needles of incrementally decreasing calibre until no large sheets of keratinocytes remained. Keratinocytes were then extracted from the enzyme mixture by centrifugation and suspended in "ectocervical culture medium" consisting of 70% DMEM, 20% Hams F-12, 9% FCS, 1% Neutridoma -SP (Boehringer Mannheim) and 0.4 μg / ml hydrocortisone (Upjohn, Rydalmere, NSW).

The human keratinocytes were suspended in ectocervical medium at a density of 1-2 x 10^5 cells /ml, with 500 μl of this cellular suspension being added to 1.5 ml culture wells (Nunc) seeded 24 hours earlier with murine 3T3 fibroblasts. These fibroblasts secrete proteins such as laminin and fibronectin, which are required for keratinocyte attachment to tissue culture plastic. The 3T3 fibroblasts were rendered mitogenically inactive by exposure to 4% mitomycin C (Sigma) for two hours before addition to culture wells at a density of 2 x 10^4 cells/well. The completed keratinocyte cultures were incubated for 5 -7 days to enable the majority of the keratinocytes to attach to the culture well floor and displace the 3T3 fibroblasts. Once attachment had occurred, the culture supernatant containing desquamated keratinocytes and 3T3 fibroblasts was removed and exchanged for fresh ectocervical medium (500μl). Twelve hrs later this "pre-treatment" supernatant was collected and replaced with 500μl of fresh medium containing either

10 ng of $rTGF\beta_1$, 10% semen or culture medium only (control), which in turn was collected 12 hrs later as the "post-treatment" supernatant. The pre and post-treatment supernatants were centrifuged to remove cellular debris before being stored at - 80 ^{0}C until GM-CSF assay.

2.7 Chromatography

2.7.1 Size exclusion chromatography

Chromatography was performed using a dual pump Fast Protein Liquid Chromatography (FPLC™) system (Pharmacia, Upsalla, Sweden) at room temperature with eluted fractions collected by an automated Frac-100 (Pharmacia) fraction collector. A continuous analysis of eluted protein content was made by measurement of UV absorbance at 280 nm, which in turn was recorded as an elution profile on a chart recorder. All elutions were performed using 6M guanidine hydrochloride, either unbuffered (pH 4.7) or buffered by the addition of 0.05M Tris-HC*l* (pH 7.4).

The chromatographic medium used for size exclusion chromatography was Sephacryl S-400 HR. This was applied to C type chromatography columns (Pharmacia), as detailed in the manufacturer's instructions, and calibrated by the application of purified proteins of known molecular weight (gel filtration calibration kit, Pharmacia). The coefficient of distribution (K_d) for each protein standard could then be calculated once its elution volume (V_e) had been measured:

$$K_d = \frac{Ve - Vo}{Vt - Vo}$$

Vo = void volume corresponding to the elution volume of Blue Dextran

Vt = total column volume measured directly before the addition of chromatography media

Once a series of K_d values had been determined for proteins of known molecular weight, a calibration curve was constructed which enabled the calculation of molecular weights of seminal vesicle proteins. Before and after every chromatography run, the column was flushed with a volume of guanidine hydrochloride equivalent to three times the void volume to remove any residual protein.

2.7.2 Desalting chromatography

Seminal vesicle secretions dissolved in guanidine hydrochloride were desalted into culture medium or PBS/0.1% BSA using HiTrap ™ 5 ml desalting columns (Pharmacia) before being applied to uterine epithelial cell cultures or assayed for TGFβ content. Desalting columns were equilibrated with culture medium or PBS/0.1% BSA prior to the application of 1.5 ml of dilute seminal vesicle secretions, with the subsequent 1.5 mls being collected as the desalted fraction. All desalting columns were stored in PBS-20% ethanol between uses to prevent bacterial contamination.

2.8 Immunohistochemistry

2.8.1 Murine immunohistochemistry

Uteri from cycling, day 1 mated or oestrous mice 16 hrs after intrauterine administration of $rTGF\beta_1$ 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 in thickness were cut on a Bright (Huntingdon, UK) cryostat, air-dried, and fixed in 96% ethanol for 10 mins at 4 ^{0}C. Following rehydration through four changes of PBS, sections were incubated with monoclonal antibodies (mAbs) specific for various leukocyte lineages. The hybridoma origins of these mAbs and their leukocyte specificity are recorded in Table 2.2.

Sections were incubated with lineage-specific mAbs (neat hybridoma supernatant containing 10 % NMS) for 16 hours at 4 °C in humidified chambers, then washed three times in PBS, followed by incubation for 90 mins with goat α rat-HRP antibody (Dako, Copenhagen), diluted 1:40 in PBS / 1% BSA/10% NMS. To detect the F4/80 antigen present at a relatively low density on cells, sections were incubated for two hours with biotinylated rabbit α rat antibody (Dako, Carpinteria, CA; 1:200 in PBS/1% BSA/10%

NMS), followed by avidin-HRP (Dako; 1:400 in PBS/1% BSA/10% NMS) for one hour. All sections were then washed three times, with HRP reactivity being visualised using Sigma Fast™ DAB peroxidase substrate tablets. Tissue was then stained in Gill's haematoxylin (Sigma), dehydrated in two changes of absolute ethanol, cleared in two changes of Safsolvent (Ajax Chemicals, Auburn, NSW), mounted in DPX (BDH) and left to dry for 24 hours before viewing or photographing.

Table 2.2 Antibodies used in murine immunohistochemistry

hybridoma	antigenic epitope	leukocyte specificity	reference
F4/80	mac-1	macrophages	Austyn & Gordon 1981
TIB120	Ia	activated macrophages, dendritic cells	Bhattacharya & Dorf 1981
TIB122	Leukocyte common antigen (CD45)	all leukocytes	Springer & Galfre 1978
TIB128	Mac-1 (CD11b)	macrophage, monocyte	Springer & Galfre 1978
RB6-8C5	RB6	neutrophil	Conlan & North 1994

Quantification of stain density for each antibody type was performed using video image analysis software (Video Pro, Faulding Imaging, Mt. Waverly, Victoria) and an Olympus BH-2 microscope. This software quantified the proportion of cells labelled with mAb by measuring the area of brown staining (antibody positive), as a percentage of total stain (haematoxylin stained cells plus antibody positive cells). Ten representative low power (20 X) fields were measured on each section, with duplicate sections being analysed from each experimental animal. Negative control sections were incubated with an irrelevant, isotype-matched rat mAb or without mAb.

2.8.2 Immunohistochemical analysis of human endometrial tissue

Human endometrial cultures and endometrial curettings were frozen in OCT and processed to produce 6 μm sections, as described in 2.8.1. In order to increase the adhesion of fragile tissue culture sections to glass, all slides and cover-slips were treated by soaking

overnight in potassium bichromate (Ajax Chemicals, Aulburn, NSW), followed by coating with 3-aminopropyl-triethoxy-silane (Sigma). Sections were then fixed and processed as described in 2.8.1. A monoclonal mouse α human cytokeratin antibody (CK-1, Dako) was used to identify uterine epithelial cells in these sections, since this antibody is specific for the type 18 cytokeratin reported to be expressed by human uterine epithelial cells, but not stromal cells (Moll *et al.* 1983). The CK-1 antibody was diluted 1:30 in PBS with 10% NHS, and a HRP-conjugated rabbit α mouse antibody (Dako; diluted 1:60 in PBS/10% NHS) was used as a secondary detection antibody.

2.9 Immunological assays

2.9.1 Anti-sperm antibody-specific ELISA

A solid phase ELISA using a modification of the protocol described by Okada *et al.* (1993) was used to quantify the maternal antibody response following exposure to sperm antigens, in an immunoglobulin isotype-specific manner. Freshly isolated sperm from stud CBA males were suspended in PBS at a density of 5×10^6 sperm / ml, disrupted using a Branson sonicator (setting 6, intermittent pulse for 2 minutes), and 50 μl of this sperm antigen suspension (membrane + cytosol antigens) was then added to polystyrene 96-well flat bottom ELISA plates (Maxisorb™, Nunc). These plates were incubated overnight at 4 °C to allow binding of sperm antigens to the plate surface. Excess sperm membrane solution was then removed from the wells, and each plate washed four times in PBS containing 0.05% Tween 20 (wash buffer), before the remaining protein binding sites were blocked by the addition of 200μl of PBS /3 % BSA for a period of 1 hr at room temperature. The plates were then "flick" dried, wrapped in aluminium foil and frozen at -20 °C until use.

Test sera were diluted 1:4 in PBS, then serially diluted 1:2 to a final dilution of 1:128, before application to the thawed antigen-coated ELISA plates. All serum samples were assayed in duplicate. After two hours incubation at room temperature, the serum was washed out of the wells using four changes of wash buffer, then 100μl of rabbit α mouse antibody (Mouse Typer™, BioRad) was added to each well and incubated for 1 hr at room temperature. Quantification of each immunoglobulin isotype (IgG_1, IgG_{2a}, IgG_{2b}) was performed in separate plates using rabbit α mouse antibodies specific for each immunoglobulin isotype. After washing each well four times, 100μl of biotinylated

donkey α rabbit antibody (Amersham,UK), diluted 1:2000 in PBS / 1% BSA, was added to each well and incubated for 1 hr at room temperature. Following the removal of unbound antibody by a further four washes, 100 μl of streptavidin-HRP (Amersham, diluted 1:4000 in PBS), was added to each well for 30 minutes, followed by a final series of four washes. Bound antibody was detected by adding 100 μl of the HRP chromogenic substrate tetra methylbenzidine (TMB, Sigma) to each well, and the green reaction product was allowed to develop over 20 minutes at room temperature. Acidification of each well was achieved by adding 50 μl of 1 M H_2SO_4, which terminated the chromogenic reaction and converted the reaction product to a yellow derivative, the absorbance of which could be measured at 450 nm (reference 660 nm). The antibody titres for individual sera was determined by plotting the optical density of each sample following subtraction of the negative control OD.

2.9.2 Delayed-type hypersensitivity (DTH) responses against sperm antigens

To measure delayed-type hypersensitivity (DTH) responses against sperm antigens, a footpad swelling assay was performed, as described by Lee and Ha (1989). Female Balb/c F1 mice, primed against sperm antigens by artificial insemination (described in 2.1.4), were lightly sedated with Avertin before measuring the thickness of their left and right hind-foot footpads with a thickness gauge (0.01 mm increments) (Mitutoyo, Tokyo, Japan). Initial measurements revealed that less than 0.02 mm variation in thickness existed between the left and right footpads of all females prior to antigen challenge. The left hind footpad was then injected with 25 μl of sperm suspension (density 1 x 10^8 sperm / ml in HBSS) using a 100μl Hamilton glass syringe (Hamilton, Nevada, USA) and a disposable 30 G needle, and the right hind footpad injected with 25μl of HBSS (negative control). A new needle was used for each injection to minimise non-specific oedema resulting from tissue damage produced by blunt needles. Measurement of footpad thickness was then repeated 24 hours later, with sperm antigen-specific swelling being calculated by subtracting the thickness change measured in the right footpad (negative control) from that measured in the left footpad (sperm injected).

Additionally, footpad oedema was quantified by measuring changes in footpad weight, since this had been reported to be a more objective measurement of DTH responses (Kitamura 1980). After footpad thickness measurements had been performed, the left and right hind footpads were removed using scissors at the level of the tarsus,

which was recognised as the indentation of bone below the joint connecting the tibia with the fibula, but above the *articulatio torsi transversa*. Each footpad was then immediately weighted to the nearest mg, with any increase in footpad weight in response to sperm challenge being calculated as the difference between the weight of the sperm-challenged footpad versus that of the HBSS injected footpad.

2.9.3 Human leukocyte chemotaxis assay

Leukocyte populations were obtained from the peripheral blood of healthy adult volunteers using Ficoll-Paque™ density gradient centrifugation, according to the method described by Boyum (1968). Briefly, 10 ml of blood was collected by standard venipuncture methods, heparinised by the addition of 150 U heparin (Beckton Dickson), diluted 1:1 in PBS, before being carefully layered over 20 ml of Ficoll-Paque™ contained within a 50 ml Falcon tube. Following centrifugation at 400 g for 30 minutes (room temperature, without use of a brake), the peripheral blood mononuclear cells (PBMC: lymphocytes and monocytes) were removed from the interface between plasma and Ficoll, washed twice in PBS, then added to culture flasks or used immediately in chemotaxis assays. Assays measuring macrophage chemotaxis were performed using *in vitro* matured monocytes according to the protocol described by Davies and Lloyd (1989). This consisted of culturing PBMCs in RPMI-10% FCS for four hours, after which time monocytes became adherent to the culture flask plastic, thereby facilitating the selective removal of non-adherent lymphocytes and platelets by washing. Adherent monocytes were cultured for a further four days in RPMI-10% FCS, during which time they matured into macrophages. Macrophages were harvested by mechanical detachment from the culture flask wall by using a sterile cell scraper (Nunc). Leukocytes used in chemotaxis assays were suspended in HBSS containing 10 % "cervical keratinocyte culture medium," at a density of 5×10^5 cells/ml.

The chemotaxis assay used in these studies is a modification of the classic Boyden chemotaxis chamber, as described by Bignold (1989). The chamber was comprised of a 30 μl well drilled into 5mm perspex, into which was placed 30 μl of cervical keratinocyte culture supernatant (diluted 1:1 with HBSS/10% Cx medium), fresh HBSS/10% Cx medium (negative control) or fresh medium containing N-formyl-methionyl-leucyl-phenylalanine (FMLP, Sigma), a peptide that is chemotactic for PBMCs and granulocytes at concentrations as low as 10^{-9} M. Each perspex well was then mounted within the screw

cap of a 10 ml plastic disposable tube (Disposable products, Adelaide, Australia). The bottom perspex well was filled with the appropriate medium, covered by a 3 μm polycarbonate filter (Nuclepore, CA), followed by a 8 μm polycarbonate sparse-pore filter (Nuclepore). A silicon rubber "O-ring" was then placed on top of the filters, and the assembly tightened into place by screwing the stage constructed from the top portion of the 10 ml tube into the cap housing the well apparatus. A schematic diagram summarising the Bignold chemotaxis chamber is depicted in Figure 8.6a. Fifty μl of PBMC suspension was then pipetted into the central hole of the O-ring, and incubation commenced. Chemotactic activity within the lower well stimulated the migration of PBMCs from the top well through the 8 μm sparse-pore filter, until they were trapped on the surface of the underlying 3μm filter (the pores being too small to allow transit of PBMCs). Assembly of the apparatus and incubations were performed in a room maintained at 37 °C, since temperature gradients interfered with leukocyte trafficking. Following an incubation period of 45-60 mins, chemotaxis was stopped by adding 1 ml of 10% formalin to each chemotaxis chamber. The individual filters with adherent PBMCs were then removed and stained with Mayer's haematoxylin before being mounted on glass slides in DPX.

Chemotactic activity was quantified by manually counting the number of PBMCs fixed to a circular area of approximately 20 mm^2 on the 3 μm catchment filter (corresponding to the chemotaxis well provided by the interior diameter of the silicon O-ring). Mean cell numbers ($\pm$ s.d.) of triplicate measurements were made using an Olympus BH-2 microscope for each test sample.

2.10 RT-PCR protocol

2.10.1 General conditions

Ribonuclease (RNAse)-free conditions were maintained for all materials, chemicals and solutions used in the generation and handling of riboprobes, and in the preparation and processing of tissue used for extraction of mRNA. Precautions taken to prevent RNAse contamination included the wearing of disposable gloves when handling reagents and apparatus, the use of autoclaved disposables (pipettes and tubes) and the baking of all glassware at 180^0 C for three hours prior to use.

2.10.2 RNA extraction from seminal vesicles

RNA was extracted from the seminal vesicles of CBA F1 stud males according to a modification of the method described by Chomczynski and Sacchi (1987). This consisted of immersing seminal vesicle glands immediately following their removal into 1.6 ml of extraction solution A, followed by disruption of these glands using an Ultra-Turrax tissue homogeniser. Solution A consisted of 10 ml phenol (water saturated), 5 ml of 4 M guanidine thiocyanate, 72 µl of 0.1 M β-mercaptoethanol, and 1 ml of 25 mM Na citrate pH 7.0. Phase separation and DNA precipitation was achieved by adding 160 µl of chloroform / isoamyl alcohol (49:1), vortexing and incubating for 15 minutes on ice, followed by centrifugation at 10,000 g for 20 minutes at 4^0 C. The resulting aqueous phase containing mRNA was removed and the remaining DNA / protein layer discarded. RNA was then precipitated from this aqueous phase by adding an equal volume of isopropanol, followed by incubation overnight at -20 ^{0}C. The RNA was pelleted (10,000 g / 20 minutes / 4^0 C), washed in 70% ethanol, dried *in vacuo,* and then dissolved in MQ water. RNA (approximately 10 µg in 50 µl) was depleted of any residual DNA by treatment with RNAse free DNAse (5 U / 50 µl RNA; Bresatec, Adelaide, Australia), in combination with 5 U of RNAse inhibitor (Promega) in Tris-HC*l* (pH 7.8) buffer for a period of 60 mins at 37 ^{0}C. The resulting DNA-free RNA was then re-extracted from phenol / chloroform (1:1 mix for 10 minutes at RT), and precipitated overnight at - 20 ^{0}C in 70% ethanol / 30mM Na acetate. After centrifugation, the RNA pellet was washed in 70% ethanol, dried *in vacuo*, then resuspended in MQ water for quantification of purity by measuring absorbance at 260 and 280 nm. An absorbance of 1.0 at 260 nm was assumed to correspond to an RNA concentration of 40 mg /ml, and an absorbance ratio of A_{260}:A_{280} in excess of 1.7 was judged to indicate the sample was acceptably free of contaminating protein.

2.10.3 cDNA generation from tissue RNA using reverse transcription

Approximately 2 µg of RNA was dissolved in 10 µl of MQ water, incubated with 1 µl of random hexamers (Bresatec, 100µg/ml) at 70^0 C for 10 minutes, and chilled on ice for 5 minutes, before addition of 4 µl RT buffer (250 nM Tris-HC*l* pH 8.3, 300mM KC*l*, 15 mMMgCl_2) and 2 µl 0.1 M DTT, 2 µl 10 mM dNTPs (10 mM each dATP, dTTP, dCTP and dGTP). The mixture was then incubated for two minutes at 43^0 C before adding

1 μl of Reverse Transcriptase (Gibco, 200 U/μl), followed by incubation at 43^0 C for 90 minutes. Finally enzyme inactivation and RNA-cDNA denaturation was achieved by heating to 94^0 C for five minutes, followed by cooling on ice for five minutes. The resulting cDNA solution was brought up to a volume of 50 μl by the addition of 30 μl MQ water. This amount of cDNA was sufficient to perform up to 20 amplification reactions.

2.10.4 Polymerase chain reaction

Primer pairs specific for murine TGFβ_1, TGFβ_2, and actin cDNA were designed with the aid of Primer Design Software (Scientific and Educational Software, State Line, PA). The TGFβ_1 5' primer (5'- GGAGGAACATGTGGAACT-3') corresponds to the murine TGFβ_1 sequence from nucleotide 844-846 and the complimentary 3' primer (5'-GGTTCATGTCATGGATGG-3') is antisense to the cDNA sequence from nucleotide 1098-1115, giving an amplified product of 273 bp in length. The TGFβ_2 5' primer (5'-AGAAATGTGCAGGATAATTGCTGC-3') corresponds to the murine TGFβ_2 sequence from nucleotide 2148-2171 and the complimentary 3' primer (5'-TTCGATCTTGGGCGTATTTCCAAT-3') is antisense to the cDNA sequence from nucleotide 2420-2397, giving an amplified product of 273 bp in length. The actin 5' primer (5'- TGTGATGGTGGGTATGGGTC-3') corresponds to the murine actin sequence from nucleotide 48-67 and its complementary 3' primer (5'-TAGATGGGCACAGTGGGT-3') is antisense to the cDNA sequence from nucleotide 419-400, giving an amplified product of 372 bp in length.

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 25 μl reaction volume, the following were combined at 4^0 C: 16 μl of MQ water, 2.5 μl 10 X PCR buffer (670 nM Tris-HC*l* pH 8.8, 166 mM $(NH_4)_2SO_4$, 2mg/ml gelatin, 4.5% Triton X-100), 2.5 μl 25 mM $MgCl_2$, 0.5 μl 10 mM dNTPs, 0.5 μl 100 μM 5' primer, 0.5 μl 100 μM 3' primer and 5 U Taq DNA polymerase in 0.1 μl. This mixture was overlaid with a drop of paraffin oil and placed into a PCR thermal-cycler (Hybaid) programmed to cycle 40 times through a thermal cycle of denaturation for one minute at 94^0 C, annealing for one minute at 58^0 C, and extension for one minute at 72^0 C.

Each amplification was followed by a final extension for seven minutes at 72^0 C. Twenty µl of the reaction product was analysed by "minigel" electrophoresis through a 2% agarose gel (Promega, Madisson, WI) containing 0.5 mg/ml ethidium bromide 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).

2.11 Human studies

2.11.1 Ethics approval

Ethics approval for conducting all human studies was obtained from the joint institutional human ethics committees of the University of Adelaide and the Queen Elizabeth Hospital. Two separate protocols were approved:

Application 2/96 The role of seminal plasma in uterine priming for successful pregnancy following *in vitro* fertilisation.

Application 3/96 Investigation of the post-coital inflammatory response in the human female.

2.11.2 IVF intercourse trial

All couples enrolled in the *in vitro* fertilisation (IVF) thawed embryo transfer program of The University of Adelaide's clinical reproductive medicine program (Repromed) were requested to become involved in this study. Fresh embryo transfer cycles were excluded, because the trans-vaginal oocyte collection procedure would have made intercourse painful for participants. Any woman requiring ovulation induction during a thawed embryo transfer cycle was also excluded from the study. Besides anovulation, no

distinctions were made based on infertility aetiology for enrolment in this study. Those couples who were interested in becoming involved were given a study information package which contained information on the purpose of the study and the involvement required of participants (Appendix A). Three days after delivery of the information package, couples were contacted by telephone to request their verbal consent for involvement, with any remaining questions being answered at the time. Written consent was also requested from each couple.

Once a couple had given their verbal consent they were randomly allocated into one of two trial arms; intercourse or abstinence. Randomisation was performed using block stratification, which guaranteed equal numbers of participants in each trail arm after every fourth enrolment. Randomisation was also stratified for age (maternal age 35 years or younger, maternal age 36 years or older), since age is reported to be the most important variable effecting IVF outcome (Smith and Buyalos 1996). Randomisation was achieved with the assistance of a member of the Departmental clerical staff (located at a different site to the clinical reproductive medicine program), who opened an opaque envelope containing the trial allocation for each couple. Once this allocation had been made, it was recorded in the couple's clinical notes so that they could be informed of their allocation by the clinic staff at the time of their next appointment.

The period of time selected for studying the effect of semen exposure on thawed embryo transfer outcome was deemed a four-day period encompassing two days before, and two days after embryo transfer. This was considered an appropriate period since it corresponded to the approximate time at which intercourse would normally occur if natural pregnancy was to ensue, and menstrual cycle tracking performed by the reproductive medicine unit (LH, oestrogen, progesterone) usually provided couples with at least two days notice of an impending embryo transfer. Those couples randomised to the intercourse group were asked to have unprotected intercourse on at least one occasion during this four day period, with couples allocated to the abstain group being asked to abstain from sex during the same period, or use a condom if they did have intercourse. Sexual intercourse was defined as penetrative vaginal intercourse resulting in orgasm of the male partner. Couples who were unable to comply with their trial allocation were requested to leave their name on a confidential answering machine so that they could be removed from the trial analysis.

Two months after trial enrolment, the clinical notes of participating couples were reviewed to enable assessment of their IVF cycle outcome, as well as to collect background information on each couple (infertility aetiology, obstetric history etc).

Chapter 3

Characterisation of the GM-CSF stimulating factors present in murine seminal vesicle fluid

3.1 Introduction

GM-CSF, a cytokine whose production by the murine uterine epithelium is dramatically increased following mating, has now been identified as one of the major pro-inflammatory cytokines accompanying the post-mating inflammatory response in the mouse (Robertson *et al.* 1990 and 1992a, 1992b). Previous studies have implicated GM-CSF as one of the key mediators of the post-mating inflammatory response, since instillation of rGM-CSF into the uterine lumen evokes cellular changes resembling those seen following mating (Robertson *et al.* 1994). The increase in uterine GM-CSF content observed following mating is not due to the introduction of GM-CSF contained within the ejaculate, is not provoked by cervical stimulation, and is independent of the presence of sperm in the ejaculate or any MHC disparity between the male and female (Robertson *et al.* 1992b). The trigger responsible for eliciting an increase in uterine epithelial GM-CSF has been localised to the seminal vesicle gland, since only the secretions from these glands are capable of increasing uterine epithelial cell GM-CSF production *in vitro,* while removal of the seminal vesicle glands renders an individual incapable of increasing uterine GM-CSF production following mating.

The aim of studies outlined in this chapter was to identify and characterise the seminal vesicle trigger responsible for eliciting an increase in uterine epithelial cell GM-CSF production. Initially this was addressed by fractionating murine seminal vesicle fluid on the basis of molecular weight using size exclusion chromatography, followed by an assessment of the capacity of each fraction to stimulate GM-CSF production in uterine epithelial cell cultures. This initial step towards isolation of the seminal vesicle trigger for the post-mating inflammatory response (here after abbreviated as PMIR) identified two distinct stimulatory factors; a high molecular weight protein of approximately 650 kDa, and a second intermediate molecular weight trigger which eluted in a broad band between

150 and 440 kDa. The focus of this chapter will be on the biochemical characterisation of the 650 kDa stimulating fraction, while Chapter 4 will describe how the intermediate molecular weight stimulating factor was eventually identified as transforming growth factor beta (TGFβ).

3.2 Chromatographic fractionation of seminal vesicle fluid under acidic conditions

3.2.1 Set-up and calibration of the chromatography column

Chromatographic separation of seminal vesicle fluid was performed initially using a 100cm x 16mm chromatography column packed with Sephacryl S-400 gel filtration media. A large chromatography column (V_t 184*ml*) and a broad resolution chromatography gel (20 000-8 000 000 M_r), were employed to enable the greatest possible separation of seminal vesicle constituents over the widest range of molecular weights, since nothing was known of the approximate molecular weight of the seminal vesicle GM-CSF stimulating factor (SGSF). Murine seminal vesicle secretions are extremely viscous, exhibiting the tendency to form a gelatinous conglomerate within minutes of their removal from the seminal vesicle glands. This characteristic presented technical difficulties for their chromatographic fractionation, since the secretions had to be fully solubilised in a liquid medium before they could be chromatographed. The strong denaturing agent guanidine hydrochloride was able to completely solubilise seminal vesicle secretions at dilutions below 25% vol/vol, and therefore was adopted as the medium for seminal vesicle secretion chromatography. Guanidine hydrochloride was used at its native pH of 4.7 in the first series of experiments outlined in 3.2.1, but once it became apparent that elutant pH had an effect on the bioactivity of the SGSF, all further chromatography was performed using neutral guanidine hydrochloride (pH 7.4).

Gel filtration chromatography enables an estimate of the molecular weight of unidentified proteins by comparing their elution volumes with that of proteins of known molecular weight. The proteins thyroidglobulin (669 kDa), ferritin (440kDa), catalase (232kDa) and aldolase (158kDa), all obtained from a Pharmacia molecular weight standard kit, were individually chromatographed on three occasions to ensure accurate measurement of their elution volume. The void volume was calculated from the elution volume of Blue Dextran (molecular weight of approximately 2 million Daltons). A

molecular weight calibration curve was then prepared by plotting the logarithms of the molecular weight of protein standards versus their calculated Kd values, as previously described (Andrews 1965, Ui 1979). This calibration curve, depicted in Figure 3.1, was relatively linear between 158 and 669 kDa, therefore allowing accurate estimates of protein molecular weight over this range. It has been reported that the elution profile of chromatography columns may change over time due to alterations in the gel structure produced by the strong denaturing effects of guanidine hydrochloride (Ansari and Mage 1977). Because of this possibility, the column was recalibrated every two months, yet no changes in the elution volume of protein standards were observed over the 6-month life of the column.

3.2.2 Fractionation of seminal vesicle fluid

Seminal vesicle secretions from 2-3 stud CBA F1 males were solubilised in 1.5 ml of guanidine hydrochloride and loaded onto the chromatography column. It was not possible to apply secretions from more than three stud males at any one time, since this overloaded the column and reduced its resolving capacity. Seminal vesicle secretions were eluted at a rate of 0.4 ml per minute, with 1.5 ml fractions being collected for assessment of GM-CSF stimulating activity. The protein content of each fraction was continuously estimated by measuring UV absorbance at 280 nm. The resulting protein elution profile contained no isolated protein peaks, but rather a pattern of multiple merged peaks that made identification of the elution positions of individual seminal vesicle proteins impossible (Figure 3.2a). Several attempts were made to improve the resolving power by applying smaller amounts of seminal vesicle fluid to the column, or eluting at a slower rate, but none were successful.

Every alternate fraction was assessed for its GM-CSF stimulating capacity, since the number of fractions obtained from each chromatography experiment was too large to enable all to be processed in one day. Before adding seminal vesicle fractions to uterine epithelial cell culture, each fraction was desalted into culture medium using 5ml Sephadex G-25 desalting columns. This step was necessary, since even trace amounts of contaminating guanidine hydrochloride were highly toxic to uterine epithelial cells. A typical elution profile of GM-CSF stimulating activity from seminal vesicle fluid is depicted in Figure 3.2b. On each of three separate chromatography runs, the major proportion of GM-CSF stimulating activity was found to elute in a peak of approximately

650 kDa in molecular weight, with a smaller and more heterogeneous peak eluting between 100-400 kDa in molecular weight.

3.2.3 Characterisation of the 650 kDa seminal trigger for uterine GM-CSF release

A serial dilution of the 650 kDa seminal vesicle fraction was performed prior to its application to uterine epithelial cell cultures. This confirmed that the observed stimulation was concentration-dependent, with maximal activity being observed at a 1:2 dilution, titrating to undetectable values at dilutions beyond 1:16 (Figure 3.3). These figures correspond approximately to a 1:20 to 1:160 dilution of neat seminal vesicle fluid respectively (seminal vesicle fluid was loaded onto the column solubilised in GuHC*l* at a concentration of ~ 10% vol/vol). Since it has been estimated that seminal vesicle fluid is diluted 1:10 to 1:20 by uterine luminal fluid following mating (section 4.6), the 650 kDa SGSF should be capable of eliciting an increase in uterine epithelial cell GM-CSF production *in vivo*. In addition, no GM-CSF bio-activity was detected in seminal vesicle secretions, thereby confirming that the observed increase in GM-CSF content within uterine epithelial cell cultures following the addition of seminal vesicle fluid must be due to an increase in uterine epithelial cell GM-CSF production, not seminal fluid-derived GM-CSF.

A series of protein denaturing and lectin binding experiments were carried out on the 650 kDa seminal vesicle fraction in order to assess its biochemical properties, with a view to using this information to assist further attempts to purify the active moiety. The results, summarised in Table 3.1, reveal that the SGSF was heat sensitive, with less than 50% activity remaining after incubation at 80^{0}C for 10 minutes. Removal of protein from the seminal vesicle fraction by acetone precipitation also resulted in a significant fall in stimulating activity. Incubation of the 650 kDa seminal vesicle fraction with trypsin for 30 minutes at 37^{0}C produced a 50% reduction in GM-CSF-stimulating activity. All these observations suggested that the 650 kDa SGSF is a protein.

A series of lectin affinity experiments were performed on the 650 kDa SGSF in order to assess whether affinity chromatography may be a useful purification step. Small affinity chambers were constructed from 2 ml syringes using Concavalin A and Heparin Sepharose affinity media, with the syringe outlet being covered by a permeable glass paper filter. The 650 kDa seminal vesicle fraction was incubated with the chromatography

Table 3.1 Identification of the biochemical properties of the 650 kDa seminal vesicle trigger for uterine GM-CSF production.

treatment group	GM-CSF output (U/ 10^5 epithelial cells)	GM-CSF output (% control output)
control	26.7 ± 6.5	100 ± 24.3%
untreated SVF	100.7 ± 11.1	377 ± 41.6%
SVF - 50°C for 10 min.	95.0 ± 16.9	355.5 ± 63.5%
SVF - 80°C for 10 min.	41.6 ± 6.9	155.6 ± 25.8%
acetone precipitated SVF	49.4 ± 0.6	184.9 ± 2.2%
acetone exposed DMEM (control)	37.6 ± 1.7	140.9 ± 6.4%
trypsinised SVF	46.4 ± 4.9	173.4 ± 18.5%
trypsin exposed DMEM (control)	28.9 ± 6.0	108.2 ± 22.5%
Concavalin A extracted SVF	59.4 ± 6.7	222.5 ± 25.1%
Heparin extracted SVF	42.3 ± 10.2	158.2 ± 38.2%

SVF represents the 650 kDa seminal vesicle fraction. All values are expressed as the mean ± s.d. of duplicate measurements, except the controls which were performed as quadruplicate measurements.

medium for one hour, after which time unbound protein was removed by centrifuging the chamber. This extraction process resulted in very little dilution of the seminal vesicle fluid, unlike conventional fluid elution techniques, with in excess of 80% of seminal vesicle fluid being recovered following centrifugation. The small decline in GM-CSF stimulating activity observed following incubation with Concavalin A probably only reflects a small loss of protein during the extraction process, however the 50% decline in stimulating activity seen following incubation with the Heparin affinity medium is more suggestive of true affinity. This observation lead to several attempts at isolating the SGSF using heparin affinity chromatography. These experiments were not very successful however, because guanidine hydrochloride could not be used as the elutant for affinity chromatography, and seminal vesicle secretions were relatively insoluble in sodium phosphate, which in turn limited the amount of seminal vesicle protein that could be

applied to each chromatography run. Furthermore, the SGSF was found to exhibit only weak affinity for heparin, which did not allowed this line of inquiry to proceed any further.

3.3 Chromatographic fractionation of seminal vesicle fluid under neutral pH conditions

3.3.1 Introduction

During the initial series of experiments, it became apparent that the elution profile and GM-CSF stimulating activity of seminal vesicle fluid was dependent upon elutant pH. Extremes of pH have been reported to denature and dissociate proteins to varying degrees, resulting in considerable changes to their gel filtration behaviour and subsequent molecular weight estimate (Andrews 1974). Even when a protein is dissolved in a strong denaturant such as guanidine hydrochloride, the conformational state can still be pH dependent, and thus profoundly influence protein elution behaviour (Osterhout *et al.* 1985). It was therefore decided to perform all future chromatography experiments using neutral guanidine hydrochloride (pH 7.4) as the elutant. This change necessitated recalibration of the molecular weight standard curve, and therefore it was decided to build a new, smaller chromatography column that would produce a more manageable number of fractions per run.

3.3.2 Calibration of the 26 ml chromatography column

A 26 ml Sephacryl S-400 chromatography column was constructed using a 40cm x 16mm chromatography column. Molecular weight protein standards varying in size from 6 kDa (insulin) to 669 kDa (thyroidglobulin) were chromatographed using neutral guanidine hydrochloride, as described in 3.2.2. The resulting molecular weight standard curve (Figure 3.4) was relatively linear between 25 and 520 kDa.

3.3.3 Chromatographic fractionation of seminal vesicle secretions under neutral pH conditions

Seminal vesicle secretions from two stud males were solubilised in 0.5 ml of neutral guanidine hydrochloride, applied to the 26 ml chromatography column, and eluted as 1 ml fractions. Each fraction was divided in two equal portions, one being transiently acidified to a pH of 2 for 10 minutes by the addition of 5M HC*l*, while the other was kept at a neutral pH. Both the acid activated and neutral samples were desalted into DMEM prior to their application to uterine epithelial cells. The pattern of seminal vesicle GM-CSF stimulating activity was observed to be vastly different for these two treatment groups. The neutral fractions had an stimulation profile similar to that seen in the previous experiments using a large chromatography column under mildly acidic conditions, with a single peak of GM-CSF-stimulating activity at approximately 650 kDa, but no second intermediate molecular weight peak (Figure 3.5b). Transient acidification of the seminal vesicle fractions resulted in loss of the 650 kDa SGSF fraction, but produced a return in stimulating activity within the 150-440 kDa intermediate molecular weight range (Figure 3.5b). These observations suggested that two moieties may exist for uterine GM-CSF release; a high molecular weight (650 kDa) acid labile protein, and a 150-440 kDa intermediate molecular weight factor that has no stimulating capacity in its neutral state, but following transient acidification can initiate an increase in uterine epithelial cell GM-CSF production.

3.4 Discussion and conclusion

The experiments outlined in this chapter support the conclusion that there are at least two proteins present in murine seminal vesicle fluid which can elicit an increase in uterine epithelial cell GM-CSF production following mating. Alternatively, it is possible that the observed pattern of GM-CSF stimulatory activity may be due to one stimulating factor, existing in two different molecular weight forms. This could occur if the active moiety is bound to different carrier proteins or latency complexes, with vastly different molecular weights. Any bonds involved in binding the active moiety to its carrier complex would have to be covalent in nature, or buried deep within the protein complex, since guanidine hydrochloride is a strong chaotrophic agent capable of cleaving non-covalent bonds typically involved in protein-protein interactions. The scenario of one active moiety

bound to several different carriers would certainly explain why the intermediate molecular weight trigger for GM-CSF production (150-440 kDa) eluted over such a broad molecular weight range. Analysis of the two SGSF fractions using polyacrylamide gel electrophoresis (PAGE) under native and denaturing conditions (SDS) would determine if these two stimulating fractions are the same moiety bound to carrier proteins of different size, since this would result in both SGSFs sharing a common molecular weight protein when analysed by PAGE under denaturing conditions. Preliminary attempts were made to characterise the SGSFs by PAGE, however these were unsuccessful due to technical problems. At this point it was decided to concentrate on identifying the 150-440 kDa, rather than the 650 kDa SGSF, since this line of investigation was producing more interesting results in relation to the primary hypothesis that seminal plasma could modify maternal immune responses towards ejaculate antigens.

Any future attempts to isolate and identify the 650 kDa SGSF should start with obtaining the 650 kDa fraction by size exclusion chromatography, since this approach has two advantages. Firstly, seminal plasma proteases, small proteins (<100kDa) capable of destroying the 650 kDa protein, are removed by this initial fractionation step. Secondly, one of the major limitations of all forms of chromatography is that only a small amount of protein is able to be loaded on to a column at any one time. This is an important consideration since the 650 kDa SGSF makes up only a small fraction of crude seminal vesicle protein (< 5%). Separating the 650 kDa fraction from the remaining 95% of seminal plasma protein using size exclusion chromatography would enable the application of 20-fold more stimulating factor protein at the next purification step, while eliminating the majority of contaminating proteins. Heparin affinity chromatography would be a useful second step, since the 650 kDa SGSF is known to bind to heparin. The resulting stimulating fraction would contain only a few proteins by this stage of purification, so additional separation techniques such as ion exchange or hydrophobic interaction chromatography are likely to produce a relatively pure SGSF. Once pure protein has been isolated it could be sequenced by automated Edman degradation to determine its N-terminal sequence, with this sequence then being compared with proteins present in the SWISS-PROT database to determine if the isolated protein had already been isolated from other tissue, or if the SGSF belongs to a larger family of related proteins.

A search of protein data bases to identify a likely candidate for the 650 kDa seminal vesicle trigger for GM-CSF release was not successful, yet did provide additional clues to the identity of the intermediate molecular weight seminal vesicle trigger. The

cytokine transforming growth factor beta (TGFβ), usually secreted as a 230 -290 kDa latent complex, also exhibits the unusual physical property of increased biological activity upon transient acidification. This biochemical property, together with similarities in molecular weight between TGFβ and the 150-440 kDa SGSF, lead to the conclusion that this seminal vesicle protein may be a member of the TGFβ family. TGFβ has not been previously identified within murine seminal plasma, but human seminal plasma is known to contain large amounts of TGFβ (Nocera and Chu 1993). These close similarities prompted a series of experiments to investigate the possibility that TGFβ, secreted in its latent state, may be the intermediate molecular weight seminal vesicle trigger responsible for initiating an increase in uterine epithelial cell GM-CSF production. The outcome of these experiments are described in Chapter 4.

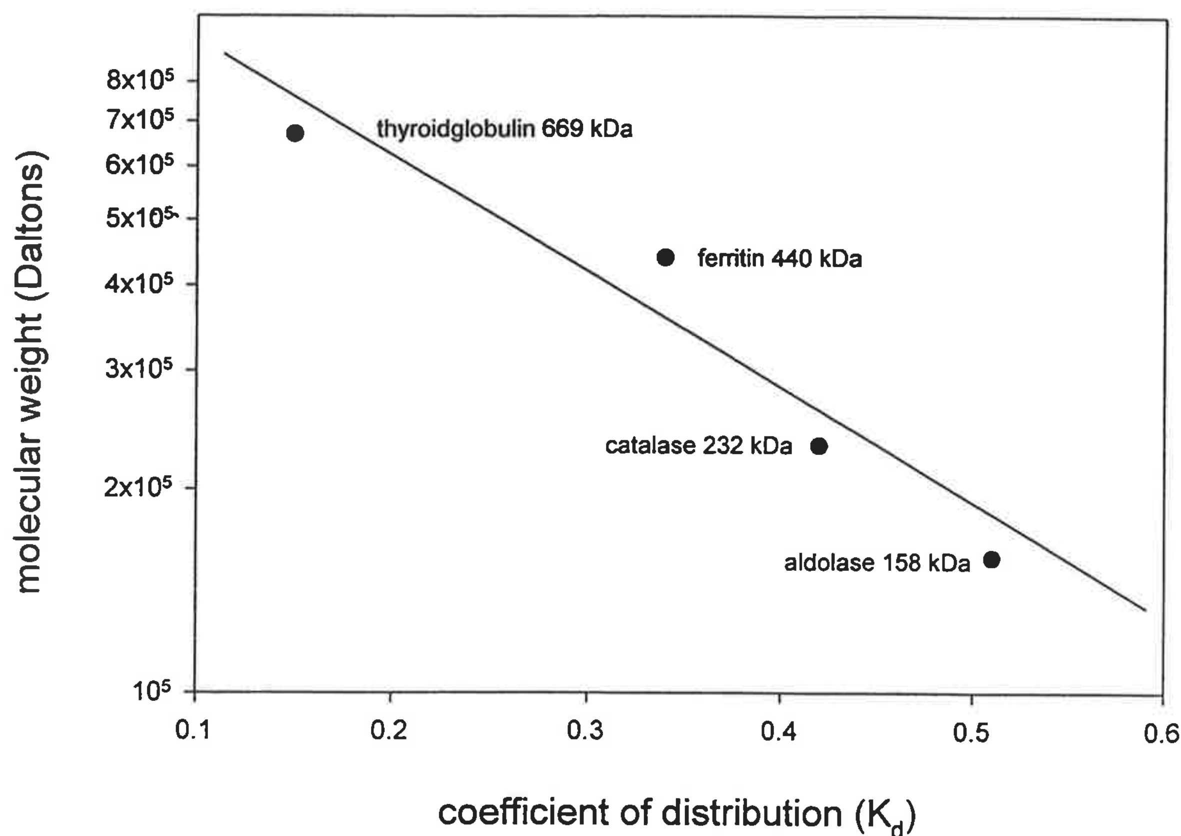


Figure 3.1 Construction of a molecular weight standard curve for the 186 ml Sephacryl S-400 column. A 100 cm x 16 mm chromatography column was packed with Sephacryl S-400 and equilibrated with 500 ml of 6 M guanidine hydrochloride (pH 4.7) before commencing calibration. Thyroidglobulin, ferritin, catalase and aldolase (0.5-1 mg) were individually dissolved in 1.5 ml of guanidine hydrochloride, applied to the column and eluted at a rate of 0.4 ml per minute. Ultraviolet absorbance (280 nm) was monitored at the fraction collection point to determine the elution volume of each protein standard. All molecular weight standards were chromatographed on three occasions, with identical elution volumes being recorded each time. Void volume was calculated from the elution volume of Blue Dextran (Vo 73 ml), and total column volume was directly measured (Vt 185 ml).

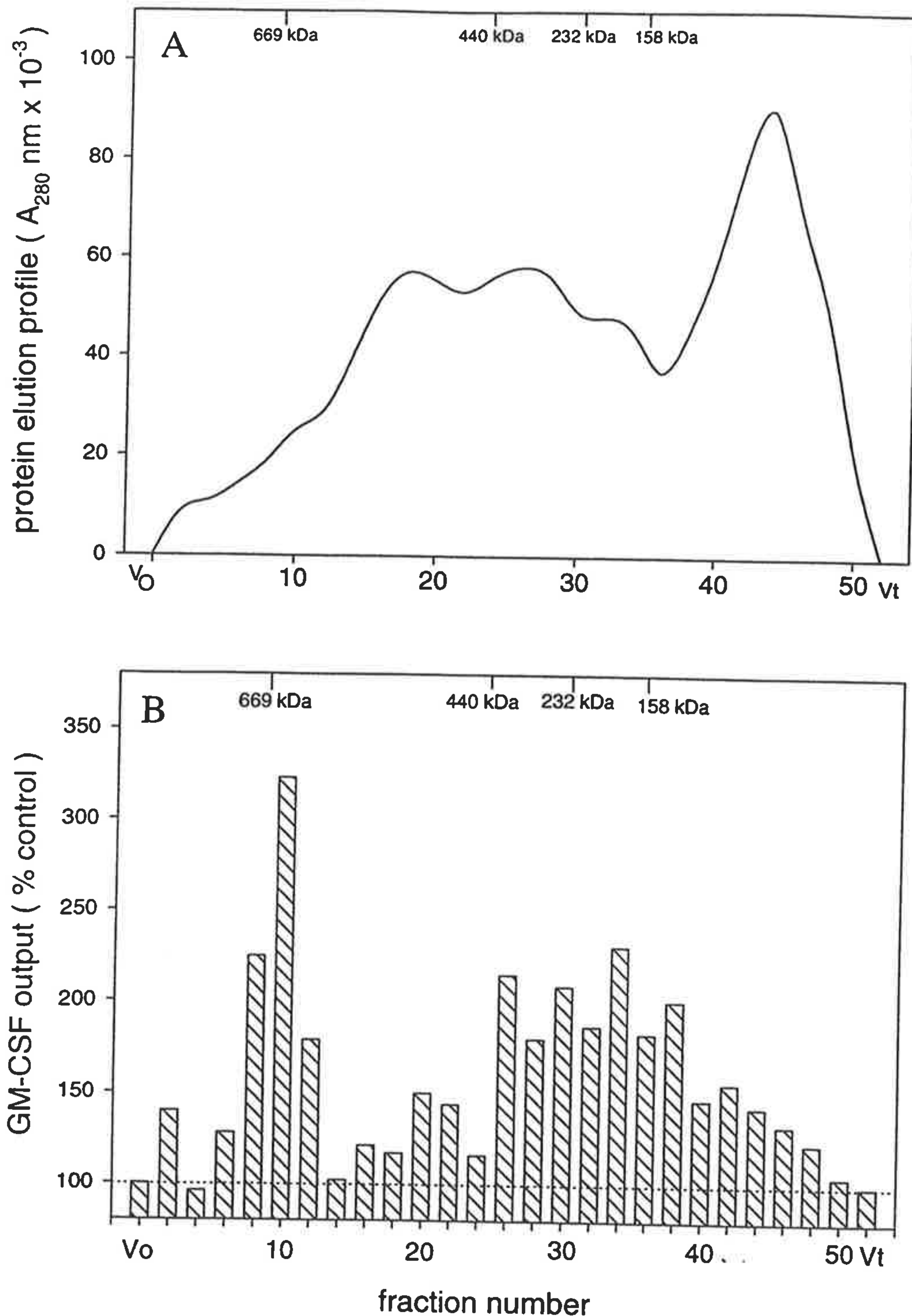


Figure 3.2 Fractionation of seminal vesicle fluid under acidic elution conditions - assessment of GM-CSF-stimulating activity. Seminal vesicle fluid from two stud CBA F1 males was solubilised in 1.5 ml of guanidine hydrochloride and applied to the 185 ml Sephacryl S-400 column. Acidic guanidine hydrochloride (pH 4.7) was used as the elutant at a flow rate of 0.4 ml per minute. The protein elution profile was assessed by monitoring UV absorbance at 280 nm (A). Every alternate seminal vesicle fraction was desalted into DMEM culture medium using Sephadex G-25 desalting columns, before being added to duplicate uterine epithelial cell cultures for assessment of GM-CSF-stimulating activity (B). The GM-CSF content within each supernatant was assessed by a FD 5/12 bioassay. All values are recorded as the mean GM-CSF output, expressed as a percentage of the GM-CSF output observed in the control uterine cultures. The pattern depicted in these graphs is typical of the results obtained in three identical chromatography experiments.

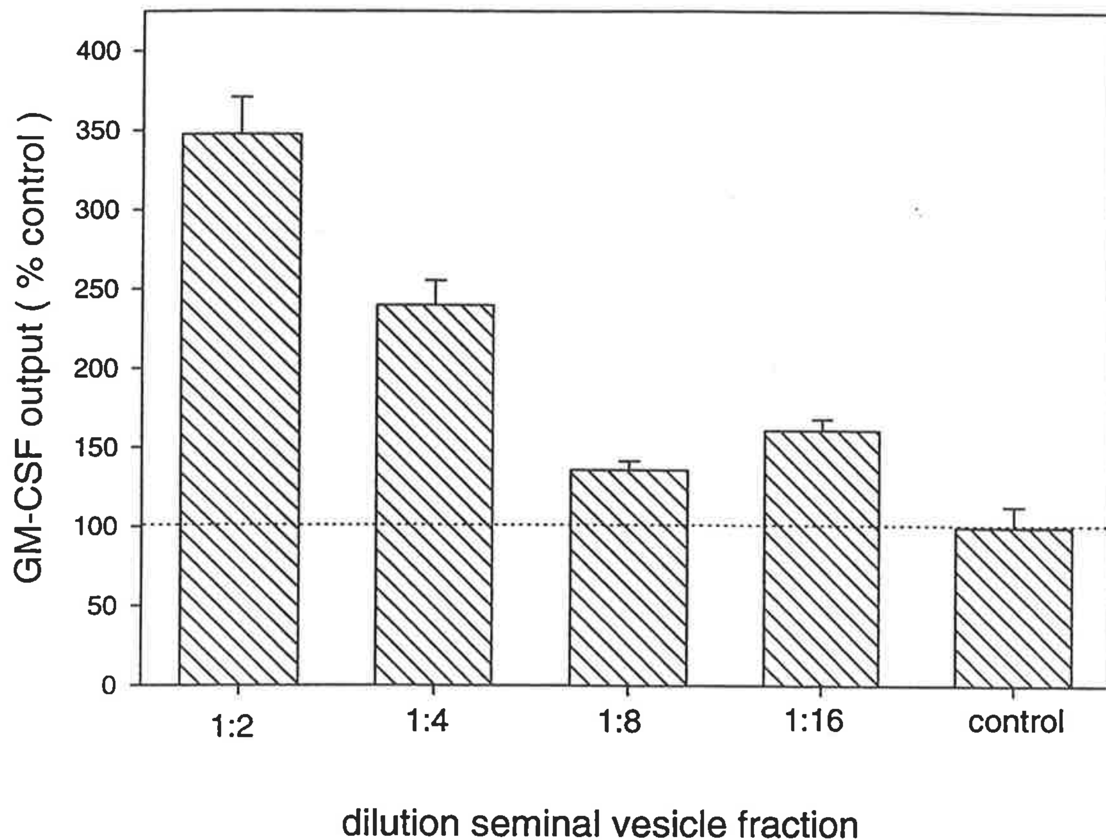


Figure 3.3 Serial dilution performed on the 650 kDA seminal vesicle trigger for uterine GM-CSF release. The 650 kDa seminal vesicle fraction, obtained from a chromatography run similar to that described in Figure 3.2, was desalted into DMEM medium and serially diluted. The resulting solutions, representing a final dilution of between 1: 20 and 1:160 of neat seminal vesicle fluid, were added to duplicate uterine epithelial cell culture wells. The GM-CSF content within uterine epithelial cell supernatants was measured by FD 5/12 bioassay and recorded as the mean $\pm$ SD of duplicate wells, expressed as a percentage of GM-CSF output in control cultures. Similar results were obtained in each of two duplicate experiments.

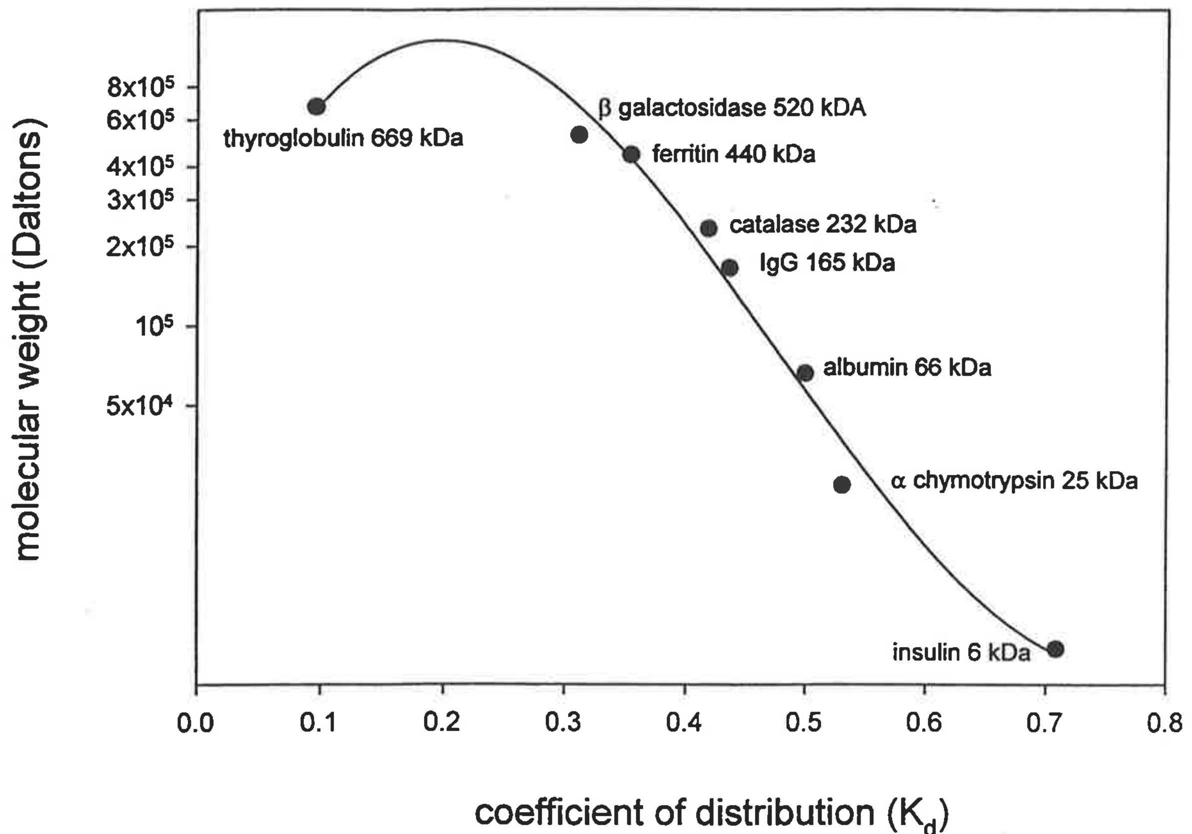


Figure 3.4 Construction of a molecular weight standard curve for the 26 ml Sephacryl S-400 column. A molecular weight standard curve was constructed for the 26 ml Sephacryl S-400 column in a similar manner to that described in Figure 3.1, with the exception that neutral guanidine hydrochloride (pH 7.4) was used as the elutant. In addition to the proteins used in Figure 3.1, β galactosidase (E. Coli derived), rabbit IgG, bovine albumin, insulin and porcine α chymotrypsin were included as molecular weight standards. All standards were eluted at a rate of 0.15 ml per minute, and elution profiles were monitored by absorbance at 280 nm. Vo = 10.5 ml. Vt = 26 ml.

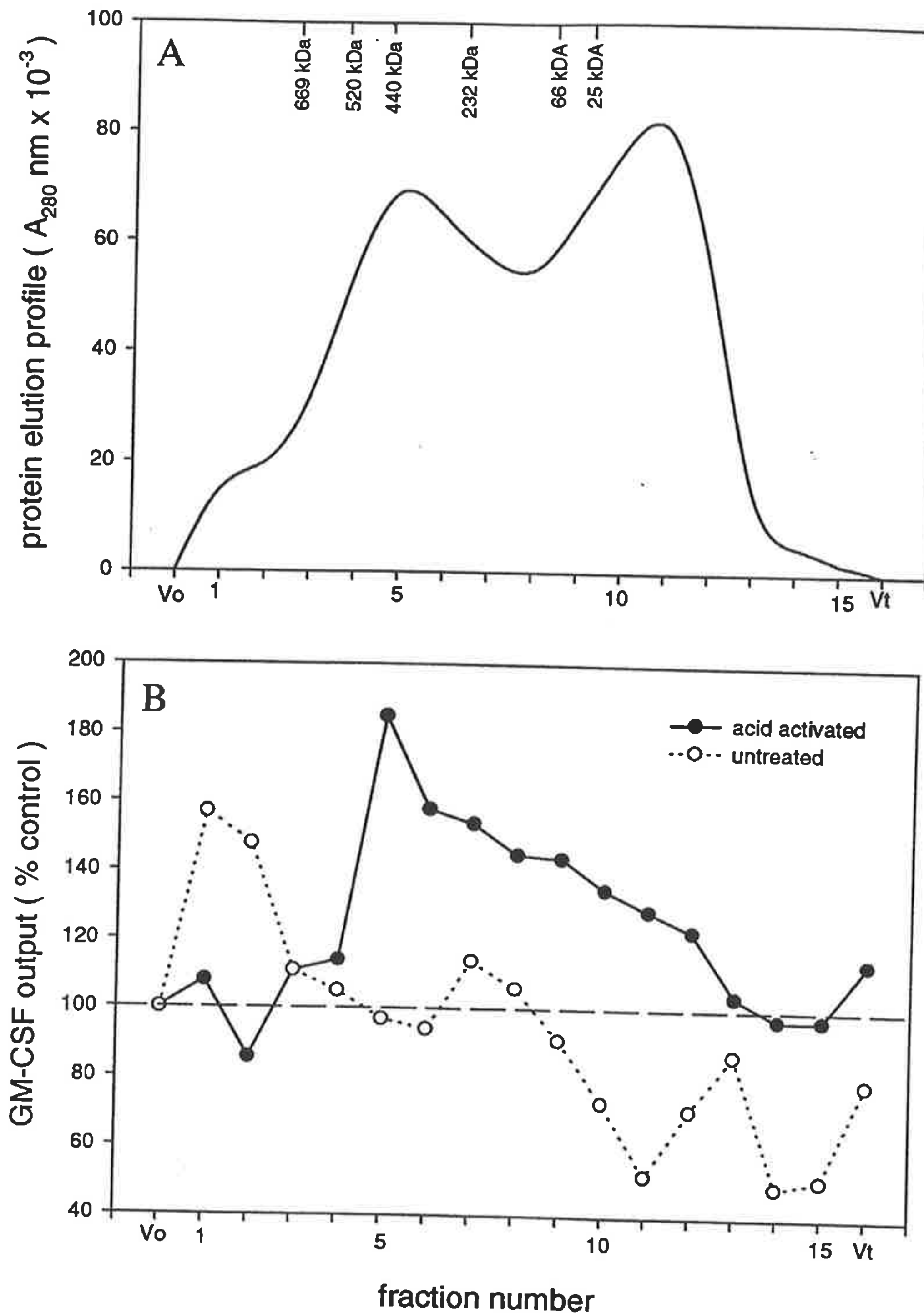


Figure 3.5 Fractionation of seminal vesicle fluid under neutral elution conditions- the effect of transient acidification on GM-CSF stimulating activity. Seminal vesicle secretions from two stud CBAF1 males was solubilised in 0.5 ml of neutral guanidine hydrochloride and applied to a 26 ml Sephacryl S-400 column. Elution in neutral guanidine hydrochloride was then performed at a speed of 0.15 ml per minute, with 1 ml fractions being collected. The protein elution profile was assessed by monitoring of UV absorbance at 280 nm (A). Each seminal vesicle fraction was divided into two equal portions, one being transiently acidified to pH 2 for 10 minutes by the addition of concentrated 5M HCl, followed by neutralisation with 5 M NaOH. The remaining portion was left untreated. Both portions were then desalted into DMEM culture medium and added to duplicate uterine epithelial cell cultures. The GM-CSF content within each culture supernatant was measured by FD 5/12 bioassay. The mean uterine epithelial cell GM-CSF output is expressed as a percentage of the GM-CSF output in control cultures (B).

Chapter 4

The role of seminal Transforming Growth Factor Beta in the post-mating inflammatory response

4.1 Introduction

The studies reported in this chapter have identified seminal vesicle-derived transforming growth factor beta (TGFβ) as the predominant trigger for uterine epithelial cell production of GM-CSF, which ultimately results in the initiation of the murine post-mating inflammatory response.

Fractionation of seminal vesicle secretions using size exclusion chromatography, as described in Chapter 3, revealed that one of the proteins with GM-CSF-stimulating activity in uterine epithelial cell cultures eluted in a broad molecular weight range between 150 and 440 kDa, and had the unusual biochemical property of minimal GM-CSF-stimulating activity in its native state, but marked stimulating activity following transient acidification. This observation raised the possibility that a TGFβ-like molecule may trigger uterine production of GM-CSF, since TGFβ is predominantly secreted as a latent complex of 230 - 290 kDa (Wakefield *et al.* 1988), with bioactive TGFβ being released under acidic conditions (Lawrence *et al.* 1985). Fractionation of human seminal plasma by size exclusion chromatography had earlier revealed that TGFβ eluted in a broad molecular weight range of between 100 and 450 kDa, with bioactivity only being exhibited following transient acidification (Nocera and Chu 1993). Given the close physical similarities between human seminal plasma-derived TGFβ and the murine seminal vesicle trigger for GM-CSF production, it was reasonable to investigate the possibility that this activity may be associated with a TGFβ-like molecule.

The studies described in this chapter describe how TGFβ was identified for the first time within murine seminal vesicle secretions. They also demonstrate that $TGF\beta_1$ is the predominant stimulus for production of GM-CSF in uterine epithelial cells, both in culture

and following *in vivo* administration. The ability of different members of the TGFβ superfamily (TGFβ_1,TGFβ_2, activin and inhibin) to generate an increase in uterine epithelial GM-CSF production was compared, as was the capacity of uterine epithelial cells from different stages of the oestrus cycle to respond to rTGFβ_1. Finally, confirmation of the physiological likelihood that seminal vesicle-derived TGFβ could initiate a post-mating inflammatory response was made by identifying an increase in uterine luminal fluid TGFβ_1 content shortly following mating, and demonstrating that intrauterine injection of recombinant TGFβ_1 is capable of creating an inflammatory response comparable with that seen following mating.

4.2 Chromatographic identification of TGFβ in murine seminal vesicle secretions

4.2.1 TGFβ bioactivity assayed by the mink lung cell assay

Fractionation of seminal vesicle secretions for determination of TGFβ bioactivity was performed using a 26 ml Sephacryl S - 400 column. Each individual 1 ml fraction was divided into two equal portions, one left untreated ("native") and the other transiently acidified to release bioactive TGFβ ("acid-activated"). All samples were desalted into DMEM / 0.1% BSA prior to their addition to a mink lung fibroblast (CCL-64) bioassay specific for TGFβ. In each of three separate chromatography experiments, no significant inhibition of CCL-64 cell growth was seen in any of the "native" samples, yet significant inhibition was seen in the "acid-activated" fractions between 150 and 440 kDa. The inhibition of CCL-64 growth by the 150 -440 kDa "acid-activated" fractions (fractions 5-7) was confirmed to be mediated by TGFβ, since this inhibition could be abrogated by the addition of TGF$\beta_{1,2,3}$-specific antisera (Figure 4.2a). Additionally, these fractions had no inhibitory effect on the growth of TGFβ-insensitive L cell fibroblasts, thereby discounting a non-specific cytotoxic effect. Given the observed high molecular weight elution profile, and the requirement of transient acidification before detection of TGFβ bioactivity, it can be concluded that TGFβ contained within the 150 - 440 kDa elution range is likely to be present in its latent form.

The addition of pooled, acid-activated fractions 5, 6 and 7 to uterine epithelial cell cultures resulted in an increase in uterine epithelial cell GM-CSF output. However, this increase was not observed if TGFβ_{123}-specific antisera was added to these seminal vesicle

fractions prior to their addition to uterine epithelial cell cultures (Fig 4.2b). This finding supports the conclusion that TGFβ, eluting between 150 and 440 kDa, is the major acid-stable seminal vesicle trigger responsible for increasing uterine epithelial cell GM-CSF production.

4.2.2 *TGFβ_1 immunoactivity in seminal vesicle secretions*

Seminal vesicle secretions were fractionated as described above, with "native" and "acid-activated" TGFβ_1 content being determined using a commercial ELISA. Peak TGFβ_1 immunoactivity was found to elute in the 150 - 440 kDa molecular weight range, as previously identified using the CCL-64 TGFβ bio-assay. Surprisingly, low levels of TGFβ_1 immunoactivity were present throughout the chromatographic elution profile, with a second peak eluting between 650-700 kDa (Fig 4.1). This finding is consistent with the observation of low levels of GM-CSF stimulating activity in fractions outside the 150- 440 kDa stimulating range (Figure 3.2 and 3.5).

4.3 Antibody neutralisation of seminal vesicle trigger for uterine GM-CSF release

In order to confirm the biological identity of the SGSF, unfractionated dilute (2% vol/vol), seminal vesicle secretions were added to uterine epithelial cell cultures in the presence or absence of TGFβ-neutralising antisera. Two different antibodies were used; one specific for TGFβ_1 and one which neutralised all mammalian forms of TGFβ (TGFβ_1, β_2 and β_3). All samples were acid-activated prior to incubation with antibody and addition to uterine epithelial cell cultures. Figure 4.3 illustrates the GM-CSF output of these cultures measured 16 and 40 hours after the addition of seminal vesicle secretions. It is clear that a significant increase in GM-CSF output was evoked by acid-activated unfractionated seminal vesicle fluid, but this increase was reduced by 60-85% after incubation with a TGFβ_1-specific neutralising antibody, and was completely lost upon the addition of a TGFβ_{123} neutralising antibody. This implies that either TGFβ_1 is vastly more prevalent in murine seminal vesicle fluid, or TGFβ_1 is a more potent SGSF compared to the other TGFβ isoforms. The decrease in baseline GM-CSF production levels seen in the controls following addition of TGFβ neutralising antibody may be due to neutralisation of

small amounts of TGFβ present in FCS (Childs *et al.* 1982) . Similar results to those depicted in Figure 4.3 were seen in three replications of the above experiment.

4.4 Uterine epithelial GM-CSF response to the TGFβ family of proteins

4.4.1 Dose response effect of rTGFβ_1 on uterine GM-CSF production in culture

Antibody neutralisation experiments identified TGFβ_1 as the dominant TGFβ isoform within seminal vesicle secretions responsible for upregulating uterine epithelial cell GM-CSF production. Given this finding, a dose response study was conducted measuring the ability of recombinant human TGFβ_1 to elicit an increase in uterine epithelial cell GM-CSF production over a broad range of concentrations. Figure 4.4 depicts the 16 and 40 hour results of one of four replications of this experiment, all of which gave similar results. The addition of increasing concentrations of TGFβ_1 (up to 5ng / ml) resulted in an incremental increase in GM-CSF output. Concentrations above 5 ng / ml produced either a plateau or decline in GM-CSF production. This observation was not unexpected, since TGFβ has been reported to have an inhibitory effect on most cell functions at supra-physiological concentrations (Moses 1985, Roberts *et al.* 1985). Cytokines such as TGFβ generally are only present at relatively low concentrations *in vivo*, so it is unlikely that the uterine epithelium would ever encounter concentrations of TGFβ sufficient to have an inhibitory effect on GM-CSF production.

4.4.2 Comparison of the potency of the TGFβ family of proteins for stimulating GM-CSF release from uterine epithelial cells

In mammals, the TGFβ family of proteins includes TGFβ_1,TGFβ_2,TGFβ_3, activin, inhibin and bone morphogenic protein (Massague 1990). Inhibin is known to be present in human seminal plasma (Sluss *et al.* 1993), and activin has been identified in testicular tissue (Lee *et al.* 1989), although it is not present in human seminal plasma (Anderson and Martin 1998). The presence of activin and inhibin in murine seminal plasma is presently unknown. Bone morphogenic protein has only been isolated from bone, therefore its effect on uterine epithelial cell GM-CSF release was not determined.

Comparison of the GM-CSF stimulating capacity of recombinant human $TGF\beta_1$ and purified porcine $TGF\beta_2$ over a wide range of concentrations reveals that these two isoforms have equal potency in regard to stimulating uterine epithelial cell GM-CSF production (Figure 4.5a). This was not unexpected, since they have almost identical bio-activities in most other systems (Jennings *et al.* 1988). The $TGF\beta_3$ isoform was not readily available at the time that this experiment was conducted, so its GM-CSF-stimulating activity was not tested. Since there is a high degree of sequence homology and similar bioactivity between $TGF\beta_3$, $TGF\beta_1$ and $TGF\beta_2$, one would expect $TGF\beta_3$ to have a similar biological effect and potency (Massague 1990).

The ability of recombinant activin and inhibin to initiate a GM-CSF response from murine uterine epithelium was compared with that of $TGF\beta_1$. Inhibin was observed to have no GM-CSF stimulating activity, while activin showed weak activity (Figure 4.5b). On a weight to weight basis, $TGF\beta_1$ was approximately 100 times more potent than activin in generating an increase in GM-CSF production from uterine epithelial cell cultures. This degree of response is similar to the relative potencies of $TGF\beta_1$ and activin in inhibiting the growth of CCL-64 cells in our laboratory. It is possible that TGFβ and activin may mediate their GM-CSF stimulatory effect by binding to a shared receptor on the uterine epithelium, since receptors capable of binding both these cytokines have been identified in the rat (Moustakas *et al.* 1995) and human (Attisano *et al.* 1993). The human TSR-I receptor is an example of a type I receptor that can bind both TGFβ and activin to produce a cellular response, provided that the cytokine-specific type II receptor is co-expressed on the cell surface (Attisano *et al.* 1993, ten-Dijke *et al.* 1994). The TSR-I receptor exhibits greater affinity for TGFβ than activin, which may explain why activin has only weak bio-activity in uterine cultures compared to $TGF\beta_1$.

4.4.3 Effect of stage of the oestrous cycle on uterine epithelial GM-CSF response to TGFβ

Cell proliferation and secretory function of the endometrial epithelium is primarily controlled by ovarian sex steroids (Martin and Finn 1968). The production of GM-CSF from the murine uterine epithelium has been shown to be influenced by the stage of oestrus cycle, with fluctuations in production being mediated by ovarian sex steroid status (Robertson *et al.* 1996). Uterine epithelial cell culture experiments have shown that these

cells produce GM-CSF in response to oestrogen, but this oestrogen-mediated surge in GM-CSF production can be diminished by progesterone. In addition, it has been shown that baseline production of GM-CSF is highest in cells harvested from oestrous uteri compared to all other stages of the oestrus cycle (Robertson *et al.* 1996).

Because of marked variations in baseline levels of GM-CSF production, the possibility that the oestrus cycle may also modify responsiveness of uterine epithelial cells to TGFβ was investigated. Uteri were excised from two groups of mice, oestrous and dioestrous (as confirmed by vaginal cytology), and processed individually. The control GM-CSF values, and those obtained following the addition of 5 ng/ml of rTGFβ_1, are recorded for the individual uteri in Figure 4.6a. Only the GM-CSF content from the first 16 hours of culture was assayed, since it is possible that culturing epithelial cells for a further 24 hours in medium containing oestrogenic activity associated with FCS or red phenol dye may have resulted in an alteration in epithelial cell function (Barnes and Sato 1980, Berthois *et al.* 1986).

It can be seen from Figure 4.6a that the baseline level of GM-CSF production in oestrous uterine cultures is approximately double that seen in dioestrous controls ($p= 0.03$), as has been previously reported (Robertson *et al.* 1996). Interestingly, both oestrous and dioestrous uterine epithelial cells had the capacity to increase their production of GM-CSF in response to TGFβ_1 exposure. The magnitude of this response was greater in the oestrous uteri (median 338 %, range 227-511%) than dioestrous uteri (median 275 %, range 198-377 %), but this trend was not statistically significant due to large variations in TGFβ_1 responsiveness within each group. A more pronounced difference in uterine GM-CSF production in response to TGFβ_1 stimulation was observed in an earlier experiment in which uterine epithelial cells from three oestrous females and three dioestrous females were pooled into two groups prior to culture (Figure 4.6b).

4.5 Investigation of the *in vivo* role for TGFβ in the generation of the post-mating inflammatory response

4.5.1 Ability of rTGFβ_1 to initiate an increase in uterine GM-CSF production in vivo

In order to conclude that the increase in GM-CSF production observed following addition of rTGFβ_1 to uterine epithelial cell cultures was not a culture artefact but a true physiological response, an experiment was conducted in which rTGFβ_1 was injected

directly into the uterine lumen of oestrous mice and the GM-CSF content measured 16 hours later. Following injection of carrier medium, uterine luminal fluid GM-CSF levels were below the minimum amount detectable by this bio-assay. In contrast, administration of either 10 or 40 ng of r$TGF\beta_1$ produced a significant increase in uterine luminal GM-CSF content (Fig 4.7). In addition, uterine fluid from mated females contained elevated levels of GM-CSF, with the magnitude of this response being similar to that previously reported (Robertson *et al.* 1990 and 1992b), and comparable to that seen following administration of r$TGF\beta_1$. These results confirm the potential capacity for seminal vesicle-derived $TGF\beta_1$ to upregulate uterine epithelial cell GM-CSF production in the physiological context of mating.

4.5.2 Measurement of uterine TGFβ content before and after mating

Mice are known to deposit a substantial portion of their ejaculate directly into the uterine lumen (Mann 1981). It was therefore postulated that the $TGF\beta_1$ content of uterine luminal fluid should be elevated following mating compared to non-mated oestrous controls. In order to investigate this, an experiment was performed in which uterine luminal fluid was harvested one hour after mating, and its $TGF\beta_1$ content compared with that from virgin oestrous mice. All uterine fluids were divided into two, with one portion being "acid-activated" (to measure total $TGF\beta_1$ levels), and the other sample assayed in its native state (naturally bioactive $TGF\beta_1$). Comparison of the relative levels of $TGF\beta_1$ within uterine fluid revealed that there was a marked increase in both naturally active and total $TGF\beta_1$ content in the mated uterus compared to unmated controls (Figure 4.8). It is likely that the observed threefold increase (250-400 pg per horn) in total and naturally active $TGF\beta_1$ levels following mating would be sufficient to elicit an increase in uterine epithelial cell GM-CSF production, since this equates with a 5-8 ng /ml increase in uterine luminal fluid $TGF\beta_1$ content, a concentration capable of increasing GM-CSF output *in vitro*.

Measurement of uterine TGFβ content following mating with vasectomised and seminal vesicle-deficient males was also performed in order to localise the origin of seminal TGFβ. No significant differences were observed in the total uterine TGFβ content of females mated with intact or vasectomised males ($p=0.234$), however there was a significantly higher level of naturally active TGFβ in the uterine fluid of females mated to

vasectomised males compared to intact males ($p=0.035$). TGFβ is reported to bind to human sperm (Chu *et al.* 1996), therefore it is possible that some TGFβ may have been bound to sperm in the intact mating group. Since sperm and cellular debris were removed by centrifugation prior to assaying for TGFβ content, it is possible that some TGFβ may have been lost in the sperm pellet in the intact mating group yet not in the vasectomised group, thereby explaining the observed discrepancy in TGFβ levels.

Females mated to males from which the seminal vesicle glands had been surgically removed did not exhibit the post-mating increase in uterine $TGF\beta_1$ content. This was despite having in excess of 10^6 sperm in their uterine fluid, implying that adequate transfer of the ejaculate into the uterine lumen had occurred (Tucker 1980). This observation confirms that the seminal vesicle gland is the major source of TGFβ introduced into the uterine lumen at the time of mating.

4.5.3 Ability of $TGF\beta_1$ to initiate a post-mating inflammatory response in vivo

In order to investigate the effect of $TGF\beta_1$ on uterine leukocyte parameters, an immunohistochemical analysis was made of the endometrial leukocyte populations of oestrous mice 16 hours following the intra-luminal administration of either 20 ng $rTGF\beta_1$ or control medium, then compared with that seen following mating. Uterine sections were stained for leukocyte common antigen (CD45), macrophage markers F4/80 and Mac-1, anti - Ia (MHC class II antigen present on activated macrophages and dendritic cells), neutrophil marker RB6-8C5, or endogenous peroxidase activity (exhibited by eosinophils). Administration of $rTGF\beta_1$ resulted in an extensive infiltration of macrophages and eosinophils into the uterine endometrium, as evident from the significant increase in endometrial staining for F4/80 and endogenous peroxidase (Table 4.1, Figure 4.9). A trend towards increased numbers of endometrial leukocytes reacting with anti-CD45 , anti-Ia and anti-Mac-1 was also evident in mice treated with $rTGF\beta_1$. The extent of infiltration of the uterine endometrium with macrophages and eosinophils after $rTGF\beta_1$ treatment was indistinguishable from that seen in uteri harvested from mice mated 16 hours earlier (Figure 4.9). However, the accumulation of neutrophils under the uterine epithelial surface and their subsequent transit into the uterine lumen, prominent following mating, was not evident following intrauterine administration of $rTGF\beta_1$.

Table 4.1 The effect of intrauterine injection of rTGFβ_1 on leukocyte recruitment into the murine endometrium.

	oestrous control	rTGFβ_1 (20 ng/ uterus)	day 1 mated
n	5	4	4
CD45 (leukocyte common antigen)	15.3 ^{a} (8.2-18.8)	27.0 ^{ab} (12.8-38.7)	40.8 ^{b} (30-58.8)
F 4/80	14.6 ^{a} (11.9-24.6)	36.8 ^{b} (30-48.1)	31.2 ^{b} (20.5-40.7)
MAC-1	8.6 ^{a} (6.7-21.4)	22.6 ^{a} (18.3-41.9)	47.7 ^{b} (46-56.3)
Ia antigen	19.7 ^{a} (7.5-23.2)	25.4 ^{b} (15.3-34.8)	26.9 ^{c} (26.1-57.3)
RB6-6C5	9.4 ^{a} (6.3-15.2)	9.4 ^{a} (4-19.7)	35.6 ^{b} (15.1-40.9)
peroxidase positive (eosinophils)	3.8 ^{a} (3.6-7)	14.7 ^{b} (11.1-19.3)	11.9 ^{b} (9.7-18.9)

All values are expressed as the mean percentage positivity (range) of immunohistochemical staining for each antibody. Duplicate slides were prepared for each antibody and ten fields from each slide were assessed by video image analysis to give a mean percentage positivity value. All data were assessed by a Kruskal Wallis one-way ANOVA, followed by Mann Whitney rank sum test, with significant differences between groups shown as different superscripts ($p<0.05$).

In summary, this immunohistochemical study confirms that intra-luminal administration of rTGFβ_1 is able to initiate a uterine inflammatory response similar to that seen following mating. Given the previously described capacity for TGFβ to upregulate uterine epithelial GM-CSF production, the known chemotactic activity of GM-CSF for macrophages and eosinophils, and the observation of an accumulation of inflammatory leukocytes directly beneath the GM-CSF-producing uterine epithelium, it is reasonable to conclude that TGFβ mediates this post-mating inflammatory response at least in part through increasing uterine epithelial GM-CSF production.

4.6 Discussion and conclusion

The experiments outlined in this chapter provide compelling evidence that the transforming growth factor β family of proteins, contained within seminal vesicle secretions, reach the uterine cavity shortly following mating where they stimulate an increase in GM-CSF production by uterine epithelial cells. This surge in GM-CSF production, probably acting in concert with other pro-inflammatory cytokines and chemokines, mediates the post-mating inflammatory response through recruitment of leukocytes into the endometrium.

Chromatographic fractionation of seminal vesicle fluid according to molecular weight provided the first indication that TGFβ may act as a trigger for uterine epithelial GM-CSF release. TGFβ is usually secreted as a biologically inactive latent complex consisting of a mature TGFβ homodimer (25 kDa) non-covalently linked with a 75-80 kDa latency-associated protein, which in turn is covalently bound to a 130-190 kDa binding protein (Wakefield *et al.* 1988). One manner in which biologically active TGFβ can be released from the 230-295 kDa latent complex is by transient acidification (Lawrence *et al.* 1985). The finding that transient acidification of the 150-440 kDa fractions could liberate SGSF activity is consistent with the postulate that this stimulating activity may be associated with TGFβ in its latent form. The ability of TGFβ-specific antisera to abrogate GM-CSF stimulating activity within the 150-440 kDa fractions also implicates TGFβ as the intermediate molecular weight trigger for GM-CSF production.

Identification of the majority of seminal TGFβ within fractions consistent with its high molecular weight latent form (~ 230-290 kDa), rather than its low molecular weight bioactive state (25 kDa), was unexpected since the chaotrophic activity of guanidine hydrochloride was expected to break the non-covalent bonds which bind active TGFβ with its latency complex. The disruption of non-covalent bonds by guanidine hydrochloride is however dependent upon variables such as protein concentration, pH and guanidine concentration (Brown and Schleich 1975). Because chromatography was conducted at a neutral pH and not the acidic pH of guanidine hydrochloride, the TGFβ complex may have been more resistant to the denaturing effect of this chaotrophic elutant. The disruption of non-covalent bonds within myoglobin (Bismuto *et al.* 1983) and cytochrome C (Tsong 1974 and 1975) by guanidine hydrochloride has been reported to be retarded at neutral pH. In addition, the high protein content (20-40 mg) applied to each chromatography run in

order to maximise GM-CSF stimulating activity may have saturated the chaotrophic capacity of guanidine hydrochloride. This protein concentration is in excess of 20 times the recommended concentration needed to ensure full dissociation of non-covalent bonds (Pharmacia protein chemistry handbook). Whatever the reason for failure of dissociation of bioactive TGFβ from its latent precursor, it is clear that peak TGFβ bioactivity and immunoactivity were co-localised to the 150-440 kDa molecular weight range, thereby implying that TGFβ contained within a latency complex is the trigger for uterine GM-CSF release.

TGFβ binds to a number of proteins such as α–2-macroglobulin (725 kDa), fibronectin (460-500 kDa), a soluble form of the TGFβ type III receptor (250-300 kDa), decorin (103 kDa), α-fetoprotein (70 kDa), type IV collagen (130 kDa) and thrombospondin (450 kDa) (reviewed in Miyazono *et al.* 1993). The binding of seminal TGFβ to several different carrier proteins may help explain why low levels of TGFβ immunoactivity elute over a broad molecular weight range. A very similar elution profile has been reported for human seminal plasma-derived TGFβ (Nocera and Chu 1993). These investigators found that peak TGFβ bioactivity eluted between 100 and 440 kDa, but some TGFβ bioactivity also eluted either side of this molecular weight range. Size exclusion chromatography of adrenocortical cell condition medium (Souchelnitskiy *et al.* 1995) and bovine milk (Rogers *et al.* 1996) has also revealed great heterogeneity in the elution profile of TGFβ, with activity being seen between 80 and 600 kDa. These observations support the concept that TGFβ is generally bound to carrier proteins or contained within a latent complex when present in biological fluids. The observation of a second peak in seminal vesicle $TGF\beta_1$ immunoactivity between 650 and 700 kDa (Figure 4.2), together with the capacity of $\alpha TGF\beta_{123}$ neutralising antisera to completely block all GM-CSF stimulating activity within acid-activated unfractionated seminal vesicle fluid (Figure 4.3), supports the possibility that the 650 kDa SGSF may also be TGFβ, perhaps bound to a large carrier protein such as fibronectin.

These studies have identified $TGF\beta_1$, $TGF\beta_2$ and activin from the TGFβ family as having the capacity to stimulate uterine epithelial cell GM-CSF production. The observation that $TGF\beta_1$ and $TGF\beta_2$ exhibit equal GM-CSF stimulating activity is noteworthy given that different members of the TGFβ family have been reported to exhibit different activity within other biological systems (Cheifetz *et al.* 1990, Joyce *et al.* 1990). The fact that the majority of GM-CSF stimulating activity in murine seminal vesicle

secretions can be blocked by $TGF\beta_1$-specific antisera indicates that $TGF\beta_2$ and activin are unlikely to play a major role in generating the post-mating GM-CSF surge. Given the equal potency of $TGF\beta_1$ and $TGF\beta_2$, this previous observation must indicate that $TGF\beta_1$ is the major member of the TGFβ family present in murine seminal vesicle fluid. A similar pattern is seen in human seminal plasma, where $TGF\beta_1$ is found at a 10 to 15-fold higher concentration than $TGF\beta_2$ (Nocera and Chu 1995).

The observed fluctuations in uterine epithelial cell responsiveness to $TGF\beta_1$, depending on oestrus cycle position, is consistent with the knowledge that sex steroids play a pivotal role in endometrial function. Both baseline and TGFβ-stimulated GM-CSF production levels were higher in oestrous than dioestrous uterine epithelial cell cultures, however both oestrous and dioestrous cultures were able to respond to $TGF\beta_1$ with an increase in GM-CSF production. Future studies investigating the relationship between steroid hormone status and uterine epithelial cell TGFβ receptor expression may help to explain these observations. Furthermore, the responsiveness of dioestrous uterine cultures to TGFβ may in part be artefactual, since culture medium and FCS both contain oestrogenic activity (Berthois *et al.* 1986).

The differences in $TGF\beta_1$ content of uterine luminal fluid recovered before and after mating with intact or vasectomised males (500-800 pg per uterus) is consistent with expected values. Measurement of $TGF\beta_1$ concentration within murine seminal plasma, detailed in Chapter 5, reveals that the median $TGF\beta_1$ content of seminal vesicle secretions is 74 ng / μl. Given that the mean ejaculate volume of a mouse is 3.5 μl (Anderson *et al.* 1983), and a male will mate with a single female several times during one oestrous period, it is reasonable to expect that approximately 10 μl of seminal plasma (mainly originating from the seminal vesicle gland) will reach the uterine lumen following a mating session. This 10 μl would contain approximately 700 pg of $TGF\beta_1$, similar to the increase in $TGF\beta_1$ content measured within uterine luminal fluid after mating.

Administration of 40 ng of $rTGF\beta_1$ to the uterine lumen was needed to produce an increase in uterine GM-CSF production comparable to that seen following natural mating (Figure 4.7). Forty ng of $rTGF\beta_1$ is approximately 50 times the amount of naturally active TGFβ that has been measured to be introduced into the uterine lumen following mating. The possible explanation for this discrepancy in potency is two-fold. Firstly, rTGFβ is not bound to a protective latency-associated protein and therefore exhibits less resistance to clearance through processes such as enzymatic breakdown and scavenging by antagonists

such as α2-macroglobulin. Seminal plasma TGFβ is predominantly released as the more stable latent form, which may allow the slow release of bioactive TGFβ over a longer period of time, thereby potentiating its effect. Secondly, latent TGFβ, but not rTGFβ, is associated with a latency binding protein which is able to bind the TGFβ complex to ECM proteins such as collagen, fibronectin and vitronectin (Taipale *et al.* 1994). This may enable the latent TGFβ complex to become anchored to the uterine epithelium, facilitating the release of active TGFβ in close proximity to its site of action (Flaumennaft *et al.* 1993).

The experiments described in this chapter provide a clear link between seminal vesicle TGFβ and the induction of GM-CSF production by uterine epithelial cells, which in turn initiates many of the cellular changes observed following mating. It is also possible that TGFβ may initiate leukocyte recruitment independent of GM-CSF production, since TGFβ has been described as being chemotactic for monocytes (Wahl *et al.* 1987) and neutrophils (Brandes *et al.* 1991) in its own right. In addition, TGFβ can induce monocytes to produce pro-inflammatory mediators such as IL-1 and TNFα (Wahl *et al.* 1987, McCartney-Francis *et al.* 1990), thereby creating a positive feedback loop to the post-mating inflammatory response. Additional chemotactic cytokines such as TNFα, IL-1, IL-6 and chemokines such as RANTES, MCP-1, MIP-1 and eotaxin have been reported to be upregulated in the murine uterus shortly following mating (Sanford *et al.* 1992, Pollard *et al.* 1998, Robertson *et al.* 1998). These cytokines / chemokines are likely to supplement the pro-inflammatory effects of GM-CSF. It is presently unknown if TGFβ has a role in triggering uterine epithelial cell production of pro-inflammatory agents other than GM-CSF. The diminished neutrophil infiltration observed following intrauterine injection of $rTGF\beta_1$, compared to that seen following mating, suggests that generation of the post-mating inflammatory response may be more complex than simply a TGFβ-induced upsurge in uterine epithelial GM-CSF production. Seminal factors other than TGFβ may be required to initiate production of the full range of inflammatory cytokines / chemokines needed to generate the post-mating inflammatory response in the murine uterus. Alternatively, the diminished neutrophil influx may simply reflect a dose-response effect, with higher doses of $rTGF\beta_1$ possibly replicating the post-mating neutrophilia in full.

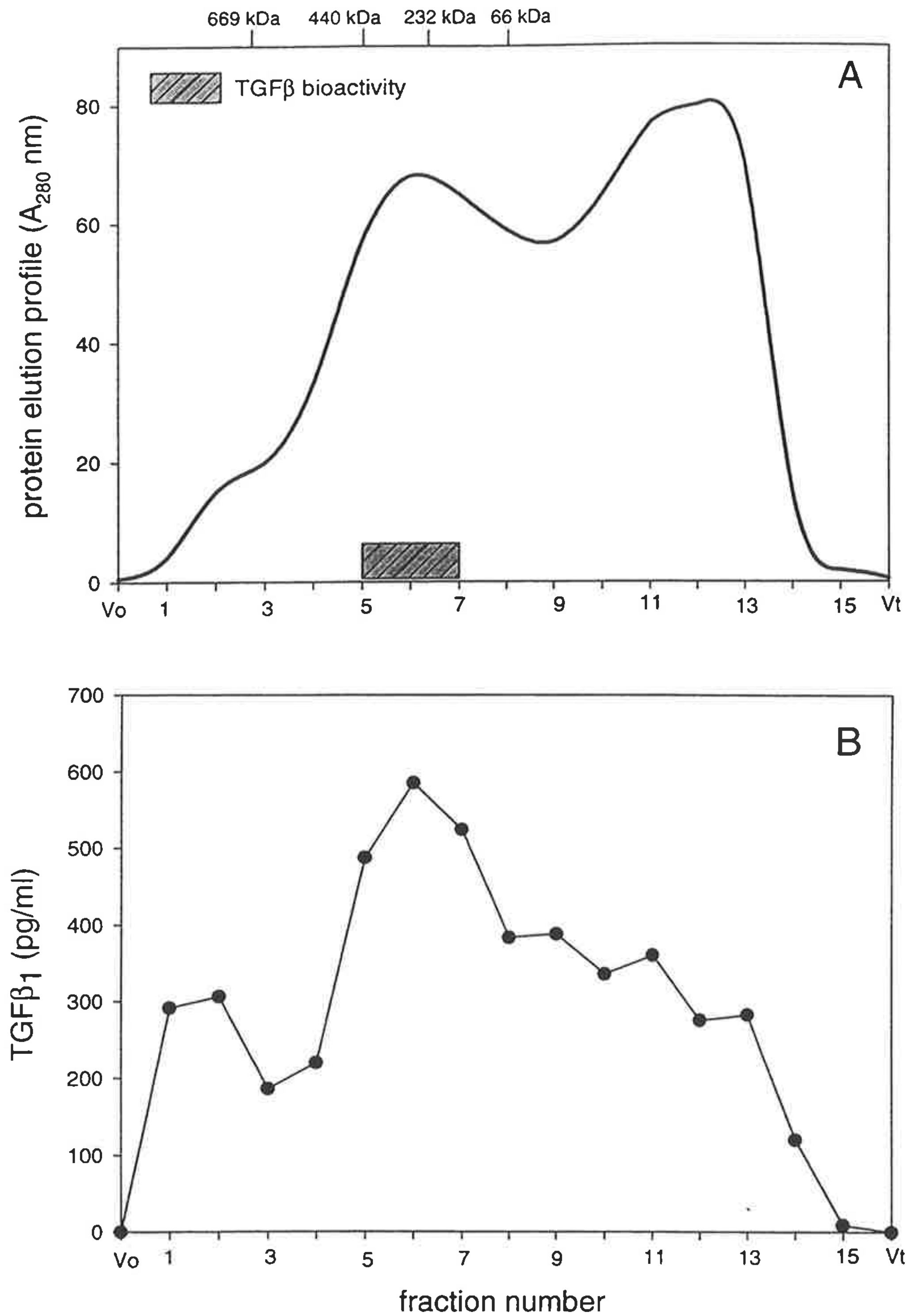
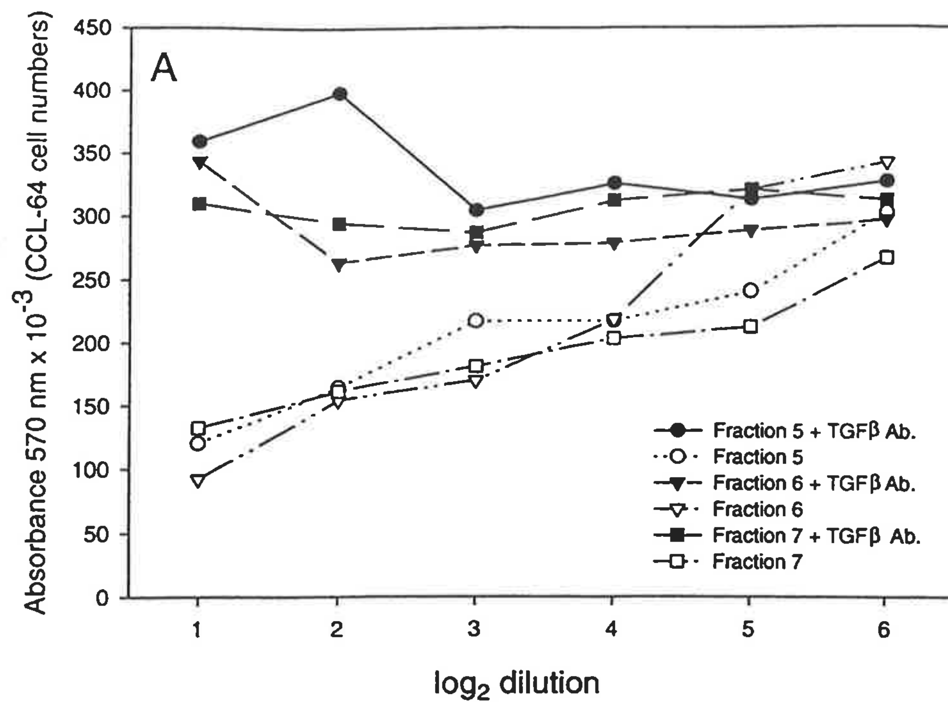


Figure 4.1 Identification of $TGF\beta_1$ within seminal vesicle secretions fractionated by size exclusion chromatography. Seminal vesicle secretions from 2 stud CBA F1 males were solubilised in 0.5 ml of 6 M guanidine hydrochloride (pH7.2) and applied to a 26 ml Sephacryl-S400 chromatography column. Proteins were collected as 1 ml fractions following elution with neutral guanidine hydrochloride running at a speed of 0.15/ml minute (A). Each fraction was transiently acidified (pH 2, 10 minutes) before being desalted into DMEM for assessment of TGFβ bioactivity or PBS / 0.1% BSA for assessment of $TGF\beta_1$ immunoactivity (B).



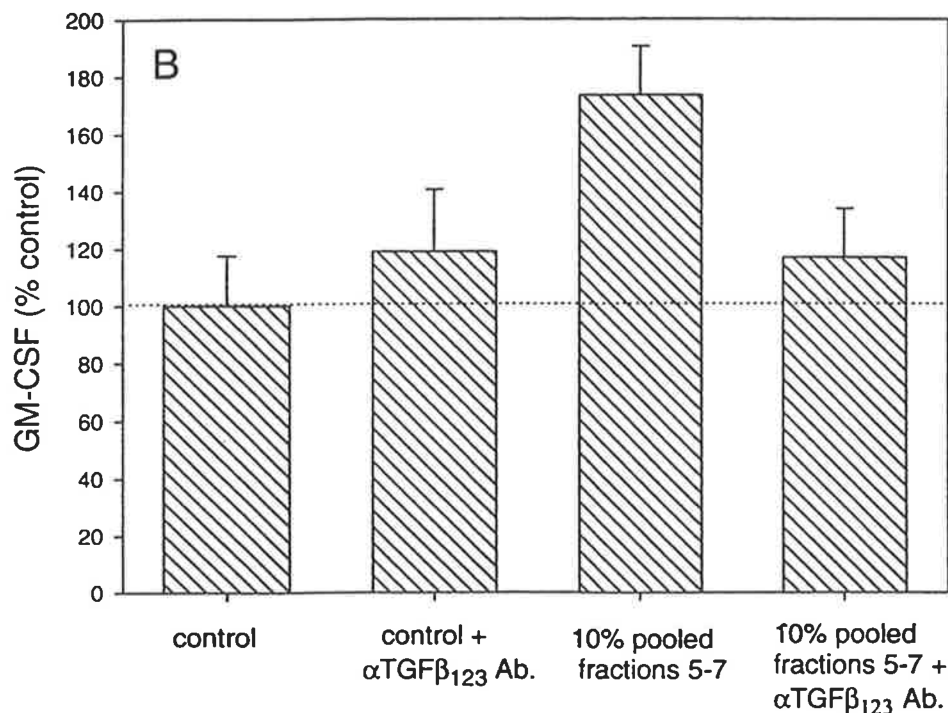


Figure 4.2 Identification of TGFβ within seminal vesicle secretions following chromatographic fractionation. Seminal vesicle fluid was fractionated as described in Figure 4.1 and TGFβ bioactivity was measured using a Mink Lung fibroblast bio-assay (CCL-64). No TGFβ bioactivity was identified in any of the non acid-activated samples, but significant bio-activity was observed in acid-activated fractions 5, 6 and 7 on three separate chromatography experiments (A). Inhibition of CCL-64 proliferation was TGFβ-dependent, since no inhibition was observed following neutralisation of TGFβ bioactivity with $\alpha TGF\beta_{123}$ antibody (4 μg/ml), and fractions 5-7 were unable to inhibit the growth of TGFβ-insensitive L cell fibroblasts. Addition of pooled fractions 5-7 to uterine epithelial cell cultures (n=5 for each group) produced a significant increase in GM-CSF production which could be blocked by the addition of $\alpha TGF\beta_{123}$ antibody (B).

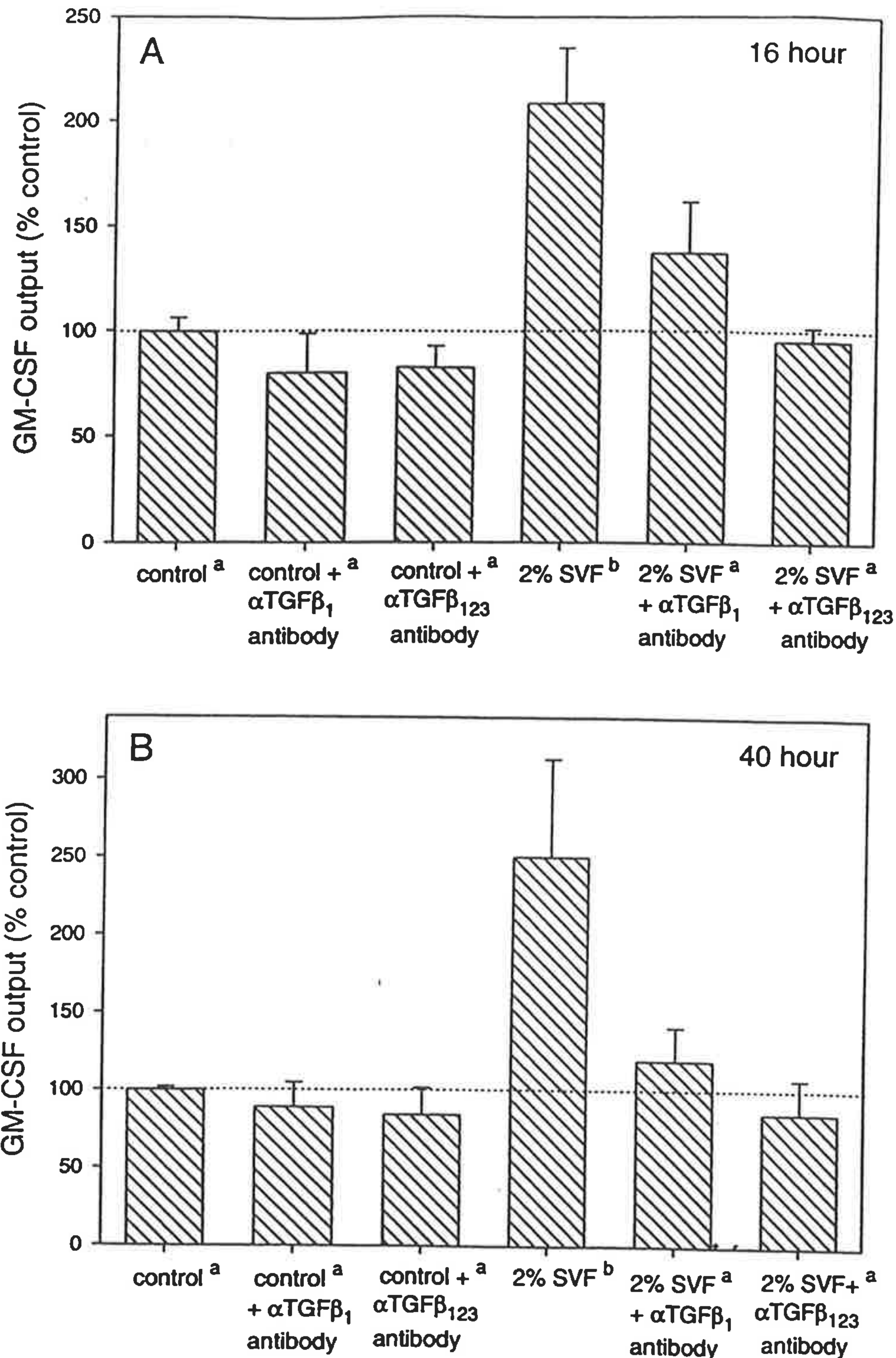


Figure 4.3 Identification of TGFβ$_1$ as the dominant TGFβ isotype responsible for increasing uterine epithelial cell GM-CSF production *in vitro*. Seminal vesicle secretions from stud CBA F1 males were solubilised into 6 M guanidine hydrochloride, acid activated and desalted into DMEM / 0.1%FCS. Dilutions equivalent to 2% v/v seminal vesicle fluid were added in triplicate to uterine epithelial cell cultures in the presence of either αTGFβ$_1$(10 μmg/ml) or αTGFβ$_{123}$(4μmg/ml) neutralising antibody. The GM-CSF content of the 16-hour (A) and 40-hour (B) culture supernatants were assayed using a FD 5/12 bio-assay and expressed as relative percentage of the GM-CSF output observed in the control cultures. The data is presented as the mean (±s.d.) of triplicate cultures with different superscripts indicating significant differences between the groups when assessed by Kruskal Wallis one-way ANOVA, followed by the Mann Whitney rank sum test ($p<0.05$).

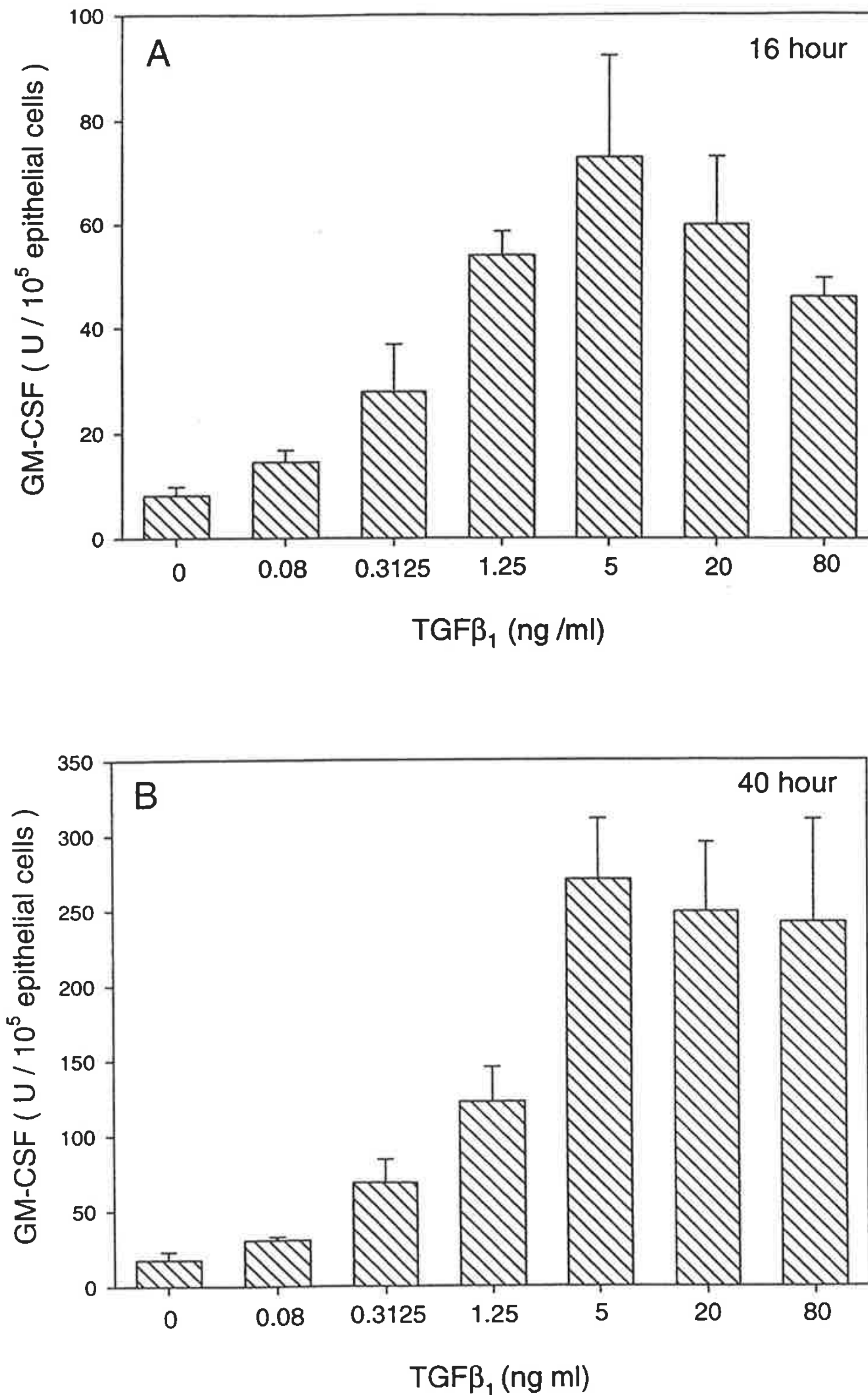


Figure 4.4 The effect of rTGFβ_1 on uterine epithelial cell GM-CSF production *in vitro*. Human rTGFβ_1 was added to oestrous uterine epithelial cell cultures over a range of concentrations (0.08-80 ng /ml) and the GM-CSF content within supernatants assessed by a FD 5/12 assay. The GM-CSF content in the 16 hour (A) and 40 hour (B) supernatants is recorded as the mean ($\pm$ SD) of triplicate wells for each concentration of rTGFβ_1. Similar patterns of increased GM-CSF output following rTGFβ_1 stimulation were seen in four replicate experiments.

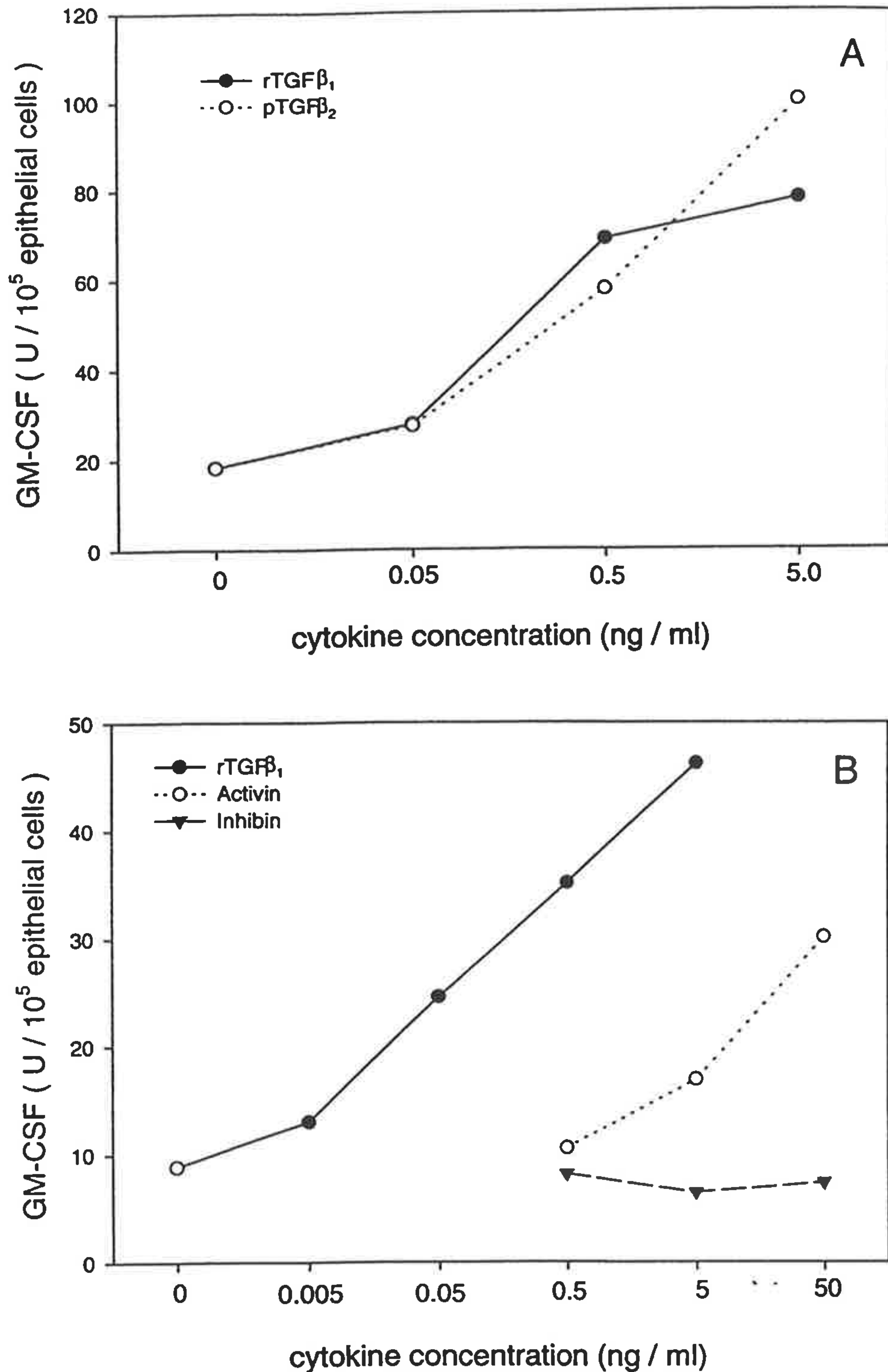


Figure 4.5 The effect of different members of the TGFβ super-family on uterine epithelial cell GM-CSF production *in vitro*. Human rTGFβ_1, porcine TGFβ_2, and human recombinant activin and inhibin were added to oestrous uterine epithelial cell cultures over a wide range of concentrations. Assessment of supernatant GM-CSF content was made 16 and 40 hours later using the FD 5/12 bio-assay. The plotted data points represent the mean GM-CSF content (n=4) of the 16-hour supernatants. The 40-hour supernatants contained similar amounts of GM-CSF to the 16-hour supernatants, however this data is not shown. Comparisons between rTGFβ_1 and porcine TGFβ_2 revealed very similar GM-CSF stimulating activity (A). Inhibin had no capacity to increase GM-CSF production,while activin had approximately one hundredth the stimulating activity of either rTGFβ_1 or pTGFβ_2 (B).

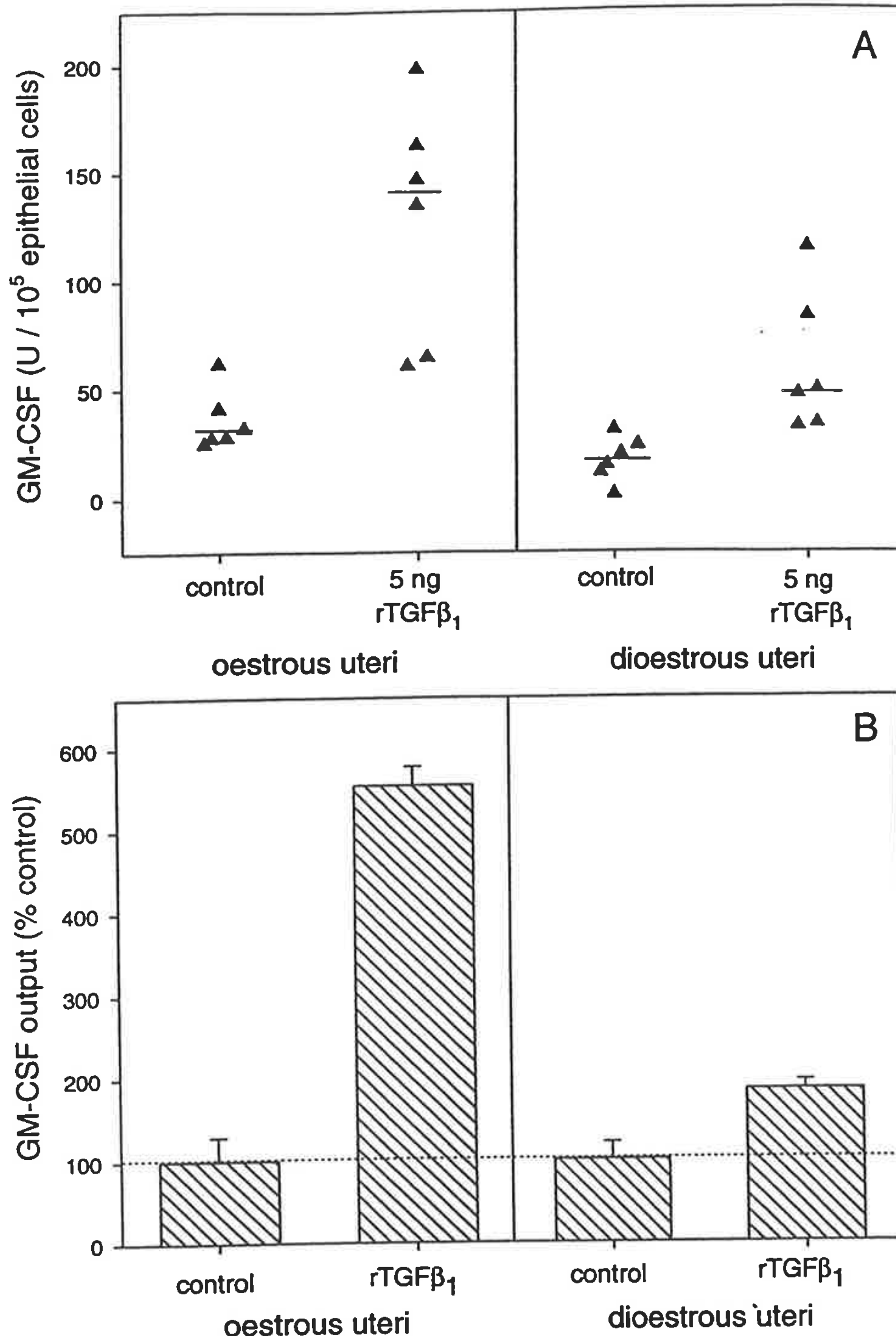


Figure 4.6 The effect of oestrous cycle stage on uterine epithelial cells GM-CSF response to rTGFβ$_1$. Vaginal smears were performed to assess stage of oestrous cycle before harvesting uterine epithelial cells from six oestrous and six dioestrous females. Culture medium (control) or 5 ng/ml rTGFβ$_1$ was then added to duplicate cultures, followed by assessment of GM-CSF content in the 16-hour supernatants using FD 5/12 bio-assay. All plotted points represent the mean of duplicate wells for individual animals (A). Analysis of the data by Kruskal Wallis one-way ANOVA, followed by Mann Whitney rank sum test, revealed that both the oestrous baseline and post-TGFβ$_1$ stimulation GM-CSF values were significantly higher than the respective controls ($p<0.05$). The percentage increase in GM-CSF production relative to the respective control did not reach significance (oestrous 364% increase, dioestrous 276% increase, $p=0.20$). When 4ng /ml rTGFβ$_1$ was added to pooled uterine epithelial cultures (n=4 per group) from three oestrous and three dioestrous females, a significantly greater increase in GM-CSF production was observed in the oestrous cultures compared to dioestrous cultures (B).

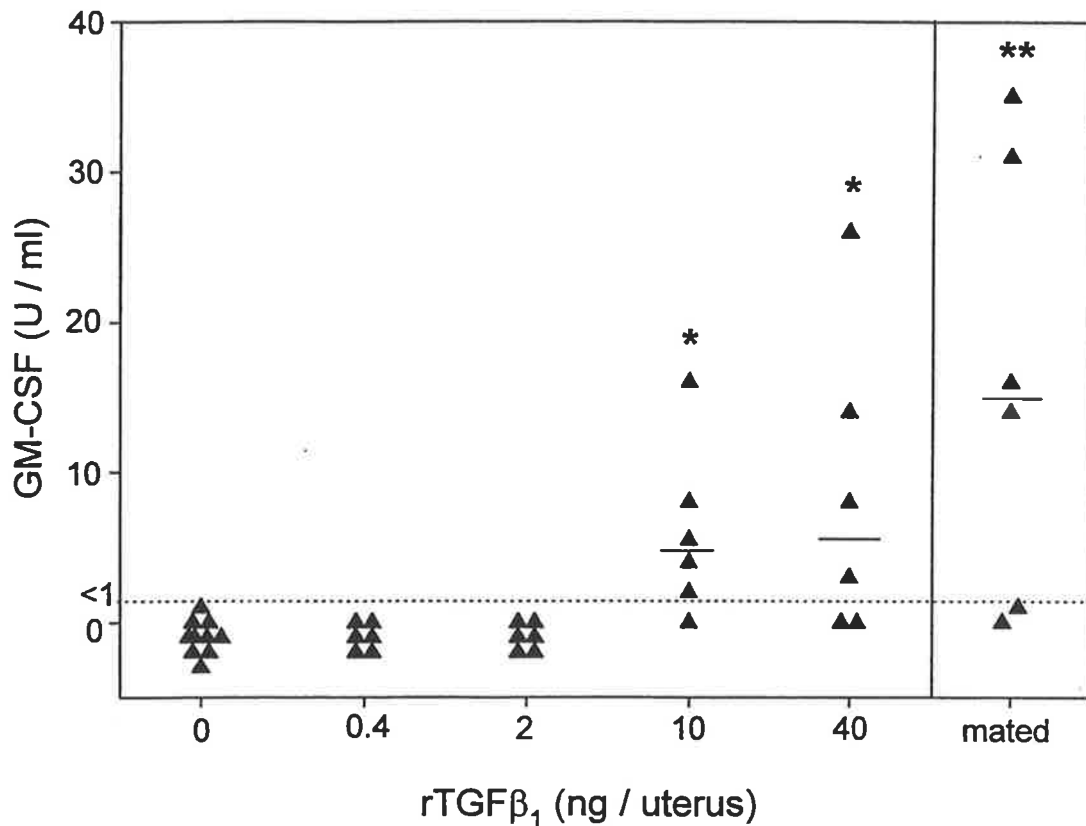


Figure 4.7 The effect of rTGFβ_1 on uterine epithelial cell GM-CSF production *in vivo*. BalbcF1 females were mated with stud CBA F1 males, or given intra-uterine injections of rTGFβ_1 contained within 50 μl of RPMI/0.1%BSA. Animals were sacrificed 16 hours later to enable collection of uterine luminal fluid which was assayed for GM-CSF content using a FD 5/12 bio-assay. Differences between the experimental groups and the control were assessed by Kruskal Wallis one-way ANOVA, followed by Mann Whitney rank-sum test (* $p<0.05$; ** $p<0.01$).

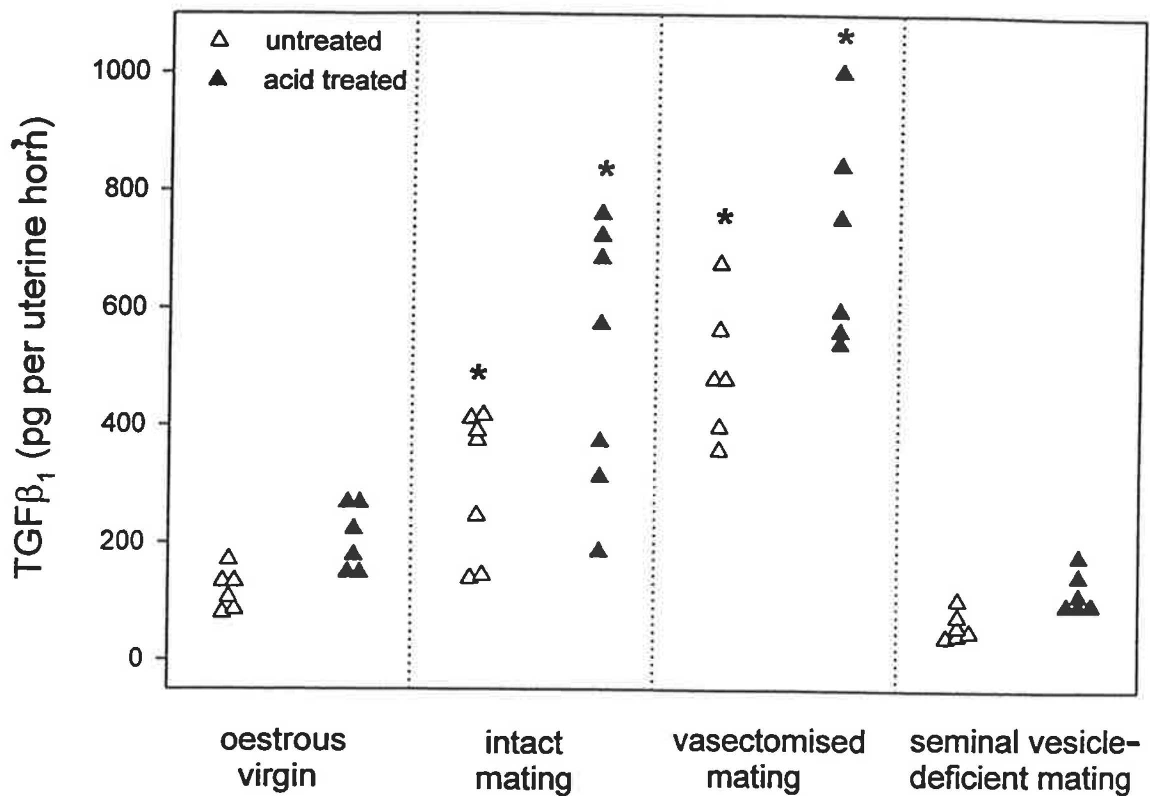
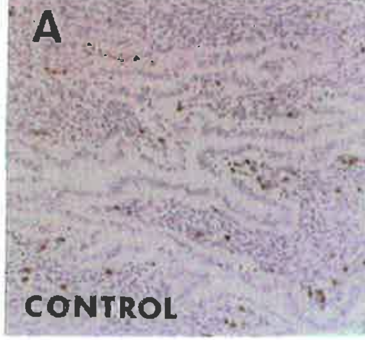
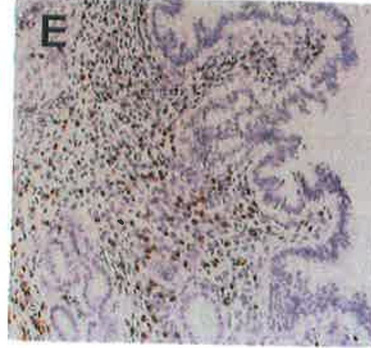


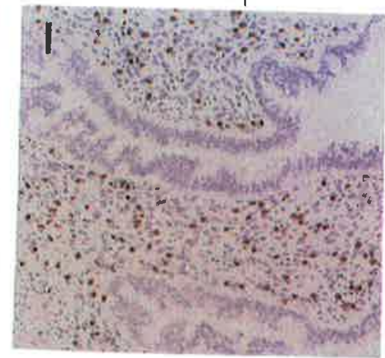
Figure 4.8 The effect of mating on uterine luminal fluid TGFβ_1 content. Balb/c F1 females were mated with intact, vasectomised or seminal vesicle-deficient CBA F1 studs before assessing uterine luminal fluid TGFβ_1 immunoactivity (total and naturally bioactive). Symbols represent the uterine luminal fluid TGFβ_1 content of individual animals, with median values being scored. Data were assessed by Kruskal Wallis one-way ANOVA, followed by Mann Whitney rank sum test, with asterisks indicating existence of significant differences between experimental groups and the appropriate virgin control.

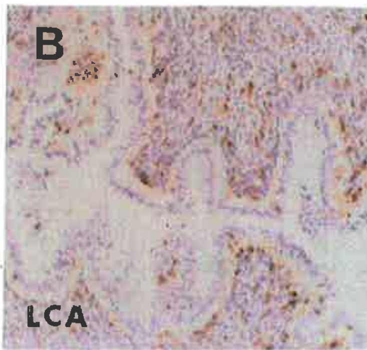
Figure 4.9. The effect of mating or intrauterine injection of rTGFβ_1 on endometrial leukocyte numbers. Sections of uterus were taken 16 hours after mating (E-H), administration of 20 ng rTGFβ_1(I-L), or carrier medium (A-D). Sections were stained with DAB and H_2O_2 to detect eosinophils (A,E,I), or incubated with mAbs specific for the different leukocyte lineages. All leukocytes are detected with the pan leukocyte marker CD45/LCA (B,F,J). The F4/80 mAbs is primarily directed against macrophages(C,G,K) and the RB6-6C5 mAb is directed against neutrophils (D,H,L). The arrow in L is highlighting the trans-epithelial migration of a neutrophil following mating.

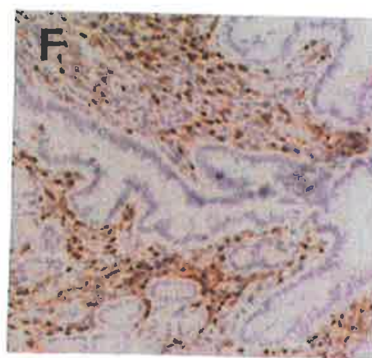
CARRIER MEDIUM | MATED | rTGFβ$_1$

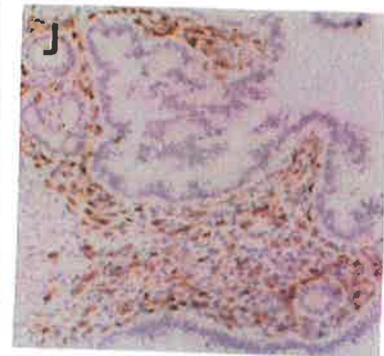


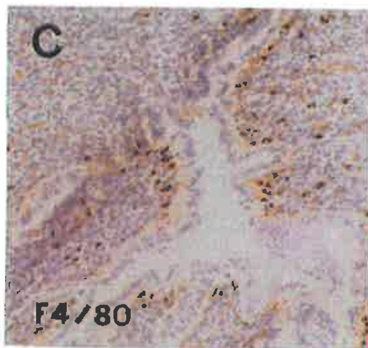


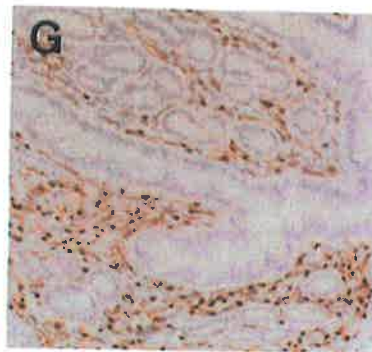


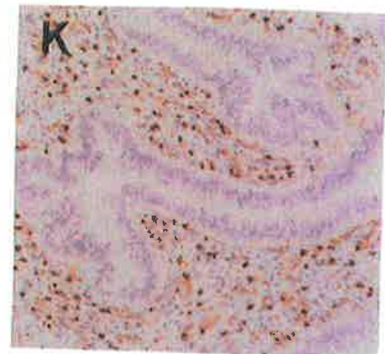


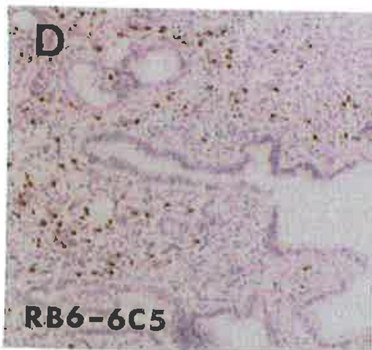


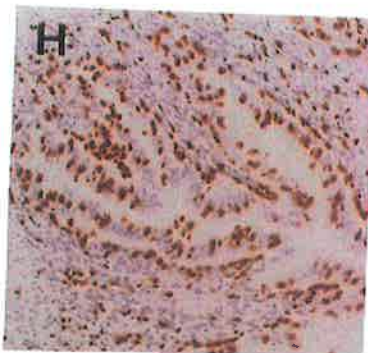


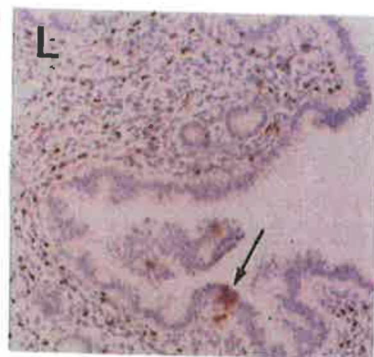












Chapter 5

TGFβ within the male reproductive tract

5.1 Introduction

Experiments described in Chapter 4 provided evidence that seminal vesicle-derived TGFβ is able to increase GM-CSF production by uterine epithelial cells, which in turn initiates an inflammatory response within the uterine endometrium. Described in this chapter are a series of experiments undertaken to investigate the presence of $TGF\beta_1$ and $TGF\beta_2$ mRNA and $TGF\beta_1$ protein within the seminal vesicle, prostate and coagulating glands of stud CBA mice. These three accessory sex gland secretions make up the bulk of seminal plasma (Mann and Lutwak-Mann 1981) and therefore are likely to be the source of TGFβ found within the male ejaculate. The analysis of $TGF\beta_1$ content within seminal vesicle secretions confirmed earlier suspicions that the majority of $TGF\beta_1$ is present as a latent form. This finding prompted a search for *in vivo* mechanisms for the release of bioactive $TGF\beta_1$ from its latent precursor. Finally, the effect of androgens on seminal vesicle $TGF\beta_1$ production was investigated, since androgens are known to have a profound effect on most aspects of seminal vesicle function (Williams-Ashman 1983).

5.2 Identification of $TGF\beta_1$ and $TGF\beta_2$ in accessory sex glands by RT-PCR

The prostate, seminal vesicle, coagulating and bulbourethral glands were excised from stud CBA males using care to avoid contamination with adjacent tissues, and individually processed to recover RNA. Testicular tissue was also included, since it is possible that some seminal plasma cytokines may originate from testicular tubule secretions. Splenic tissue was included as a positive control. Primers specific for murine $TGF\beta_1$ and $TGF\beta_2$ were generated and employed in a qualitative analysis of the presence of $TGF\beta_1$ and $TGF\beta_2$ mRNA within these tissues. The results of the RT-PCR (Figure 5.1) clearly show that both $TGF\beta_1$ and $TGF\beta_2$ mRNA are present in all accessory sex gland tissue. This observation is in keeping with the earlier description of $TGF\beta_1$ mRNA within neonatal mouse seminal vesicles (Tanji *et al.* 1995) and adult prostatic tissue (Timme *et al.*

1994). However, the presence of TGFβ mRNA does not conclusively prove that significant amounts of TGFβ protein are produced by these tissues, since the control of TGFβ protein production is predominantly made at the post-transcriptional level (Kim *et al.* 1992, Mahmood *et al.* 1995). In addition, TGFβ mRNA isolated from seminal vesicle tissue may be derived from cells not directly involved in exocrine function (eg stromal leukocytes). Because of these limitations, it was necessary to measure $TGF\beta_1$ protein content within all accessory sex gland secretions.

5.3 The $TGF\beta_1$ content of male accessory sex gland secretions

Since $TGF\beta_1$ is the major stimulus for uterine GM-CSF release, it was decided to concentrate on quantifying this isoform of TGFβ within accessory sex gland secretions. Extraction of seminal vesicle secretions was feasible since they could be readily extruded from the thin walled saccular glands by gentle pressure. Extraction of prostate and coagulating gland secretions was more problematic because these secretions could not easily be expressed from the tissue. The entire prostate and coagulating glands were therefore homogenised in order to recover the majority of their secretions. This procedure has previously been used successfully for the extraction of cytokines from gastric mucosa (Gionchetti *et al.* 1994) and skin (Skoglund *et al.* 1991). An estimate of extraction efficiency was calculated by comparing the total protein recovered in each homogenate with the original gland weight (μg of protein per mg of gland tissue). Provided the protein recovery was comparable between glands, it was judged reasonable to compare relative $TGF\beta_1$ content between the gland secretions and homogenates. No attempt was made to measure bulbourethral gland $TGF\beta_1$ content because of the minute size of these glands and the fact that their secretions account for less than 1 % of ejaculate volume (Mann and Lutwak-Mann 1981).

Quantification of gland $TGF\beta_1$ content was performed using a $TGF\beta_1$-specific ELISA. TGFβ bioactivity was not determined because unfractionated seminal vesicle secretions were found to have a non-specific inhibitory effect on the proliferation of CCL-64 cells used in the TGFβ bio-assay. This inhibition was not reversed by the addition of TGFβ neutralising antisera, and was observed in the TGFβ-insensitive L fibroblast line, thereby indicating that a TGFβ independent mechanism was responsible (data not shown).

Table 5.1 summarises the $TGF\beta_1$ immunoactivity, gland weight, secretion volume and total protein content within the secretions / homogenate of seminal vesicle, prostate and coagulating glands of six 3-month old stud CBA males.

Table 5.1 $TGF\beta_1$ content within accessory sex glands of CBA stud male mice.

	seminal vesicle glands	prostate	coagulating glands
total gland weight (mg)	284 (228 - 379)	84 (67 - 109)	38.5 (29 - 49)
volume of secretions* (μl)	113 (71 - 134)	ND	ND
total protein content (μg)	17 015 (4500 - 24 200)	1082 (775 - 1574)	1542 (675 - 1950)
extraction efficiency (μg protein per mg of tissue)	64.8 (11.9 - 76.1)	12.81 (8.24 - 22.27)	40.09 (15.41 - 65.42)
$TGF\beta_1$ content (pg per mg tissue)	27.5 (11.08 - 37.9)	1.97 (1.61 - 2.35)	1.516 (0.726 - 2.08)
$TGF\beta_1$ content (pg per μg of extracted protein)	0.435 (0.251 - 0.933)	0.138 (0.109 - 0.206)	0.041 (0.025 - 0.088)
total $TGF\beta_1$ concentration in seminal vesicle fluid (ng/ml)	73.7 (36 - 104)	ND	ND
% $TGF\beta_1$ present in its active state	28.1 (8.8 - 41)	ND	ND

* Seminal vesicle secretion volume was estimated by weighing the seminal vesicle secretions and then calculating the volume, given the density of 1.068 gm /ml.

Data are expressed as median (range) observed in six stud males

ND = not determined

The volume of seminal vesicle secretions could not be directly measured because of the viscosity of these secretions and their propensity to gel. Good estimates of secretion volume were made by weighing the secretions from each pair of glands prior to solubilisation in guanidine hydrochloride. The density of seminal vesicle secretions had earlier been measured as 1.068 g / ml, which then allowed an estimate of secretion volume to be made using the mass of the secretions (mass = density x volume). The observed

median seminal vesicle secretion volume of 113 μl per pair of glands is in agreement with values reported by other investigators (Lee *et al.* 1991).

The median $TGF\beta_1$ content within stud seminal vesicle secretions was found to be 73.7 ng / ml (range 36 - 104 ng / ml). This concentration of $TGF\beta_1$ is similar to that reported within human seminal plasma by Nocera and Chu (238 ng / ml, 1995) and Srivastava *et al.* (80 ng / ml, 1996).

Measurement of protein content within seminal vesicle secretions using a micro bicinchoninic acid assay revealed that between 4 500 and 24 200 μg of protein was present in each pair of glands (median 17 000 μg). Normand *et al.* (1989) had earlier reported a similar total protein content in the seminal vesicle secretions of the Swiss mouse (22000 $\pm$ 1200 μg). These values are comparable with the amount of protein recovered from prostate and coagulating gland homogenate when expressed as μg of extracted protein per mg of tissue. When $TGF\beta_1$ content was compared on the basis of glandular weight, it was clear that the seminal vesicle gland was by far the major source of $TGF\beta_1$ production, containing approximately 15-fold more $TGF\beta_1$ on a weight to weight comparison than either the prostate or coagulating glands. When glandular $TGF\beta_1$ content was compared in terms of total protein content, the seminal vesicle secretions contained three times more $TGF\beta_1$ than prostate tissue, and ten times more than the coagulating gland. Given these observations, and the fact that seminal vesicle secretions comprise the majority of seminal plasma, it is clear that the seminal vesicle gland is the major source of $TGF\beta_1$ within the ejaculate.

Both the bioactive and total $TGF\beta_1$ content of seminal vesicle secretions was measured. The majority (median 71.9%, range 59- 91.2%) of seminal vesicle $TGF\beta_1$ was secreted in a latent form. This is comparable to the situation reported for human seminal plasma, where 84% of $TGF\beta_1$ and 53% of $TGF\beta_2$ is present as a latent form (Nocera and Chu 1995). The identification of the majority of seminal vesicle $TGF\beta_1$ in a latent form is consistent with the chromatography experiments described in Chapter 4, where transient acidification of seminal vesicle fractions was required to release GM-CSF stimulating activity.

5.4 Release of bioactive TGFβ_1 from its latent precursor

In order for seminal vesicle-derived TGFβ_1 to have a biological effect on the murine uterine epithelium, a mechanism for the release of bioactive TGFβ must exist within the female reproductive tract. Two different experimental approaches were used to test this hypothesis.

An initial attempt at elucidating the mechanisms behind the release of bioactive TGFβ consisted of incubating seminal vesicle secretions with uterine luminal fluid obtained from unmated oestrous females. It was hypothesised that the oestrous uterine fluid, known to contain enzymes such as plasmin (Danglot *et al.* 1986), may facilitate the release of bioactive TGFβ_1 from its latent precursor. A mixture of uterine fluid and seminal vesicle secretions were incubated for 60 minutes at 37 ^{0}C before the relative amounts of bioactive and latent TGFβ_1 were compared with that present in seminal vesicle fluid incubated with PBS. This experiment was unable to confirm the presence of any TGFβ_1 activating factors within uterine luminal fluid, since no increase in bioactive TGFβ_1 was seen in the uterine fluid treated samples compared to PBS-treated controls (data not shown). A similar experiment in which seminal vesicle fluid was incubated with prostatic enzymes (tissue homogenate) also was unable to increase the release of bioactive TGFβ_1 from seminal vesicle secretions. Paradoxically, incubation of seminal vesicle fluid with either uterine luminal fluid or prostate homogenate reduced the concentration of bioactive and total TGFβ_1 below that measured in fresh seminal vesicle fluid. This observation raises the possibility that uterine luminal fluid and prostatic secretions may release bioactive TGFβ_1, but because bioactive TGFβ is not bound to a latency complex, it is more vulnerable to proteolytic destruction by enzymes and scavenging by proteins such as α_2-macroglogulin, thereby preventing its accumulation (Crookston *et al.* 1994).

Given the difficulties of working with complex biological fluids such as uterine luminal fluid and prostate homogenate, the ability of purified human plasmin to mediate the release of bioactive TGFβ_1 was investigated. Plasmin is known to release bioactive TGFβ_1 from latent complexes trapped within thrombi (Grainger *et al.* 1995a). It was therefore proposed that the vaginal plug, consisting mainly of coagulated seminal vesicle secretions, may be susceptible to plasmin proteolysis and provide a "slow release depot" for the sustained delivery of bioactive TGFβ_1 to the female reproductive tract. In order to test this hypothesis, equal portions of vaginal plug were broken up into small fragments

and incubated at 37 ^{0}C in PBS / 0.1% BSA, with or without 0.1 U of plasmin. Aliquots of this reaction mixture were then assayed for bioactive TGFβ_1 over the eight hour incubation period. The time course of release of bioactive TGFβ_1, depicted in Figure 5.2, shows that plasmin was unable to accelerate the release of bioactive TGFβ_1 from the vaginal plug. However, bioactive TGFβ_1 was liberated by transient acidification of the plug fragments at the completion of the experiment, confirming the presence of latent TGFβ_1 within the vaginal plug.

5.5 Effect of androgen status on TGFβ_1 content within murine seminal vesicle secretions

Androgens are known to be essential for the growth and function of many tissues in the male reproductive tract, including the seminal vesicle glands. The exocrine activity of the seminal vesicles is known to be positively influenced by androgen status (Lee *et al.* 1991), with production of many murine seminal vesicle proteins being regulated by testosterone (Normand *et al.* 1989).

The effect of testosterone status on seminal vesicle gland TGFβ_1 production was investigated. Adult CBA stud males were castrated, allowed to recover for two weeks, then given a 7-day course of testosterone (100 or 500 μg testosterone per day, delivered s/c in peanut oil) or peanut oil only (castrated controls). This dose and duration of testosterone treatment has previously been shown to restore seminal vesicle epithelial cell growth and function in castrated males (Ayata *et al.* 1988, Lee *et al.* 1991). All animals were sacrificed at the completion of the experimental protocol, followed by measurement of each animal's seminal vesicle secretion volume, total protein content, and total TGFβ_1 content. The effect of androgen status on these parameters is summarised in Figures 5.3 and 5.4. As expected, both secretion volume and total protein content fell considerably following castration, but returned to normal levels with testosterone treatment in a dose responsive manner (Figure 5.3). This observation confirms that the doses of testosterone administered must have been sufficient to induce a physiological response in the glandular epithelium. When TGFβ_1 was measured in seminal vesicle secretions and expressed as a concentration (to correct for androgen-related changes in secretion volume), testosterone status did not appear to have any significant influence on the concentration of TGFβ_1 present in seminal vesicle secretions (Figure 5.4a). However, when TGFβ_1 production was

analysed in terms of total TGFβ_1 content, it became evident that production of TGFβ_1 by the seminal vesicle epithelium was extremely androgen sensitive (Figure 5.4b), with very little being produced in the castrated state without exogenous testosterone therapy .

5.6 Discussion and conclusion

The experiments described in this chapter have identified the seminal vesicle gland as the major male accessory sex gland responsible for the production of seminal plasma-derived TGFβ_1. The quantification of TGFβ_1 content within murine seminal vesicle secretions has shown that these glands produce similar amounts of TGFβ_1 to that found within human seminal plasma (Nocera and Chu 1995, Srivastava *et al.* 1996). The TGFβ_1 content of murine seminal vesicle secretions is relatively high compared to the levels found in other biological fluids such as tears (2.3 ng / ml, Gupta *et al.* 1996), aqueous humour from the anterior chamber of the eye (1.5 ng / ml, Tripathi *et al.* 1994) and human serum (5.6 ng / ml, Grainger *et al.* 1995b). However, the TGFβ content of human milk exceeds that found within seminal plasma, with colostrum containing 1365 ng / ml TGFβ and late milk 952 ng / ml TGFβ (Saito *et al.* 1993c). The relative abundance of TGFβ_1 in seminal plasma certainly suggests a significant role for this cytokine in mammalian reproductive physiology. While it has been established that seminal TGFβ_1 can initiate a post-mating inflammatory reaction through inducing uterine epithelial cell GM-CSF synthesis, it is possible that seminal TGFβ may play other roles in murine reproduction. These are discussed further in Chapter 6.

The observation that at least 90% of seminal plasma TGFβ_1 is derived from the seminal vesicle gland is in accordance with experiments reported in Chapter 4, where it was observed that mating with intact, but not seminal vesicle-deficient males, lead to an increase in uterine TGFβ_1 levels. This observation also explains why only secretions from the seminal vesicle gland have the ability to increase uterine GM-CSF production *in vitro* (Robertson *et al.* 1990).

The presence of seminal TGFβ_1 in a predominantly latent form may have several physiological advantages, but requires mechanisms to be in place for the release of bioactive cytokine within the female reproductive tract. One advantage of latency is that it prevents TGFβ from having an immediate biological effect at its site of production, an important consideration given that TGFβ has been reported to influence a huge array of

cell types (Moses *et al.* 1987). The biological activity of TGFβ is therefore in part controlled by the availability of activating mechanisms within various tissues. The secretion of TGFβ as a latent complex may also protect it from proteolysis, thereby sustaining its biological effect (Wakefield *et al.* 1990). The copulatory plug, comprised mainly of seminal vesicle proteins, presents an ideal depot for the slow release of TGFβ, since it remains within the cervix and vagina for between 12 and 24 hours after mating. While the *in vitro* experiments described in this chapter were unable to confirm the ability of plasmin to release bioactive $TGF\beta_1$ from the copulatory plug, it is possible that plasmin, or other female reproductive tract-derived enzymes, may evoke the release of bioactive TGFβ under *in vivo* conditions.

Several mechanisms for the release of bioactive $TGF\beta_1$ have been described in the literature, some which may apply to the female reproductive tract. Acidification is a common *in vitro* technique for activating TGFβ, but the recorded pH of the murine female reproductive tract ranges from 7.0 within the vagina, to 7.5 within uterine luminal fluid (Dmitrieva *et al.* 1971), making acidification an unlikely mechanism for the release of bioactive TGFβ *in vivo*. However, the enzyme plasmin is a possible candidate since it can release bioactive TGFβ from its latency complex (Lyons *et al.* 1988), and plasminogen (the precursor of plasmin) is known to be present within the uterus. The plasminogen content of the murine uterus is second only to that found in the lung (Danglot *et al.* 1986), peaking around the time of oestrous under the influence of oestrogen (Finlay *et al.* 1983). Plasminogen is readily converted into plasmin by plasminogen activator, the production of which also peaks at oestrous (Peltz *et al.* 1983). Peak uterine plasmin activity would therefore correspond with mating, being ideally placed to facilitate the release of bioactive TGFβ from seminal plasma. Plasminogen activator, derived predominantly from the prostate and bulbourethral glands, is also present in human seminal plasma (Kester 1971, Tauber *et al.* 1976). While its presence in murine seminal plasma is unknown, seminal plasminogen activator may provide an additional mechanism for the activation of uterine plasminogen after mating.

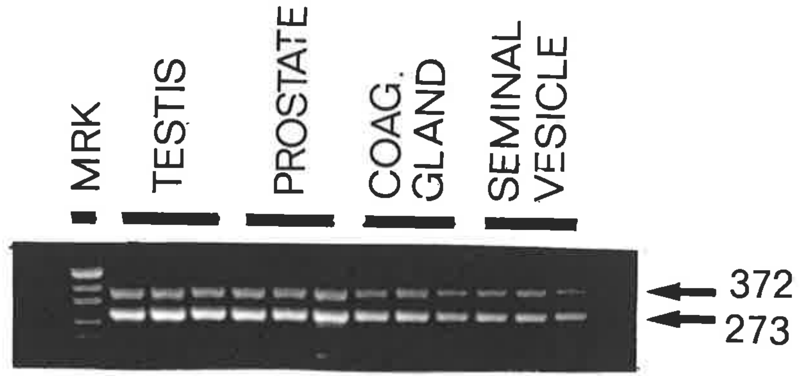
Leukocytes invading the endometrium in response to the semen-induced expression of pro-inflammatory cytokines may further promote the release of bioactive TGFβ through their production of proteases within an acidic lysosomal micro-environment (Silver *et al.* 1988, Nunes *et al.* 1995). This may contribute to a positive feedback loop to ensure that the majority of seminal plasma TGFβ is converted into its bioactive form.

While all these mechanisms are currently speculative, some mechanism for the release of bioactive TGFβ must exist, since the majority of $TGF\beta_1$ present within uterine luminal fluid shortly following mating is bioactive (Figure 4.8), unlike fresh seminal vesicle secretions.

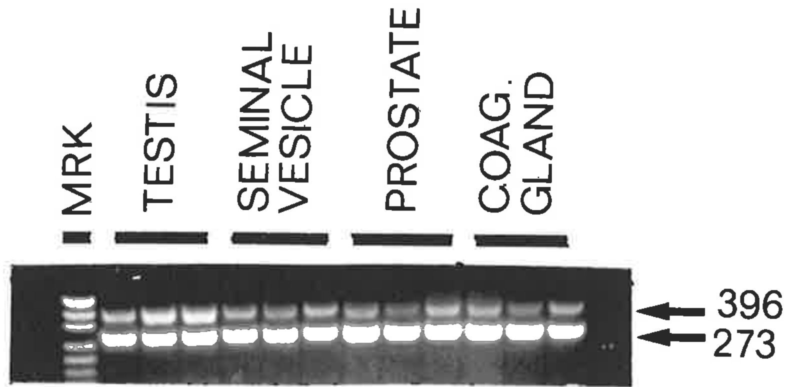
The development and function of all male accessory sex glands is testosterone-action dependent (Williams-Ashman 1983). The growth and function of the seminal vesicle glands is known to be influenced by androgen status (Lee *et al.* 1991), with castration of adult animals resulting in diminished gland weight and a marked decline in secretion volume. These changes can be reversed by administration of exogenous testosterone, thereby confirming the central role of testosterone in seminal vesicle function. Analysis of seminal vesicle protein synthesis in castrated mice by SDS gel electrophoresis has shown that the majority of seminal vesicle proteins, although not all, require testosterone for their synthesis (Normand *et al.* 1989). One previous study investigating the effect of androgen status on seminal vesicle TGFβ production reported that castration of neonatal mice resulted in a transient increase in $TGF\beta_1$ mRNA, with a return to levels comparable with intact males within 6 days of castration (Tanji *et al.* 1995). Since the control of TGFβ production is usually made at the post-transcriptional level (Kim *et al.* 1992, Mahmood *et al.* 1995), it is not possible to draw firm conclusions about the role of androgens in TGFβ production from this study. In the current experiment, TGFβ protein production was observed to fall to very low levels following castration, yet could be restored to pre-castration levels with exogenous testosterone therapy, thereby confirming that TGFβ production is under testosterone control. This finding is congruent with earlier reports of heightened GM-CSF-stimulating activity in seminal vesicle secretions obtained from stud males, compared to secretions from sexually immature unseparated males with low serum testosterone levels (Robertson *et al.* 1996b).

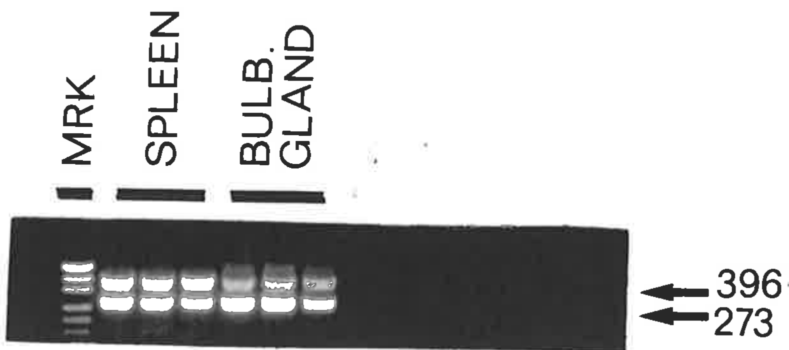
Figure 5.1 RT-PCR analysis of $TGF\beta_1$ and $TGF\beta_2$ mRNA expression in male accessory sex gland tissue. RNA was isolated from the prostate, coagulating gland, seminal vesicle and testis of stud CBA male mice. First strand cDNA was reverse transcribed using oligo-dT primers and amplified using primers for actin (372 or 396 bp), $TGF\beta_1$ (273 bp) and $TGF\beta_2$ (273 bp). Reaction products were analysed by gel electrophoresis (1% agarose gel containing ethidium bromide) and photographed under UV illumination.

TGFß$_1$



TGFß$_2$





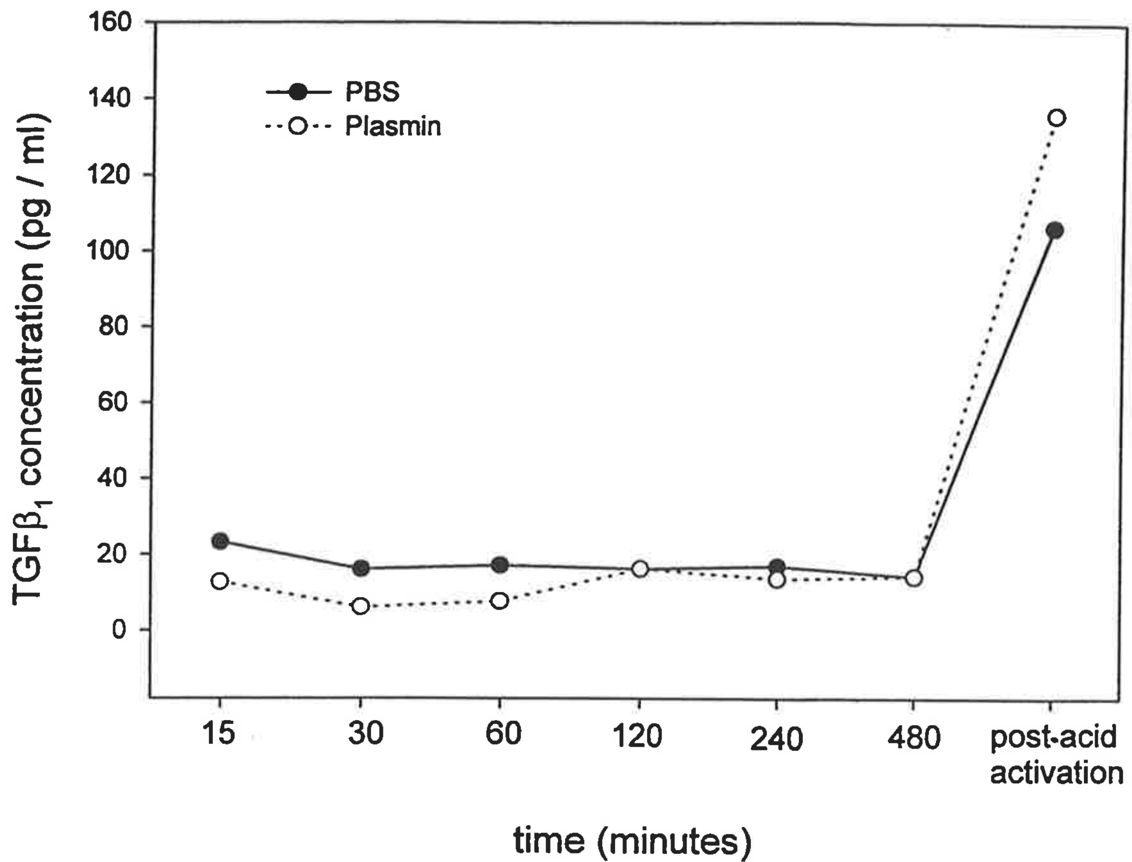


Figure 5.2 Release of active TGFβ$_1$ from coagulated semen by plasmin digestion. A fresh vaginal plug (Balb/c F1 female x CBA male) was removed from a female 8 hours after copulation, crushed into small fragments and then divided into two equal parts. Both samples were immersed in 500 μl of PBS / 0.1% BSA and incubated for 8 hours at 37 °C in the presence or absence of 0.1 U purified human plasmin. Aliquots (25 μl) were sequentially removed over the next 8 hours and assayed for bioactive TGFβ$_1$ content by ELISA. At the completion of the experiment the remaining solutions were transiently acidified prior to assaying for TGFβ$_1$. Plasmin had no significant effect on the rate of release of bioactive TGFβ$_1$.

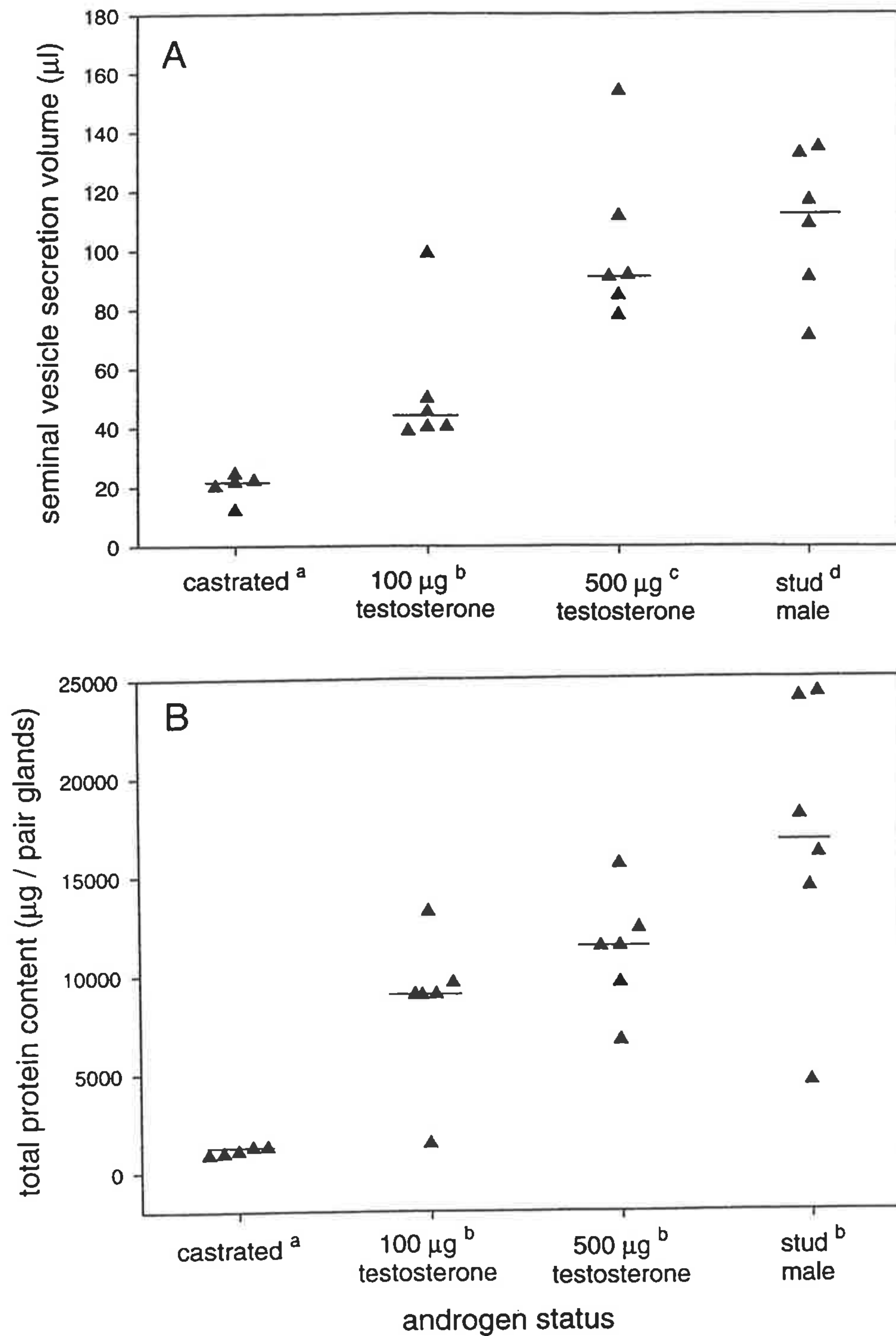


Figure 5.3 Effect of androgen status on seminal vesicle exocrine function. A group of stud CBA males were surgically castrated and allowed to recover for two weeks. Testosterone (100 or 500 µg in peanut oil) was injected subcutaneously daily for 1 week. Control mice received s/c peanut oil only. All males were sacrificed on day 8 and seminal vesicles were excised to measure the volume of glandular secretions (A) and total protein content (B). Symbols indicate values for individual animals (n=6 per experimental group) and median values are scored. Data was analysed by Kruskal Wallis one-way ANOVA, followed by Mann Whitney rank sum test. Different superscripts denote significant differences between groups ($p<0.05$).

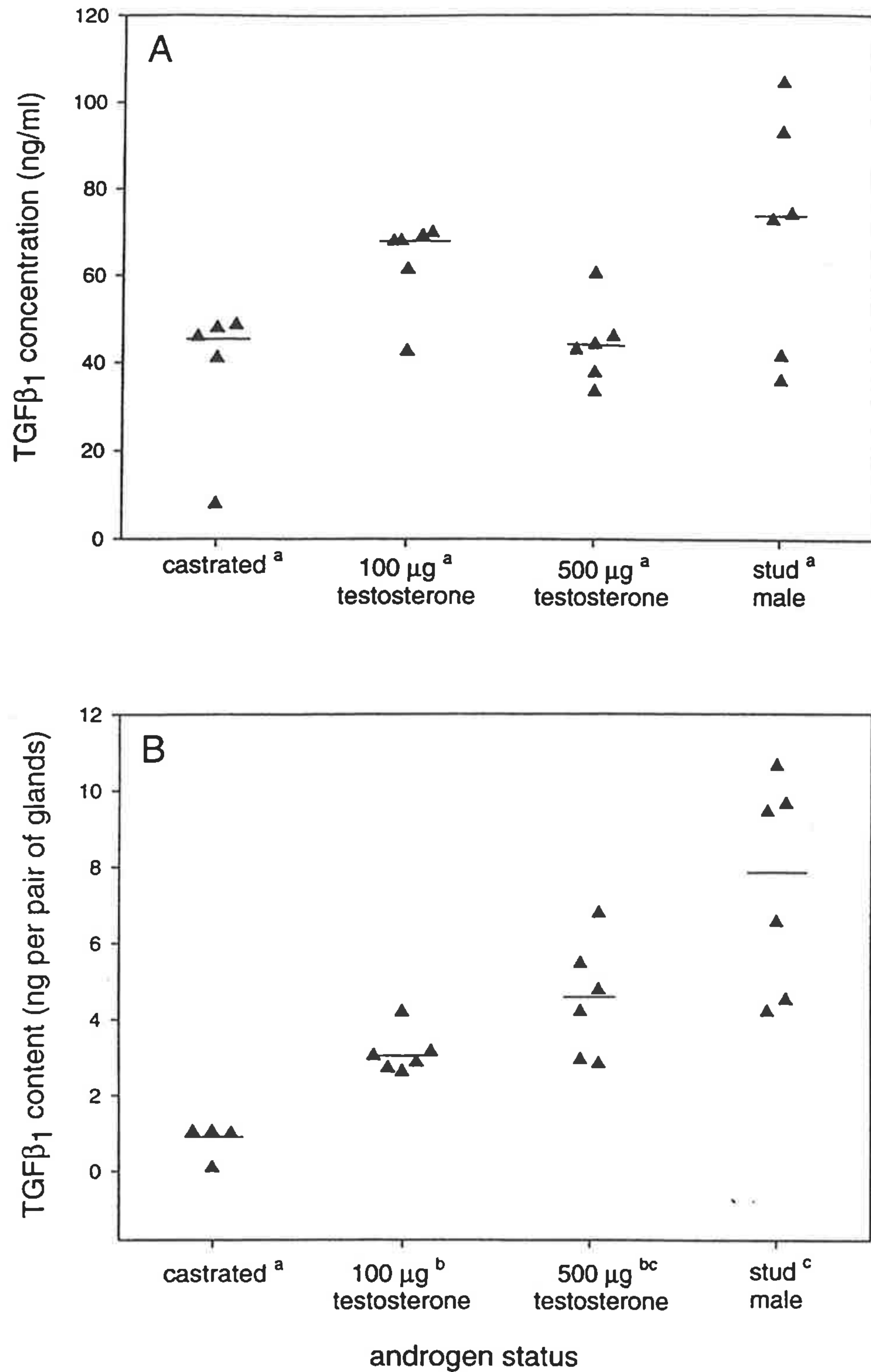


Figure 5.4 Effect of androgen status on seminal vesicle TGFβ$_1$ production. Stud CBA males were castrated and treated with testosterone, as described in Figure 5.3. Seminal vesicle glands were removed on day 8, followed by measurement of TGFβ$_1$ content. The TGFβ$_1$ content of individual glands was recorded as a concentration (A) and total TGFβ$_1$ content (B). Symbols represent individual animals (n=6 per group) and median values are scored. Data were analysed by Kruskal Wallis one-way ANOVA, followed by Mann-Whitney Rank sum test. Different superscripts denote significant differences between groups ($p<0.05$).

Chapter 6

The role of seminal vesicle secretions and TGFβ in murine pregnancy

6.1 Introduction

The physiological role that seminal vesicle secretions play in mammalian reproduction has been under debate for several years. The skeptic's view is that seminal vesicle secretions are not important for reproductive outcome other than promoting sperm survival and transport within the female reproductive tract. This view is supported by the observation that epididymal sperm or washed ejaculated sperm are capable of yielding viable pregnancies following artificial insemination or *in vitro* fertilisation / embryo transfer (Williams-Ashman 1983). While it is clear that pregnancy is possible without exposure to seminal plasma, several studies in rodents have revealed that pregnancy outcome is inferior when a prospective mother is not exposed to seminal vesicle secretions (Pang *et al.* 1979, Peitz and Clarke 1986). It is therefore probable that seminal vesicle secretions play a non-essential, yet supportive role that helps maximise rodent reproductive potential. The exact mechanism by which seminal plasma mediates this beneficial effect has yet to be identified.

The aim of the studies described in this chapter was to determine the role that seminal vesicle secretions play in murine reproduction. This was investigated by comparing the outcome of pregnancies sired by seminal vesicle-deficient males with intact males. The poor pregnancy outcome observed following mating with seminal vesicle-deficient males prompted further investigation of possible causes, one being the absence of a post-mating inflammatory reaction due to a lack of exposure to seminal $TGF\beta_1$. The PMIR is postulated to initiate a "immuno-permissive" environment that may favour the growth and survival of the semi-allogenic conceptus. Experiments were conducted to investigate the possibility that creating an artificial PMIR through intrauterine administration of $rTGF\beta_1$ may improve the outcome of pregnancies sired by seminal vesicle-deficient males.

6.2 Reproductive outcome in seminal vesicle-deficient matings

6.2.1 Seminal vesicle-deficient CBA males mated with Balb/c F1 females.

Seminal vesicle glands from stud CBA males were surgically removed, while ensuring that the adjacent coagulating glands and nerves were not damaged. The CBA x Balb/c F1 mating combination was used to achieve 100% allogenicity for Class I MHC antigens, since it was postulated that seminal vesicle secretions may induce a "permissive" maternal immune response to paternal antigens that may prevent rejection of the semi-allogenic conceptus, an effect expected to be more pronounced in a fully allogenic mating pair.

Balb/c F1 females mated with intact CBA males exhibited a 100% pregnancy rate, with all "plugged" females carrying fetuses on day 17 of pregnancy. The mean (± s.d.) litter size for these pregnancies was 11.42 (± 1.1) with 98.5 % viability. No viable pregnancies were sired by seminal vesicle-deficient CBA males when assessed on day 17 p.c., despite conformation of the presence of sperm in post-mating vaginal smears.

In order to investigate the extent to which fertilisation failure may have contributed to this result, oviducts from females mated with intact, seminal vesicle-deficient and vasectomised males were flushed on day 3 of pregnancy (1600hrs) to enable assessment of embryonic development (Figure 6.1). This revealed that 60% of embryos derived from seminal vesicle deficient matings were at a 2-cell stage of development or greater, but the majority of these embryos were retarded in their development compared to intact matings. The possibility that a large proportion of these retarded embryos may be parthogenically activated (non-fertilised) was supported by the observation that 54% of embryos derived from vasectomised matings were also 2-cell or greater. However, a significant proportion of day 3 embryos from seminal vesicle-deficient matings (33%), but not vasectomised matings, were as equally well developed as embryos derived from intact matings, suggesting that fertilisation of some oocytes does occur after mating with seminal vesicle-deficient males. This was confirmed by the observation that 22.2% (4/18) of females mated with seminal vesicle-deficient males had signs of implantation on day 7 of pregnancy. Furthermore, collection of day 2 embryos from females mated with intact and seminal vesicle-deficient males, followed by embryo culture and assessment until day 6 of pregnancy, revealed that 25% of embryos from seminal vesicle-deficient matings reached the blastocyst stage of development at a similar rate as embryos derived from intact

matings (Figure 6.2). This observation would indicate that the rate of embryonic development is not altered by exposure to seminal vesicle secretions, but oocyte fertilisation is reduced by approximately 75% in the absence of exposure to seminal vesicle secretions.

A possible mechanism for the reduced fertilisation rates seen in females mated with seminal vesicle-deficient CBA males is a reduction in the number of sperm reaching the oviduct, since seminal vesicle secretions are reported to increase the forward motility of sperm (Peitz 1988). To investigate this potential mechanism, uterine sperm counts were performed 8 hours after mating with intact and seminal vesicle-deficient males. Females mated with intact CBA males had in excess of 1 million sperm per uterus, but females mated with seminal vesicle deficient males had no identifiable sperm (n=4 per group). This observation confirms that the reduction in fertilisation seen in females mated with seminal vesicle deficient males is due to insufficient numbers of sperm reaching the oviducts.

6.2.2 *Seminal vesicle-deficient CBA F1 males mated with Balb/c F1 females*

To investigate the strain specificity of the effect of seminal vesicle removal on pregnancy outcome, the experiment described in 6.2.1 was repeated using CBA F1 males. These males are larger and more robust than their CBA counterparts, but are only semi-allogenic for Class I MHC antigens when paired with Balb/c F1 females because of a common C57Blk background. Table 6.1 summarises the data on pregnancy outcome from mating CBA F1 males (intact and seminal vesicle deficient) with Balb/c F1 females.

Mating with intact CBA F1 studs resulted in a 100% pregnancy rate, with seminal vesicle gland removal reducing this rate to 66.6% (10 / 15 females pregnant). This difference in the pregnancy rate was statistically significant ($p = 0.021$, $X^2 = 5.27$), thereby confirming the need for exposure of the female to seminal vesicle secretions, in order to achieve maximal reproductive potential. In those females who did become pregnant, there was a significant difference in the viable litter size between pregnancies sired by intact (9.30 ± 1.79 fetuses) and seminal vesicle-deficient males (7.70 ± 1.76 fetuses, $p = 0.044$). No significant difference was observed in the number of late fetal resorptions between the two groups, thereby implying that either fertilisation rates were reduced, or early resorptions were increased in the absence of exposure to seminal vesicle secretions.

Table 6.1 The effect of rTGFβ_1 on reproductive outcome in pregnancies sired by seminal vesicle-deficient males

	Intact mating	Seminal vesicle-deficient matings		
		control	+ PBS	+ TGFβ_1
number of mated females pregnant at day 17 (%)	100% (13/13)^{a}	66.6% (10/15)^{b}	46.1% (6/13)^{b}	38.5% (5/13)^{b}
fetal numbers	127	84	31	36
viable fetuses per litter (% total)	9.31 ± 1.79^{a} (95.3%)	7.70 ± 1.76^{b} (91.7%)	4.67 ± 3.32^{c} (90.3%)	6.80 ±3.83^{b} (94.4%)
non-viable fetuses per litter (% total)	0.46 ± 0.52^{a} (4.7%)	0.70 ± 0.97^{a} (9.3%)	0.50 ± 0.83^{a} (9.7%)	0.40 ± 0.55^{a} (5.6%)
fetal weight (mg)	727.3 ± 111.1^{a}	718.2 ± 114.9^{a}	706.1 ± 107.0^{a}	661.1 ± 60.9^{b}
placental weight (mg)	101.7 ± 14.9^{a}	101.1 ± 15.9^{a}	106.9 ± 18.8^{ab}	109.5 ± 20.2^{b}
fetal-placental ratio	7.30 ± 1.0^{a}	7.19 ± 1.1^{a}	6.83 ± 1.65^{a}	6.22 ± 1.17^{b}

Balb/c F1 females were mated with stud CBA F1 males (intact or seminal vesicle-deficient). Between 12 and 14 hours after mating, some of the females paired with the seminal vesicle-deficient males received an intrauterine injection of 20 ng rTGFβ_1 or PBS. All females were sacrificed on day 17 of pregnancy. Data is expressed as the mean ± s.d. Statistical analysis was initially performed using a two-way ANOVA, followed by students t test, with different superscripts indicating significant differences between groups ($p<0.05$).

Analysis of mean fetal weight, placental weight and the ratio of fetal to placental weight (a measure of placental function) revealed no statistically significant differences between pregnancies sired by intact or seminal vesicle-deficient males (Table 6.1). Fetal and placental weight data were also analysed according to litter size, particularly the number of fetuses within ipsilateral uterine horns, since this has been reported to be an important variable affecting fetal and placental weight (Healy 1961). This observation is attributed to competition for nutritional supplies from a common arterial supply. All fetal and placental weights were analysed in relation to the total number of fetal-placental units (viable and non-viable) within each uterine horn, since mid-term resorptions are still capable of effecting the weight of viable siblings (Healy 1961). This analysis, graphically depicted in

Figures 6.3 and 6.4 , revealed no significant differences in mean fetal or placental weight between intact and seminal vesicle-deficient matings, even after accounting for differences in fetal numbers (p= 0.093 fetal weight, p= 0.472 placental weight).

6.3 Role of $TGF\beta_1$ in pregnancy outcome following seminal vesicle-deficient matings

Because seminal vesicle secretions are the major source of seminal plasma $TGF\beta_1$, it was hypothesised that the decline in fertility observed in matings with seminal vesicle-deficient males may be the result of a lack of exposure to $TGF\beta_1$, possibly mediated by the absence of a post-mating inflammatory reaction. In order to test this hypothesis, 20 ng of $rTGF\beta_1$ was injected trans-cervically into Balb/c F1 females eight hours after mating with seminal vesicle-deficient CBA F1 males. The dose of 20 ng was chosen since it had been shown to increase uterine epithelial cell GM-CSF production and initiate an inflammatory reaction comparable to that seen following an intact mating.

The traditional technique for delivery of fluid to the uterus is by direct intra-uterine injection at laparotomy. This method requires manual handling of the uterus with forceps and insertion of a needle through the uterine wall, both likely to result in an inflammatory reaction that may be detrimental to pregnancy outcome. To avoid these problems, a less traumatic trans-cervical method was used to deliver $rTGF\beta_1$ to the uterine lumen. Trans-cervical delivery of fluid into the murine uterus has been used previously to transfer sperm during artificial insemination (Wolfe 1967, Leckie *et al.* 1973) and for embryo transfer (Moler *et al.* 1979). The administration of 20ng of $rTGF\beta_1$ contained within 50 µl of PBS / 0.1% BSA was delayed until eight hours after mating to ensure that fluid injection did not interfere with the distribution of sperm in the reproductive tract or with fertilisation of oocytes, which is generally complete within two hours of mating (Hogan 1986). A third group of females mated with seminal vesicle-deficient males were trans-cervically injected with 50 µl of PBS / 0.01% BSA to enable an assessment of the effect of intrauterine injection on pregnancy outcome. A summary of the pregnancy outcomes for this experiment is contained in Table 6.1.

The intrauterine administration of $rTGF\beta_1$ was unable to reverse the decline in pregnancy rates observed in females mated to seminal vesicle-deficient males. No significant difference in the pregnancy rates was observed between any of the three

seminal vesicle-deficient experimental groups, although both the rTGFβ_1 and PBS-treated groups had a lower pregnancy rate (38.5% and 46.2% respectively) compared to the untreated controls (66.6%). The number of viable fetuses per uterine horn was significantly smaller in all three seminal vesicle-deficient experimental groups when compared to intact controls ($p<0.0001$). Females administered rTGFβ_1 had significantly larger numbers of viable fetuses than PBS-treated females ($p= 0.042$), but viable fetal numbers were not significantly different between untreated and rTGFβ_1-treated females ($p= 0.142$).

Recorded in Table 6.1 are the mean fetal weight, placental weight and the fetal : placental weight ratios for treated and untreated groups compared with pregnancies sired by intact CBA F1 males. A significant decline in mean fetal weight and an increase in mean placental weight was observed in the TGFβ_1-treated group when compared with all other groups (Figure 6.5 and 6.6). The increase in placental weight was still significant after analysing the data with fetal numbers as a covariate ($p=0.044$). The mean fetal weight of pregnancies treated with rTGFβ_1 and PBS was lower than that observed in untreated seminal vesicle-deficient pregnancies, with fetal numbers having no significant effect on fetal weight ($p=0.13$). Since lack of exposure to seminal vesicle secretions had previously been shown to have no significant effect on fetal weight, it is probable that this fall in fetal weight is a result of harm produced by the process of trans-cervical cannulation and intra-uterine fluid injection. In light of this technical difficulty, it is impossible to draw firm conclusions about the role that TGFβ_1 plays in pregnancy outcome from this data.

6.4 Discussion and conclusions

The experiments described in this chapter confirm that lack of exposure to seminal vesicle secretions can have a detrimental effect on reproductive outcome, with a decline in pregnancy rates and litter size being observed. The magnitude of this detrimental effect appears to be extremely variable depending on the strain of male being studied. Removal of the seminal vesicle glands from CBA males produced complete infertility, yet the same procedure in CBA F1 males produced only a 33% decline in pregnancy rates in the same strain of females. Similar divergent findings have been reported by other investigators. Pang *et al* (1979) reported that only 7% of female albino mice became pregnant when mated to seminal vesicle-deficient albino males, with a significant fall in litter size being observed in those females who did become pregnant; whereas Peitz and Clarke (1986)

reported that mating seminal vesicle-deficient (C3H x C57BL6)F1 males with B6D2F1/J females resulted in a 77.8% pregnancy rate, with no significant decline in litter size. It is unclear why such large variations in fertility occur in different strains of male mice, since their reproductive physiology is almost identical.

Sperm derived from seminal vesicle-deficient males is reported to exhibit slower forward progression, with more side to side displacement, compared to sperm derived from intact males (Peitz and Clarke 1986). These alterations in sperm motility may result from a lack of exposure to an unidentified pro-motility factor present within murine seminal vesicle secretions (Peitz 1988). A reduction in sperm forward motility would result in a decrease in oocyte fertilisation, since fewer sperm would reach the oocyte and be physically capable of penetrating the zona pellucida. It is possible that sperm from different strains of mice vary in their dependence on seminal vesicle secretions to maintain normal motility, thereby explaining the different fertilisation rates exhibited by CBA and CBA F1 seminal vesicle-deficient males. This concept is supported by the observation that removal of seminal vesicle glands from CBA males produced a dramatic decline in the number of sperm present within the uterine lumen following mating, yet this procedure had no significant effect on the number of uterine sperm present following mating with CBA F1 males (section 4.5.2). Future experiments investigating the effect of seminal vesicle secretions on sperm motility from different strains of mice may help explain these differences.

An alternative explanation for the observed differences in fertility between the two strains of males may be related to the extent of MHC antigenic disparity between males and the Balb/c F1 females. CBA F1 males and Balb/c F1 females both express the H-2b antigen due to their common C57Blk parentage, whereas CBA males and Balb/c F1 females are completely H-2 disparate. Fetuses resulting from a CBA F1 x Balb/c F1 mating would therefore be less allogenic than those resulting from a CBA x Balb/c F1 mating, and perhaps less susceptible to immune rejection (Aguilar 1997). It is postulated that immuno-suppressive components within seminal vesicle secretions may help initiate an "immuno-permissive" environment, which prevents hostile immune responses towards paternal antigens (discussed in Chapter 7). Such an "immuno-permissive" state would not develop following mating with a seminal vesicle-deficient male, placing the more antigenically foreign CBA x Balb/c F1 embryo at greater risk of immune destruction. This may explain why all the fertilised embryos sired by seminal vesicle-deficient CBA males

were lost shortly after implantation (22.2% implantation rate day 7 of pregnancy, no implantation sites seen on day 17 of pregnancy).

A reduction in oocyte fertilisation due to decreased sperm motility may account for some of the adverse pregnancy outcomes seen following mating with seminal vesicle-deficient males, but it does not appear to be the sole mechanism. Embryo transfer experiments, in which oocyte fertilisation was not an experimental variable, have shown that transfer of embryos to recipient female rats made pseudopregnant through mechanical stimulation of the cervix results in inferior pregnancy rates when compared to transfers performed on females made pseudopregnant by mating with vasectomised males (Vickery *et al.* 1969). Since mechanical induction of pseudopregnancy is comparably effective at replicating the hormonal profiles of early pregnancy, it can be concluded that the superior embryo survival observed in females mated to vasectomised males is likely to be mediated by exposure to seminal plasma. A study comparing early embryo implantation and survival rates in pregnancies sired by intact and accessory sex gland-deficient hamsters revealed that despite equal fertilisation rates, a large increase in early embryonic loss occurred 72 hours after mating in the females not exposed to seminal plasma (46.9 v 9.4%). As a result, only 31.6% of embryos survived to implant by day 5 of pregnancy, compared with 68.2% in the intact mating group (O *et al.* 1988). These observations are consistent with the experimental findings described in 6.2.1, where all of the fetuses sired by seminal vesicle-deficient CBA males were lost shortly after implantation.

Since seminal vesicle secretions have been shown to contain large amounts of TGFβ, it was postulated that the decline in reproductive outcome seen in pregnancies sired by seminal vesicle-deficient males may be the result of a lack of exposure to TGFβ. As TGFβ has no effect on sperm motility nor sperm penetration of zona-free hamster eggs (Naz and Kumar 1991), TGFβ-mediated changes in sperm activity are unlikely to be responsible for the decline in fertility seen in seminal vesicle-deficient mice. An alternative mechanism may involve the effect of TGFβ on early embryo growth and attachment. Murine embryos express receptors for TGFβ from the 8-cell stage of development (Paria *et al.* 1992, Roelen *et al.* 1994). Addition of TGFβ to culture medium has been reported to accelerate the development of murine pre-implantation embryos (Paria and Dey 1990, Lim *et al.* 1993) and increase production of oncofetal fibronectin (Feinberg *et al.* 1991, 1994), a protein reported to assist attachment of the pre-implantation embryo to the uterine epithelium. While it is possible that seminal vesicle-derived TGFβ may influence early murine embryonic development directly, it is unlikely to do so *in vivo,*

since the pre-implantation embryo does not leave the oviduct until day 4 of pregnancy (Hogan 1986). Seminal plasma proteins do not reach the oviduct, and have only been identified within the rat uterus up to eight hours after mating (Carballada and Esponda 1997), making it unlikely that seminal vesicle derived-TGFβ would persist within the uterus for long enough to have a direct influence on embryo development.

An effect of seminal plasma TGFβ on embryo implantation and development may occur through the generation of the post-mating inflammatory reaction. The inflammatory cells which infiltrate the uterus after mating may influence endometrial events such as angiogenesis and degradation of the ECM, which in turn may influence placental development after implantation. Migration of leukocytes into the endometrium may also favour the generation of a permissive immune response towards the semi-allogenic fetus (Robertson *et al.* 1997), a topic that will be discussed further in Chapter 7.

Despite identifying several mechanisms by which TGFβ may influence early murine embryonic development, these mechanisms presently remain speculative because the experiment designed to test this hypothesis was found to have major technical flaws. Dilation of the murine cervix, together with intrauterine injection of PBS, resulted in a decrease in the pregnancy rate and litter size. A similar detrimental effect has been reported following cervical dilation and intrauterine injection of fluid during early equine pregnancy (Weber 1996). It is possible that an inflammatory reaction produced by mechanical dilation of the cervix may have interfered with early embryo development. Therefore, it is impossible to draw any firm conclusions on the effect of intrauterine TGFβ instillation on murine reproduction from the current set of experiments.

The studies described in this chapter have confirmed previous reports that pregnancies sired by seminal vesicle-deficient males have an inferior outcome compared to pregnancies sired by intact males. The main cause for this reduction in pregnancy success appears to be a decline in fertilisation, possibly related to a reduction in sperm motility. However, an increase in mid-term fetal resorption may also play a role in the reproductive failure of seminal vesicle-deficient CBA males.

Using TGFβ knock-out mice on a SCID background, the role of seminal vesicle TGFβ in murine reproduction may soon be elucidated. These knock-out mice do not die from multi-focal inflammatory disease before reaching sexual maturity (Diebold *et al.* 1995), unlike their immuno-competent relatives, and therefore may provide a useful experimental model for elucidating the role that TGFβ plays in the establishment of

murine pregnancy, without the confounding variables produced by trans-cervical cannulation.

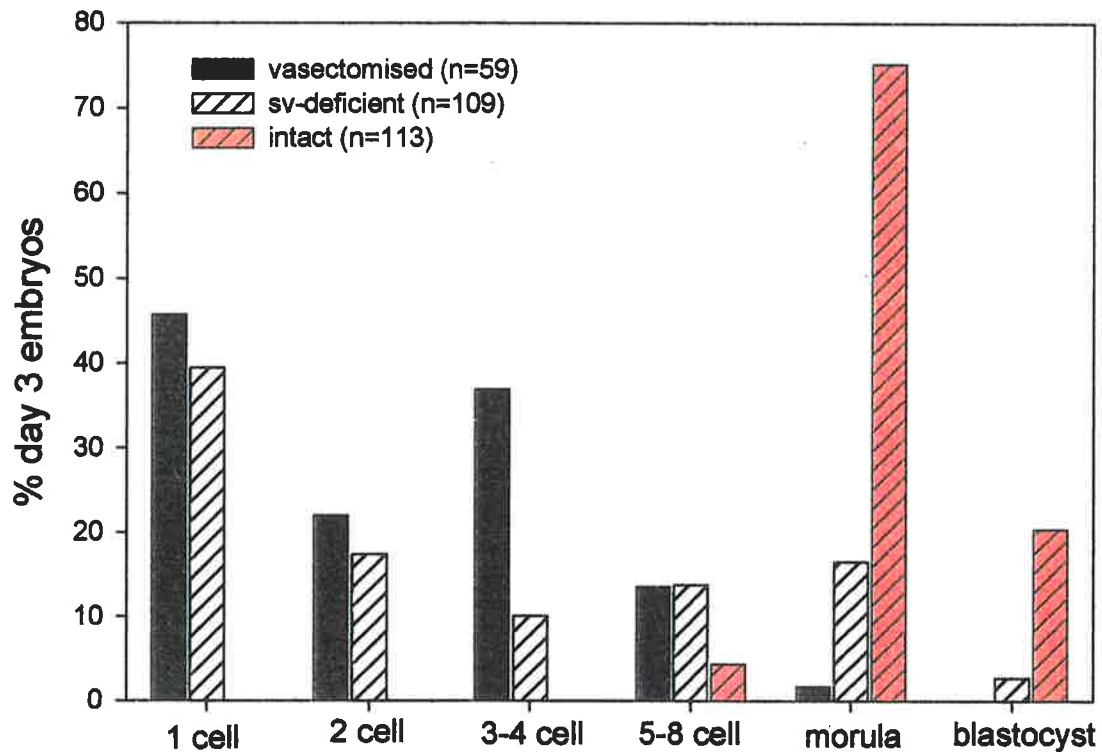


Figure 6.1 The pre-implantation development of Balb/c F1 x CBA embryos *in vivo*. Balb/c F1 females were mated with intact, vasectomised and seminal vesicle-deficient CBA males. *In vivo* development was assessed by flushing embryos from the uterine cavity at 1600 hrs on day 3 of pregnancy, followed by an assessment of their morphology.

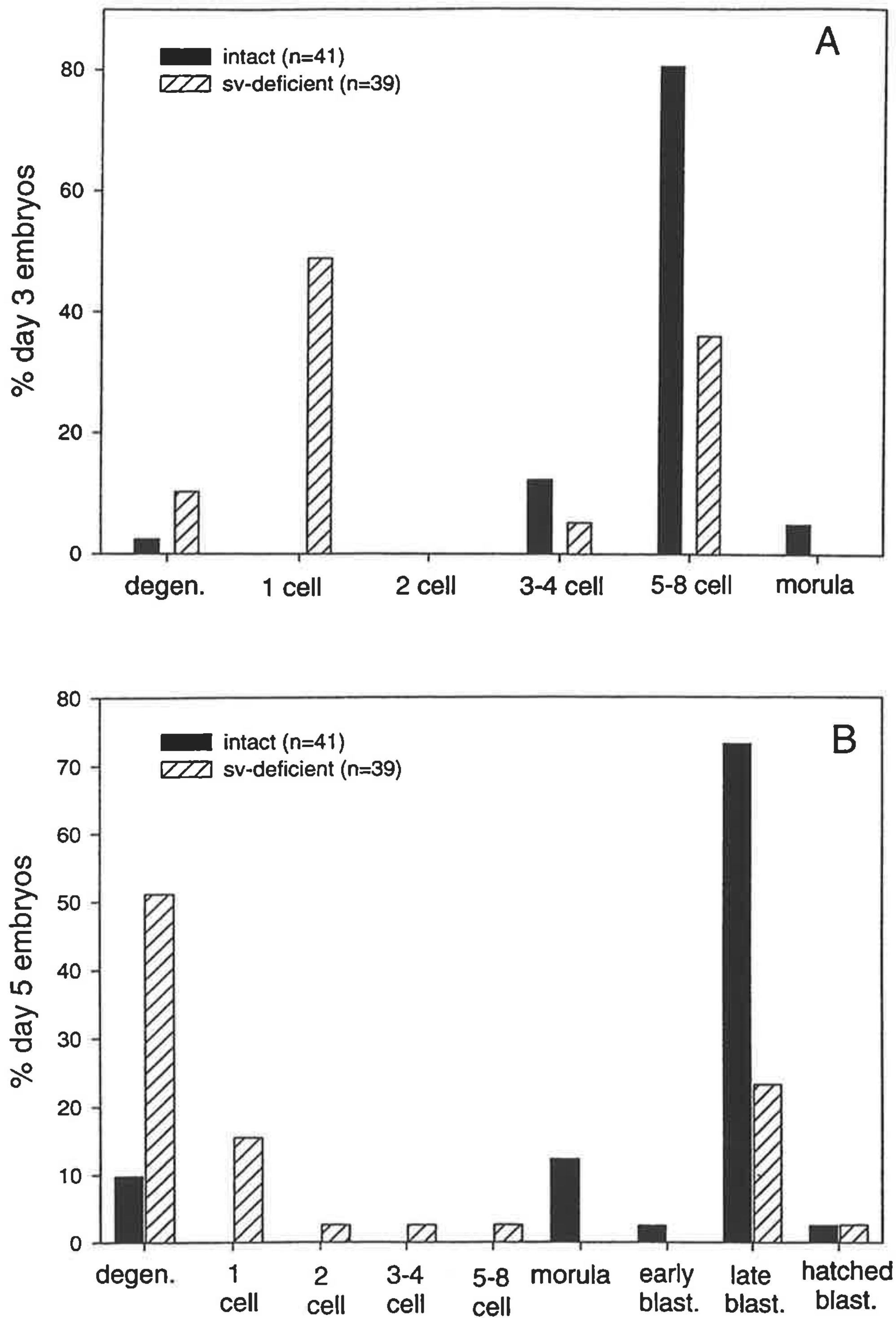


Figure 6.2 The development of Balb/c F1 x CBA embryos *in vitro*. Balb/c F1 females were mated with intact, vasectomised and seminal vesicle-deficient CBA males. Oviducts were flushed at 1400 hrs on day 2 of pregnancy and the resulting embryos placed into culture. Embryonic development was then scored at 1000 hrs on day 3- 6 of pregnancy, with the day 3 (A) and day 5 (B) results graphed.

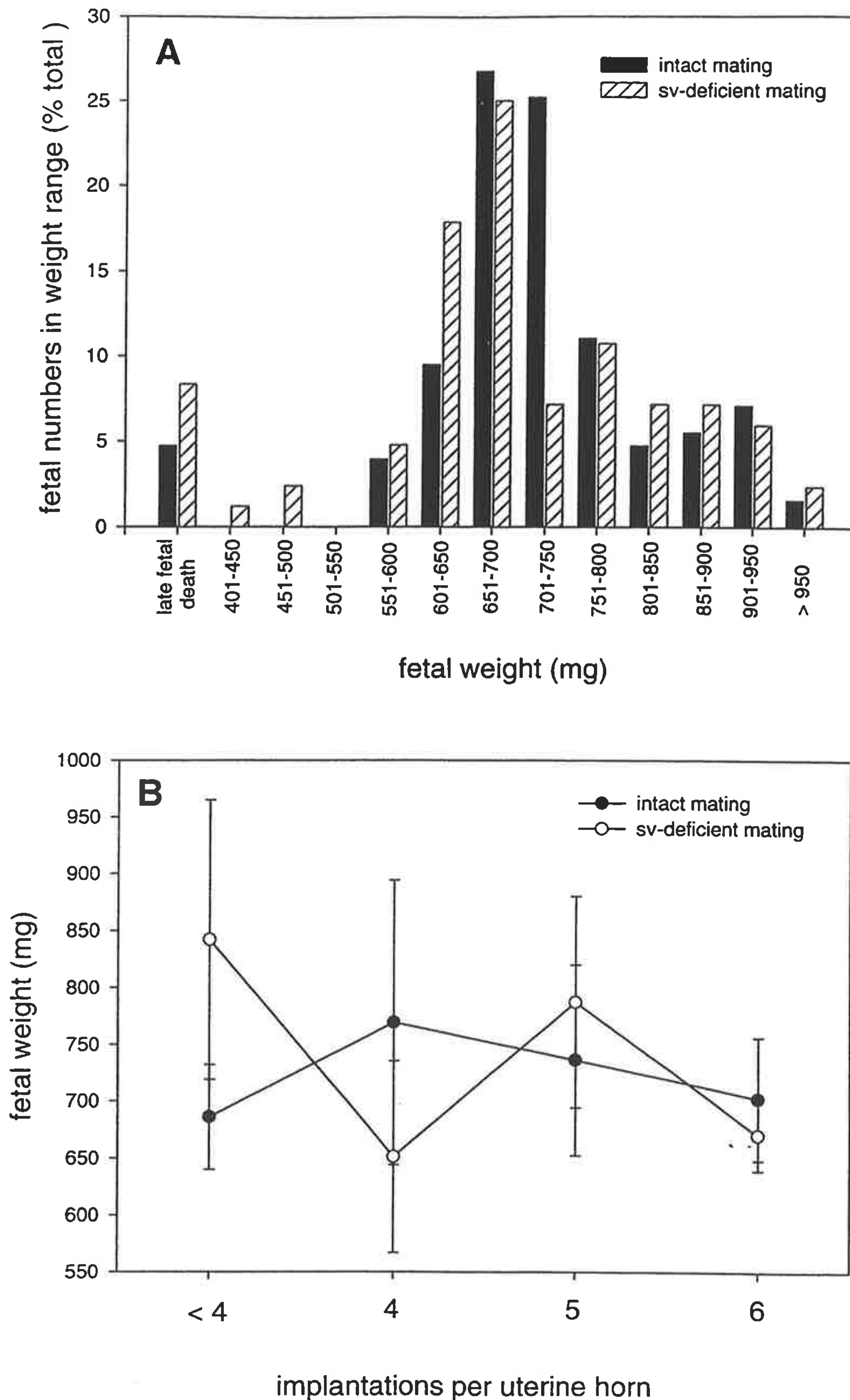
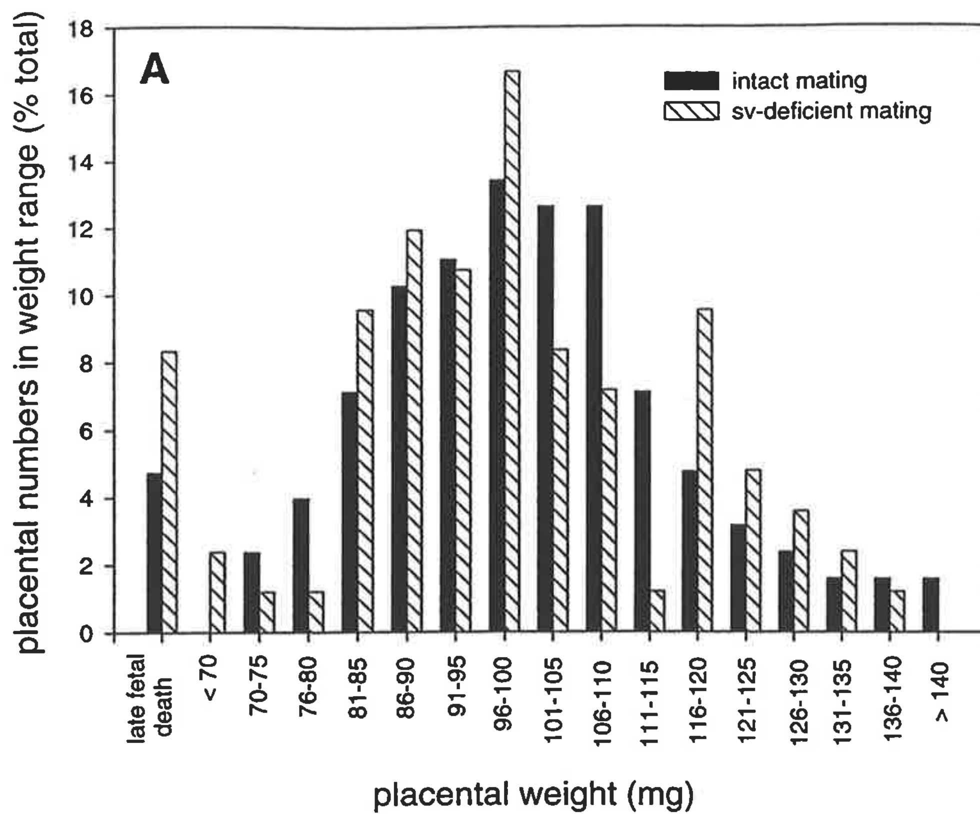


Figure 6.3 The effect of seminal vesicle secretions on fetal weight. Balb/c F1 females were mated with intact or seminal vesicle-deficient CBA F1 males and the fetuses weighed on day 17 of pregnancy. No significant difference in fetal weight distribution was seen between litters sired by intact or seminal vesicle-deficient males (A). Analysis of fetal weight according to the number of implantations per uterine horn did not reveal any significant differences between the two groups (B, ANOVA p= 0.093).



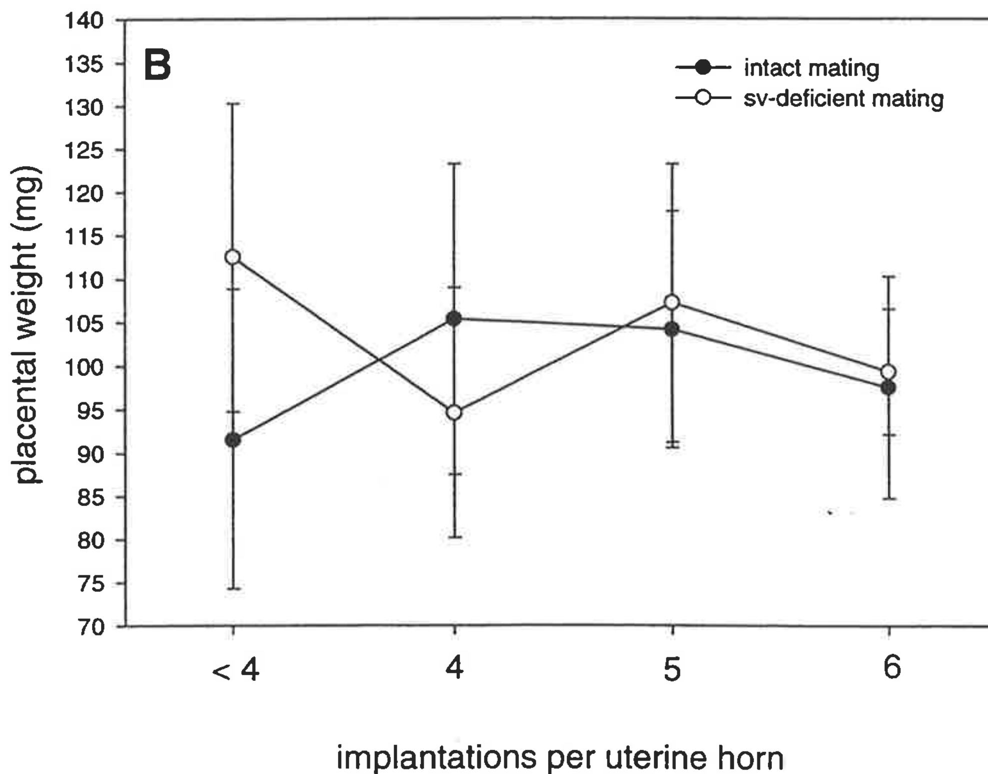
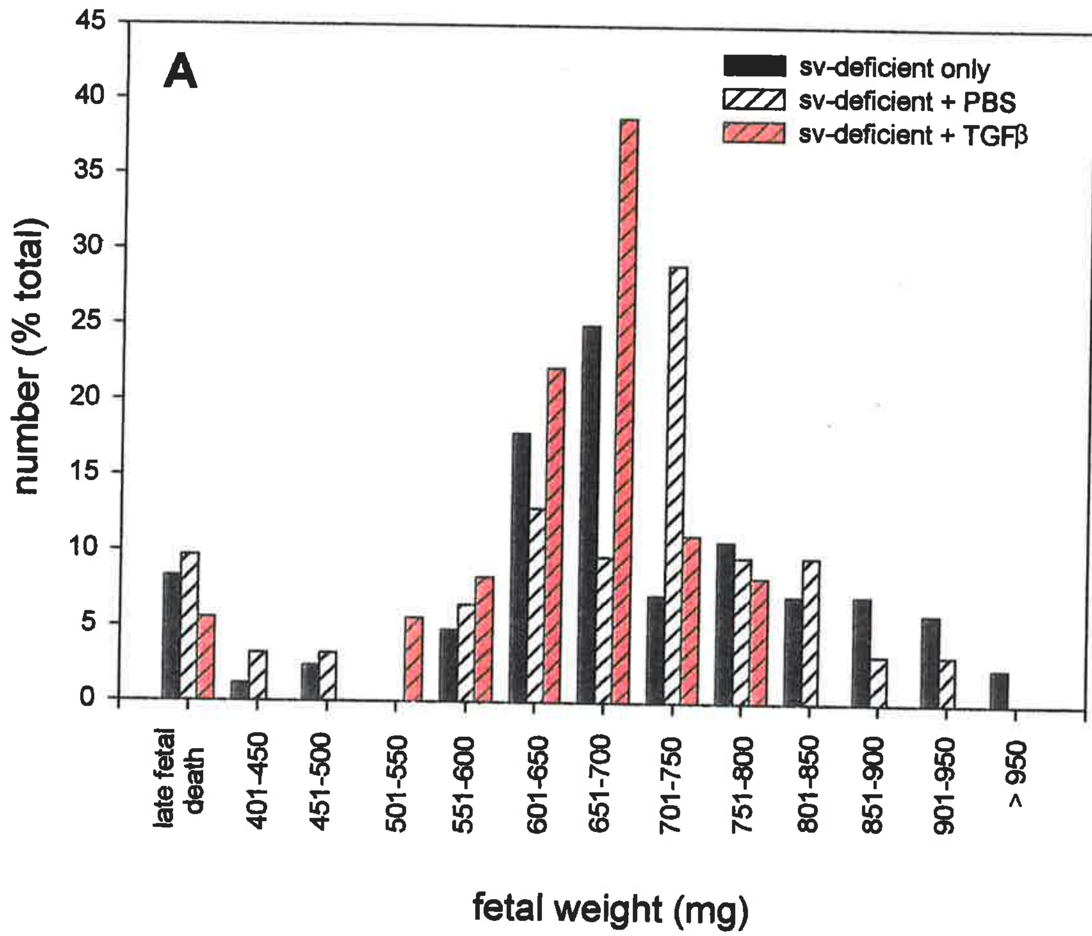


Figure 6.4 The effect of seminal vesicle secretions on placental weight. Balb/c F1 females were mated with intact or seminal vesicle-deficient CBA F1 males and the placentae weighed on day 17 of pregnancy. No significant difference in placental weight was observed between litters sired by intact or seminal vesicle-deficient males (A). Analysis of placental weight according to the number of implantations per uterine horn did not reveal any significant differences between the two groups (B, ANOVA p= 0.472).

Figure 6.5 The effect of intra-uterine TGFβ_1 supplementation on fetal weight. Balb/c F1 females were mated with seminal vesicle deficient CBA males. Some of these females then received an intra-uterine injection of PBS or PBS containing 20 ng of rTGFβ_1 12-14 hours after mating. All females were sacrificed on day 17 of pregnancy to enable fetal weighing. TGFβ_1 supplementation produced a significant decrease in mean fetal weight compared to PBS supplementation or untreated females (A). No significant differences were apparent when comparing the PBS and TGFβ_1 supplemented groups once differences in litter sizes between the two groups were accounted for (B).


A
sv-deficient only
sv-deficient + PBS
sv-deficient + TGFβ
number (% total)
45
40
35
30
25
20
15
10
5
0
late fetal death
401-450
451-500
501-550
551-600
601-650
651-700
701-750
751-800
801-850
851-900
901-950
> 950
fetal weight (mg)

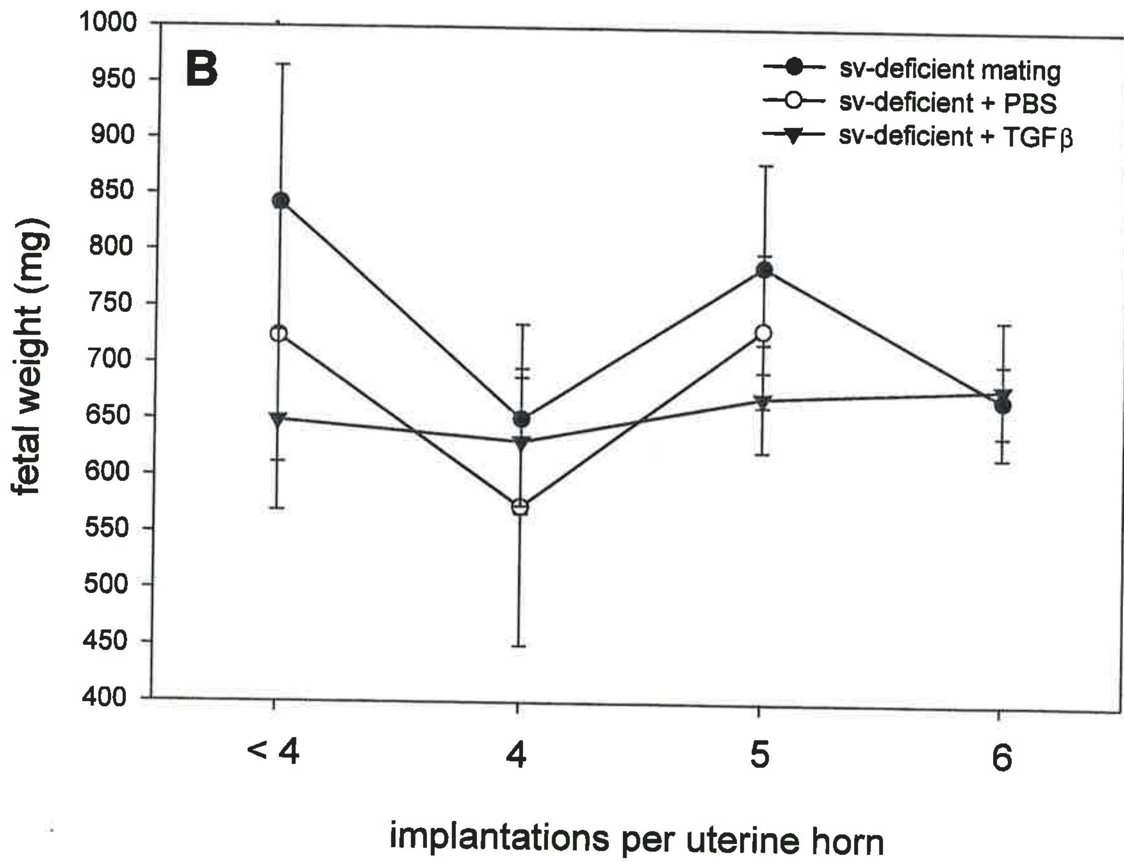
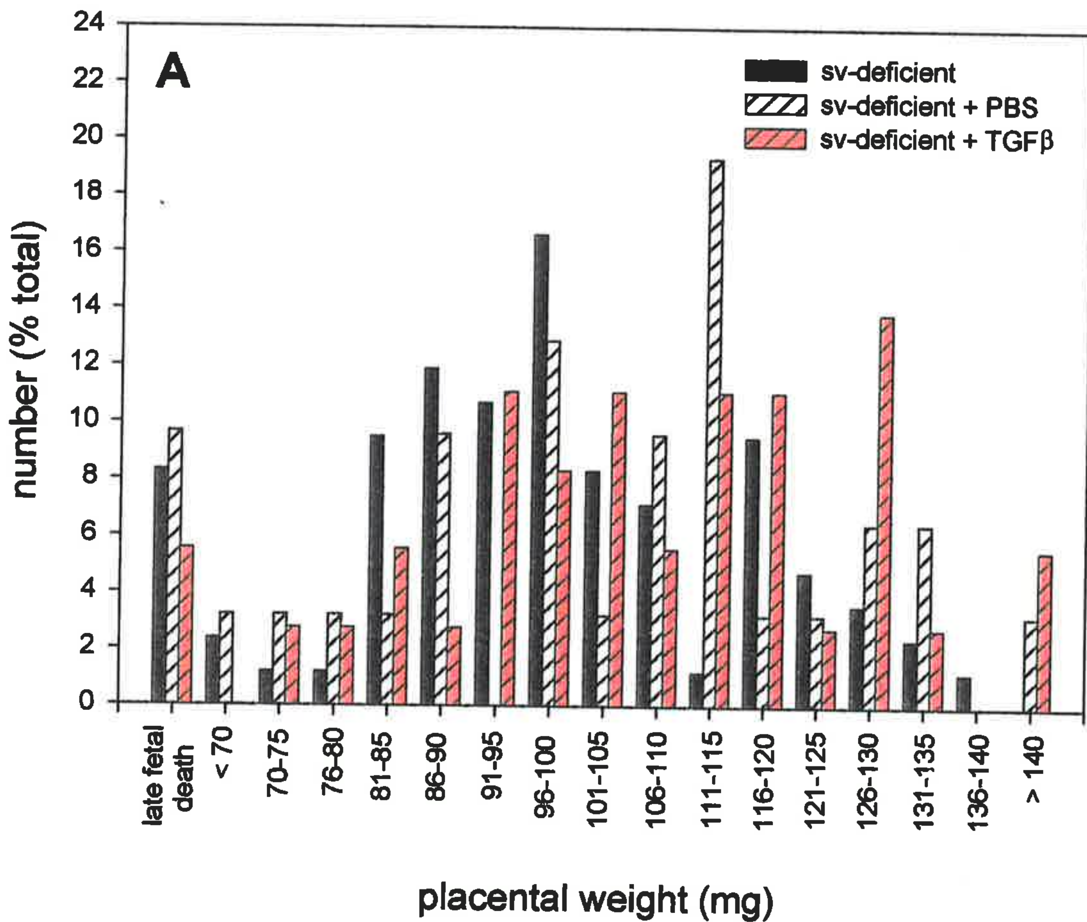
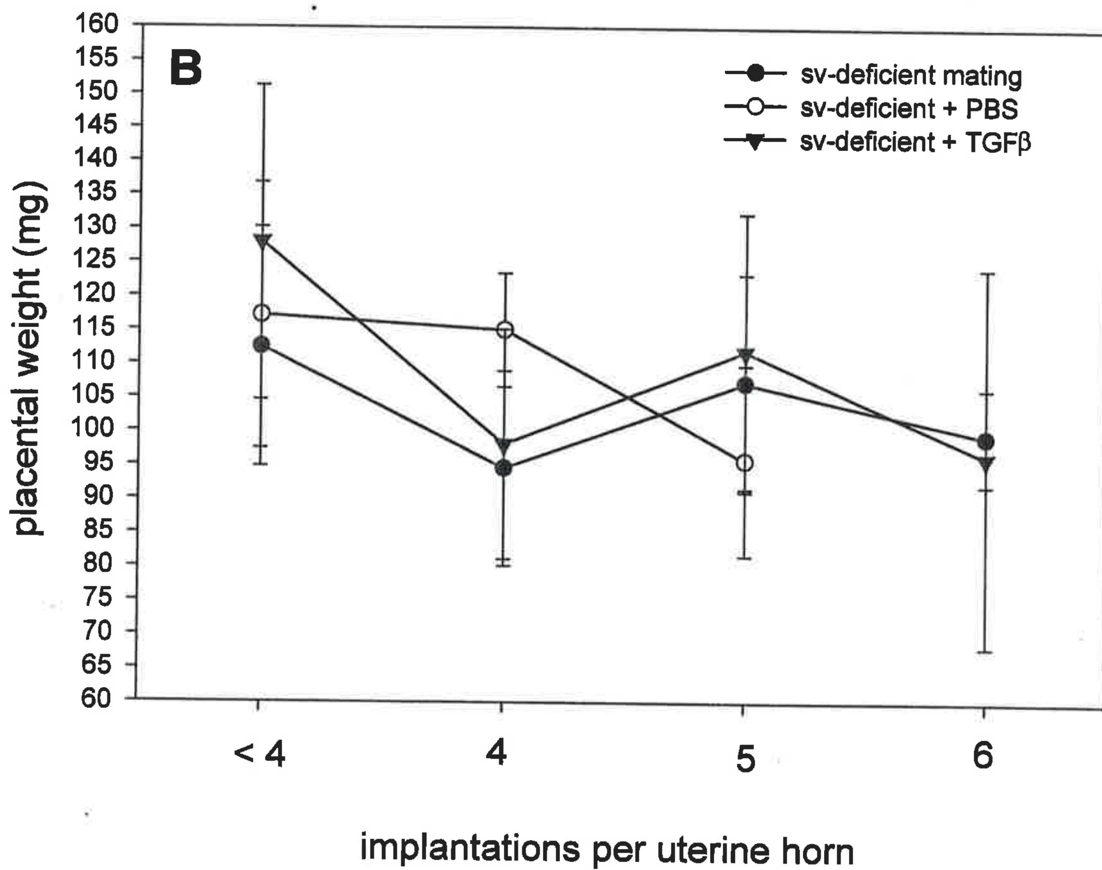

B
sv-deficient mating
sv-deficient + PBS
sv-deficient + TGFβ
fetal weight (mg)
1000
950
900
850
800
750
700
650
600
550
500
450
400
< 4
4
5
6
implantations per uterine horn

Figure 6.6 The effect of intrauterine TGFβ_1 supplementation on placental weight. Balb/c F1 females were mated with seminal vesicle-deficient CBA males. Some of these females then received an intrauterine injection of PBS or PBS containing 20 ng of rTGFβ_1 12-14 hours after mating. All females were sacrificed on day 17 of pregnancy to enable placental weighing. TGFβ_1 supplementation produced a significant increase in mean placental weight compared to the non-supplemented females (A), but this difference was not apparent once differences in litter sizes between the three groups were accounted for using covarient ANOVA analysis (B).


A
sv-deficient
sv-deficient + PBS
sv-deficient + TGFβ
number (% total)
placental weight (mg)
late fetal death
< 70
70-75
76-80
81-85
86-90
91-95
96-100
101-105
106-110
111-115
116-120
121-125
126-130
131-135
136-140
> 140


B
sv-deficient mating
sv-deficient + PBS
sv-deficient + TGFβ
placental weight (mg)
implantations per uterine horn
< 4
4
5
6

Chapter 7

The role of TGFβ in maternal immune deviation during early pregnancy

7.1 Introduction

The concept that semen exposure may provide a "paternal antigenic inoculum" was first mooted by Breyere and Barrett (1960) when it was observed that female rats developed immunological awareness towards paternal antigens after mating, which in turn rendered them incapable of rejecting paternal skin allografts from these males. Surprisingly, this immuno-protective response was also observed in mated tubal-ligated females, thereby indicating that semen exposure, not pregnancy itself, was responsible for generating paternal antigen immune deviation (Lengerova *et al.* 1963). The inability of mated females to reject a foreign graft was viewed as analogous to successful pregnancy, in which maternal rejection of the semi-allogenic conceptus is inhibited. These early studies prompted a series of pioneering experiments in which intrauterine immunisation of female rats with paternal cells resulted in an increase in litter size and fetal-placental weight in all subsequent pregnancies sired by males sharing the same MHC antigens as the initial donor (Beer and Billingham 1974, Beer *et al.* 1975). The mechanism responsible for this improvement in reproductive outcome was unknown, however the absence of a reproductive advantage following removal of lymph nodes draining the uterus, or immunisation with cells antigenically disparate to the prospective father, implies that a maternal immune response towards paternal antigens must be responsible.

An immune response towards paternal ejaculate antigens can be beneficial or detrimental to pregnancy outcome, depending upon the type of response. Previous investigators have observed that Th1(cell-mediated) immune responses against paternal antigens are detrimental to the survival of the semi-allogenic conceptus, while Th2 (humoral) immune responses are beneficial (Wegmann *et al.* 1993, Raghupathy and Tangri 1996). It has been suggested that seminal plasma may play a role in initiating Th2 immune

deviation towards paternal antigens, since seminal plasma is reported to suppress Th1 delayed-type hypersensitivity (DTH) responses towards foreign antigens administered to the murine vagina (Lee and Ha 1989), and is capable of generating a Th2 bias in cytokine production from lymphocytes exposed to seminal plasma *in vitro* (Kelly *et al.* 1997a).

The principal aim of the studies described in this chapter was to investigate whether $TGF\beta_1$ may be one of the components of seminal plasma that plays a role in initiation of the Th2 immune response towards sperm antigens. This hypothesis is supported by the observation that TGFβ is a potent Th2 immune-deviating agent in other tissues (Wilbanks and Streilen 1992). In addition, experiments investigating the effect of intrauterine immunisation with sperm plus $TGF\beta_1$ on subsequent pregnancy outcome were performed.

7.2 Delayed-type hypersensitivity response to sperm antigens

Delayed-type hypersensitivity (DTH) is an *in vivo* Th1 immune response mediated by CD4+ Th1 lymphocytes (Vadas *et al.* 1976). It is characterised by a delayed inflammatory reaction (infiltration of mononuclear leukocytes peaking about 24 hours after injection) at the site of antigenic challenge. Such cellular reactions are typical of Th1 immune responses (Cher and Mosmann 1987), and play a central role in immunological processes such as allograft rejection and resistance to tumour growth (Crowle 1975).

The most commonly used technique for assessment of DTH is the measurement of footpad swelling 24 hours after injection of antigen into the hind footpad of an immunised animal (Miller and Jenkins 1988). In this series of experiments, 5×10^6 CBA sperm plus 20 ng of $rTGF\beta_1$ or control medium was injected into the uterine lumen of Balb/c F1 females on two occasions separated by four weeks. Two weeks after the second immunisation, the DTH response to sperm antigens was assessed in these mice and a third group of non-immunised virgin females, by injecting a suspension of CBA sperm (25 μl per footpad, 1×10^8 sperm / ml) into the left hind footpad and measuring the change in footpad thickness 24 hours later. A fourth group of mice (positive control) were immunised on two occasions by subcuticular injection of CBA sperm in Freund's complete adjuvant (CFA) to produce a strong DTH response. A fifth group of mice were ligated at the uterine-oviduct junction before mating with CBA males. The results of these experiments are summarised graphically in Figure 7.2a.

As expected, virgin mice generated a weak DTH reaction following challenge with sperm, since they had not been previously exposed to these antigens. In contrast, mice sensitised to sperm in CFA produced a marked DTH response upon re-challenge, confirming that epididymal-derived CBA sperm are capable of eliciting a cell-mediated immune response in Balb/c F1 mice. Females sensitised by intrauterine injection of sperm produced a DTH response upon subsequent footpad challenge that was significantly greater than that seen in the virgin controls, with the magnitude of this response being similar to that seen in the group sensitised through subcuticular immunisation. This result confirmed that the uterus can act as an inductive site for cell-mediated immune reactions. However, the key finding was that intrauterine delivery of sperm in combination with $TGF\beta_1$ resulted in a relative inhibition of the DTH response to sperm upon subsequent footpad challenge. This result indicates that $TGF\beta_1$ has the capacity to diminish the induction of DTH responses against sperm antigens. Naturally mated females exhibited a DTH response to sperm that was significantly less than that seen following intrauterine immunisation with epididymal sperm alone, but not significantly different from that observed following immunisation with sperm in combination with $TGF\beta_1$, implying that seminal plasma must also have the capacity to dampen the induction of DTH immune responses to vaginally administered antigens.

In addition to measuring footpad thickness, an assessment of footpad weight was performed, since this has been reported to be a more objective assessment of DTH responses (Kitamura 1980). Pre-challenge weight of the left and right hind footpads did not differ significantly for individual mice, hence comparison of left hind footpad weight (injected with sperm suspension) with right hind footpad weight (injected with control medium) enabled an assessment of DTH-mediated regional inflammation. The results of this analysis, depicted in Figure 7.2b, shows similar trends to that described for the footpad thickness, although these trends were less pronounced. This probably reflects the fact that a large portion of total footpad weight is not subject to DTH-mediated oedema (bone, connective tissue), whereas the low density tissue in the distal footpad (where thickness measurements are taken) is the principal site at which DTH oedema occurs.

7.3 Humoral immune response to sperm antigens

Deviation of the immune system towards a Th2 response results in the production of large amounts of IL-4 and IL-5, which promote antibody production ahead of cell-mediated immunity (Mosmann and Coffman 1989). In addition, Th2 immune responses are characterised by a shift in antibody iso-type profiles characterised by an increase in IgG1 and a decrease in IgG2a and IgG2b production (Finkelman *et al.* 1990).

In order to assess the ability of TGFβ_1 to modify the humoral immune response to sperm antigens, serum was collected from Balb/c F1 females following intrauterine inoculation with epididymal-derived CBA sperm (in combination with 20 ng of rTGFβ_1 or PBS), following natural mating (tubal-ligated) or after s/c immunisation with sperm plus CFA. The resulting antibody response was compared to that seen in virgin non-immunised females. The protocol for sperm immunisation was identical to that used in the previous DTH experiments, with serum being collected 14 days after the second immunisation. Antibody responses were assessed using a modification of a previously described ELISA assay (Okada *et al.* 1993) in which iso-type specific secondary antibodies were substituted to enable measurement of antibody iso-type responses. Three different secondary antibodies specific for murine IgG1, IgG2a and IgG2b were used. No anti-sperm IgG2a was detected in any of the experimental animals and therefore the results focus on IgG1 and IgG2b titres.

To confirm that the ELISA was specific for sperm antibodies, the ability of purified CBA sperm to absorb sperm antibodies from test sera was examined. Purified sperm was collected by a combination of centrifugation and "swim up", before being incubated with serum from virgin or naturally mated mice. This resulted in a significant fall in anti-sperm IgG2b titres in both virgin and naturally mated sera (Figure 7.1), thereby confirming that the antibodies measured by this ELISA were predominantly directed against sperm.

IgG1 anti-sperm antibody titres from the various experimental groups are graphically summarised in Figure 7.3a. Serum from virgin females contained very low or undetectable levels of anti-sperm IgG1. While the presence of any anti-sperm antibody in "naive" animals seems counter-intuitive, other investigators have reported the presence of "natural" anti-sperm antibodies in the serum of virgin females (Edwards 1960, Hancock 1975). It has been suggested that a physiological role for these antibodies may be to mask potentially antigenic sites on sperm. No significant differences in anti-sperm IgG1 titre

were apparent between virgin, sperm or sperm plus TGFβ_1 immunised groups, all yielding very low IgG1 titres. The lack of a relative increase in anti-sperm IgG1 production following immunisation with sperm plus TGFβ_1 is still consistent with a Th2 response, since other investigators have reported a decrease in IgG2b, with no increase in IgG1, following the initiation of a Th2 immune response (Brown *et al.* 1995, Myers *et al.* 1997). Interestingly, a significant increase in anti-sperm IgG1 was observed in mated females compared to the virgin controls.

The IgG2b antibody response to sperm was far greater than that observed for IgG1 (Figure7.3). The intrauterine immunisation of females with sperm in combination with TGFβ_1 produced a significant two to four fold decrease in IgG2b titres compared to immunisation with sperm alone. Since no significant differences in IgG1 production existed between these two experimental groups, it is possible that TGFβ_1 selectively inhibits IgG2b production.

7.4 Effect of sperm immunisation on subsequent reproductive performance

Immunisation of female mice (intrauterine or intra-peritoneal) with cells from a prospective father can result in an increase in litter size and fetal and placental weight (James 1965, Beer and Billingham 1974, Beer *et al.* 1975). Seminal vesicle-derived TGFβ_1 may initiate Th2 immune deviation towards paternal ejaculate antigens, which in turn may benefit the growth and survival of the semi-allogenic conceptus. To test this hypothesis, Balb/c F1 females were immunised with epididymal CBA sperm in combination with either 20 ng of rTGFβ_1 or control medium, then mated two weeks later with CBA males. Immunisation of females with sperm did not result in pregnancy because immunisation was delayed until 1500 hrs on the day of oestrous, at which stage fertilisation of oocytes is no longer possible (Sakai and Endo 1988). On day 17 of pregnancy, these females and a group of non-immunised controls were sacrificed to assess fetal numbers, viability and fetal and placental weights. The results are summarised in Table 7.1 and illustrated in Figures 7.4-7.6.

Immunisation of females with sperm, with or without TGFβ_1, produced a significant decline in mean litter size of approximately one pup per litter. Earlier investigators have reported a similar decline in litter size following immunisation of

females with paternal antigen-bearing cells (Clarke 1971, Hetherington *et al.* 1976). It is unclear

Table 7.1 The effect of intrauterine sperm immunisation on Balb/c F1 reproductive outcome

	non-immunised controls	sperm + TGFβ_1-immunised	sperm-immunised
total fetal numbers	139	144	103
total litter size (viable + non-viable)	11.4 ± 1.0^a	10.4 ± 1.2^b	10.3 ± 0.9^b
number viable pups per litter	11.2 ± 1.3^a	10.1 ± 1.5^b	10.1 ± 0.9^b
number resorptions per litter	0.17 ± 0.58^a	0.21 ± 0.58^a	0.20 ± 0.42^a
fetal weight (mg)	645.2 ± 61.2^a	677.6 ± 56.6^b	646.1 ± 49.9^a
placental weight (mg)	97.7 ± 12.1^a	105.2 ± 12.4^b	101.8 ± 9.8^b
fetal: placental ratio	6.7 ± 0.9^a	6.5 ± 0.8^{ab}	6.4 ± 0.8^b

Values are mean ± s.d., unless otherwise stated. All data was initially analysed by a Kruskal Wallis one-way ANOVA, followed by a students t test if significant differences existed between the immunised groups. Different superscripts indicate significant differences ($p<0.05$) between groups as assessed by t tests.

why immunised females produced slightly smaller litters than their non-immunised controls, since no significant difference in the rate of late fetal reabsorption was observed. The decline in litter size may therefore result from either a small decrease in the ovulation or fertilisation rate, or an increase in early fetal loss. Immunisation of mice with epididymal sperm has been reported to generate anti-sperm antibodies (Tung *et al.* 1979) (as was confirmed by the current set of experiments). It is possible that these antibodies may have interfered with sperm motility or sperm-oocyte interaction, thereby reducing fertilisation, and creating a smaller litter size. This adverse antibody response may not

occur at the time of natural mating, perhaps because of the presence of additional immuno-suppressive seminal plasma constituents such as prostaglandin E (Kelly 1995).

Despite this, analysis of fetal and placental weight by simple-factorial ANOVA revealed that immunisation status had a significant beneficial effect on both fetal and placental weight, even after accounting for differences in litter size. Immunisation of females with sperm in combination with $TGF\beta_1$ resulted in a 5% increase in mean fetal weight compared to all other experimental groups ($p<0.001$, Table 7.1). When the weights of individual fetuses from the three study groups are plotted as a histogram (Figure 7.4), it is apparent that immunisation with sperm plus $TGF\beta_1$ produces a weight advantage for all litter members, not just those of low weight, since the entire distribution curve is skewed to the right. Analysis of the mean placental weight (Table7.1, Figure 7.5) reveals that sperm and sperm plus $TGF\beta_1$ immunised groups exhibited a significant increase in mean placental weight compared to the non-immunised controls ($p< 0.001$ and $p=0.005$ respectively). This increase was more pronounced in the sperm plus $TGF\beta_1$ immunised group, however it did not quite reach statistical significance ($p= 0.072$).

Litter size is known to be a variable affecting both fetal and placental weight, with heavier fetuses and placentae occurring in small litters due to reduced competition for nutrients. It is therefore possible that the increase in fetal and placental weights seen following immunisation may simply reflect a reduction in litter size, rather than an immunologically mediated reproductive advantage. To account for differences in litter size, fetal and placental weights were analysed using a simple factorial ANOVA test, with the number of fetuses per uterine horn being analysed as a covariate. This analysis revealed that both immunisation status and fetal numbers had a significant effect on fetal weight, with immunisation status producing a significant effect on fetal weight even after accounting for differences in fetal numbers ($p<0.0001$). An analysis of the effect of immunisation status on placental weight revealed a significant difference between groups ($p< 0.0001$), but fetal numbers did not have a significant influence on placental weight ($p=0.158$).

The effect of immunisation status on fetal and placental weights was also analysed according to the number of implantation sites per uterine horn, as described in Chapter 6. This analysis, graphically depicted in Figure 7.6, clearly shows that immunisation with sperm in combination with $TGF\beta_1$ results in an increase in fetal and placental weight irrespective of litter size.

7.5 Discussion and conclusion

The experiments described in this chapter have identified two novel effects of $TGF\beta_1$. Firstly, it has been shown that intrauterine administration of sperm in combination with $TGF\beta_1$ may skew the immune response to sperm antigens towards the Th2 compartment. Secondly, it has been identified that $TGF\beta_1$ potentiates the beneficial effect of intrauterine injection of sperm, resulting in a greater increase in fetal and placental weight than immunisation with sperm alone. This alteration in reproductive performance is likely to be mediated through effects on the maternal immune system, specifically resulting in the induction of a Th2 immune response to paternal antigens.

The suppression of cell-mediated DTH responses against sperm antigens following mating is consistent with earlier reports suggesting that females were unable to mount a cell-mediated immune response to reject paternal skin grafts once they had been mated with a male of the same strain (Breyere and Barrett 1960, Lengerova *et al.* 1963). The realisation that seminal plasma may be responsible for inhibiting cell-mediated immunity became apparent when Lee and Ha (1989) reported that intra-vaginal delivery of foreign antigens to mice (sheep red blood cells or human sperm), in combination with seminal plasma, resulted in the suppression of DTH immune responses upon subsequent footpad challenge. Various components of seminal plasma such as prostaglandin E, spermidine, and many as yet unidentified factors have been suggested to be responsible for this immunosuppression (Alexander and Anderson 1987, Kelly 1995). The experiments outlined in this chapter indicate that $TGF\beta_1$ may be one important immuno-suppressive compound within seminal plasma, which can prevent the generation of potentially hostile cellular immune responses against paternal antigens introduced at mating.

TGFβ has previously been identified as having the capacity to suppress DTH responses following administration of foreign antigens to the anterior chamber of the eye. The anterior chamber of the eye is an immunologically privileged site in which administration of foreign antigen results in a systemic shift towards Th2 immunity (Streilein 1993, Li *et al.* 1996). This shift is characterised by suppression of DTH responses against the immunising antigen, and an increase in production of non-complement fixing IgG1 antibody - so called "anterior chamber-associated immune deviation" (ACAID). TGFβ is known to be produced in abundance within the anterior chamber of the eye and has been identified as being principally responsible for initiating the Th2 immune deviation seen in ACAID (Wilbanks *et al.* 1992). The ability of TGFβ to

initiate Th2 immune deviation is not peculiar to this site since peritoneal macrophages pulsed *in vitro* with bovine serum albumin (BSA) in combination with TGFβ, then adoptively transferred to naive mice, can also suppress DTH immune responses to later challenge with BSA (Hara *et al.* 1993).

Mucosal surfaces other than the female reproductive tract rely on TGFβ to suppress unwanted, potentially destructive cellular immune responses against benign antigens. Both the lung and the gut are exposed to a wide range of antigens, many of which are not pathogenic (eg food proteins, dust particles), so mechanisms for circumventing cellular immune responses exist in these tissues. Alveolar macrophages are known to produce $TGF\beta_1$, which acts to suppress allogenic T cell priming against antigens presented by lung dendritic cells *in vitro* (Lipscomb *et al.* 1993). TGFβ has also been identified as playing a major role in suppressing colitis-inducing Th1 immune responses in the gut (Powrie *et al.* 1996), and is involved in the induction of oral tolerance to foreign antigens (Miller *et al.* 1992). It is apparent that significant mechanistic parallels may exist at the mucosal surfaces of the uterus, lung and gut in regard to the manner by which TGFβ can modify potentially harmful cellular immune responses to non-pathogenic antigens.

TGFβ has previously been reported to generate a shift in antibody production towards Th2 iso-types (Finkelman *et al.* 1990). Injection of foreign antigens into the TGFβ-rich environment of the anterior chamber of eye initiates the production of non-complement-fixing IgG1, but not complement fixing IgG2 (Wilbanks and Streilein 1990). Administration of sperm to the uterus in combination with $TGF\beta_1$ resulted in a decrease in the production of IgG2b antibody compared to immunisation with sperm alone. This is an important observation, since IgG2b is the most potent complement-fixing antibody isotype and therefore potentially the most destructive humoral immune response that can be generated against paternal antigens (Oi *et al.* 1984). The experiments reported in this chapter are not the first to describe effects of seminal plasma on antibody production, but they are the first to observe a switch to a Th2 antibody isotype profile mediated by seminal $TGF\beta_1$. Anderson and Tarter (1982) reported that seminal plasma has the ability to suppress anti-sperm IgG and IgM production following subcutaneous immunisation, while Head and Smith (1982) reported that allogenic mating diminished the generation of haem-agglutinating antibodies following re-challenge with paternal skin grafts in rats. While neither of these experiments measured antibody iso-types, it is clear that natural exposure to paternal antigens in combination with seminal plasma resulted in a change in

humoral immunity towards these antigens, which was qualitatively similar to that observed in the current set of experiments.

There is abundant evidence that pregnancy is characterised by a Th2 deviation in humoral immunity against paternal antigens (Wegmann *et al.* 1993). It has been reported that anti-paternal antibodies produced during murine pregnancy are mainly of the IgG1 isotype, with very little or no IgG2 production (Bell and Billington 1980). The finding that anti-paternal IgG1 binds to paternal antigens present on the placenta (Voisin 1974) prompted the proposal that production of non-complement fixing antibodies against paternal antigens may actually be beneficial to pregnancy by masking paternal antigens, thereby preventing their recognition by more hostile components of the maternal immune system. While these earlier reports did not investigate the role of semen in the initiation of anti-paternal IgG production, it is possible that presentation of paternal antigens in combination with seminal plasma TGFβ_1 at the time of mating may contribute to the generation of this potentially protective immune response. Indeed, such a mechanism may be advantageous in that a "permissive" maternal immune response would then be in place by the time of implantation.

The moderate increase in fetal and placental weights seen following intrauterine immunisation with sperm or sperm plus TGFβ_1 concur with earlier reports by Beer *et al.* (1975) who showed that immunisation with paternal antigens can increase fetal and placental weights. The current experiments build on these early findings by showing that administration of paternal antigens in combination with TGFβ_1 can result in a more significant increase in fetal and placental weight, compared to immunisation with antigen only. This observation may be explained by two possible mechanisms. Earlier in this thesis it was reported that intrauterine injection of rTGFβ_1 elicits an increase in uterine epithelial cell GM-CSF production, with a resultant influx of antigen-presenting leukocytes into the uterus. It is possible that by increasing the number of activated APCs within the uterus, TGFβ_1 may increase maternal processing of paternal antigens and thereby augment the ensuing immune response towards these antigens. GM-CSF has been identified as a potent biological adjuvant at other anatomical sites (Jones *et al.* 1994). Although the mechanical trauma produced by trans-cervical cannulation, or direct injection at laparotomy (as performed by Beer and Billingham, 1974), may have initiated an inflammatory response within the uterus, this response may have been inferior in magnitude and qualitatively different to that mediated by TGFβ_1. Thus the processing of

paternal antigens in this situation may be diminished compared to that which occurs in the presence of rTGFβ_1, or naturally following mating.

A second mechanism by which TGFβ may potentiate the beneficial effect of paternal antigen immunisation is based on the concept of "immunotrophism." This term describes the situation in which T cell awareness of paternal antigens results in the generation of a Th2 immune response, which in turn promotes fetal and placental growth (Wegmann *et al.* 1993, Chaouat *et al.* 1997). This theory was proposed when it was noted that a reduction in fetal loss could be achieved in the abortion-prone CBA/J x DBA/2J mating combination if the female was immunised with paternal MHC antigens prior to mating (Clark *et al.* 1980, Chaouat *et al.* 1983). More recently it was discovered that immunisation of these abortion-prone females results in a decline in the number of decidual and splenic gammadelta T cells producing abortogenic cytokines such as TNFα and IFNγ, while increasing the number of gammadelta T cells producing anti-abortive IL-10 and TGFβ_2-like molecules (Arck *et al.* 1997). This immunisation mediated shift in gammadelta T cell behaviour from a Th1 to a Th2 pattern is associated with a marked improvement in fetal survival. Thus, although the precise mechanisms remain to be determined, a mating-initiated expansion of Th2 lymphocyte subsets specific for paternal antigens may directly or indirectly result in increased placental growth, in turn facilitating fetal development.

In summary, the results described in this chapter support the proposal that generation of paternal antigen tolerance may be initiated at the time of mating, and this may in turn favour the growth and survival of the semi-allogenic conceptus. Seminal plasma TGFβ_1 increases uterine epithelial cell GM-CSF production, which then results in an influx of antigen-presenting leukocytes (APCs) into the uterus that are capable of processing paternal ejaculate antigens. The processing of antigen in a micro-environment containing high levels of TGFβ is reported to result in an autocrine increase in production of TGFβ_1 and TGFβ_2 by these macrophages (Takeuchi *et al.* 1997). These macrophages may then travel from the uterus to the draining lymph nodes, where they would present paternal antigens to regulatory T cells. Peritoneal macrophages exposed to antigen *in vitro* in the presence of TGFβ_2, then injected into naive recipients, have been reported to trigger a Th2 shift in responding T cells, resulting in the selective suppression of delayed-type hypersensitivity and IgG2a antibody production (Zhang *et al.*1995, Takeuchi *et al.* 1997). Seminal plasma TGFβ may be able to elicit a similar Th2 immune response towards ejaculate antigens that could prevent destructive immune reactions against the semi-

allogenic conceptus. In addition, T or NK cells may directly benefit placental growth, perhaps through the release of cytokines such as CSF-1, GM-CSF and IL-3, which are known to promote trophoblast proliferation and differentiation (Athanassakis *et al.* 1987, Armstrong and Chaouat 1989). An alternative mechanism may lie in the protective effect of non-complement fixing antibodies which may act to mask alloantigens on the surface of trophoblast cells.

In summary, it may be postulated that in addition to providing male gametes necessary for fertilisation, the act of mating may prime the maternal immune system in a manner that promotes growth and survival of the semi-allogenic conceptus.

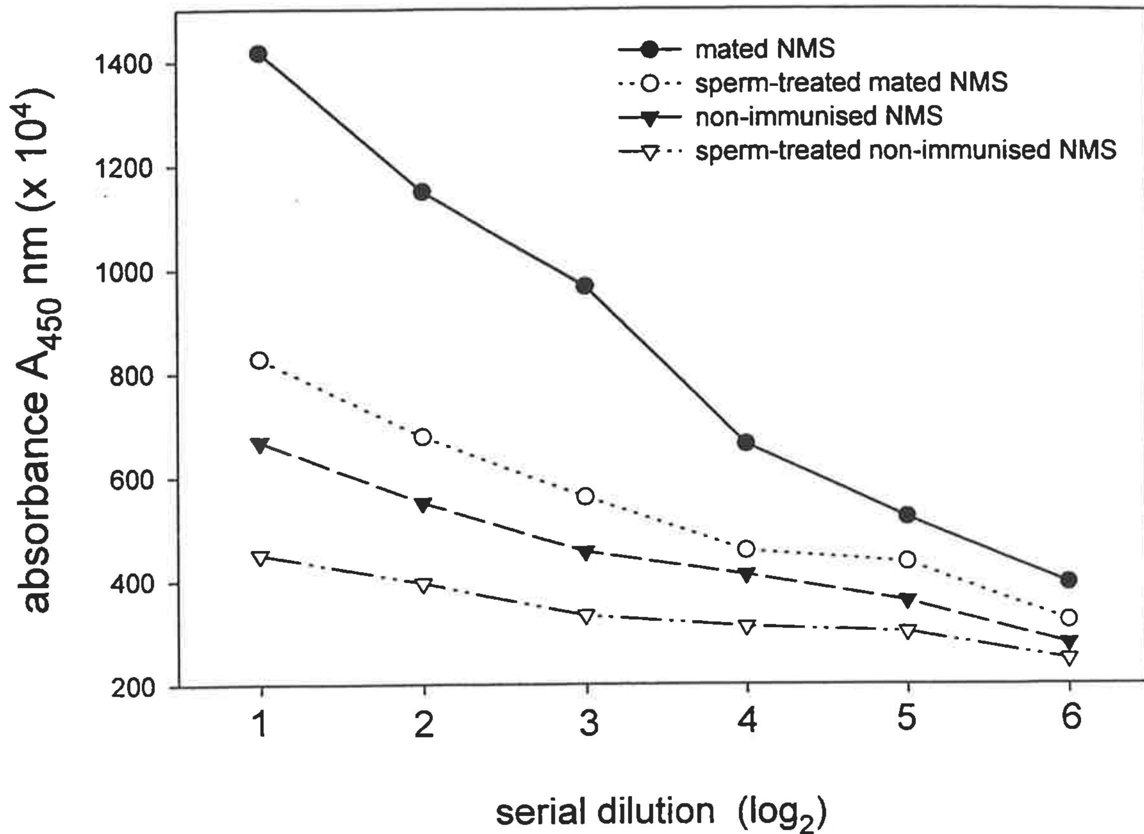


Figure 7.1 Validation of anti-sperm antibody ELISA. To confirm that the ELISA was specific for CBA sperm antibodies, mouse serum obtained from either mated or non-immunised virgin females was absorbed with highly purified CBA sperm. The titre in the remaining NMS was then compared with the untreated matched sera. A significant reduction in titres was observed following sperm incubation, confirming the specificity of the anti-sperm antibody ELISA.

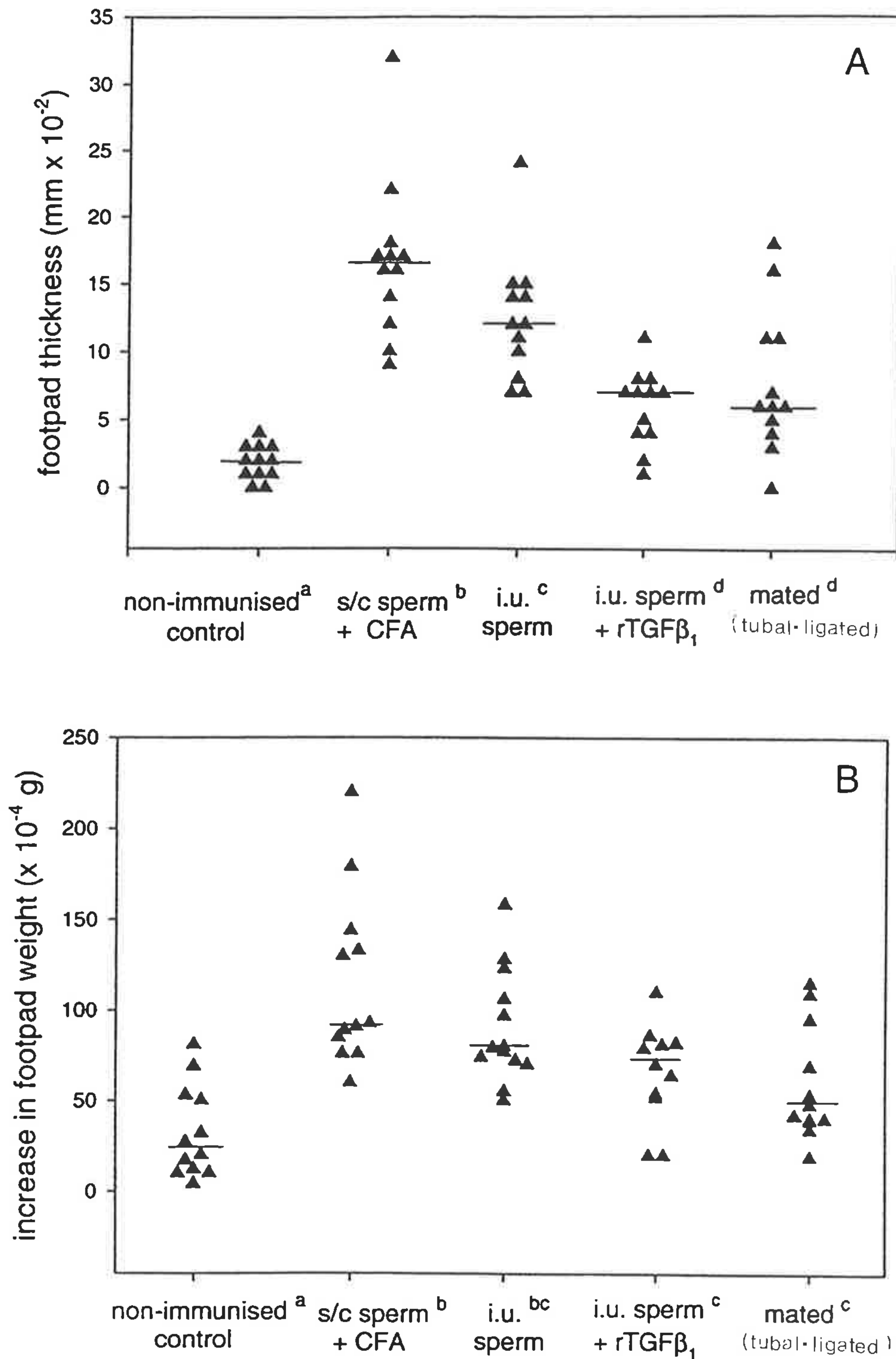


Figure 7.2 DTH response to sperm antigens. Female Balb/c F1 mice were immunised with 5 x 10^6 CBA sperm, as specified on the abscissa, then a DTH response was elicited two weeks later by injecting 8 x 10^6 CBA sperm into the left hind footpad. The magnitude of the DTH response was measured by either an increase in footpad thickness (A) or weight (B) 24 hours post injection. Each symbol represents an individual animal with median values scored. Data was compared by Kruskal Wallis one-way ANOVA ($p< 0.0001$ for both A and B), followed by Mann Whitney rank sum test. Different superscripts denote significant differences between groups ($p<0.05$).

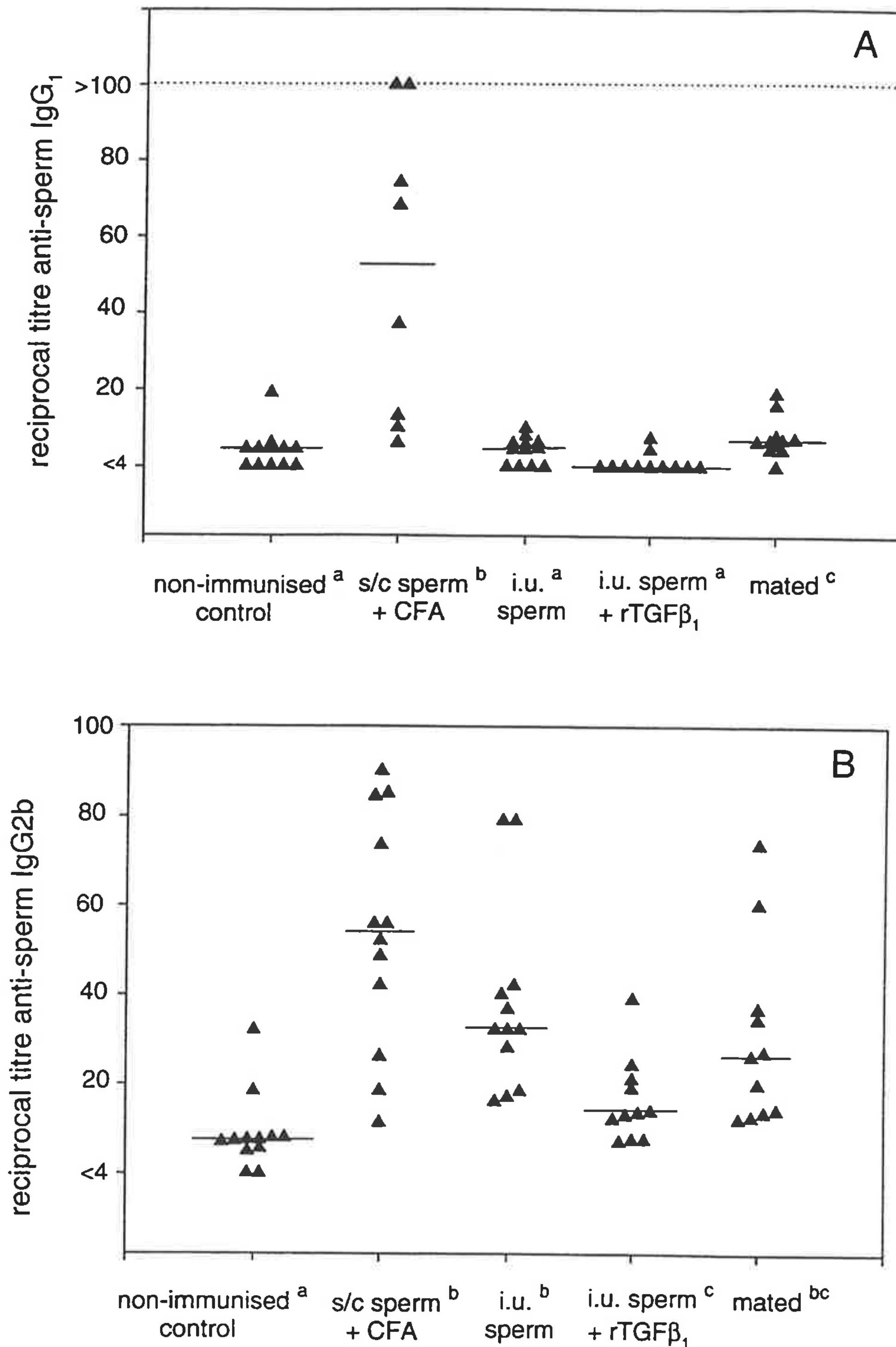


Figure 7.3 Production of anti-sperm antibodies. Female Balb/c F1 mice were immunised with CBA sperm on two separate occasions separated by 4 weeks, as outlined on the abscissa, before collecting serum 2 weeks later for determination of anti-sperm antibody content. Both IgG1 (A) and IgG2b (B) anti-sperm antibodies were measured by ELISA. The symbols represent individual titres and are expressed as the reciprocal titre at which anti-sperm antibody was no longer detectable on a serial dilution series. Data was compared by Kruskal Wallis one-way ANOVA ($p < 0.0001$ for both A and B), followed by Mann Whitney rank sum test. Different superscripts denote significant differences between groups ($p<0.05$).

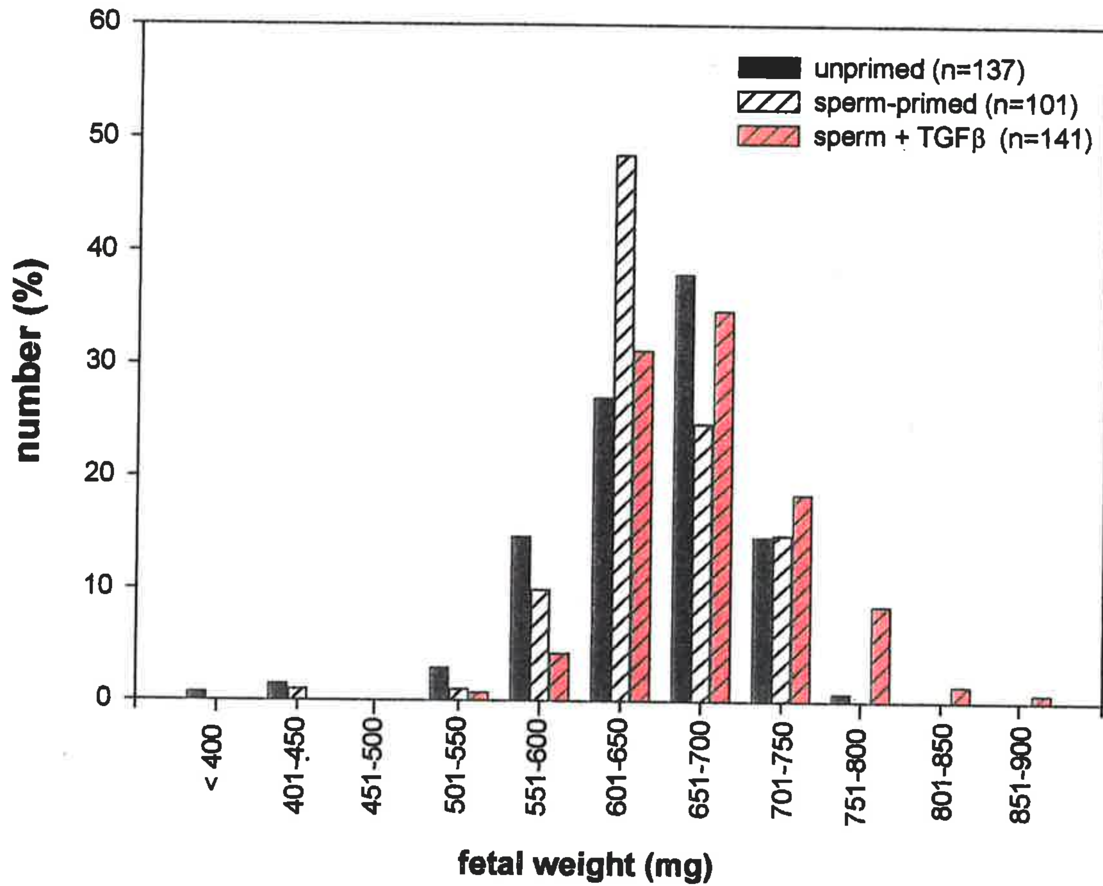


Figure 7.4 The effect of pre-immunisation of females with sperm on fetal weight. Female Balb/c F1 mice were intrauterine immunised with CBA sperm in combination with 20 ng rTGFβ_1 or control medium, then mated with CBA males two weeks later. Pregnant females were sacrificed on day 17 of pregnancy and fetal weights were measured. A significant increase in mean fetal weight was observed in the rTGFβ_1 / sperm immunised mice compared to the other groups (statistics summarised in Table 7.1).

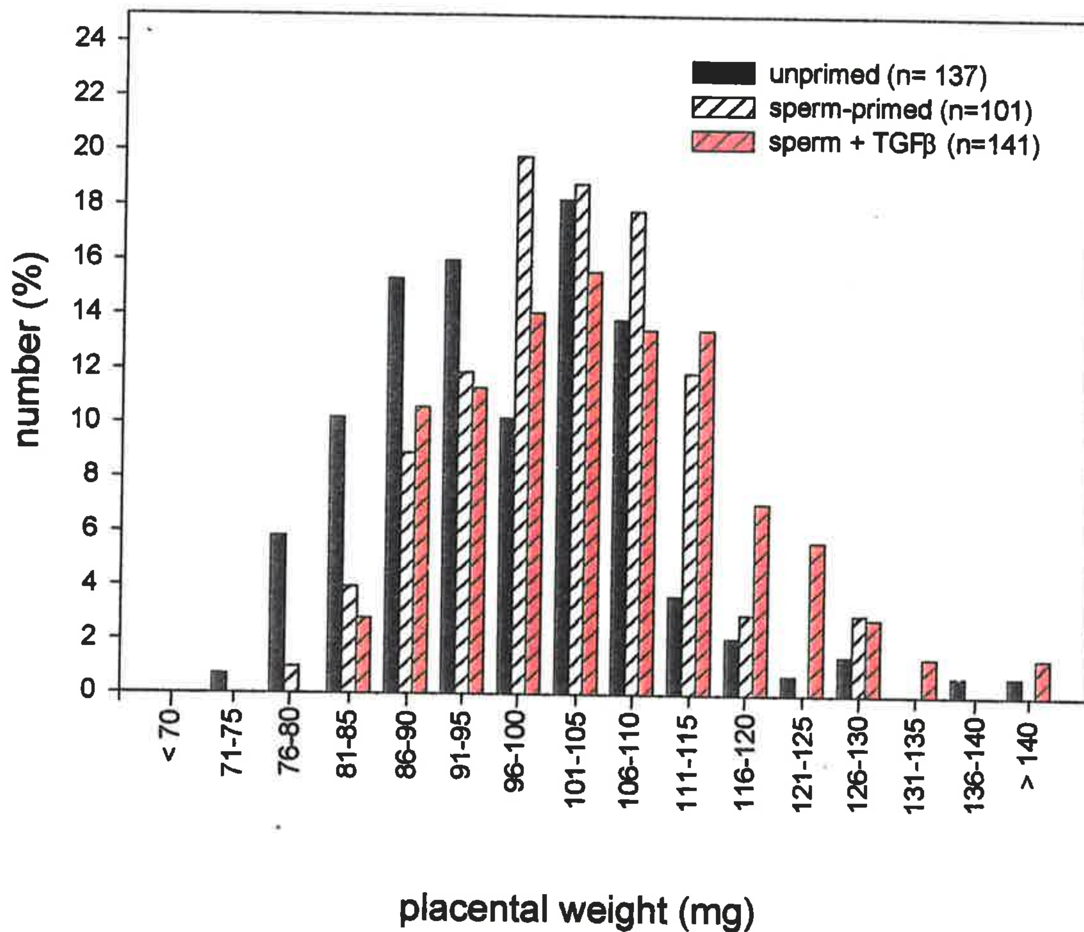
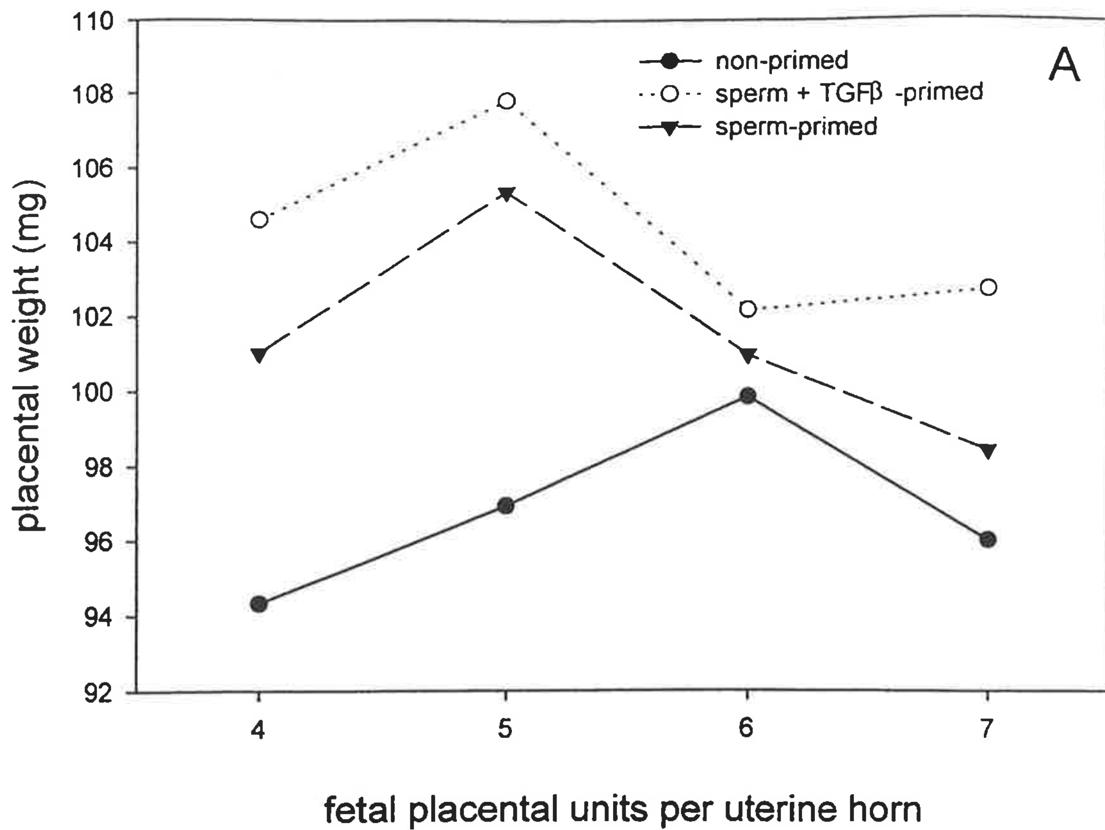


Figure 7.5 The effect of pre-immunisation of females with sperm on placental weight. Female Balb/c F1 mice were intrauterine immunised with CBA sperm in combination with 20 ng rTGFβ_1 or control medium, then mated with CBA males two weeks later. Pregnant females were sacrificed on day 17 of pregnancy and placental weights were measured. The mean placental weight of the sperm and the rTGFβ_1 / sperm immunised groups was significantly greater than that observed in the non-immunised animals (statistics summarised in Table 7.1).



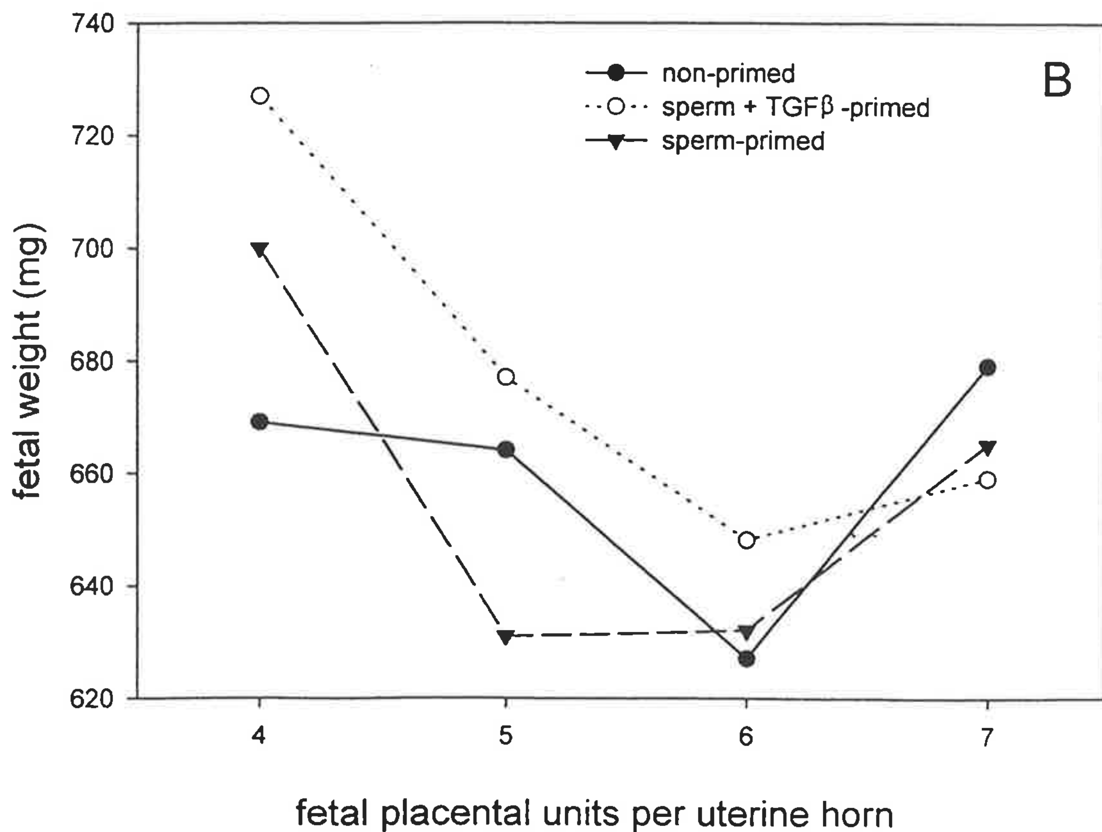


Figure 7.6 Effect of litter size on fetal and placental weights following intrauterine sperm immunisation. Female Balb/c F1 mice were immunised with CBA sperm ± rTGFβ_1 before mating with CBA males, as described in Figure 7.5. Values represent the mean fetal (B) and placental (A) weights for litters of a given size in each of the experimental groups.

Chapter 8

The post-coital inflammatory response in the human female

8.1 Introduction

Investigation of the post-coital inflammatory response in women has been limited by ethical and logistic constraints surrounding human experimentation in such a sensitive field. Very little is known about the mechanisms leading to the generation of the post-coital inflammatory response, with even less being understood about its potential function. Two reports to date have confirmed the presence of an inflammatory reaction within the human cervix following sexual intercourse or artificial insemination (Pandya and Cohen 1985, Thompson *et al.* 1992). Both groups reported a large influx of neutrophils into the cervix, together with a smaller contingent of monocytes and lymphocytes. Until recently it was believed that this response did not involve the endometrium, since the majority of sperm and almost all of the seminal plasma is contained within the cervix and vagina. However, this assumption may not be correct since one group of investigators have reported an increase in leukocyte numbers within uterine luminal fluid following intercourse (Williams *et al.* 1993). Whether this observation is due to the introduction of cervical leukocytes at the time of uterine cannulation, or truly associated with an inflammatory response in the endometrial stroma, is yet to be confirmed by histological analysis of uteri obtained by hysterectomy.

The aim of the studies described in this chapter was to investigate if similarities exist between mice and humans in regard to the manner by which they generate a post-mating inflammatory response. Such similarities may exist, since the human female genital tract does produce GM-CSF (Giacomini *et al.* 1995), and human seminal plasma contains large amounts of TGFβ_1 (Nocera and Chu 1993). It was therefore decided to investigate whether human semen or recombinant TGFβ_1 could initiate an increase in GM-CSF production from human cervical and endometrial cells *in vitro*. In addition, leukocyte chemotactic activity within these culture supernatants was measured to determine if exposure to semen or TGFβ_1 could induce an inflammatory response.

8.2 Production of GM-CSF by human endometrial cells in response to semen or rTGFβ_1

The ability of human endometrial tissue to produce inflammatory cytokines was investigated because the endometrium is the site of initiation of the post-mating inflammatory response in mice. Endometrial tissue was obtained from consenting women by curettage at the time of laparoscopy for assessment of non-endometrial pathology (tubal ligation or assessment of fallopian tube patency). Endometrial tissue was taken from the endocervical canal and the lower uterine segment, since this area is most likely to be exposed to semen *in vivo.* Indeed, sperm are concentrated within the endocervical gland crypts (Insler *et al.* 1980), so seminal plasma bound to the surface of sperm may come in contact with endometrial tissue at this site. In addition, endometrial tissue protruding outside the external cervical os (cervical ectropion) would come into direct contact with seminal plasma.

Endometrial samples were obtained during the mid-follicular to mid-luteal phase of the menstrual cycle and processed to produce three-dimensional endometrial cultures. Representative samples of endometrial cultures were immunohistochemically stained for the presence of type 18 cytokeratin, reported to be produced by human endometrial epithelial cells, but not endometrial stromal cells (Moll *et al.* 1983, Weikel *et al.* 1987). This confirmed that cultured endometrial epithelial cells form a discrete layer above the stromal cell matrix, in a manner comparable to that seen in *ex vivo* endometrial biopsies (Figure 8.1).

Two days after initiation of the cultures, medium was replaced with fresh medium, and twelve hours later this "pre-treatment" supernatant was collected and replaced with either fresh medium (control), medium containing 5 ng /ml rTGFβ_1 or medium containing 10% semen. Cultures were incubated for a further 12 hours before collecting the "post-treatment" supernatant. All supernatants were centrifuged to remove cellular debris prior to GM-CSF assay.

The GM-CSF content of endometrial cultures was initially assayed using a TF-1 cell GM-CSF bio-assay. No increase in endometrial GM-CSF production was observed following exposure to either rTGFβ_1, semen or the potent pro-inflammatory agent LPS, using endometrial tissue obtained from three different women at different stages in their menstrual cycle (data not shown). However, rTGFβ_1 was later found to inhibit TF-1 cell proliferation (Figure 8.2), and therefore this bio-assay was not used any further. All

subsequent measurements of GM-CSF content were performed using a commercial GM-CSF ELISA.

The addition of rTGFβ_1 to endometrial cultures obtained from peri-ovulatory and mid-luteal phase endometrial tissue produced a moderate (140-160%) increase in the production of GM-CSF immuno-activity (Figure 8.3). The GM-CSF immunoactivity released after treatment with dilute semen was equal to or less than that seen in the control wells in each of two experiments. Given that semen is known to contain TGFβ_1, and recombinant TGFβ_1 was able to increase GM-CSF production, it is possible that an unidentified factor(s) within seminal plasma may be inhibitory to endometrial cells in culture, thereby preventing their response to seminal TGFβ_1. Alternatively, endometrial cells in culture may not have the capacity to release bioactive TGFβ_1 from seminal plasma. This was not investigated any further, since it appeared that endometrial tissue was not strongly responsive to seminal induction of GM-CSF production.

8.3 Production of GM-CSF by cervical keratinocytes in response to semen or rTGFβ_1

Human cervical keratinocytes were obtained by enzymatic digestion of ectocervical biopsies collected from premenopausal women undergoing hysterectomy for benign, non-cervical pathology. Keratinocytes were layered over murine 3T3 fibroblasts and allowed to attach to the culture plate, then desquamated keratinocytes were removed from the wells and fresh culture medium added (Figure 8.1). This medium was collected 12 hours later as the "pre-treatment" supernatant and replaced with either medium (control), medium containing 10ng / ml rTGFβ_1, or medium containing 10% semen. Twelve hours later this "post-treatment" supernatant was collected. All supernatants were centrifuged to remove cellular debris. Comparison of the GM-CSF content within pre-treatment and post-treatment supernatants from each individual culture well enabled the calculation of any increase in cervical keratinocyte GM-CSF production in response to exposure to semen or rTGFβ_1. Since TGFβ does not increase keratinocyte proliferation (Moses 1985), any relative increase in GM-CSF content could be ascribed to an increase in GM-CSF production on a per cell basis, rather than an increase in cell number.

The GM-CSF output from cervical keratinocytes was increased following exposure to either dilute semen or rTGFβ_1 when compared to control cultures (Figure 8.4). In three separate experiments, a two to three-fold increase in GM-CSF production was

observed following exposure to $rTGF\beta_1$. The application of dilute semen produced a two-fold increase in GM-CSF production on two occasions, but no increase in a third experiment. In this later experiment, $rTGF\beta_1$ was able to produce a 260% increase in GM-CSF output (data not shown), thereby confirming the good health of the culture. Interestingly, semen used in this experiment had inadvertently been stored at room temperature for six hours before delivery to the laboratory, whereas all other semen samples were less than two hours old when used. Since the number of sperm within fresh and aged semen was comparable, it is possible that the active seminal constituent responsible for increasing GM-CSF production is labile, or associated with more metabolically active fresh sperm.

Since seminal plasma-derived $TGF\beta_1$ had been identified as the main stimulus for GM-CSF production by the murine uterine epithelium, it appeared reasonable to postulate that $TGF\beta_1$ may also be the main trigger for the release of GM-CSF from human cervical keratinocytes. An experiment was initiated to investigate whether neutralising antibodies towards $TGF\beta_1$ could block the GM-CSF-stimulating activity of semen on cervical keratinocytes. Unexpectedly, semen was found to initiate an increase in GM-CSF production, but very little of this stimulatory activity could be blocked by $TGF\beta_1$-neutralising antibody (Figure 8.5). The addition of 10 μg of $TGF\beta_1$-neutralising antibody to each culture well should have completely neutralised all 10 ng of seminal plasma $TGF\beta_1$ estimated to be present (Nocera and Chu 1995). It would therefore appear that $TGF\beta_1$ is not the only trigger for the release of GM-CSF from human cervical keratinocytes. This is in direct contrast to the mouse, where $TGF\beta_1$ is responsible for at least 75% of the post-mating surge in GM-CSF production.

An attempt was made to measure the *in vivo* GM-CSF response of cervical keratinocytes following exposure to semen at the time of intercourse. Ethical approval was granted to take cervical biopsies from pre-menopausal women at the time of hysterectomy in order to compare GM-CSF mRNA content of biopsies from women who had recently had sexual intercourse with those who abstained. Unfortunately this study did not reach fruition due to difficulties in recruiting appropriate volunteers.

8.4 *In vitro* chemotactic activity within human cervical keratinocyte supernatants

Since GM-CSF is chemotactic for human neutrophils, monocytes and macrophages (Wang *et al.* 1987), an increase in cervical keratinocyte GM-CSF production may contribute to the generation of the post-coital inflammatory response. In order to test this claim, the chemotactic activity within cervical culture supernatants was measured using an *in vitro* chemotaxis assay. This chemotaxis assay system consisted of a lower well containing potentially chemotactic culture supernatant, separated by two thin membranes from an upper well containing human leukocytes (Figure 8.6). The upper sparse pore membrane contained 8 μm pores which were large enough to permit leukocyte trans-migration, but too small to allow leukocytes to passively fall through (Figure 8.6). The presence of chemotactic activity within the lower well produces a chemotactic gradient that attracts leukocytes from the upper well, through the 8 mm sparse pore membrane, until they become trapped on the lower 3 μm membrane. After an appropriate incubation period, chemotaxis was halted by fixing the leukocytes with formaldehyde and then quantifying the chemotactic activity by counting the number of leukocytes on the lower membrane. In every assay, the potent chemotactic peptide N-f-methionyl-leucyl-phenylalanine (FMLP) was included as a positive control (Nielsen 1990) and fresh ectocervical culture medium was included as a negative control to measure random non-chemotactic cell migration.

The first series of chemotaxis assays examined the migration of mononuclear leukocytes separated from fresh blood by Ficoll-Paque density centrifugation. This leukocyte preparation consisted predominantly of monocytes and lymphocytes, with a lesser proportion of neutrophils and basophils (Boyum 1968, Miroli *et al.* 1986). The unstimulated (control) keratinocyte supernatants were found to have considerably more chemotactic activity than medium alone, a finding that was not unexpected given that cervical keratinocytes had earlier been shown to produce GM-CSF (Figure 8.7). The rTGFβ_1 and semen-treated cervical supernatants contained significantly more chemotactic activity than the control cervical supernatants. The increase in leukocyte chemotaxis was not directly mediated by TGFβ_1 (itself a potent chemo-attractant), but rather due to the release of pro-inflammatory intermediatories from cervical keratinocytes, since addition of TGFβ_1-neutralising antibody to the culture supernatants did not result in a significant decline in chemotactic activity (Figure 8.7).

The neutralisation of GM-CSF bio-activity within culture supernatants resulted in a large decrease in chemotactic activity, implying that GM-CSF is largely responsible for leukocyte recruitment *in vitro*. The chemotactic activity within these supernatants treated with GM-CSF-neutralising antibody still exceeded that found in the negative control, indicating that cervical keratinocytes must release chemotactic factors other than GM-CSF. While GM-CSF may be partially responsible for the post-coital inflammatory response in women, other pro-inflammatory mediators may also be involved, as has been shown to be the case in the murine post-mating inflammatory response (Pollard *et al.* 1998, Robertson *et al.* 1998b).

Macrophages are a major component of the post-mating inflammatory response in the mouse (De and Wood 1991), however it is uncertain what role they play in the human post-coital inflammatory response, given that they only make up 3.5% of all leukocytes within cervical mucous following intercourse (Thompson *et al.* 1992). This type of sampling technique may underestimate the magnitude of a macrophage influx, because macrophages tend to remain within the stromal tissue, rather than traversing epithelial surfaces like neutrophils (Robertson *et al.* 1996b). Given that macrophages are likely to be pivotal to antigen processing and presentation, it was felt important to assess the chemotactic activity of cervical culture supernatants for human macrophages. Macrophages, present in very low numbers in fresh peripheral blood, were produced from blood monocytes using appropriate culture conditions (Davies and Lloyd 1989). Chemotaxis assays using these monocyte-derived macrophages confirmed that cervical keratinocytes produce more chemotactic activity following stimulation with $rTGF\beta_1$ (Figure 8.8). The semen-treated keratinocyte supernatants contained only marginally more chemotactic activity than untreated controls, with this difference not reaching statistical significance. This finding was unexpected, because semen-treated keratinocyte cultures had earlier been shown to produce increased amounts of GM-CSF (a potent macrophage chemo-attractant), and exhibited increased chemotactic activity for fresh PMN cells compared to controls. It is possible that unidentified factors within human seminal plasma reported to inhibit macrophage chemotaxis *in vitro* (Mallmann *et al.* 1991) may be responsible for this discrepancy.

8.5 Discussion and conclusion

The experiments described in this chapter have shown that considerable similarities are likely to exist between humans and mice in regard to the mechanisms by which they generate their post-coital inflammatory response. While seminal $TGF\beta_1$ has been shown to increase the production of GM-CSF in both the human and murine female reproductive tract, differences in coital physiology dictate that some differences still exist. The mouse, an intrauterine inseminator, deposits the bulk of its ejaculate within the uterine cavity, and therefore the endometrium is the dominant site of the post-mating inflammatory response. Conversely, humans are intra-vaginal inseminators, where the ejaculate is largely contained within the cervix and vagina, with only the small portion of seminal plasma bound to sperm reaching the uterine cavity. It therefore is not surprising that semen and $rTGF\beta_1$ elicit a greater increase in GM-CSF production from cervical keratinocytes than endometrial cells. The ability of seminal $TGF\beta_1$ to elicit an endometrial inflammatory response can not be ruled out, since $TGF\beta_1$ bound to the surface of sperm (Chu *et al.* 1996) may "hitch-hike" a ride to the uterine cavity, where it may elicit an increase in GM-CSF production.

As was found to be the case in the mouse, the vast majority of $TGF\beta_1$ contained within human seminal plasma is present in a latent form (Nocera and Chu 1995). Several potential mechanisms exist for the release of bioactive $TGF\beta_1$ within the female reproductive tract. Firstly, the production of lactic acid by vaginal bacteria (lactobacillus) makes the vagina an acidic environment (Mackay *et al.* 1992), which may facilitate the release of bioactive TGFβ. Within seconds of ejaculation, the buffering effect of seminal plasma neutralises vaginal acidity, but within a few hours the native acidic environment is restored (Fox *et al.* 1973). This gradual return to an acidic pH may favour the slow release of TGFβ from seminal plasma retained within the vagina. A second potential mechanism for the conversion of latent TGFβ into its bioactive form is via the enzymatic activity of plasmin. Exposure of keratinocytes to TGFβ, IL-1β or TNFα (Keski-Oja and Koli 1992, Bechtel *et al.* 1996), all present within human seminal plasma, has been reported to increase keratinocyte production of plasmin, raising the possibility of a positive feedback loop potentiating the release of TGFβ within the vicinity of the cervix. Plasmin is also produced by the uterine endometrium, with peak plasmin activity coinciding with ovulation (Casselin and Ohlsson 1981, Casselin *et al.* 1986).

While the TGFβ / GM-CSF axis may play a significant role in the human post-coital inflammatory response, other mechanisms are also likely to exist. Human seminal plasma contains IL-1 and TNFα (Huleihel *et al.* 1996), both reported to increase cervical keratinocyte GM-CSF production (Woodworth and Simpson 1993), which may explain why $TGF\beta_1$-neutralising antisera was unable to block the semen-induced increase in keratinocyte GM-CSF production. In addition, GM-CSF is unlikely to be the only chemotactic factor responsible for evoking a post-coital cervical leukocytosis, since GM-CSF-neutralising antibody blocked only half of the chemotactic activity contained within cervical keratinocyte supernatants. In the mouse, cytokines such as IL-1, IL-6, TNFα, CSF and the chemokines RANTES, MCP-1 and MIP-1 (Sanford *et al.* 1992, Pollard *et al.* 1998, Robertson *et al.* 1998b) are implicated in the post-mating inflammatory response. It is possible that these cytokines may also play a role in the human post-coital inflammatory reaction.

Human seminal plasma contains pro-inflammatory cytokines such as IL-1β, IL-2, IL-4, IL-6, TGFβ, IL-8 and MIP-1α (Shimoya *et al.* 1995, Srivastava *et al.* 1996). These seminal cytokines may directly initiate an inflammatory response without triggering an increase in GM-CSF production. This direct effect is likely to be responsible for the early phase of the post-coital inflammatory reaction, evident within 15 minutes of exposure to semen (Pandya and Cohen 1985).

The current observations conflict with earlier reports that sperm, not seminal plasma, elicits a cervical leukocytosis *in vivo* (Pandya and Cohen 1985, Thompson *et al.* 1992). The experiment in which aged semen failed to elicit an increase in cervical keratinocyte GM-CSF production indicates that labile factors within seminal plasma are more likely to be responsible for increasing cervical keratinocyte GM-CSF production than sperm. Further *in vivo* studies to investigate the cervical inflammatory reaction following exposure to epididymal sperm (no contact with seminal plasma), seminal plasma, or whole semen are needed to provide a definitive answer to what triggers the post-coital cervical leukocytosis.

Potential roles for the human post-coital inflammatory response were not investigated, but it is worthwhile to speculate on possible physiological functions. Firstly, an influx of leukocytes into the cervix / cervical mucous may assist in the removal of potentially pathogenic bacteria and viruses introduced at the time of intercourse. Highlighting the importance of this response is the observation that smoking women, a group known to have reduced cervical immunity due to the deleterious effects of cigarette

toxins, have an increased risk of contracting cervical Human Papilloma Virus infection (a sexually transmitted pathogen) when compared to non-smokers ((Hawthorn *et al.* 1988, Poppe *et al.* 1995). Secondly, a cervical leukocytosis may assist in the removal of dead or immotile sperm. Phagocytic clearance of sperm by leukocytes has been postulated to act as an immunological filter, the function of which is to recognise and select the "best" sperm for fertilisation (Tomlinson *et al.* 1992).

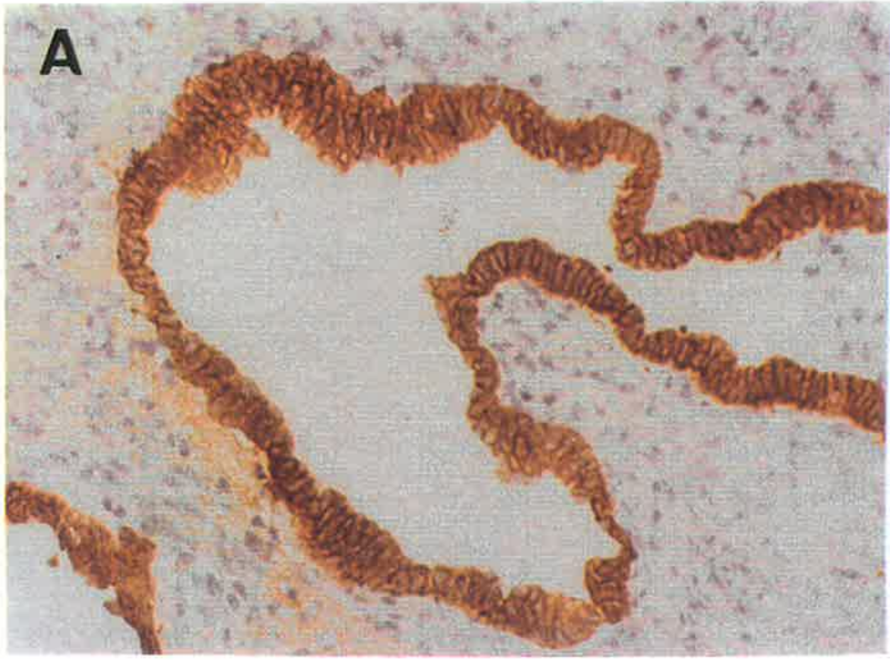
Passage of sperm through the leukocyte-rich environment provided by the cervix is clearly not harmful to sperm, since conception has been recorded in the presence of a cervical leukocytosis (Thompson *et al.* 1992). The passage of sperm through a cervical leukocytosis may actually assist fertilisation, since contact with leukocytes has been reported to stimulate capacitation of rabbit sperm (Soupart 1970). It is uncertain whether this occurs in humans, since supernatants from lectin-stimulated monocytes, as well as recombinant IFNγ, TNFα and IL-8, are reported to have no effect on the acrosome reaction in human sperm (Fedder *et al.* 1995).

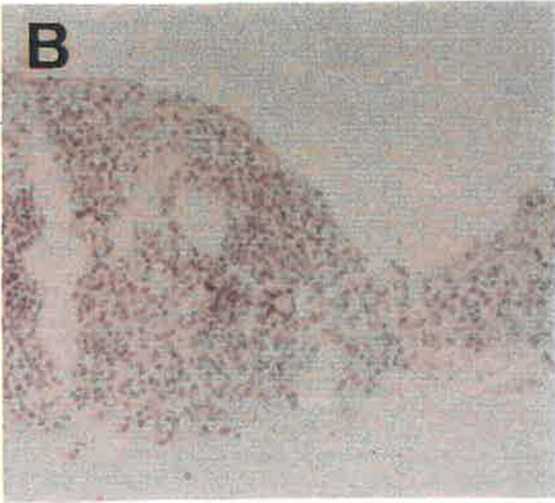
Pre-eclampsia is a disorder characterised by a shallow placental development (Khong *et al.* 1986) , possibly due to an overly aggressive maternal immune response towards trophoblast antigens (Sibai 1991, Redman 1992). Maternal immune responses to paternal antigens are believed to play a role in the development of pre-eclampsia since primiparity, change of partner, or conception with the aid of donor semen, all increase the risk of developing pre-eclampsia (Need *et al.* 1983, Trupin *et al.* 1996). In contrast, a previous pregnancy to the same partner, or exposure to foreign antigens through blood transfusion, decreases this risk (Sibai 1991). It has been suggested that these "immunisation events" may initiate paternal antigen tolerance, thereby limiting maternal immune destruction of the invading trophoblast, and allowing normal placental development to proceed (Clark 1994).

The human post-coital inflammatory response is ideally positioned to act as an induction site for maternal immune responses to paternal ejaculate antigens. Sexual intercourse may sensitise a woman to her partner's antigens in a cumulative manner, since a prolonged period of sexual cohabitation significantly decreases the risk of developing pre-eclampsia (Robillard *et al.* 1994). Conversely, a lack of exposure to ejaculate antigens due to the use of barrier contraception before conception increases the risk of developing pre-eclampsia (Marti and Herrmann 1977, Klonoff-Cohen *et al.* 1989). These epidemiological studies do not provide conclusive proof that the post-coital inflammatory response is involved in the initiation of an immuno-permissive environment towards

paternal antigens. However, the existence of similarities between mice and women in regard to generation of the post-mating inflammatory response, in combination with the ability of semen to initiate paternal antigen-specific tolerance in mice (Breyere and Barrett 1960, Prehn 1960), suggests that cumulative exposure of women to paternal antigens through sexual intercourse may facilitate the development of an immuno-permissive maternal environment that may favour the growth and survival of the fetal allograft.

Figure 8.1 Human female reproductive tract cultures. (A) Human endometrial biopsy taken during the early luteal phase and stained for cytokeratin type 18 (haematoxylin section, x 20 magnification). Sections from human endometrial cultures stained with haematoxylin only (B) or haematoxylin and α cytokeratin type 18 (C), both at x 20 magnification. (D) Human cervical keratinocytes 7 days after the initiation of culture (magnification x 10). Note the cobblestone appearance of attached keratinocytes and the "cornflake" outline of desquamated keratinocytes (shown with an arrow in D).


A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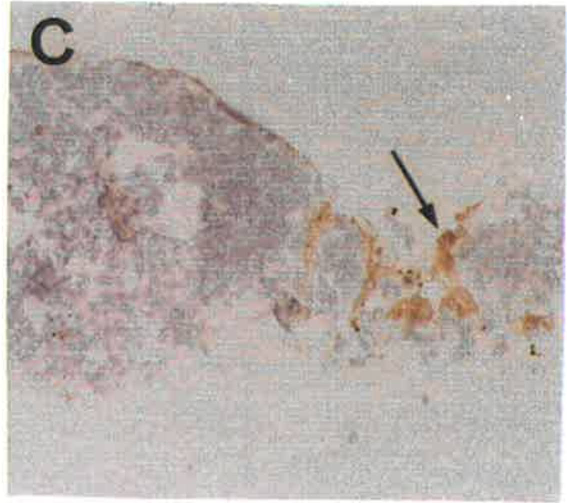
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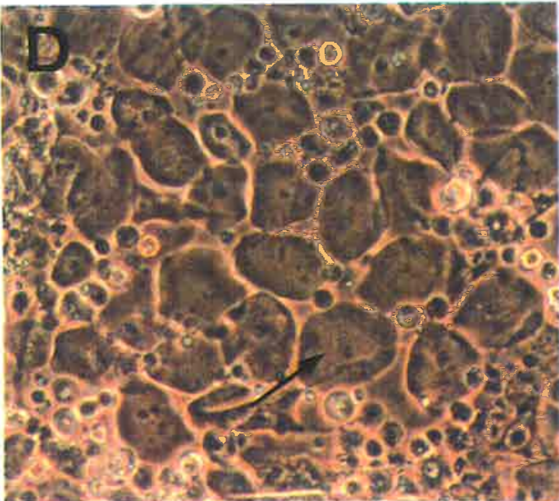
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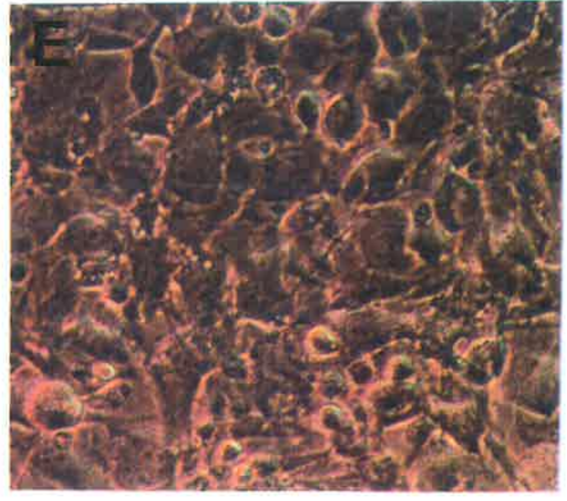
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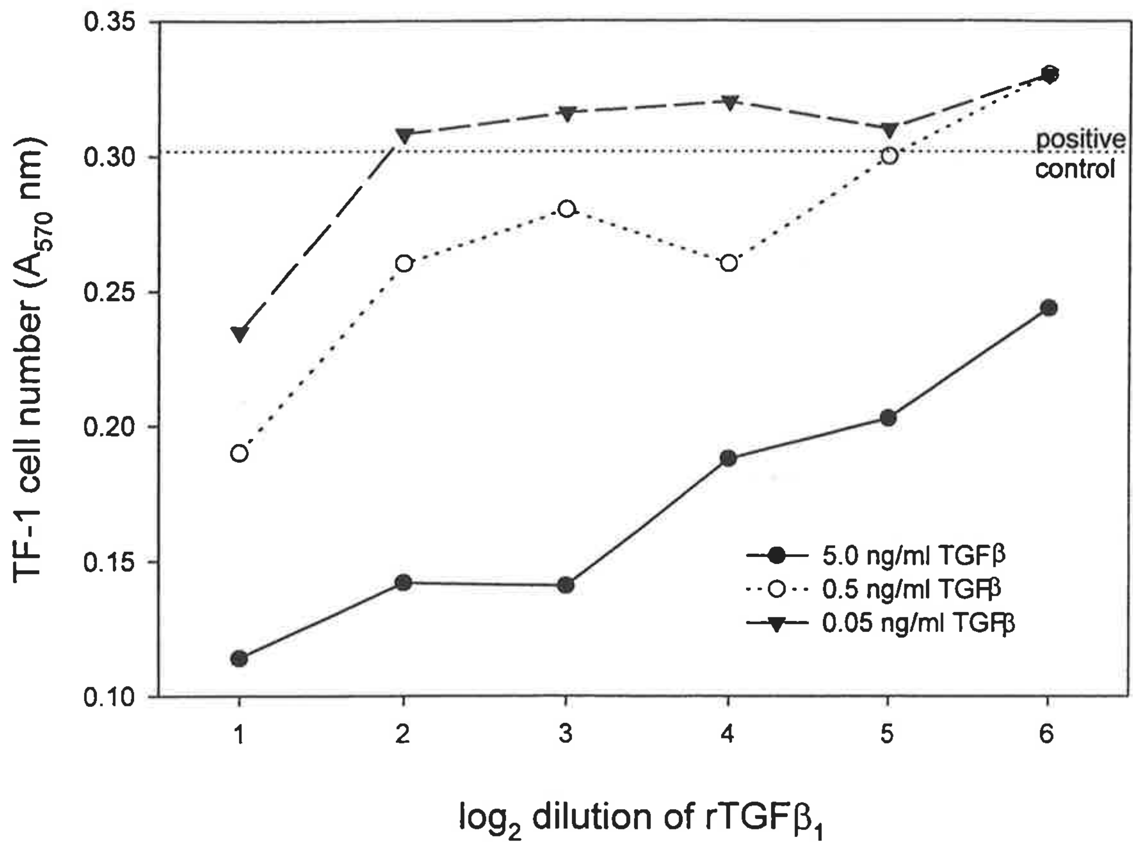


Figure 8.2 Effect of rTGFβ_1 on TF-1 cell proliferation. Serial 1 in 2 dilutions of rTGFβ_1 were made in RPMI-5% FCS using a starting concentration of 0.05 , 0.5 or 5.0 ng/ml of rTGFβ_1. Each well was then seeded with 200 TF-1 cells (suspended in culture medium containing 1% cervical keratinocyte supernatant) and incubated for 24 hours before addition of Alamar Blue. Cell proliferation was quantified 24 hours later by measurement of absorbance at 570 nm. Symbols represent the mean of duplicate wells. The mean rate of proliferation of TF-1 cells in the absence of rTGFβ_1 (positive control) is indicated by the horizontal dotted line.

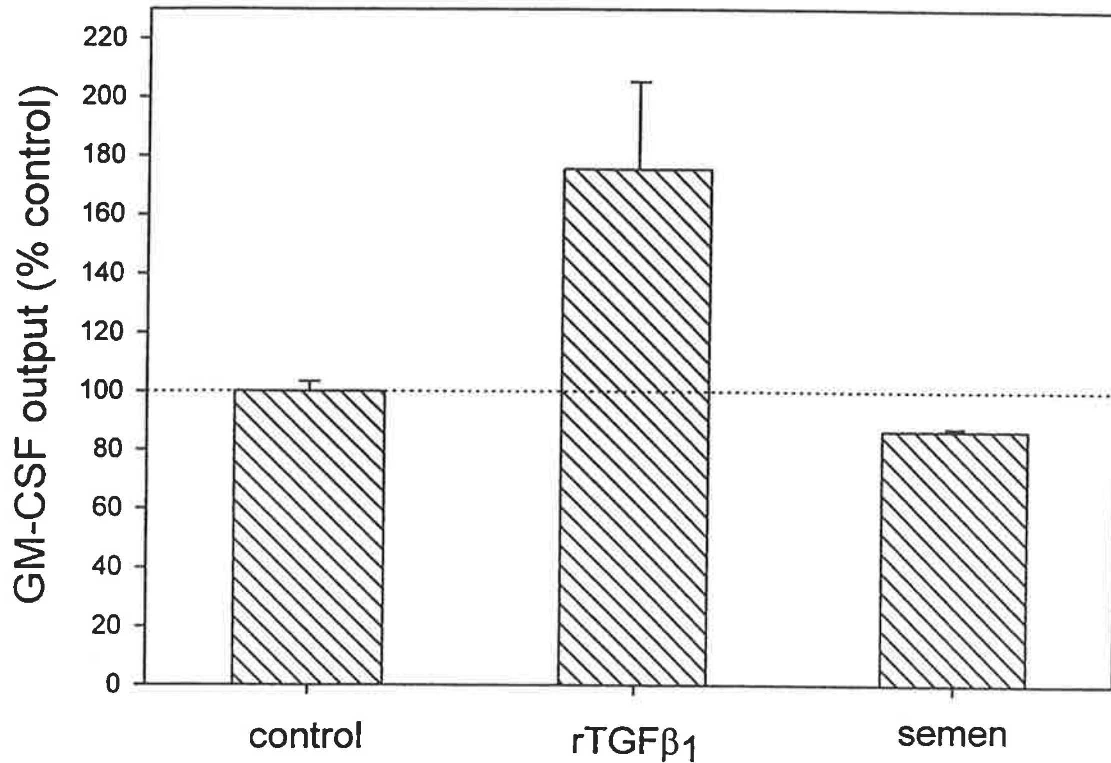


Figure 8.3 Effect of rTGFβ_1 and semen on human endometrial cell GM-CSF production *in vitro*. Cells were prepared from peri-ovulatory endometrium, and the GM-CSF content of these pre and post-treatment supernatants was measured by ELISA. Post-treatment data was normalised to pre-treatment values and expressed as a percentage of the GM-CSF output in the control. All measurements are the mean $\pm$ s.d. of duplicate wells in each experimental group. A significant effect of treatment group was confirmed by Kruskal-Wallis one-way ANOVA ($p=0.043$), with the control and rTGFβ_1 groups being significantly different when analysed by the Mann-Whitney Rank sum test ($p=0.0495$).

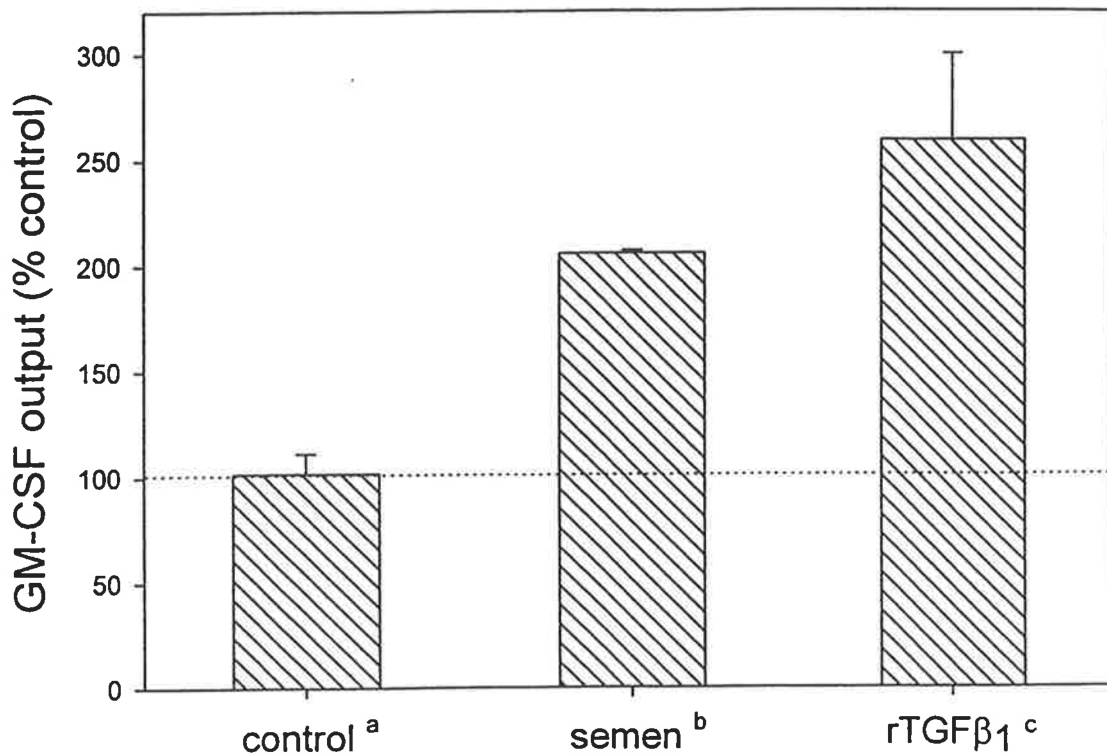


Figure 8.4 Effect of rTGFβ_1 and semen on human cervical keratinocyte GM-CSF production *in vitro*. Cervical keratinocytes were cultured in combination with murine fibroblasts for 7 days before adding either culture medium (control), dilute semen (10% v/v) or rTGFβ_1 (10 ng/ml). The GM-CSF content in 12-hour culture supernatants taken before and after treatment was compared and normalised to that seen in the control wells, as outlined in Figure 8.3. The data expressed in this figure were obtained by pooling two separate experiments using keratinocytes from two different women. All measurements are the mean $\pm$ s.d. of quadruplicate wells in each experimental group. Data was compared by Kruskal-Wallis one-way ANOVA ($p=0.015$), followed by Mann-Whitney Rank Sum test. Different superscripts indicate significant differences ($p<0.05$) between groups.

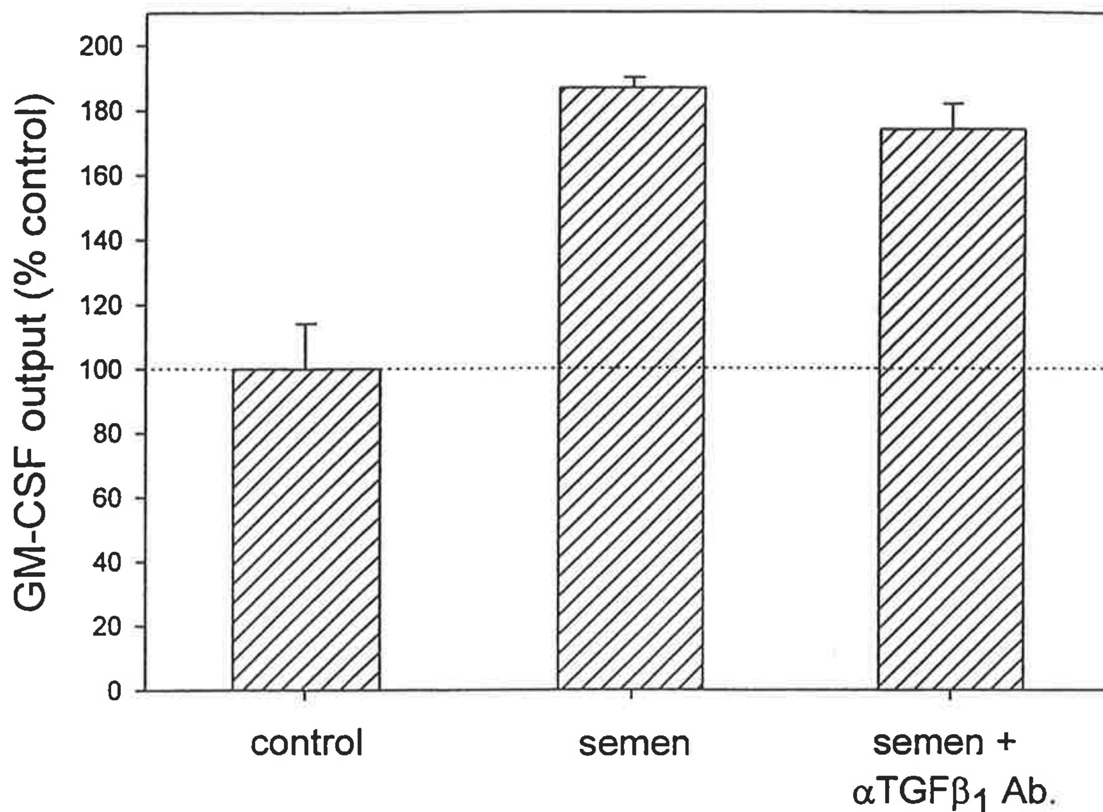
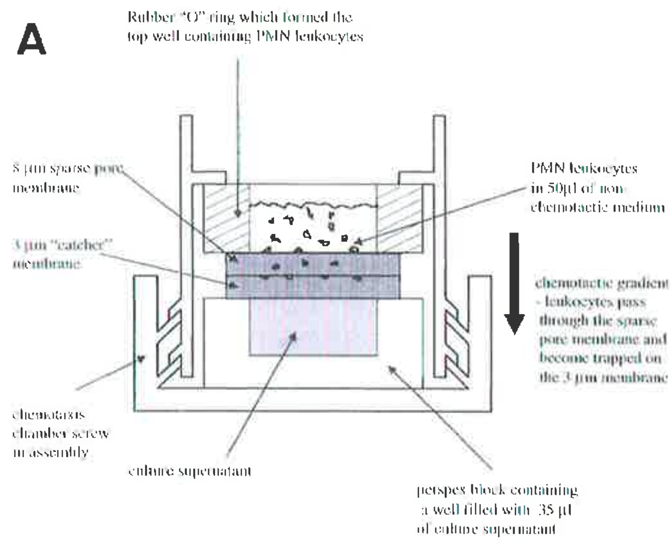
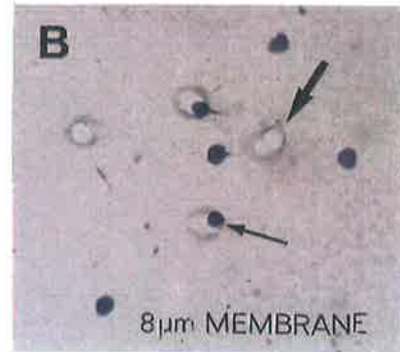
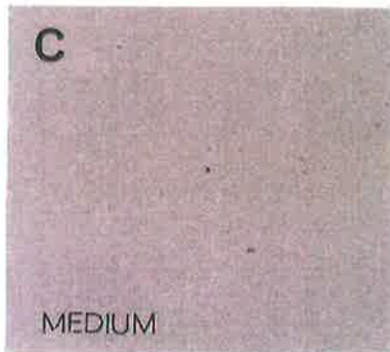


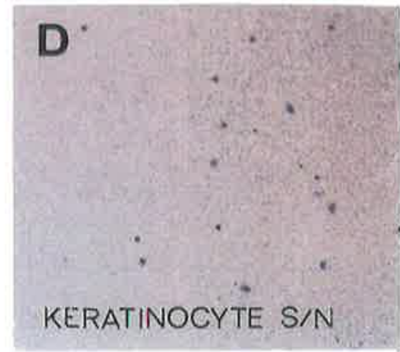
Figure 8.5 Effect of TGFβ_1 neutralising antisera on the semen-induced increase in cervical keratinocyte GM-CSF production. Cervical keratinocyte cultures containing either culture medium (control), semen (10% vol / vol) or semen in combination with TGFβ_1-neutralising antisera (10μg / ml) were assayed for GM-CSF content by ELISA. All measurements are the mean ± s.d. of duplicate wells in each experimental group. The increase in GM-CSF production seen following exposure to semen was not abrogated by neutralisation of TGFβ_1 in either of two replicate experiments.

Figure 8.6 Assessment of MN Leukocyte chemotaxis. (A) Schematic diagram of a chemotaxis chamber. (B) MN leukocytes passing through pores in the 8 μm sparse-pore membrane. Leukocytes trapped on the 3 μm catcher membrane (magnification x10) from chemotaxis wells containing either fresh medium (C), or keratinocyte supernatant from unstimulated (D), semen-stimulated (E) or rTGFβ_1-stimulated cultures (G). The addition of αGM-CSF antibody (0.2 μg/ml) to semen (F) and rTGFβ_1 (H)-treated cervical supernatants significantly reduced their chemotactic activity.


A
Rubber "O" ring which formed the
top well containing PMN leukocytes
8 µm sparse pore
membrane
PMN leukocytes
in 50µl of non
chemotactic medium
3 µm "catcher"
membrane
chemotactic gradient
- leukocytes pass
through the sparse
pore membrane and
become trapped on
the 3 µm membrane
chemotaxis
chamber screw
in assembly
culture supernatant
perspex block containing
a well filled with 35 µl
of culture supernatant

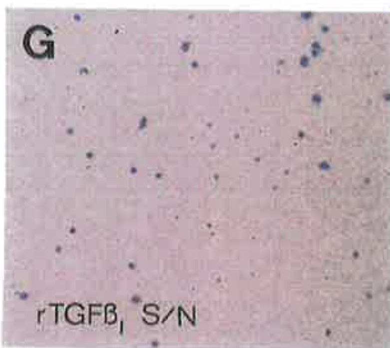

B
8µm MEMBRANE


C
MEDIUM


D
KERATINOCYTE S/N

E
SEMEN S/N

F
SEMEN + GM-CSF Ab.


G
rTGFß1 S/N

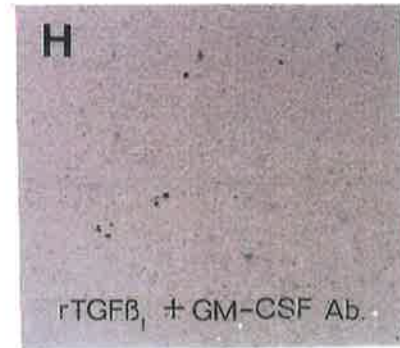
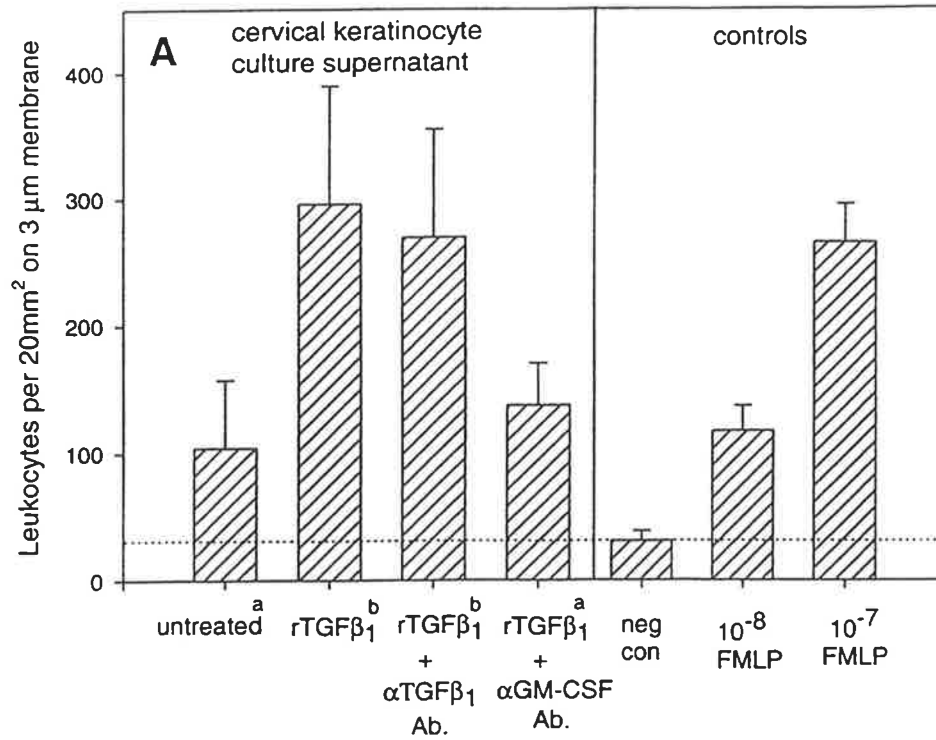
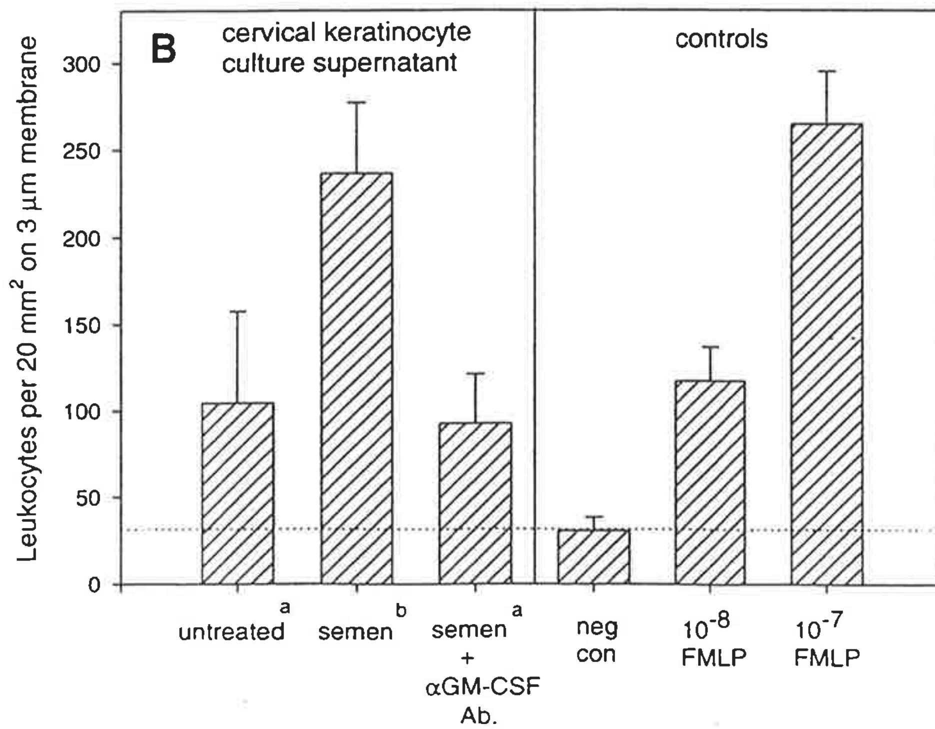

H
rTGFß1 + GM-CSF Ab.

Figure 8.7 Chemotactic activity of cervical keratinocyte culture supernatants for fresh mononuclear (MN) leukocytes. Post-treatment culture supernatants were diluted 1:2 before addition to chemotaxis chambers. In some experimental groups, the GM-CSF or $TGF\beta_1$ bioactivity within these supernatants was blocked by neutralising antibodies (0.2 μgm / ml final concentration). Chemotaxis chambers were assembled as shown in Figure 8.6a, incubated for 45 minutes, and the leukocytes fixed to the "catcher" membrane with gluteraldehyde before staining with haematoxylin. Chemotaxis was quantified by counting the number of leukocytes fixed to the 3 μm "catcher" membrane (circular field of $20mm^2$). Treatments were assessed in duplicate, with each duplicate being assayed in triplicate (n=6 chemotaxis assays per group). FMLP, a potent leukocyte chemotactic agent, served as a positive control, and fresh ectocervical culture medium diluted in HBSS was the negative control. Data were initially compared by Kruskal-Wallis one-way ANOVA ($p=0.0001$), followed by the Mann-Whitney rank sum test . Significant differences ($p<0.05$) are indicated by different superscripts. The chemotactic activity contained within $rTGF\beta_1$ treated cervical culture supernatants is depicted in (A) and the activity within semen-treated cultures in (B).


A
cervical keratinocyte culture supernatant
controls
Leukocytes per 20mm² on 3 µm membrane
400
300
200
100
0
untreated a
rTGFβ1 b
rTGFβ1 + αTGFβ1 Ab. b
rTGFβ1 + αGM-CSF Ab. a
neg con
10-8 FMLP
10-7 FMLP


B
cervical keratinocyte culture supernatant
controls
Leukocytes per 20 mm² on 3 µm membrane
300
250
200
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100
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untreated a
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10-8 FMLP
10-7 FMLP

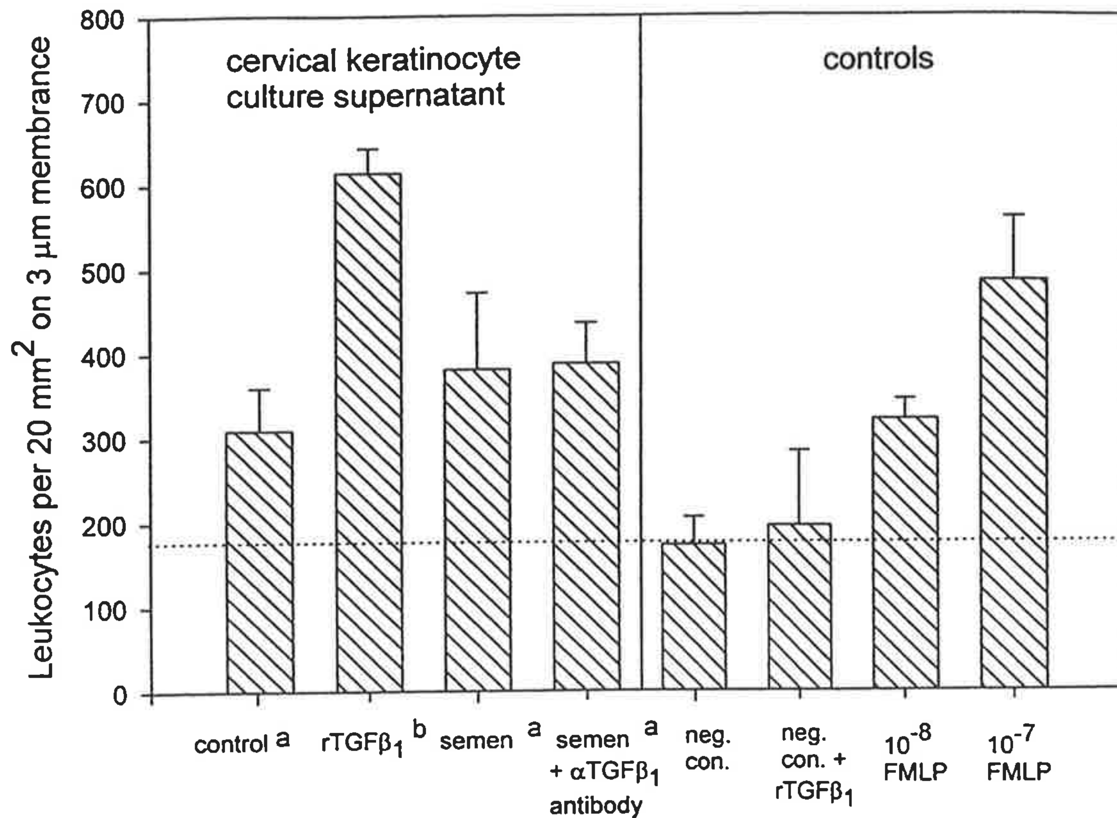


Figure 8.8 Chemotactic activity of cervical keratinocyte culture supernatants for macrophages. Peripheral blood monocytes were isolated from other PBM leukocytes by virtue of their selective capacity to adhere to plastic. The resulting monocyte-enriched population were cultured in RPMI-10% FCS / 1% Cx medium for four days to promote transformation into macrophages. Chemotaxis assays were then performed as outlined in Figure 8.7, with the exception that only macrophages were added to the upper chemotaxis well. Differences between the treatment groups were initially analysed by a Kruskal-Wallis one-way ANOVA ($p=0.007$), followed by Mann-Whitney rank sum test. Significant differences between the groups are indicated by different superscripts.

Chapter 9

The effect of semen exposure in assisted human reproduction

9.1 Introduction

The development of techniques for achieving artificial fertilisation (IUI, IVF, ICSI, GIFT) have resulted in great advances being made in the treatment of infertility. Despite this, pregnancy rates from "artificially created" embryos are still inferior to that seen following natural conception. The rate of early embryo (pre-menstrual) loss following assisted reproduction technology (ART) treatment far exceeds that seen following natural conception (Lenton *et al.* 1988, Weinberg and Wilcox 1988), with up to 70% of transferred embryos being lost around the time of implantation, despite the majority of these embryos being chromosomally normal (Creasy 1988).

It has been proposed that immune rejection of "normal" embryos may contribute to the high rate of embryo wastage seen following IVF treatment (Stern 1997). Because a large proportion of couples abstain from sex during IVF treatment (Read *et al.* 1996), it is possible that lack of exposure to semen, perhaps compromising the development of an immuno-permissive response, may precipitate the rejection of transferred embryos. While it is presently uncertain if semen exposure can help generate an immuno-permissive response, exposure to semen or seminal plasma has been reported to improve pregnancy rates following embryo transfer (Bellinge *et al.* 1986, Marconi *et al.* 1989, Tucker *et al.* 1990), and increase the number of viable pregnancies conceived naturally by women experiencing idiopathic recurrent spontaneous abortion (Coulam and Stern 1995).

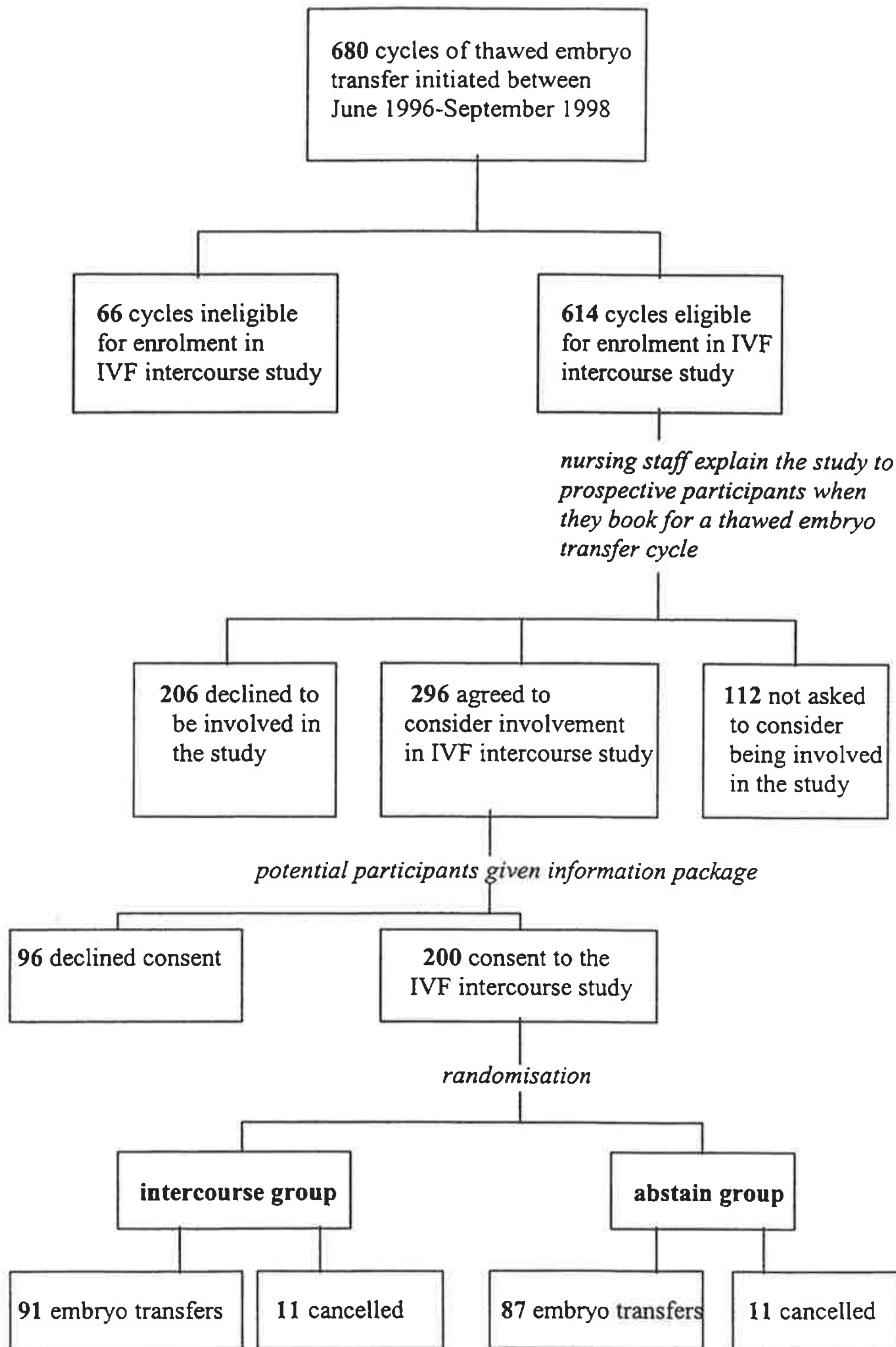
The aim of the clinical study outlined in this chapter was to perform a prospective randomised control trial to investigate whether semen exposure alters the success of thawed embryo transfer. The rationale behind this trial was three-fold. Firstly, while several studies have reported that exposure to semen or seminal plasma improves pregnancy outcome, others have reported no significant benefit (Fishel *et al* 1989, Qasim *et al* 1996). One inconclusive study did show a declining trend in the incidence of fetal loss following semen exposure (Qasim *et al* 1996), but suffered from the potential for type II statistical error because of small numbers. Secondly, randomisation procedures used in

the majority of these earlier trials were non-existent or unblinded, raising the potential for bias. Finally, a large study by Fishel *et al.* (1989) reported no improvement in IVF pregnancy outcome following artificial insemination, however these investigators made no comment on whether couples had been asked to refrain from intercourse around the time of embryo transfer. If this instruction was not made clear to participants, the "no insemination" control group may have been exposed to semen through intercourse, thereby invalidating the study conclusion. It was therefore proposed that a prospective randomised trial that addressed the deficiencies of these earlier studies may help to determine whether "acute" exposure to semen plays a role in human reproductive success.

9.2 Study enrolment

Between June 1996 and September 1998, 680 cycles of thawed embryo transfer were performed by the University of Adelaide's reproductive medicine program. Exclusion of women that were non-English speaking, enrolled in the donor embryo program, who required ovulation induction or who resided in rural areas resulted in 614 cycles being eligible for enrolment in the trial (90.3 %). Rural women were excluded, because separation from their partner may have made compliance with the intercourse trial allocation difficult. Women requiring ovulation induction were excluded, because many had polycystic ovary syndrome, a condition reported to significantly increase the risk of miscarriage (Balen *et al.* 1993). Forty-eight percent of couples eligible to be enrolled agreed to receive an information package about the trial (Appendix A). When later contacted by telephone, 68 % of these couples gave their final consent to be involved (n=200). The remaining eligible couples declined to be involved from the outset, or were not invited to participate (Figure 9.1). Couples were not asked why they declined, but many volunteered that they were unable to cope with any further intrusion into their private lives. Once a couple had agreed to be involved, they were randomised into one of two trial arms; intercourse or abstinence. Roughly equal numbers of cycles reached embryo transfer in both trial groups. Eleven percent of participants were removed from the trial analysis because they did not reach embryo transfer (no embryo survival upon thawing, anovulation, missed ovulation, illness), or because of non-compliance with their trial allocation (illness or travel precluding intercourse).

Figure 9.1 Accrual of volunteers for the IVF intercourse study



9.3 Trial participant characteristics

The characteristics of participants in the two trial groups are summarised in Table 9.1. No significant differences in maternal age, past obstetric history, aetiology of infertility, number of previous thawed embryo transfers, or number of embryos transferred were identified between the two groups. Maternal age, history of previous miscarriages, duration of infertility treatment and aetiology of infertility are major risk factors for miscarriage (Wilcox *et al.* 1990). The absence of any significant differences between the two study groups permits a meaningful analysis of the effect of acute semen exposure on patterns of pregnancy loss.

Table 9.1 Characteristics of study participants undergoing thawed embryo transfer

	intercourse group	abstain group	p value
number of cycles reaching embryo transfer	91	87	-
age (years)	33.8 ± 4.4	33.1 ± 4.4	0.648
median number of previous live births (range)	0 (0-5)	0 (0-5)	0.554
median number of previous non-viable pregnancies (range)	0 (0-5)	0 (0-5)	0.903
duration of infertility (years)	5.0 ± 2.5	5.1 ± 2.8	0.707
aetiology of infertility			
1. male factor	48.4 %	39.1 %	
2. female factor	22.2 %	21.8 %	X^2= 2.95 p= 0.624
3. combined	13.1 %	12.7 %	
4. unexplained	16.5 %	26.4 %	
median number of thawed embryo transfers completed (range)	2 (1-10)	2 (1-9)	0.893
median number of embryos transferred during trial cycle (range)	2 (1-3)	2 (1-3)	0.163

All values expressed as mean ± s.d, unless otherwise shown. Data were analysed with a students t test or Chi-square, as indicated.

Since only one third of eligible couples agreed to participate in this study, it was prudent to ensure that the study cohort was still typical of the general infertile population. This was achieved by comparing data on maternal age, obstetric profile and treatment outcome of trial participants with that from patients undergoing IVF / thawed embryo transfer at other reproductive medicine programs in Australia (Hurst *et al.* 1996). This analysis, outlined in Table 9.2, confirmed that the study cohort was representative of the infertile population in general.

Table 9.2 Comparison of characteristics between the IVF intercourse trial cohort and the Australian infertile population in general

	study cohort	Australian infertile population
maternal age	33.5 years	< 29 years - 20.2 % 30-34 years - 41.3 % 35-39 years - 29.7 % > 40 years - 8.8 %
number of previous pregnancies (viable and non-viable)	1.35	none - 52.9% one - 26.9% two - 11% > three - 9.2%
duration of infertility	5.1 years	< 2 years - 6.8% 2-3 years - 39.1% 4-5 years - 28.6% > 6 years - 25.5%
aetiology of infertility	male - 44% female - 22% combined - 13% unexplained -21%	male - 30% female - 20.1% combined - 34.9% unexplained- 15%
average number of embryos transferred per treatment cycle	1.90	2.3
viable pregnancy rate per 100 embryo transfer cycles	11.2	11.1

Data for the Australian infertile population was obtained from Assisted Conception Australia and New Zealand 1996 (Hurst *et al.* 1997). All data is expressed as a mean value, except for some of the Australian population data that is presented as a range (mean values not published).

9.4 Pregnancy outcome

Assessment of early implantation was monitored by performing a serum hCG assay (lower limit of detection 5 mIU/L) on the 16th day following embryo transfer. Any patient who had at least one hCG reading above 20 mIU/L was considered to have evidence of early implantation. Because exogenous hCG was not used in any of the treatment cycles, it was deemed unnecessary to limit biochemical pregnancies to the traditional definition of a rising trend in hCG, since this more restrictive criteria may have led to an under estimation of the incidence of early implantation. Table 9.3 reveals that 15.4 % of couples in the intercourse group showed evidence of early implantation per treatment cycle, compared to 16.1 % of couples in the abstain group (p=0.896). No significant difference in the rate of early pregnancy wastage (proportion of biochemical pregnancies progressing to viable pregnancies), nor late fetal demise (fetuses non-viable on ultrasound) was observed. The viable pregnancy rate (proportion of treatment cycles resulting in a viable pregnancy at 6-week ultrasound) was 12.1 % in the intercourse group and 10.3 % in the abstain group (p=0.712). Pregnancies were not followed beyond this point, as the aim of the study was to determine if acute exposure to semen could increase early pregnancy success. Furthermore, it has been reported that once fetal cardiac activity is recorded at six weeks, 93% of these fetuses will continue to be viable (Molo *et al* 1993). Analysis of IVF outcome according to the number of transferred embryos that were viable at the 6-week scan revealed a trend towards an inferior pregnancy outcome in patients who abstained, yet this difference was not statistically significant (intercourse = 7.7 viable pregnancies per 100 transferred embryos, abstain = 5.3 viable pregnancies per 100 transferred embryos, p=0.355).

Table 9.3 Pregnancy outcome in trial participants

	intercourse group	abstain group	p value
number of cycles enrolled	102	98	-
number of cycles reaching embryo transfer (% cycles cancelled)	91 (10.8 %)	87 (12.6 %)	-
total number of embryos transferred	168	171	-
biochemical pregnancies	3	3	-
viable singleton on ultrasound	9	9	-
viable twin pregnancy on ultrasound	2	0	-
non-viable fetus on ultrasound	1*	2	-
implantation rate per 100 transferred embryos	10.1	8.2	0.537
viable pregnancy rate per 100 transferred embryos	7.7	5.3	0.355
percentage of transfer procedures resulting in implantation	15.4 %	16.1 %	0.896
percentage of transfer procedures resulting in a viable pregnancy	12.1 %	10.3 %	0.712
percentage of biochemical pregnancies that progress to viability	78.6 %	64.3 %	0.402

* signifies a twin pregnancy on ultrasound, one fetus viable and the other non-viable.

9.5 Discussion and conclusion

From the results of this trial, it can be concluded that acute exposure to semen does not have a major effect on early pregnancy success in humans, at least in the context of thawed embryo transfer. Statistical power analysis performed before the commencement of this trial estimated that 800 treatment cycles (400 in each trial arm) would need to be recruited before it could be concluded that a minimum 25% difference in viable pregnancy rates did not exist between the two groups (α=0.05, β=0.8). Since only 200 cycles were recruited, it is impossible to exclude that a small (<25%) difference may exist between the trial groups, a "type II statistical error" (Mittendorf *et al.* 1995). Comparison of the "take-home baby rate"(proportion of treatment cycles resulting in a viable pregnancy) revealed that couples who did have sex around the time of embryo transfer were 15% more likely to achieve a successful pregnancy per treatment cycle than couples who abstained. The small number of pregnancies in each group makes it impossible to draw firm conclusions from these figures, however this data does raise the possibility that acute exposure to semen may have a small beneficial effect on early implantation success. A large multi-centre trial investigating the effect of intercourse on fresh embryo transfer success is presently being conducted in Spain (Carlos Simon, *personal communication*). This trial has enrolled 400 cycles to date, and therefore may provide a more definitive answer on whether semen exposure has any role in assisted reproduction.

No formal assessment of participant's compliance with trial allocation was made during this trial, since this was felt to be too intrusive. All couples received a letter stressing the need for strict adherence to their trial allocation (Appendix A), a point that was also reiterated by telephone around the time of embryo transfer. A confidential answering machine was set up to take messages from trial participants who wished to be removed from the trial because of non-compliance with their trial allocation. Those couples who did not adhere to their trial allocation were not included in the final statistical analysis. However, it is possible that some non-compliant couples may still have been included in the trial analysis. The presence of prostate-specific antigen within vaginal secretions is reported to indicate that vaginal intercourse has occurred within the last 24 hours (Kamenev *et al.* 1989, Roach and Vladutiu 1993). This assay could have been used to monitor trial compliance, but was not used since this may have been perceived as an invasion of privacy by some patients.

The inability of the current trial to identify a significant effect of acute semen exposure on human implantation does not refute the concept that an immuno-permissive state may be generated by exposure to semen. Women, unlike mice, are usually exposed to their partner's semen on multiple occasions prior to conception, especially if there is some difficulty in conceiving. Therefore, it is possible that "chronic" semen exposure may have a cumulative affect on the generation of an immuno-permissive response, with a single additional exposure being unlikely to have any significant further effect. The observation of a reduction in the incidence of pre-eclampsia with increasing duration of sexual cohabitation (Robillard *et al.* 1994) supports the concept that chronic semen exposure is more important than acute exposure in the generation of an immuno-permissive response.

Differences in the timing of reproductive events between thawed embryo transfers and natural conception make it impossible to conclude from the current study that acute semen exposure does not play a role in natural reproduction beyond fertilisation. Following natural conception, the pre-implantation embryo remains within the fallopian tube for four days, not implanting until six days after fertilisation. Since natural conception can result from an episode of intercourse up to five days before ovulation (Wilcox *et al.* 1995), the uterine cavity may not be exposed to sperm for up to ten days before the blastocyst makes contact with the endometrium. This is in direct contrast to the IVF situation, where embryos are directly transferred to the uterine cavity, where they may come into contact with a recent post-coital inflammatory response (<48 hours old). Furthermore, the post-ovulatory surge in progesterone makes cervical mucous impenetrable to semen during a natural conception cycle (Carlstedt and Sheehan 1989), thereby preventing extension of the post-coital inflammatory response to the endometrium during the luteal phase. Cannulation of the cervix during an embryo transfer however may weaken this cervical mucous plug, raising the possibility that seminal plasma may reach the uterine cavity to create an endometrial inflammatory response that could influence embryonic development.

A significant proportion of couples begin to experience sexual difficulties once they have been diagnosed as infertile (Fagan *et al.* 1986, Hurwitz 1989, Gervaize 1993). These sexual difficulties are exacerbated by the anxiety associated with ART treatment and the loss of privacy in the area of sexuality (Lamont and Anderson 1993). Many couples allegedly experience feelings of guilt associated with sexual pleasure once it becomes obvious that sex will not result in conception (Meyers *et al.* 1995), and in turn abstain to minimise these feelings. A recent questionnaire examining sexual activity

during IVF treatment found that 40% of couples did not have sexual intercourse at all during the luteal phase of an IVF cycle (Read *et al.* 1996). Since only a small proportion of couples returned this questionnaire, it is likely that an even larger proportion of couples abstain during IVF treatment, given the inherent bias in such studies. Anecdotal reports from couples involved in this study suggest that apart from the psychological reasons for avoiding sexual activity, many couples did not have intercourse around the time of embryo transfer because of a fear of "dislodging" the embryo or introducing infection. The current data suggests that having intercourse around the time of embryo transfer does not increase the risk of pregnancy loss. This clearly is useful information to convey to patients, since it may reduce anxiety and improve couple's relationships during ART treatment.

Using ultra-sensitive hCG assays, Lenton *et al* (1988) found that 70% of IVF pregnancies ended in fetal loss, with three quarters of these losses being early biochemical pregnancies. This compares unfavourably with the pregnancy-loss rate of 31% seen in fertile women following natural conception (Lancaster 1985, Wilcox *et al.* 1988). Until recently, it was believed that the high rate of early embryo wastage observed during IVF treatment was due to deficiencies in embryo culture conditions or stimulation protocols. However, it has now been recognised that sub-fertile women experience a very high rate of early pregnancy loss, even during natural conception cycles (70% loss v 21% controls, Hakim *et al.* 1995). This suggests that the underlying cause of infertility, not its treatment, may be responsible for the poor pregnancy rates observed during IVF treatment. The loss of many "normal" embryos in infertile women has been suggested to be due to maternal immunological rejection of the conceptus (Stern 1997). Evidence supporting this theory includes the observation that administration of immunosuppressive drugs such as methyl-prednisone or intravenous immunoglobulin can increase viable pregnancy rates by reducing the incidence of embryonic loss (Polak de Fried *et al.* 1993, Coulam *et al.* 1994, Mottla *et al.* 1996). In addition, sensitisation of a mother to her partner's antigens by injection of paternal leukocytes has been reported to dramatically improve the viable pregnancy rate in a cohort of women who experienced multiple biochemical pregnancy losses during IVF treatment (Carp *et al.* 1994).

A cohort of infertile women experience recurrent pregnancy losses because of an adverse Th1 immune response against their partner's antigens (Sargent *et al.* 1988, Hill *et al.* 1995). It is possible that failure of these women to induce a Th2 immune response towards their partner's antigens may result from abnormalities in their partner's semen. The partners of a group of women experiencing idiopathic recurrent spontaneous abortion

have been found to express abnormally low levels of soluble HLA antigens in their semen (Gus Dekker, *personal communication*), which supports the postulate that a lack of antigenic priming during intercourse may lead to immune rejection of the conceptus. Similarly, a lack of seminal TGFβ, or a woman's inability to activate latent seminal TGFβ, may lead to an inappropriate immune response to her partner's seminal antigens. Ongoing studies comparing the level of soluble HLA antigen, $TGF\beta_1$ and PGE in the seminal plasma of fertile and infertile cohorts are currently being conducted (Gus Dekker, *personal communication*). This type of study may help determine whether exposure to semen does indeed play a role in human pregnancy outcome.

Chapter 10

General discussion and conclusion

10.1 The post-mating inflammatory response in mice and humans

In mice and humans, deposition of semen in the female reproductive tract provokes a cascade of cellular and molecular events that resemble a classic inflammatory response. The trafficking of leukocytes within the murine endometrium and their phenotypic behaviour appear to be under the control of steroid hormones and seminal factors acting through cytokines emanating from the uterine epithelium. The production of one of these cytokines, GM-CSF, is increased 20-fold following mating and is believed to play a central role in the recruitment of endometrial leukocytes.

One of the principal aims of the studies described in this thesis was to investigate the nature of the seminal vesicle-derived trigger(s) that stimulate GM-CSF release from the murine uterine epithelium. Fractionation of seminal vesicle secretions by size exclusion chromatography identified two moieties which could trigger the release of GM-CSF; an unidentified 650 kDa protein and the cytokine $TGF\beta_1$. These studies are the first to describe the presence of $TGF\beta_1$ within murine seminal vesicle secretions. Interestingly, seminal vesicle-derived $TGF\beta_1$ is secreted predominantly in a latent form. The murine uterus has the capacity to release bioactive $TGF\beta_1$ from seminal vesicle secretions since uterine luminal fluid collected from recently mated females contains significantly more bioactive $TGF\beta_1$ than fluid from unmated oestrous controls. The mechanism(s) behind activation are presently unknown, but may involve the enzymes plasmin and leukocyte glycosidase. Addition of $rTGF\beta_1$ to oestrous uterine epithelial cell cultures, or its intrauterine administration *in vivo*, resulted in an increase in GM-CSF production that was sufficient to initiate an endometrial leukocytosis comparable with that seen following mating. The receptivity of uterine epithelial cells to $TGF\beta_1$ stimulation fluctuates during the oestrus cycle, with oestrous cells releasing more GM-CSF following $rTGF\beta_1$ stimulation than dioestrous cells. In addition to initiating GM-CSF production, $TGF\beta_1$ may also directly mediate an endometrial inflammatory response through its own chemotactic activity for macrophages and neutrophils.

Subtle differences in endometrial leukocyte recruitment were observed between mated and rTGFβ_1-injected animals, highlighting the possibility that seminal plasma components other than TGFβ_1, such as the 650 kDa stimulating protein, may be required to initiate production of the full array of cytokines / chemokines needed to generate a post-mating inflammatory response.

At the outset of these studies, very little was known of the nature, location and molecular biology of the human post-coital inflammatory reaction. The presence of a cervical leukocytosis after intercourse had been reported, however the trigger(s) responsible for initiating this response had not been conclusively identified. From the experiments described in Chapter 8, it appears that mechanistic parallels do exist between mice and humans in regard to the manner by which the post-mating inflammatory response is generated. Human cervical and endometrial cell cultures were found to produce significant amounts of GM-CSF, with supernatants from cervical keratinocyte cultures possessing potent chemotactic activity *in vitro*. The addition of rTGFβ_1 to cervical cultures produced an increase in keratinocyte GM-CSF production and chemotactic activity. Neutralisation of GM-CSF bioactivity within these supernatants resulted in a 50% decline in chemotactic activity, implying that GM-CSF is one of a multitude of pro-inflammatory agents involved in the human post-coital inflammatory reaction, similar to the situation in the mouse.

10.2 Immune-deviating activity of seminal TGFβ_1

The introduction of TGFβ_1 to the murine uterus, in combination with paternal ejaculate antigens, may favour the growth and survival of the semi-allogenic fetus in two ways. Firstly, by initiating a post-mating inflammatory reaction, TGFβ_1 increases the ability of the maternal immune system to sample and process paternal antigens contained within the ejaculate. Beer *et al.* (1975) reported that initiation of maternal awareness towards paternal antigens through intrauterine immunisation of the mother with paternal cells resulted in an increase in fetal and placental weight. In Chapter 5, it was reported that intrauterine immunisation of females with sperm in combination with rTGFβ_1 resulted in a significant increase in fetal and placental weight compared to immunisation with sperm alone. Since the sperm inoculum used in these two groups was identical, it would appear that intrauterine administration of rTGFβ_1 may augment maternal processing of paternal antigens, thereby providing a reproductive advantage.

The second novel concept derived from these studies was that seminal $TGF\beta_1$ appears to initiate Th2 immune deviation. This finding was not unexpected, since $TGF\beta$ has been reported to favour induction of Th2 immunity following injection of foreign antigen into the anterior chamber of the eye. By initiating a Th2 immune response towards paternal ejaculate antigens, seminal $TGF\beta_1$ may inhibit the induction of Th1 responses against the semi-allogenic conceptus that are thought to be associated with poor placental development and fetal loss. Furthermore, the generation of non-complement fixing anti-paternal IgG_1 antibodies following mating may help mask paternal trophoblast antigens, thereby preventing their recognition by more destructive components of the maternal immune system. In addition, a Th2-bias favouring the generation of non-compliment fixing antibodies against ejaculate antigens may help prevent destructive humoral immune responses against sperm.

10.3 Role of seminal plasma in early murine and human pregnancy

From the studies described in Chapter 7, it is apparent that seminal vesicle fluid plays several roles in mammalian reproduction. Firstly, pregnancies sired by seminal vesicle-deficient males resulted in smaller litters than those sired by intact males. This reduction in litter size was principally due to a decrease in oocyte fertilisation secondary to reduced sperm numbers within the upper portion of the female genital tract. The mechanisms behind this observation were not explored, however the absence of a vaginal plug (Carballada and Esponda 1992), in combination with a lack of exposure to seminal vesicle-derived pro-motility factors (Peitz 1988), may account for the reduced number of sperm reaching the oviduct following mating with seminal vesicle-deficient males.

A second cause for reduced reproductive efficiency in pregnancies sired by seminal vesicle-deficient males may be an increase in implantation failure. The majority of semi-allogenic fetuses sired by seminal vesicle-deficient CBA F1 males were viable on day 17 of pregnancy, yet none of the embryos sired by fully allogenic seminal vesicle-deficient CBA males successfully implanted. Since both groups of fetuses were exposed to the same uterine environment, it is possible that differences in fetal "antigenicity" may play a role in fetal loss. Future experiments in which fully allogenic and semi-allogenic embryos are transferred to pseudopregnant females (mechanically induced) may help to further ascertain the role of seminal plasma in preventing rejection of the conceptus.

The IVF-intercourse study reported in Chapter 9 is the largest randomised control trial to date investigating the effect of semen exposure on early human pregnancy. Unfortunately, the predetermined sample size was not achieved because of low trial enrolment rates, thereby severely limiting the studies ability to identify small significant differences in pregnancy outcome between the two study groups. A greater proportion of transferred embryos implanted and progressed to viable pregnancies in women who were exposed to semen around the time of embryo transfer, but this trend was not statistically significant because of the low numbers of pregnancies in both groups. If the current non-significant differences in pregnancy outcome were maintained, a large study comprising of approximately 1000 IVF cycles would be required before statistical significance would be achieved. Such a study is currently under way in Spain (Carlos Simon, *personal communication*), and hopefully will determine if acute semen exposure has any role in human reproduction beyond fertilisation. Differences in reproductive physiology between "natural" and assisted conception cycles (eg the period of time between intercourse and arrival of the blastocyst in the uterine cavity, as well as differences in permeability of cervical mucous), may still make it impossible to draw firm conclusions on the role of acute semen exposure during natural conception.

The observation of an inverse relationship between duration of sexual co-habitation and the incidence of pre-eclampsia suggests that long-term semen exposure may be more important for human implantation success than acute exposure. This makes physiological sense since the human female is one of the few mammals exposed to her partner's semen on multiple occasions prior to conception. From an evolutionary perspective, it can be argued that induction of paternal antigen tolerance through repeated semen exposure may have reproductive advantages, perhaps by promoting implantation and survival of embryos conceived in long-term relationships where it could be argued that the male parent may be more committed to the well-being of the resultant child.

The immune-deviating activity of human seminal plasma may have some adverse consequences for reproductive health. It has been postulated that by inhibiting Th1 responses towards ejaculate antigens, seminal plasma may actually weaken the body's immune defences against sexually transmitted pathogens, thereby increasing the incidence of genital infections and viral-related disorders such as cervical dysplasia and condylomata (Kelly *et al.* 1997b).

10.4 Proposed model for the induction of an immuno-permissive environment favouring the growth and survival of the semi-allogenic mammalian conceptus

Survival of the conceptus from the time of its exposure to maternal tissues at implantation is dependent upon its ability to evade rejection by the maternal immune system. Because implantation does not occur until day 4 of pregnancy in mice and day 6-7 in humans, there is a short window of opportunity to initiate protective responses to prevent immune rejection of the conceptus. From a temporal view point, mating provides the ideal trigger for initiation of an immuno-permissive response. Inoculation with paternal antigens, in combination with tolerising agents such as seminal plasma TGFβ_1 and / or PGE, would occur in sufficient time to allow the activation and proliferation of appropriate cellular mediators so that protective immune responses would be in place by implantation. A schematic outline of the current working model surrounding the immunological events of early murine pregnancy is presented in Figure 10.1.

Mating results in the deposition of sperm, paternal somatic cells (leukocytes, genital tract epithelial cells), and latent TGFβ within the uterine lumen. TGFβ may become closely approximated to the uterine epithelium because of binding between extra-cellular fibronectin and the TGFβ latency protein. Plasmin produced by the oestrous uterine epithelium would be capable of enzymatically cleaving active TGFβ from the latency complex, thereby releasing bioactive TGFβ in close proximity to the uterine epithelium. Bioactive TGFβ could then initiate an influx of leukocytes into the endometrium by three mechanisms: increasing uterine epithelial cell GM-CSF production, increasing monocyte production of pro-inflammatory agents such as IL-1 and TNFα, and the direct chemotactic activity of TGFβ for macrophages and neutrophils.

A post-mating inflammatory response is likely to serve several roles. Firstly, neutrophils may prevent intrauterine infection by phagocytosing bacteria introduced at the time of mating, while also scavenging dead or dying sperm ("uterine housekeeping" role). Secondly, under the influence of the local cytokine environment, antigen presenting cells such as macrophages, dendritic cells and possibly even uterine epithelial cells may take up, process and present ejaculate antigens (sperm, somatic cells and soluble antigens) to T cells in the draining lymph nodes or endometrial stroma. The processing of antigen by APCs in an environment containing TGFβ is likely to initiate a Th2 phenotype within these responding T cells. Th2-deviated T cells could benefit the growth and survival of the semi-allogenic conceptus by preventing destructive Th1 cellular immune responses, while

favouring the generation of protective Th2 humoral responses. The production of non-complement fixing IgG_1 antibody specific for paternal placental antigens is reported to occur during murine pregnancy. By triggering the production of IgG_1, seminal TGFβ may mask paternal transplantation antigens, thereby preventing their recognition by more destructive elements of the maternal immune system such as cytotoxic T cells.

Decidual macrophages, present in an immuno-suppressive phenotype from the time of implantation, may inhibit NK cell lytic activity (Malygin *et al.* 1993, Lauzon and Lemaire 1994) through their release of molecules such as TGFβ, IL–10 and prostaglandin E_2. The local cytokine milieu created by exposure to semen increases the number of decidual macrophages, while also driving these cells towards an immunosuppressive phenotype, thereby inhibiting NK cell-mediated destruction of the conceptus. Furthermore, the presence of abnormally small placentae in NK cell-deficient mice (Guimond *et al.* 1998) suggests that appropriately activated uterine NK cells may play a role in the control of placental development, possibly by releasing growth promoting cytokines such as GM-CSF (Jokhi *et al.* 1994b).

Immunosuppressive macrophages within the lung, analogous to those found in the decidua, have been reported to inhibit the activation and proliferation of T cells, while maintaining T cell production of cytokines (Bilyk and Holt 1993, Upham *et al* 1995). Whilst the number of T cells within the decidua is minimal compared to NK cells, it is possible that T cell-derived cytokines may also play a role in the control of placental development, through direct effects of molecules such as TGFβ, or indirectly through immunoregulatory behaviour.

10.5 Future perspectives

The studies described in this thesis have identified $TGF\beta_1$ as a key component of murine seminal plasma that has a principal role in initiating the post-mating inflammatory response, and is implicated in inducing an immuno-permissive uterine environment favouring the growth and survival of the semi-allogenic conceptus. Additional studies are clearly required to investigate whether human seminal plasma TGFβ can initiate a post-coital inflammatory response in women, and whether this response has any role in the generation of paternal antigen-specific tolerance. It is possible that sub-optimal levels of $TGF\beta_1$ within human seminal plasma, or an inability of the female genital tract to release bioactive TGFβ, may inhibit the development of an immuno-permissive environment at

implantation, which in turn may have serious reproductive consequences (increased fetal loss, pre-eclampsia and IUGR). If this hypothesis is confirmed, intra-vaginal administration of $rTGF\beta_1$ shortly after intercourse, or in combination with exogenous paternal antigen such as paternal leukocytes, may favour the development of protective immunity. This may eventually prove to be a useful therapy for the prevention of pre-eclampsia and idiopathic recurrent spontaneous abortion, which at least in a proportion of women is associated with an overly aggressive maternal immune attack on trophoblast cells. Future therapies aimed at improving uterine receptivity, such as vaginal immunisation with paternal antigens in the presence of $TGF\beta_1$, may offer hope to infertile couples who are capable of conceiving naturally or through assisted reproductive techniques, yet are unable to carry the fetus beyond the first trimester because of hostile immune responses towards the semi-allograft. Therapies aimed at improving uterine receptivity are likely to provide the next major breakthrough in reproductive endocrinology now that the various techniques for achieving fertilisation (IUI, IVF and ICSI) have exhausted their developmental potential. Any therapy that increases uterine receptivity would have major therapeutic advantages over current assisted reproduction techniques, since acceptable pregnancy rates could be achieved from the transfer of a single embryo, thereby protecting the prospective mother and child from the risks associated with multiple birth, a common "side-effect" of current infertility treatment.

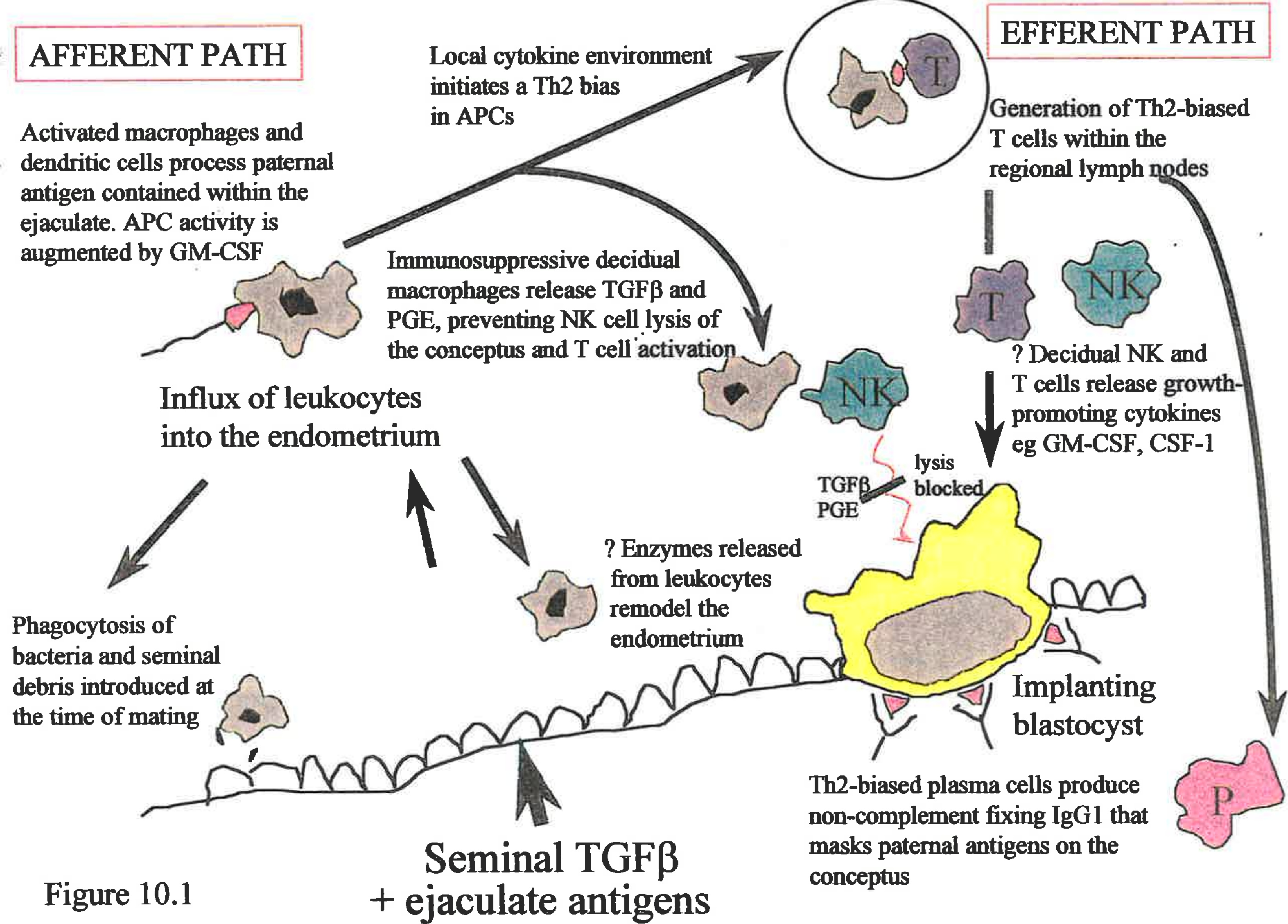


Figure 10.1

APPENDIX A

REPROMED
THE REPRODUCTIVE MEDICINE UNIT

Ref:RH/KT/mu/h24

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Dr T J Mudge
(Prov 0324887T)
Dr M L Bowen
(Prov 002986L)
Dr T G Davies
(Prov 045059AH)
Dr S L Girle
(Prov 497496F)

Dear

We would like to invite you to take part in a research project which is currently being undertaken by the Reproductive Medicine Unit in collaboration with the Department of Obstetrics and Gynaecology, University of Adelaide.

The enclosed information sheet outlines the aim of the project and the manner in which it is going to be conducted. Briefly, the aim of the study is to investigate a *possible* improved pregnancy rate if intercourse occurs at the time of embryo transfer. This benefit has in no way been proven so we need to do this study so that we can correctly advise IVF couples on the safety of intercourse while having treatment.

Please be assured that whether you decide to take part in the study or not will in no way affect the type of care that you receive.

If you have any further questions please do not hesitate to contact Dr. Tremellen (principal researcher) at the Department of Obstetrics and Gynaecology, University of Adelaide on 8303 5100 or email: ktremell@medicine.adelaide.edu.au.

If you are happy to join our study could you *both* sign the consent form and return it to Dr. Tremellen in the reply paid envelope.

With the very best of wishes,

Kind regards,

Yours sincerely,

ASSOC. PROF. R.J. NORMAN
Gynaecologist Repromed,
Associate Professor in the
Department of Obstetrics & Gynaecology
University of Adelaide

DR. KELTON TREMELLEN
Dept. of Obstetrics and Gynaecology
University of Adelaide

encl.

The University of Adelaide's clinical reproductive medicine programs are conducted by its wholly owned subsidiary company REPROMED PTY LTD ACN 008 123 466. Any profits generated are used to fund further research and development in reproductive medicine

Patient Information Sheet – Seminal Plasma Priming Study

Thank you for taking the time to consider your possible involvement in this new study. The purpose of this study is to investigate the possibility that normal substances in semen may improve the growth of the early embryo and help this embryo attach to the wall of the uterus. Several small studies in humans have a suggested that this may be the case but before we can make any firm decisions we need to investigate this in a much larger study. Animal studies in mice and pigs have shown that the presence of semen (the fluid that sperm swim in) may increase the size and numbers of babies born to the mothers. Because of all these studies suggesting a benefit we are eager to test this idea on a large group of couples. We have estimated that we will need to have the help of a few hundred couples so that we are able to answer the question once and for all.

If you decide to join our study you will be randomly (like the toss of a coin) put into one of two groups. The first group will be asked to have sexual intercourse (without a condom) at least once within 48 hours before or after the embryo is placed inside your uterus; while the second group will be asked not to have intercourse, or to use a condom if they do, for 48 hours before and after the embryo transfer. Neither the researcher nor yourself will be able to dictate which group you are allocated to since this must be done randomly. You will be informed of which group that you have been allocated to when you present for your routine blood tests a few days prior to embryo transfer. It is very important that you stay in this group and do not swap groups. If for any reason you are not able to stay in your allocated group (eg you had intercourse when you were in the abstain group or visa versa) it is very important that you contact us so that we can remove you from our analysis for that cycle. A confidential answering machine has been set up (phone 83690751) to enable you to easily contact us to remove you from the study for the above mentioned reasons (just leave name and date).

Before you consider being involved in this study we would like to make clear several points:

1. You will not be pressured into joining our study and if you do not wish to you will still get the usual excellent care from all the doctors and staff.
2. Even if you agree today to join the study you can change your mind later and leave with no problems.
3. It must be understood that this study is not going to definitely improve your chance of falling pregnant because the benefit of seminal plasma has not yet been proven (hence this study) and you have a 50% (half) chance that you will be placed in the group which receives no seminal plasma. There is however no scientific proof to show that seminal plasma is harmful to IVF pregnancies.
4. Your privacy will be respected at all times. We will need to look at your case notes 3 months after you have an IVF cycle so we can count the number of successful pregnancies in each of the study groups, but all the information that we take out of these notes will remain confidential and only be used for research purposes.

If you have any further questions about this study please fell free to ring either of the following researchers.

Dr. Kelton Tremellen	83035100 (work) 83691101 (home) or fax/answering machine 83690751
Sr. Helen Alvino	82226782 (work)

This study has been reviewed by The Queen Elizabeth Hospital Human Ethics Committee and has been felt to be safe and ethical. If you would like to speak to a person from The Queen Elizabeth Hospital who is not directly involved with the study you may speak to Mr. Paul Miller (Executive Officer of the Ethics Committee) on 8222 6871

Thank you for taking the time to consider helping us with this important study.

Chapter 11

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Errata

1. *page 58, line 13.* Insert the following text:

a. Sperm collection and preparation

Sperm used in immunisation studies were collected from stud CBA males. Males were killed by cervical dislocation and their reproductive tract (vas deferens, epididymis) removed and placed in sterile PBS. Gentle pressure was placed on the vas deferens with fine forceps to extrude sperm. The epididymis and vas were then cut into small sections and incubated at 37 ^{0}C for 15 minutes to enable sperm to leave these structures. The remaining solution was allowed settle at 1g for 5 minutes, before the sperm rich supernatant was harvested.

Human semen was collected by masturbation, placed in a sterile container and delivered to the laboratory within 3 hours of production. Once liquefaction had occurred, the semen was diluted 10-fold by the addition of human cervical keratinocyte culture medium (10% vol / vol).

2. *page 99, line 18.* At the end of this paragraph the following sentence should be added. " In the other three replicate experiments peak GM-CSF stimulation was achieved with similar doses of rTGFβ_1. (exp.1, 4 ng / ml rTGFβ_1 gave a peak stimulation of 800 % at 16 hours; exp. 2, 5 ng / ml rTGFβ_1 gave a peak stimulation of 525 % at 16 hours; exp. 3, 2 ng / ml rTGFβ_1 gave a peak stimulation of 305 % at 16 hours)."

3. *page 105, line 19.* At the end of this paragraph the following sentence should be added. " Purification of the intermediate molecular weight trigger, followed by amino acid sequencing, would be required before its identity can be conclusively identified as TGFβ."

4. *page 110, line 4.* Insert the phrase "(data not shown)" after the sentence " No TGFβ bioactivity was identified in any of the non-acid activated samples".

5. *page 167, line 10.* Insert the following sentence. "Oxygen free radicals generated by the action of FCS derived monoamine oxidase on seminal plasma spermine / spermidine may be responsible for this inhibition of cellular function (Allen and Roberts 1986)."

6. *page 204, line 15.* Insert the following citation. " Allen RD, Roberts TK. Seminal plasma immunosuppression: an irrelevant biological phenomenon? Clin. Reprod. Fert.; 4: 353-355."