REGULATION OF INFLAMMATORY MEDIATORS BY ANTIGESTAGENS AT THE UTEROPLACENTAL INTERFACE.

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CONTENTS

ABSTRACT.

The onset of parturition involves both cervical ripening and co-ordinated myometrial activity. There is increasing evidence that inflammatory mediators such as cytokines and prostaglandins are involved in this process. Cervical ripening is associated with degradation of the collagen by neutrophil derived matrix metalloproteinases. The cytokine interleukin-8 (IL-8) is a potent neutrophil chemoattractant and thus could be pivotal to the events leading to the onset of cervical ripening.

It is clear that progesterone is central to the maintenance of pregnancy and therefore antigestagens, such as mifepristone, have been utilised for termination of pregnancy in the first and second trimesters of pregnancy. There is also some evidence to suggest that the withdrawal of progesterone results in the onset of labour. The antigestagens provide a means to study the effects of progesterone withdrawal on the production of cytokines and prostaglandins by uterine tissues and thus to further elucidate the nature of the interaction between these mediators and progesterone at the time of parturition.

This thesis contains a randomised placebo-controlled clinical trial to assess the effectiveness of mifepristone in causing cervical ripening in women at term. This showed that the administration of 200mg mifepristone to primigravid pregnant women with unfavourable cervices at term significantly improved cervical ripening as compared with placebo ($p=0.01$), whereas the improvement following the administration of 50mg mifepristone to comparable women was just below the level of significance.

The role of antigestagens in the regulation of inflammatory mediators in uterine tissues is as yet unclear. Laboratory studies were performed to investigate the effects of antigestagens on the production of IL-8 and other mediators by placenta, decidua and cervix. These studies showed that third trimester placental and decidua obtained in the third trimester and cervical tissue from the first trimester all produced IL-8. In the third trimester, IL-8 was demonstrated in placenta and decidua by immunohistochemistry and shown to be localised to perivascular regions, which is in keeping with its putative role in neutrophil migration into uterine tissues. The production of IL-8 by placental explants was significantly increased ($p<0.05$) in samples collected after spontaneous vaginal delivery as compared with those from pre-labour caesareans. Culture of placental explants with antigestagens produced a variable, but generally increased effect on IL-8 production. For example, onapristone caused a significant increase in IL-8 production by explants from spontaneously delivered placentae. The effects of progesterone, dexamethasone and mifepristone on production of IL-8 by decidual explants was examined. Progesterone and dexamethasone caused a suppression of IL-8 production which was partially reversed by culture with mifepristone. These results did not however reach a statistically significant level, possibly due to the large variation in production between the samples. Cervical biopsies were obtained in the first trimester of pregnancy following pre-treatment with mifepristone at varying time-points and production of IL-8, monocyte chemoattractant peptide-1 (MCP-1, a cytokine), prostaglandin E_2 and $PGF_{2\alpha}$ was determined. The first trimester cervix produces all the mediators studied, regardless of whether mifepristone had been administered to the women prior to sample collection or not. There was an increase noted in the production of these mediators, particularly MCP-1, but again this did not reach statistical significance.

This thesis thus demonstrates that the inflammatory mediators studied, in particular IL-8, are produced in the placenta, decidua and cervix The alterations in this production shown after labour or antigestagen administration provide further evidence that these cytokines are integral to the mechanism by which parturition is initiated. The significant effect of mifepristone in promoting cervical ripening in women at term supports the hypothesis that progesterone withdrawal alters the production of inflammatory mediators so that parturition can ensue.

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Clinical study.

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Laboratory work.

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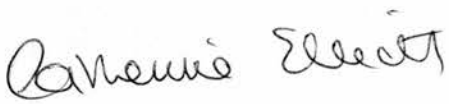
I would also like to acknowledge the unfailing support of my parents for which I am deeply grateful.

DECLARATION

This thesis has been composed by the undersigned. It is my own work, with the exceptions noted above. This thesis has not been submitted in candidature for any other degree, diploma or professional qualification.



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ABBREVIATIONS.

ACTH	adrenocorticotrophic hormone
COX	cyclo-oxygenase
FSH	follicle stimulating hormone
IL	interleukin
LH	luteinising hormone
MCP	monocyte chemotactic protein/peptide
MMP	matrix metalloproteinase
PBS	phosphate buffered saline
PG	prostaglandin
PGDH	15-hydroxyprostaglandin dehydrogenase
PGI	prostacyclin
PR	progesterone receptor
RU486	mifepristone
TNF	tumour necrosis factor
TXA	thromboxane
ZK98299	onapristone
ZK98734	lilopristone

AIMS

The hypothesis for these studies is that use of antigestagens, in particular mifepristone, will increase the production of IL-8 and other inflammatory mediators in uterine tissues increasing the influx of neutrophils into the uterine cervix allowing release of their lytic enzymes and leading to cervical ripening. The studies described examine the production of IL-8 by placenta. decidua and first trimester cervix and the effects of antigestagens on this phenomenon. The *in vivo* effects of the antigestagen mifepristone in ripening the third trimester cervix are studied in a clinical trial comparing the effectiveness of two doses compared with placebo.

Chapter I:

THE PHYSIOLOGICAL REGULATION OF PARTURITION.

Introduction.

The structure of the uterus and its modification for parturition.

1.1: The cervix uteri

1.1 i: Structure of the cervix

1.1 ii: Cervical modification associated with parturition.

1.1 iii: Role of collagenases and leucocytes.

1.2: The myometrium

1.2 i: Structure of the myometrium

1.2 ii: Myometrial contractions - intracellular events

1.2 ii: Myometrial contractions - intercellular events

1.2 iii: Modifications of myometrial structure and responsiveness associated with parturition

1.3: The fetal membranes

Regulation of the onset of parturition.

1.4: Prostaglandins

1.4 i: Structure and action of prostaglandins.

1.4 ii: Prostaglandin production by uterine tissues and alterations at parturition.

1.5: The chemokines

1.5 i Interleukin-8

1.5 ii: Monocyte chemotactic peptide-1 (MCP-1)

1.6: White cell populations in the uterus during pregnancy

1.7: The role of progesterone in the onset of parturition

INTRODUCTION.

The progression of pregnancy demands that the function of each component of the uterus alters as gestation progresses. With the continuation of pregnancy from implantation to parturition, the roles of the various parts of the uterus are required to reverse completely. The uterine cervix, for example, must remain closed and non-compliant throughout gestation to prevent premature delivery. During parturition, by contrast, it must efface and dilate to allow delivery of the fetus. Similarly, myometrial contractions prior to full term would threaten the continuation and viability of the pregnancy but become essential at term to effect delivery. The fetal membranes provide protection for the fetus, both as a physical barrier and by cushioning it in amniotic fluid, their preterm rupture can have severe effects on fetal growth and development or may indeed lead to delivery of the fetus. Around the time of parturition, however, they will usually rupture spontaneously.

The regulation of these functions during pregnancy and parturition remains to be fully elucidated. There appears to be a cascade of events culminating in the onset of labour in which prostaglandins, particularly prostaglandins E_2 (PGE_2), $F_{2\alpha}$ ($PGF_{2\alpha}$) and cytokines, such as interleukin-8 (IL-8), interleukin-1β (IL-1β) and tumour necrosis factor-α, (TNF-α) have been implicated. The effect of these and the many other factors involved in maintaining pregnancy and causing its termination at parturition are hypothesised to be under steroid and in particular progesterone, control. The physiology of the events leading to fetal delivery and the evidence for their control by endocrine and paracrine factors is reviewed in the following discussion.

1.1: THE CERVIX.

1.1i: Structure of the cervix.

The composition of the cervix is markedly different from the corpus of the uterus, in that it is composed mainly of a connective tissue of collagen and elastin embedded in a proteoglycan matrix[1] with only a small amount of smooth muscle present (<15%) as a thin layer at the periphery of the non-pregnant cervix[2,3]. By contrast, the corpus consists mainly of smooth muscle. The junction between the two uterine components is at the fibromuscular junction, which lies close to but is not synonymous with the internal os.

The majority of the connective tissue in the cervix is formed by collagen as was first described by Danforth in 1947[1] and confirmed by a further report in 1954[4]. There are five types of collagen which each contain three polypeptides coiled into a triple stranded helix. These molecules are extracellularly ordered into fibrils. About one third of the amino acid residues in collagen are glycine with proline and the 3- and 4-hydroxyprolines providing another 21 to 23%. The polypeptide helices are composed such that every third residue is glycine, the intervening residues vary between the types of collagen. Glycine does not contain a bulky terminal group and thus a tightly coiled helix can form. In the human cervix the collagen present is of types I (70%) and III (30%) [3,5,6]. In these types of collagen the collagen molecules are organised into a staggered longitudinal pattern. Collagen types II, IV and V are predominantly found in cartilage and basement membranes respectively, but small amounts of type IV collagen have been found in the human cervix [7]. Collagen is produced by fibroblasts, although smooth muscle may also be capable of its synthesis. It is synthesised on membrane bound ribosomes as pre-procollagen. This contains a signal peptide that ensures the passage of the peptide through the endoplasmic reticulum and which is then cleaved off to produce procollagen [8]

(molecular weight around 150,000), containing an N-terminal peptide extension and a COOH-terminal domain. Post-translational modification occurs intracellularly with hydroxylation of proline and lysine residues and glycosylation of hydroxylysine prior to the complete molecule being secreted from the cell via Golgi-derived vesicles. Once in the extracellular space, the procollagen extensions are enzymatically cleaved by NH_2-terminal and COOH-terminal procollagen peptidases and the collagen molecules thus produced aggregate into fibrils by intermolecular cross linking of lysine and hydroxylysine residues which are converted to active groups by the enzyme peptidyl lysine oxidase [7,9-11].

Another small component of the cervical connective tissue is elastin. Like collagen this consists of one third glycine residues but it has a lower content of proline and hydroxyproline and a higher proportion of valine and alanine residues. Unlike collagen, elastin is not formed into a triple helix. The elastin chains are cross linked by desmosines which form between lysine and allysine (formed by the action of lysyl oxidase on lysine). It is proposed that the elastin fibril contains regions known as 'oiled coils' which are spring-like and can stretch to 2 to 2.5 times the length of the molecule [10]. It is believed that it is the elastin in the cervix which is responsible for its elastic recoil during uterine contractions [12]. Elastin is degraded by elastases.

The proteoglycan matrix of the cervix, in which the collagen bundles and elastin are embedded, consists of heteropolysaccharide side-chains (glycosaminoglycans or GAGs) covalently linked to a central polypeptide core. The glycosaminoglycans all consist of repeating disaccharide groups, typically containing a large number of sulphate groups which endow the molecule with highly hydrophilic characteristics. The exception is hyaluronic acid which is not sulphated and not attached to a core protein. The predominant GAGs in the cervix are chondroitin-4 and chondroitin-6 sulphate and

dermatan sulphate (its epimer) [13,14], the attachment of either one of these to a small protein core forms decorin [7,15], the most abundant proteoglycan in the cervix. Other GAGs present in the cervix include keratan sulphate, heparan sulphate and hyaluronic acid. Hyaluronic acid is a much larger molecule than the other GAGs present with a molecular weight of up to $80x10^6$. It forms a backbone for the proteoglycan complex with core proteins and their attached glycosaminoglycan side chains attached to it [16]. The arrangement of the proteoglycans with their peripheral GAGs is thought to be essential to maintain the collagen alignment within, and thus the rigidity of, the cervix. The binding of GAGs to collagen is dependent on their length and their charged density. Those which contain iduronic acid i.e. dermatan sulphate and heparan sulphate bind more strongly than those lacking this residue. Dermatan sulphate has the capability to bind orthogonally to collagen fibrils and thus is important for the stabilisation of collagen whereas both hyaluronic acid and chondroitin sulphate bind weakly to collagen. Hyaluronic acid has also been noted to have a particularly high affinity to water [11,17].

1.1ii: Cervical modification associated with parturition.

The collagen of the cervix changes both qualitatively and quantitatively as labour approaches. The fibrils become dissociated from their tightly organised bundles to appear more widely scattered in an increased amount of ground substance [3,5,18]. The quantitative changes in the amount of collagen present has been reported to be of varying extents. The hydroxyproline content of the cervix has been measured as an equivalent for collagen concentration and a moderate reduction in the concentration following cervical dilatation has been reported in several studies [1,5,14,19]. This biochemical method of detection would also be expected to detect the depolymerised fibrils of collagen which are still biochemically intact although they have lost their structural rigidity and so it may not be the most accurate method by which to assess the functional effect of alterations in collagen

structure. In the sheep cervix during parturition, scanning electron microscopy showed a loosening of the collagen bundles in the cervical stroma [20]. In a refinement of this technique, Junqueira et al [21] described a histochemical method using a Picrosirius red stain which when viewed through a polarising microscope shows only oriented polymerised collagen as bright red or yellow bifringence but does not highlight dissociated collagen. Using this method they compared the histology of non-pregnant and intrapartum human cervices [22] and found that during labour the appearance of the collagen fibres showed irregularities, such as lack of continuity and variations in thickness that suggested the collagen was being corroded by a process of local degradation. Areas of amorphous material which were seen around these degraded fibrils were thought to be conglomerations of partially depolymerised collagen. Further evidence for the occurrence of collagenolysis in the cervix at term has been provided by the use of antibodies specific to the degraded $\alpha 2$ chain of type I collagen. Using this antibody an increase in this product was demonstrated by immunoassay and immunoblotting in the guinea pig cervix during labour [23]. These findings provide support for the hypothesis that cervical dilatation is associated with significant collagenolysis. The absolute concentration of collagen (as measured by extractable hydroxyproline) in the cervix has been shown to be inversely proportional to the degree of 'ripeness' of the cervix[24], In contrast the proportion of elastin in the uterus increases dramatically during pregnancy to about eight times the non-pregnant state, this increase rapidly reverses following delivery of the fetus[25]. These changes in structure of the connective tissue of the cervix seem likely to be causative of at least some of the functional changes in the cervix at term and during parturition.

There are also alterations in the GAGs of the cervical matrix at the time of parturition. The overall concentration of these substances has been described as rising 2.5-fold before the

onset of labour with an associated relative increase in the amount of hyaluronic acid (which is relatively more hydrophilic than other GAGs). This increase was noted in human cervical biopsies taken when the cervix was two to three centimetres dilated following which there was a decrease in the GAG content of cervices taken at six to eight centimetre dilatation with an associated decrease in the hyaluronic acid concentration[26]. Early work by Danforth et al[18] initially suggested an increase in GAG levels during labour but other groups suggest a precipitous fall in GAG levels after the onset of parturition, the first to decrease being dermatan sulphate, followed by decreasing amounts of hyaluronic acid and chondroitin sulphate[13,26] with a rise in heparan sulphate during the active phase of labour[17,18]. As heparan sulphate is localised mainly in vessel walls its increase during pregnancy and during parturition may be a result of the increasing vascularity of the cervix at this time[26]. In the guinea pig, circulating levels of hyaluronic acid significantly increase at parturition to around five times the antenatal levels, decreasing to the pre-pregnancy levels within two days post-partum[27]. These findings of marked alterations in the GAG content of the cervix in the course of labour have however, been disputed by a study which examined biopsies from non-pregnant and intrapartum cervices[22]. The relative quantities of glycosaminoglycans were determined after proteolytic digestion and microelectrophoresis. Although there was a relative increase in chondroitin sulphate and decrease in dermatan sulphate in the intrapartum samples it was commented that these were minor changes relative to the dramatic changes in histology and biochemistry of the cervix at this time and so of "relatively little importance in this process"[22]. Further studies, however,[17,28] have confirmed marked decreases in GAG levels in cervical tissue and in the lower uterine segment around the time of parturition. In a study of human uteri the concentrations of hyaluronic acid in the cervix, fundus and isthmus of the uterus were found to be decreased in term pregnant women as compared with the non-pregnant state[19]. Some of the discrepancies between these studies may have arisen due to the

precise timing at which the samples were taken. As described above in several studies there is an increase in the GAG content of the cervix prior to the onset of labour and during its initial active phase followed by a decrease in the concentration during the later phases of labour[26]. It follows that conflicting results could arise if the precise point during labour at which samples were taken is not clearly defined.

It is therefore apparent that there are changes occurring in the glycosaminoglycan content of the cervix associated with the development of a looser stroma with more hydrophilic properties. It is evident that rapid changes in the concentrations of GAGs can occur and that marked differences have been observed in the concentrations of these substances in late pregnancy and during parturition[26]. The precise nature and alterations in the relative concentrations of the individual GAGs and in particular the extent of any decrease in hyaluronic acid levels remains controversial. The cervix at term and during labour contains a higher proportion of water than in the non-pregnant state, reported in one study to be 78.4% compared with 74.4% [18] and in other work to increase to as much as 86% of the cervical content[14].

These biochemical changes in the cervix are accompanied by changes in its physical properties as parturition approaches and throughout labour. The cervix softens, shortens (effaces) and then dilates to allow passage of the fetus. In the sheep cervix the stretch modulus of the cervix (that is the ratio of stress to strain) decreases gradually during pregnancy and then exhibits a marked decrease immediately prior to the onset of labour[29].

The changes in structure of the cervix at the time of parturition, with a decrease in the concentration of collagen and its dissociation, a looser matrix and an increase in the water content means that the cervical tissue offers low resistance to force applied and the fibres

will move under tension. During myometrial contractions cervical tissue is thus drawn up into the lower segment of the uterus, in the processes of effacement and dilatation[11].

1.1iii: Role of collagenases and leucocytes.

Studies such as that of Junqueira [22] described above confirm that collagenolysis occurs in the term cervix which in all probability facilitates cervical ripening and dilatation. Collagen extractabilty is the solubility of collagen following standard pepsin digestion and there is a four-fold increase in this parameter during cervical ripening suggesting that there is active lysis of collagen occurring at this juncture. The main lytic enzymes with effect in the cervix are collagenase and elastase.

Collagenases are members of the family of matrix metalloproteinases (MMPs). The MMP family are responsible for the degradation of components of the extracellular matrix. All members of the MMP family contain three domains, the 'pre' domain signalling for cellular export, the 'pro' domain, the removal of which activates the enzyme, allowing zinc binding to the 'catalytic' domain[30]. Some of the MMP members involved in the degradation of collagen also contain a haemopexin domain which allows them to cleave fibrillar collagen. MMP-1 (interstitial collagenase) has specificity for collagens type I, II, III and X, MMP-8 (neutrophil collagenase) is specific for collagens I, II and III and MMP-13 for collagen I. The specificity of MMPs is diverse and other MMPs are also involved in collagen and elastin degradation such as MMPs 2, 3, 9, 10, 11 and 12[30]. The activity of the MMPs is inhibited by tissue and plasma inhibitors, the former being known as TIMPs (tissue inhibitors of metalloproteinases).

The MMPs catalyse the initial hydrolysis of collagen by cleaving each of its three chains between residues 771 and 772 into two fragments. The cleavage products thus produced

are more susceptible to thermal denaturation than intact collagen and it is proposed that they denature at body temperature to gelatin which is further degraded by MMPs acting as gelatinases. The small peptides thus produced are then be further cleaved.

MMPs have been demonstrated in human fetal membranes and it has been shown, in particular, that MMP-3 and 9 increase as labour begins and then decrease following delivery[31,32]. Levels of collagenase, both active and latent forms, extracted from the uterine lower segment (which was assumed to correlate with levels in the cervix in the study) increased thirteen to fourteen fold during cervical dilatation at term as compared with prior to labour[2] During labour there is a significant increase in the levels of circulating collagenase[33]. In samples of pregnant guinea pig cervices, levels of procollagenase increase 6-fold and levels of its net activity double at parturition[34]. The role of collagenase has been studied further using a technique by which active collagenase was extracted from cervical biopsies and its activity in degrading radiolabelled collagen assayed. Biopsies were collected from the posterior cervical lip of non-pregnant and pregnant women at differing stages of gestation and labour[35]. There were no significant difference in the levels of active collagenase in non-pregnant women, those in the first trimester of pregnancy and those in the early stages of labour. However in the final stages of cervical dilatation (from 6 to 8cm) collagenase activity rose significantly as compared with the first trimester of pregnancy and as compared to the third trimester levels pre-labour. This increase in cervical levels of collagenase during labour has been confirmed in a more recent study[28] using biopsies from the posterior cervical lip during pregnancy and from the lower uterine segment at elective caesarean.

In addition to the increase in collagenase noted during active labour there has also been shown to be an increase in the degradation product DNP-peptidase which has been

reported as between 3 to 4 times the levels found pre labour[19,36] and in PZ-peptidase, also a product of collagen catabolism, which doubles its levels in the cervix at term.[37].

Fibroblasts, which are present in the cervix are capable of synthesising collagenase, but there is accumulating evidence that other cells, in particular neutrophils are implicated in the increased collagenase release into the cervix around the time of parturition. Neutrophil collagenase, also known as MMP-8[38] was sequenced in 1990[39] and found to have 72% chemical similarity with fibroblast collagenase with four additional glycosylation regions.[39]. In contrast to fibroblasts which constitutively synthesise and release collagenase, neutrophils synthesise and store collagenase in intracellular granules during their development in the bone marrow. MMP-8 has been sequenced and found to contain 467 amino acids with areas of homology to both fibroblast collagenase and other metalloproteinases[39]. MMP-8 has a different rate of hydrolysis of collagen substrates to tissue collagenase (MMP-1). MMP-1 cleaves collagen type III about 15 times more rapidly than it cleaves collagen type I whereas MMP-8 cleaves collagen type I about 20 times faster than it cleaves collagen type III[40]. This specificity of MMP-8 correlates with the proportions of collagen types I and III present in the cervix. In biopsies of non-pregnant cervices, the most common cell population is fibroblasts whereas in the intrapartum period there is a heavy infiltration of neutrophils into the cervical stroma, in addition to which there are increased numbers of mast cells and macrophages in the intrapartum cervical stroma[22]. In this study the neutrophils were particularly seen in association with venules and appeared to be migrating from them. These histological findings support the proposal by Liggins that cervical ripening resembles an inflammatory reaction and that an explanation of the process of cervical ripening must account for this similarity[41]. The increase in collagenase described during labour has been examined[26,35] using in-situ hybridisation to fibroblast collagenase specific mRNA. Using messenger for

fibroblast collagenase this was not demonstrated in cervical biopsies obtained from women in labour. Examination of the polymorphonuclear leucocyte population in the cervix, however, showed a marked accumulation of these cells in the capillaries of the cervix at term, with massive stromal infiltration occurring at the onset of cervical dilatation and subsequent immunofluorescent evidence of degranulation of these leucocytes to release human leucocyte collagenase detectable with specific antibody[26,35]. An increase in leucocyte elastase activity in the cervix as pregnancy progresses has also been described[42]. It thus seems that the collagenase demonstrated in the cervix at term is derived from neutrophils. Cultures of rabbit cervical explants have been described as releasing a neutrophil chemoattractant agent which is thought to be interleukin-8 (IL-8), a potent attractor and activator of neutrophils. IL-8 is also present in and released by many uterine tissues, including human cervix[43]. The potential role for this cytokine in neutrophil recruitment and activation at the onset of parturition is explored further below.

During cervical ripening and dilatation at term in human pregnancy therefore, there is degradation of collagen which is evident both histochemically and biochemically, the former method of investigation providing a truer representation of the dramatic alterations in collagen structure during this period. These catalytic changes in collagen are mediated by collagenase, the levels of which have been demonstrated to increase both peripherally and in the cervix during labour. Further examination of the source of this collagenase is supportive of its being released from the specific granules of neutrophils as opposed to being synthesised by the cervical stromal fibroblasts. This hypothesis is further supported by the marked infiltration of neutrophils into the cervical stroma at term and demonstration of their subsequent degranulation.

1.2: MYOMETRIUM

Alterations in myometrial activity occur concurrently with cervical ripening and dilatation, the two being facets of the same process which leads to delivery of the fetus. The factors that regulate the timing of parturition must therefore act on both uterine areas. The aim of the studies described in this thesis was not to investigate the mechanisms of myometrial contractions but the alterations in production of mediators examined in the cervix, decidua and placenta could be expected to affect the myometrium. The physiology of myometrial contractions is therefore considered below and the changes at the time of parturition discussed.

1.2 i: Structure of the myometrium

Myometrium consists of smooth muscle fibres embedded in an extracellular material that is mainly composed of collagen, this connective tissue matrix facilitates transmission of the contractile force generated by the muscle fibres. In contrast to striated muscle, smooth muscle fibres exist in irregular bundles, this organisation enables the contractile force of the myometrium to be exerted in any direction and ensures that the uterine morphology can alter to accommodate a fetus of varying size and position[44]. The contractile unit of muscle is composed of actin and myosin which slide along each other to generate contraction by the formation of cross-bridges between the myosin heads and the actin molecules. Myosin (molecular weight about 500kDa) acts as both a structural protein and as an enzyme which utilises the chemical energy of ATP to generate the mechanical force of a contraction. The myosin molecule contains a head, a region where the ATPase sites are located and where interaction with actin occurs and a tail region. Myosin consists of two 'heavy" chains (around 200kDa) and two pairs of "light" chains (15 to 20kDa)[44]. It is the phosphorylation and dephosphorylation of these light chains that regulates the interaction

between myosin and actin. The latter is a protein with molecular weight of 45kDa. Monomers of actin polymerise to form long thin filaments.

1.2 ii: Myometrial contractions - intracellular events.

For contraction to occur, the myosin light chains must be phosphorylated prior to their interaction with the actin molecules[45,46]. This reaction is catalysed by myosin light chain kinase (MLCK). This enzyme is, in turn, activated by the combination of calcium with calmodulin[47], the binding of these two agents causes a configurational change in calmodulin exposing a hydrophilic domain that interacts with MLCK[46]. When calcium is removed from the system dephosphorylation occurs rapidly leading to muscle relaxation[12]. Relaxation can also occur by the action of myosin light chain phosphatase which removes the phosphate group from myosin light chains[48]. There is a strong positive correlation in human myometrium between the levels of intracellular calcium and the rate of myosin light chain phosphorylation[47]. Stimulation of myometrial cells in culture with oxytocin produces wave-like changes in the levels of intracellular calcium as shown by fluorometry[12]. In order for a contraction to occur intracellular calcium levels need to increase from 10^{-7}M to 10^{-5} M[49].

The system of myometrial contractility is controlled via a subtle network of interacting second messengers. Calcium is available within the cell either following its release from vesicles in the intracellular sarcoplasmic reticulum which is the main intracellular calcium store, or after entry into the cell across extracellular channels. Release of intracellular calcium from the sarcoplasmic reticulum is promoted by inositol 1,4,5-triphosphate ($InsP_3$) acting to open a calcium channel. Phospholipase C acts on phosphatidylinositol 4,5-biphosphate (PIP_2) to hydrolyse it releasing $InsP_3$ and 1-2-diacylglycerol (DAG). The uptake of calcium (and therefore its removal from availability to activate MLCK) into

the sarcoplasmic reticulum is facilitated by cyclic AMP via the activation of protein kinase A (PKA). PKA also inhibits contractions by phosphorylating MLCK thereby decreasing its affinity for the calcium-calmodulin complex. In addition to its release from intracellular stores calcium can also enter the myometrium cell across the cell membrane via receptor or voltage mediated channels. Depolarisation of the membrane to the required extent activates the voltage regulated channels allowing calcium influx into the cell. The plasma membrane also contains calcium/sodium ion 'pumps' which remove calcium from the intracellular space during relaxation of the muscle cell. Exogenous agents such as hormones or drugs acting on the myometrial cell do so via cell membrane receptors that are coupled via G-proteins to enzymes such as adenylyl cyclase which produces cAMP from ATP or phospholipase C converting PIP_2 to $InsP_3$ and DAG. Adrenaline, for example can act via β-receptors to increase intracellular cAMP causing muscle relaxation or via α-receptors to increase $InsP_3$ and decrease cAMP leading to contraction.

1.2 ii: Myometrial contractions - intercellular events.

Calcium can enter myometrial cells via voltage dependent channels when the resting potential of the membrane is altered as occurs when waves of depolarisation spread between myometrial cells. This spread of depolarisation is facilitated by intercellular connections or gap junctions that have low electrical resistance[50,51] and which also enhance metabolic coupling between cells[52]. Gap junctions are composed of six identical proteins, connexins, arranged in a hexameric array which are functional when paired across an intercellular membrane [49]. They were first described in 1967[50] and identified by Garfield in human myometrium in 1977[53]. The myometrial gap junction protein is coded for by the connexin 43 gene[54] which is found on chromosome 6. Agents such as β-adrenergic agonists that relax the myometrium are capable of closing gap junctions[49].

The regulation of changes in the gap junctions around the time of parturition is discussed further below.

Oxytocin (from the Greek 'rapid birth') is a nonapeptide secreted from the posterior pituitary and is structurally similar to vasopressin, the other nonapeptide secreted from this site. Both are formed from large precursor polypeptides in the supraoptic and paraventricular nuclei of the hypothalamus from whence they are transported to the pituitary gland via the axons of hypothalamic neurones bound to carrier proteins (neurophysins) and are then stored in the pituitary prior to their release [10]. The major actions of oxytocin are to cause expulsion of milk by means of the neurogenic reflex, the 'milk letdown response' and to stimulate of uterine contractions. It also weakly mimics the effects of vasopressin, such as inhibition of water diuresis, an important consideration if large amounts of oxytocin are to be administered exogenously to stimulate uterine contractions. The 'Ferguson reflex'[55] whereby oxytocin release would be increased by cervical stimulation was postulated by Ferguson in 1941 but has not been proven to occur in humans in whom vaginal distension may have a more potent effect than cervical stretch[56]. Following its release into the bloodstream oxytocin is carried to the uterus where it exerts its uterotonic effects via membrane receptors in addition to that produced locally. Once bound to its receptor oxytocin inhibits the ATPase membrane pump which moves calcium from the intracellular to the extracellular area [46]. It also promotes the influx of calcium from both the sarcoplasmic reticulum and the extracellular area into the intracellular area [44]. Oxytocin receptors are also found in the decidua, in increasing numbers at the time of parturition[57] and are thought to interact with other agents involved in the onset of parturition, in particular the prostaglandins. Oxytocin receptors have also been described in the amnion and choriodecidua of human fetal membranes at term[58].

1.2 iii: Modifications of myometrial structure and responsiveness associated with parturition.

It was first noted by Braxton-Hicks in 1872 [59] that from early pregnancy the uterus exhibits uncoordinated myometrial activity. More recently a pattern of contractility termed contractures has been observed in pregnant animals[60]. These contractures were initially described in sheep from around day 60 of gestation (term being about 150 days)[59]. They are prolonged low amplitude increases in intrauterine pressure and have subsequently been demonstrated in many other species including non-human primates such as the Rhesus monkey [61,62]. They seem to be part of the intrinsic activity of uterine muscle as they continue to occur in strips of sheep myometrium transplanted away from the uterus [63]. As pregnancy progresses contractures eventually switch to uterine contractions which are more regular, shorter lasting and are associated with greater intrauterine pressures. In sheep and cows this switch from contractures to contractions occurs once prior to labour and delivery whereas in non-human primates it occurs several times commencing a few days prior to the onset of labour. These switches demonstrate a photoperiodicity, generally occurring when lights are switched off [64]. There is also a circadian rhythm to these switches demonstrable even when animals are kept at the same light intensity throughout an experimental period[65]. The onset of parturition in many animals, including non-human primates, is therefore associated with an alteration in the pattern of uterine activity to produce stronger more co-ordinated contractions, which in the presence of a ripening cervix will effect labour and delivery of the fetus. In mechanical terms it is more efficient to cause deformation of a visco-elastic structure, such as the cervix, by applying loads of a small amplitude over an increased length of time, such as with regular myometrial contractions, rather than a larger load over a shorter time period[66]. It will, however, be more difficult to establish such changes in the human female as to do so would require prolonged insertion of intrauterine pressure recording devices which could

potentially introduce infection and may not be ethically acceptable. External monitoring of uterine activity via a tocograph is, unfortunately, not accurate enough to reliably detect these changes.

Intercellular communication.

The onset of regular co-ordinated uterine activity at the time of parturition requires the myometrial cells to function as a syncytium with rapid communication between them. In rat myometrial strips the diffusion of 2-deoxy-D-glucose through cells is greater in myometrium from parturient rats than from ante or post-partum ones[67]. Samples from the rats in labour were also noted to have an increase in the area of gap junctions, a finding confirmed by another study[68] which showed an increase in gap junctions in the myometrium of rats commencing six hours prior to delivery. The area of gap junctions underwent a rapid decline post-partum. Communication of electrical signals between myometrial cells also increased at the time of parturition. Increased propagation of electrical excitation was shown in myometrial strips from uteri during parturition as compared to those at earlier gestations[69]. In women the appearance of gap junctions in myometrium sampled at the time of caesarean section has been described by Garfield and Hayashi[70]. In myometrium obtained from women having an elective caesarean section four of eleven samples contained demonstrable gap junctions whereas there was a significant increase in the area of tissue containing gap junctions found when tissue was obtained at caesarean during labour. A similar increase in the number of gap junctions was seen in tissue from women in preterm labour as compared with those not in labour at term. In the same study correlations were shown between the area of gap junctions and the frequency of uterine contractions and the area of gap junctions and the stage of cervical dilatation. It was estimated that around 250 gap junctions per cell appear at the time of parturition in women[70]. Parturition is therefore associated with an increase in intercellular

coupling via gap junctions which will decrease the impedance to the flow of electrical current between the cells and which also facilitates the diffusion of metabolites between cells. The formation of this functional syncytium at the time of labour explains the gradual synchronisation and co-ordination of the myometrium that is seen preceding and during labour.

Oxytocin and oxytocin receptors.

There has been some controversy over the alterations in oxytocin levels during pregnancy and parturition, indeed oxytocin physiology has been described as 'a graveyard of scientific reputations'[64]. Initial studies in monkey[71] and human[72] females prior to and during labour did not demonstrate convincing alterations in oxytocin levels. In 1991 Fuchs et al readdressed this issue[56]. They suggested that the measurement of oxytocin levels may not have been accurate in previous work for several reasons such as the low physiological levels of oxytocin, the limitations of previously used assays and the rapid degradation by oxytocinase of oxytocin in stored plasma. In their study they performed serum sampling at one minute intervals over a thirty minute period in women prior to labour and in the first, second and third stages of labour. They found that oxytocin was released in a pulsatile manner and that there was a significant increase in the frequency of these pulses during labour and a further rise in their frequency during the second and third stages of labour. There was also a less marked increase in the amplitude and duration of the pulses in the second and third stages of labour.

The response of the myometrium to oxytocin is dependent not only on the absolute serum levels of oxytocin but also on the concentration and affinity of the tissue receptors for oxytocin. The uterus demonstrates increasing sensitivity to oxytocin as pregnancy progresses, for example in the non-pregnant state 100mU of oxytocin is required to

produce a contractile response in the myometrium, whereas at term the same response can be elicited by 1 or 2 mU of oxytocin[73]. This increased sensitivity could be mediated by an alteration in the receptors for oxytocin in the myometrium. Oxytocin specific binding sites have been described in human myometrium and decidua[73,74]. A specific G protein receptor for oxytocin was described in 1992 containing seven trans-membrane domains[75]. When myometrial strips were taken from uteri at the time of hysterotomy or caesarean section at varying gestations it was found that the concentrations of oxytocin receptors rose markedly as gestation progressed. In the second trimester the concentrations were six times those found in the non-pregnant state, at 37 to 41 weeks they were 80 times higher and in early labour there was a further 2.5 increase in the pre-labour levels [74]. When tissue was obtained from caesarean sections in the later stages of labour (after 7cm dilatation) much lower concentrations, around 2.5 times the early pregnancy levels, of receptors were found. One explanation for this was postulated to be due to the lower levels of oxytocin receptors in the isthmus of the uterus as compared with the fundus and corpus. Tissue collected from caesarean sections in the later stages of labour may have been taken from an incision lower in the uterus with lower levels of receptors than the higher regions. The affinity of oxytocin receptors for the hormone was not found to alter during gestation. An increase in the mRNA for the oxytocin receptor has also been demonstrated in human myometrium at the time of parturition with levels over 300 times higher than those found in the non-pregnant myometrium[76]. This study also demonstrated that the distribution of the mRNA for the receptor was heterogeneous in the myometrial cells. Similar findings of increased mRNA for oxytocin at the time of labour have been reported in sheep[77] and rat[78] myometrium. The decidua also contains oxytocin receptors[73,74] which also increase in number, but not affinity, from early to late pregnancy. Decidua parietalis obtained from the uterine surface was found to have a greater concentration of receptors than the decidua vera from the surface of the chorion[74].

An increase in the number of oxytocin receptors is also seen in the fetal membranes, both amnion and chorio-decidua, during labour[58].

There is therefore an increase in the frequency of oxytocin pulses and an increase in the myometrial and uterine responsiveness to oxytocin, via an increase in the number of receptors at the time of parturition. There may also be an alteration in the G protein coupling of the receptor at this time which would further increase the uterine response to oxytocin[12].

1.3: THE FETAL MEMBRANES.

The fetal membranes consist of the inner amnion and the outer chorion. The latter is in close association with the decidua and cells derived from this layer are often termed choriodecidual. The tensile strength of the fetal membranes is due to their connective tissue component which has a high concentration of collagen[79] which as in the cervix is predominantly types I and III[80]. The amnion is thinner than the choriodecidua but it is stronger[81]

In the majority of women the membranes rupture late in the first stage of labour but in around 10 per cent they rupture at term prior to the onset of established labour[82]. Furthermore, it is estimated that 40 per cent of preterm labours are preceded by rupture of the membranes[83]. In one study in which membranes that had ruptured before term were compared with those that had ruptured at term it was found that the former were thinner near the rupture site with a lower modulus of elasticity near the placenta[84]. It was not apparent however whether these changes occurred before or after the membranes had ruptured at this site. A later study[85] describes an area of altered morphology in the

membranes which forms part but not all of the rupture site. As this area is smaller than the ruptured area it was suggested that it existed prior to and indeed may have been the focus for the membrane rupture. The area of altered morphology contained thicker connective tissue layers with a thickened and swollen spongy layer seen between the membranes. Despite this layer, in this area the membranes showed an overall decrease in thickness of 35% due to the marked thinning of the decidual and trophoblast layers. It is proposed that in this area of altered morphology the increase in separation of the two membranes means that they act as separate components thus having decreased strength as compared to their action when functioning as a unit. This study excluded any membranes with evidence of infection which is a factor that may be associated with abnormal membrane rupture. Contact with group B streptococci or with Staphylococcus *aureus* will decrease membrane integrity as measured by *in vitro* outcomes, such as bursting tension, elasticity and work required to rupture the membranes. This effect is enhanced by the addition of neutrophil elastase[86].

Rupture of the fetal membranes may therefore occur at an area with altered collagen composition, infection may act on this site or on a normal area to cause distortion of the collagen. The role of collagenases, in particular those derived from neutrophils in causing this distortion remains to be more fully elucidated.

REGULATION OF PARTURITION.

Many and various factors have been implicated in the onset and establishment of labour and delivery. There would appear to be a complex interaction of local factors which act in a cascade with internal feedback mechanisms to culminate in cervical dilatation, regular myometrial contractions and delivery. In some animals, such as the sheep, the mechanism for the initiation of parturition has been much more clearly elucidated than in the human. In sheep it is known that alteration of fetal steroid production acts via ovine placental steroid production to cause increased prostaglandin production from the fetal cotyledons and chorioallantois[87]. In the human female, however, such a clear pathway has not been proven and the mechanism of parturition cannot be assumed to follow this model. It is much more difficult to obtain information as to the levels of prostaglandins and other hormones at various stages of normally developing pregnancies in women than in experimental animals. In many species, but not in women, a decrease in serum progesterone or in the progesterone : oestrogen ratio is seen immediately prior to the onset of labour.

Prostaglandins, in particular PGE_2 and $PGF_{2\alpha}$ occupy a pivotal role in this system and their local concentrations may be at least partially under progesterone control as will be discussed further below. In addition to the prostaglandins, it seems that other local effectors participate in the cascade leading to parturition. These are the cytokines, which are involved in local inflammatory responses and also in altering cell populations and activities.

There are therefore many agents to be considered in a discussion of the events leading to parturition. In the following it is proposed to focus on the alterations in local levels of

prostaglandins, their interaction with the cytokine IL-8 in recruiting neutrophils into uterine tissues and the possible control over this system exerted by progesterone.

REGULATION OF THE ONSET OF PARTURITION.

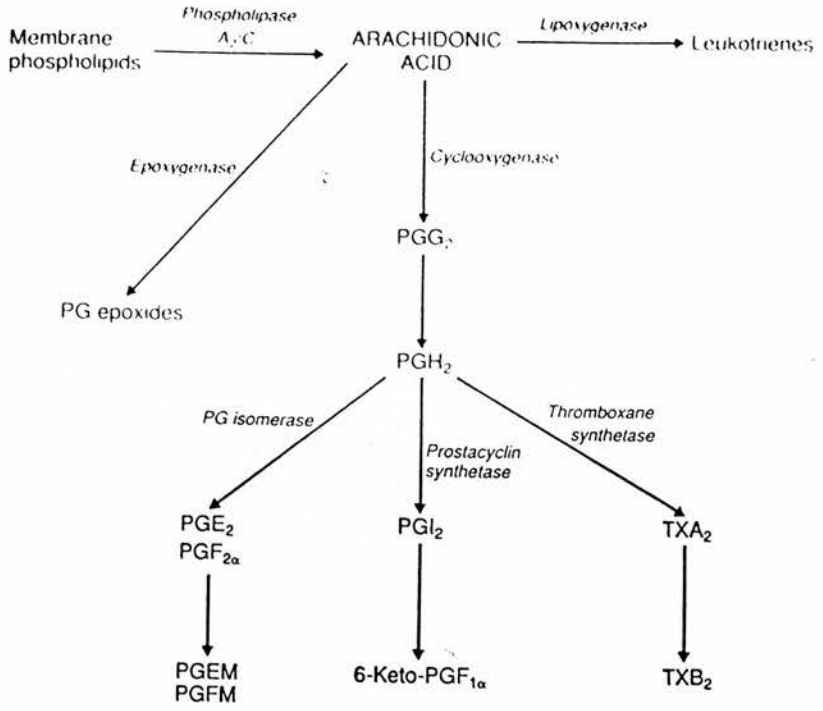
1.4: Prostaglandins

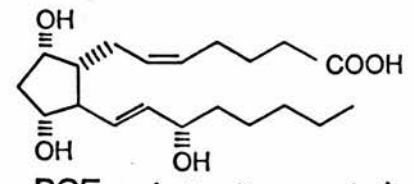
1.4 i: Structure and action of prostaglandins.

Arachidonic acid is the precursor of several families of compounds that exert diverse biological effects in many systems including the reproductive organs. These include prostaglandins, thromboxanes and leukotrienes as well as the hydroperoxy and hydroxy fatty acid derivatives. These agents are not typical of endocrine hormones as they are produced in almost all tissues rather than in specialised glands, they are not stored to any great extent and they generally exert local effects[10]. The prostaglandins were named by von Euler in 1935 as a term for prostatic extracts and their configuration was determined in 1966 by Nugteren[88]. They are a family of related C_{20} carboxylic acids which contain a cyclopentane ring from which two aliphatic side chains protrude ending in a carboxyl and a methyl group. Prostaglandins are divided into the A, B, C, D, E, F and J series. Prostaglandins G and H are unstable endoperoxides that are intermediates in prostaglandin biosynthesis. In addition are prostacyclin (PGI_2) and thromboxane (TXA_2) which are derived from the same endoperoxides but which do not fit into the simplified scheme of nomenclature[88].

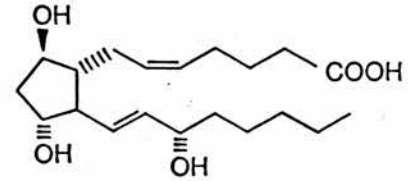
The two series of most relevance in reproduction are the E and F series (figure 1.1). These each consist of three members i.e. E_1, E_2, E_3, $F_{1\alpha}$, $F_{2\alpha}$ and $F_{3\alpha}$. The subscript numerals in this classification refer to the number of double bonds in the side chains. These two series of prostaglandins differ from one another in that the PGEs have a C-9 keto oxygen and a C-11 hydroxyl group in the cyclopentane ring whereas the PGFs

Figure 1.1: The synthesis and structure of prostaglandins.

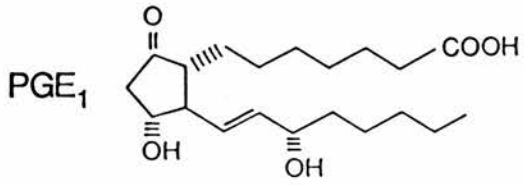


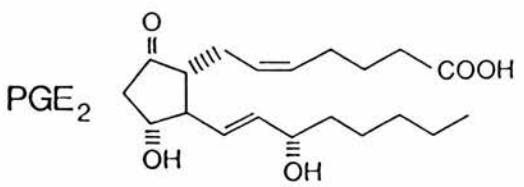


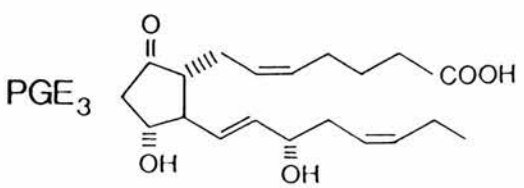
$PGF_{2\alpha}$ (naturally occurring)



$PGF_{2\beta}$







contain a hydroxyl group at both positions[10]. Of these compounds it is PGE_2 and $PGF_{2\alpha}$ which have the most biological and clinical importance. Prostaglandins of the '$_2$' series are derived from arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid). This is either ingested in the diet or formed from linoleic acid. It is derived as a precursor for prostaglandin synthesis from membrane phospholipases. As prostaglandins are not stored to any appreciable extent in tissues their rate of release is regulated by the availability of arachidonic acid and thus by the actions of the phospholipases that liberate it from membrane phospholipids. The main phospholipase is phospolipase A_2 but phospholipase C can also act to release arachidonic acid in conjunction with diacylglycerol lipase.

Synthesis.

The conversion of arachidonic acid to prostaglandins is via the action of prostaglandin synthetase which is a membrane bound multi-enzyme complex. One component of this is cyclooxygenase (COX) or prostaglandin endoperoxide synthetase which catalyses the conversion of the substrate into the unstable intermediates PGG_2 and PGH_2 by oxygenation. This step requires the removal of a hydrogen ion at C13 and the addition of an oxygen molecule at C11[10,88]. These intermediates are then converted into the prostaglandin series by prostaglandin endoperoxide isomerases in the case of PGE or reductases for PGF or into prostacyclins or thromboxanes by prostacyclin or thromboxane synthetases respectively. Other metabolites of arachidonic acid such as the leukotrienes and the hydroxyeicosatetraenoic acids are formed in a pathway which utilises lipooxygenases rather than cyclooxygenase in the initial step.

There are now known to be two forms of the COX enzyme (otherwise known as prostaglandin endoperoxide synthase PES), COX-1 and -2. The former is a constitutive enzyme that is present in most tissues. COX-2 is a rapidly induced and short-lived form

of the enzyme[89] the message for which can be induced by phorbol ester and inhibited by glucocorticoid[89,90]. It has been demonstrated in amnion where its expression rises rapidly after treatment with IL-1β[91] and has also been demonstrated immunocytochemically around the vessels of first trimester decidual vessels[89].

Pharmacological inhibition of prostaglandin synthesis can be achieved using the non-steroidal anti-inflammatory drugs (NSAIDs). These agents inhibit the action of cylooxygenase and thus reduce prostaglandin synthesis. It has been found however that their effects are less potent in inhibiting COX-2 as compared with their effects on COX-1. Indomethacin, for example, has an ID_{50} which is 22 times less for COX-1 than for COX-2[92]. The finding of this transient inducible enzyme for prostaglandin synthesis has many implications for understanding the mechanisms of parturition. It seems likely that the rapid changes in uterine prostaglandins and their synthesis that are postulated to occur at this time could be modulated by induction or inhibition of this enzyme. In laboratory studies of prostaglandins non-steroidal agents such as indomethacin are often used to antagonise their production, however their lower efficacy against COX-2 means that this suppression of prostaglandin synthesis may not be absolute.

Metabolism.

The major site for prostaglandin metabolism in all species is the lung although local mechanisms for degradation also exist in some tissues[88]. The initial steps in the catabolism of prostaglandins E_2 and $F_{2\alpha}$ are mediated by 15-hydroxy prostaglandin dehydrogenase (PGDH) and Δ^{13}-reductase to produce the biologically inactive 15-keto-13,14-dihydro prostaglandin metabolites. Two types of dehydrogenase have been described which use either NAD^+ or $NADP^+$ as cofactors and which have greater affinities for PGE_2 or $PGF_{2\alpha}$ respectively[10].

General effects of prostaglandins.[10]

i: Vascular: In general PGEs will cause vasodilatation whereas PGFs promote vasoconstriction. Prostacyclin (PGI_2) is a potent vasodilator whereas thromboxane (TXA_2) is a vasoconstrictor.

ii: Platelet aggregation: TXA_2 decreases cAMP levels and thus promotes platelet aggregation. Conversely PGEs raise intracellular cAMP levels and inhibit aggregation. Vascular endothelial cells generate PGI_2 which inhibits platelet aggregation protecting vascular walls from such aggregates.

ii: Bronchial system: PGEs relax and PGFs constrict the smooth muscle present in bronchi and trachea.

iv: Gastrointestinal effects: Prostaglandins inhibit gastric secretion but increase intestinal secretions and motility. They also alter the electrolyte balance in the intestinal cells. They may thus be protective against gastric ulceration but can cause marked diarrhoea.

These general effects can be of relevance when considering the therapeutic uses and potential side-effects of prostaglandins.

1.4 ii: Prostaglandin production by uterine tissues and alterations at parturition.

Prostaglandins are widely accepted as playing a key role in the onset of parturition[46,89]. This conclusion arises from three main avenues of research, namely that the administration of exogenous prostaglandins results in uterine activity and cervical ripening at any gestation [93,94], that there is an increase in the production of prostaglandins by uterine

tissues during pregnancy and that an increase in the concentrations of prostaglandins in amniotic fluid, blood and urine occurs during pregnancy and, particularly, at the time of parturition [95-97].

The effects of prostaglandins in the uterine tissues will be governed, as elsewhere, by the rate of their synthesis and degradation. It is the rapidly induced COX-2 isoenzyme which may be of most relevance to short term alterations in the synthesis of prostaglandins. Their catabolism is initially by prostaglandin dehydrogenase (PGDH) and so alterations in the levels of this enzyme will also affect the concentrations of prostaglandins available locally.

$PGF_{2\alpha}$ is a potent uterotonic agent both *in vitro* and *in vivo*. PGE_2, also a uterotonin, causes marked cervical softening and ripening and is widely used clinically for this purpose as discussed in Chapter II[93,98]. Blocking of prostaglandin synthesis with indomethacin has been shown to abolish contractures in monkeys thereby promoting uterine quiescence[99].

Human amnion and chorion produce mainly PGE_2 whereas the decidua can produce both this and $PGF_{2\alpha}$. An increase in the production of PGE_2 and $PGF_{2\alpha}$ *in vitro* by fetal membranes, placenta and decidua at term has been described[100,101]. More specifically after spontaneous labour there is an increase in the production of PGE_2 and $PGF_{2\alpha}$ by the amnion, a lesser increase in the decidual production of these prostaglandins and a marked increase in the release of PGF metabolite by the chorion[102]. This increase in prostaglandin production by human uterine tissues at term has been suggested to derive from a fetal signal excreted via the fetal kidneys into the amniotic fluid where it could affect the production of prostaglandins by the amniotic membranes[89,103]. The role of the

prostaglandin produced by the fetal membranes has yet to be determined, in particular its potential effect on the adjacent decidua. Although the amnion produces substantial amounts of PGE_2 the chorion has been shown to contain high levels of PGDH (the main catabolising enzyme)[102,104,105]. In one *in vitro* study of intact amnion and chorion it was shown that PGE_2 could cross these membranes at a similar rate to sucrose [100] supporting a possible *in vivo* effect of amniotic prostaglandins on the decidua or myometrium. A later study, however, showed that while both the amnion and chorion could produce PGE_2 only a small proportion of that added to the amnion side of a perfusion chamber could cross to the decidual side[106]. There is therefore debate as to how much influence the PGE_2 that is undoubtedly produced by the amnion can exert on the decidua and myometrium.

The levels of PGDH activity in the fetal membranes has been compared in different areas of the uterus using both immunoassay for enzyme activity and immunocytochemistry. The levels of activity are highest in the region of the cervix in the non-labouring uterus but are significantly lowered in this area after the onset of labour [107]. A reduction in the levels of the catabolic enzyme present in this tissue would provide a local mechanism for an increased effect of the prostaglandins in the lower uterine segment and the cervix.

The increase in levels of prostaglandins in uterine tissues could alternatively or additionally be the result of an increase in the liberation of arachadonic acid mediated by phospholipases or an increase in the action of the cyclooxygenase enzymes or to both of these effects[46]. In a study of the enzyme kinetics of PGHS in human amnion a significant increase in the V_{max} was noted in amnion obtained after spontaneous deliveries as compared to that from caesarean delivery[108]. COX-1 gene expression has been demonstrated in human endometrium and decidua using reverse transcription PCR[109], in

this study endometrial tissue was found to express much higher levels (two to three times higher) of mRNA than decidual tissue obtained at elective caesarean section. This study did not demonstrate any COX-2 RNA in decidua or indeed in endometrium, a finding previously reported in a study showing that COX-2 expression *in vitro* occurred only in response to inducing agents[110] and confirmed by a study which demonstrated PGHS-2 by immunocytochemistry and in situ hybridisation in amnion and chorion but not in decidua. These findings are in keeping with the proposed role of COX-2 as a rapidly inducible enzyme which can cause marked but short-lived and easily modulated changes in the uterine levels of prostaglandins.

The cervix produces PGE_2, PGI_2 and lesser amounts of $PGF_{2\alpha}$. Production of these prostaglandins by the cervix has been shown to increase at term[111] and receptors for both PGE_2 and $PGF_{2\alpha}$ have been demonstrated in the cervix[112]. The levels of COX-2 double in the rat cervix biopsied in spontaneous labour[113].

Although the pharmacological effects of prostaglandins in ripening the cervix are undisputed there has been much debate about the physiological effects of prostaglandins in causing cervical ripening. Indirect evidence of the effects of prostaglandins on the process of cervical ripening comes from a study in which the administration of aspirin, which blocks prostaglandin synthesis, delayed parturition and also prevented cervical ripening[114]. There have been conflicting reports on the effects of prostaglandins on collagenase activity in the cervix but a study using improved methodology to detect collagenase activity found no alteration in this after treatment with sulprostone (a prostaglandin analogue) despite the cervical ripening produced in the treated group. It was also found that there was no difference in the levels of collagen degradation products detected in the cervix after prostaglandin treatment as compared with those in a placebo

group[115]. In animal studies, prostaglandins have been found to increase the production of GAGs, in particular the production of hyaluronic acid from fibroblasts and this is associated with a decrease in collagen production and an increase in circulating levels of chondroitin sulphate [116]. The effects on the cervix of prostaglandins administered *in vivo* are considered further in Chapter 2.

The precise method of action of the prostaglandins on uterine tissues and in particular the signals for their production remain to be fully clarified. There appears to be much interaction between the various areas of the uterus and in their production of eicosinoids. For example, the vaginal administration of PGE_2[46] leads to an increase in plasma levels of PGF metabolite, suggesting that increased levels of PGE_2, administered to the cervix increases the production of $PGF_{2\alpha}$ either by the cervix itself or elsewhere. It has also been found that PGE_2 increases the expression of intercellular adhesion molecules in endothelial cells, a finding which may indicate a role for this prostaglandin in recruitment of leucocytes into the cervix prior to ripening[117].

Although prostaglandin production from uterine tissues has been demonstrated to be increased following spontaneous labour it is possible that this is an effect rather than a cause of labour. This argument was expanded by MacDonald and Casey [118] who found that fluid from the forebag of amniotic fluid (i.e. that presenting in front of the baby's head) contained higher concentrations of PGE_2 and $PGF_{2\alpha}$ than that obtained from behind the presenting part. They concluded from this that the increase in prostaglandins measured during labour was an effect of the trauma of cervical dilatation rather than a causative factor. However, as discussed in a review by Kelly[89] the area of decidua exposed during cervical ripening may undergo activation and selective induction of PG synthesis around blood vessels and thus there could be particular amplification of

inflammatory signals in the region of the cervix and the forebag of membranes. This area of controversy highlights the important issue that although an increase in the production by uterine tissues of many factors can be demonstrated following parturition this does not necessarily mean that their production has increased prior to and with a causative role in the initiation of labour.

Role of oestrogen and progesterone.

In the myometrium oestrogen increases the responsiveness of α-adrenergic receptors which leads to an increase in production of the uterotonin $PGF_{2\alpha}$ whereas progesterone stimulates β-adrenergic receptors causing a preponderance of prostacyclin synthase and eventual relaxation of myometrial cells via cyclic AMP[46,119]. Further evidence of the actions of progesterone in modulating prostaglandin production has been elucidated following the use of antiprogestins and is considered in detail below. Oestradiol can stimulate cervical prostaglandin production following progesterone exposure and has been used clinically to cause cervical ripening[120].

1.5: CHEMOKINES

As discussed above, the exact mechanism by which prostaglandins facilitate cervical ripening is not yet known. However it seems that although PGE_2 does cause ripening this is not due to a direct effect on the collagen degradation in the cervix. The actions of prostaglandins on the cervix can be postulated to be via a series of intermediary substances. In recent years attention has focused on the possible role of cytokines in this process. These intermediaries act as local signals in inflammation and can have both stimulatory and inhibitory effects. In the following review the role of interleukin-8 will be concentrated on.

Interleukin-8 (IL-8) is a member of the chemokine family. This is a group of structurally and functionally related cytokines which all contain four conserved cysteine residues which form two intra-chain disulphide bridges. They can be sub-divided into two families according to whether the first two cysteine residues are adjacent (CC proteins) or separated by an amino acid (CXC or α- chemokine proteins)[121]. IL-8 is a member of the latter family as are platelet factor 4 (PF4) and β-thromboglobulin. Monocyte chemoattractant peptide-1 (MCP-1) is a member of the CC group which has also received recent interest as having a possible role in the initiation of parturition. Many of the genes coding for α-chemokines have been localised and assigned to chromosome 4q 12 - 21.

1.5 i: Interleukin-8

Interleukin-8 (IL-8) was first described[122] as a peptide, also known as neutrophil-attractant peptide-1 (NAP-1) which was released from monocyte cultures stimulated by lipopolysaccharide. It has since been found to be released by many other cell types[123,124] including endothelial cells, fibroblasts, some epithelial cells and also by neutrophils following phagocytosis[125]. The latter finding suggests a feedback mechanism whereby the effects of IL-8 in the target tissue can be amplified.

The initial description of IL-8 was of a 72 amino acid peptide with a molecular weight of 8383[122] but it has since been found to exist in other forms. It is produced from a 99 amino acid precursor which is processed to release a 79 amino acid peptide. Another biologically active form has also been described which contains 77 amino acids[126]. In concentrated solution or on crystallisation IL-8 exists as a dimer which is stabilised by hydrogen bonds and side chain interactions forming two parallel α-helices lying on an antiparallel β-pleated sheet[124].

Effect on neutrophils

As indicated by its synonym one effect of IL-8 is to attract neutrophils. In addition to this it can activate these cells. It has also been found to act as a chemoattractant for T cells, basophils and smooth muscle cells [124] and to cause endothelial cell proliferation in blood vessels [127]. The extravasation of neutrophils takes place by their adherence to and subsequent diapedesis through the endothelial wall of blood vessels. For this to occur the neutrophil first rolls along the endothelial cell surface, a process mediated by endothelial P-selectin. It then adheres to the endothelial cell by means of an interaction between a CD11-CD18 integrin heterodimer on the neutrophil surface with endothelial intercellular adhesion molecule (ICAM-1). Chemoattractants, such as IL-8, facilitate this adhesion by inducing the translocation of CD11-CD18 from its intracellular stores to the surface of the neutrophil. It also causes the shape change and shedding of L-selectin from the neutrophil surface which are prerequisites for neutrophil extravasation [128]. It has been proposed that IL-8 does not attract neutrophils by creating a simple diffusion gradient but rather that it binds to the endothelial cell walls of small venules (but not arterioles) promoting neutrophil adhesion to endothelial cells and directly stimulate their emigration [128].

Following the extravasation of neutrophils into tissues IL-8 causes their activation. This produces a change in their shape, exocytosis of intracellular enzymes from their storage granules and a respiratory burst [129]. The shape change is partially caused by the formation of large thin cytoplasmic lamellae which alter the transmission of light by neutrophil cell suspensions once they are activated [130]. Neutrophils contain two types of storage granules the azurophil and the specific granules. The contents of the azurophil granules include elastase, myeloperoxidase and lysozyme while the specific granules contain collagenase, lactoferrin, B_{12}-binding proteins and lysozyme. The contents of these granules can be released without phagocytosis occurring. The third event in the activation of neutrophils

is the generation of a respiratory burst. This results from the activation of NADPH-oxidase, which is a trans-membrane complex which on assembly (this can occur within 2 seconds of neutrophil activation) transfers electrons from cytosolic NADPH to extracellular oxygen[129]. This respiratory burst results in the formation of hydrogen peroxide and superoxide radicals which are involved in the killing of pathogens.

IL-8 thus acts as a classical chemotaxin in that it both attracts and activates neutrophils causing all of these characteristic events to occur [124,128,129]. It has also been shown to have chemotactic action on T-lymphocytes[131].

Regulation of interleukin-8 production.

Il-8 production is induced by LPS[106] and also by other cytokines. including IL-1 (α and β) and TNFα [124,132]. These cytokines have been shown to stimulate production of IL-8 in endothelium, epithelium and in fibroblasts [133]. Dexamethasone is a potent inhibitor of IL-8 production by monocytes [53,124] and other cell types including fibroblasts [124,134].

Interleukin-8 receptors.

Two specific IL-8 receptors have been described termed IL-8 receptor A (RA or 1) and B (RB or 2)[135-138] or alternatively CXCR1 and CXCR2[139]. IL-8 RA is a 350 amino acid molecule which has a configuration characteristic of a G-protein linked receptor with seven trans-membrane domains. IL-8 RB has 77% homology with the RA receptor and consists of 355 amino acids. Both these receptors have been shown to be present in large numbers on the human neutrophil[137,138]. The genes for these receptors have been mapped to chromosome 2q35.

The effects produced by IL-8 are initiated by its binding to the specific cell surface receptors, IL-8 R1 and 2 which are coupled to G-proteins which dissociate into their α and $\beta\gamma$ subunits when the ligand is bound. The α subunits then activate phospholipase C which leads to the generation of inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 diffuses into the cytosol and facilitates calcium release from intracellular stores and DAG activates protein kinase C.

IL-8 receptors types 1 and 2 have been described in human fetal membranes, placenta and myometrium. The levels in placenta and amnion were noted to be increased in samples taken after spontaneous labour as compared with those obtained at elective caesarean [141].

IL-8 can also be bound by the human erythrocyte chemokine receptor which also binds a wide variety of other chemokines, including MCP-1. This receptor has been shown to be the Duffy blood group antigen which is also a receptor for the malarial parasite *Plasmodium vivax* [135]. It does not appear that the binding of IL-8 to this erythrocyte receptor has any effect on the red blood cell and so it has been proposed that this receptor acts to bind excess IL-8 in the blood and thus act as a clearance mechanism for high levels of IL-8 in the circulation[135].

Biological Effects of Interleukin-8.

In rabbits intradermal injection of IL-8 induces plasma exudation and a massive local infiltration of neutrophils but not of other leukocyte cells[142,143]. This accumulation of neutrophils is particularly marked around the venules and is strongly enhanced by the concomitant administration of PGE_2[142-144]. In the absence of this vasodilator the threshold for infiltration of neutrophils is about 10^{-11} mol IL-8, with the addition of PGE_2 this threshold becomes around 10^{-14} mol IL-8 per site[142,143]. In addition to lowering the

threshold level of IL-8 necessary to cause neutrophil infiltration the presence of PGE_2 also leads to a marked increase in the number of accumulating neutrophils and in the volume of the associated plasma exudation[144]. A similar effect is seen following the injection of IL-8 into human skin[145]. A perivascular neutrophil infiltration is demonstrated that is time-dependent and persists for several hours. This is similar to animal studies in which the effects of IL-8 persist for up to eight hours[143,144], suggesting that IL-8 is resistant to inactivation for at least this time period. IL-8 can bind to glycosaminoglycans in tissues and its chemotactic activity is increased following binding to heparan sulphate [128].

A further biological role for IL-8 has been postulated following the finding that *in vitro* and *in vivo* it acts as a potent angiogenic factor. When IL-8 was implanted into a rat cornea, which is normally avascular, it caused an angiogenic response in all ten corneas studied and produced no evidence of non-specific inflammation [127].

Interleukin-8 is therefore a chemokine which has been shown to aid the emigration of neutrophils from blood vessels and to activate them in tissues, causing a shape change, the release of storage granules they contain and a respiratory burst. It has a prolonged effect in tissues, due to its resistance to inactivation and to its binding to glycosaminoglycans. Its effectiveness in causing neutrophil movement is significantly increased in the presence of PGE_2. IL-8 is thus an attractive proposition as a mediator in the process of cervical ripening and in the onset of parturition. As discussed above neutrophils are intimately involved in the cervical changes at parturition and PGE_2 is known to play a key role in the mechanisms operating at this time. The possible steroid control of IL-8 production in reproductive tissues would mean that its concentration could alter at the time of labour.

Interleukin-8 in uterine tissues: Endometrium.

Human endometrium, which becomes decidualised during pregnancy, consists of glandular epithelium and stroma. Both of these cell types have been shown to produce IL-8 *in vitro*. This production was significantly increased after addition of either TNF-α or IL-1α to the culture medium and this increase was associated with an increase in IL-8 mRNA production[146]. Kelly *et al* confirmed this production of IL-8 by endometrial cells and also demonstrated that it was significantly decreased by the addition of either progesterone or dexamethasone to the culture medium [147].

Using immunohistochemical techniques IL-8 in the human endometrium has been localised to the perivascular areas of the endometrium[148,149]. The specific immunostaining did not occur in all vessels of the endometrium and was found in both the proliferative and secretory phases of the menstrual cycle with the more recent study [149] demonstrating an increase in immunostaining for both IL-8 and PGE_2 premenstrually supporting the hypothesis of a role for neutrophil migration in the onset of menstruation. This localisation was not endothelial but was associated with the smooth muscle cells in the vascular wall.

Choriodecidua.

Decidual cells can be found on the chorionic surface of the fetal membranes and when these cells were obtained from elective caesarean sections at term they produced high amounts of IL-8 in culture[150]. The addition of progesterone to the culture medium significantly inhibited the production of IL-8 whereas the concomitant addition of the antiprogesterone mifepristone overcame this effect. The production of IL-8 was also stimulated by the bacterial product lipopolysaccharide (LPS). A separate study confirmed that group-B streptococcus stimulates IL-8 production by chorion cells [151]. These studies

suggest that a decrease in the effects of progesterone or the presence of significant bacterial invasion of the decidua could increase levels of IL-8 leading to an increase in the numbers of neutrophils present and to their activation. A study of the expression of IL-8 mRNA, however, using decidua, amnion and chorion from term and preterm labours in the presence and absence of chorioamnionitis, did not find a difference in the mRNA expression between any of these tissues [152]. These tissues were all collected after delivery and so the changes at the onset of parturition may have been masked by a subsequent inflammatory cascade. The production of IL-8 by choriodecidual cells has also been shown to be inhibited by dexamethasone [147].

In another study the production of IL-8 by choriodecidual cells was confirmed in samples of both decidual and chorionic cells obtained from placentae at the time of elective Caesarean section. In both cell types production was increased significantly and in a dose dependent manner by the addition of either IL-1β or TNF to the culture medium. This increase was associated with an increase in mRNA for IL-8[153]. In this study Dudley *et al* proposed that a cytokine network exists in gestational tissues with intercommunication between maternal and fetal tissues producing a cascade of cytokines. IL-1 and TNF have been shown to stimulate PGE_2 production by chorion, amnion and decidual cells[145,154] and may thus amplify the effectiveness of IL-8, the production of which they also increase. These findings were corroborated in a further study of choriodecidua and amnion in which production of IL-8 by both tissues was demonstrated and it was shown that production by the former was increased by addition of lipopolysaccharide to the culture medium[155].

Fetal membranes.

IL-8 mRNA has been demonstrated in fetal membranes and it has also been found that the level of this is increased by stretching the membranes in vitro using mechanical force. This study also demonstrated an increase in IL-8 receptors in decidua (but not amnion-chorion) following the application of a mechanical stretch [156]. It was therefore suggested that the physical stretching caused by the expansion of the intrauterine contents may provide a mechanism via IL-8 production to favour the initiation of parturition.

Amniotic fluid

The levels of IL-8 in amniotic fluid have been studied in samples acquired by amniocentesis[133,157,158]. It has been suggested that while most samples from mid-trimester and term patients do not contain detectable IL-8, a greater number of samples in the term group do contain detectable IL-8[133,157]. A more recent study however showed IL-8 to be present in the amniotic fluid of all women tested at term, whether they were labouring or not[159]. In one study using samples tested from women in threatened preterm labour all of those with positive microbiological amniotic fluid culture had quantifiable levels of IL-8 whilst of those with negative amniotic fluid cultures higher IL-8 levels were found in samples from those who delivered preterm than in those who did not[157]. Amniotic fluid levels of IL-8 increase during term labour, even when there was no bacteriological invasion of the amniotic fluid[133,157].

Amniotic fluid IL-8 levels therefore increase during parturition, are associated with positive amniotic fluid microbiological culture and are associated with an increased risk of preterm delivery in the presence of sterile amniotic fluid cultures. In preterm labour, the levels of amniotic fluid IL-8 were shown to be predictive of histological chorioamnionitis

[158] and may thus have a potential use as an indicator for the likely success of tocolysis in this clinical situation.

The levels of other inflammatory cytokines, TNFα and IL-1 and -6, in amniotic fluid have been shown to be inter-related and to correlate with histological signs of inflammation in the placenta[160]. IL-1 and -6 were found to be present in all the samples tested from women after spontaneous rupture of the membranes, whereas TNFα was present only in some of the samples. The presence of these inflammatory agents after membrane rupture may however be a result of contact of the fluid with cervical or vaginal secretions.

Placenta.

Placental tissue culture in any stage of pregnancy produces IL-8 in increasing amounts with advancing gestation or in the presence of chorioamnionitis[161]. IL-8 has been localised immunohistochemically to the trophoblast cells of the placenta with no staining seen in endothelial or mesenchymal cells.

Cervix.

Cultures of fibroblasts from the cervices of pregnant rabbits produce IL-8[162]. This is increased by IL-1 α and β and is inhibited in a dose dependent manner by the addition of progesterone. This inhibition of IL-8 production is reversible by the addition of mifepristone. These changes are all associated with similar changes in the levels of mRNA for IL-8.

Human cervices from first trimester pregnancies and from non-pregnant, pre- and post-menopausal women all produce IL-8 in culture. This production was significantly increased by the addition of phorbol myristate acetate (PMA, a protein kinase C activator)

except in the post-menopausal group. Progesterone did not inhibit this stimulation. There was no difference in the production by cervices from women in the first trimester who had received a PGE analogue prior to sampling and those who had not [43].

In biopsies of cervices of women at term IL-8 production has been demonstrated. This production is increased as compared to non-pregnant control biopsies and is further increased in biopsies obtained after vaginal delivery[163].

In both guinea pigs[164] and rabbits[165] the direct application of IL-8 to the cervix causes softening and dilatation. In rabbits this is associated with a significant increase in the water content, the glycosaminoglycan concentration and the mean number of neutrophils of the cervices. There is a decrease in the collagen content of the cervices. These findings were found in both pregnant and non-pregnant animals. In guinea pigs the effects of IL-1β and TNF-α were also studied. It was found that the local application of these cytokines to the cervix of the guinea pig caused histological changes similar to those found in physiological cervical ripening. TNFα, however, produced a more severe inflammatory type reaction with infiltration of lymphocytes and macrophages in addition to the neutrophil invasion seen in all of the cervices treated with cytokines. Of the three cytokines, only IL-8 caused a significant alteration in the cervical extensibility as measured by mechanically testing to determine its elastic properties[164]. In a study where both IL-8 and dehydroepiandrosterone sulphate (DHEAS) were applied to rabbit cervices, it was found that both treatments reduced the collagen content of the cervix (as assessed by staining with picrosirus red) but that this decrease was greatest when the two were used in combination. Influx of neutrophils into the cervical tissue was noted after IL-8 application but again this was greater when IL-8 and DHEAS were applied concomitantly [166].

The cervix is therefore capable of producing IL-8 in both human and animal models. When IL-8 is applied to the cervix in animals it causes changes equivalent to physiological ripening, including neutrophil invasion.

Plasma

An increase in plasma levels of IL-8 during pregnancy as compared with the non-pregnant state has not been found[133]. An increase in serum IL-8 has however been demonstrated in women at term with histologically confirmed chorioamnionitis as compared with serum from non-affected women. This was a significant increase and was compared with other markers of infection such as white cell count, C-reactive protein and the cytokines IL-1 IL-6 and TNF-α which did not show a consistent rise in the presence of chorioamnionitis [167]. In preterm women with chorioamnionitis a similar increase in plasma IL-8 has not been demonstrated, whereas in this group an increase in IL-6 has been shown [168].

1.5 ii: Monocyte chemotactic peptide-1 (MCP-1).

This is a 76 amino acid protein that was initially called lymphocyte derived chemotactic factor (LDCF). It is a C-C cytokine (i.e. there is no residue between the first two cysteines in the protein) and is a heterogeneous protein existing in both α and β forms. It was first described in 1989 on being obtained from lymphocyte culture[169] and as its name implies is able to attract blood monocytes which are also capable of its production in large amounts. In addition to its effects on monocytes it is chemotactic for basophils, eosinophils and lymphocytes but not for neutrophils. *In vivo*, however, its effects on monocytes are the most marked. Its chemotactic effects seem to be limited to blood macrophages, tissue macrophages being unresponsive[170]. MCP-1 also causes an increase in monocyte secretion of IL-1 and IL-6 [171]. IL-1 in particular is known to stimulate IL-8 production and so release of MCP-1 could act as an indirect stimulant of IL-8 production.

As with IL-8 its production by cells has been shown to be stimulated by IL-1, TNFα and LPS [124].

1.6: WHITE CELL POPULATIONS IN THE PREGNANT UTERUS.

The changes in the immune environment of the uterus at all stages of gestation are complex and the results of studies are at times contradictory. It does appear however that, both in early and late pregnancy there are altered immune responses in the uterus. In early pregnancy these changes may protect the fetus from rejection by the maternal immune system, whereas in later pregnancy a cascade of inflammatory events may precipitate labour and the ultimate expulsion of the fetus.

The leucocyte populations of the uterus alter during pregnancy. These populations have mainly been studied by the identification of their cell surface markers which are classified according to the CD (cluster of differentiation) type[172]. The common marker for all leucocytes is CD45 which can exist in RB, RA and RO forms. Leucocytes are abundant in the first trimester decidua, with over 30% of stromal cells expressing the leucocyte common antigen[348]. These decidual leucocytes consist of three main types: macrophages, granulated lymphocytes and T lymphocytes.

Macrophages

There are significant numbers of tissue macrophages present throughout pregnancy in the placenta and decidua which can elaborate cytokines, such as IL-1 and TNF-α and also PGE_2[124,160,173,174]. These cells may thus have a central role in the proposed inflammatory cascade of parturition, their activity resulting in an increase in IL-1, TNF and thus IL-8 production. In the human cervix, a ten-fold increase in the number of

macrophages present was noted in immunohistochemical studies of biopsies from term as compared with first trimester pregnancies[175]. This increase in numbers could be pivotal in amplifying the proposed inflammatory cascade leading to cervical ripening and dilatation.

The prostaglandins produced by decidual tissue appear to be derived from a macrophage population[176,177], in contrast to the endometrium in which the source of prostaglandins is the stromal cells[178]. Lopez Bernal and Watson used flow cytometry to separate decidual cells from fetal membranes and then labelled them immunocytochemically to determine their antigen markers. The subsequent analysis of prostaglandin (PGE and PGF) production from these cells showed that CD45 positive cells (i.e. bone marrow-derived) produce more prostaglandins than CD45 negative cells and that it is the macrophage subgroup (labelled with HLA-DR) which produce the most prostaglandins[179]. The PGE_2 produced may have direct effects, for example on the myometrium and could also act synergistically with IL-8 to facilitate neutrophil recruitment into the uterine tissues.

Lymphocytes

During early placentation the number of large granular lymphocytes (LGLs) in the decidua increases markedly.[146] These are cells of an unusual phenotype which may aid in the protection of the fetal tissue from immune surveillance. They account for up to 75% of leucocytes in the first trimester decidua[349]. LGLs are strongly positive for CD56, an antigen expressed by natural killer (NK) cells but not for the other NK antigens, CD16 or CD3 nor the T lymphocyte antigens CD3, 4 or 8. Despite their NK lineage these first trimester LGLs do not lyse trophoblast cells[350]. Their number increases markedly in the first trimester and then shows a significant decrease as pregnancy advances[174]. Decidual

CD56+ cells from early pregnancy have been shown to be capable of producing a large amount of IL-8 and to express IL-8 mRNA[180].

The proportion of lympnocytes that are T-cells (CD3 positive) increases during pregnancy, doubling by the third trimester[174], as the proportion of CD-56 positive cells decreases.

In addition to their altered concentrations in pregnancy, decidual lymphocytes isolated from term human placentae respond differently to antigenic stimulation than peripheral lymphocytes from the same women[181]. The decidual cells were found to produce granulocyte-macrophage colony- stimulating factor (GMCSF) both spontaneously and on stimulation whereas peripheral cells did not. This finding suggests that lymphocytes from gestational tissues may respond in a different way to their immunological environment than plasma cells and thus that the uterus may function as a separate immunological environment.

During early placentation the number of large granular lymphocytes in the decidua increases markedly[146] These are cells of an unusual phenotype which may aid in the protection of the fetal tissue from immune surveillance. The number of T-cells (CD3 positive) increases during pregnancy, doubling by the third trimester, whereas the number of cells that are CD-56 positive increases markedly in the first trimester and then shows a significant decrease as pregnancy advances. CD3 is a polypeptide marker of T-lymphocytes and CD56 a marker for natural killer (NK) cells [172]. Decidual CD56+ cells from early pregnancy have been shown to be capable of producing a large amount of IL-8 and to express IL-8 mRNA[180].

In addition to their altered concentrations in pregnancy, decidual lymphocytes isolated from term human placentae respond differently to antigenic stimulation than peripheral lymphocytes from the same women[181]. The decidual cells were found to produce granulocyte-macrophage colony- stimulating factor (GMCSF) both spontaneously and on stimulation whereas peripheral cells did not. This finding suggests that lymphocytes from gestational tissues may respond in a different way to their immunological environment than plasma cells and thus that the uterus may function as a separate immunological environment.

Neutrophils

The above study which described the leukocyte populations in early and late pregnant cervices showed an increase in the neutrophil concentrations during pregnancy but not during labour in the cervical biopsies studied[175]. This indicates the possibility that the increase in neutrophil population precedes and is not an effect of labour.

Eosinophils

An increase in the concentration of eosinophils in the cervix during pregnancy has been noted in a study comparing biopsies from women who delivered vaginally or at elective caesarean with those in early pregnancy and with non-pregnant controls [182]. Although the sample studied was small, an increase in the number of eosinophils was noted after vaginal deliveries. This may reflect the inflammatory changes in the cervix during labour or could, as the authors of this study suggest, indicate a role for eosinophils in the onset of cervical ripening at term.

Although the changes in maternal peripheral white cells populations may not be an accurate reflection of changes in their concentrations or activity in uterine tissues, there are

marked changes evident in maternal plasma at the time of parturition. A consistent rise in the neutrophil count has been found, with a concurrent lymphopaenia as compared with non-pregnant women [183]. This lymphopaenia was found to be a result of a decrease in almost all types of T-and B-cells studied.

1.7: THE ROLE OF PROGESTERONE IN THE ONSET OF PARTURITION

In 1975 Csapo described the "see-saw" theory of pregnancy regulation in which there is a balance between the factors that promote pregnancy maintenance and those which favour its termination[184]. This theory related in particular to the effects of $PGF_{2\alpha}$ which is an intrinsic stimulator of uterine activity, the effects of which are decreased when the uterus is dominated by progesterone.

Progesterone and oestradiol have been shown to affect the function of the components of the uterus, often in opposite ways. In the guinea pig cervix, for example, oestradiol stimulates the degradation of type I collagen whereas this effect is blocked by the addition of progesterone[23]. In the myometrium the changes associated with parturition, such as the increase in gap junctions and the change from the contracture to a contraction pattern of activity have been related to alterations in the hormonal environment. In monkeys, there is a pre parturient increase in oestrogen prior to the onset of switches from contractures to contractions[61]. This may be due to a combination of the effects of oestrogen which stimulates oxytocin receptor production in the myometrium and decidua, stimulates prostaglandin production and also stimulates the formation of gap junctions. Other factors which may also be involved in this switch include oxytocin, the antagonism of which decreases the frequency of contractures[64] or even physical stretching of the myometrium

as in experimental conditions expansion of the uterus with saline infusion increases the frequency of contractures and eventually results in a switch to contractions[64].

In rat myometrium there is a significant increase in nuclear oestrogen receptors and decrease in progesterone receptors at the time of parturition. These alterations correlate with an increase in the area of gap junctions in the myometrium. but not with the levels of steroids in the serum[68]. Administration of oestradiol to immature or to mature ovariectomised rats[185] caused an increase in the numbers of myometrial gap junctions similar to that seen in parturient animals. The gap junctions thus induced were however only about half of the area of those demonstrated at the time of labour. Administration of progesterone inhibited this effect of oestradiol. Indomethacin enhanced the effect of oestradiol in inducing gap junctions. The latter finding seems paradoxical if prostaglandins are required for the increase in gap junctions seen at labour. It may be however that indomethacin differentially inhibits the production of prostaglandins that suppress gap junction formation such as prostacyclin has been proposed to do. Connexin production is thought to be upregulated by progesterone withdrawal from the myometrium[53,186]. The increase in gap junctions at the time of labour that has been demonstrated in many species including humans[70] may therefore be due to an increase in connexin production facilitated by decreasing local levels of progesterone and enhanced by rising levels of oestrogen and alterations in prostaglandins present.

In many of the events in the cervix and myometrium associated with parturition, progesterone acts to promote quiescence whereas oestradiol can act in an opposing manner. It is thus proposed that a decrease in available progesterone levels at the time of the onset of parturition enables cervical ripening and myometrial contractions to proceed.

Csapo also described the delicate nature of pregnancy maintenance by progesterone in the rat in which a gradual decrease in progesterone in the mid-trimester allowed the continuation of the pregnancy whereas an abrupt withdrawal led to termination of the pregnancy[187]. In sheep, there is a well defined pathway by which increased fetal secretion of ACTH stimulates an increase in fetal adrenal production of cortisol which acts as a substrate for increased placental oestradiol production[188,189]. A recent study in Rhesus monkeys found that the administration of epostane (which inhibits progesterone production by selectively inhibiting 3-beta-hydroxysteroid dehydrogenase) caused cervical ripening, uterine activity and delivery within 48 hours. This was associated with an increase in the amniotic fluid levels of PGE_2. This study also showed that the administration of progesterone alone to these animals did not inhibit spontaneous parturition at term[190].

In humans, however, such a clear mechanism has not yet been described. It has been shown, however, that there is an increase in the salivary oestriol / progesterone ratio in the three weeks preceding the onset of spontaneous term labour in women[191] and that this increase does not occur in women whose labours are induced post-term[192]. This increase in the ratio of these hormones in saliva is caused by an increase in oestrogen levels rather that by an absolute decrease in the progesterone level[191,192]. The peripheral plasma levels of progesterone, oestradiol, oestriol and dehydroepiandrosterone sulphate (DHEAS: a precursor of oestrogen) were measured in women with ripe (i.e. with a modified Bishop score[193] of greater than 4) and unripe cervices prior to the induction of labour and it was found that the group with ripe cervices had a significantly raised level of DHEAS but not of the other steroids[194]. These results are similar to those obtained from rhesus monkeys in which DHEAS levels rise in the latter stages of pregnancy with an acceleration in this increase before parturition[195]. There may be an alteration in the ratio of oestrogen to

progesterone prior to the onset of labour in humans, possibly due to an increase in the production of oestrogen precursors.

Recent work has shown that preterm labour is associated with an increase in corticotrophin releasing hormone (CRH) and a fall in its binding protein, in both preterm and term deliveries[196,197]. It has also been found that plasma CRH concentrations are higher in those women who eventually deliver preterm than in those who deliver post-term[197]. A 'placental clock' is proposed which acts to govern the gestational length of the pregnancy[197,198]. This 'clock' could presumably be over-ridden by events such as infection that could bypass the steroid balance and cause direct stimulation of the inflammatory cascade leading to labour.

Progesterone Receptors.

The effects of progesterone on uterine tissues are caused via its receptors, either nuclear or cytosolic, the structure of which is considered further in the following chapter. Progesterone down-regulates the levels of both progesterone and oestradiol receptors[199] in the non-pregnant and pregnant uterus.

The number of nuclear progesterone and oestrogen receptors in human amnion, chorion, decidua and myometrium decreases as pregnancy advances[200-202]. Indeed, in one study[200] there were no detectable receptors in the term tissues studied. A possible explanation suggested was that these specific receptors are replaced by less specific binding sites in these tissues at later gestations. This lack of nuclear progesterone receptors in placenta and membranes was also found in another study of tissue obtained from elective caesarean sections prior to labour at term[201]. This group did, however, find detectable progesterone receptors in the decidua and myometrium. These receptor levels

contrasted with the high levels of progesterone found in placenta and fetal membranes, the possibility being suggested that the lack of receptors at these sites allowed progesterone to reach the decidua and myometrium to maintain uterine quiescence.

In a more recent and detailed study of the receptors in the specific cell types of the endometrium and decidua[202] progesterone receptors were demonstrated in the nuclei of decidual stromal cells and in the walls of spiral arteries throughout pregnancy but were down-regulated in the epithelial cells. Oestradiol receptors were down-regulated in all the endometrial cell types at each gestation and increased following mifepristone treatment indicating that their down-regulation was due to the action of progesterone.

Understanding of the effects of progesterone on prostaglandin metabolism has been improved by the antagonist mifepristone and a full discussion of these effects is considered below. In summary the effects of the antigestagen are to increase prostaglandin production and decrease its metabolism by PGDH. The effect of progesterone in many reproductive tissues is to inhibit the production of IL-8. A decrease in the local action of progesterone, either due to a fall in its proportion relative to oestrogens or to a decline in the number of available receptors could thus lead to an increase in the levels of prostaglandin E_2 and IL-8 in the uterine tissues. An increase in these levels would favour neutrophil recruitment into the uterine environment, including the cervix, where they could then promote the events leading to cervical ripening. This would occur in an environment of increased inflammatory activity in which other cytokines, such as MCP-1, IL-1 and TNF, elaborated by lymphocytes and macrophages could act in a positive cascade promoting the events of parturition.

Chapter II:

THERAPEUTIC MODULATION OF THE PREGNANT UTERUS.

Introduction.

2.1: Termination of pregnancy.

2.1 i: Mechanical cervical ripening.

2.1 ii: Pharmacological cervical ripening.

2.2: Cervical ripening and induction of labour.

2.2 i: Historical considerations.

2.2 ii: Indications for and risks of labour induction.

2.2 iii: Cervical ripening prior to labour induction.

2.2 iv: Induction of labour.

2.3: Mifepristone.

2.3 i: Mechanism of action and effects on uterine tissues.

2.3 ii: Clinical uses of mifepristone.

2.4: Current clinical practice.

INTRODUCTION.

It is possible to use pharmacological or mechanical means to interrupt pregnancy at any gestational stage. To accomplish this the events of normal term parturition must be imitated such that the cervix softens and dilates and myometrial contractions are produced. In early pregnancy the alternative is to physically empty the uterine cavity by surgical methods such as vacuum aspiration or the less commonly used dilatation and evacuation. Both these techniques use physical dilatation of the cervix, usually with a series of dilators of increasing circumference. Regardless of the stage of interruption of pregnancy the degree of softening of the cervix is of paramount importance. In surgical procedures preoperative softening of the cervix decreases the risk of damage to the cervix. In medical terminations in the second trimester it is advantageous to have improved cervical compliance to reduce the duration of the process and also reduce the risk of uterine rupture[116]. In the third trimester the induction of labour also necessitates cervical dilatation and, as will be discussed below, the degree of cervical ripeness prior to labour induction is predictive of the ease of that induction. When considering the dilatation of the cervix at any stage of pregnancy there is an essential difference between forced dilatation where force is applied along the length of the circumferential fibres against the strength of the fibres and the ground substance and the dilatation achieved by the action of uterine contractions in which the force is from above at right angles to the fibres separating them "like a trellis"[11].

This review will focus mainly on methods of promoting cervical ripeness, with reference also to the production of uterine contractions in the third trimester. The effects of the antiprogesterones, in particular mifepristone, are central to the elaboration of the hypothesis that progesterone antagonism will lead to cervical ripening and plays a role in

the onset of normal parturition. These agents are considered together in the final section of this chapter.

2.1: TERMINATION OF PREGNANCY.

If pregnancy is interrupted before the fetus is viable (currently adjudged to be at 24 weeks), the pregnancy is regarded as having been terminated, rather than labour having been induced. This may legally be performed, in Great Britain according to the 1968 Abortion Act, if there is a substantial risk of fetal anomaly or if the mothers well-being is at risk if the pregnancy continues or if the well-being of existing children would be compromised by the continuation of the pregnancy. Although the legalisation of abortion has greatly reduced the maternal morbidity and mortality of the procedure there are still associated complications. In the period of 1988 to 1990 one death was recorded as a consequence of a termination of pregnancy which was due to a cervical tear during a mid-trimester surgical procedure thus emphasising the potential hazards of forced cervical dilatation[203]. In uteri removed immediately following cervical dilatation tears of the internal os were found around 40% of the specimens examined[204].

Cervical ripening may also be used prior to surgical evacuation of retained products of conception in a non-viable pregnancy. This may be of particular benefit in a primigravida whose cervix will often be less distensible during mechanical dilatation than that of a parous woman.

Cervical ripening prior to surgical techniques.

The techniques developed are either physical employing intracervical devices or pharmacological using prostaglandins. The more recent use of the antiprogesterone

mifepristone is discussed below. Dilatation can be traumatic to the non-compliant cervix causing trauma or lacerations[205] and has been implicated in the aetiology of an incompetent cervix, which causes mid-trimester pregnancy losses and preterm delivery. Difficult dilatation of the cervix is also associated with uterine perforation, necessitating laparoscopy or even laparotomy and with an increase in perioperative blood loss[206].

2.1i: Mechanical cervical ripening.

The use of intracervical dilators has been practised for over 100 years, the first such devices being prepared from seaweed[116]. There are three types of intracervical tents currently available[207]. The laminaria tent is composed of sticks of seaweed which act as an osmotic dilator which expands as it absorbs water to exert a considerable force per unit area on the cervix[208]. This is no longer used in the UK in view of the length of time it takes to act. Dilapan is a synthetic form of this compound which is claimed to have a faster action, acting in two to four hours compared with four to twelve hours[116]. Lamicel, another synthetic product, is a poly-vinyl alcohol polymer impregnated with magnesium sulphate which acts as a sponge absorbing water from the cervix. Unlike the former two devices it does not exert a physical force on the cervix[207] but may act by altering the matrix of the cervix[209]. The use of all these devices effectively decreases the cervical resistance to subsequent dilatation[210,211] and also decreases the incidence of complications such as bleeding or uterine perforation[211]. The mechanism of action of these tents may not be due simply to a mechanical effect as it has been found that following insertion of laminaria tents the plasma levels of $PGF_{2\alpha}$ metabolite are increased suggesting that the mode of action may involve increased synthesis of prostaglandins. Histologically after dilatation with Lamicel the changes are similar to those seen in physiological cervical ripening with a decrease in the collagen, an increase in the water content and infiltration of inflammatory cells [212].

Lamicel tents have been compared with the prostaglandin analogue gemeprost (16, 16-dimethyl-transΔ_2 PGE_1 methyl ester, Cervagem) pessaries prior to surgical termination of pregnancy but conflicting results have been reported. In an open study both treatments made cervical dilatation easier to perform as compared with placebo with no difference between the two interventions. The prostaglandin pessaries caused more pain following their insertion than the Lamicel[213]. In a later blinded study, however, it was found that cervical dilatation was easier following the administration of gemeprost than Lamicel with no difference in their side-effect profile[214].

2.1 ii: Pharmacological cervical ripening.

The potential role of prostaglandins in cervical ripening has been discussed in the previous chapter. Prostaglandins have been utilised to terminate pregnancies for the past 30 years either in conjunction with surgery as a preoperative ripening agent or to effect medical termination of pregnancies, particularly at later gestations[116]. Pre-operative treatment with prostaglandins has been shown to soften the cervix leading to easier dilatation and decreased peri-operative blood loss[215-218].

$PGF_{2\alpha}$

Both intravenous and intrauterine infusions of $PGF_{2\alpha}$ will cause abortion in some women they are administered to but even with large doses only around half or less of those treated will abort, the intravenous route being rather more effective. Administration of these large doses caused marked rises in intrauterine pressure but did not always lead to cervical dilatation. These regimes were associated with severe gastrointestinal side-effects[219]. In 1976, improved results were reported after administration of extraovular $PGF_{2\alpha}$ in a 'double impact' regimen in which an initial prostaglandin dose was used to destabilise the

regulatory mechanisms of the uterus followed by further boluses as necessary. Patients required sedation prior to this procedure[220].

PGE_2.

The local or systemic administration of PGE_1 or PGE_2 has now replaced $PGF_{2\alpha}$ in the management of pregnancy termination. In the first or mid-trimester of pregnancy the primary prostaglandin is less effective than its synthetic analogues which have been designed to hinder inactivation by prostaglandin dehydrogenase. Examples of these analogues include sulprostone (16-phenoxy-tetranor-PGE_2-methyl sulphonamide) given intramuscularly, gemeprost (16, 16-dimethyl-transΔ_2 PGE_1 methyl ester, Cervagem) given intravaginally or misoprostol (a PGE_1 analogue) given orally or intravaginally. The latter has the advantage of being cheap and has been shown to be equally as effective as gemeprost in producing cervical ripening[221].

In the first eight weeks of pregnancy use of these preparations will result in about 90% success in effecting termination of pregnancy avoiding the need for anaesthesia and in-patient hospital admission. Some patients, however may experience the predictable gastrointestinal side-effects caused by prostaglandins and some will have severe uterine pain[116]. The use of mifepristone as an adjunct to medical termination of pregnancy is discussed below.

At eight to twelve weeks of gestation most clinicians will employ surgical termination of pregnancy. The use of prostaglandins preoperatively will however increase the initial dilatation of the cervix, the ease with which it can subsequently be dilated and reduce the blood loss during the procedure[215-218]. These effects have been demonstrated with vaginal PGE_2[215], gemeprost pessaries[216,218] and more recently vaginal or oral

misoprostol, a PGE_1 analogue[217]. In 1984, the Medical Advisory Committee of the International Planned Parenthood Federation recommended that these agents should be used routinely prior to suction termination[116].

At later gestations the use of PGE_2 either vaginally in the form of gemeprost pessaries or by the extra-amniotic route[222] will produce termination in most cases. The use of PGE_2 by either route rather than $PGF_{2\alpha}$ is associated with improved cervical softening and is less likely to cause uterine rupture as compared with intraamniotic $PGF_{2\alpha}$ [223].

The histological changes associated with the administration of prostaglandins have been reported in several studies[218,224,225]. Gemeprost administration prior to taking a cervical biopsy at the time of surgical termination in the first trimester was found to cause a significant reduction in the polymerised collagen concentration of the cervix (as measured by the optical densitometry of Picrosirius red staining) and a modest neutrophil influx as compared with placebo administration. No change in the GAG content of the cervix was noted following prostaglandin administration in this study[218]. Microscopy of the cervix following PGE induced mid-trimester termination showed dispersed collagen fibrils in an increased amount of ground substance with neutrophil invasion noted in samples of the cervix obtained from more proximal regions[225]. This effect of prostaglandin administration on the collagen concentration has been shown to be associated with an increase in the activity of collagenase and elastase in cervical biopsies. The levels of these enzymes increase with the length of time after PGE administration that biopsies were taken[224]. In this study an increase in the hyaluronic acid concentration was described in around half of the cervices examined. No alteration has been found in the collagen structure of the amnion following mid-trimester termination of pregnancy using a vaginal

PGE_1 analogue, suggesting that the uterine tissues can respond differently to the local administration of prostaglandins[225].

The histological effects of prostaglandin E or its analogues on the cervix therefore closely resemble the collagen dissociation and inflammatory infiltrate seen in physiological cervical ripening giving a good basis for its use for this purpose in the first and second trimesters of pregnancy and also supporting the role of endogenous prostaglandins in the normal process of cervical ripening.

2.2: CERVICAL RIPENING AND INDUCTION OF LABOUR.

2.2i: Historical considerations

Many methods have been tried over hundreds of years to induce labour. These have been extensively reviewed by Thiery[231]. The first recorded attempts used manual dilatation of the cervix which was superseded by the use of intracervical tents, such as the laminaria. Mechanical methods to induce labour also included the insertion of bougies or catheters into the cervix or the extra-amniotic space. An alternative was to infuse large amounts of fluid into the vagina or uterine cavity. The procedure of amniotomy was first utilised to promote the onset of labour in the mid- 18th century. Both high (or hindwater) and low amniotomy techniques were described but neither was consistently effective and as they were associated with a high incidence of intra-uterine infection their use was discontinued for many years. In the last forty years the use, particularly of low amniotomy, has once again become part of obstetric practice, most often in conjunction with oxytocin infusions as first described in 1968 [227]. High amniotomy is very rarely performed now but a Drew-Smythe catheter may still be occasionally employed for this purpose.

The earliest non-surgical methods of inducing labour used cloths which were soaked in plant extracts and then packed into the vagina. The administration of ergot, also used as an abortifacient, was revived during the early 19th century but was discontinued due to the severe risks of hypertonus. The discovery of ergometrine in the 1930s led to a re-evaluation of its use in labour induction but it is now solely used to control third stage haemorrhage.

During the 19th century most attention was focused on the mechanical methods of inducing labour but in the early twentieth century a 'medical induction' was proposed by Watson [228]. This method involved the administration of castor oil, quinine and pituitary extract. This was further combined with a hot bath and a hot soap enema. Although the first two elements and baths and enemas are no longer used, the purification of pituitary extract to produce oxytocin was a major milestone in the development of pharmacological methods to induce labour. Indeed those responsible for the characterisation and purification of oxytocin and antidiuretic hormone were awarded a Nobel prize in 1955. Oxytocin was initially used in continuous infusions but the use of a titrated regimen as described by Turnbull and Anderson[227] in combination with amniotomy improved its efficacy and decreased the risk of uterine hypertonus. At this time use of the prostaglandins to induce labour was also being reported[229]. The initial work reported in 1968 used $PGF_{2\alpha}$ with the similar action of PGE_2 being reported in the following year[230].

'Natural' methods to induce labour have also been advocated, for example nipple stimulation and sexual intercourse. Nipple stimulation has been shown to provoke uterine contractions by causing release of oxytocin from the posterior pituitary but it has been suggested to have a risk of uterine hypertonus[231]. A study of 300 women of at least 39

weeks gestation who performed this for 3 hours per day showed a significant reduction in the incidence of post-term pregnancies with no alteration in the rate of caesarean section[232]. Sexual intercourse has theoretically been proposed to increase the likelihood of labour occurring due to the prostaglandin content of semen and the suggestion that female orgasm is associated with oxytocin release but this has not been formally evaluated as a method of labour induction[231].

There have therefore been numerous methods utilised in the attempt to induce labour over centuries of obstetric practice. As the physiological mechanisms of cervical ripening and dilatation and the control of myometrial contractions are more clearly understood such methods can hopefully be refined to mimic the normal physiology of parturition as closely as possible to minimise the risks of side-effects and the chance of the procedure failing.

2.2 ii: Indications for and risks of labour induction.

As the methods described above have been developed and refined it has become apparent that none are without risks to the mother and to the fetus. The use of either prostaglandins or oxytocin can cause uterine hyperstimulation resulting in hypertonic contraction of the myometrium. This can contribute to fetal hypoxia and, if prolonged, necessitate emergency caesarean delivery. Oxytocin and its synthetic derivatives have weak anti-diuretic hormone actions and thus administration of large amounts over time in fluid may provoke fluid retention and pulmonary oedema. Prostaglandins, even when administered vaginally, can cause gastrointestinal side-effects, such as vomiting and diarrhoea and uncomfortable uterine contractions.

In addition to the side-effects inherent in the use of these agents the process of induction of labour carries additional drawbacks. The procedure may take a variable length of time

during which the mother will be an in-patient in the maternity unit, in primigravidae in particular it may take at least 24 hours for labour to become established after the initial attempt to ripen the cervix. It may also be that the procedure will fail to induce labour within a given time span or with a given regimen of drugs. In such cases clinicians must then decide whether to abandon the attempted induction or to proceed to caesarean section.

It is therefore of vital importance that, in addition to the development of safe and effective methods of inducing labour, the conditions in which labour should be induced are carefully evaluated. There are many clinical scenarios in which the risks to the mother or to the fetus of the pregnancy continuing outweigh the potential risks of induction or of delivery of a possibly preterm infant. Such a decision will be made in a situation where it is on balance safer to induce labour but where the condition of neither the fetus nor the mother requires the rapid and less stressful delivery of a caesarean section.

In many cases the risks to mother or fetus of the pregnancy continuing are fairly clear. However, many inductions are performed for the reason that the pregnancy is 'post-term' and this indication has received much recent attention. The definition by the World Health Organisation of a post-term pregnancy is one which exceeds 42 completed weeks or more. There has been evidence to suggest that perinatal mortality is slightly increased after this time [233]. The question of whether to induce labour after 41 weeks or whether to monitor the pregnancies and only deliver those in which there is evidence of compromise has been addressed by several studies, the largest being reported by the Canadian MultiCentre Post-term Pregnancy Trial Group [234]. This group studied 3407 women at gestations of over 41 weeks and randomly allocated them to either having labour induced or to serial antenatal monitoring with Caesarean delivery being performed in the presence of any evidence of fetal compromise. There was no significant difference in the perinatal

mortality rate between the two groups nor in the neonatal morbidity. There was a significant reduction in the rate of caesarean sections in the induced group but this may be due in part to the rather rigid policy of performing caesarean rather than inducing labour if fetal compromise was suspected[235]. The low rate of perinatal loss of two in over three thousand cases reflects the low rate of perinatal mortality in developed countries which at 41 weeks is estimated at 2 per 1000 rising to 3 per 1000 at 42 weeks and 4 per 1000 at 43 weeks[233,236,237]. These figures are however based on small numbers of cases and may not be a true representation of the figures. [233,235] This low rate of an outcome measure is one reason that the use of meta-analysis, such as in the Cochrane database is a significant advance in the evaluation of clinical trials. Using this technique to examine randomised controlled trials comparing elective induction after 41 weeks with conservative management, it was found that induction decreased the incidence of perinatal death in normal babies (relative risk 0.23; confidence interval 0.76 - 0.98)[238]. In practical terms it has been calculated that this means inducing 500 women to prevent one perinatal death occurring[235]. This meta-analysis indicates that routine induction of labour is not associated with any major adverse events for mother or fetus, reduces the already small risk of perinatal death and may be associated with a reduction in the caesarean section rate[233,239].

In view of the potential problems of labour induction it is therefore essential that before induction is embarked upon the reasons for this are carefully evaluated and the potential risks and benefits of the procedure explained to the mother.

2.2 iii: Cervical ripening prior to labour induction.

Induction of labour requires progressive cervical dilatation and regular uterine contractions. It has been found that the success of induction relates to the 'ripeness' of the

cervix prior to the onset of the induction procedure. The ripeness of the cervix can be quantified by scoring of various criteria on vaginal examination. such as the system described by Bishop in 1964 which was originally designed for use in multiparous patients[193]. Scores were assigned according to the cervical dilatation, percentage effacement, consistency and position and by the level of descent of the fetal head out of a total of 13. Modifications of this system have been proposed by several groups including Calder et al [240] (shown below) in which a modified score is obtained by quantifying the cervical length rather than expressing it as percentage effacement and giving less emphasis to the position of the cervix. The total score in this system is 12:

	0	1	2	3
Dilatation (cm)	< 1	1 - 2	2 - 4	> 4
Length (cm)	> 4	2 - 4	1 - 2	< 1
Consistency	Firm	Average	Soft	
Position	Posterior	Mid - Anterior		
Level *	- 3	- 2	- 1; 0	Below spines

* Level of presenting part in relation to the maternal ischial spines

In the initial proposal of a cervical score Bishop showed that in multiparae there was a correlation between this score and the length of time to the spontaneous onset of labour[193]. Calder *et al* subsequently described a group of 125 primigravid women undergoing induction of labour by amniotomy and oxytocin infusion in whom there was a significant increase in the length of time to delivery and in the rates of caesarean section,

maternal pyrexia and birth asphyxia in those women whose initial modified Bishops score was less than 3[241]. It has been known for over 30 years that the presence of an unripe cervix increases the uterine work needed to effect dilatation three to four fold[242]. It thus seems that production of uterine contractions alone is not necessarily sufficient to successfully and safely induce labour particularly in the presence of an unripe cervix. It has been suggested more recently that the presence of fetal fibronectin in vaginal secretions may act as a reliable predictor of the ease of labour induction, in terms of interval to delivery and caesarean section rates, independent of the Bishops' score[243].

Methods to ripen the cervix prior to the onset of uterine contractions have therefore been studied. Although these methods may also result in some women in myometrial contractions and labour their primary purpose is to ripen the cervix to the extent that has been associated with a more successful outcome of subsequent induction.

The techniques employed to ripen the cervix are similar in principle to those described above used to terminate pregnancy in the first trimester. When such methods are used in the third trimester, however, fetal well-being must also be considered in addition to that of the mother.

Mechanical devices and cervical ripening.

The oldest methods of attempting to ripen the cervix are by digital means or by the insertion of devices into the cervix, such methods were described by Hippocrates as described in a review by Trofatter [244]. These methods have been refined into the insertion of either a balloon catheter or a hydroscopic dilator such as Lamicel or Dilapan into the cervix. A balloon catheter can be inserted into the cervix and the balloon inflated above the level of the cervical os, with the catheter then being withdrawn until the catheter is held

against the internal os. Additional traction can be applied by the use of a weight attached to the end of the catheter[244]. While this method may be effective at promoting cervical ripening it is a rather awkward method and has largely been replaced by more recent developments. The use of synthetic hygroscopic dilators, such as Lamicel placed in the cervix does result in gradual cervical ripening[93,244]. It is debatable as to whether the effect of these devices in promoting cervical ripening is a result of the mechanical force exerted as they swell or of the resultant increase in the local production of prostaglandins or to a combination of these effects[93,245]. Although they have a small effect on cervical ripening the use of Lamicel has been shown in several studies to be less effective than prostaglandins at increasing the cervical ripeness as assessed by the pelvic score and to be less likely to result in labour onset during the ripening period[93]. Meta-analysis of trials of mechanical methods for cervical ripening concluded that "the data limited as they are, do not suggest that the insertion of various mechanical devices into the cervix, or the extra-amniotic space, is a useful approach to ripening the cervix prior to the induction of labour"[93]. This negative conclusion may be due, at least in part, to the lack of large studies with well defined outcome measures addressing the effects of mechanical devices. Such devices, however, may still have a place in obstetric practice, for example in patients in whom the use of prostaglandins is contra-indicated .

Prostaglandins and cervical ripening.

The initial clinical study of the use of prostaglandins for induction of labour reported in 1968 used intravenous $PGF_{2\alpha}$ [229]. In 1973 it was reported that the use of a lower dose of prostaglandin than that needed to induce labour had a marked effect on cervical ripeness[246]. In subsequent years studies have addressed the optimal dose, route and regimen of administration. Meta-analysis of all such randomised controlled trials confirms that the use of either prostaglandin E or $F_{2\alpha}$ by all the studied routes (oral, vaginal,

extraamniotic or intracervical) results in a significant increase in the cervical score and an increased chance of delivery during the ripening procedure as compared with placebo[93]. These beneficial effects are offset by an increase in the incidence of uterine hypertonus with the use of prostaglandins. Analysis of the five trials which used $PGF_{2\alpha}$ showed that this compound is effective at improving the cervical score, at producing delivery during the ripening period but also carries a risk of uterine hypertonus. There are only two studies comparing the effects of PGE_2 with $PGF_{2\alpha}$ directly and these do not suggest a significant difference between the two agents but PGE_2 is five to ten times more effective than $PGF_{2\alpha}$ and a lower dosage can thus be used that is associated with fewer side-effects[93,96].

PGE_2 can be administered orally but this is not as effective in producing cervical ripening than local routes and may be expected to cause more systemic side-effects[93]. Local administration may be either extra-amniotic, intracervical or vaginal. Extraamniotic infusion of PGE is achieved by the insertion of a transcervical Foley catheter into the extraamniotic space and the delivery of PGE_2 in a gel into this space. The production of cervical dilatation associated with mild uterine contractions means that ideally the catheter will be expelled once the cervix is around 3cm dilated and therefore ripened in preparation for formal induction of labour. In a study of primigravidae with unripe cervices the use of this technique prior to amniotomy and oxytocin infusion produced a decrease in the length of the induction period and a marked decrease in the rates of maternal pyrexia, caesarean section and Apgar scores of ≤ 4 at one minute after delivery[247]. Although this method was effective it has largely been superseded by the use of either vaginal or intracervical preparations due to the increased ease of administration of these preparations.

Use of a vaginal gel or tablet to administer PGE_2 is a simple method which does not involve the inconvenience to the patient of an intra-uterine catheter. In a comparison of the vaginal with the extra-amniotic route for PGE_2 administration in primigravidae[248] they were found to be comparable in terms of their effectiveness in producing cervical ripening and in the number of women subsequently requiring oxytocin. Women who received the prostaglandin as a vaginal tablet required less analgesia during the period of cervical ripening. These results are similar to those found by meta-analysis of the available trials comparing extra-amniotic with vaginal administration of PGE_2 which also found no difference between the efficacy of the two methods in promoting cervical ripening. There is a slightly higher likelihood of labour occurring during ripening with extra-amniotic than with vaginal PGE_2 (odds ratio 2.71 with 95% confidence interval 1.34 - 5.51). In this analysis there was no difference in the incidence of side-effects or of maternal pyrexia between the two routes[93]. The plasma levels of prostaglandin E and F metabolites significantly increase following the administration of PGE_2 by either route with a greater increase seen following extra-amniotic administration. This increase directly correlated with the alteration in cervical score[96]. The greater increase in PGFM production after extra-amniotic administration of PGE_2 may reflect an increase in the production of $PGF_{2\alpha}$, a uterotonin, explaining the increased need for analgesia with this route of administration.

PGE_2 administration by the endo-cervical route is comparable with the vaginal route in terms of its efficacy with a slight increase in the number of women delivering during the ripening period following the use of endo-cervical PGE_2[93]. A smaller dose is utilised with this method, 0.5mg compared with 1 or 2mg when given vaginally as a gel preparation or 3mg in the tablet formulation. In a double blind comparison between the two methods the intravaginal route was found to be more effective at increasing the

cervical score than the endo-cervical route with no difference in the incidence of side-effects. This study did not report on the incidence of delivery during the ripening period. The study groups consisted of nulliparous and multiparous patients and the intravaginal group did contain more parous patients which may have biased the results[249]. Although prostaglandins are generally safe, in addition to their potential systemic side-effects their oxytocic action means that their is a small risk of uterine rupture following application. This complication has been reported after all methods of prostaglandin administration [250].

The vaginal route of administration is therefore as effective as either extra-amniotic or endocervical administration in causing cervical ripening, although the latter two routes are slightly, but significantly, more likely to result in the onset of labour or delivery during the ripening period. This slight advantage of these two routes is however balanced by the relative ease with which vaginal forms of PGE_2 can be given, without a need to visualise or cannulate the cervical os. Repeated doses of vaginal prostaglandin may be needed to produce sufficient dilatation to allow induction to proceed. The development of a controlled release preparation of PGE_2 (Propess, Ferring, UK) may replace such repeated doses and this preparation has been shown to produce plasma levels of PGE metabolite comparable with those seen in spontaneous labour and to be effective in the production of cervical ripening[96,251]. There have however been concerns regarding the predictability of the prostaglandin release from the pessary *in vivo* and the possibility of a high incidence of uterine hypertonus [96].

Oxytocin and cervical ripening.

In view of the physiology of parturition the use of oxytocin would be expected to produce uterine contractions but not to effect significant cervical changes other than by the action of such contractions. Myometrial contractions in the presence of an unripe cervix require

more work than when the cervix is ripe and so the use of oxytocin for this purpose does not seem to offer much advantage. This theoretical view is confirmed by the few studies that examined the effects of oxytocin on cervical ripening, the conclusion from the meta-analysis of these trials being that use of such treatments "serves no useful purpose....This practice should be abandoned"[93].

Oestrogens and cervical ripening

Oestradiol, oestriol and the oestrogen precursor dehydroepiandrosterone sulphate have all been studied as potential agents to ripen the cervix. In 1977 the extra-amniotic administration of oestradiol valerate to primigravidae was compared with placebo administered by the same route and shown to be more effective at increasing the cervical score, at reducing the caesarean section rate and at decreasing the incidence of maternal pyrexia[120]. However, when a further two placebo controlled trials were also taken into accout in a meta-analysis the use of oestrogen was not found to have a significant benefit on cervical ripening or on the onset of labour [93].

Relaxin and cervical ripening.

Relaxin is a hormone which is produced in humans by the corpus luteum, decidua and chorion. That in use for therapeutic purposes is derived from the corpora lutea of pigs and has been known for seventy years to relax the pelvic ligaments of the guinea pig. In view of its effects on connective tissue it has been proposed to play a role in cervical ripening. In the 1950s it was claimed to cause cervical softening but paradoxically it was also claimed to prevent preterm labour. Placebo controlled trials did not confirm any benefit on cervical softening[252]. More recently relaxin has become available in a purified form and has been re-evaluated as a method of promoting cervical ripening. The small studies performed suggest a beneficial effect on cervical score and an increase in the incidence of

labour onset during ripening[93,252]. A randomised trial of 96 women, however, failed to demonstrate any significant effect of recombinant human relaxin, administered as an intravaginal gel, on ripening the cervix at term [253].

The current method of choice for cervical ripening prior to labour induction is therefore PGE_2. The initial route of administration was extra-amniotic and while this is effective in producing a ripened cervix it has largely been replaced by the use of the endo- cervical and vaginal routes of administration. The latter are both simpler to employ and involve less discomfort for the mother. While the vaginal route has been suggested by meta-analysis of the available data to be marginally less effective, it is the simplest method of administration.

2.2 iv: Induction of labour.

Although the process of cervical ripening may sometimes induce labour and delivery the methods considered below are those used with the intention of causing uterine contractions with concomitant cervical dilatation and thus leading to vaginal delivery.

'Sweeping the membranes'.

This refers to the mechanical process of digitally stripping the membranes off the decidua during vaginal examination. It will only be possible if there is already some degree of cervical dilatation. This procedure has been shown to cause a significant rise in the maternal plasma levels of prostaglandin metabolites [254] but despite its common use in obstetric practice its use has not been formally evaluated since 1958 [94].

Amniotomy

The fetal membranes may be ruptured using the technique of either high or low amniotomy. The latter method is almost exclusively used nowadays. Although this procedure may induce labour it does so in an unpredictable fashion and there may be a long delay before the onset of contractions causing a risk of ascending intrauterine infection[94]. Combination of low amniotomy with oxytocin increases the effectiveness of this method at inducing labour [94].

Oxytocin

In the late 1940s the use of pituitary extract to induce uterine contractions was studied, this extract was further purified to give oxytocin as discussed above. This is now usually administered intravenously and titrated against the apparent uterine activity[94,227]. Controlled delivery of a titrated dose is used to decrease the risk of uterine hypertonus and to prevent fluid retention resulting from the administration of a significant dose of oxytocin (which has an anti-diuretic effect) in a large volume of fluid. Analysis of available data indicates that the use of oxytocin is associated with an increased incidence of neonatal jaundice. It cannot be concluded whether this is in fact a causal relationship or a reflection of increased risk of jaundice in situations in which oxytocin is used[94].

Prostaglandins

In addition to their proven efficacy in ripening the cervix, prostaglandins have also been studied as potential agents to induce labour. In a review of trials of prostaglandins for this purpose by Keirse and Chalmers [94] it was confirmed that prostaglandin administration is significantly more effective than placebo in effecting labour induction and delivery with a low incidence of maternal side-effects. While both $PGF_{2\alpha}$ and PGE_2 have been studied in this context it appears that the latter is at least as efficacious as the former and has a

lower incidence of side-effects. In the United Kingdom PGE_2 is now almost exclusively used for this purpose. Topical or oral preparations have largely replaced intravenous ones as they are equally effective with a lower incidence of systemic side-effects. The use of misoprostol (PGE_1) intravaginally for labour induction has been studied and is of interest as it is much cheaper than the topical preparations currently available. At present, however, misoprostol is only licensed for use orally in the prevention and treatment of gastric ulcers. In a comparison between intravaginal misoprostol and dinoprostone (PGE_2) the oral prostaglandin was shown to improve the Bishops score significantly more than the vaginal tablet while interval to delivery was unchanged between the two groups and there was no difference in the methods of delivery [255]. A later study, however, while confirming the improved efficacy of misoprostol as compared with dinoprostone at ripening the cervix and inducing labour, reported an increase in the number of caesarean sections performed for fetal distress in the group that received misoprostol [256]. It is of note that currently no synthetic prostaglandins are used for the induction of labour with a live fetus and it may be that their increased resistance to catabolism is a disadvantage in this context.

When all available trials comparing the use of prostaglandins with oxytocin by any route were collated using meta-analysis[257] it was found that the use of prostaglandins carried a higher probability of delivery within 24 and 48 hours but no increase in the probability of delivery after 12 hours. Less analgesia was required after induction with prostaglandins. Use of prostaglandins, however, had an increased risk of gastrointestinal side-effects and of uterine hyperstimulation. When the use of vaginal prostaglandins was directly compared with oxytocin the only significant benefit of the former was a small increase in the incidence of delivery within 12 hours. The analysis of these trials was confounded, however, by the wide variety of induction schedules that were followed. When the

intrauterine pressure was studied during induction of labour in women with either vaginal PGE_2 gel or with amniotomy and oxytocin infusion it was found that the use of PGE_2 was associated with a longer pre-established phase and a shorter established phase of labour than oxytocin with lower uterine activity integrals (the intrauterine pressure over 15 minute periods) being observed in the nulliparous patients. This means that in the prostaglandin treated group less overall uterine activity was required to attain the same degree of cervical dilatation than in the oxytocin treated group[258]. This is consistent with the effect of prostaglandins in promoting cervical compliance prior to the onset of regular contractions which would be expected to decrease the myometrial work needed to cause subsequent dilatation. A comparison has been performed of labour induction with intravaginal misoprostol or intravenous oxytocin with cervical ripening with PGE_2 if needed. The women given misoprostol delivered within a shorter time interval and there was no difference in the rate of intrapartum complications, neonatal or maternal adverse effects between the groups despite a greater frequency of uterine tachysystole after misoprostol administration [259]. A meta-analysis of studies comparing the use of intravaginal misoprostol with placebo which included 966 patients, confirmed the efficacy of misoprostol for cervical ripening and that it increased the rate of vaginal delivery within 24 hours and decreased the caesarean rate[260]. This cheap and easily administered prostaglandin may therefore be an alternative to current induction regimens.

2.3: MIFEPRISTONE (RU486).

2.3 i: Mechanism of action and effects on uterine tissues.

Mifepristone (RU38486, now shortened to RU486) was first described by Philibert in 1981[261]. Although its main initial clinical application was as an abortifacient [262,263] many other uses have subsequently been investigated, both in gynaecology, for example as a contraceptive[264] and in other areas of medicine, such as in the management of meningiomas [265].

RU486 is a derivative of norethindrone containing a 4-(dimethylamino) phenyl group at the 11ß position and a 17α 1-propynyl chain. It is thought that the 17 substitution increases its binding affinity to the progesterone receptor as compared with progesterone and that the 11ß branched aminophenyl group induces and stabilises the formation of inert receptor complexes[264]. Mifepristone binds to progesterone and glucocorticoid receptors with a high affinity, 5 times greater than the binding of progesterone to the rabbit uterus receptor and 3 times more than that of dexamethasone to the rat thymus glucocorticoid receptor. It also shows weak binding to the androgen receptor but does not bind to oestrogen or to mineralocorticoid receptors[264]. Other synthetic antigestagens have since been synthesised including onapristone (ZK98299) and lilopristone (ZK98734) which both have fewer antiglucocorticoid effects than mifepristone. Onapristone shows stronger synergism than mifepristone with prostaglandins and oxytocin and has a shorter half life[266].

The progesterone receptor

The progesterone receptor (PR) is capable of 'shuttling' between the cytoplasm and the nucleus but resides mainly in the nucleus in normal circumstances (in contrast to the

glucocorticoid receptor which is cytosolic). It is a ligand-activated transcription factor which consists of four regions[264,267]. These are a DNA binding area which is characterised by two 'zinc fingers', a hinge region which is probably involved in the nuclear localisation of the receptor, the hormone binding domain and a transactivation area which increases gene transcription once the hormone is bound. It seems that the glycine residue at position 722 of the progesterone receptor is essential for the binding of RU486, as in the chick receptor this is substituted with a cysteine and RU486 does not bind to this receptor[264]. In vivo the unbound receptor is associated with cytosolic proteins including heat shock protein 90 (hsp90) which inactivate the receptor. Following progesterone binding the receptor is activated (transformed) and the hsp90 is dissociated exposing the DNA binding area. The activated hormone-receptor complexes then dimerise. These dimers then bind to the progesterone response elements (PRE) which are transcriptional enhancer regions of progesterone responsive genes causing increased rates of transcription of regulated proteins.

When RU486 binds to the PR it causes a very similar transformation of the receptor[268] and dimerisation of the complexes also occurs, however the hsp90 does not seem to then dissociate from the receptor. The dimers may still bind to the PRE of responsive genes, but in the presence of progesterone the drug-receptor complexes are inactive, which may be due to a difference in the structure of the receptor once bound to RU486 [268]. If, however, there is no progesterone present the drug-receptor complexes may have partial agonistic effects on the DNA transcription of regulated proteins [264]. This partial agonistic effect of RU486 has been demonstrated clinically in post-menopausal women who are receiving oestrogen replacement[269]. When RU486 is administered to such women it causes endometrial changes similar to those seen with progesterone administration,

however if RU486 is given with progesterone to this group of women it will antagonise the effects of the progesterone .

Pharmacology of mifepristone.

RU486 is currently used as an oral preparation and although one study reported intravaginal use in humans this resulted in inadequate absorption of the drug as compared with vaginal use in rats and monkeys[270]. Following oral administration of RU486 metabolism in the splanchnic circulation results in a bioavailability in humans of 40% whereas in monkeys the bioavailability is 15%. Clearance in humans is slow, around 0.55L/kg/day which is less than in other animals such as monkeys (36L/kg/day) or rats (72L/kg/day)[264]. Part of the reason for the decreased clearance in humans is that in humans mifepristone binds with a high affinity to orosomucoid (a1 acid glycoprotein)[271]. It does not however bind to either sex hormone binding globulin (SHBG) or to corticosteroid binding globulin (CBG). The maximum plasma concentrations of RU486 in women are reached about one hour after oral administration. The pattern of kinetics seen varies with the dose of mifepristone used, after low doses of 50mg to 100mg first order kinetics are displayed with a half-life of 20 to 25 hours [264,272] but at higher doses of 100mg to 800mg two phase kinetics are seen with a redistribution phase lasting 6 to 10 hours followed by a plateau in serum levels for at least 24 hours [273]. During this plateau a dose-dependent change in serum levels is not seen but metabolite levels do increase with higher doses. These metabolites also exhibit some antagonistic effects which, although weaker than those of RU486, may have biological effects due to their relatively high concentration in serum [273]. Metabolites are formed by a 2 step demethylation of the C11 ring and hydroxylation of the 17-propynyl side chain followed by further hydroxylation and acetylation. Excretion of drug and metabolites is mainly faecal [274].

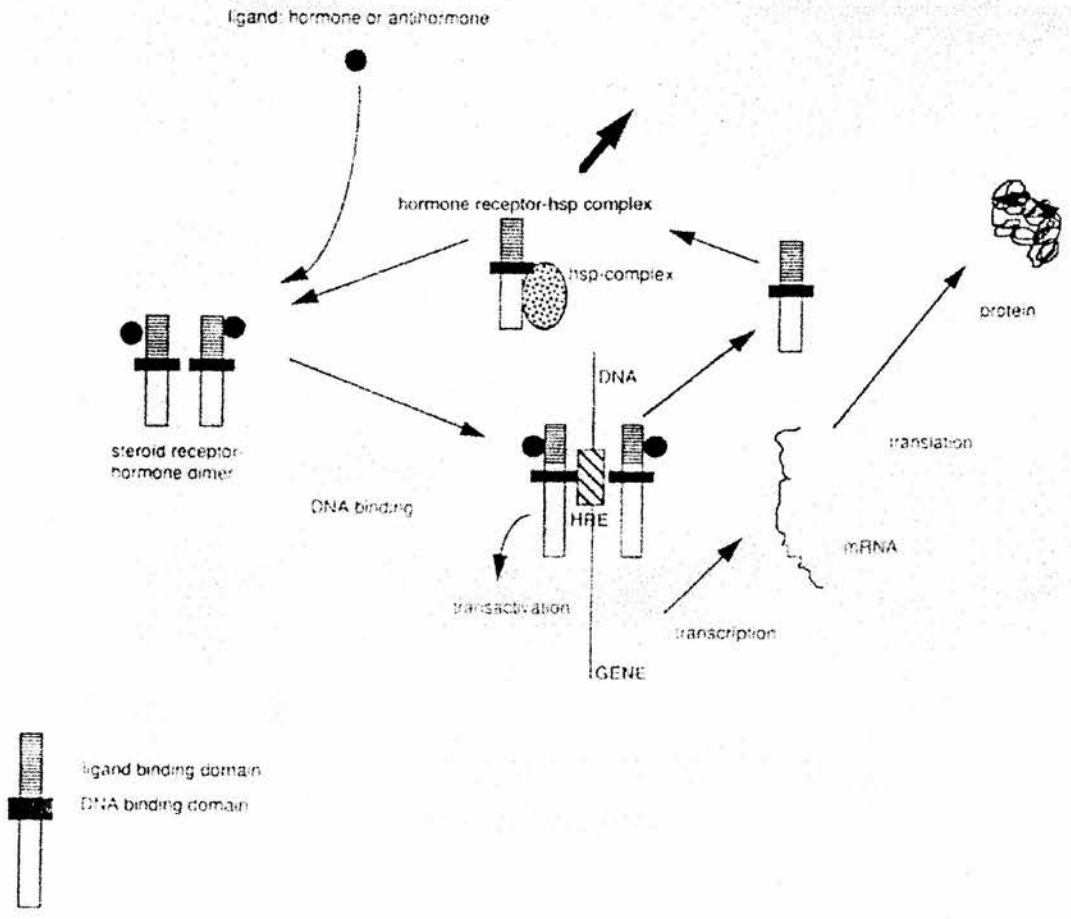
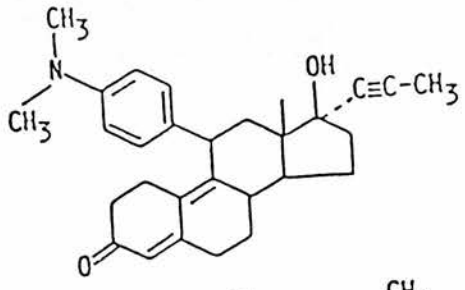
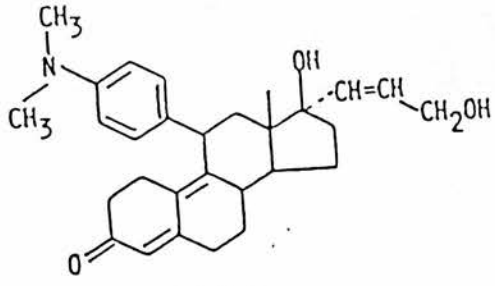


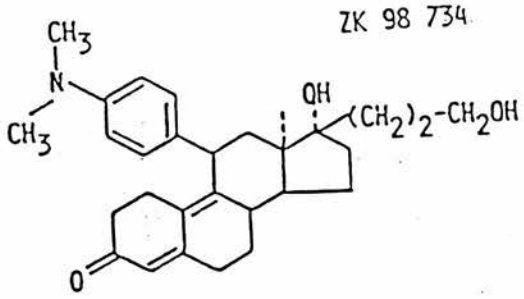
Figure 2.1: The mechanism of activation of steroid hormone receptors



RU 38486



ZK 98 734



ZK 98299

Antiglucocorticoid Effect

Mifepristone shows a dose-dependent inhibition of the negative feedback effect of cortisol on ACTH secretion meaning that increasing levels of peripheral cortisol can overcome the antagonistic effects of mifepristone [275]. With a relatively low dose of mifepristone (6mg/kg) this effect is most marked during the morning hours when the negative feedback of cortisol on the hypothalamic-pituitary is inhibited leading to increased circulating cortisol levels[276]. When 600mg of mifepristone is administered to women to induce termination of pregnancy, the subsequent rise in peripheral cortisol levels is observed throughout the day. Short term courses of RU486 have not been shown to cause symptoms of glucocorticoid deficiency [277]. In Rhesus monkeys a low dose of 1mg/kg of mifepristone was sufficient to cause elevation of plasma ACTH whereas higher doses of 5mg/kg and 10mg/kg also caused increased plasma cortisol and arginine vasopressin levels[278]. The effect on vasopressin is postulated to be due to inhibition of the glucocorticoid provoked rise in the osmotic threshold for vasopressin release [279]. In these monkeys mifepristone administration did not affect the plasma levels of FSH, LH, GH or TSH[280]. Use of mifepristone has been reported in patients with Cushings syndrome[281,282] who have increased ectopic ACTH secretion or carcinomas secreting cortisol. In such patients, who have a fixed rise in their cortisol levels, mifepristone reverses most of the symptoms of Cushings syndrome. This benefit is not reported in patients with Cushings disease in whom cortisol secretion is variable.

Mifepristone and prostaglandins.

The effects of mifepristone on prostaglandin production and metabolism are relevant to many of its potential uses in reproductive medicine. As described above, endogenous prostaglandins are implicated both in first trimester interruption of pregnancy, whether spontaneous or induced, and in the initiation of labour. It has long been postulated that

progesterone has a key role in the maintenance of pregnancy. In the early 1970s, Csapo[219,283] proposed a 'see-saw' theory for pregnancy regulation whereby a balance is maintained during pregnancy between progesterone and tocomimetic substances, such as PGF2α and oxytocin. His hypothesis was that at the initiation of parturition it is the withdrawal of progesterone which stimulates the events leading to labour by promoting levels of prostaglandins and other oxytocics. Antagonism of progesterone by an exogenous agent, such as mifepristone, might therefore be expected to promote termination of pregnancy at any stage.

Prostaglandins such as PGE_2 and $PGF_{2\alpha}$, which are of most relevance in the context of the pregnant uterus are metabolised by 15-hydroxyprostaglandin dehydrogenase (PGDH). The catabolism involves initial oxidation of the alcohol group at C-15 by PGDH, followed by reduction of the double bond at C-13 and then further oxidative steps. Prostaglandin metabolism mainly occurs in the pulmonary cells but PGDH also occurs in other tissues, such as endometrium [284] and decidua [104,285] suggesting it has a role in the control of local prostaglandin concentrations. The short half life of this enzyme of 47 minutes would provide a relatively rapid control mechanism of local prostaglandin concentrations. PGDH has been suggested to be under progesterone control as an increase in levels of progesterone cause a corresponding increase in PGDH activity[286]. Progesterone control of local prostaglandin levels by an increase in their metabolism could provide a mechanism for the maintenance of uterine quiescence during pregnancy until the time of parturition. Antagonism of this effect by mifepristone would thus favour events leading to termination of pregnancy or parturition by increasing locally available levels of prostaglandins.

The levels of PGDH in first trimester human decidua have been shown to be markedly reduced following mifepristone administration when assessed by either assay for enzyme

activity or by the level of immunohistochemical staining intensity[287]. PGDH is immunolocalised in the decidual stroma and gland cells and in the endothelial cells of the decidual blood vessels. The effect of mifepristone in decreasing the staining intensity for PGDH appears to be most marked around the blood vessels. This decrease in the catabolic enzyme following mifepristone administration in the first trimester of pregnancy is accompanied by an increase in immunostaining for PGE_2, also in decidual glands and small vessels and with a parallel decrease in PGEM immunostaining [287].

In vitro endometrial cell cultures treated with mifepristone exhibited increased $PGF_{2\alpha}$ and PGE_2 production which was inhibited in a dose-dependent manner by progesterone. In these cell cultures progesterone led to increased conversion of prostaglandins to their 13,14-diydro-15-keto metabolites[288]. A similar effect was observed in cultures of decidual cells[289]. In the guinea pig uterus pretreatment with mifepristone leads to decreased PGDH levels in subsequent tissue culture but no alteration in the prostaglandin production by these tissues[290]. Similar findings have been reported for women in the first trimester of pregnancy. Culture of decidual tissue collected from women who had been given mifepristone prior to first trimester termination of pregnancy showed increased $PGF_{2\alpha}$ production and decreased PGFM (the metabolite of $PGF_{2\alpha}$) levels compared with control tissue [291]. While it is apparent that mifepristone does affect prostaglandin production and metabolism by uterine tissues, its clinically observed effects do not seem to be solely mediated via this effect. For example, in a further study[292] women were treated with mifepristone prior to termination of pregnancy in the first trimester and one group were also given indomethacin (a prostaglandin synthase inhibitor). As expected from previous work, mifepristone pre-treatment increased the *in vitro* production of $PGF_{2\alpha}$ by decidual cells and this increase was significantly reduced by concomitant indomethacin administration. However, the observed uterine activity (as measured by an

intrauterine pressure recorder and expressed in Montevideo units), increased after mifepristone treatment, regardless of indomethacin administration. Similar findings have been reported in studies of varying tissues, species and prostaglandin synthase inhibitors. In the cervix, as further discussed below, the effects of mifepristone on cervical ripening in the late pregnant rat are not reversed by diclofenac administration[293] nor is the effect of mifepristone in causing increased cervical softening and dilatation in women in the first trimester of pregnancy reversed by the concomitant administration of naproxen (a cyclooxygenase inhibitor) [294].

Mifepristone therefore increases production of prostaglandins by uterine tissues and decreases their metabolism, however inhibition of this action by cyclooxygenase inhibitors may not prevent most of the events provoked by mifepristone. One reason for this could be that, as discussed above, there are at least two forms of the cyclooxygenase enzyme: COX-1 and COX-2. The latter is less inhibited by agents such as diclofenac and may mediate at least some of the actions of mifepristone that are not inhibited by currently used cyclooxygenase inhibitors. Alternatively, the observed effects of mifepristone on uterine contractility and cervical ripening may be mediated by other pathways involving local factors such as cytokines or relaxin. In the late pregnant monkey, oral administration of mifepristone led to an increase in the amniotic fluid levels of 6-keto-$PGF_{1\alpha}$, PGFM, $PGF_{2\alpha}$ and PGEM which increased in that order over time[295]. This increase was not observed, however, until at least 40 hours after mifepristone administration by which time an increase in uterine contractile activity was already evident. This observation is in contrast to the finding in the same study that in spontaneous labour intra-amniotic prostaglandins and their metabolites increase before the onset of regular uterine activity. These results suggest that the alteration in prostaglandin metabolism provoked by

mifepristone may not necessarily result in the sequence of events observed in normal labour.

Mifepristone and interleukin-8

Interleukin-8 (IL-8) is a chemokine which was initially described as 'neutrophil attractant peptide'. As discussed above, it is hypothesised to have a role in the onset of parturition by acting synergistically with PGE_2 to attract neutrophils into uterine tissues, such as the cervix. It also activates neutrophils and thus could facilitate collagen dissociation such as is seen during cervical ripening. IL-8 has been demonstrated in several uterine tissues, including choriodecidua[150], endometrium[147], cervix[43] and placenta[161]. *In vitro*, production of IL-8 by cultured choriodecidual cells is inhibited by progesterone, this inhibition being overcome by the addition of mifepristone[150]. Similar results have been described from cultures of rabbit cervical fibroblasts[162] whose production of IL-8 in culture is also inhibited by physiological levels of progesterone, an effect which is overcome by the addition of mifepristone. In human endometrial cells, IL-8 production is also inhibited by the addition of medroxyprogesterone acetate and dexamethasone to the cell cultures[147]. The IL-8 gene has been shown to contain a glucocorticoid response element[124] and it has been hypothesised that the inhibitory effect of progesterone could be effected via the glucocorticoid receptor or that it could act on the gene via its own intracellular receptors[89].

These in *vitro* results suggest that *in vivo* mifepristone may be capable of overcoming an inhibitory effect of endogenous progesterone on IL-8 production. Its use would therefore lead to increased local levels of IL-8 which could act synergistically with prostaglandins (the levels of which are also increased by mifepristone) in the recruitment of neutrophils into uterine tissues.

Effects of antiprogesterone on uterine tissues: Endometrium

In women, administration of varying doses of mifepristone during days 6 to 8 of the luteal phase of the menstrual cycle causes menstrual bleeding within 72 hours and results in changes in the endometrial histology consistent with progesterone withdrawal[296,297]. This is due to a direct action on the endometrial progesterone receptors as is shown by a study in which pregnancy was mimicked by the administration of hCG during the luteal phase followed by the concomitant administration of RU486. In these women menstrual bleeding still occurred following the RU486 treatment [298]. Interestingly, in contrast with monkeys, in humans mifepristone causes irregular shedding of the endometrium so implantation may still occur following its use [299].

Decidua

In the early pregnant uterus, mifepristone directly blocks the supportive action of progesterone on the decidua leading to bleeding and interruption of placental function. The decrease in hCG that is observed following RU486 administration is probably consequent to this disruption of chorio-decidual function and contributes to luteolysis, further decreasing the hormonal support for the early pregnancy [264].

In the termination of pregnancy at less than six weeks gestation, administration of mifepristone prior to vacuum aspiration leads to a dose dependent decrease in the level of cytosolic progesterone receptors and an increase in the levels of cytosolic and nuclear oestrogen receptors. The effect on receptor levels was greater after 36 hours than after 12 hours[300]. In late pregnant macaque monkeys, treatment with mifepristone also caused an increase in decidual oestrogen receptors which it was noted does not occur during spontaneous labour[301]. In these animals mifepristone administration does not alter the

maternal plasma concentration of oestrogen but it leads to increased levels of progesterone after 72 hours [295].

Some of the effects of mifepristone on the decidua may therefore be mediated in alterations in local levels of steroid receptors. Alterations in local receptor concentrations could in turn modulate differential effects of progesterone and oestrogen on uterine tissues without a change in peripheral hormone levels being observed.

Myometrium.

Treatment with mifepristone increases the contractile response of the uterus in early pregnancy after 24 to 36 hours, both in terms of the basal tonicity and in the establishment of contractions. This effect is particularly marked if prostaglandins are administered following pretreatment with mifepristone, which facilitates increased contractility and more co-ordinated contractions[302]. Administration of oxytocin in early pregnancy does not give rise to similar co-ordinated activity regardless of whether or not mifepristone is administered. This may be due to lack of functional oxytocin receptors in early decidua[74].

In late pregnant macaque monkeys, administration of mifepristone led to an increase in uterine activity as compared with control animals. However, the contractions stimulated by mifepristone were of a long duration and high amplitude and occurred with low frequency as compared with those of normal labour which occur with high frequency, have a short duration and a much greater amplitude [295]. Another study in macaques also describes mifepristone as causing increased uterine activity and a 'hyperexcitable' uterus but not the regular uterine activity seen in normal spontaneous labour[303]. The administration of mifepristone to these animals does however greatly improve the effectiveness of oxytocin at inducing labour. Mifepristone alone was found to cause

delivery in two of 19 treated monkeys, oxytocin alone led to one out of seven treated monkeys delivering whereas 12 out of 14 animals who received mifepristone and oxytocin in combination delivered[304]. It seems, therefore, that at least in non-human primates mifepristone does not cause sufficient myometrial activity to establish labour but that it does increase the sensitivity of the myometrium and its responsiveness to oxytocin. The mechanism by which this could occur may involve the gap junctions between the myometrial cells. These intercellular communications are composed of connexin 43 and are responsible for increased electrical coupling between myometrial cells. In guinea pigs treated with subcutaneous onapristone (another antigestagen) there was an increase in the myometrial responsiveness to oxytocin which was associated with an increase in the immunolocalisation of gap junctions in the myometrium but not with an increase in oxytocin receptors [305]. Similarly, in the late pregnant rat, administration of subcutaneous mifepristone led to a significant increase in the area and number of myometrial gap junctions from 24 to 48 hours following treatment[306]. All of these treated rats delivered within 72 hours of mifepristone administration compared with no deliveries of the control animals. The time interval for all pups to be delivered was however longer than that seen in spontaneous labour, possibly due to lack of endogenous uterotonic agents at this stage of pregnancy.

Antagonism of progesterone, therefore, may act on the myometrium by increasing intercellular communication via gap junctions but not by altering the oxytocin receptor levels resulting in increased myometrial responsiveness to endogenous or exogenous stimulation but not to the establishment of effective uterine activity. This postulate is presented by Garfield *et al* [306] as "Perhaps after RU486 treatment, the myometrium is prepared for labour with gap junctions and appropriate receptors for stimulants but lacks the necessary stimulation provided by oxytocin or excitatory prostaglandins".

Cervix

Mifepristone causes cervical softening and dilatation of the cervix in the first trimester of pregnancy even if termination of the pregnancy does not occur. Its clinical use for this purpose is discussed further below. In guinea pigs, administration of onapristone (ZK98299) in late pregnancy led to cervical ripening which was associated with histological changes similar to those seen in spontaneous ripening, such as oedema, dissociation of collagen and infiltration of leucocytes into the cervical stroma[307].

In the late pregnant rat, the administration of mifepristone leads to preterm delivery[293,306], this is associated with an increase in cervical hydration and levels of hyaluronic acid. This effect is not inhibited by the cyclooxygenase inhibitor diclofenac which does however prevent the animals delivering preterm when administered with mifepristone[293]. This suggests either that the mechanism of action of mifepristone in causing these effects is not solely mediated through prostaglandins or that other synthetic pathways producing prostaglandins unaffected by diclofenac are involved.

Several studies have reported the effects of mifepristone on the cervices of non-human primates near term. The normal gestational length in these animals is 167 days ± 3 days. In one such study[295] 20mg/kg/day mifepristone was administered orally for three days to macaque monkeys at gestations of 125 to 133 days. There was no evidence of progressive cervical effacement and dilatation despite an increase in uterine activity in these animals. However, in a separate study of the same species[304] at day 160 of pregnancy a significant increase in cervical dilatation was noted following oral administration of 25mg mifepristone. This alteration was not associated with a significant increase in the number of animals delivering but concomitant administration of oxytocin with mifepristone did lead to a significant increase in the number of monkeys delivering as

compared with the control group and with animals given mifepristone or oxytocin alone. These findings were confirmed by a further study in which oral or intramuscular mifepristone administration at gestations of 150 to 158 days caused cervical ripening but not delivery in macaque monkeys [303].

In the human cervix in the first trimester *in vivo* administration of mifepristone did not alter the subsequent *in vitro* bioconversion of radiolabelled arachodonic acid to thromboxane, PGE_2 or $PGF_{2\alpha}$ as compared with placebo[308] again supporting the hypothesis that the effect of mifepristone on the cervical structure is not solely mediated through prostaglandins. When other metabolites of the lipooxygenase chain were examined the administration of mifepristone was not found to alter the production of cervical 12-hydroxyeicosatetraenoic acid (HETE) or leukotrienes and so its action does not seem to be mediated by alterations in these substances[283].

In early pregnancy, the administration of mifepristone to women leads to a decrease in the ratio of α-2 to β-adrenoceptors[309] in the cervix, a similar effect to that observed in rabbits[310]. This ratio is high during normal pregnancy and has been implicated in maintaining the tonus of the internal cervical os [311]. The administration of mifepristone to women prior to vacuum aspiration has not been shown to affect either the spontaneous activity or the contraction frequency of cervical smooth muscle *in vitro* [312]. In this study there was no effect demonstrated on the *in vitro* inhibition of cervical smooth muscle contractions by PGE_2 in the cervices pre-treated with mifepristone. The action of mifepristone on the cervix does not seem, therefore to be associated with alterations in stromal smooth muscle behaviour despite the alteration in proportions of adrenoceptor types. These receptor changes could potentially affect blood vessel contractility and it may

be that alterations in the control of vascular smooth muscle are of more importance in the control of cervical ripening than effects on the stromal smooth muscle.

2.3 ii: CLINICAL USES OF MIFEPRISTONE.

Mifepristone has therefore been shown to have varied actions on pregnant and non-pregnant tissues. At least some of these effects are mediated by an increase in the production and a decrease in the catabolism of prostaglandins, in particular PGE_2 and $PGF_{2\alpha}$. The concomitant increase of local levels of IL-8 by antagonism of the inhibitory effects of progesterone could lead to a significant effect on the recruitment of neutrophils into uterine tissues. Activation of these neutrophils would release matrix metalloproteinases, such as collagenase capable of degrading the extracellular matrix of these tissues. Other actions of mifepristone relate to its effects on myometrial sensitivity to tocomimetic substances, such as oxytocin, which is increased possibly via an effect on intercellular gap junctions.

Mifepristone And Termination Of Pregnancy.

Since 1982 various doses and regimens of mifepristone administration have been studied to determine their effectiveness in interrupting early pregnancy. There are several advantages of an effective method of medically terminating pregnancy. In developing countries where surgical abortions are a major factor in maternal mortality and morbidity, an effective medical alternative would decrease the risks from surgery and its potential sequelae such as sepsis or haemorrhage[313]. In the developed countries medical abortion offers an alternative to women who may not wish or may not be suitable for anaesthesia and surgical termination.

Initial studies of the potential use of RU486 for pregnancy termination with doses varying from 140mg to 1600mg over up to 7 days administration[314-317] reported a success rate of 64 to 85%. This is significantly less than the rate achieved by vacuum aspiration of the uterus and so did not present a satisfactory alternative. The failure rate of mifepristone in inducing abortion increases with rising gestation[315,317]. In one study in which women of up to 55 days amenorrhoea or of 56 to 70 days amenorrhoea were given 200mg or 100mg mifepristone daily for four days in the nine patients at gestations greater than 56 days only three had successful termination of pregnancy compared with 29 of the 35 patients with less than 55 days amenorrhoea[317]. In a study comparing three dosage regimens of mifepristone (100mg; 50mg or 20mg for seven days) the effectiveness in the three groups was 73%, 66% and 64% respectively. It was found that for each treatment group the chance of successful termination of pregnancy was significantly increased with lower pre-treatment hCG levels[316]. There may also be individual variations in the metabolism of mifepristone between women, in particular in its binding by orosomucoid[271] although serum levels of this protein are the same in women who did and who did not have a successful termination of pregnancy with mifepristone[318].

Although the efficacy of mifepristone at inducing termination of pregnancy is not equal to that of vacuum aspiration this efficacy has been improved by combining mifepristone with various prostaglandins, such as the intramuscular PGE_2 analogue sulprostone[302], the vaginal PGE_1 analogue gemeprost [262,319] and the oral PGE_1 analogue misoprostol[320]. It has been found that combination of mifepristone with a prostaglandin, such as gemeprost, is more effective at causing complete abortion than gemeprost alone. In a study of 301 women with less than 56 days of amenorrhoea, complete abortion occurred in 87% who were given gemeprost alone compared with 98% of those who received mifepristone in addition. Pre-treatment with mifepristone also decreased the analgesic requirements of the

women following prostaglandin administration[319]. Similar results have been shown in large French [262] and British [321] multi-centre trials in which combination of prostaglandin with mifepristone resulted in complete abortion rates of 96% and 94% respectively. Subsequent work[322] has found that a lower dose of gemeprost (0.5mg) is of equal efficacy at inducing abortion as the higher dose (1mg) initially investigated. The other advantage of combining mifepristone with a prostaglandin is that the decreased dose of prostaglandin required leads to a decrease in the mainly gastrointestinal side-effects that are associated with the use of prostaglandin analogues. The analgesic requirements of women undergoing termination of pregnancy are also reduced when mifepristone is concomitantly administered with a prostaglandin analogue[263,319]. The combination of mifepristone with a prostaglandin also increases the gestation at which successful termination of pregnancy can be induced. Mifepristone alone markedly decreases in effectiveness after seven weeks amenorrhoea[317], whereas in combination with a prostaglandin it remains effective at up to nine weeks of amenorrhoea [321]. In the second trimester of pregnancy mifepristone reduces the dose of prostaglandin required to cause termination of pregnancy and decreases the time taken from prostaglandin administration to abortion [323].

Mifepristone for cervical ripening prior to vacuum aspiration.

Administration of mifepristone in early pregnancy causes cervical softening in many species including guinea pigs[307] and rats[293]. This is associated with similar morphological changes in the cervix to those observed during spontaneous cervical ripening at term [293]. This effect is beneficial in promoting termination of the pregnancy and has also been examined further as a means of preparing the cervix prior to surgical dilatation. The cervix, particularly in nulliparous women, is dilated prior to the performance of intrauterine operative procedures including vacuum termination of

pregnancy. Such dilatation can be traumatic to the non-compliant cervix causing trauma or lacerations[205]. Difficult dilatation of the cervix is associated with uterine perforation, necessitating laparoscopy or even laparotomy and with an increase in perioperative blood loss[206]. Pre-operative treatment with prostaglandins has been shown to soften the cervix leading to easier dilatation and decreased peri-operative blood loss[215-218]. Prostaglandins, however, when given systemically can cause marked gastrointestinal side-effects and even local administration can cause these effects and uterine pain as a result of contractile activity.

In addition to causing termination of pregnancy in the first trimester of pregnancy, mifepristone was also noted to cause marked cervical softening[294]. This effect has been demonstrated following the administration of doses of mifepristone ranging from 50mg to 600mg at time intervals from 12 to 48 hours prior to vacuum aspiration of the uterus[294,308,311,324-327]. Mifepristone decreases the initial cervical resistance to dilatation (as objectively measured with a mechanical dilator)[325] and increases the initial cervical dilatation[311]. In addition mifepristone causes improved cervical compliance during subsequent dilatation which has also been reported subjectively[311] and objectively[308,325]. After 600mg of mifepristone 48 hours preoperatively the force required to dilate the cervix to 9cm was 9.5N compared with 24.5N after administration of placebo[326]. One of the benefits of pre-operative treatment with prostaglandins prior to suction termination is a decrease in subsequent peri-operative blood loss. In a large multi-centre study[311] comparing placebo with 25mg, 50mg or 100mg of mifepristone given 12 and 24 hours pre-operatively although mifepristone improved the clinical assessment of cervical compliance, it did not significantly alter peri-operative blood loss. The significant improvement in cervical compliance was less than that previously reported for prostaglandins by the World Health Organisation[328,329] A further study, however,

directly compared by blinded randomisation mifepristone (200mg administered 36 hours preoperatively) with gemeprost (16, 16-dimethyl-trans Δ-2 PGE_1 methyl ester, Cervagem) and placebo for preoperative cervical ripening prior to vacuum aspiration in the first trimester of pregnancy[327]. The findings were that compared with placebo, both treatments significantly improved the baseline dilatation of the cervix and the subsequent objectively measured force required to dilate the cervix. The intraoperative blood loss was significantly decreased by both treatments compared with placebo with no difference between their effectiveness. The use of prostaglandins was associated with significantly more abdominal pain than mifepristone or placebo. Mifepristone could be expected to cause preoperative bleeding as a result of its abortifacient effect and this has been observed in some studies. The effect, however, would seem to be time dependent as 62% of women experienced preoperative bleeding following the administration of mifepristone 48 hours before surgery[330] compared with 20%[325] when it was given 24 hours preoperatively. This is as would be expected as with increased time there will be an increase in the effect of mifepristone on the decidua leading to uterine bleeding.

Mifepristone, when given prior to first trimester termination of pregnancy thus causes an increase in cervical dilatation prior to dilatation and a subsequent improvement in the ease of dilatation of the cervix. Administration of mifepristone can be on an out-patient basis unlike prostaglandin pessaries which are used during in-patient admission Due to the logistics of the timing of ward admissions and scheduling of theatre lists there may be insufficient time between the administration of prostaglandin pessaries and their maximal effect. As mifepristone can be administered at least 12 hours prior to surgery and is effective over a relatively wide time-span it could provide a more convenient method of cervical preparation. The benefit of mifepristone in decreasing cervical resistance could also be utilised prior to other procedures such as insertion of intrauterine devices or

hysteroscopy. However, it may not be appropriate for use prior to endometrial sampling as it may alter endometrial histology.

Mifepristone for induction of delivery following intrauterine death.

The induction of delivery following an antenatal intrauterine death is a distressing experience for parents and for the staff involved. It can be achieved by combinations of prostaglandin analogues (e.g. cervagem), amniotomy and oxytocin administration but can be difficult and slow to induce. In a study of 94 women with an intrauterine death at a mean gestation of 198 days randomised to receive 600mg of mifepristone or placebo, delivery of the fetus occurred within 72 hours in 63% of those who received mifepristone, compared with 17.4% after placebo[331].

This success rate is less than that observed after prostaglandin administration in a similar situation of 91 to 100%. There may be a role for mifepristone in cases where prostaglandins are contraindicated or when treatment is preferred initially on an out-patient basis or as an adjunct to prostaglandin use to shorten the length of time to delivery.

Mifepristone for the induction of labour.

In the rat[293,306] and the sheep[305] administration of mifepristone induces preterm labour. However there are substantial differences between these species and primates in the site of progesterone synthesis and the mechanisms by which parturition occurs. In non-human primates mifepristone promotes cervical ripening at term but does not lead to delivery of the fetus due to the lack of co-ordinated uterine activity[303,304]. Following its administration to monkeys there is an increase in uterine contractility that has been referred to as 'hyperexcitability'[303] but the establishment of regular tonic uterine contractions that are associated with spontaneous labour does not occur[295,303]. It has been shown,

however, that in combination with oxytocin mifepristone will significantly increase the numbers of monkeys delivering at term[304]. It thus seems that mifepristone administration at term will cause cervical ripening and facilitate the subsequent induction of labour with oxytocin. In rats and guinea pigs this effect on the myometrium is associated with an increase in the number of intercellular gap junctions[305,306]. In humans therefore it is reasonable to propose that mifepristone could cause cervical ripening at term and while not necessarily inducing labour could facilitate its' induction by other agents such as prostaglandins or oxytocin.

The safety of mifepristone for pregnancy induction is dependent on whether it is transferred across the placenta and on its potential effects on the fetus. In the late second and third trimester of pregnancy in monkeys intravenous injection of mifepristone was followed by rapid establishment of a steady state equilibrium with a gradient between mother and fetus possibly limiting flux[32]. These results suggested that mifepristone initially crosses the placenta by simple diffusion with the subsequent development of a gradient between mother and fetus. It was also noted that the efficiency with which RU486 crosses the placenta was significantly decreased in the third as compared to the second trimester of pregnancy. In women in the second trimester of pregnancy, a single dose of 600mg RU486 was found to give a maternal : fetal ratio of circulating mifepristone levels of 9:1. The ratio of the metabolite RU42633 was 17:1. This dose caused no alteration in fetal progesterone, oestradiol or cortisol levels but an increase in fetal aldosterone levels was noted which was only just significant in this small study of 6 women[333].

There has been one study reported of the administration of mifepristone to women at term to induce labour [334]. This was a double-blinded placebo controlled trial in which 120

women at gestations of 37.5 to 41.4 weeks were randomised to receive either placebo or 200mg of mifepristone orally for two days. In women who had not delivered four days after treatment administration labour was induced. Significantly more of the women who had received mifepristone went into spontaneous labour than those in the placebo group. Treatment with mifepristone also caused a significant decrease in the number of women with an unfavourable cervix after four days, in the length of the subsequent labour and in the total amount of oxytocin required during labour. There were no serious maternal side effects after mifepristone administration. Fetal distress was not increased after mifepristone treatment nor was the rate of operative delivery. There were no differences in the neonates of the treated and placebo groups in terms of birth weights, Apgar scores blood pH nor in the incidence of hypoglycaemia. This study, however was of a heterogeneous group of women in terms of their parity 65% and 60% of the treated and placebo groups respectively being nulliparous.

There is therefore theoretical and practical support for the use of mifepristone as an agent for cervical ripening at term and a need for further study of its effectiveness for this purpose in defined groups of women including the evaluation of lower doses than that found to be effective in the study described above [334].

2.4: CURRENT CLINICAL PRACTICE.

There is wide variation between different centres and indeed between different clinicians in the approach to pregnancy interruption. The following are therefore broad statements intended to summarise current practice.

Termination of pregnancy.

The preferred method used will depend on the gestation of the pregnancy, on the womans' wishes, on the methods available and on the clinicians judgement of the most suitable method to be used. If surgical methods are employed most clinicians will use pre-operative cervical ripening, at least in primigravid patients. This may be with prostaglandin analogues or less commonly with tents or more recently with mifepristone. The most effective medical technique for termination of pregnancy utilises mifepristone followed by a prostaglandin analogue. At less than seven weeks gestation either medical or surgical methods are possible but medical methods are particularly effective and perhaps preferable. Between seven and twelve weeks the effectiveness of medical termination decreases and so surgical methods may be recommended if appropriate. As the risks of surgery increase with advancing gestation after twelve weeks medical methods are of increasing value.

Cervical ripening.

Prostaglandin E_2 is the agent of choice at present for this purpose with units varying in their choice of either intracervical or vaginal preparations, the latter being rather simpler to apply. Extra-amniotic prostaglandins are rarely employed at present. Prostaglandin analogues were not previously used in the third trimester due to concerns about their fetal effects but recent work suggests that misoprostol when given vaginally is effective and

safe for this purpose. Mifepristone has been reported in small studies to be beneficial for improving cervical ripening prior to formal labour induction.

Induction of labour.

Although labour can be effectively induced with prostaglandins the current standard method of induction is by low amniotomy followed by titrated oxytocin infusion. The infusion is started either at the time of amniotomy or at a variable time after it depending on the parity of the patient and the existing uterine activity.

Chapter III:

CLINICAL STUDY: THE EFFECTS OF 50mg AND 200mg OF MIFEPRISTONE ON CERVICAL RIPENING AND LABOUR INDUCTION IN PRIMIGRAVIDAE AT TERM WITH UNFAVOURABLE CERVICES.

3.1: INTRODUCTION

Induction of labour involves promoting softening and dilatation ("ripening") of the cervix and producing effective myometrial contractions. It has been shown that induction is more likely to have a successful outcome if the cervix can be ripened prior to the onset of contractions, whether spontaneous or augmented[96,193]. PGE_2 is now widely used to prepare the 'unfavourable' cervix for parturition[112,247,335]. However, prostaglandin preparations may cause problems of uterine hyperstimulation[336] or may fail to produce sufficient cervical ripening for labour induction to proceed.

Mifepristone (RU486) is a potent progesterone and glucocorticoid antagonist acting on the progesterone receptor[264]. Since it was first described by Philibert in 1981[261] its' main clinical application has been as an abortifacient although it has been noted that its' administration causes marked cervical softening in the first trimester, either in conjunction with prostaglandins promoting medical abortion[319] or when used alone before vacuum aspiration of the uterus[327, 311]. Mifepristone shows synergism with prostaglandins in causing termination of pregnancy in the first or second trimester[319, 323]. It seems reasonable, therefore, to propose mifepristone as an agent for cervical ripening in the third trimester, particularly in conjunction with prostaglandins. In primates, mifepristone has been demonstrated to be efficacious, in combination with oxytocin, in achieving cervical dilation and induction of labour[304]. In humans, Frydman et al have shown that administration of 200mg mifepristone on 2 consecutive days to women at term significantly increased the number entering labour and decreased the prostaglandin requirements of the remainder, as compared with placebo [334].

Our study aimed to evaluate two doses of mifepristone (50mg and 200mg) as compared with placebo for their effect on cervical ripening and subsequent induction of labour in primigravid women whose cervices were initially very unfavourable for induction. We also evaluated the maternal and neonatal safety of mifepristone when used for this purpose. Mifepristone is also a potent glucocorticoid antagonist [264] and as it is known to cross the placenta[332,333] we particularly wished to assess whether there was an increased risk of neonatal hypoglycaemia following its' antepartum use.

3.2: METHODS

This study was a placebo controlled double-blinded trial in which two single oral doses (50mg and 200mg) of mifepristone were compared with placebo in a dose escalation study to assess their efficacy and safety for the induction of labor in unfavorable primigravidae at term. The study was designed such that in the first part the efficacy of a dose of 50mg mifepristone was compared with placebo. It was then determined on the basis of the an interim efficacy analysis of this dose compared with the placebo whether a higher or a lower dose of mifepristone should be used in the second part of the study. Twenty-five women were included in each treatment arm in this first part and on the basis of the interim efficacy analysis an increased dose of 200mg was used in the second part of the study. In the second part of the study twenty-five women received this higher dose of mifepristone and a further five were randomised to receive the placebo (the placebo groups from both parts of the study were pooled for the final analysis). The sample size was calculated to detect a 40% difference in the number of patients who went into spontaneous labour or who had a Bishops score greater than or equal to 6 between patients receiving mifepristone and patients receiving placebo with a power of 90% at the 5% significance level. Randomisation was by pre-determined randomisation code, patients were allocated a

number, and therefore treatment, in strict numerical order as they entered the study. The study had ethical approval from the Lothian Research Ethics Reproductive Medicine Sub-Committee.

Patient Recruitment

The inclusion criteria for the study were that patients were primiparous women aged 18 - 40 with an indication for labour induction and a normal live single cephalic presentation. The subjects were all of gestations between 37 weeks to 41 weeks and 4 days. Labour induction was scheduled 72 hours following treatment administration so this maximum gestation ensured that no patient was induced at a gestation later than 42 weeks. (There is a small rise in perinatal mortality after this gestation[235]) Gestational age was assessed by a first trimester ultrasound scan. Women were not included in whom there was an obstetric or medical reason to induce labour within 72 hours. Patients who met the above criteria had a cervical assessment performed and were offered the option of inclusion in the study if they had a modified Bishop score[247] of 4 or less (Table 1). Following written informed consent the patient was blindly randomised to receive either mifepristone or placebo. The placebo tablets were identical to those containing mifepristone.

Patients were excluded who showed signs or symptoms of the onset of labour or of placental insufficiency. They were also excluded if they had any contraindication to a vaginal delivery, if a previous attempt had been made to induce labour or if the membranes had ruptured. Medical contraindications to inclusion were renal failure, hepatic disorder, adrenal insufficiency, a history of abnormal coagulation or treatment with anticoagulants or diabetes mellitus. Patients who during their pregnancy had received chronic corticosteroid treatment (including inhaled preparations) or a treatment, that may have interfered with the mode of action of mifepristone, such as aspirin, were not included.

Study Procedure.

Before taking the study medication a maternal medical and gynaecological history was taken and a general, abdominal and vaginal examination performed. A modified Bishops' score (BS)[247] was assigned according to the state of the cervix as described in table 3.1. A cardiotocograph was obtained, entry to the study being conditional on a reactive tracing.

After entering the study the women were reviewed as out-patients after 24 and 48 hours. Labour induction was scheduled for 72 hours post treatment if the patient was not in labour by that time. Fetal well-being was assessed by the women keeping a 'kick-chart' (on which she recorded the time it took to feel the baby move or kick ten times) for each 24 hour period and by a further cardiotocograph being performed at each review visit. At the 24 and 48 hour reviews maternal blood pressure and pulse were recorded, the vaginal examination was repeated and a Bishops score calculated.

Labour Induction

If labour had not occurred within 72 hours after taking the allocated treatment the woman was admitted for induction of labour. If the cervix was not 3cm dilated and fully effaced (i.e. favourable for artificial rupture of the membranes, ARM) then an initial dose of 1mg PGE_2 gel was inserted into the posterior fornix of the vagina. The cervix was reassessed after 6 hours and (unless labour was established) a further dose of 1 or 2mg of PGE_2 given intra-vaginally. The examination was repeated 4 hourly and when appropriate an ARM was performed. Oxytocin was administered as clinically indicated following the ARM. Labour management thereafter was at the discretion of the labour ward medical staff. The fetal heart rate was monitored continuously once labour was established.

At delivery a cord blood sample was obtained for blood gas analysis, biochemical and haematological parameters and for assay of cortisol, ACTH and mifepristone levels. Serum for the latter three investigations was stored at -20°C until the assays were performed.

Post Delivery.

Neonatal observations

Neonatal blood glucose was monitored 1, 3 and 12 hours post delivery by means of a heel prick sample tested with BMStix. Neonatal weight, pulse and temperature were recorded after 24 and 48 hours as were any adverse clinical findings

Maternal observations

Maternal pulse, temperature and blood pressure were recorded 24 and 48 hours following delivery and again adverse events were noted. A further sample of maternal blood was taken 24 hours after delivery. Both mother and baby were reviewed one week and one month following delivery, again standard observations were recorded and any adverse events noted.

Statistical Analysis

Data were stored on computer database, analysis was performed using the Statistical Analysis Software (SAS) package. (SAS Institute Inc., Cary, North Carolina, USA). Differences between the treatment groups were compared using the t-test for continuous data and the χ^2 test for categorical data.

3.3: RESULTS.

Patient Characteristics.

These are outlined in table 3.2. Those in the placebo group had a significantly lower booking weight than those in either treatment group (p = 0.01 for the 50mg treatment group and 0.02 for the 200mg treatment group). This was not significantly related to the efficacy outcomes The patients in the 50mg mifepristone group had a slightly higher pre-treatment Bishops' score (median of 4 compared to 3) and this was accounted for in the efficacy analysis. The median Bishops' score in the 200mg group was 3. In all three treatment groups the most common reason for inclusion was prolonged pregnancy (that is of duration greater than 40 weeks) (table 3).

Efficacy

The main outcome measures are summarised in table 3.4. Cervical ripening was deemed to be successful if spontaneous labour had ensued or if the BS was ≥ 6 prior to induction of labour 72 hours after the treatment administration. This was the case for 64%, 48% and 30% of the patients treated with 200mg, 50mg mifepristone and placebo respectively. This difference was significant for the 200mg group (p=0.01) with an odds ratio of 4.15 (95% confidence interval 1.34, 12.84). In the 50mg treatment group the difference was not significant when the pre-treatment BS was included in the analysis but the odds ratio was 2.36 (95% confidence interval 0.66, 8.37). The numbers of patients in spontaneous labour after 72 hours were 9 (36%), 8 (32%) and 7 (23.33%) in the 200mg, 50mg and placebo groups respectively.

The subsequent course of labour is summarised in table 3.5 and the modes of delivery in Graph 1. Similar numbers of women in all three groups were given PGE_2, had an

artificial rupture of the membranes and received oxytocin. The amount of oxytocin required was 1095mU, 5198mU and 5780mU for the 200mg, 50mg and placebo groups respectively. This represents a significant reduction in the requirement by the former group. Significantly fewer women who received 50mg of mifepristone had a caesarean delivery than those who were given placebo ($p=0.033$, $\chi^2=4.55$), however in the group given 200mg of mifepristone there was no difference from the placebo group in the number requiring caesarean section. In the 200mg treatment group 8 of the 9 caesareans were performed for fetal distress and the other one for failure to progress. In the placebo treated group 3 of the 8 caesareans were for distress and 5 for failure to progress.

Only one patient in the 200mg mifepristone group and three in the placebo group required manual removal of the placenta. Post partum haemorrhage (blood loss $\geq$ 500ml) was reported in 2, 1 and 6 patients who received 200mg mifepristone, 50mg mifepristone and placebo respectively. The mean estimated blood loss for these patients was 600ml, 850ml and 940ml respectively.

Analysis of Safety.

Maternal Safety.

There was a wide range of maternal minor adverse events reported in all three treatment groups, most of which were complications of pregnancy, such as haemorrhoids or headache. There were no marked differences across the groups in the reporting of these. One patient in the 200mg mifepristone treatment group had transiently elevated liver function tests.

Fetal Safety.

There were no episodes of antepartum fetal distress following recruitment to the study. All of the patients had satisfactory CTGs and normal fetal movement at each antenatal assessment.

In labour, fetal distress was considered as severe if it required medical intervention (such as fetal blood sampling) or delivery of the baby. This was the case for 12 (48%), 6 (24%) and 4 (13.33%) in the 200mg mifepristone, 50mg mifepristone and placebo groups respectively.

Neonatal Safety.

Blood was taken from the umbilical vein at delivery, the blood gas parameters, ACTH and cortisol values from these samples are summarised in table 3.6. Samples were not obtained from all deliveries due to logistical problems in an investigator being present at delivery and due to technical problems with the equipment to analyse blood gases. The pO_2 was slightly higher and the base excess slightly lower in the 200mg mifepristone group, however there were no statistically significant differences between the groups in any of the cord gas parameters. The analysis of ACTH and cortisol in the cord blood showed a wide range of values with no significant differences between the three groups in their values or the ratio between them, however, there is a trend for the ACTH to be lower and the cortisol to be higher across the groups from 200mg to 50mg mifepristone to placebo. There were no differences between the groups in the renal or liver function tests nor in haematological or coagulation parameters. The mean levels of mifepristone in the cord blood were 0.020mg/l (sd=0.034) and 0.036mg/l (sd=0.046) after 50mg and 200mg of mifepristone respectively. Graph 2 illustrates the relationship between the length of

time to delivery after mifepristone administration and the cord blood levels of mifepristone.

The Apgar scores of the babies were recorded at 1 minute and 5 minutes in all the babies and at 10 minutes in 8 babies. The scores are summarised in table 3.7 from which it can be seen that there was very little difference between the treatment groups.

Monitoring for hypoglycaemia in the neonates after delivery was by means of a BM stick test of a heel-prick sample 1, 3 and 12 hours following delivery. Hypoglycaemia was defined as a BM reading of ≤ 2.2, this was the case in a total of 10, 11 and 10 infants of mothers who received 200mg, 50mg and placebo respectively. Only one infant in the study population was admitted to the Special Care Unit as a result of hypoglycaemia, the mother of this infant had received placebo. Neonatal jaundice was reported in 7 (28%), 2 (8%) and 2 (6.7%) of the infants in the 200mg mifepristone, 50mg mifepristone and placebo groups respectively. All of these cases resolved spontaneously.

3.4: DISCUSSION

Mifepristone was deemed to have successfully caused cervical ripening in primigravid women at term with initially unfavourable cervices if after 72 hours the woman was in spontaneous labour or the cervix had become favourable (Bishops' score ≥ 6). By these criteria 200mg mifepristone had a significant effect on cervical ripening, while the effect of 50mg was just below significance level, as compared with placebo. An improvement in the Bishops' score confers an increased chance of successful labour induction[193] which may be of particular benefit to this group of women. These findings confirm those of Frydman et al [334] who gave 200mg of mifepristone on two consecutive days to women at

term . In their study, using a higher dose of mifepristone than reported here, 54% of those treated with mifepristone went into labour spontaneously and a further 23% had a Bishops' score greater than 4 in the four days following treatment administration compared with 18% and 24% in the placebo treated group. The higher number in that study entering labour spontaneously, as compared with our data, is most probably a result of the higher dose administered, it may also be related to the extra day allowed before labour induction began or to the fact that some multiparous women were included in the study group. As the effect of 50mg mifepristone in our study only just failed to reach significance levels it seems likely that a slightly increased dose, such as 75mg or 100mg may also significantly ripen the cervix and that doses greater than 200mg could have a more pronounced effect.

In the first trimester of pregnancy, mifepristone promotes cervical ripening[311] and acts by way of the decidua [264] to effect termination of the pregnancy. The concomitant administration of prostaglandins significantly improves the efficacy with which abortion is induced[319]. In the second trimester, after intrauterine death, mifepristone was found to significantly increase the incidence of miscarriage as compared with placebo[331]. As pregnancy progresses mifepristone becomes less efficacious at inducing abortion but its action in promoting cervical ripening is maintained[264]. This effect has previously been noted in Rhesus monkeys, in whom mifepristone alone induced cervical ripening but did not produce sufficient myometrial activity to effect delivery[303]. In combination with oxytocin, however, mifepristone was effective at inducing delivery in these monkeys[304]. It is thus to be expected, as is shown in this study, that the most pronounced effect of mifepristone in the third trimester will be in causing cervical ripening and increased sensitivity to oxytocin rather than labour induction.

The cervical changes following mifepristone administration have clinical implications but in addition this finding may help to elucidate the processes whereby cervical ripening and the onset of labour occur spontaneously. The effect of antiprogesterones in causing cervical softening indirectly supports the role of progesterone in the maintenance of pregnancy, as has long been postulated[184]. During cervical ripening there is an influx of neutrophils into the cervical matrix[22] and an increase in neutrophil specific collagenase[35]. Progesterone may prolong pregnancy by the suppression of intermediary factors. For example, production of the neutrophil attractant IL-8 *in vitro* is inhibited by progesterone[147,150]. In guinea pigs the administration of IL-8 or IL-1β vaginally resulted in cervical ripening with associated histological changes[164]. The ripening action of mifepristone on the human cervix demonstrated here may be mediated by such cytokines or by an alteration in local prostaglandin production and catabolism. In first trimester decidua, mifepristone administration causes a decrease in prostaglandin dehydrogenase (the principal catabolic enzyme) and an increase in PGE_2 around blood vessels[287,337]. If mifepristone causes similar changes in the third trimester cervix this would provide a mechanism whereby the blood vessels could become more permeable for neutrophil extravasation.

As with prostaglandins, it would seem preferable to administer mifepristone vaginally if this method could maintain the ripening effects demonstrated with oral dosage. In one study in the first trimester of pregnancy [270] it was found that intravaginal administration of mifepristone did not lead to termination of the pregnancy, this was thought to be due to insufficient absorption of the drug. However, it may be, as discussed above, that in the third trimester a systemic effect on the decidua is not integral to the action of mifepristone and that this route could provide a local ripening effect on the cervix. It may be that a

suitable preparation of mifepristone could be administred vaginally if a suitable formulation could be developed to cause local effects on the cervix.

In labour following mifepristone administration a marked reduction was seen in the mean total oxytocin dose required, although there was no alteration in the number of women requiring augmentation. A similar finding was reported by Frydman et al [334]. This decreased requirement may arise because mifepristone administration leads to a more compliant cervix, thus reducing the myometrial work needed to effect dilatation. A further possibility is that mifepristone increases the baseline contractility of the myometrium thus reducing the oxytocin requirement. In the late pregnant guinea pig[305] administration of onapristone, (another antiprogesterone), improved the sensitivity of the myometrium to oxytocin by increasing the number of intercellular gap junctions which facilitate intercellular communication. A similar effect on human myometrial gap junctions could explain the decreased oxytocin dose described in our results. In a small study in early human pregnancy[302] mifepristone caused a slow rise in uterine contractility but no change in the response to oxytocin, it may be that the sensitising effect of mifepristone on the myometrium becomes more pronounced with increasing gestation. In our study the women were initially given only 1mg PGE_2 following treatment administration due to concerns that increased uterine responsiveness and synergism between the two agents could lead to a risk of hyperstimulation. However, there were no episodes of hyperstimulation reported in any group. At the out-patient antenatal assessments following mifepristone administration very little uterine activity was observed tocographically.

In addition to efficacy, the other main outcome measures of the study were fetal and neonatal safety. Mifepristone has already been used extensively in women at higher doses

than employed here without major concerns regarding its' safety. Our data showed no serious maternal side effects.

Previous studies of placental transfer in the late second and third trimester of pregnancy in monkeys[332] showed that a steady state equilibrium was relatively quickly established with a gradient between mother and fetus possibly limiting flux. It was also noted that the efficiency with which RU486 crossed the placenta was significantly decreased in the third as compared to the second trimester of pregnancy. In women in the second trimester of pregnancy, a single dose of 600mg RU486 was found to give a maternal : fetal ratio of circulating mifepristone levels of 9:1.The ratio of the metabolite RU42633 was 17:1. This dose caused no alteration in fetal progesterone, oestradiol or cortisol levels but an increase in fetal aldosterone levels was noted which was only just significant in this small study of 6 women[333]. In our study the values of ACTH and cortisol do not show any significant differences across the groups nor do any of the other biochemical or haematological markers analysed. As would be expected the levels of mifepristone in the cord blood samples were higher in the group treated with 200mg mifepristone than in those given 50mg. The levels of mifepristone in cord serum are inversely correlated with the length of time after administration that delivery occurs.

Fetal and neonatal well-being were assessed at each stage of the study. There was no antepartum evidence of fetal distress following mifepristone administration, as assessed by CTG and by maternal record of fetal movement. Intrapartum distress was assessed clinically and was more often diagnosed in the women who received 200mg of mifepristone than the other two groups. In this group of women there was an increase in caesarean deliveries for fetal distress and a decrease in the number performed for failure to progress, although there was no overall increase in the number of caesarean sections

performed. In the group of women who received 50mg of mifepristone there was a reduction in the number of caesarean deliveries. No difference was found in the cord pH values between the three groups nor in the Apgar scores. These findings may be related to the small numbers in each group undergoing caesarean section but they could reflect an effect of the higher dose of mifepristone on fetal distress, possibly related to the antiglucocorticoid action of this substance. There is a decreased incidence in each group receiving mifepristone in the number of women requiring caesarean section as a result of failure to progress in labour as compared with placebo. It may be that labour induction following mifepristone administration results in a more efficient process thus decreasing dystocia in the subsequent labour.

In addition to its antigestogen action, mifepristone is also a glucocorticoid antagonist and indeed has been used in the treatment of Cushings' syndrome[281]. There is therefore a theoretical risk that following delivery the neonates could become hypoglycaemic, if there is a decrease in neonatal glucocorticoid action. In this study, we therefore measured the blood glucose levels of the babies 1, 3 and 12 hours post-partum. There were no differences between the groups in terms of clinically significant hypoglycaemia. The only baby in the study group who needed admission to the neonatal unit with hypoglycaemia had received placebo.

3.5: CONCLUSION

These results therefore confirm previous work that mifepristone does promote cervical ripening, in this case in term primigravidae with unfavourable cervices. Two doses, 200mg and 50mg were studied and it was the larger dose that had a significant effect on improving cervical ripening. However, this dose was also associated with an increase in clinical suspicion of fetal distress, an effect not seen with the lower dose. It seems likely

that a dose such as 100mg could provide the beneficial effects on cervical ripening with a lower incidence of fetal distress but the possibility of fetal distress must be carefully evaluated in further studies of mifepristone.

3.6: TABLES AND FIGURES.

Table 3.1: MODIFIED BISHOP SCORE

Score	0	1	2	3
Dilatation (cm)	<1	1 - 2	2 - 4	>4
Length (cm)	>4	2 - 4	1 - 2	<1
Consistency	Firm	Average	Soft	
Position	Posterior	Mid; anterior		
Level	sp -3	sp -2	sp -1; 0	

From Calder et al [247]

Table 3.2: PATIENT CHARACTERISTICS

	PLACEBO	MIFEPRISTONE 50mg	MIFEPRISTONE 200mg
AGE (years) mean ± SD	26.2± 5.9	25.8 ± 4.5	25.6 ± 3.3
GESTATION (weeks + days) mean (sd days)	40+6 (3.6)	40+5 (5.5)	40+6 (5.1)
BOOKING WEIGHT (kg) mean ± SD	62.2 ± 8.9	71.8 ±13.6	69.8 ±13.3
INITIAL CERVICAL SCORE median (range)	3 (1 - 4)	4 (2- 4)	3 (1-4)
PREVIOUS PREGNANCY			
a) TOP	2	1	4
b)miscarriage	4	5	1
c) ectopic	0	1	0

Table 3.3: REASON FOR INCLUSION

	PLACEBO	MIFEPRISTONE 50mg	MIFEPRISTONE 200mg
POST TERM	27 (90%)	18 (72%)	23 (92%)
HYPERTENSIVE DISORDER	1 (3.3%)	5 (20%)	1 (4%)
OTHER	2 (6.6%)	2 (8%)	1 (4%)
TOTAL	30 (100%)	25 (100%)	25 (100%)

Table 3.4: OUTCOME AT 72 HOURS AFTER TREATMENT.

	PLACEBO (n=30)	MIFEPRISTONE 50mg (n=25)	MIFEPRISTONE 200mg (n=25)
SPONTANEOUS LABOUR	7 (23.33%)	8 (32%)	9 (36%)
INDUCED WITH A BS $\geq$ 6	2 (6.7%)	4 (16%)	7 (28%)
'SUCCESSFUL' CERVICAL RIPENING	**9 (30%)**	**12 (48%)**	**16 (64%) ***

* p=0.01 compared with placebo

Table 3.5: COURSE OF SUBSEQUENT LABOUR

	PLACEBO (n=30)	MIFEPRISTONE 50mg (n=25)	MIFEPRISTONE 200mg (n=25)
TIME TO ONSET OF LABOUR median (range)	81h 15m (17h 40m to 104h 30m)	80h 20m (6h 55m to 100h)	75h 50m (9h 45m to 101h 15m)
TIME TO DELIVERY median (range)	88h 14m (27h 21m to 113h 35m)	85h 15m (15h 12m to 113h 47m)	84h 6m (13h 1m to 110h 49min)
PGE_2 GIVEN	23 (76.7%)	17 (68%)	16 (64%)
TOTAL DOSE PGE_2 (median)	3mg	3mg	3mg
OXYTOCIN RECEIVED	14 (46.7%)	8 (32%)	12 (48%)
TOTAL DOSE OXYTOCIN median (range)	5780mU (210 - 19650)	5198 mU (2060 - 20800)	1095 mU * (210 - 14220)
ARM PERFORMED	20 (66.7%)	20 (80%)	16 (64%)

* $p < 0.05$ compared with placebo

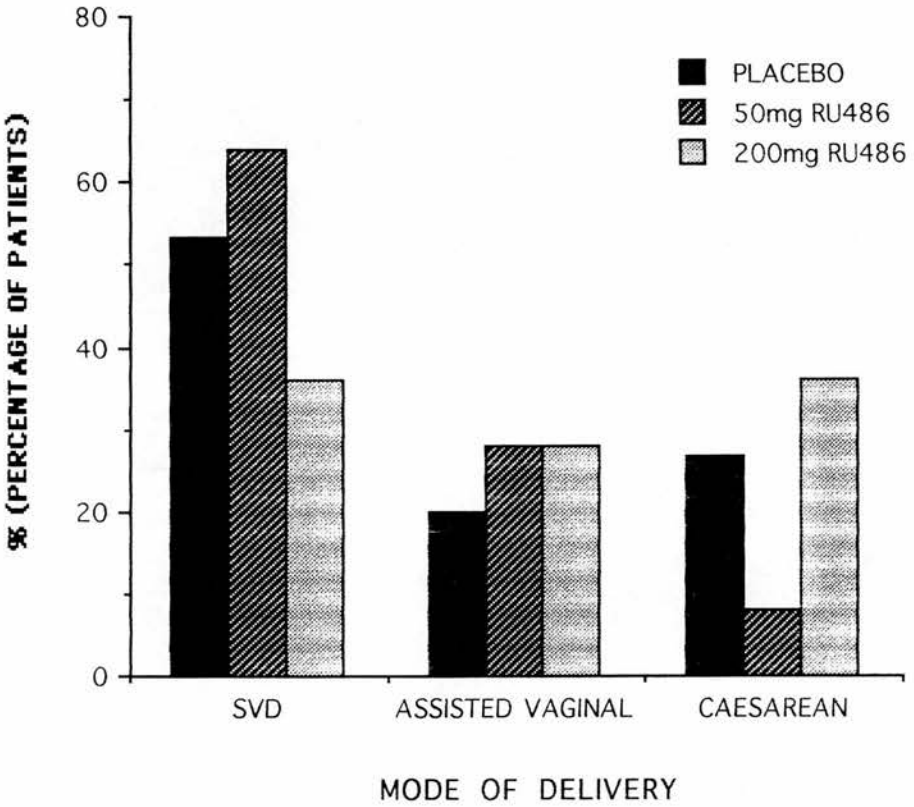
Table 3.6: UMBILICAL VEIN BLOOD PARAMETERS.

	PLACEBO N=30	MIFEPRISTONE 50mg N=25	MIFEPRISTONE 200mg N=25
pO_2 (mmHg) mean (SD) range	n=6 32.3 (11.0) 22.5 - 53.3	n=6 30.3 (6.0) 24.0 - 39.8	n=12 38.9 (36.5) 9.0 - 137.3
pCO_2 (mmHg) mean (SD) range	n=6 39.5 (9.6) 24.0 - 52.0	n=6 40.9 (40) 34.3 - 45.3	n=12 41.4 (10.5) 13.1 - 52.0
pH mean (SD) range	n=16 7.3 (0.1) 6.9 - 7.4	n=21 7.3 (0.1) 6.9 - 7.4	n=14 7.3 (0.1) 7.1 - 7.4
BASE EXCESS(mmol/L) mean (SD) range	n=5 -4.7 (5.8) -14.6 - -0.7	n=6 -5.3 (2.1) -7.9 - -2.3	n=12 -7.7 (5.5) -22.2 - -1.7
ACTH (mU/L) mean (SD) range	n=25 51.5 (91.5) 3 - 433	n=21 41.8 (43.9) 3 - 208	n=19 20.7 (22.6) 3 - 84
CORTISOL (mmol/L) mean (SD) range	n=24 484.5 (223.2) 234 - 1037	n=21 498.6 (158.9) 295 - 894	n=19 522.1 (190.2) 211 - 885

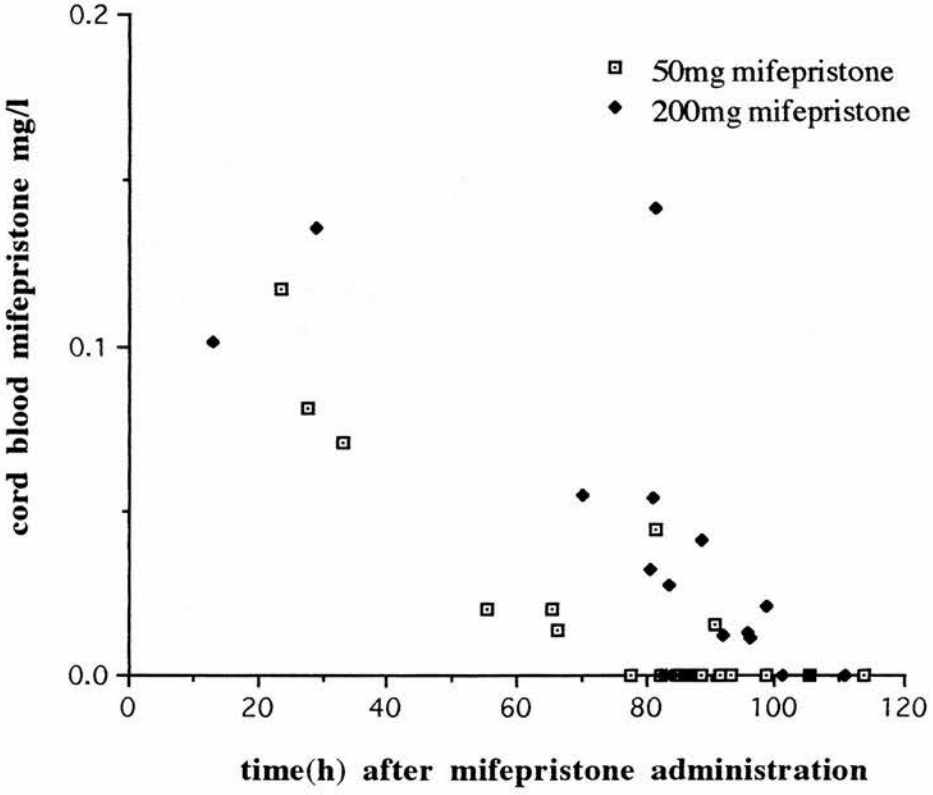
Table 3.7: APGAR SCORES AT DELIVERY

		PLACEBO	MIFEPRISTONE 50MG	MIFEPRISTONE 200mg
1 minute	number	30	25	25
	median (range)	8 (1 -9)	8 (2 - 9)	8 (3 -9)
5 minutes	number	30	24	25
	median (range)	9 (5 - 10)	9 (8 - 9)	9 (7 - 10)
10 minutes	number	4	2	2
	median (range)	9 (9 - 9)	9 (9 - 9)	9 (9 - 9)

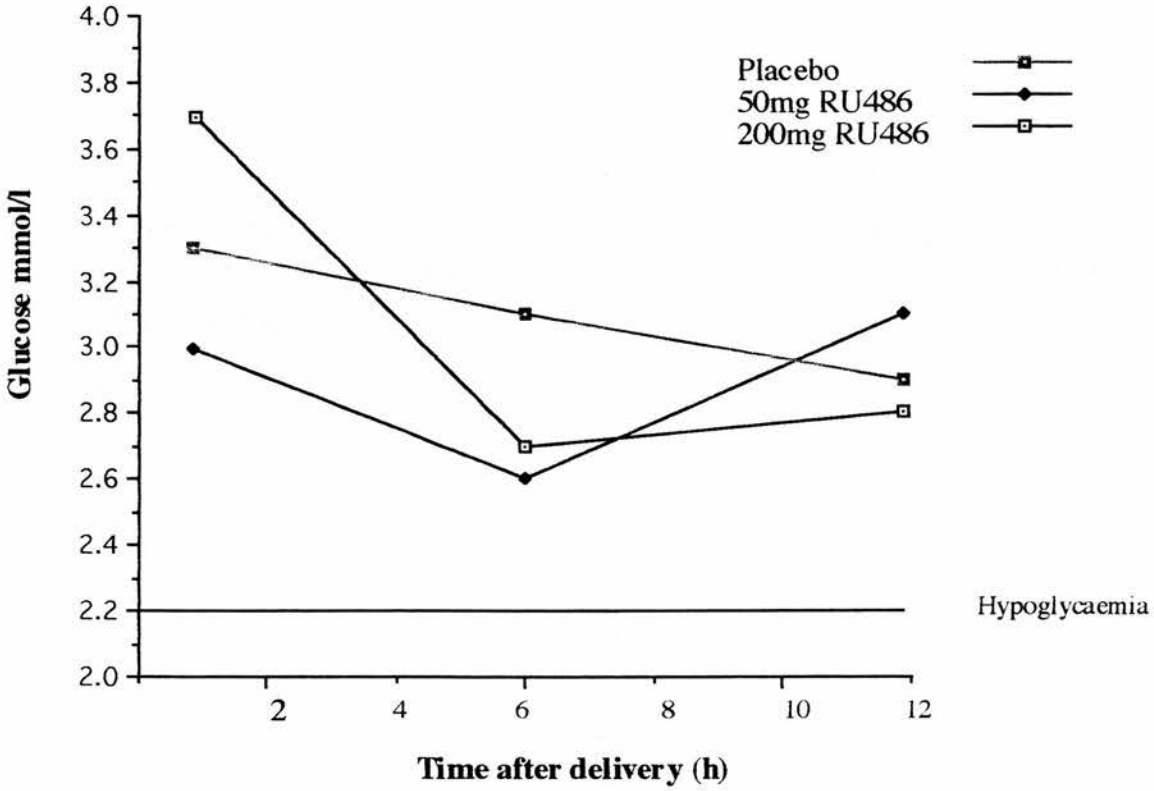
Graph 3.1: Mode of delivery after treatment with 200mg or 50mg mifepristone (RU486) or placebo.



Graph 3.2: Mifepristone levels in cord blood obtained at the time of delivery compared with the time to delivery after administration of 50mg or 200mg mifepristone .



Graph 3.3: Mean neonatal blood glucose levels 1, 3 and 12 hours after delivery following antenatal administration of 50mg or 200mg mifepristone (RU486).



Chapter IV:

LABORATORY METHODS

4.1: Tissue explants.

4.1 i: Culture of placental explants.

4.1 ii: Culture of decidual explants.

4.1 iii Culture of cervical explants

4.2: Immunohistochemistry.

4.2 i: Technique.

4.2 ii: General protocol.

4.2 iii: Specific protocols.

4.2 iv: Tissue preparation for immunohistochemistry.

4.2 v: Image analysis of decidual samples.

4.3: Radioimmunoassay.

4.1: TISSUE EXPLANTS

In order to examine the in vitro tissue production of IL-8 and to assess the effects of various steroid treatments on this cultures of tissue explants were used. The advantage of this method of tissue culture is that it maintains the tissue architecture to a greater extent than cell culture and so is a closer mimic of the physiological relations between various cell types. Culture in this manner is also less prone to contamination by infection as is the case with the longer term cell cultures. The tissues studied in this way were placenta, decidua and cervix. The details of their collection and incubation are given below. In general, the tissues were collected and then immediately transported to the laboratory in sterile 0.9% saline. Under sterile conditions in a tissue culture hood the specimens were prepared for culture in the various regimes described below. As IL-8 is known to be produced by many cell types, including monocytes and activated neutrophils care was taken to carefully wash all the tissue explants at least three times in PBS prior to culture. Obvious blood vessels, which were particularly seen in the placental explants, were also dissected out prior to culture. The basic culture medium was 'complete' medium which is RPMI 1640 (Dulbecco) supplemented with fetal calf serum and a combination of antibiotics (see appendix for full details). The steroids used were all diluted with complete medium from solution in ethanol. After being dissected into appropriately sized sections the explants were placed on CellGraft capillary matting, to aid removal of culture medium, in the wells of 24 well plates. These were placed in an incubator at 37°C in 5% CO_2. Medium removed during culture was stored at -20^{0}C until the immunoassays were performed.

All of the studies described below were approved by the Lothian Region Ethics Committee.

4.1 i: Culture of placenta explants

Placentae were collected from women at elective caesarean section or after spontaneous delivery at term (37 to 41 weeks gestation, n=5 in each group). No woman had been exposed to prostaglandins or oxytocics prior to delivery, although all patients routinely received 10iu oxytocin (Syntocinon, Sandoz) at caesarean or 5iu of oxytocin and 500μg of ergometrine (Syntometrine, Sandoz) at vaginal delivery, prior to the delivery of the placenta. Immediately following delivery, a piece of villous tissue approximately 1 to 2 cm in diameter was dissected out of each placenta from a central cotyledon, avoiding any obvious large stem blood vessels or areas of calcification and this was transported to the laboratory in sterile 0.9% saline.

Under sterile conditions the basal plate of the placenta was dissected off and large vessels were dissected out. Pieces of placenta measuring approximately $5mm^3$ were cut from the remaining tissue and washed three times in phosphate buffered saline (PBS, Dulbecco) to remove maternal and fetal blood cells. One piece was placed in each well of a 24 well plate on sterile capillary matting (CellGraft) containing 1 ml of culture medium (RPMI 1640 with fetal calf serum, L-glutamine, penicillin, streptomycin and gentamicin). Forty-eight explants were prepared in this manner from each placenta, these were divided into four treatment groups, each comprising twelve replicates. The four treatments used were control (i.e. no antigestagen added to the culture medium) and the antigestagens mifepristone (RU486) (10^{-6}M), onapristone (10^{-6}M) or lilopristone (10^{-6}M). The plates were incubated at 37°C in 5% CO_2 in humidified air. After 24 hours, the medium was collected, replaced with the same treatment and collected again after a further 24 hours. This medium was stored at -20°C until the assay for IL-8 was performed.

After 48 hours the medium was pipetted off each well and stored as described above. The samples were then washed twice with PBS and then each was treated with 1ml of 1N NaOH overnight. The protein content of each was then estimated using the BioRad assay according to the manufacturers instructions (see appendix for full protocol). This assay is a colorimetric assay which estimates the total protein concentration in tissue by comparison with a standard curve prepared from serial dilutions of bovine serum albumin. The IL-8 content of the culture medium was determined by radioimmunoassay (see appendix) using an in-house rabbit anti-IL8 antibody and the IL-8 produced per μg of protein was thus calculated.

4.1 ii: Culture of decidual explants

Decidua was collected from four elective caesarean deliveries at 37 to 41 weeks gestation after uncomplicated singleton pregnancies. After delivery of the placenta decidua was obtained by curettage of the non-placental area of the uterus (decidua parietalis). This decidua was either immediately placed in sterile saline for transport prior to culture.

Twelve sections of approximately equal size (about $3mm^2$) were dissected from each sample of decidua, these were placed in four treatment groups so that each treatment group contained three replicates. All the explants were individually placed in 1ml of complete culture medium. The explants were cultured for 72 hours with medium being replaced and stored at 24 hour intervals. The first group, A, was the control group and received no additional treatments during culture. This treatment regimen for groups B, C and D is summarised below. This regimen was used as it is known that manipulation of explants and their culture can increase the basal production of cytokines therefore the production of IL-8 was initially suppressed using dexamethasone and progesterone prior to the effects of culture with progesterone and mifepristone being evaluated.

	0 - 24h	24 - 48h	48 - 72h
A	Control	Control	Control
B	Prog 10^{-6} + Dex 10^{-7}	Control	Control
C	Prog 10^{-6} + Dex 10^{-7}	Prog 10^{-6}	Prog 10^{-6}
D	Prog 10^{-6} + Dex 10^{-7}	Prog 10^{-6} + RU486 $5x10^{-7}$	Prog 10^{-6} + RU486 $5x10^{-7}$

After culture the medium was stored at -70°C until immunoassay was performed. The tissue sections were dried in air before being weighed.

4.1 iii: Culture of cervical explants.

The study group consisted of thirty women of less than 9 weeks amenorrhoea who were to have a suction termination of pregnancy. None of the women included had had any previous pregnancies. Informed consent was obtained from all those who participated. The women were randomised into five treatment groups, each consisting of six women, to receive mifepristone at 6, 12, 24 or 36 hours or no treatment prior to the termination. The surgeons performing the terminations were blinded to the treatment allocation of the women. Women were not included if they had serious medical conditions, were less than 16 years old or were otherwise unable to give informed consent. At the time of suction termination a biopsy of the cervix was taken using punch biopsy forceps and was

transported in complete culture medium for incubation. The biopsies thus obtained measured about 3mm by 5mm. These samples were obtained in conjunction with studies being undertaken with the support of an MRC Project Grant (G9406438PA).

The cervical biopsies were cultured in 0.5ml culture medium for 24 hours at 37°C in 5% CO_2 in humidified air.. The medium was pipetted off and analysed by radioimmunoassay. The biopsies were then weighed and the production of the prostaglandins PGE_2, $PGF_{2\alpha}$, their metabolites and the cytokines IL-8 and MCP-1 was calculated by radioimmunoassay as detailed below.

4.2: IMMUNOHISTOCHEMISTRY

4.2 i: Technique.

The technique of imunocytochemistry involves detection of the desired antigen with a specific primary antibody, this localisation is then amplified by a secondary antibody directed against the first. This secondary antibody will be from a different species to the first. In the method described below an enzyme is then used together with a chromagen whose colour alters when the enzyme catalyses a reaction. The most commonly used enzymes are horseradish peroxidase (HRP) and alkaline phosphatase, the former was used in all the staining procedures described here. The peroxidase may be linked to a third antibody directed against the secondary antibody (and from the same species as the first antibody enabling the secondary antibody to act as a link between them) as in the PAP (peroxidase - antiperoxidase) method, where three molecules of peroxidase are complexed with two antiperoxidase antibodies. Alternatively, an avidin and biotinylated horseradish peroxidase complex may be used[338,339] in the ABC method described below. This method is more sensitive and requires a shorter incubation period than the PAP method as

well as ensuring less background staining. This method utilises the high affinity of avidin for biotin (10^{15} M^{-1}). Both the secondary antibody and the peroxidase can be biotinylated and then be linked together by avidin. The ABC complex thus binds via the avidin to a biotinylated secondary antibody, so the peroxidase is indirectly bound to the antigen to be detected. Addition of an electron donating chromagen, such as DAB (3-diaminobenzidrine tetrahydrochloride) and peroxide facilitates the enzyme reaction causing oxidation and colour change of the DAB. Other potential chromagens, such as AEC (3 - amino - ethylcarbazole) could be used but DAB has advantages in that it is not alcohol soluble and remains well localised to the site of precipitation.

Background staining.

Staining of the tissue at sites other than those containing the desired antigen may occur for several reasons. These are often due to the formation of an immune complex at sites other than those containing the targeted antigen. This may be due to binding of either the secondary antibody or the ABC complex directly to the tissue. Endogenous peroxidase present in the tissue may catalyse the third stage enzymatic stage and cause colour change in the DAB at the site of endogenous enzyme. The secondary antibody may bind to epitopes in the original tissue as well as to the primary antibody from the species it was raised against. To decrease the likelihood of background staining thus occurring, certain steps were provided in all the following protocols. Prior to the addition of antibodies the tissue was exposed to dilute hydrogen peroxide to saturate any endogenous peroxidase rendering it inactive and therefore decreasing the incidence of background staining. Normal serum of the species from which the second antibody was raised was added prior to antibody application to decrease non-specific secondary antibody binding. Following incubation with the DAB sections are immersed in tap water to stop the reaction and then stained with haematoxylin, dehydrated and mounted as described in the protocols.

Controls

Each staining procedure was performed using a tissue and a reagent control:

Positive tissue control: A tissue, in this case, tonsil known to contain the desired antigen and therefore expected to stain positively. Tonsil has previously been demonstated to produce many cytokines, including IL-8[352], *in vivo* and is known to contain the leucocyte populations being studied.

Negative tissue control: Sections of the tissue being stained which are treated in exactly the same manner except that they receive either non-immune serum or immunoglobulins from the species providing the primary antibody in its place in the protocol. These sections should therefore exhibit minimal staining.

4.2 ii: General protocol.

1. Fixation: Frozen sections: These were fixed in 10% neutral buffered formalin (NBF) for 10 minutes and then washed in PBS prior to staining.

Paraffin sections: These were fixed in NBF for 24 hours then washed twice in 70% alcohol, dehydrated in sequential increasing concentrations of alcohol and xylene and embedded in paraffin wax.

2. Peroxidase block: Sections were immersed in 3% hydrogen peroxide diluted in distilled water for 10 minutes at room temperature to saturate endogenous peroxidase. They were then washed twice in PBS.

3. Non-immune block: Using normal serum from the same species as the secondary antibody to decrease background staining. Sections were not washed prior to the next step.

4. Application of the primary antibody or negative control: The antibodies used were either monoclonal (i.e. raised from the same cell line and targeted against a specific epitope on the antigen) or polyclonal and therefore directed against several epitopes of the targeted antigen. The negative tissue controls were treated with either immunoglobulins or serum from the same species as the primary antibody. Tissues were protected from drying out during incubation with the primary antibody by covering them with cover slips, using a humidity chamber or covering with Cling Film. Following incubation, tissues were washed, ensuring that the negative tissues were washed separately in PBS with Tween.

5. Application of the second non-immune block: In the IL-8 protocol a second block was employed at this stage using identical means as the first one.

6. Application of the secondary antibody: The biotinylated antibody was applied and washed off (with PBS Tween) after a suitable incubation period.

7. Application of ABC - HRP: The complex was mixed from the commercially available kit (Vectastain or Vectastain Elite, Vector Laboratories), allowed to stand for 30 minutes according to the manufacturers' instructions and then applied to the tissues. It was washed off with PBS(Tween).

8. DAB application: Again, this was prepared according to instructions from a commercially available kit. After an 8 minute application the reaction was stopped by immersing the tissue in tap water.

9. Counterstain and mounting: Haematoxylin was used as counterstain, after washing off excess the tissue was dehydrated with alcohols and then immersed in xylene prior to mounting.

4.2 iii: Specific protocols.

Detailed protocols for the immunohistochemistry are given in the appendix.

IL8

The method utilised for immunocytochemistry is as previously described by Critchley et al[148]. The polyclonal rabbit antibody was produced in-house (kindly supplied by Dr R. Kelly) and demonstrated specific immunoreactive binding to sites on frozen tissue only. It was used in conjunction with a biotinylated swine anti-rabbit secondary antibody (E353, DAKO, Glostrup, Denmark) and the ABC peroxidase system (Vectastain ABC kit, Vector laboratories, Burlinghame, USA). The non-immune block was performed with normal swine serum. The chromagen was DAB (Vector, SK - 4100). The negative control in this protocol was the IL-8 antibody preabsorbed with IL-8 protein. This was produced by overnight incubation of the IL-8 antibody with an excess of IL-8 protein (provided by Schering). Tonsil tissue was used as a positive tissue control as abundant IL-8 is found in the vascular regions in this tissue.

CD45

This antigen, also known as leukocyte common antigen (LCA) was used as commercially available mouse monoclonal antibody (DAKO) labelling the RB epitope (see above). Paraffin fixed sections were used for this protocol. A biotinylated horse anti-mouse secondary antibody (Vector) was used and the ABC-HRP complex for the third stage, as in the IL8 protocol. Prior to the application of the primary antibody, a non-immune block was performed using horse normal serum (Vector 4002).

CD68

This was also a commercially produced mouse monoclonal antibody (Mac CD68, DAKO) raised against Gaucher's cells which was used in conjunction with a biotinylayed horse secondary antibody (Vector), horse normal serum (Vector 4002) and an ABC peroxidase detection system (Vector). In this protocol a trypsin digestion stage was included prior to the first non-immune block. The tissue sections were paraffin fixed following collection.

Neutrophil elastase

A commercial mouse monoclonal antibody to α - neutrophil elastase (DAKO) was used with horse biotinylated secondary antibody and horse normal serum (Vector 4002) and the ABC peroxidase detection system (Vector) as before.

4.2 iv. Tissue preparation for immunohistochemistry.

Placental tissue

Tissue samples of placentae were collected after elective caesarean section at term (37 to 39 weeks gestation) from singleton pregnancies uncomplicated by diabetes or hypertensive disorders. Sections from each placenta, including the basal plate, were dissected, immediately embedded in OCT (Miles Inc., Elkhart, IN, USA.) and placed in

an isopentone tray which was surrounded by liquid nitrogen. The frozen OCT block was then stored at -70°C prior to cutting into approximately 5μm sections using a microtome. Sections for paraffin sections were placed in 10% neutral buffered formalin and processed as described above.

First trimester villi were collected from four women during surgical termination of pregnancy and processed in the same manner.

Decidual tissue.

Decidual tissue was collected at elective caesarean section by curettage of the non-placental area of the uterus (decidua parietalis) as described in preparation for tissue culture. This was placed in 10% formalin (for paraffin fixed sections) or frozen in embedding medium (OCT, TissueTek, Miles Inc., Elkhart, USA) in a block placed in isopentone which was in turn placed in liquid nitrogen (for frozen sections). The frozen OCT blocks were then stored at -70C until 5μm sections were cut with a microtome. First trimester decidua was collected for immunocytochemistry at the time of surgical termination of pregnancy and frozen as described above for IL-8 immunocytochemistry.

4.2 v: Image analysis of decidual samples.

Ten fields from each decidual sample stained for CD45 were analysed using image analysis. The fields were visualised with an Olympus BH2 microscope (x 20 objective) and analysed as previously described[17] with Colour Vision software (Improvision, UK). A digital image of the field was thus obtained for analysis. Areas containing no cells were deleted from the computer image. The software package discriminated areas of brown

(positive) from blue (haematoxylin)) staining measuring all areas in pixels. The proportion of positive staining cell area compared with total cell area was thus calculated.

4.3: RADIOIMMUNOASSAY

This technique is used to determine the concentration of a given substance in a solution by comparison with a standard curve created from solutions of known concentrations. Antibody radiolabelled with ^{125}I is added to the sample solution and a second antibody conjugated to a magnet is added. The magnetic complex thus created is precipitated by placing the test tube on a magnetic plate (see appendix for protocol). Excess solution is discarded and the radioactive count in the remaining pellet is counted using a multigamma counter and the concentration of the substance tested for calculated by comparison with the standard curve. The standard curve is prepared by assay of serial 1:2 dilutions of a known concentration of the test antigen and then plotting a curve of the radioactive count emitted after assay. Each assay includes a test of the total count which contains only the radiolabelled antibody, the non-specific binding (NSB) found by testing buffer and label, to assess any binding of the buffer to the label and the B_O found by saturating the antiserum with label. In these studies all the immunoassay results were calculated using Assay Zap (Biosoft, Cambridge).

Immunoassay For IL-8.

A rabbit antibody, which was raised against the intact 72 amino acid IL-8 peptide synthesised using fluorenyl methoxy carbonyl (FMOC) chemistry, was used in a radioimmunoassay as has been previously described[147]. A 1 in 60 000 dilution of the antiserum was used. Recombinant IL-8 was used for the standard curves. A top standard of 25ng/ml was diluted by two and serial dilutions of two were continued to give ten

standards. The assay was performed overnight with a 'non-stick' buffer containing serum albumin and tween (see appendix) to minimise the non-specific binding. A magnetic anti-rabbit antibody was then added and the tubes used then placed on a magnet. The tubes were then counted using a mutiganna counter for ^{125}I for 60 seconds and analysed using AssayZap.

The assay has cross reactivities of < 0.25% against IL-1, IL-2, IL-6 and IL-10. The intra-assay variation was 7% and between-batch assay variation was 12%.

Immunoassay for MCP-1.

This was by radioimmunoasay as described for IL-8. The antibody was raised against the intact 77 amino acid MCP-1 and recombinant MCP-1 (R and D Systems) was used as the standard.

Immunoassay for PGE / M and PGF / M.

These assays are as described in previous publications[340,341]. Antiserum and label were both mixed with assay buffer (see appendix) prior to use. Samples were moxed using mox B solution (see appendix) immediately after thawing. The standard curve was constructed using ten serial x2 dilutions of a top standard (5120pg/ml). After overnight incubation magnetic second antibody was added and counting performed as described for IL8

Chapter V

REGULATION OF INTERLEUKIN - 8 PRODUCTION IN THE TERM HUMAN PLACENTA DURING LABOUR AND BY ANTIGESTAGENS

5.1: INTRODUCTION.

Interleukin-8, (IL-8) has been shown both to attract and activate neutrophils[342]. It is a 72 amino acid chemokine that has subsequently been demonstrated to be released by many cell types, including fibroblasts[343], macrophages, endothelial cells and even phagocytosing neutrophils[125]. IL-8 is also present in and released by many uterine tissues, including placenta[161], choriodecidua[150] and cervix[43]. The influx of neutrophils has been postulated to be an integral part of the onset of parturition as described above, particularly in the cervix, where the collagenase involved in ripening appears to be derived from peripheral neutrophils which increase in number in the cervix during this process[2,22,35]. The presence of IL-8 in these diverse reproductive tissues and its known synergism with prostaglandin E_2 (PGE_2) in attracting neutrophils to skin sites[144,344] suggests a wider role for IL-8 in the initiation of labour than in cervical ripening alone. Furthermore, IL-8 production in other uterine tissues, such as cervix[43], choriodecidua[150] and endometrium[147] is suppressed by progesterone. This effect may be reversible by antigestagens, such as mifepristone[150] which may have an inducing effect on the initiation of labour.

The aim of this study was therefore to examine the release of IL-8 by term human placenta prior to and after labour had ensued. The regulation of IL-8 production by three antigestagens was studied to asses a possible regulatory role in the induction of the labour process. Furthermore, the localisation of IL-8 in the first and third trimester placenta was determined using immunohistochemical techniques. The localisation of cells showing positive staining for CD-45 (pan-leucocyte marker), CD-68 (monocyte-macrophage marker) and neutrophil elastase was also examined.

5.2: SUMMARY OF METHODS.

These are described in detail in Chapter 4 and are summarised below.

Placental Explants

Placentae were collected after spontaneous and caesarean delivery at term (n=5 in each group). After transport to the laboratory they were cultured for 24 and 48 hours in complete medium to which was added mifepristone (RU486) (10^{-6}M), onapristone (10^{-6}M) or lilopristone (10^{-6}M), a fourth group was the control i.e. no antigestagen added to the culture medium. The plates were incubated at 37°C in 5% CO_2 in humidified air. The production of IL-8 after 24 and 48 hours by each group was analysed by radioimmunoassay. The protein content of each placental tissue sample was assayed using the BioRad method as described in Chapter 4.

Data analysis.

The IL-8 production was expressed as ng per μg of protein. The results obtained were tested for significance using a parametric test, Fishers protected least significant difference (StatView) and significance at the level of $p< 0.05$ was determined.

Immunocytochemistry

Tissue samples of placentae were collected after elective caesarean section at term (37 to 39 weeks gestation) from singleton pregnancies uncomplicated by diabetes or hypertensive disorders. Frozen and paraffin sections were prepared from these samples. First trimester villi were collected from four women during surgical termination of pregnancy and processed in the same manner. Sections were stained for IL-8, CD45, CD-68 and neutrophil elastase positive cells.

5.3: RESULTS

Production Of Interleukin-8 By Placenta

All the explants of placental tissue released interleukin-8 into the culture medium, irrespective of the presence or absence of labour or treatment with antigestagens.

The culture medium was replaced after 24 hours and in all samples IL-8 release increased in the second 24 hours of culture. This increase was significant ($p<0.05$) for the control samples from both elective caesarean and spontaneous deliveries and also for the caesarean samples treated with onapristone and the spontaneously delivered placentae that were treated with mifepristone or with lilopristone.

After spontaneous delivery, for each treatment group and at each time point, all the placental explants produced more IL-8 than the explants collected from women not in labour. As illustrated for the control groups in graph 1, this was a significant increase ($p< 0.05$) at both time points.

The effects of three antigestagens, onapristone, mifepristone and lilopristone on IL-8 production in both types of placenta were also examined and are shown in graph 2. In the spontaneously delivered group those treated with onapristone produced significantly more IL-8 than the control samples at 24 and 48 hours while in the electively delivered group it was lilopristone at 24 hours which caused a significant increase in production over control samples ($p< 0.05$ in each case).

Localisation Of Interleukin-8 In Placenta.

In the third trimester villi IL-8 positive immunostaining was found to be localised in the perivascular walls of fetal vessels as shown in figure 5.1 . A negatively stained section of third trimester placenta is shown in figure 5.2 . In the first trimester IL-8 was shown peripherally in the developing syncytiotrophoblast of the villi (figure 5.3).

White cell populations in the placenta.

Few cells in the third trimester placentae studied showed positive staining for CD-45, those seen were mainly localised in the blood pools outside the trophoblast tissue (figure 5.4). There were CD-68 cells localised within the trophoblast tissue, mainly within the cytotrophoblast (figure 5.5). Hardly any cells showing positive staining for neutrophil elastase were seen and, as with the CD-45 positive cells, those present were in blood outside the trophoblast (figure 5.6).

The populations studied were also found to be immunolocalised around vessels in the basal plate of the placenta as in the sections shown from a spontaneously delivered placenta. CD-45 positive cells were seen in association with this vessel (figure 5.7), both within the lumen and within the vessel walls (figure 5.8). These cells were not predominantly CD-68 positive (figure 5.9), but exhibited strongly positive staining for neutrophil elastase (figure 5.10). The decrease in concentration of these cells with increasing distance from the vessel may suggest a gradient of cells from the vessel.

Tonsil was used as a tissue control for all of the staining protocols. Positive staining was seen in the tonsil with all the protocols used, for example IL-8 was immunolocalised to the areas around blood vessels (figure 5.11). No staining was demonstrated in the negative control sections of either tonsil or of placenta.

5.4: DISCUSSION

These studies demonstrate that the human placenta produces IL-8 and that this production is increased during labour and also by culture with some of the antigestagens studied.

An increase in IL-8 prior to the onset of spontaneous labour could facilitate recruitment of neutrophils into the uterine environment. It has been shown that in skin sites IL-8 and PGE_2 act synergistically to increase the influx of neutrophils[144,344] and it may be that such synergism extends to the reproductive tissues where at the time of parturition there is an increase in local levels of prostaglandins which have an established role in the initiation and progression of labour. Once recruited into the uteroplacental tissue neutrophils could be activated by IL-8 causing release of lytic enzymes, such as collagenase and elastase, which may cause loosening of the tissue matrix in the cervix[2,35], choriodecidua[157] and fetal membranes[85]. IL-8 may therefore be part of a cascade of inflammatory agents, including other cytokines and prostaglandins involved in the initiation of parturition. An immunohistochemical study of IL-8 receptors (types 1 and 2) in the placenta has found that these are also increased after spontaneous delivery as compared with after elective caesarean section[141]. This indicates a parallel increase in the production of IL-8 and in its functional effects via these receptors.

It is also possible that the increase in IL-8 production seen after vaginal delivery is an effect and not a cause of labour. It may be that the mechanical stress of contractions leads to enhancement of the capability of the placental tissue to produce IL-8. To further clarify the role of IL-8 in parturition more detailed study of the time course of its production during labour would be required. However, the placenta cannot be directly sampled during spontaneous labour in women and although tissue can be obtained at emergency

caesarean delivery performed during labour these cases may differ inherently from those resulting in vaginal delivery.

There were no other obvious significant differences in the placentae to account for the difference in production following labour, as the elective deliveries were all at term and were not performed for placental insufficiency, growth retardation or metabolic abnormalities. The spontaneously delivered placentae resulted from labours that were not induced or augmented with prostaglandins or oxytocics. All women delivering vaginally in our unit routinely receive oxytocin and ergometrine after delivery of the fetus, at caesarean oxytocin alone is given. It seems unlikely that ergometrine would have a significant effect on IL-8 production.

It has previously been shown that IL-8 production is down-regulated by both progesterone [43,147] and dexamethasone [345]. The addition of antigestagen to the culture medium increased IL-8 production to a significant degree in the spontaneously delivered placentae treated with onapristone and in the caesarean delivered ones treated with lilopristone for 24 hours. Currently, antigestagens are being studied in clinical trials for cervical ripening and induction of labour in the third trimester [334]. The local antagonism of progesterone in uterine tissues may increase IL-8 levels permitting recruitment of neutrophils and also lead to increased local levels of PGE_2 [89]. In first trimester decidua, mifepristone treatment increases perivascular PGE_2 levels and decreases its metabolism[287,337]. A parallel increase in IL-8 and PGE_2 levels in placenta at term would enhance the synergistic effects of these two agents on neutrophil recruitment. A significant increase in IL-8 production may not have been demonstrated in this study as the amount of antigestagen used may not have been sufficient to antagonise the large endogenous progesterone production of the placenta. It could also be that, particularly

after spontaneous delivery the response of the placental cells to exogenous agents has altered such that an effect *in vivo* is masked.

These results confirm the findings of Shimoya et al [161] that the third trimester placenta can produce IL-8 constitutively without in vitro stimulation by other agents. Using immunohistochemistry we have demonstrated that cells showing positive IL-8 staining are localised in the perivascular area. This is the same location as that described for IL-8 in the endometrium [148]. This supports the hypothesis that IL-8 may have a role in the migration of neutrophils from blood vessels around the time of parturition. This localisation is in contrast to that of the above study [161] which described IL-8 immunostaining in both first and third trimester placentae in trophoblastic cells and macrophage-like cells. This difference may be due to an intrinsic difference in the samples studied or in the antibodies used.

Leucocytes staining with CD-45, CD-68 or with neutrophil elastase were not widely seen in the placental samples studied. The antibody against neutrophil elastase does not detect this after degranulation of the cells and so it may be that there have been greater numbers of neutrophils present but that they are not detected due to their rapid degranulation. However, one would expect, if this were the case to see cells present in the tissue prior to degranulation. The photomicrographs of the vessel in the basal plate do suggest an emigration of neutrophils into the tissue, however this cannot be directly inferred from a static view of a vessel in one section.

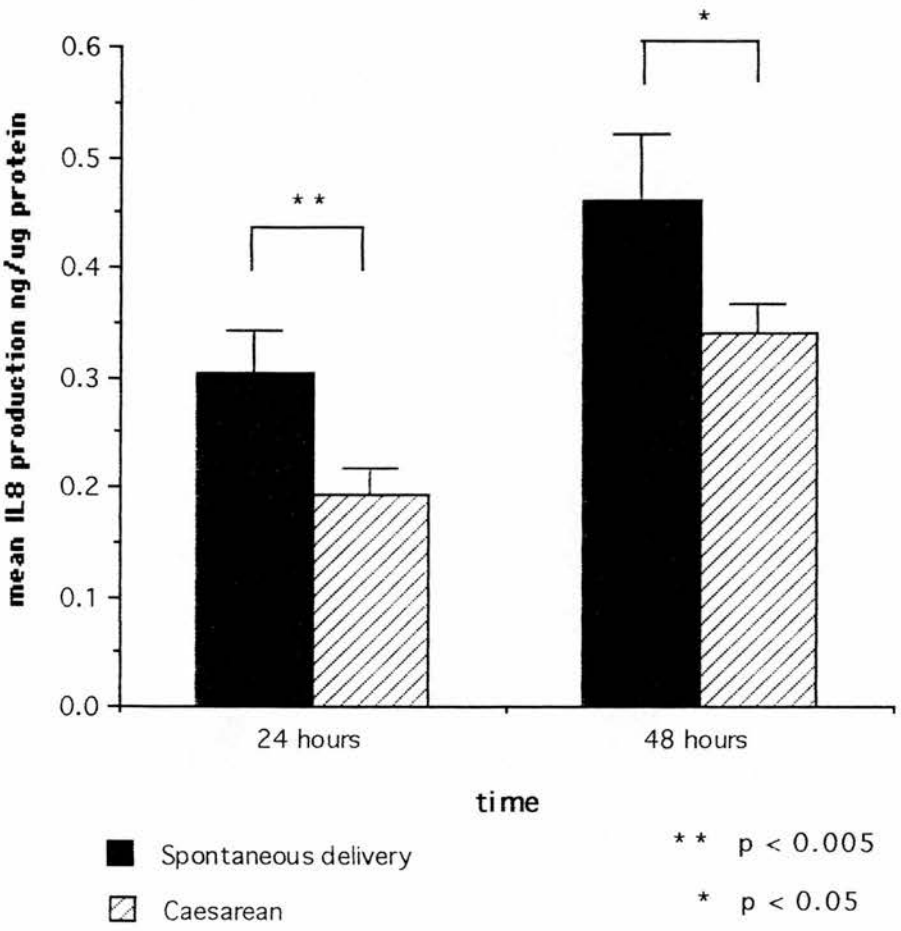
In addition to its effects on neutrophils, IL-8 has been associated with an angiogenic action [127]. It has been shown here to be present in first trimester villi and around the villous vessels in the third trimester. If IL-8 were to have an effect on angiogenesis as the

placenta develops it could have a potential role in conditions involving abnormal placental vascularisation, such as intrauterine growth retardation and pre-eclampsia.

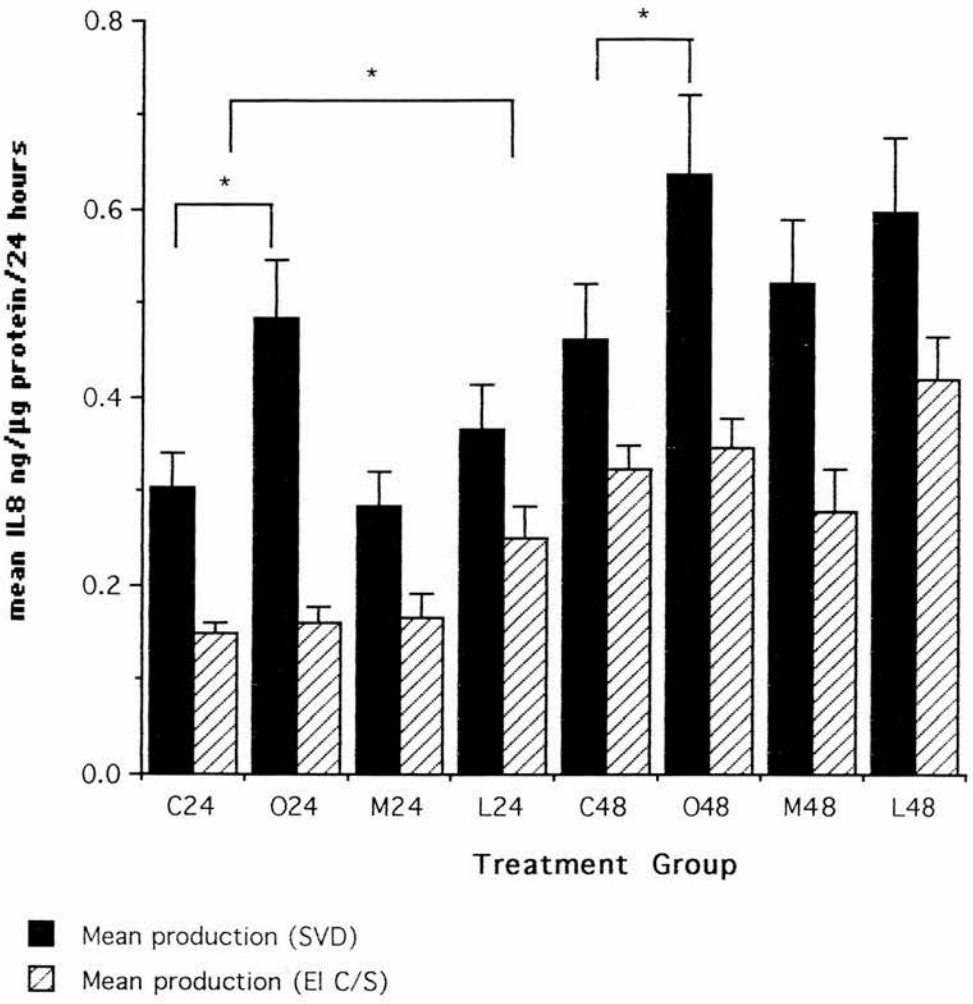
5.5: CONCLUSION.

In summary, these studies demonstrate that the term placenta constitutively produces IL-8 which is localised principally around the perivascular area of the villi. This production of IL-8 is increased following spontaneous labour, increases with culture time and is stimulated to varying degrees by some antigestagens. IL-8 may have a role in the onset of parturition by recruiting and activating neutrophils at the placental site facilitating release of lytic enzymes, hence antigestagens may aid the induction of labour by increasing the influx of neutrophils into the placenta. Further work is now underway to establish the controlling mechanisms involved in the stimulation of IL-8 during labour and the potential role of antigestagens in regulating IL-8 production and the timing of the onset of parturition.

Graph 1: Interleukin-8 (IL-8) production (mean ± SEM; ng/μg protein) by explants of placental villous tissue obtained after spontaneous (solid bars) and elective caesarean (hatched bars) delivery and maintained in tissue culture for 24 and 48 hours. Statistical significance is denoted by: ** $p<0.005$ and * $p<0.05$.



Graph 2: Interleukin-8 (IL-8) production (mean ± SEM; ng/μg protein) by explants of placental villous tissue obtained after spontaneous (solid bars) and elective caesarean (hatched bars) delivery, untreated (control medium, C) or treated with onapristone (O, 1μM), mifepristone (M, 1μM) or lilopristone (L, 1μM) for 24 and 48 hours in culture.



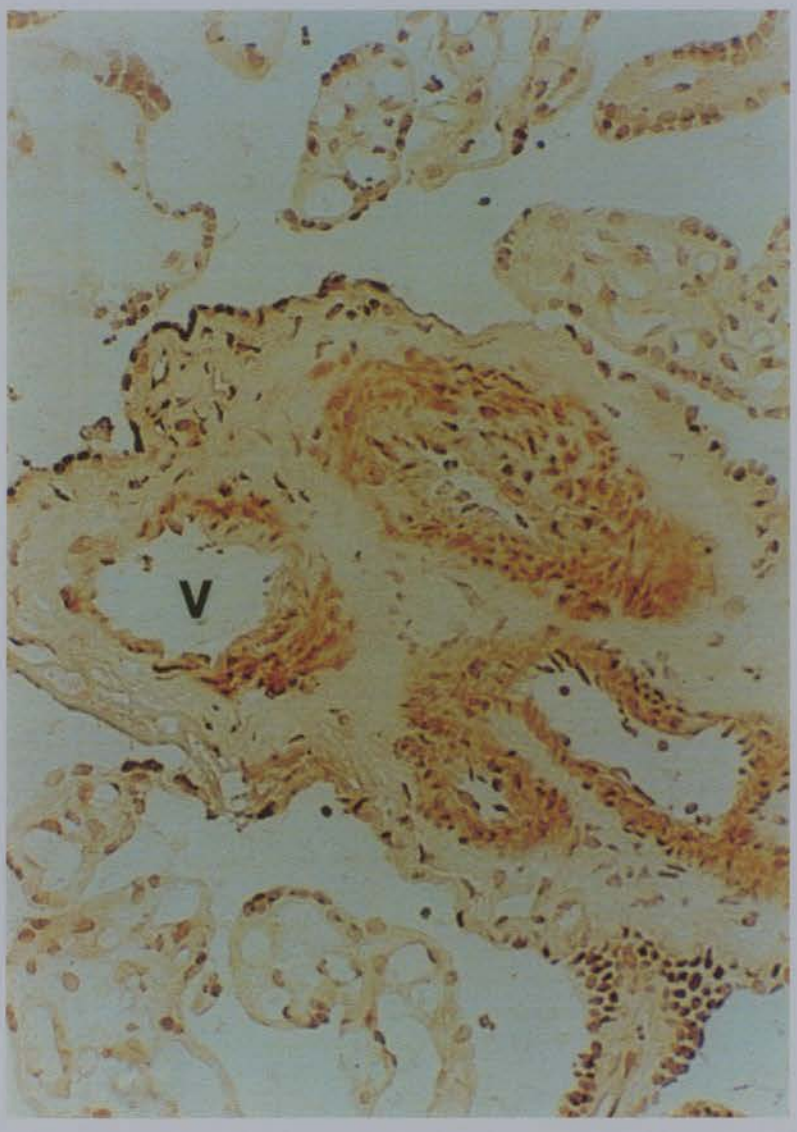


Figure 5.1: IL-8 positive immunostaining in a section of third trimester placenta following spontaneous delivery (x20) showing staining in the perivascular area. v= fetal vessel

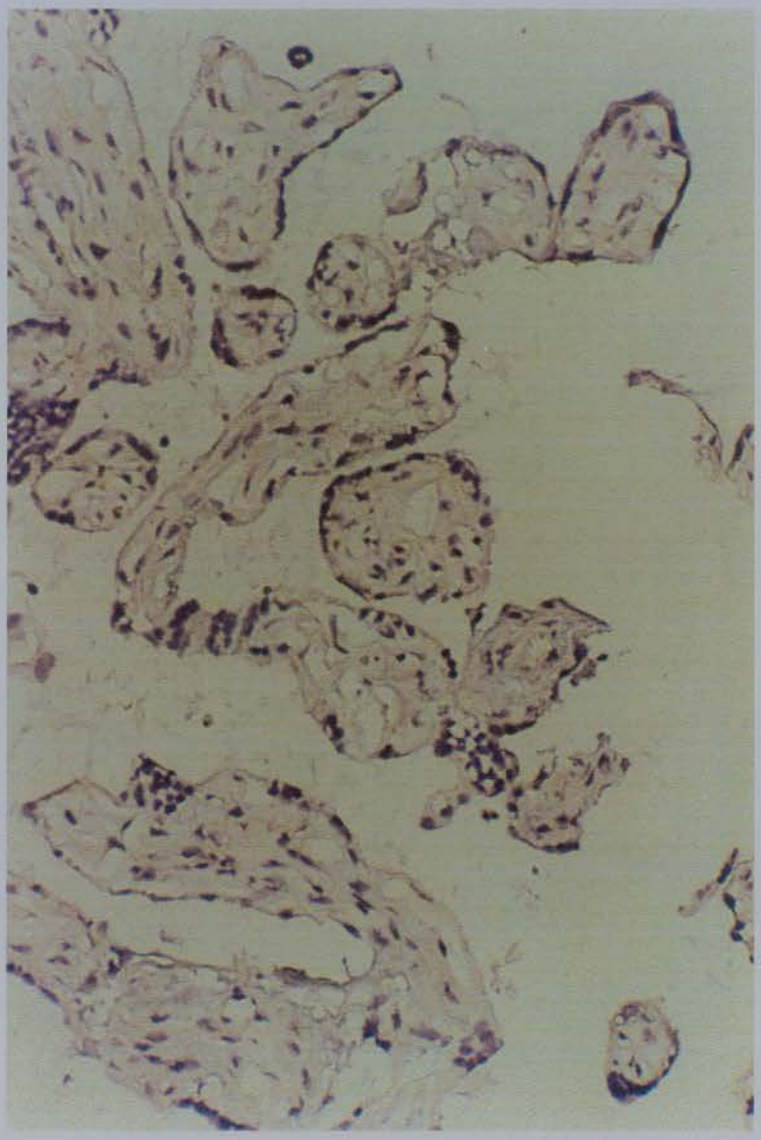


Figure 5.2: Il-8 negative immunostaining in a section of third trimester placenta following spontaneous delivery (x10).

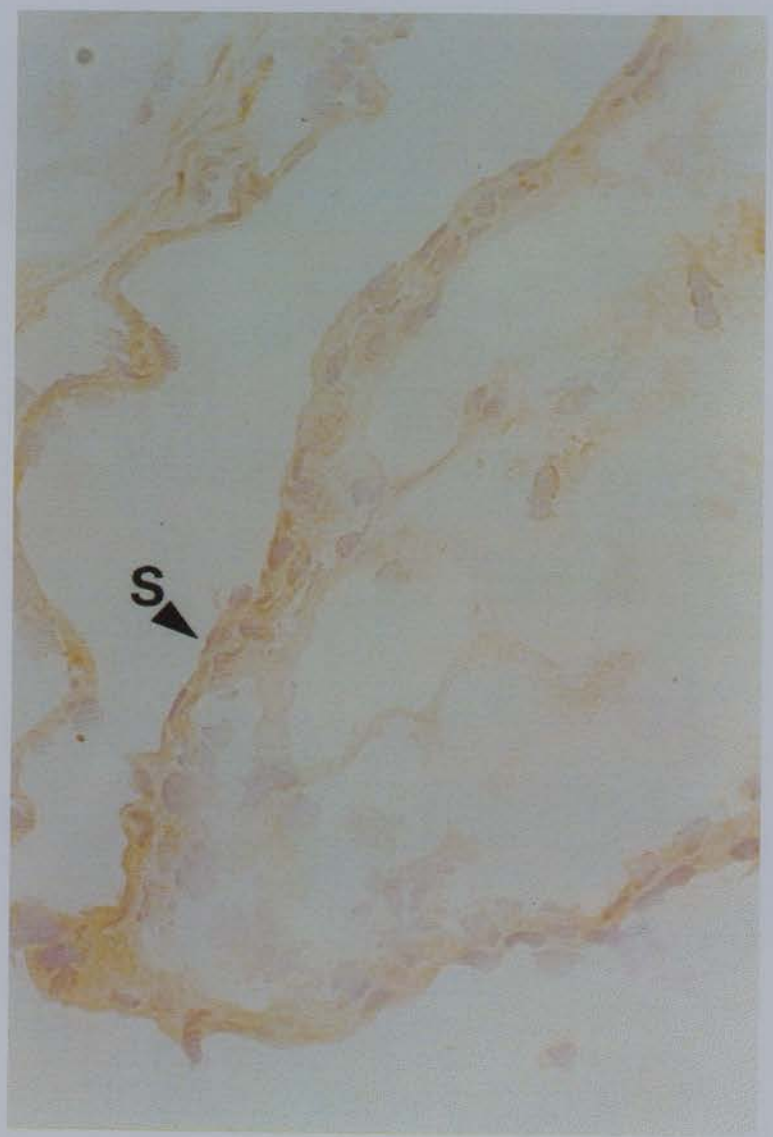


Figure 5.3: IL-8 positive immunostaining
in a section of first trimester placenta (x20)
showing staining in the syncytiotrophoblast (s).

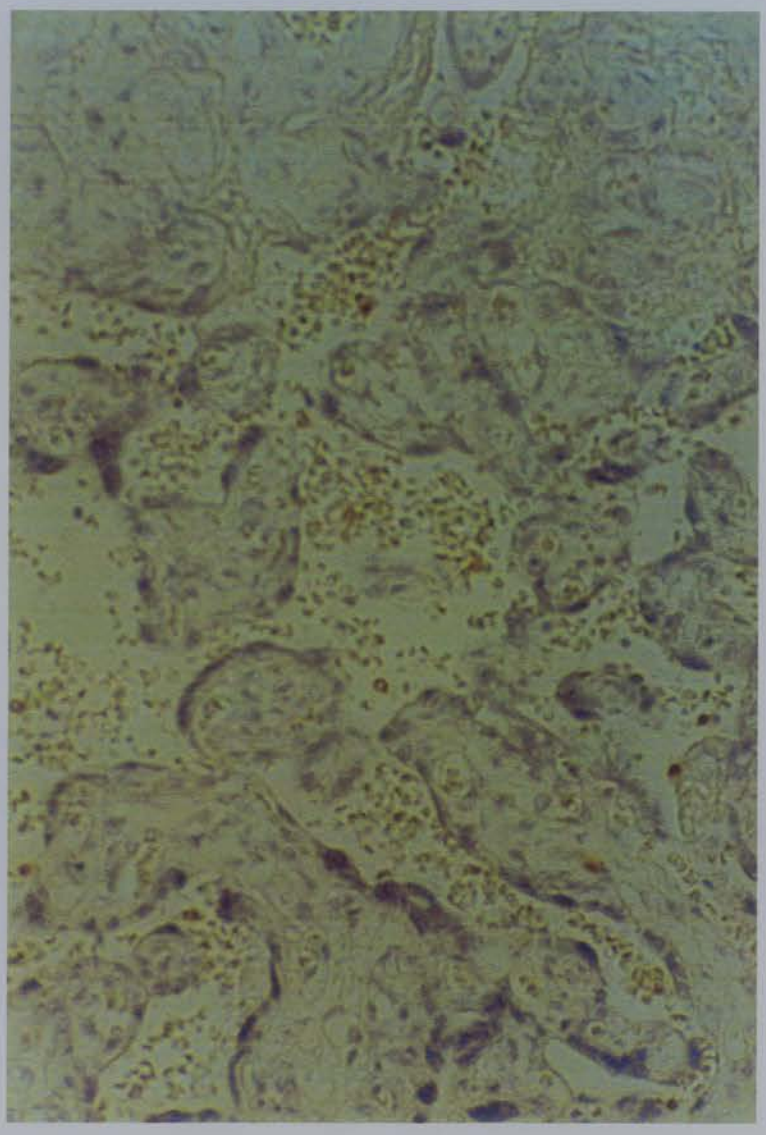


Figure 5.4: CD45 positive immunostaining in a section of third trimester placenta following spontaneous delivery (x20)

Figure 5.5: CD68 positive immunostaining in a section of third trimester placenta following spontaneous delivery (x40).

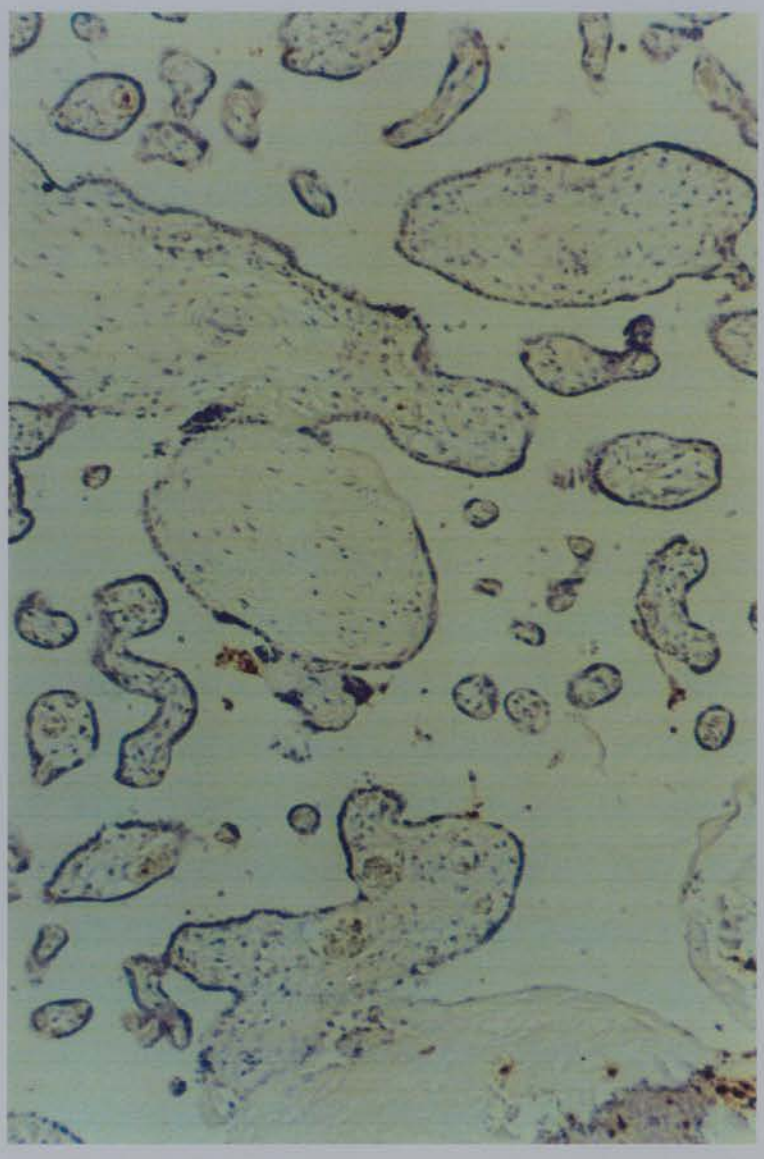


Figure 5.6: Neutrophil elastase positive immunostaining
in a section of third trimester placenta
following spontaneous delivery (x10)

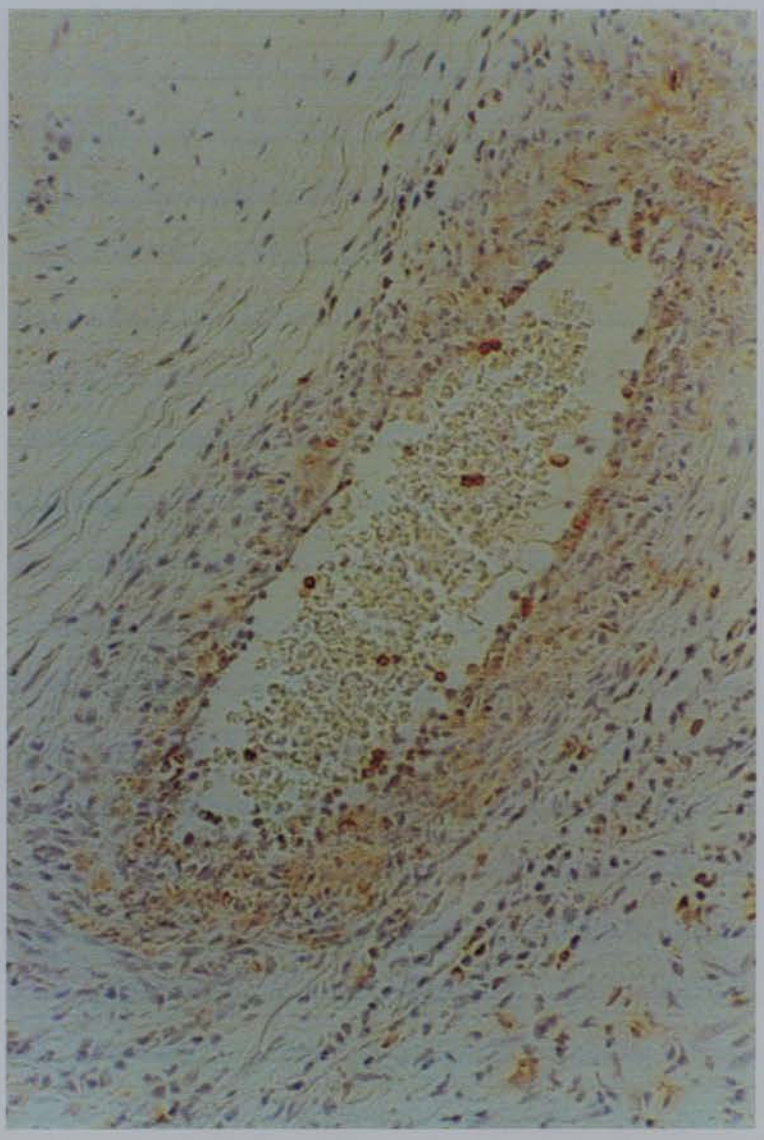


Figure 5.7: CD45 positive immunstaining in a section of third trimester placenta in the region of the chorionic plate following spontaneous delivery (x20) showing staining in the region of a vessel.

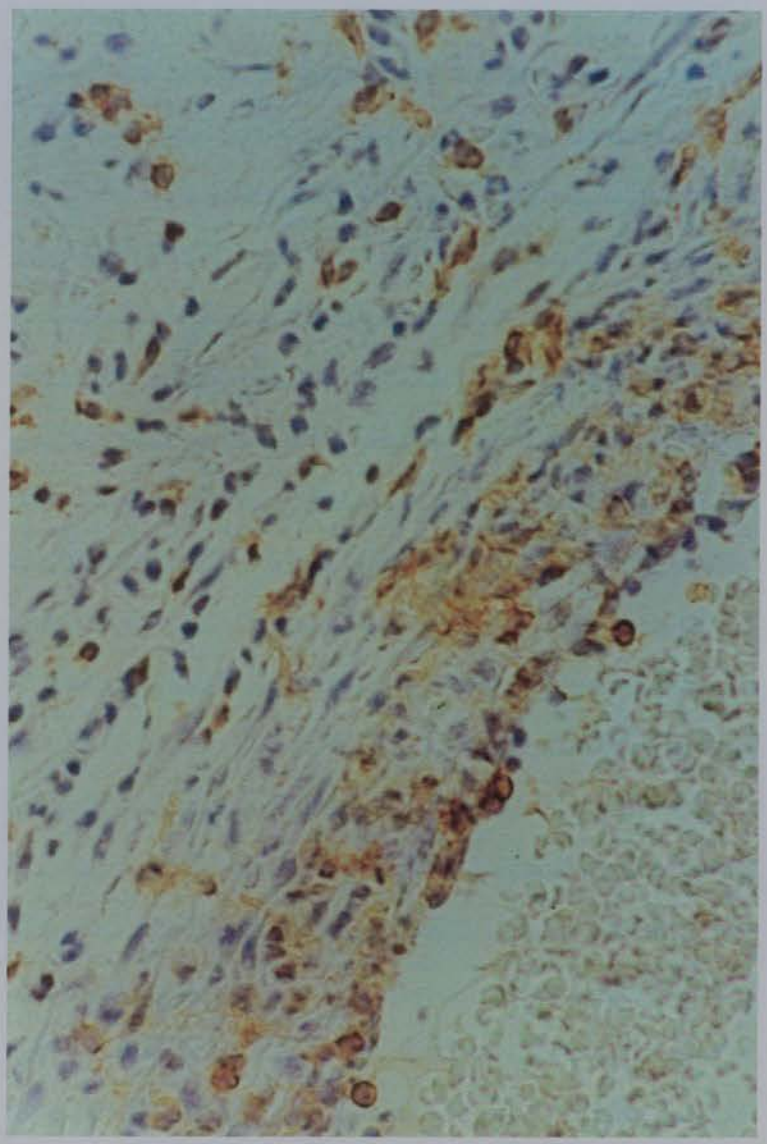


Figure 5.8: CD45 positive immunostaining in a section of third trimester placenta in the region of the chorionic plate following spontaneous delivery (x40) showing staining in the region of a vessel.

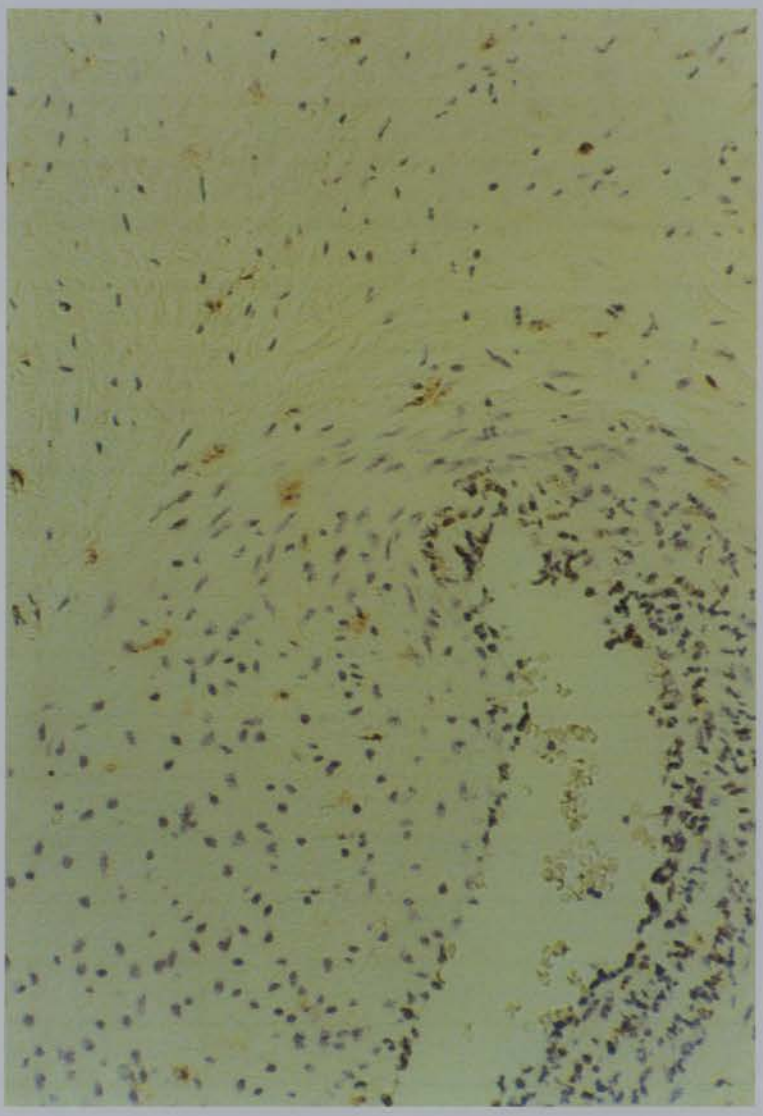


Figure 5.9: CD68 positive immunostaining in a section of third trimester placenta in the region of the chorionic plate following spontaneous delivery (x20) showing little staining in the area adjacent to the vessel.

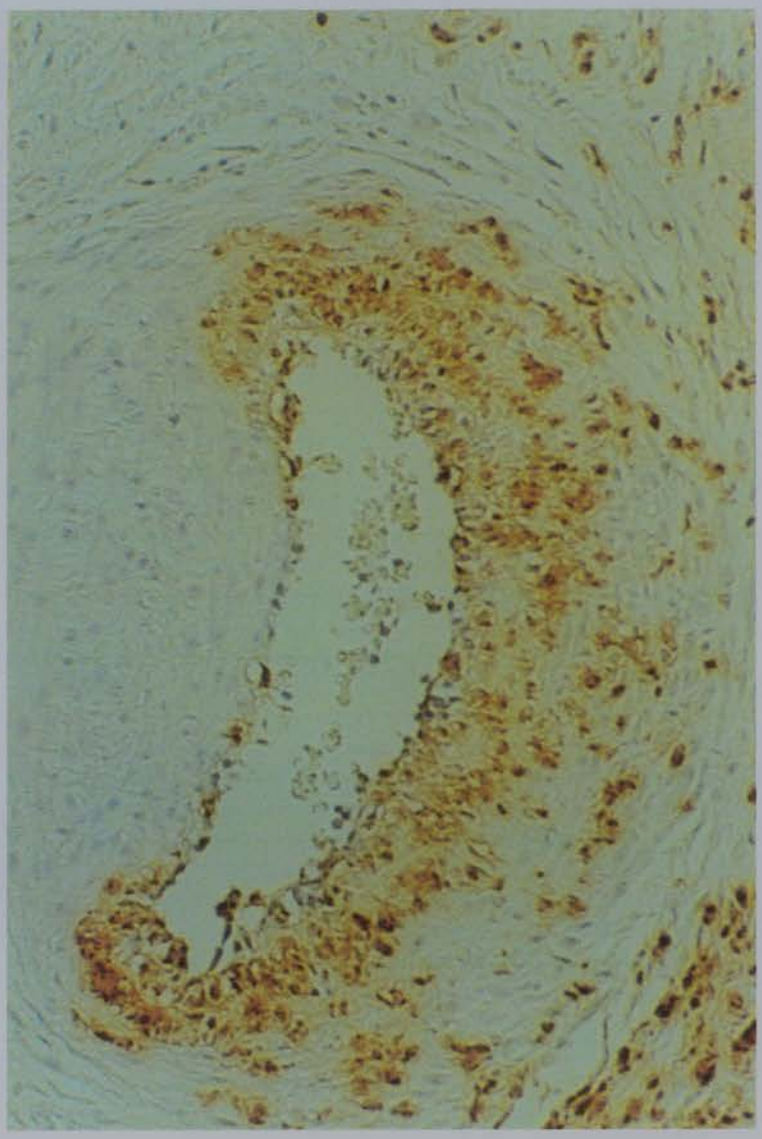


Figure 5.10: Neutrophil elastase positive immunostaining in a section of third trimester placenta in the region of the chorionic plate following spontaneous delivery (x20) showing increased staining in the region of a vessel.

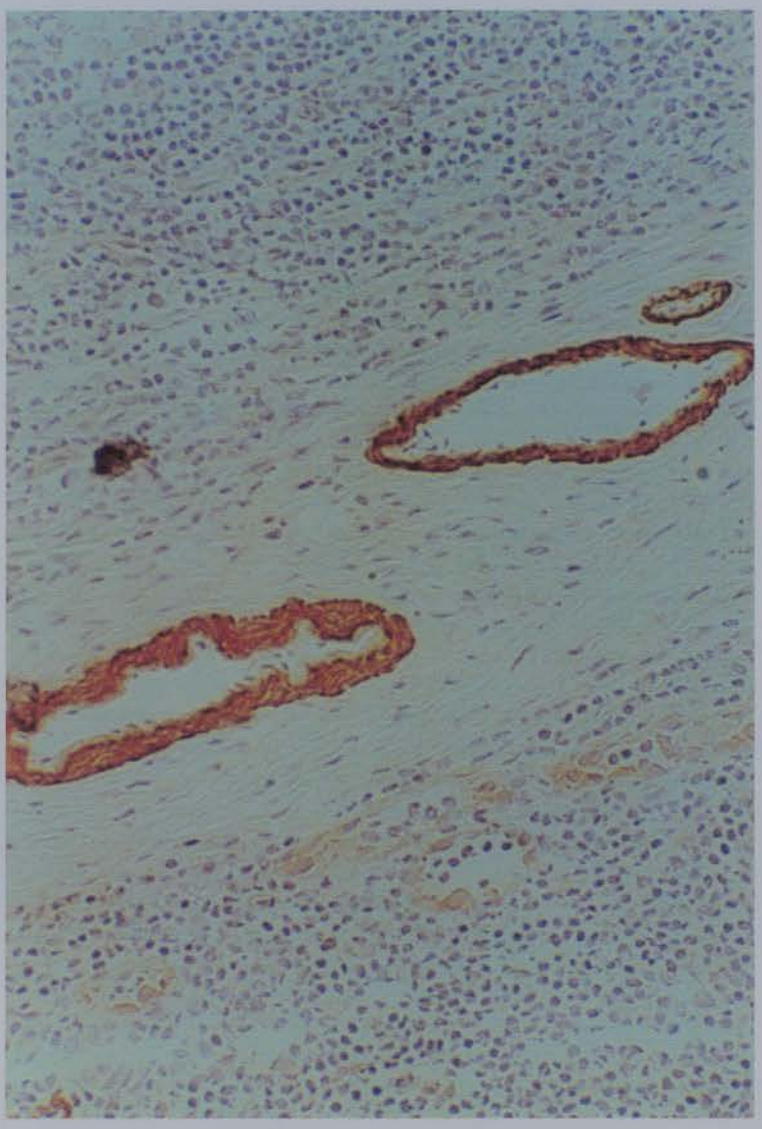


Figure 5.11: IL-8 positive control in a section of tonsil (x20) showing positive immunostaining in the vessels.

Chapter VI:

PRODUCTION OF INTERLEUKIN-8 BY DECIDUAL TISSUE: REGULATION BY STEROIDS AND MIFEPRISTONE.

6.1: INTRODUCTION

Decidual tissue occupies a pivotal position in the parturient uterus, being in contact with the myometrium, adjacent to the cervix and separated only by the fetal membranes from the amniotic fluid. Products of the decidua could therefore potentially influence and be influenced by all of these areas.

Interleukin - 8 (IL-8) is a cytokine which has been shown to be produced by cervical tissue [43], by choriodecidual cells obtained from the chorionic surface [147] and by placental tissue [161]. It is also present in the amniotic fluid in amounts that have been demonstrated to be increased in association with both term and preterm labour and it has been proposed that at least some of this IL - 8 is of decidual origin [157].

Influx of neutrophils appears to be a key event in the onset of cervical ripening and of parturition. It has been hypothesised that PGE_2 aids this influx of neutrophils, possibly synergistically with IL-8 [89] which is a potent attractor and activator of neutrophils. It has previously been shown that IL-8 production is down-regulated by both progesterone [43,147] and dexamethasone [345]. The events of parturition have been linked to a decrease in local levels of progesterone, although this has not been conclusively demonstrated in the human. A decrease in the effect of progesterone could lead to an increase in the expression of IL-8 enhancing neutrophil influx and activation. Use of antigestagens such as mifepristone (RU486) may also activate this process.

This study examines the production of IL - 8 by the decidua parietalis in contact with the myometrium and the effects of dexamethasone, progesterone and mifepristone on this.

The leukocyte populations present in this tissue prior to the onset of labour are studied immunohistochemically.

6.2: SUMMARY OF METHODS

Decidua was collected from four elective caesarean deliveries. After delivery of the placenta the decidua was obtained by curettage of the non-placental area of the uterus (decidua parietalis) and prepared as described in chapter 4 for culture and for immunohistochemistry. First trimester decidua was collected for immunohistochemistry at the time of surgical termination of pregnancy.

Twelve sections from each sample of third trimester decidua were placed in four treatment groups so that each treatment group contained three replicates, these were cultured according to the protocol described above. The supernatant was removed after 24, 48 and 72 hours and IL-8 production determined by radioimmunoassay. The percentage change in production for each treatment group at 48 and 72 hours as compared with the 24 hour production was calculated for each well and the median thus obtained. Statistical significance was tested using Fishers protected least significant difference (StatView) and significance at the level of $p < 0.05$ was determined.

Sections of first trimester decidua were stained using immunohistochemistry to localise IL-8. Sections of third trimester decidua were examined to determine the populations of CD-45, CD-68 and neutrophil elastase positively staining cells. Ten fields from each decidual sample stained for CD45 were analysed using image analysis as described in section 4.2v to calculate the proportion of positive staining cell area.

6.3: RESULTS

Explant Culture

All the explants of decidua parietalis obtained at elective caesarean section in the third trimester produced IL-8. Culture with progesterone 10^{-6} and dexamethasone 10^{-7} for 24 hours (groups B, C and D) caused a decrease in this production, as compared with the control samples which were not treated with steroids (group A) as is shown in graph 6.1. This reduction was, however, not statistically significant ($p = 0.08$).

The median percentage change in production of IL-8 at 48 and 72 hours as compared with the 24 hour production in each treatment group was calculated and is shown in graph 6.2. There were wide variations between the decidual samples in their production of IL-8 and also in the alteration of this in response to treatments and it may be, at least in part, a reflection of these wide variations that none of the differences between the treatment groups or over the two time points are significant.

Group A was the control group in which there was a non-significant increase in production at 48 and 72 hours as compared to the 24 hour production. In group B, which was treated initially with progesterone and dexamethasone and then with no further steroids for the 48 hour and 72 hour timepoints there was a non-significant decrease in production at 72 hours as compared with that at 24 hours. In group C which was treated with progesterone 10^{-6} alone after 24 hours treatment with progesterone and dexamethasone the pattern of IL-8 production is similar to that seen in group B. The addition of mifepristone 5×10^{-7} and progesterone 10^{-6} to the culture medium in group D was associated with an non-significant increase in IL-8 production, as compared with the addition of progesterone alone.

Immunocytochemistry.

IL-8

In the first trimester decidua, positive IL-8 staining was seen in the perivascular areas, this area was negative in tissue stained with rabbit IgG. (figures 6.1, 6.2). A similar pattern was seen in some of the third trimester decidua with clear localisation of the staining in the perivascular area (figure 6.3).

CD45

Cells with positive staining for this marker were evident in the third trimester decidua. A field containing a large number of such cells is shown. (figure 6.4) By image analysis (as described above) the overall percentage of cells staining positively for this marker was 6.7% ± 8.0. This analysis is calculated on the basis of the *area* of cells staining positively and negatively. The tissue negative for CD45 showed no staining.

CD68

There appear to be fewer of these cells in the decidual tissue, many fields contained less than that shown here (figure 6.5). The cells staining appeared rather fragmented in their morphology.

Neutrophil Elastase

Virtually no cells in the decidual stroma stained positively with this antigen (figure 6.6). Positively staining cells were seen in small numbers in blood vessels within the decidua.

6.4: DISCUSSION

These results confirm that decidua parietalis collected from the myometrial aspect of the uterus produces IL-8. This production seems to be affected by the steroid environment in which the explants are cultured. A suppression in production is seen during culture with dexamethasone and progesterone as compared with control samples but this is not significant in the above results, possibly due to the large variations between the samples. Suppression of production by dexamethasone and progesterone would be expected from work in similar tissues, such as choriodecidua, endometrium [147,150] and cervix [43]. In this study the suppression observed appeared to continue after removal of the steroid medium and careful washing of the explants (group B). This could be due to a continued alteration in the intracellular release or production of IL-8 or may be because the suppression is not a direct effect of the steroids but is mediated by other factors, such as IL-1 or TNFa both of which have been shown to increase IL-8 production [153]. A destabilisation of the mRNA for these cytokines by the steroids used could have a relatively long acting effect on decreasing IL-8 production.

The use of explants, rather than cell cultures in this study is supported by work demonstrating that decidual tissue explants produce lower levels of IL-8 (and many other inflammatory cytokines) than decidual cells cultured in the same conditions, suggesting that the preparation of cell cultures may stimulate cytokine production [346].

Treatment with progesterone alone after initial suppression of production by dexamethasone and progesterone (Group C) did not cause a significant recovery in IL8 production. The explants that received no further progesterone or dexamethasone showed a similar continued suppression of production (as described above), so the effect of

progesterone may be weak. However when interpreting the effect of progesterone on this tissue it may be significant that it had been exposed to high levels of progesterone in utero prior to culture.

Addition of mifepristone and progesterone after initial suppression (group D) did not produce a significant rise in IL-8 production as compared with addition of no further steroids or the addition of progesterone alone although a non-significant rise in production was seen. This may again be due to the wide variation in these samples but it suggests that the initial suppression by dexamethasone and progesterone is not overcome by the addition of mifepristone. Further studies could be performed to assess if increasing concentrations of mifepristone would produce a significant increase in IL-8 production. Mifepristone has an antiglucocorticoid action and it may be that this is of more importance in overcoming the continuing suppression of IL-8 production seen in group B than its antiprogesterone effects.

These results do not show a significant effect of mifepristone on increasing IL-8 production in third trimester decidua. This is in contrast to recent work showing that administration of mifepristone *in vivo* in the first trimester results in a time-dependent increase in IL-8 production by cultured decidua[351]. This difference could be due to the differing progesterone concentrations between the first and third trimesters. It may also be that there are other factors necessary for the effect of mifepristone on IL-8 production, such as other cytokines which are modulated following administration of mifepristone to women but which are not affected during *in vitro* culture with mifepristone. In choriodecidual cells from the maternal surface of third trimester chorion IL-8 production was decreased by progesterone and increased by mifepristone[150]. This may represent a difference between choriodecidual cells and decidua parietalis or be due to the smaller

inter-sample variability found in this study. As mentioned above decidual cell culture production of IL-8 is greater than explant production and the effect of steroids may also vary between the two preparations.

The perivascular location of IL-8 in the first and third trimester decidua mirrors the position of PGE_2 in early decidua as shown by Cheng et al in their studies [337] [287] where it was shown that treatment with RU486 caused a decrease in local PGDH and an increase in PGE_2 in the perivascular area. In view of the synergism that has been shown to exist between IL-8 and PGE_2 in other tissues [144] their similar localisation in decidua suggests a similar synergism may occur there. This in turn could relate to recruitment of neutrophils from the decidual vessels around the time of parturition. In the staining for neutrophil elastase little positive staining was seen in the decidua obtained from elective caesarean sections. There could be several reasons for this. The antibody used may not stain the elastase once the neutrophils are activated which may occur rapidly in tissue with potentially high levels of IL-8. Alternatively, as these decidual samples are pre-labour the tissue levels of IL-8 may either not be sufficient or may be prevented by some other factor from recruiting neutrophils. High local levels of endogenous progesterone may inhibit the production of IL-8.

There are leucocytes present in the third trimester decidua, which are CD45 +ve. This is a non-specific marker for leucocytes (the leucocyte common antigen or LCA). The cells thus stained are generally CD68 -ve. The latter antibody is a macrophage marker raised against Gaucher cells. In this study the proportion of CD 56+ cells in the decidua was not assessed but as discussed in Chapter 1, this has previously been found to reduce in the third trimester as compared with the first trimester.

The decidua in this study was obtained by curettage at the time of elective caesarean section. Decidua collected in this way contains fewer chorionic cells than that taken off the chorion and is a closer representation of the decidual tissue in direct contact with the myometrium. Factors released by decidua may have an effect on the myometrium and thus could have a role in stimulating contractions. The decidua parietalis is also in close contact with the cervix and so there may exist a network of local factors between these two tissues integral to the initiation of both cervical ripening and myometrial contractions. It has previously been shown that the collagenase involved in cervical ripening is derived from neutrophils from blood vessels [22] [35]. IL-8 from decidua, in addition to that produced by the cervix [43] may have a role in recruiting these neutrophils and in their activation causing release of collagenase. IL-8 has also been shown to be present in amniotic fluid [157] and at least some of this is thought by that group to be of decidual origin. In this context IL8 has been postulated to have a role in 'clearing debris' post-partum.

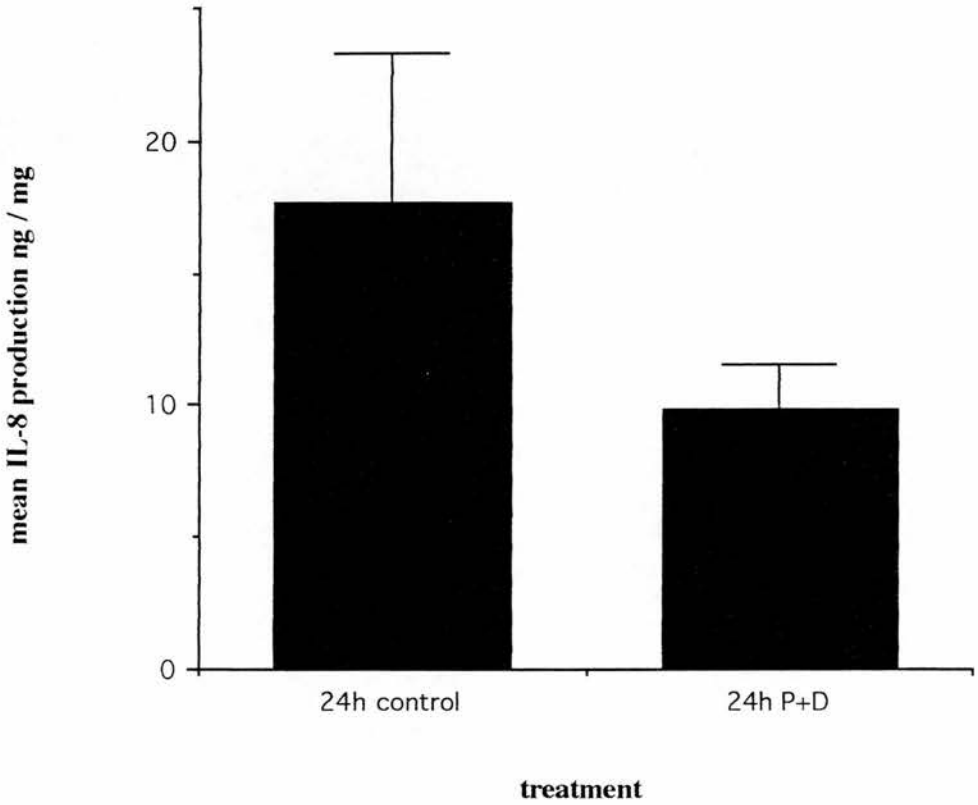
There are, therefore, many potential roles for IL-8 being produced by decidua in the third trimester, including effects on the cervix and myometrium. In order to clarify these further, samples of decidua are required from other stages of labour. However, it is not easily possible to collect decidua during normal labour and decidua collected from intrapartum caesareans may differ intrinsically from that found in labour following a more 'natural course'.

6.5: CONCLUSION

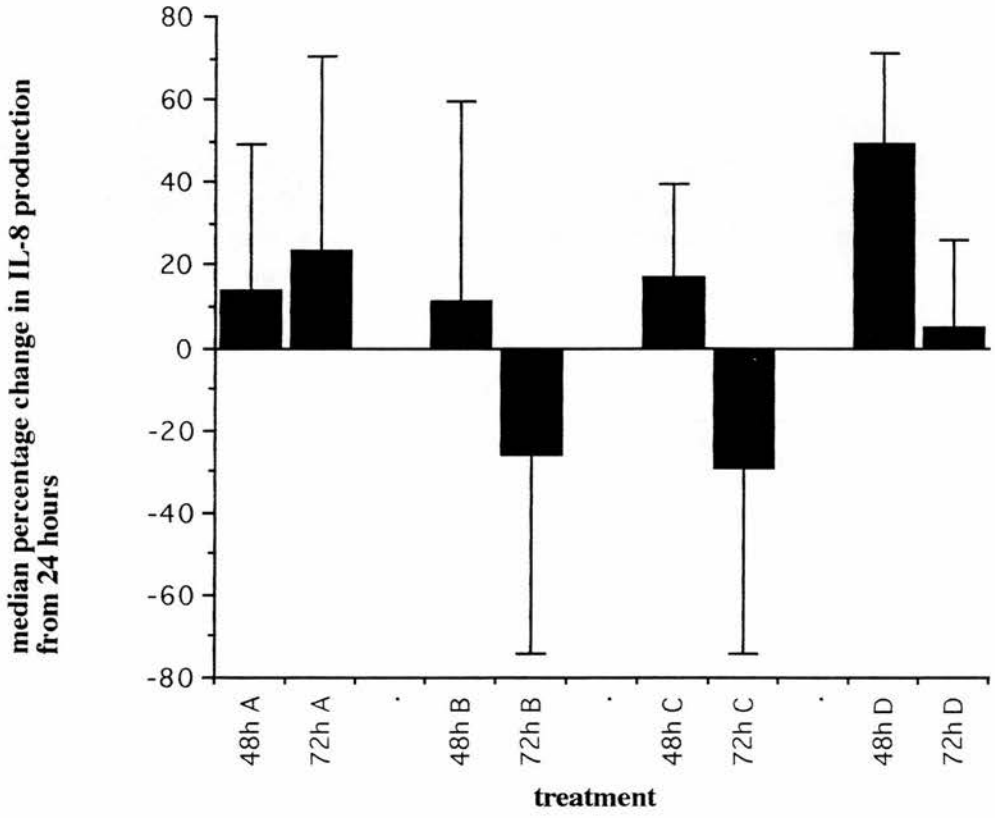
IL-8 is produced by the decidua parietalis in the third trimester prior to labour. This production may be related to an ability of the decidua to recruit neutrophils from blood

vessels around which it has been immunolocalised. There are leucocytes present in the decidua, of which some are CD45 and some CD68 positive. In this study few neutrophils were identified in the decidual tissue.

Graph 6.1: Effect of progesterone and dexamethasone on IL-8 production (mean±sem, ng / mg) as compared with control treatment over 24 hours. (p= 0.08).



Graph 6.2: Median percentage change (±sem) in IL-8 production in each treatment group at 48 and 72 hours as compared with production at 24 hours.



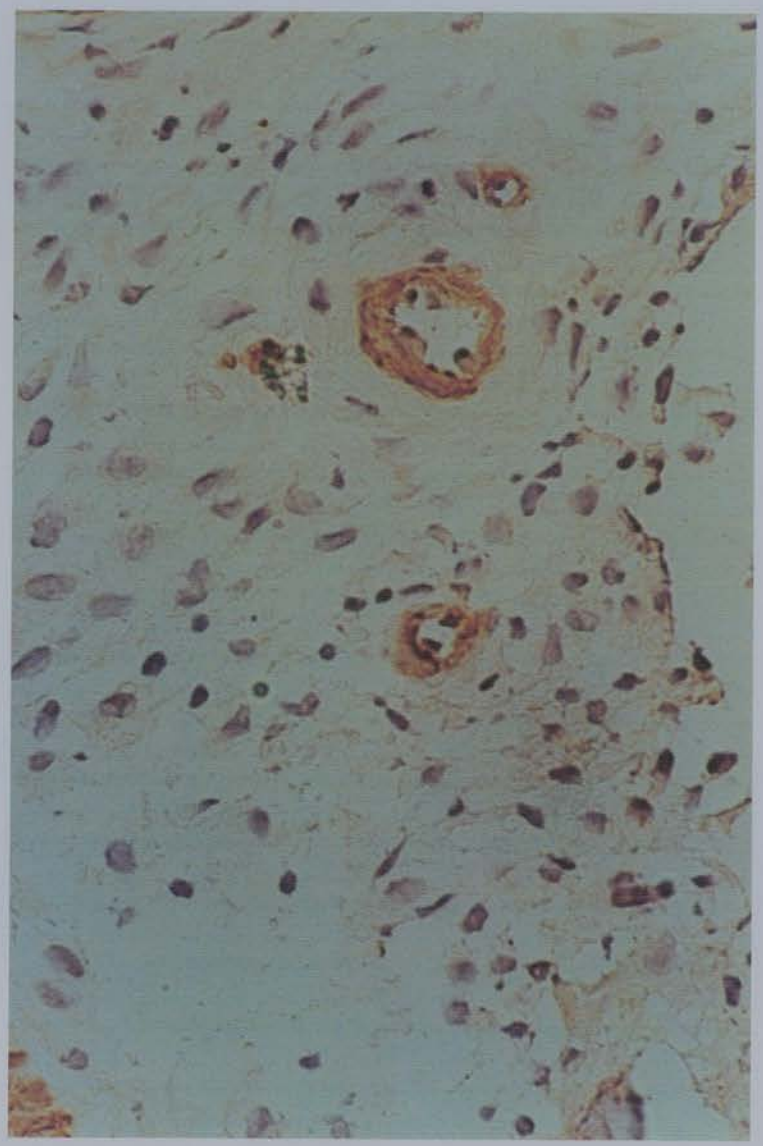


Figure 6.1: IL-8 positive immunostaining in a section of first trimester decidua (x40) showing staining in the perivascular area.

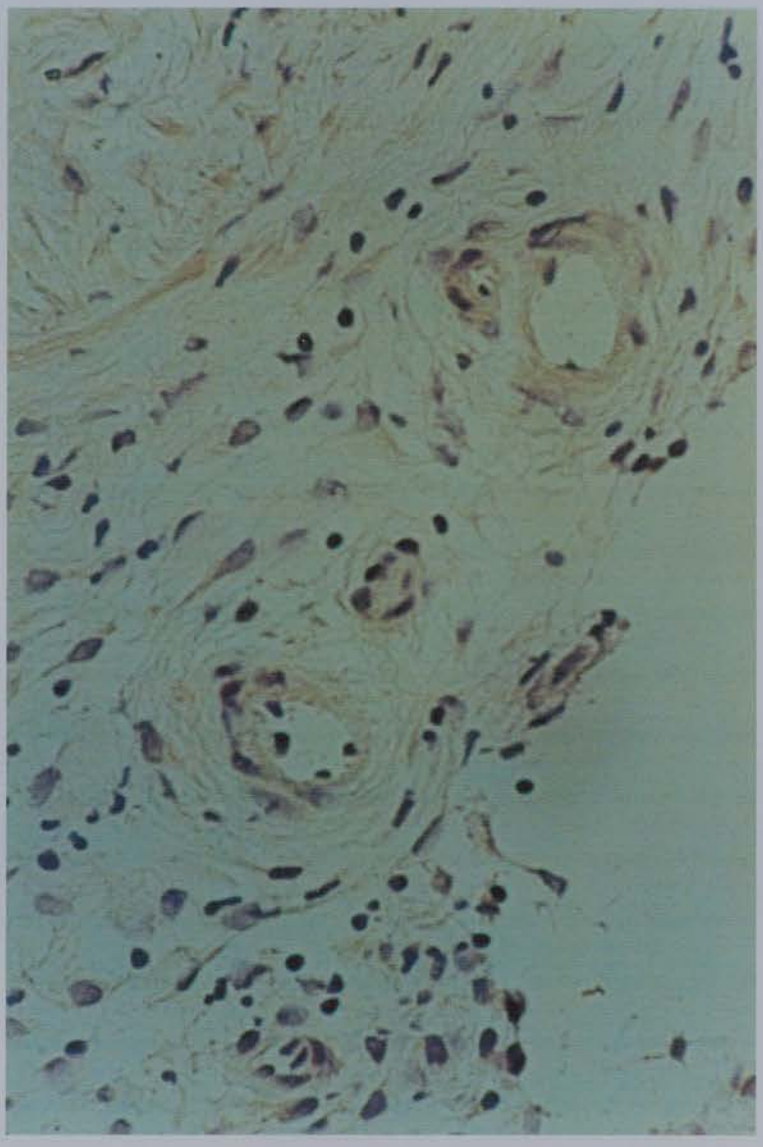


Figure 6.2: IL-8 negative immunostaining in a section of first trimester decidua (x40) showing absence of staining.

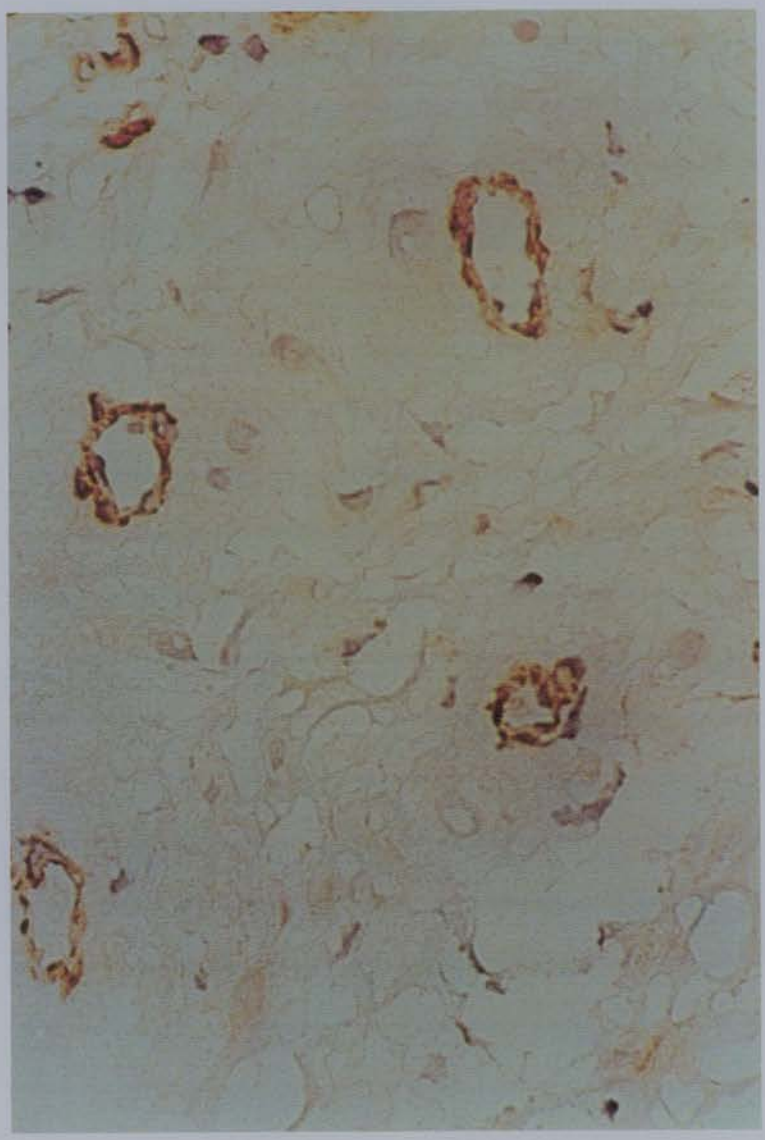


Figure 6.3: IL-8 positive immunostaining in a section of third trimester decidua obtained at elective caesarean (x40) showing staining in the perivascular area.

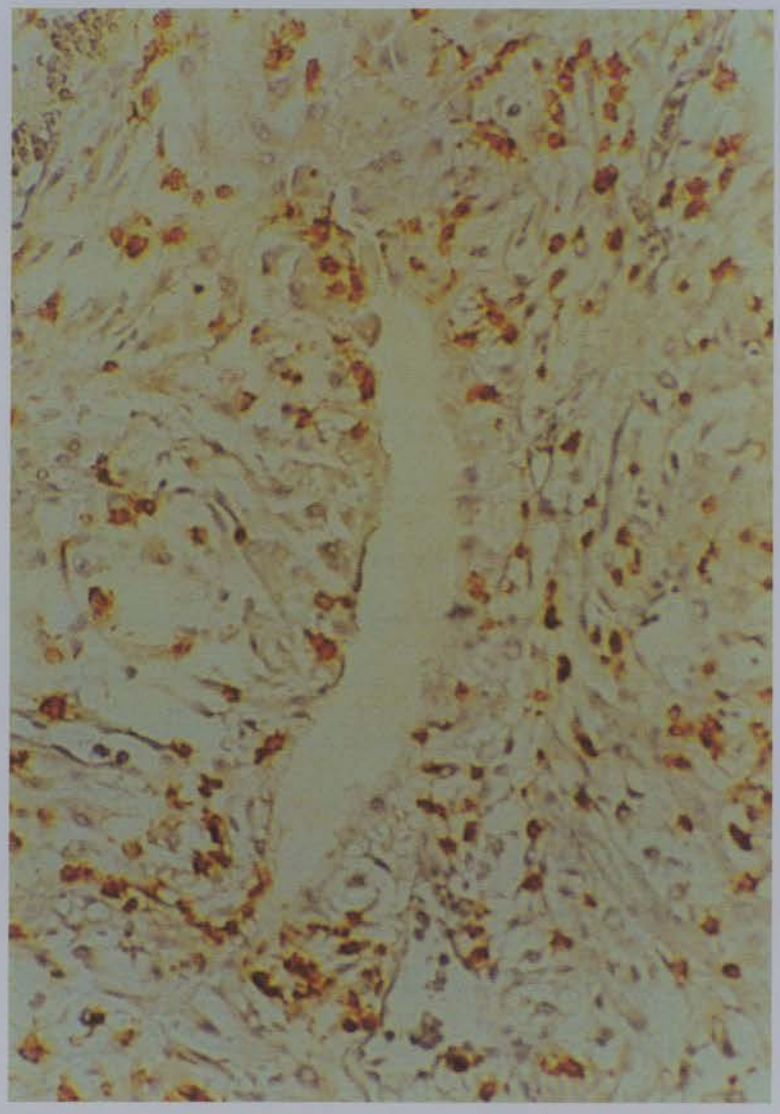


Figure 6.4: CD45 positive immunostaining in a section of third trimester decidua obtained at elective caesarean (x20).

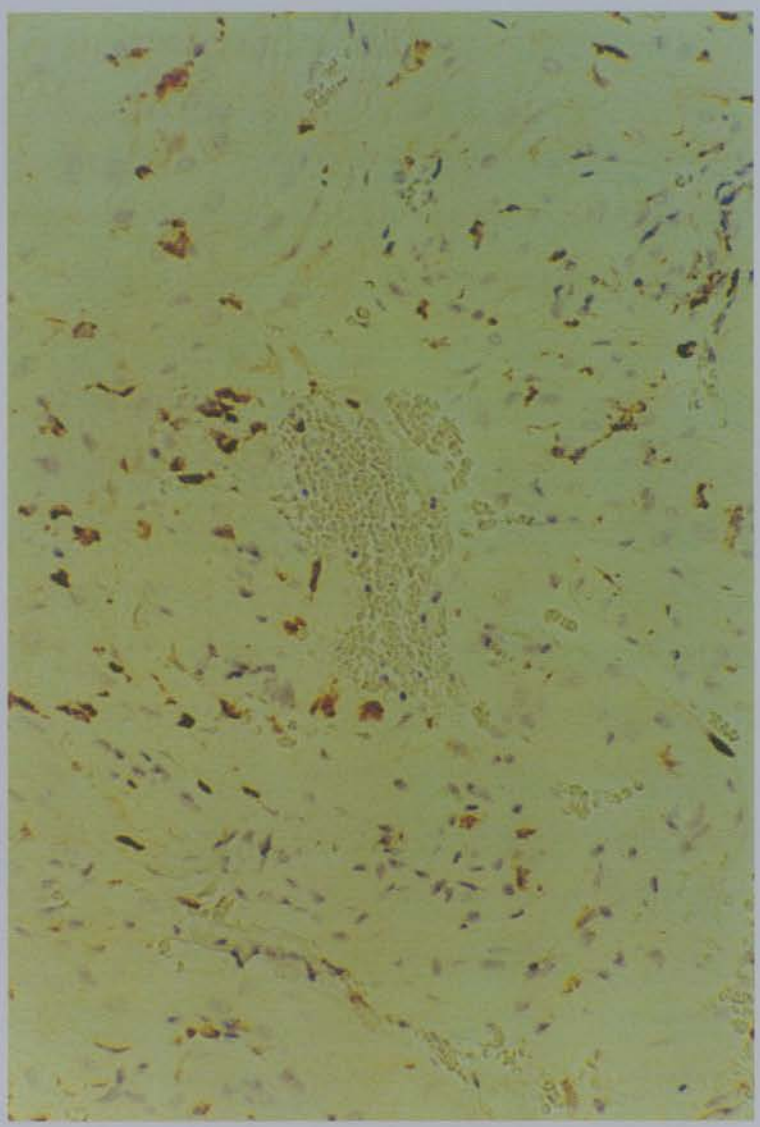


Figure 6.5: CD68 positive immunostaining in a section of third trimester decidua obtained at elective caesarean (x20).

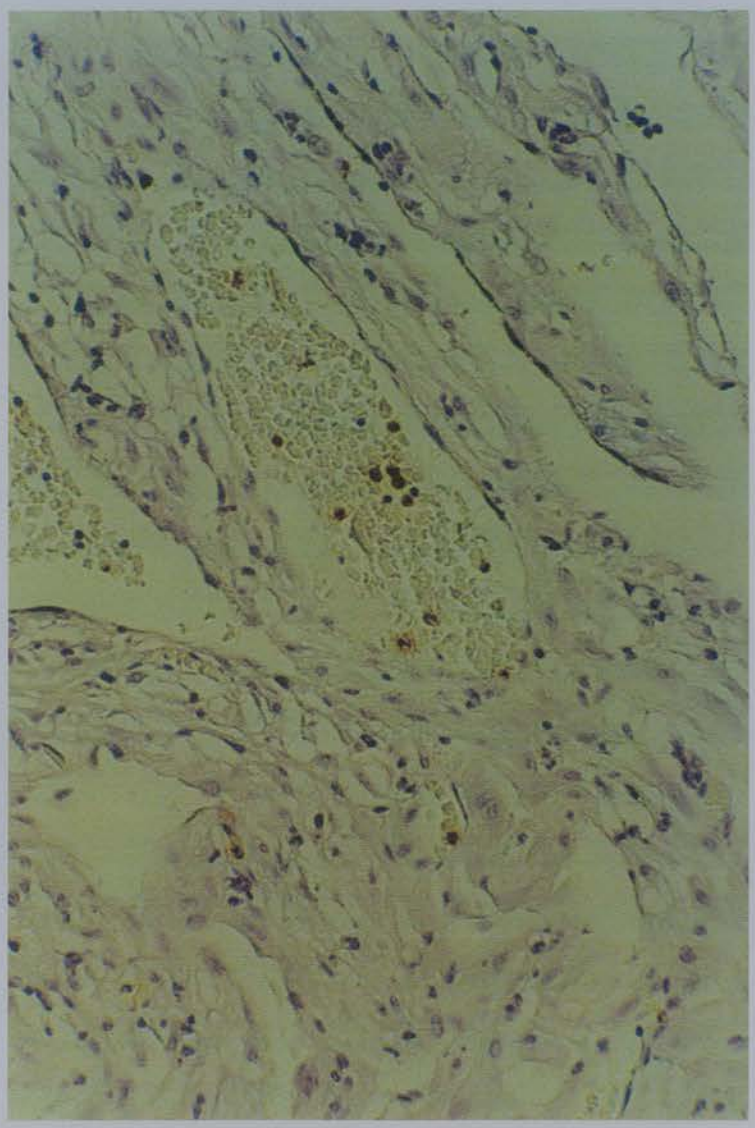


Figure 6.6: Neutrophil elastase positive immunostaining
in a section of third trimester decidua obtained at elective caesarean (x20).

Chapter VII:

THE EFFECT OF MIFEPRISTONE ON THE PRODUCTION OF INFLAMMATORY MEDIATORS BY FIRST TRIMESTER CERVIX

7.1: INTRODUCTION

The progesterone antagonist mifepristone has previously been shown to soften the cervix in the first trimester as an adjunct to medical [262,321] or surgical termination [327] of pregnancy and to promote cervical ripening in the third trimester of pregnancy in monkeys [303] and in humans [334]. The exact mechanism by which this occurs is unclear although it is known that mifepristone acts on the progesterone receptor to antagonise the effects of progesterone [264].

Cervical ripening prior to parturition at term is associated with an influx of neutrophils into the cervical stroma with an associated release of the collagenase they contain which causes dissociation of the collagen fibres [26,35]. This has been proposed to occur in the presence of an inflammatory type reaction in which the cytokine interleukin-8 (IL-8) and prostaglandin E_2 (PGE_2) interact to attract and activate neutrophils in the cervical stroma[89]. Monocyte chemoattractant peptide-1 (MCP-1) has been proposed to be involved in the recruitment of inflammatory cells which could produce IL-8 and other cytokines such as IL-1 and IL-6 in the cervix during ripening. Mifepristone may act by directly increasing the levels of such mediators in the cervix or by antagonising an inhibition of their production by progesterone.

This study aimed to examine the production of inflammatory mediators by cervical biopsies obtained at surgical termination of first trimester pregnancies in primigravid women who had received mifepristone 6, 12, 24 or 36 hours prior to termination. These levels were compared to those in a control group of women who were not given any preoperative treatment. The mediators studied were PGE_2, $PGF_{2\alpha}$, their metabolites and the cytokines IL-8 and MCP-1.

7.2: SUMMARY OF METHODS

The study group consisted of thirty women of less than 9 weeks amenorrhoea who were to have a suction termination of pregnancy. None of the women included had had any previous pregnancies. Informed consent was obtained from all those who participated. The women were randomised into five treatment groups, each consisting of six women, to receive 200 mg of mifepristone at 6, 12, 24 or 36 hours or no treatment prior to the termination. The surgeons performing the terminations were blinded to the treatment allocation of the women. Women were not included if they had serious medical conditions, were less than 16 years old or were otherwise unable to give informed consent. At the time of suction termination a biopsy of the cervix was taken using punch biopsy forceps and was transported in complete culture medium for incubation. The biopsies thus obtained measured about 3mm by 5mm. The study was approved by the Lothian Ethics Committee.

Tissue culture and radioimmunoassay protocols.

The cervical biopsies were cultured for 24 hours in complete medium as described in chapter 4. Their production of the mediators studied was then analysed by radioimmunoassay.

Statistical analysis.

Statistical significance was tested using Fishers protected least significant difference (StatView) and significance at the level of $p < 0.05$ was determined.

7.3: RESULTS.

Patient Characteristics.

All the patients had surgical termination of pregnancy within 45 minutes of the allocated time according to their treatment group. The patient characteristics are shown in figure 1. There were no significant differences between the groups ($p > 0.05$) in terms of their age, body mass index or gestation at the time of termination of pregnancy. Three patients (one in the control group, one given mifepristone 6 hours and one 24 hours prior to termination) were of gestations greater than 63 days but less than 68 days.

Radioimmunoassay results.

The results of the assays for levels of PGE_2, PGEM, $PGF_{2\alpha}$, PGFM, IL-8 and MCP-1 are shown in Graphs 1, 2 and 3. None of the differences demonstrated reached significance at the stated level (< 0.05).

The production of PGE_2 shows a slight increase across the treatment groups but this was not significant. One biopsy produced a large amount of $PGF_{2\alpha}$ after pretreatment with mifepristone 6 hours prior to sampling. There was an increase in production of $PGF_{2\alpha}$ in samples obtained when mifepristone was given 36 hours prior to sampling as compared to when it was given 12 hours before but again this did not reach a statistically significant level. The production of PGF metabolite showed little variation across the treatment groups.

7.4: DISCUSSION

These first trimester cervical biopsies all demonstrated production of the inflammatory mediators studied, regardless of whether mifepristone was given prior to sampling and of the timing of mifepristone pretreatment. It has been postulated that cervical ripening occurs by an inflammatory process [41] and there is accumulating evidence of a cascade of inflammatory mediators that interact during cervical ripening both at term and at other stages of pregnancy. During cervical ripening the collagen of the stroma is degraded by collagenase which appears to be derived from neutrophils recruited into the cervical stroma[26,35]. IL-8 is a potent attractor and activator of neutrophils and it has been shown by this study that the first trimester cervix is capable of producing this cytokine, confirming the findings of Barclay et al [43]. The effects of IL-8 in attracting and activating neutrophils have been demonstrated in rabbit skin to be increased by PGE_2 [142,143]. If such synergism occurs in the cervix it may that administration of mifepristone, while not causing a statistically significant increase in either mediator could cause sufficient alterations in their production to affect neutrophil activation in the cervix.

Monocyte chemotactic peptide-1 (MCP-1) is a 76 amino acid cytokine which attracts blood monocytes and is also chemotactic for basophils, eosinophils and lymphocytes but not for neutrophils [124]. Tissue macrophages are unresponsive to the actions of MCP-1 whereas blood monocytes respond to its chemotactic effect[170]. MCP-1.is produced in large amounts by monocytes and also causes an increase in monocyte secretion of IL-1 and IL-6[171]. IL-1 in particular is known to stimulate IL-8 production [121] and so release of MCP-1 could act as an indirect stimulant of IL-8 production. MCP-1 also acts on basophils to stimulate histamine and leukotriene release [124]. As with IL-8 its production by cells has been shown to be stimulated by IL-1, TNFα and LPS [124]. All the biopsies which had

been exposed to mifepristone produced more MCP-1 than the control group. MCP-1 production may lead to an increase in the recruitment of monocytes into the cervix and the release of further immunoactive mediators such as IL-1 and IL-6 which could in turn increase IL-8 production and stimulate degradation of the cervical stroma. PGE_2 limits MCP-1 expression in lung fibroblasts[124] and if it has a similar action in the cervix could act as a 'brake' on the inflammatory cascade.

Mifepristone is known to soften the cervix in the first trimester of pregnancy and has been shown to be as effective as gemeprost (16, 16-dimethyl-trans Δ 2 PGE_1 methyl ester, Cervagem) in a blinded study comparing them with placebo for preoperative cervical ripening prior to vacuum aspiration in the first trimester of pregnancy[327]. The intraoperative blood loss was significantly decreased by both treatments as compared with placebo with no difference in their effectiveness. In our study it was found that the degree of softening following mifepristone administration was so marked that it affected the type of biopsy obtainable. It was for this reason that a punch biopsy was taken rather than a Trucut needle biopsy. The latter method may have been advantageous as it would have been expected to obtain a sample from above the ectocervix from which at least some of the punch biopsy was obtained. It proved impossible however to obtain an adequate specimen using the Trucut needle following mifepristone administration.

In women in the first trimester of pregnancy administration of mifepristone prior to sampling and culture of decidual tissue has been shown to decrease the levels of prostaglandin dehydrogenase (PGDH) and increase the tissue levels of PGE_2 and PGEM present [287]. Culture of decidual tissue collected from such women showed increased $PGF2\alpha$ production and decreased PGFM (the metabolite of $PGF2\alpha$) levels compared with control tissue [291]. Although mifepristone has been shown to increase prostaglandin levels

in these studies its action in causing cervical ripening in the first trimester may not be directly related to alterations in prostaglandin production. In this study we have found no significant alterations in prostaglandin production by the cervix following mifepristone administration. Other studies of the human cervix in the first trimester have shown that administration of mifepristone does not alter the subsequent *in vitro* bioconversion of radiolabelled arachodonic acid to thromboxane, PGE_2, $PGF_{2\alpha}$ [308] or the production of 12-hydroxyeicosatetraenoic acid (HETE) or leukotrienes [283]. In women, the effect of mifepristone in causing increased cervical softening and dilatation in the first trimester of pregnancy is not reversed by the concomitant administration of naproxen (a cyclooxygenase inhibitor). [294] These studies suggest that either the mechanism of action of mifepristone in causing cervical softening is not solely mediated through prostaglandins and also that other synthetic pathways producing prostaglandins unaffected by diclofenac and naproxen may involved.

In the late pregnant monkey oral administration of mifepristone led to an increase in the amniotic fluid levels of 6-keto-$PGF1\alpha$, PGFM, $PGF2\alpha$ and PGEM which increased in that order over time [295]. This increase was not observed, however, until at least 40 hours after mifepristone administration by which time an increase in uterine contractile activity was already evident. This observation is in contrast to the finding in the same study that in spontaneous labour intra-amniotic prostaglandins and their metabolites increase before the onset of regular uterine activity. It may be that if we had administered mifepristone at time points longer before termination of pregnancy significant differences in cervical production of prostaglandins and their metabolites may have been found.

Mifepristone is known to cause clinically significant softening and dilatation of the cervix of women in the first trimester of pregnancy. Further work is needed to demonstrate

whether alterations in the monocyte or neutrophil populations of the cervix occur following mifepristone administration.

Figure 1: Patient age (years, mean±SD), body mass index (BMI) (mean±SD) and gestation (days, mean ±SD) for each of the treatment groups.

	Age	BMI	Gestation
Control	21.33 (3.27)	23.52 (4.15)	58.67 (5.35)
6h	22.17 (5.00)	24.42 (3.15)	60.33 (4.41)
12h	20.50 (3.56)	23.57 (3.77)	53.50 (7.61)
24h	22.83 (2.64)	25.48 (3.78)	57.17 (5.85)
36h	20.67 (4.41)	22.25 (2.99)	50.67 (12.11)

Figure 2a: Production of PGE_2, PGEM, $PGF_{2\alpha}$ and PGFM (ng / mg, mean±sem) by cervical biopsies from each of the treatment groups.

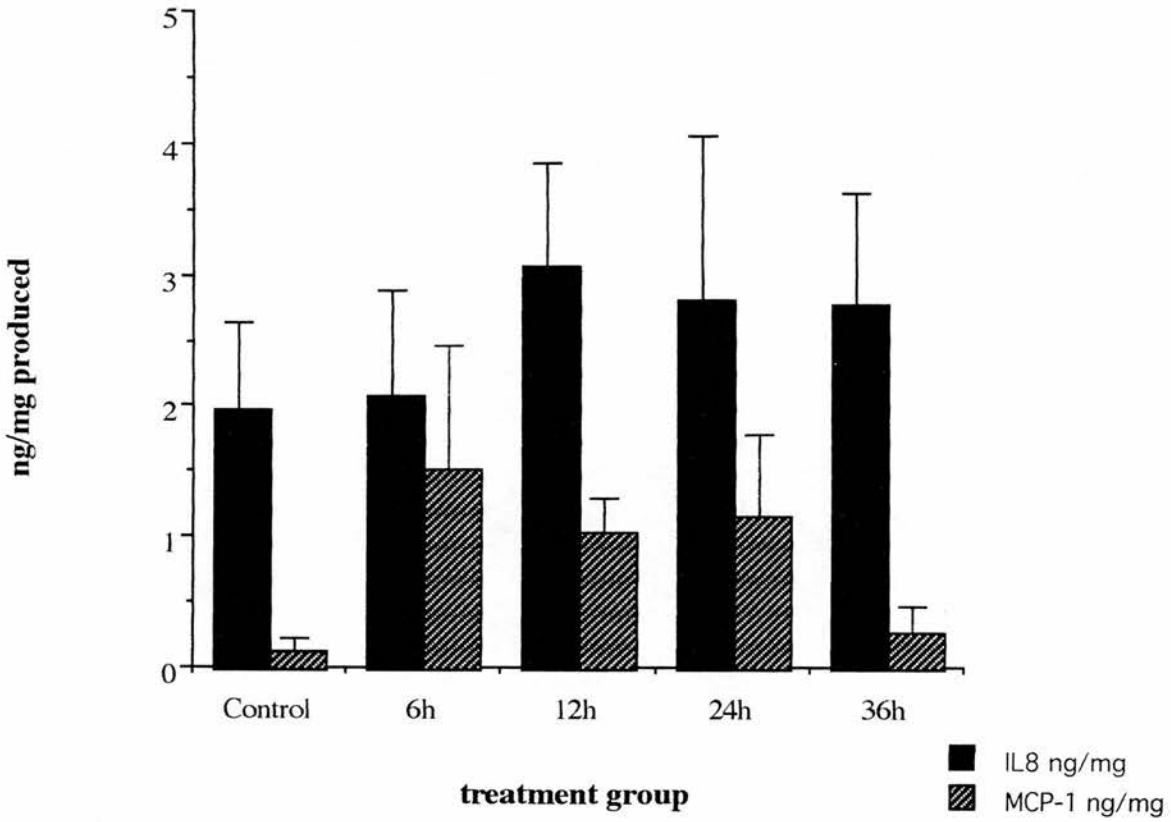
	PGE_2	PGEM	$PGF_{2\alpha}$	PGFM
Control	0.99 (0.39)	0.45 (0.17)	1.85 (0.71)	0.52 (0.17)
6 hours	1.05 (0.69)	0.59 (0.30)	5.20 (4.31)	0.55 (0.30)
12 hours	1.62 (0.56)	0.82 (0.50)	1.47 (0.60)	0.50 (0.18)
24 hours	1.83 (0.61)	0.24 (0.05)	2.03 (0.81)	0.65 (0.17)
36 hours	2.02 (0.68)	0.60 (0.20)	3.18 (1.27)	0.74 (0.29)

Figure 2b: Production of IL-8 and MCP-1 (ng / mg, mean±sem) by cervical biopsies from each of the treatment groups.

	IL-8	MCP-1
Control	1.99 (0.64)	0.15 (0.08)
6 hours	2.09 (0.78)	1.52 (0.93)
12 hours	3.07 (0.76)	1.05 (0.23)
24 hours	2.82 (1.21)	1.16 (0.61)
36 hours	2.77 (0.82)	0.29 (0.18)

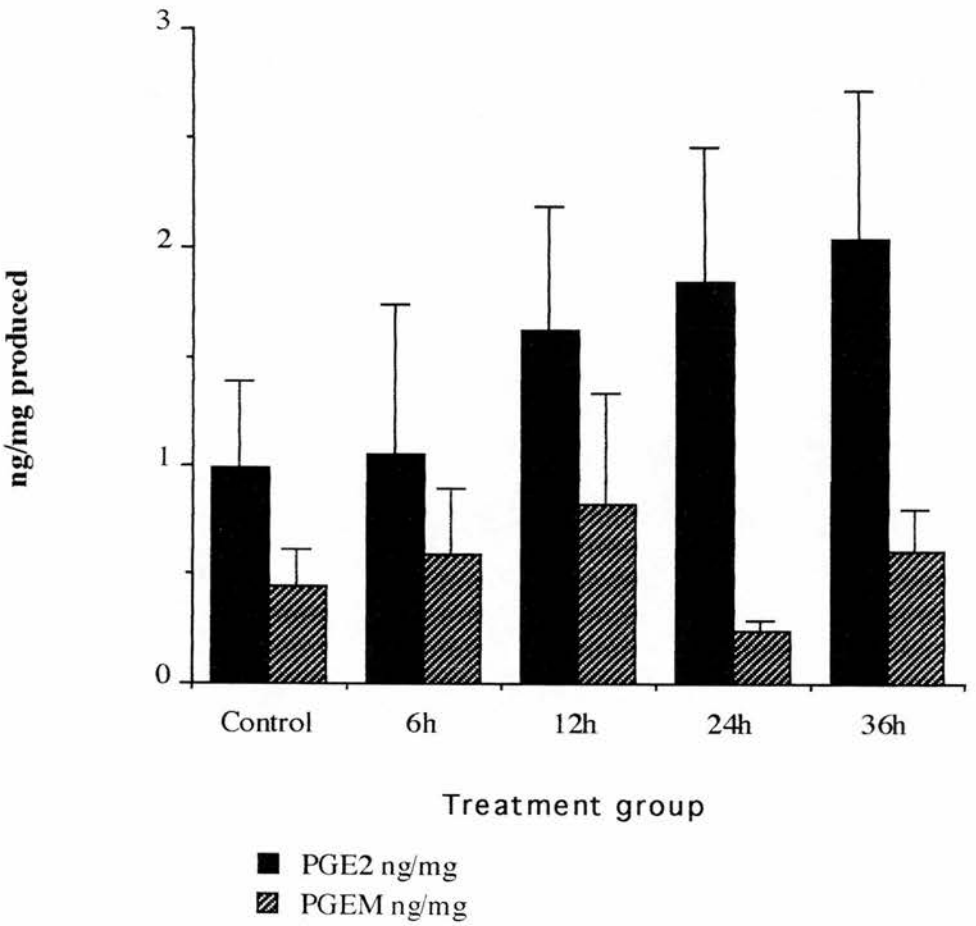
Graph 1:

Mean (± sem) production of IL-8 and MCP-1 (ng / mg) by cervical biopsies after pretreatment with mifepristone 6, 12 , 24 or 36 hours prior to biopsy as compared with control group. (n=6 for each treatment group).



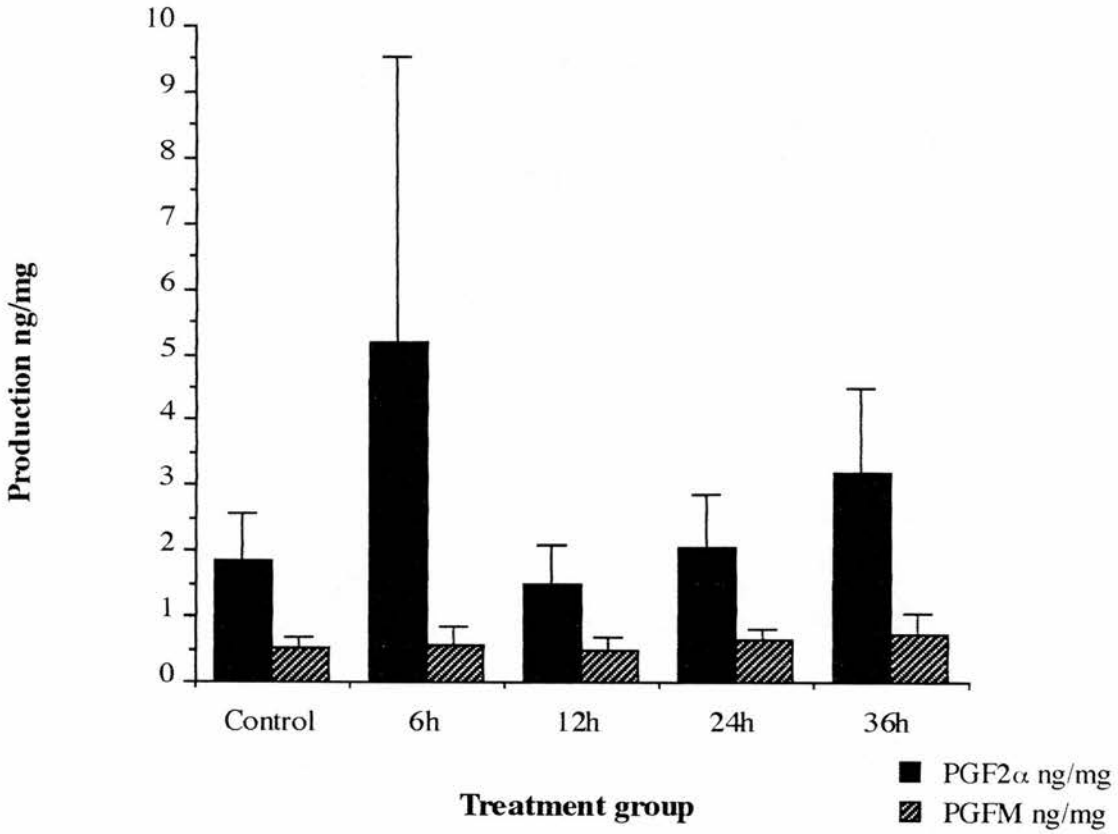
Graph 2:

Mean (±sem) production of PGE_2 and PGEM (ng / mg) by cervical biopsies after pretreatment with mifepristone 6, 12 , 24 or 36 hours prior to biopsy as compared with control group. (n=6 for each treatment group)



Graph 3:

Mean (± sem) production of $PGF_{2\alpha}$ and PGFM (ng / mg) by cervical biopsies after pretreatment with mifepristone 6, 12 , 24 or 36 hours prior to biopsy as compared with control group. (n=6 for each treatment group)



Chapter VIII

CONCLUSIONS AND DISCUSSION OF RESULTS OF CLINICAL AND LABORATORY STUDIES.

The process of parturition is evidently a complex procedure involving the interaction of many factors which appear to act in a cascade with the potential for amplification and dampening of the ensuing events. The mechanism by which this occurs must eventually lead to cervical effacement and dilatation and to the establishment of regular effective uterine contractions.

As discussed in the initial two chapters, this process seems likely to be regulated, at least in part, by the locally available concentration of progesterone. There is accumulating evidence of an inflammatory type cascade, in which increased production of mediators, such as the chemokines, IL-8 and MCP-1, and prostanoids, such as PGE_2 and $PGF_{2\alpha}$ interact to produce an influx of lymphocytes and neutrophils. The former cells can elaborate other chemokines, such as IL-1, IL-6 and TNF-α which are capable of perpetuating this cascade. Neutrophils, when activated, release collagenases and elastases, which as discussed above, are thought to be essential for the collagen dissociation associated with cervical ripening. The presence of this inflammatory environment may stimulate myometrial contractions which are favoured by an increase in the number of intercellular gap junctions, the presence of which enhances coupling between these cells.

In the studies described in the preceding chapters it has been attempted to further elucidate the mechanism of such a hypothesis. The role of progesterone in maintaining uterine quiescence has been examined by studying the effects of its antagonist mifepristone on the cervix in the first and third trimesters of human pregnancy. The capability of placental, decidual and cervical tissue explants to release IL-8 has also been studied and the effects of steroids on this production examined. The role of MCP-1 in promoting cervical ripening in the first trimester after mifepristone administration has also been assessed. In

the placental and decidual tissue studied the localisation of IL-8 has been determined immunohistochemically and the associated population of white cells has been broadly assessed.

Mifepristone has been shown to increase cervical ripening in primigravid women prior to labour induction. This effect was significant when a dose of 200mg was used and was just below significance when 50mg was used. The use of mifepristone prior to labour induction also decreased the subsequent requirement for oxytocin, as was reported in a previous study [334]. However, in our results a small increase in the number of Caesareans performed for fetal distress after the use of 200mg mifepristone as compared to placebo was found. While this may be a function of the relatively small numbers involved, it may also be a true effect and further studies of the use of mifepristone for this purpose must carefully evaluate this.

The positive effect of progesterone antagonism in promoting cervical ripening in the third trimester adds support to the proposition that it is a decrease in functional local progesterone that stimulates naturally occurring cervical ripening at term. It also seems that antagonism of progesterone may prime the myometrium to the effects of oxytocin, whether endogenous or exogenous. This may occur by an increase in the number of gap junctions present, as is seen in the guinea pig after onapristone administration[305]. In the first trimester administration of mifepristone prior to termination of pregnancy was found to cause a time-dependent increase in the production of MCP-1 by cervical explants. Withdrawal of progesterone may thus act to promote influx of monocytes which are capable of maintaining an inflammatory environment. If this effect is also seen in the third trimester the increase in chemokine concentration could provide an explanation for the

clinically confirmed promotion of cervical ripening observed following mifepristone administration.

These studies confirmed that the cervix in the first trimester is capable of synthesising prostanoids and the chemokines IL-8 and MCP-1. The placenta and decidua at term have also been shown to produce large amounts of IL-8 in culture. In the placental tissue studied, the production of IL-8 was significantly increased after labour as compared with the production by tissue obtained at elective Caesarean section. This increase is supportive of the hypothesis that parturition is associated with an increase in IL-8 which would be capable of attracting neutrophils into the stroma of the uterine tissues where release of their lytic enzymes could alter the tissue structures and favour parturition. The time course of this increase was not however determined and it remains possible that the increase in IL-8 production seen after labour is an effect, rather than a cause of parturition. The effects of dexamethasone, progesterone and antigestagens on IL-8 production were not consistently demonstrated in the above studies. This was, at least in part, due to the wide variations in IL-8 production by the explants. This was particularly seen in the decidual explants, in which the production of IL-8 varied widely between the four samples as did the change seen in this production on treatment with steroids. The decidual samples studied were all obtained at the time of elective Caesarean, but although all were obtained at similar gestations the actual proximity of the onset of parturition cannot be known and may have had a profound effect on the response of the tissue. Although the samples were washed it may be that variations in the amount of blood cells retained by the tissue altered their IL-8 production. Despite this, a trend was observed whereby IL-8 production was suppressed by dexamethasone and progesterone, the effect of the former being more pronounced and was stimulated by treatment with mifepristone. If these suppositions were to prove to be real effects this would be supportive of the role of progesterone

withdrawal and IL-8 production at the time of labour. However, such an inference cannot be drawn from these results.

In both the placenta and the decidua in the third trimester IL-8 was found to be localised around blood vessels. This is as been previously reported in the endometrium [148] and is in keeping with its putative role in neutrophil recruitment into these tissues. In neither tissue were significant quantities of cells found that stained positively with the neutrophil elastase protocol. This commercial antibody did stain the tonsil tissue used as a positive control. This may reflect a true lack of neutrophils in these tissues or it may be that the antibody did not detect neutrophils after activation and degranulation had occurred. It may be that the leucocytes present in uterine tissues are atypical and are not as easily detected with the standard techniques and markers used here. There are leucocytes, stained positive with CD-45, present in small concentrations in the placenta and in larger amounts in the decidual tissue. Cells staining with CD-68, a macrophage marker, were also found in the tissue stroma. It may be that these tissues contain other, more atypical leucocytes that would require more detailed study to localise.

It is therefore proposed that at the time of parturition the withdrawal of progesterone promotes a local increase in inflammatory mediators and cell types. The changes seen as the cervix ripens are associated with an influx of neutrophils which release lytic enzymes, such as collagenase, that cause loosening of the collagen stroma. IL-8, an inflammatory chemokine, is a potent attractor and activator of neutrophils and has been found to be released by the cervix, placenta and decidua. Its production could be increased by a cascade of effectors, in which monocytes, attracted into the cervical or uterine tissues by MCP-1, could elaborate other mediators, such as IL-1, IL-6 and TNF-α .

Further work is needed to test this hypothesis. The effects of mifepristone administration on third trimester uterine tissues could be studied, possibly by its administration prior to elective Caesarean sections. The effects of steroids on the production of IL-8 and other inflammatory mediators such as MCP-1 by uterine tissues also requires further study with large numbers of samples to decrease the effects of individual variation.

It will only be once the mechanisms by which labour occurs are more fully understood that more effective methods can be developed to induce labour in pregnancies that are post-term or in which continuation of the pregnancy poses a significant risk to the fetus or the mother. Many and various methods have been proposed to induce labour, with prostaglandins and oxytocin currently being the most effective options available. However, these methods are not always successful and can involve risks to the mother and fetus during their administration. It may be that administration of effectors proposed to be part of the inflammatory cascade prior to labour, such as IL-8 or MCP-1 could result in more efficient and successful induction of labour.

FUTURE WORK.

Based on the findings reported in this thesis there are several potential areas for further study. Further clinical studies of the use of mifepristone in the third trimester could be undertaken. It would be important that these assessed fetal well-being following treatment administration. Mifepristone may be of benefit in inducing labour in multiparous women and in higher doses than studied herein may be able to induce labour without the addition of prostaglandin pessaries.

The role of IL-8 in causing neutrophil influx into the cervix during parturition requires further study. In many tissue types IL-8 has been found to be regulated by dexamethasone. The potential regulation of IL-8 by steroid hormones, in particular progesterone could be examined in more detail in human uterine tissues. The molecular mechanisms whereby its production is regulated in these tissues also remains to be determined

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APPENDICES.

1. Reagents

   i: complete medium

   ii: IL8 non-stick buffer

   iii: Prostaglandin assay buffer

   iv: MOXB

2. Assay protocols

   i: BioRad protein assay

   ii: Radioimmunoassay protocols

3: Immunohistochemistry protocols

   i: IL8

   ii: CD45

   iii: CD68

   iv: neutrophil elastase

1. REAGENTS

i. **Complete medium:** RPMI 1640 supplemented with:

10% fetal calf serum

0.3 mg/ml L-glutamine

100IU/100μg penicillin and streptomycin

20μg/ml gentamicin

ii. **IL8 non-stick buffer:**

6.05g trizma base

2.5g bovine serum albumin (BSA)

10ml 10% tween 80

0.5g sodium azide

pH to 7.6

iii. Prostaglandin assay buffer:

1 sachet Sigma buffer powder

1g gelatin

1g sodium azide

pH to 7.2

iv. MOX B

164g anhydrous sodium acetate

20g methoxyamine hydrochloride

1200ml distilled water

200ml ethanol

2. ASSAY PROTOCOLS

i. "BioRad" protein assay.

After 48 hours of treatment the samples were washed twice with PBS and were then treated with 1ml of 1N NaOH overnight. The protein content of each was estimated using the BioRad assay according to the manufacturers instructions. This utilises a colorimetric assay to estimate the total protein concentration in tissue by comparison with a standard curve prepared from serial dilutions of bovine serum albumin. Following removal of the second application of culture medium, the placental explants were treated overnight with 1N NaOH. A standard curve was prepared from 1.4mg/ml bovine serum albumin by serial dilution 1:1 with 1N NaOH. 20μl of sample or standard had 100μl Biorad reagent added to it and colorimetry performed at 620nm. Results were again analysed using AssayZap (Biosoft, Cambridge, UK).

ii. Radioimmunoassay protocol.

Standards: Serially diluted in 0.5ml of buffer + tween

Prepare tubes as below:

	std/sample	buffer	antiserum	label	non-stick buffer
TOTAL				100μl	100μl
NSB		200μl		100μl	100μl
B_o		100μl	100μl	100μl	100μl
Standard	100μl		100μl	100μl	100μl
Sample	50μl	50μl	100μl	100μl	100μl

Cover tubes and incubate overnight.

Add 0.5ml of second antibody (anti-rabbit), this does not get added to the 'total count' tube

Leave for 15mins

Place on magnetic trays for 5mins

Tip excess of all tubes (except total count) and count using multigamma counter.

Radioimmunoassay for PGE and F

Samples were moxed immediately after collection using 1x Mox B solution (see above).

The standards are prepared using buffer with an equal percentage of mox to the samples.

3. IMMUNOHISTOCHEMICAL PROTOCOLS.

i. IL-8 Protocol:

(All steps at room temperature unless otherwise indicated)

1. Frozen sections of tissue and control tissue (tonsil) were fixed in 10% neutral buffered formalin (NBF). 10 minutes
2. Sections washed in phosphate-buffered saline 2 x 5 minutes
3. Endogenous peroxidase block with 3% hydrogen peroxide diluted in distilled water 10 minutes
4. Sections washed in phosphate-buffered saline 2 x 5 minutes
5. First non-immune block with normal swine serum (1:10 dilution) 20 minutes
6. Primary antibody application using rabbit anti-IL8 diluted 1: 500 in PBS or (as negative) rabbit IgG 1 : 500. Incubated at 37C. 60 minutes:
7. Sections washed in phosphate-buffered saline (tween) with the negative controls being washed separately. 2 x 5 minutes
8. Second non-immune block with normal swine serum (1:10 dilution) 20 minutes
9. Secondary antibody, biotinylated swine anti-rabbit 1: 300, applied. 30 minutes
10. Sections washed in phosphate-buffered saline (tween) 2 x 5 minutes
11. ABC complex applied 30 minutes
12. Sections washed in phosphate-buffered saline (tween) 2 x 5 minutes
13. DAB applied 8 minutes
14. Reaction stopped by immersing sections in tap water 5 minutes
15. Sections stained with haematoxylin 8 seconds:
16. Excess washed off in tap water 5 minutes
17. Dehydration by sequential immersion in 70%, 90%, absolute alcohol 2minutes each
18. Section immersed in xylene for at least 5 minutes and then mounted.

ii. CD45 Protocol:

(All steps at room temperature unless otherwise indicated)

1. Sections dewaxed in Histoclear	5 minutes
2. Sections rehydrated with absolute, then 90%, then 70% alcohol	2minutes each
3. Wash with distilled water	2 x 5 minutes
4. Wash with PBS	2 x 5 minutes
5. Endogenous peroxidase block with 3% hydrogen peroxide diluted in distilled water	10 minutes
6. Sections washed in phosphate-buffered saline	2 x 5 minutes
7. First non-immune block with normal horse serum (1:10 dilution)	20 minutes
8. Primary antibody application using mouse anti CD45-RB diluted 1: 50 in PBS or (as negative) mouse IgG 1 : 50. Incubated at 37C	60 minutes
9. Sections washed in phosphate-buffered saline (tween) with the negative controls being washed separately.	2 x 5 minutes
10. Secondary antibody, biotinylated horse anti-mouse applied.	30 minutes
10. Sections washed in phosphate-buffered saline (tween)	2 x 5 minutes
11. ABC complex applied	30 minutes
12. Sections washed in phosphate-buffered saline (tween)	2 x 5 minutes
13. DAB applied	8 minutes
14. Reaction stopped by immersing sections in tap water	5 minutes
15. Sections stained with haematoxylin	8 seconds:
16. Excess washed off in tap water	5 minutes
17. Dehydration by sequential immersion in 70%, 90%, absolute alcohol	2minutes each

18. Section immersed in xylene for at least 5 minutes and then mounted.

iii. CD68 Protocol:

(All steps at room temperature unless otherwise indicated)

1. Sections dewaxed in Histoclear	5 minutes
2. Sections rehydrated with absolute, then 90%, then 70% alcohol	2minutes each
3. Wash with distilled water	2 x 5 minutes
4. Wash with PBS	2 x 5 minutes
5. Endogenous peroxidase block with 3% hydrogen peroxide diluted in distilled water	10 minutes
6. Sections washed in phosphate-buffered saline	2 x 5 minutes
7. Trypsin digestion using 0.1% trypsin in 0.1% Ca Cl_2 in distilled water at pH 7.8. Incubated at 37C.	15 minutes
8. Wash with tap water	2 x 5 minutes
9. Wash with PBS	2 x 5 minutes
10. First non-immune block with normal horse serum (1:10 dilution)	20 minutes
11. Primary antibody application using mouse anti CD68 diluted 1: 50 in PBS or (as negative) mouse IgG 1 : 50.	60 minutes
12. Sections washed in phosphate-buffered saline (tween) with the negative controls being washed separately.	2 x 5 minutes
13. Secondary antibody, biotinylated horse anti-mouse applied.	30 minutes
14. Sections washed in phosphate-buffered saline (tween)	2 x 5 minutes
15. ABC complex applied	30 minutes
16. Sections washed in phosphate-buffered saline (tween)	2 x 5 minutes
17. DAB applied	8 minutes
18. Reaction stopped by immersing sections in tap water	5 minutes
19. Sections stained with haematoxylin	8 seconds:
20. Excess washed off in tap water	5 minutes

21. Dehydration by sequential immersion in 70%, 90%, absolute alcohol 2minutes each

22. Section immersed in xylene for at least 5 minutes and then mounted.

iv. Neutrophil elastase protocol:

(All steps at room temperature unless otherwise indicated)

1. Sections dewaxed in Histoclear 5 minutes

2. Sections rehydrated with absolute, then 90%, then 70% alcohol 2minutes each

3. Wash with distilled water 2 x 5 minutes

4. Wash with PBS 2 x 5 minutes

5. Endogenous peroxidase block with 3% hydrogen peroxide diluted in distilled water 10 minutes

6. Sections washed in phosphate-buffered saline 2 x 5 minutes

7. First non-immune block with normal horse serum (1:10 dilution) 20 minutes

8. Primary antibody application using mouse anti α - neutrophil elastase diluted 1: 50 in PBS or (as negative) mouse IgG 1 : 50. Incubated at 37C 60 minutes

9. Wash in PBS (tween) negative controls being washed separately. 2 x 5 minutes

10. Secondary antibody, biotinylated horse anti-mouse, applied. 30 minutes

10. Sections washed in phosphate-buffered saline (tween) 2 x 5 minutes

11. ABC complex applied 30 minutes

12. Sections washed in phosphate-buffered saline (tween) 2 x 5 minutes

13. DAB applied 8 minutes

14. Reaction stopped by immersing sections in tap water 5 minutes

15. Sections stained with haematoxylin 8 seconds:

16. Excess washed off in tap water 5 minutes

17. Dehydration by sequential immersion in 70%, 90%, absolute alcohol 2minutes each

18. Section immersed in xylene for at least 5 minutes and then mounted.

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Regulation of interleukin 8 production in the term human placenta during labor and by antigestagens

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OBJECTIVE: Our purpose was to assess the effects of labor and antigestagens on production of interleukin 8 by the term human placenta and to localize interleukin 8 in first- and third- trimester placentas.

STUDY DESIGN: The study was conducted by the Department of Obstetrics and Gynaecology of the University of Edinburgh. Five placentas were collected after spontaneous and cesarean deliveries. Explants were cultured in the presence of mifepristone, lilopristone, or onapristone. The production of interleukin 8 was determined by specific radioimmunoassay, and the immunolocalization of interleukin 8 was determined in sections of first- and third-trimester placentas.

RESULTS: All explants produced interleukin 8. Production was significantly increased ($P < .05$) after spontaneous delivery. In placentas delivered spontaneously, onapristone significantly increased production of interleukin 8 ($P < .05$), whereas in those from cesarean deliveries lilopristone caused a significant increase in production ($P < .05$). In the third-trimester placenta interleukin 8 was localized in the perivascular area of fetal vessels. In first-trimester villi it was peripherally located in syncytiotrophoblast.

CONCLUSION: The human placenta at term is capable of producing interleukin 8, which is localized around the perivascular area of the villi. Production is increased after spontaneous labor and to varying degrees by the antigestagens studied. Interleukin 8 may have a role in the onset of parturition by recruiting and activating neutrophils at the placental site. (Am J Obstet Gynecol 1998;179:215-20.)

Key words: Interleukin 8, placenta, labor, cytokines

Interleukin 8 (IL-8) was first described[1,2] as a peptide released by stimulated monocyte cultures and was shown both to attract and activate neutrophils.[3] It is a 72-amino-acid chemokine that has subsequently been demonstrated to be released by many cell types, including fibroblasts,[4] macrophages, endothelial cells, and even phagocytosing neutrophils.[5] IL-8 is also present in and released by many uterine tissues, including placenta,[6] chorion-decidua,[7] and cervix.[8] The influx of neutrophils has been postulated to be an integral part of the onset of parturition, particularly in the cervix, where the collagenase involved in ripening appears to be derived from peripheral neutrophils that increase in number in the cervix during this process.[9-11] The presence of IL-8 in these diverse reproductive tissues and its known synergism with prostaglandin E_2 (PGE_2) in attracting neutrophils to skin sites[12,13] suggests a wider role for IL-8 in the initiation of labor than in cervical ripening alone. Furthermore, IL-8 production in other uterine tissues, such as cervix,[8] chorion-decidua,[7] and endometrium[14] is suppressed by progesterone. This effect may be reversible by antigestagens, such as mifepristone,[7] which may have an inducing effect on the initiation of labor.

The aim of this study was to examine the release of IL-8 by term human placenta before and after labor had ensued. The regulation of IL-8 production by three antigestagens was studied to determine a possible regulatory role in the induction of the labor process. Furthermore, the localization of IL-8 in the first- and third-trimester placenta was determined by immunohistochemical techniques.

Material and methods

Placental explants. Placentas were collected from women at elective cesarean section or after spontaneous delivery at term (37 to 41 weeks' gestation, $n = 5$ in each group). No woman had been exposed to prostaglandins or oxytocic agents before delivery, although all patients routinely received 10 IU oxytocin (Syntocinon, Sandoz) at cesarean delivery or 5 IU of oxytocin and 500 μg of ergometrine (Syntometrine, Sandoz) at vaginal delivery before the delivery of the placenta. Immediately after delivery, a piece of villous tissue approximately 1 to 2 cm in diameter was dissected from each placenta from a

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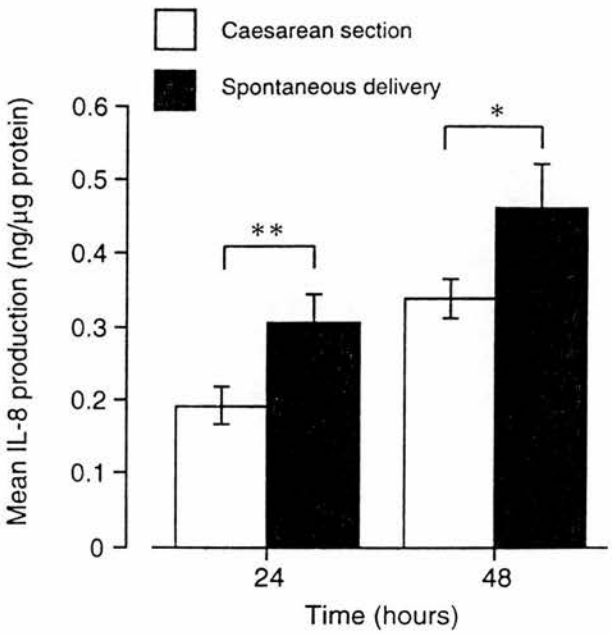

Fig 1. IL-8 production (mean ± SEM) by explants of placental villous tissue obtained after spontaneous *(solid bars)* and elective cesarean *(open bars)* delivery and maintained in tissue culture for 24 and 48 hours. Statistical significance is denoted by *symbols*: *Two asterisks*, $P < .005$; *asterisk*, $P < .05$.

central cotyledon, avoiding any obvious large-stem blood vessels or areas of calcification; the tissue was transported to the laboratory in sterile 0.9% saline solution.

Under sterile conditions the basal plate of the placenta was dissected off and large vessels were removed. Pieces of placenta measuring approximately 5 mm^3 were cut from the remaining tissue and washed 3 times in phosphate-buffered saline solution (Dulbecco's medium) to remove maternal and fetal blood cells. One piece was placed in each well of a 24-well plate on sterile capillary matting (CellGraft) containing 1 mL of culture medium (RPMI 1640 with fetal calf serum, L-glutamine, penicillin, streptomycin, and gentamicin). Forty-eight explants were prepared in this manner from each placenta; these were divided into 4 treatment groups, each comprising 12 replicates. The 4 treatments used were control (ie, no antigestagen added to the culture medium) and the antigestagens mifepristone (RU486) (10^{-6} mol/L), onapristone (10^{-6} mol/L), and lilopristone (10^{-6} mol/L). The plates were incubated at 37°C in 5% carbon dioxide in humidified air. After 24 hours, the medium was collected, replaced with the same treatment, and collected again after a further 24 hours. This medium was stored at −20°C until the assay for IL-8 was performed.

Immunoassay for IL-8. A rabbit antibody, which was raised against the intact 72-amino-acid IL-8 peptide synthesized by use of fluorenyl methoxy carbonyl chemistry, was used in a radioimmunoassay as previously described.[14] Recombinant IL-8 was used to produce data for the standard curves. The assay was performed overnight with the addition of a buffer containing serum albumin and Tween to minimize nonspecific binding. The counts were analyzed with the AssayZap program (Biosoft, Cambridge, United Kingdom).

Estimation of protein content. After 48 hours of treatment the samples were washed twice with PBS and were then treated with 1 mL of 1*N* sodium hydroxide overnight. The protein content of each was estimated with the BioRad assay according to the manufacturer's instructions. This method uses a colorimetric assay to estimate the total protein concentration in tissue by comparison with a standard curve prepared from serial dilutions of bovine serum albumin. After removal of the second application of culture medium, the placental explants were treated overnight with 1*N* NaOH. A standard curve was prepared from 1.4 mg/mL bovine serum albumin by serial dilution 1:1 with 1*N* sodium hydroxide. Next 100 µL Biorad reagent was added to 20 µL of sample or standard and colorimetry performed at 620 nm. Results were analyzed with AssayZap.

Data analysis. The IL-8 production was expressed as nanograms of IL-8 per microgram of protein. The results obtained were tested for significance using a parametric test, Fisher's protected least significant difference (StatView), and significance at the level of $P < .05$ was determined.

Immunocytochemistry. Tissue samples of placentas were collected after elective cesarean section at term (37 to 39 weeks' gestation) from singleton pregnancies uncomplicated by diabetes or hypertensive disorders. Sections from each placenta, including the basal plate, were dissected, immediately embedded in OCT (Miles Inc, Elkhart, Ind) and placed in an isopentone tray that was surrounded by liquid nitrogen. The frozen OCT block was then stored at −70°C before it was cut into approximately 5-µm sections with a microtome. First-trimester villi were collected from 4 women during surgical termination of pregnancy and processed in the same manner.

The method used for immunocytochemistry is as previously described by Critchley et al.[15] Frozen sections of tissue were fixed in 10% neutral buffered formalin for 10 minutes and then washed in phosphate-buffered saline solution before staining. The primary antibody was a polyclonal rabbit antibody as described for the immunoassay. This primary antibody was preabsorbed with synthetic IL-8 peptide (100 µg/mL) and this and rabbit IgG were used as negative antibody controls. Sections of tonsil were used as positive tissue controls, as in the above-mentioned study.[15]

Endogenous peroxidase activity was blocked with 3% hydrogen peroxide diluted in distilled water. The sections were then washed in phosphate-buffered saline

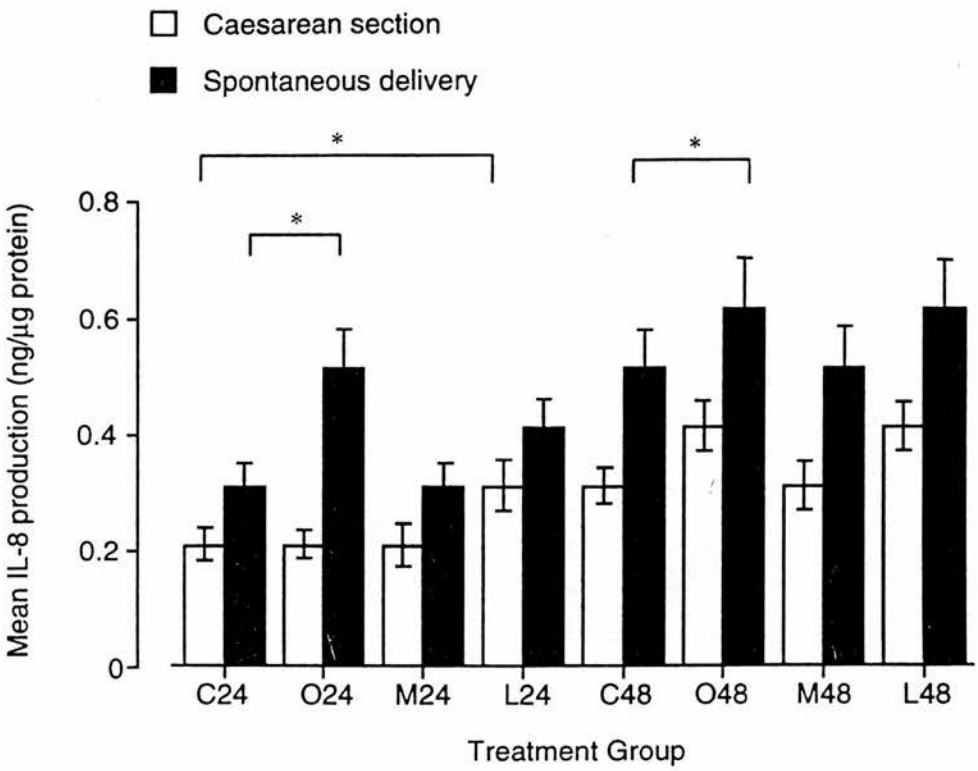

Fig 2. IL-8 production (mean ± SEM) by explants of placental villous tissue obtained after spontaneous *(solid bars)* and elective cesarean *(open bars)* delivery, untreated (control medium, *C*) or treated with onapristone (*O*, 1 µmol/L), mifepristone (*M*, 1 µmol/L), or lilopristone (*L*, 1 µmol/L) for 24 and 48 hours in culture.

solution, after which the first nonimmune block (normal swine serum diluted 1:10) was applied for 20 minutes at room temperature. Without washing, the primary antibody was then applied at a 1:500 dilution to the tested sections and the negative antibody control was applied at the same dilution to the negative controls. All sections were incubated at 37°C for 60 minutes. After a further wash in phosphate-buffered saline solution (the negative controls were washed separately to avoid contamination with the primary antibody), a second nonimmune block was applied in the same manner as the first followed by application of the secondary bridging antibody (biotinylated swine α-rabbit 1:300, DAKO, Glostrup, Denmark). The third stage in the staining used an avidin-biotin-complex peroxidase (Vectastain ABC kit, Vector Laboratories, Peterborough, United Kingdom) prepared according to the manufacturer's instructions with 3,3′-diaminobenzidine tetrahydrochloride as the chromagen, giving a brown coloration. The sections were then counterstained with Harris' hematoxylin, dehydrated in ascending alcohol concentrations, cleared with xylene, mounted, and examined by light microscopy.

Results

Production of interleukin 8 by placenta. All explants of placental tissue released IL-8 into the culture medium, irrespective of the presence or absence of labor or treatment with antigestagens (Fig 1).

The culture medium was replaced after 24 hours and IL-8 release increased in all samples in the second 24 hours of culture. This increase was significant ($P < .05$) for the control samples from both elective cesarean and spontaneous deliveries and for the cesarean samples treated with onapristone and the spontaneously delivered placentas that were treated with mifepristone or with lilopristone.

After spontaneous delivery, for each treatment group and at each time point, all placental explants produced more IL-8 than the explants collected from women not in labor. As illustrated for the control groups in Fig 1, this was a significant increase ($P < .05$) at both time points.

The effects of three antigestagens—onapristone, mifepristone and lilopristone—on IL-8 production in both types of placenta were also examined and are shown in Fig 2. In the spontaneously delivery group those treated with onapristone produced significantly more IL-8 than the control samples at 24 and 48 hours, whereas in the elective delivery group lilopristone at 24 hours caused a significant increase in production over control samples ($P < .05$ in each case).

Localization of interleukin 8 in placenta. In the third-trimester villi IL-8-positive immunostaining was found to

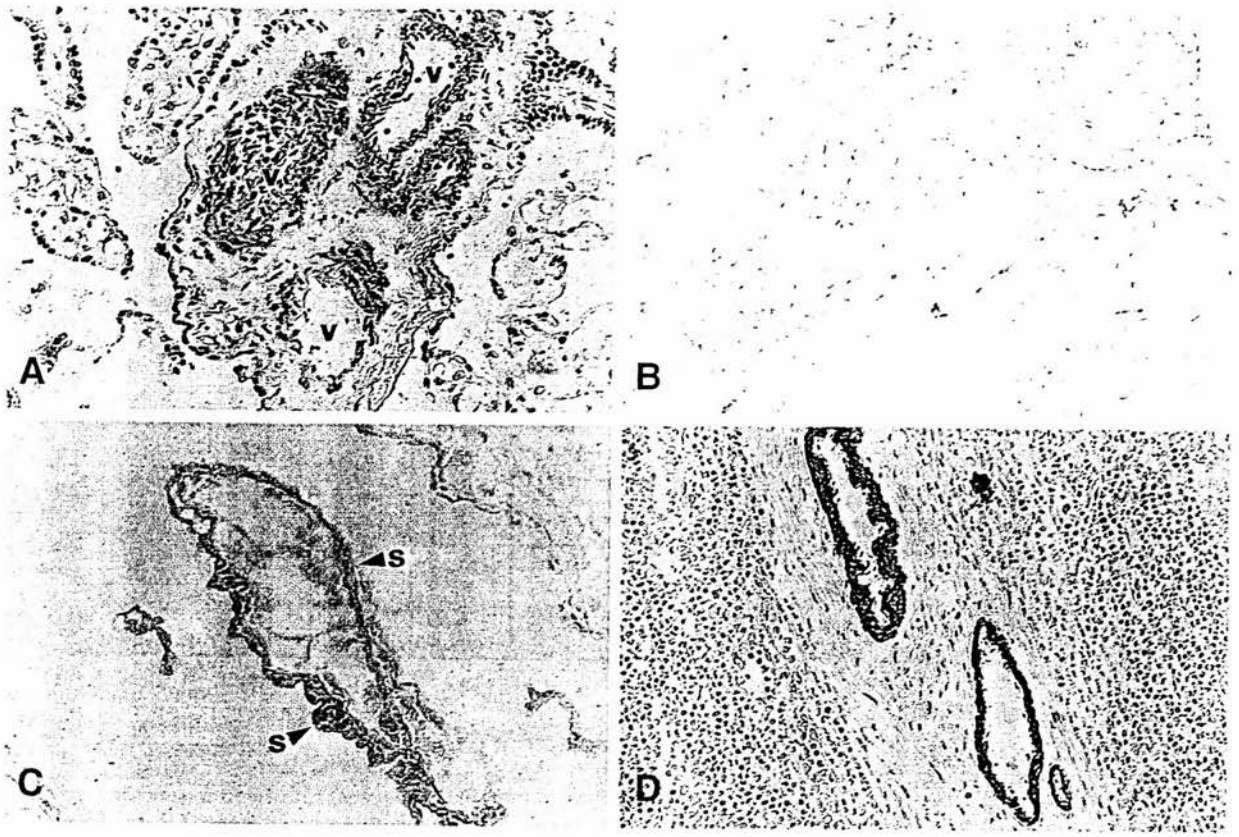

Fig 3. IL-8 immunolocalization. **A**, Third-trimester placenta after spontaneous delivery showing positive IL-8 immunostaining around larger stem vessels of fetal vasculature (*V*); **B**, third-trimester placenta after spontaneous delivery—negative control showing no specific immunostaining in stem vessels; **C**, first-trimester placenta showing positive IL-8 staining in syncytiotrophoblast (*S*); **D**, tonsil, positive tissue control. (Original magnifications ×20.)

be localized predominantly in the perivascular walls of larger (stem) fetal vessels as shown in Fig 3, *A*, with little positive IL-8 immunoreactivity in capillaries or other villous core tissue or trophoblast. A negative control section of third-trimester placenta demonstrating no specific IL-8 immunoreactivity in larger fetal vessels or other villous tissues is shown in Fig 3, *B*. In first-trimester placentas, IL-8 was localized predominantly in the developing syncytiotrophoblast of placental villi (Fig 3, *C*). Tonsil was used as a tissue control for IL-8 immunostaining, with IL-8 localized to the areas around blood vessels (Fig 3, *D*). No staining was demonstrated in any negative control sections of placenta or tonsil (Fig 3, *B*).

Comment

These studies demonstrate that the human placenta produces IL-8 and that this production is increased during labor and also by culture with antigestagens.

An increase in IL-8 before the onset of spontaneous labor could facilitate recruitment of neutrophils into the uterine environment. It has been shown that in skin sites IL-8 and PGE_2 act synergistically to increase the influx of neutrophils,[12,13] and it may be that such synergism extends to the reproductive tissues where at the time of parturition there is an increase in local levels of prostaglandins that have an established role in the initiation and progression of labor. Once recruited into the uteroplacental tissue, neutrophils could be activated by IL-8 causing release of lytic enzymes, such as collagenase and elastase, which may cause loosening of the tissue matrix in the cervix,[9,11] chorion-decidua,[16] and fetal membranes.[17] IL-8 may therefore be part of a cascade of inflammatory agents, including other cytokines and prostaglandins involved in the initiation of parturition. However, it is also possible that the increase in IL-8 production seen after vaginal delivery is an effect and not a cause of labor. It may be that the mechanical stress of contractions leads to enhancement of the capability of the placental tissue to produce IL-8. More detailed study of the time course of IL-8 production during labor would be required to

further clarify its role in parturition. However, the placenta cannot be directly sampled during spontaneous labor in women and although tissue can be obtained at emergency cesarean delivery performed during labor, these cases may differ inherently from those resulting in vaginal delivery.

There were no other obviously significant differences in the placentas to account for the difference in production after labor, as the elective deliveries were all at term and were not performed for placental insufficiency, growth retardation, or metabolic abnormalities. The spontaneously delivered placentas resulted from labors that were not induced or augmented with prostaglandins or oxytocics. All women whose infants are delivered vaginally in our unit routinely receive oxytocin and ergometrine after delivery of the fetus; at cesarean oxytocin alone is given. It seems unlikely that ergometrine would have a significant effect on IL-8 production.

It has previously been shown that IL-8 production is down-regulated by both progesterone[8,14] and dexamethasone.[18] The addition of antigestagen to the culture medium increased IL-8 production to a significant degree in the spontaneous delivery placentas treated with onapristone and in the cesarean delivery placentas treated with lilopristone for 24 hours. Currently antigestagens are being studied in clinical trials for cervical ripening and induction of labor in the third trimester.[19] The local antagonism of progesterone in uterine tissues could increase IL-8 levels permitting recruitment of neutrophils and also lead to increased local levels of PGE_2.[20] In first-trimester decidua, mifepristone treatment increases perivascular PGE_2 levels and decreases its metabolism.[21,22] A parallel increase in IL-8 and PGE_2 levels in placenta at term would enhance the synergistic effects of these two agents on neutrophil recruitment. A significant increase in IL-8 production after all the antigestagen treatments may not have been demonstrated in this study as the amount of antigestagen used may not have been sufficient to antagonize the large endogenous progesterone production of the placenta. It could also be that particularly after spontaneous delivery the response of the placental cells to exogenous agents has altered.

In summary, these studies demonstrate that the term placenta constitutively produces IL-8, which is localized principally around the perivascular area of the villi. This production of IL-8 is increased after spontaneous labor, increases with culture duration, and is stimulated to varying degrees by some antigestagens. IL-8 may have a role in the onset of parturition by recruiting and activating neutrophils at the placental site facilitating release of lytic enzymes; hence antigestagens may aid the induction of labor by increasing the influx of neutrophils into the placenta. Further work is now under way to establish the controlling mechanisms involved in the stimulation of IL-8 during labor and the potential role of antigestagens in regulating IL-8 production and the timing of the onset of birth.

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