An Investigation of Antenatal Infection & Inflammation and their contribution to Preterm Delivery & Lung Disease of Prematurity

Thesis submitted for the degree of
Doctor of Medicine
at the University of Leicester

by

Robin Miralles
Clinical Research Fellow,
University of Leicester.

August 2006

Supervisor:
Professor Sailesh Kotecha PhD.
Professor of Child Health,
University of Cardiff.
formerly Senior Lecturer,
University of Leicester.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To my parents, who spent a significant amount of time in hospital during this period of research, and to my children Aoife, Úna and Brendan.

Abstract

Antenatal infection and inflammation may have a number of effects on the fetus. The same infection and inflammation that initiates a spontaneous preterm delivery may damage the fetal lung and lead to white matter brain injury. Despite the evidence linking intrauterine infection (IUI) to preterm labour and chronic lung disease, antibiotics have failed to have much impact on these clinical problems.

Polymerase chain reaction (PCR) -based techniques of microbial detection have a greater sensitivity than culture methods without sacrificing specificity. Discrepancies in the diagnosis of IUI may have affected the accuracy of previous studies. In this thesis a methodology for the molecular detection of IUI and inflammation was successfully developed without the need for amniocentesis.

PCR-based microbial detection was applied to a range of samples harvested from preterm deliveries. Inflammation was detected by histological examination and by measuring fluid and tissue cytokines. Clinical effects on the cohort of infants were also assessed.

The majority of spontaneous singleton preterm deliveries were associated with the detection of microbial genes by PCR. These genes were still detectable even after maternal antibiotic treatment. The findings of this work support the contention that high levels of intrauterine inflammation usually result from IUI, a matter which has been the subject of debate. Gastric fluid appeared to be a potentially useful sample for the microbial detection in future studies.

A univariate analysis of the clinical data demonstrated a reduction in radiological respiratory distress syndrome following fetal exposure to IUI, as well as increase in intraventricular haemorrhage. Both fetal inflammation and pulmonary inflammation were associated with fetal exposure to IUI. A strong fetal cortisol response correlated with fetal inflammation. Mechanisms by which neonatal lung disease may be modified by IUI may include a direct effect of pro-inflammatory cytokines or an increase in fetal cortisol.

Layout of the thesis

An introduction to the relevant topics is given in the first chapter. These topics include the consequences of preterm delivery and evidence for the proposed effects of intrauterine infection and inflammation. To avoid repetition in subsequent chapters, the methods and principles of the techniques used are explained in the second chapter, with section listings in the subsequent chapters. The results are presented in Chapters 3 to 9 accompanied by a brief introduction and discussion. An overview and discussion of the main results is presented in the final chapter.

Literature review tables are presented at the back of the thesis. Other tables accompany the main text.

Contents

Chapter 1: Introduction

Chapter 5: The measurement of fetal systemic inflammation

Chapter 9: Antenatal infection and pulmonary inflammation

Chapter 10: Discussion

Figures

Tables

Appendices

Chapter 1: Introduction

1.1 Intrauterine infection and Premature Delivery

1.1.1. The consequences of premature delivery

Premature delivery is defined as delivery prior to 37 weeks gestation, and is the single most important cause of perinatal mortality and morbidity. Over the last 20-30 years preterm birth has overtaken congenital malformation as the leading cause of death and handicap in industrialised nations (Lamont *et al.,* 2003). There are an estimated 13 million preterm births worldwide each year. In Europe 5-8% of births are preterm although half of these are beyond 35 weeks gestation (Friese *et al.,* 2003). It is known that there is a wide range of geographical variation in rates of premature delivery. In the USA rates are as high as 12% (Andrews *et al.,* 2000). Even within a smaller geographical area such as the Trent region of the UK there are variations in preterm delivery rates (Field 2000).

Recent years have seen a number of improvements in neonatal intensive care such as antenatal steroids, exogenous surfactant therapy and newer techniques of mechanical ventilation. As a result of such innovations, almost all infants at greater than 32 weeks gestation and around 80% of infants 500-1000g survive (Andrews *et al.,* 2000).

Mortality and morbidity are concentrated in the more immature infants (Draper *et al.,* 1999, Manktelow *et al.,* 2001). The immaturity of organ systems at this gestation can lead to broad spectrum of complications (Ward *et al.,* 2003). The pathologies that are most likely to have an impact on mortality and future quality of life include chronic lung disease of prematurity (CLD) and neurological injury. Neurodevelopmental outcome of these infants is a major concern as disability is common in extremely premature infants (Victorian Collaborative study 1997, Tin *et al.,* 1997, Wood *et al.,* 2000). Such infants are also at increased risk of school difficulties including behavioural problems (Pharoah *et al.,* 1994, Botting *et al.,*1997), fine motor difficulties (Elliman *et al.,*1991, Powls *et al.,*1995) and educational difficulties (Hack *et al.,* 1994, Botting *et al.,*1998).

Other consequences of prematurity which can affect long-term outcome are retinopathy of prematurity, an important cause of visual difficulties in the preterm infant, and a much increased incidence of necrotising enterocolitis, an inflammatory condition of

the gastrointestinal tract which can be associated with severe systemic illness and death (Ward *et al.,* 2003).

Neonatal intensive care is expensive particularly for the extremely premature infants. At the time of writing, to keep a baby in a neonatal unit for a week costs around 10,000 US dollars in the US and around £7000 in the UK. German hospital expenditure on preterm deliveries is estimated at 300 to 400 million euros annually (Friese *et al.,* 2003). These costs do not include the subsequent management of neonatal morbidity and do not take account of the psychological effects on the families which are much harder to quantify.

1.1.2. Categories of preterm delivery

Approximately one third of preterm deliveries present with prelabour premature rupture of membranes (pPROM: where rupture of membranes precedes the onset of contractions by a significant period) and one third with preterm labour with intact membranes (Romero *et al.,* 2002). These are grouped together as spontaneous preterm births. The remaining third of preterm deliveries are indicated deliveries i.e. deliveries by caesarean section for maternal or fetal compromise (e.g. due to pre-eclampsia or placental insufficiency).

Further complications may arise if preterm labour is associated with pPROM. Particularly severe consequences occur with earlier rupture of membranes particularly if this occurs before 25 weeks gestation. The associated oligohydramnios may result in limb contractures and pulmonary hypoplasia. It is the pulmonary hypoplasia and associated abnormal pulmonary blood vessel development that may have the most significant effect on mortality. Lack of amniotic fluid also means that cord compression necessitating emergency delivery occurs more frequently with pPROM (Kilbride *et al.,* 2001).

1.1.3. The association between intrauterine infection and spontaneous preterm births

Intrauterine infection (IUI) is now established as a cause of spontaneous preterm births (Goldenberg *et al.,* 2000). In 1977, Bobitt *et al.* found that bacterial organisms could be cultured from amniotic fluid from 7 out of 10 women in preterm labour (Bobitt *et al.,* 1977). Further studies have also demonstrated a relationship between preterm delivery and positive amniotic fluid cultures (Miller *et al.,* 1980, Bobbit *et al.,* 1981, Gravett *et al.,* 1986). For most women in preterm labour the IUI is clinically silent, so the study of such infection has depended on the results of laboratory culture. Gonçalves *et al.* reviewed studies which had looked at the microbiology of preterm labour with and without intact membranes. The mean rate of positive cultures in pPROM was 32.4% (473/1462 in 18

studies) and 12.8% (379/2963 in 33 studies) in preterm labour with intact membranes (Gonçalves *et al.,* 2002). In one report, IUI was associated with up to 40% of spontaneous preterm deliveries (Lettieri *et al.,* 1993). The lower the gestational age, the more likely the frequency of IUI (Watts *et al.,* 1992). It is thought that organisms invade the upper genital tract or amniotic cavity by ascending from the lower vaginal tract (Goldenberg *et al.,* 2000).

Upper genital tract infection (i.e. intrauterine infection) can occur in a number of locations, the choriodecidual space (lying between maternal and fetal tissues), the fetal membranes (the amnion and the chorion) within the placenta, within the amniotic fluid and the umbilical cord and fetus (Goldenberg *et al.,* 2000).

Most bacteria found in the uterus in association with preterm labour are vaginal organisms of usually low virulence (Gibbs *et al.,* 1992, Carroll *et al.,* 1996, Goldenberg *et al.,* 2000). The commonest organisms isolated from women in preterm labour with intact membranes are *Ureaplasma urealyticum* (*Uu*), *Mycoplasma hominis*, *Gardnerella vaginalis*, *Peptostreptococci* species and *Bacteroides* species. Organisms such as group B *streptococci* and *E. coli* are more often found after membrane rupture has occurred.

1.1.4. The association between intrauterine inflammation and spontaneous preterm birth

A relationship between preterm delivery and intrauterine inflammation has also been demonstrated (Hillier *et al.,* 1988). Histological examination of the fetal membranes has demonstrated that inflammation (termed histological chorioamnionitis) is often present after spontaneous preterm birth before 30 weeks. More recently, elevated concentrations of pro-inflammatory amniotic fluid cytokines, including interleukin-6 (IL-6) and interleukin-8 (IL-8), have been found in association with preterm labour, confirming the association of preterm labour with intrauterine inflammation. The advantage of the measurement of cytokines in preterm labour is that this measurement is available before delivery while histological examination of the placenta can only take place after delivery. In addition it is thought the pro-inflammatory cytokines have a central part to play in the pathway that leads from intrauterine infection to preterm labour. The increased concentrations of such cytokines can stimulate prostaglandin synthesis leading to preterm uterine contractions. In addition they attract neutrophils which can infiltrate the decidua and chorioamnion. Activation of the neutrophils leads to release of protease enzymes, including the metalloproteases, which contribute to weakening of the fetal membrane and cervical ripening. In this way intrauterine inflammation can trigger three main components involved

in preterm labour, uterine contractions, cervical ripening together with membrane weakening and rupture (Goldenberg *et al.,* 2000).

1.1.5. Animal studies and preterm birth

A number of animal studies have confirmed that IUI and inflammation are not just associated with preterm labour, but actually cause preterm labour. Bacterial products and cytokines have been administered to pregnant animals and have provoked preterm parturition. (Dombroski *et al.,* 1990, Romero *et al.,* 1991, Hirsch *et al.,* 1995, Gravett *et al.,* 1996, Fidel *et al.,* 1998).

1.1.6. Antibiotics in the prevention of spontaneous preterm births

Given that a significant number of cases of preterm labour are associated with IUI, attempts have been made to prevent preterm labour with antibiotics. In the 1970s it was reported that a prolonged course of tetracycline reduced the frequency of preterm deliveries (Elder *et al.,* 1971). However, this treatment never became established due to the potential side effects on the fetus and newborn infants (tooth and bone dysplasias).

Ureaplasma urealyticum (*Uu*) is the most commonly isolated organisms in amniotic fluid from women in preterm labour. Because mycoplasmas lack cell walls, they are resistant to beta-lactam antibiotics (Wang *et al.*, 1997). Therefore some trials have included the bacteriostatic macrolide antibiotic, erythromycin.

The results of the treatment of preterm labour with erythromycin have been mixed. The largest randomised multicentre trials using erythromycin have been the ORACLE trials, the findings of which have superseded those of previous smaller trials. In the ORACLE I trial, women with pPROM were treated with antibiotics (oral erythromycin or co-amoxiclav or both) or placebo. A composite neonatal outcome (neonatal death, CLD or major cerebral abnormality) was reduced in singleton infants born to mothers who received erythromycin ($p=0.02$) (Kenyon *et al.*, 2001a) but not in twins. In the ORACLE II trial the study group consisted of mothers with suspected preterm labour but with intact fetal membranes. There were no significant benefits following treatment with antibiotics in this group (Kenyon *et al.*, 2001b).

The trials to date have not shown a consistent benefit in preterm spontaneous births. It should also be noted that in two previous randomised controlled trials of antibiotic treatment, erythromycin failed to clear *Uu* from the genital tract (Wang *et al.*, 1997). Other trials have used antibiotics such as metronidazole to target the organisms of bacterial

vaginosis. It has been suggested that alternative antibiotic regimes may be more effective clinically, particularly if used in combination with initial intravenous antibiotics (Ehrenberg *et al.*, 2001). IUI may have been present for some time before preterm labour is established. It may therefore be difficult to alter the course of preterm labour once established, as irreversible changes may have already occurred in the uterine and cervical tissues.

1.1.7. Second trimester amniocentesis: chronic fetal exposure to inflammation

Analysis of cytokine levels in amniotic fluid obtained from genetic amniocentesis in the second trimester has demonstrated that elevated IL-6 levels correlate with subsequent preterm labour, spontaneous abortion and fetal death (Wenstrom *et al.*, 1996, 1998). In addition, microbiological studies have shown that microorganisms such as *Uu* may be isolated as early as the second trimester (Cassell *et al.*, 1983) a finding that has also been associated with 'adverse outcomes' (Gray *et al.*, 1992, Horowitz *et al.*, 1995). The detection of second trimester inflammation and bacterial invasion suggests that the fetal exposure to inflammation and infection occur over a number of weeks and not just prior to preterm delivery.

1.1.8. Fetal systemic inflammation

In some cases, intrauterine inflammation may also be associated with a fetal inflammatory response. This has been termed fetal systemic inflammation and is characterised by increased pro-inflammatory cytokine concentrations in umbilical cord blood (Gomez *et al.*, 1998). The cytokines include IL-6 (Gomez *et al.*, 1998, Romero *et al.*, 1998) as well as other cytokines (Berry *et al.*, 1998, Romero *et al.*, 2000). There is evidence of neutrophil and monocyte activation (Berry *et al.*, 1995). An increase in IL-6 in the fetal circulation is usually followed by the onset of spontaneous preterm labour (Romero *et al.*, 1998). The histological correlate of fetal systemic inflammation is funisitis, a neutrophil infiltrate of the umbilical cord or umbilical vasculitis (Yoon *et al.*, 2000b, Naccasha *et al.*, 2001, Kim *et al.*, 2001, Pacora *et al.*, 2002). Infants born with elevated cytokine concentrations in umbilical cord blood or funisitis are at increased risk of adverse neonatal outcome, including chronic lung disease / bronchopulmonary dysplasia (Matsuda *et al.*, 1997, Yoon *et al.*, 1999), and neurological consequences including the development of periventricular leukomalacia (PVL) and cerebral palsy (Yoon *et al.*, 1996, 2000a, 2003a).

Intrauterine infection and inflammation may lead to the complications of prematurity by provoking preterm labour. It is also possible that infection and inflammation may have direct effects on the fetus. The evidence for this will now be discussed.

1. 2 Antenatal infection and inflammation as a direct cause of neonatal morbidity

1.2.1. Introduction

Intrauterine inflammation frequently accompanies preterm delivery before 30 weeks gestation. It has been estimated that fifty percent or more fetuses delivered before 30 weeks gestation are exposed to intrauterine infection and antenatal inflammation. This inflammation within the intrauterine cavity may be prolonged, as discussed above (Section 1.1.7.), and adversely affect the fetus. It has been proposed that exposure of the fetus to a cytokine-enriched environment prior to birth may contribute to the development of chronic lung disease of prematurity and may also cause brain injury. The role of IUI in the development of lung pathology and brain pathology will be considered in turn.

1.2.2. An introduction to chronic lung disease of prematurity

Many preterm infants are admitted to neonatal units with acute respiratory difficulties, usually with surfactant-deficient respiratory distress syndrome (RDS). The lungs are often in an early stage of development and such infants may require ventilation and the administration of surfactant. Fluorinated steroids such as betamethasone or dexamethasone given to the mother prior to preterm delivery have reduced the frequency of RDS by 50% but have not eliminated it altogether (Crowley, 2000). In some cases, the acute lung difficulties do not resolve (O'Brodovich *et al.*, 1985). The clinical manifestation of this is a prolonged need for oxygen or ventilatory support. The terms bronchopulmonary dysplasia (BPD) and chronic lung disease (CLD) have both been applied and in this thesis the terms will be used interchangeably.

Northway who was the first to describe bronchopulmonary dysplasia defined this as a persisting oxygen requirement at 28 days of age (Northway *et al.*, 1967). It was described as lung injury secondary to ventilation and oxygen. The 28 day definition was suitable at the time of description of BPD when the median weight and gestation of the treated infants was significantly greater (Northway *et al.*, 1967). Advances in perinatal care, have meant

that the disease now primarily occurs in more immature extremely low birthweight (ELBW) infants (less than 1000g) (Jobe & Bancalari, 2001). Because of the immaturity of these infants it is not surprising that they are often in oxygen at 28 days as the lungs are still in an early stage of development. Therefore the definition of BPD has been re-evaluated and the diagnosis is now also made when there is a persisting oxygen requirement at 36 weeks gestation (Shennan *et al.*, 1988). This definition is more predictive of respiratory difficulties in the first year of life, although both definitions are still used. The Trent Region Neonatal Survey, thought to be representative of England and Wales, has indicated that the incidence of CLD (defined as oxygen requirement at 28 days) varies between 40 and 50% of infants born at less than 32 weeks gestation who are ventilated (Field, 2000). It is thought that the incidence of CLD has continued at that rate over recent years, mainly because neonatal intensive care is being offered to smaller more immature infants (Manktelow *et al.*, 2001).

The long-term follow up of infants who developed CLD has demonstrated that problems may persist into young adulthood. A study comparing lung function in CLD infants (a cohort from 1964 to 1973) with matched preterm and term controls demonstrated measurable abnormalities in 76% including airway obstruction, airway reactivity and hyperinflation (Northway *et al.*, 1990).

Since the initial description of CLD, the population of susceptible infants has changed, and the clinical definition modified. The pathology has also altered. The original description of the pathology of BPD involved airway injury with epithelial metaplasia, variable inflation with areas of fibrosis and areas of emphysema and inflammation. This was as a result of alveolar repair and fibrosis following lung injury (Toews *et al.*, 1999). More recently infants that have died with BPD have had less airway injury but a striking decrease in the number of alveoli (Husain *et al.*, 1998). CLD uniquely occurs as a result of injury to lungs at an early stage of development prior to alveolar formation. At 24 weeks gestation (the current limit of viability in the UK) the potential airspaces are saccular. Alveoli start to develop from 30 weeks gestation. The canalicular stage of vascular proliferation in the mesenchyme is completed at around 26 weeks (Kotecha, 2000). The pathological findings suggest that there is a disturbance in the normal process of alveolar development (Husain *et al.*, 1998) and vascularisation (Thibeault *et al.*, 2000, Jobe and Bancalari, 2001).

1.2.3. Inflammation and infection and the development of CLD

Studies on lung cytokines using bronchoalveolar lavage (BAL) have demonstrated that infants who are ventilated and subsequently develop BPD have increased concentrations of inflammatory mediators in their BAL fluid than infants who have respiratory distress which resolve (Bagchi *et al.*, 1994, Groneck *et al.*, 1994, Kotecha *et al.*, 1995, Kotecha *et al.*, 1996). The disturbance of normal alveolarisation seen in CLD is thought to be mediated by pro-inflammatory cytokines which can also act as developmental factors (Jobe, 1999). The effect of inflammation on the ventilated lung has been termed 'biotrauma' (Attar, 2002). The observed inflammation may be induced by ventilation and oxygen. Post-mortem investigations have confirmed that inflammation in the amniotic cavity can potentially result in fetal lung inflammation (Schmidt *et al.*, 2001).

1.2.4. Evidence for the possible role of antenatal inflammation in the development of CLD

The first clinical observation that antenatal inflammation might contribute to CLD came from Watterberg *et al.* (1996) before surfactant therapy was established (exogenous surfactant was not available in that unit at the time of the study). Histological chorioamnionitis was found to be associated with less acute lung disease in infants less than 2000g but with an increase in oxygen requirement at 30 days of life. This was an interesting finding as it had previously appeared that severe RDS was the main predictor of the subsequent development of CLD. In the same study, the concentrations of the inflammatory mediator IL-1β were found to be raised at birth in the endotracheal aspirates of those infants who subsequently developed CLD. Further evidence comes from studies measuring amniotic fluid cytokines. As discussed above, elevated pro-inflammatory interleukins in amniotic fluid correlate with chorioamnionitis. Amniotic fluid concentrations of cytokines (IL1β, IL-6, TNF-α and IL-8) were higher in pregnancies in which infants subsequently developed CLD (Yoon *et al.*, 1997d). Fetal systemic inflammation is associated with an increased risk of CLD as well other neonatal morbidities (Appendix 1.1). Infiltration of the umbilical cord with neutrophils, the histological correlate of fetal systemic inflammation has also predicted the development of CLD (Matsuda *et al.*, 1997).

1.2.5. The contribution of antenatal infection to lung inflammation

IL-1 has been found in high concentrations in BAL fluid on day 1 in association with the presence of microorganisms within the lung (Groneck *et al.*, 1996). Eighty-one ventilated preterm infants with mean gestation age of 28 weeks underwent bronchoalveolar lavage. The presence of bacteria including *Uu* at birth was associated with a heightened inflammatory response. An increase in the pro- and anti-inflammatory cytokine ratio has been demonstrated in the airways in infants colonised with *Uu* (Patterson *et al.*, 1998). These finding suggest that the lung at birth in infants exposed to antenatal infection is already a cytokine-rich environment. The resulting cytokine response may inhibit alveolisation.

1.2.6. Animal models and the role of inflammatory cytokines in lung development

Evidence that pulmonary inflammation may be important in disrupting alveolisation also comes from animal studies. Transgenic mouse models with over-expression of various interleukins (tumour necrosis factor alpha TNFα, transforming growth factor alpha TGFα, and IL-6) in the pulmonary epithelium have given rise to various pathologies, but all with disturbance of alveolisation (Jobe, 1999). This provides evidence that cytokines may serve as signals that disturb normal lung development. Lung damage due to inflammation appeared to be equivalent to the effects of overventilation in a perfused mouse lung (Held *et al.*, 2001).

In addition there is good evidence from animal studies that inflammation specifically before birth can have significant impact on the development of the lung. Interleukin-1α (IL-1α) injected into the amniotic cavity induced an increase in lung maturity in the immature rabbits (Bry *et al.*, 1997). This maturation was indicated by increases in surfactant associated proteins and surfactant lipid. Intra amniotic IL-1α administered to pregnant sheep also increased the concentrations of surfactant lipids and improved the pressure-volume curves of preterm sheep (Emerson *et al.*, 1997, Appendix 1.2). These findings seem to agree with the clinical observation that histological chorioamnionitis is associated with a reduced incidence of surfactant deficient RDS (Watterberg *et al.*, 1996). Unfortunately these studies indicate the short-term effects of inflammation on the fetal lung rather than long-term effects of exposure to chronic inflammation. The mechanisms by which the lung function might be modified require

Special Note

Page 31 missing from the original

One of the main problems in interpreting the results is that *Uu* is more likely to be present at the earliest preterm deliveries and so can be isolated from the most immature infants. It is the same immature infants who are most at risk of developing CLD. Hence it has been argued that the association of *Uu* with CLD simply reflects the association of *Uu* with increasing early preterm delivery (van Waarde *et al.*, 1997). Differences in the results of clinical studies may have arisen through a number of factors, including population differences and a failure to take into account the effects of other bacteria. The samples used to determine airway colonisation have also varied, with some studies only using endotracheal aspirates, while others have included surface swabs and nasopharyngeal aspirates. In addition, difficulties in accurately diagnosing *Uu* by laboratory culture may have contributed. It is now recognised that culture methods may underestimate the presence of *Uu* (Yoon *et al.*, 2000c).

Further evidence of the impact of *Uu* also comes from animal studies. Preterm baboons experimentally colonised with *Uu* develop an acute bronchiolitis with epithelial ulceration (Walsh *et al.*, 1993) while newborn mice develop an interstitial pneumonia (Rudd *et al.*, 1989).

A reduction in CLD by an antibiotic effective against *Uu* would suggest a causative role for *Uu*. However, the treatment of *Uu* with erythromycin has not prevented the development of CLD in small studies (Lyon *et al.*, 1998; Bowman *et al.*, 1998; Jonsson *et al.*, 1998). It may be the case that damage to the fetal lungs from infection has already occurred before birth (antenatal infection) so that postnatal treatment would be less effective (Lyon 2000). This lack of benefit may also have resulted from a failure of erythromycin to clear *Uu* from the airway of preterm infants (Baier *et al.*, 2003). In addition, a Cochrane review of the subject concluded that a beneficial effect may have been missed as the studies were underpowered (Mabanta *et al.*, 2003).

It is disappointing that these clinical studies have not come to a clear conclusion even though support for a causative role in the development of CLD has come from other investigations.

1.2.10. Inflammation and CLD summary

Studies show that the exposure of premature lungs to inflammatory conditions increases the risk of developing CLD. These include exposure to postnatal sepsis (Rojas *et al.*, 1995), chorioamnionitis (Watterberg *et al.*, 1996), and increased pro-inflammatory cytokines in the amniotic fluid (Yoon *et al.*, 1997d). This may occur because prolonged inflammation is

generally damaging to tissues (Weiss, 1989). In addition, inflammation may alter the response to subsequent challenges (Jobe, 2005). It may be that the antenatal infection primes the fetal lung, so that it is more vulnerable to injury from ventilation or oxygen, in the same way that results from exposure of the lung to endotoxin. In addition inflammatory mediators may act as developmental mediators 'switching off' alveolisation at a critical stage in development (Jobe 1999).

1.2.11. Antenatal infection, inflammation and brain injury

The risk of cerebral palsy and other neurodevelopmental problems is higher in the preterm infant. In one study premature infants born below 28 weeks gestation were 70 times more likely to have cerebral palsy than the normal birthweight term infant (Cummins *et al.*, 1993). The increased survival of very low birth weight (VLBW) infants has led to an increase in the prevalence of cerebral palsy.

Epidemiological information from the Collaborative Perinatal Project in the US identified intrauterine infection as a risk factor for cerebral palsy (Nelson and Ellenberg, 1986). In infants below 33 weeks gestation chorioamnionitis increased the risk of cerebral palsy with an OR of 2.4 (O'Shea *et al.*, 1998). As discussed above, the infant born by early spontaneous preterm labour is more likely to be exposed to intrauterine infection and inflammation (Goldenberg *et al.*, 2000).

Cerebral white matter damage detected on cranial ultrasound scanning is termed periventricular leukomalacia (PVL). PVL is a form of brain injury that occurs in very preterm infants. It represents damage to white matter neuronal tracts which run adjacent to the lateral ventricles and can often be detected on cranial ultrasound scans either as cysts or as areas of increased echogenicity. White matter damage has been found to be highly predictive of subsequent cerebral palsy (Guzzetta *et al.*, 1986, Stewart *et al.*, 1987, Pinto-Martin *et al.*, 1995, Holling *et al.*, 1999). Therefore studies have looked at this form of brain injury as PVL is a strong predictor of the subsequent development of cerebral palsy. Elevated cytokines in both amniotic fluid and umbilical cord blood (Appendix 1.3) are associated with white matter damage and subsequent cerebral palsy (Yoon *et al.*, 1996, Yoon *et al.*, 1997a, Martinez *et al.*, 1998). In another study, umbilical cord inflammation was associated with an 11-fold increase in periventricular leukomalacia (Leviton *et al.*, 1999). However, this finding only held true for infants who were exposed to chorioamnionitis and born within an hour of rupture of membranes. Cytokine immunocytochemistry has showed more frequent positivity in 60-80% of brains with white

matter damage as compared to 0 to 20% suggesting the inflammatory component of this lesion (Yoon *et al.*, 1997b, Deguchi *et al.*, 1997). However, not all studies have shown a relationship between elevated cord cytokines, intrauterine infection and cerebral palsy (Nelson *et al.*, 2003, Grether *et al.*, 2003) despite a positive association with PVL.

1.2.12. Animal studies

A number of animal models have provided evidence of link between inflammation and white matter damage (Appendix 1.4). Inflammation has been induced by the intra-peritoneal administration of bacterial endotoxin to newborn kittens and dogs (Gilles *et al.*, 1976, Young *et al.*, 1983). In the dog model grey matter damage was seen in addition to white matter necrosis. Maternal bacterial infection in animal models has also been shown to cause white matter damage in the newborn animals (Ornoy *et al.*, 1976, Yoon *et al.*, 1997c, Debillon *et al.*, 2000). It should be noted though that these models have used either *E. coli* or endotoxin and therefore the results may not be representative of infection with other bacteria. However, the evidence for a link between antenatal infection and white matter injury fits in with clinical observations and has biological plausibility.

1.2.13. Possible mechanisms of brain injury

There are a number of possible explanations for the neurological effects of antenatal infection. Direct cytokine injury is possible. It is known that cytokines may increase the permeability of the blood brain barrier, allowing microbial products and cytokines to pass through. Further cytokines may be produced within the brain as a result of cytokine stimulation of microglia (Raivich *et al.*, 1999). TNF-α can cause injury to the oligodendrocyte, the cell type responsible for the deposition of myelin within the brain (Volpe 2001, Peebles *et al.*, 2002). In the study by Debillon the mechanism was thought to be induction of apoptosis (Debillon *et al.*, 2000).

It is also recognised that an important mechanism for the development of PVL is ischaemia within the cerebral arterial watershed area. Therefore it has been suggested that infants exposed to fetal systemic inflammation might have circulatory instability that would lead to brain injury as a result of hypoperfusion (Volpe 2001). Maternal pyrexia complicating chorioamnionitis may aggravate hypoxic ischaemic insults by increasing the cerebral metabolic rate.

1.2.14. The detrimental effects of antenatal infection: a summary

Antenatal infection and inflammation appear to have a range of detrimental effects. There is now evidence to support a common cause for prematurity, chronic lung disease and white matter damage. The same inflammation that initiates a spontaneous preterm delivery may also be predisposing the fetal lung to injury and causing white matter brain injury. In clinical practice this may be an important factor in deciding whether to attempt delay preterm labour, as the fetus may be escaping a hostile cytokine-enriched environment. In spite of the evidence linking intrauterine infection to preterm labour, chronic lung disease and white matter injury, antibiotics have not had a major impact on these clinical problems.

1.3 Difficulties in the investigation of antenatal infection and inflammation

1.3.1. Antenatal infection: gestation age as a confounder

The likelihood that a preterm infant born after spontaneous onset of labour has been exposed to intrauterine infection increases with decreasing gestational age at delivery (Watts *et al.*, 1992). Infants born before thirty weeks gestation are particularly likely to have been exposed to infection. The more immature an infant the more likely that adverse sequelae will occur, particularly when close to the lower limit of viability, perhaps reflecting delivery at a more vulnerable stage of development. These two factors alone could potentially be enough to explain the association between lung and brain pathology and intrauterine infection. This is an important consideration as some studies looking at exposure of the fetus to inflammation have used univariate analysis only (Appendix 1.6 e.g. Tauscher *et al.*, 2003, An *et al.*, 2004). Gestational age, an important cofactor may not have been excluded. The importance of this is well-illustrated in the recent study by Goepfert *et al.* (2004) in which some outcomes are associated with increased cord IL-6 concentrations when univariate analysis is performed but not when other factors are taken into account with multivariate analysis. To assess the true effect of intrauterine infection independent of gestational age would need studies with larger numbers of infants to allow multivariate analysis (Fig. 1.1).

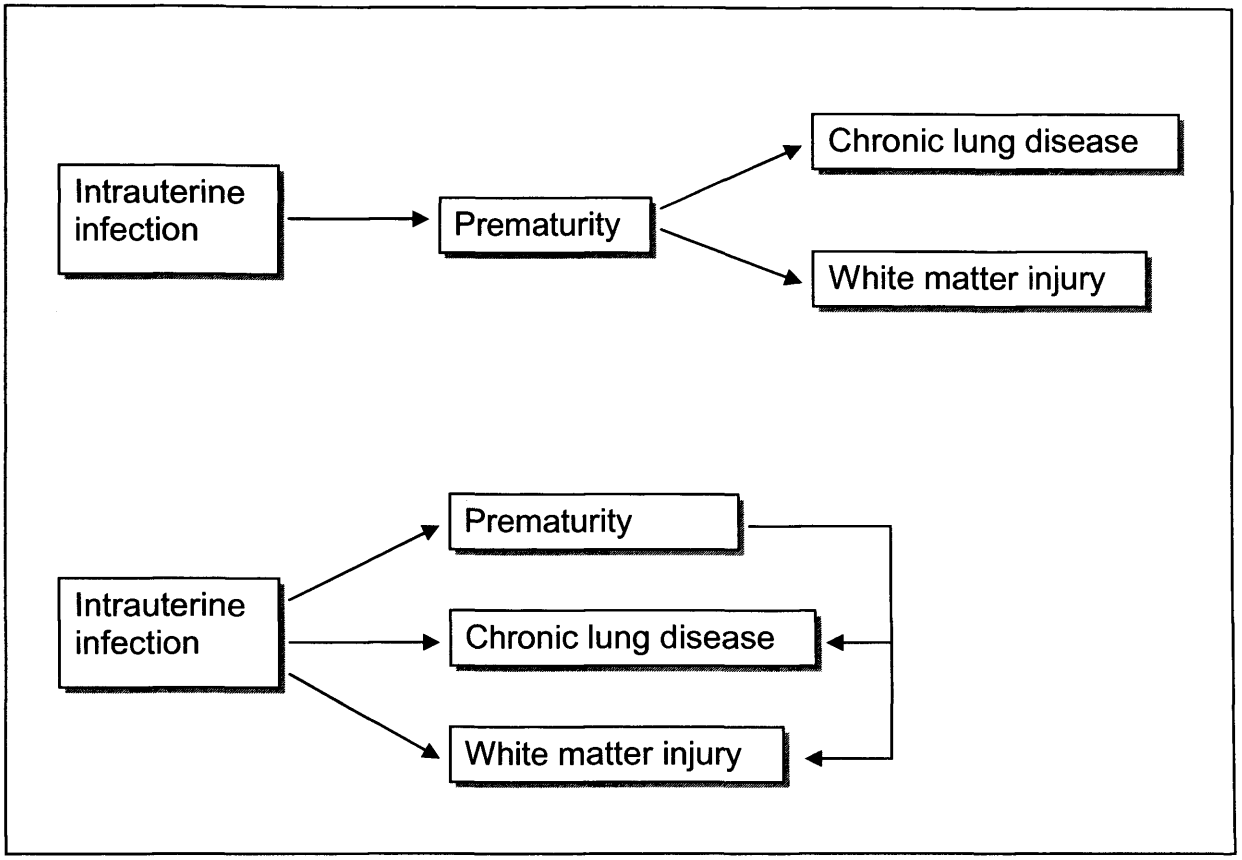


Figure 1.1. Diagram showing the competing explanations for the association between intrauterine inflammation and neonatal morbidity. The traditional stepwise view (upper) is that chronic lung disease and white matter injury occur as a result of prematurity and that prematurity is provoked by intrauterine inflammation. The more recent suggestion is that intrauterine infection also makes an additional direct contribution to chronic lung disease and white matter injury as well as prematurity.

1.3.2. The need for amniocentesis

To date all studies using PCR detection of antenatal infection have used amniotic fluid. A number of centres have performed amniocentesis in preterm labour (Gonçalves 2002). In some centres, amniocentesis has been routinely offered by the clinical service for the determination of infection (Gomez *et al.*, 1998, Romero *et al.*, 1998).

The use of amniocentesis to detect infection in patients following pPROM was put forward by Garite (Garite *et al.*, 1979). The success of amniocentesis in pPROM has been reviewed (Asrat, 2001) and found to vary from 49% to 96%. An advantage of amniocentesis is that information regarding infection can be obtained prior to delivery.

However, the safety of the procedure has been an important consideration. Complications can include leakage or injury to the fetus, umbilical cord or placenta. The risk of fetal injury has been estimated at 0.6 to 2%. The chance of injury to the placenta or cord is 0.3 to 1.1% (Galle & Meis, 1982). In one report, urgent delivery was required for a fetal bradycardia following amniocentesis (Stark *et al.,* 2000). At this stage there is no evidence to show that diagnosing infection by amniocentesis improves the outcomes of women in preterm labour. In view of the risks, and with no clear benefit, amniocentesis is not routinely performed in pPROM or preterm labour in most centres (Asrat, 2001). In addition, as it is an invasive procedure, it has not been ethically acceptable in the UK to perform amniocentesis for research purposes. This is reflected in the fact that UK studies employing molecular detection of intrauterine infection have used alternative approaches. In one study, amniotic fluid was obtained at caesarean section and did not relate to premature delivery (Bearfield *et al.,* 2002). In another *in situ* hybridisation was used to detect the presence of 16S ribosomal genes in the fetal membranes (Sullivan *et al.,* 2004).

1.3.3. Discrepancies between cytokines and culture in the detection of antenatal infection and inflammation

Chorioamnionitis has previously been associated with 36-60% of placentas resulting from preterm labour with intact membranes (Pankuch *et al.,* 1984, Hillier *et al.,* 1993, Odibo *et al.,* 1999) compared to 42-80% of placentas following pPROM (Hillier *et al.,* 1988, Mueller Huebach *et al.,* 1990, Rogers *et al.,* 2002). There is a strong association between inflammatory lesions of the placenta and isolation of organisms from the chorioamnion and amniotic fluid. However, microbes have only been isolated from either placenta (Pankuch *et al.,* 1984) or amniotic fluid (Hillier *et al.,* 1993) in a proportion of cases of chorioamnionitis (Appendix 1.7).

El-Bastawissi *et al.* reviewed studies that had measured amniotic fluid IL-6 in the context of preterm labour and found that elevated concentrations of IL-6 were seen more frequently than positive amniotic fluid cultures and were a better predictor of preterm delivery than positive amniotic fluid microbiology (El-Bastawissi *et al.,* 2000). Bacteria have only been isolated in around three-quarters of cases in which there is an elevated amniotic fluid IL-6 (Appendix 1.8). It has therefore been suggested that an inflammatory response and chorioamnionitis may occur in the absence of infection (Hillier *et al.*, 1993). Interestingly, the outcomes of pregnancies in women with an intrauterine inflammatory

response but negative amniotic fluid culture did not differ from those with a positive amniotic fluid culture (Yoon *et al.*, 2001).

A number of reasons have been put forward to explain the consistent presence of intrauterine inflammation in the absence of detectable infection. There may be the failure to detect infection by culture methods from the amniotic fluid because of the limitations of laboratory culture, the use of antibiotics and the bacteriostatic properties of amniotic fluid. It is also thought that studies looking at amniotic fluid alone may be limited by the fact that micro-organisms causing inflammation may be residing in the decidua before invading the amniotic fluid.

1.4 New approaches to the investigation of antenatal infection and inflammation

1.4.1. Introduction

To overcome the difficulties above requires new approaches.

1. Large studies would be required to allow multivariate analysis to eliminate gestation age as a confounder.
2. It appears that molecular methods such as PCR would be required to maintain a sensitive level of detection of micro-organisms.
3. Because amniocentesis is not a routine procedure, alternative ways to determine the microbiology of amniotic fluid in preterm labour membrane would be required to allow larger numbers to be studied. Proxies such as gastric fluid, bronchoalveolar fluid and fetal membrane will be considered.

1.4.2. Using the Polymerase Chain Reaction: greater sensitivity in the detection of infection

It is now recognised that laboratory culture has limitations in the detection of infection. It relies on the ability of the organism to adapt to the culture environment and reproduce. However the ability of organisms to grow on culture may be hampered by a low infectious inoculum or decreased viability due to antibiotics. Some organisms have fastidious culture requirements that are costly and labour intensive. Therefore some investigators have turned to molecular techniques for the detection of infection. Furthermore, the most recent developments in the detection of infectious pathogens have depended on the molecular

identification of bacterial or viral genomes (Relman *et al.,* 1999). For instance, molecular detection of bacterial genomes has been able to identify the pathogenetic organisms of bacillary angiomatosis (Relman *et al.,* 1990) and Whipple's disease (Relman *et al.,* 1992). These were organisms that had proved difficult to culture. A further example of a fastidious organism is *Ureaplasma urealyticum* (Wang *et al.,* 1997). Failure to detect this organism in any study of antenatal infection would be of importance, in particular because *Uu* has been implicated in the genesis of both preterm labour and chronic lung disease.

The process of detecting bacterial genomes involves the polymerase chain reaction as a first step. The polymerase chain reaction (PCR) is a technique for amplifying a strand of DNA to which a primer sequence binds (Lodish *et al.,* 1999). The sequence of the amplified strand of DNA can than be analysed. For example universal bacterial primers can anneal to conserved bacterial sequences such as those found on the gene which codes for the 16S ribosomal RNA (rRNA). By amplifying the fragments of the 16S rRNA gene, all bacteria can be detected without regard to culture requirements. The 16S rRNA gene also contains variable regions from which the specific bacteria can be identified by obtaining the sequence. There is now an extensive database of 16S rRNA which allows identification of bacteria from the sequence. This database, which is accessible on the internet, makes 16S rRNA the currently most useful target for PCR-based identification of bacterial organisms (Section 2.2.2).

The identification of conserved microbial genes (e.g. 16S ribosomal RNA genes) to detect bacteria is a technique that has been successfully applied to amniotic fluid. The yield has been compared to the results of laboratory culture. Several studies have applied universal bacterial PCR to amniotic fluid obtained by amniocentesis from women in preterm labour (Hitti *et al.,* 1997b, Markenson *et al.,* 1997) or following premature rupture of membranes (Jalava *et al.,* 1996). In one study, the sensitivity for the detection of infection by PCR was 95% compared to 76% by bacterial culture (Hitti *et al.,* 1997). In another study there was also a large discrepancy in detection of bacteria between PCR (55.5%) and culture (9.2%) (Markenson *et al.,* 1997). PCR *positive*/culture negative samples had a significantly shorter amniocentesis to delivery time than PCR *negative*/culture negative samples (Markenson *et al.,* 1997). Both studies demonstrated that bacteria could often be detected by PCR in amniotic fluid samples showing evidence of inflammation (increased amniotic fluid IL-6 concentrations) in spite of negative bacterial cultures (Hitti *et al.,* 1997, Markenson *et al.,* 1997).

PCR-based techniques also appear to have greater sensitivity in the detection of

specific organisms in amniotic fluid such as *Uu*, an organism implicated in neonatal morbidity (Yoon *et al.,* 2000c). Yoon *et al.* applied specific primers to identify *Uu* in amniotic fluid following pPROM and found that in over 40% of cases the presence of *Uu* was missed by culture (Yoon *et al.,* 2000c). Patients with a PCR positive/culture negative had a shorter amniocentesis to delivery interval and higher amniotic fluid IL-6 concentrations than those with negative PCR and negative culture. Clearly, discrepancies in the diagnosis of infection of this order may have had a significant impact on accuracy of previous investigation of antenatal infection. In a further study Nelson *et al.* (1998) applied specific PCR to 225 ET aspirates from 103 infants. The PCR was used to detect the multiple banded (MB) antigen gene of *Uu*. The ET aspirates were also sent for culture. 73 samples were positive by PCR and 60 by culture. The explanation given for the shortfall in detection by culture was that if *Uu* is vertically transmitted it would be expected to be in lower concentrations shortly after birth. It was suggested that PCR was especially useful for the early detection of *Uu* in neonatal ET aspirates (Nelson *et al.*, 1998).

One concern about molecular methods of detection of bacteria is that the increased sensitivity might give rise to false positive results. For instance bacteria have been detected in amniotic fluid samples from women who subsequently delivered at term. However the correlation with clinical outcomes described above seems to suggest that the increased sensitivity does in fact represent true invasion of the amniotic cavity (Markenson *et al.,* 1997, Yoon *et al.,* 2000).

1.4.3. Gastric fluid as a proxy for amniotic fluid

As a result of fetal swallowing, it is known that gastric fluid is effectively in continuity with amniotic fluid. Indeed, it has been postulated that the aspiration of amniotic fluid *in utero* is an important route for the transmission of amniotic fluid infection to the fetus. Gastric aspirate has been used for many years in the diagnosis of amniotic fluid infection (Blanc, 1959). More recently it has been evaluated for the ability to predict neonatal sepsis. A review of studies on the use surface swabs showed that the probability of neonatal infection was increased with white cells and bacteria in gastric fluid (Fowlie *et al.*, 1998). However in the clinical setting it may be more useful marker in neonatal sepsis when combined with other markers such as full blood count and C-reactive protein (Garland *et al.*, 2003). In view of the difficulties of obtaining amniotic fluid I planned to use gastric fluid for PCR detection of microbial genes. Gastric fluid has to date not been utilised for the molecular detection of intra-amniotic infection.

1.4.4. Use of fetal membrane tissue

In studies where chorioamnion cultures and amniotic fluid cultures have been performed in the same patients, bacteria have been isolated more often from the chorioamnion than from amniotic fluid (Hillier *et al.*, 1993, Andrews *et al.*, 1995). It is thought that in some cases bacteria may reside in the decidua and provoke an inflammatory response but without invading the amniotic cavity. Hence I planned to include PCR on fetal membranes for the detection of intrauterine infection. Microbiological analysis by PCR has not been performed on fetal membranes, placental tissue and cord blood. Therefore this represents a novel approach to the investigation of intrauterine infection. There are concerns that surface swabs of fetal membranes after the delivery of the placenta are of limited value because positive results may represent contamination with vaginal flora (Gonçalves *et al.*, 2002). It is therefore possible that this contamination could limit the use of bacterial detection in fetal membrane.

1.5 Hypothesis

- I have hypothesised that increased concentrations of inflammatory mediators in the amniotic cavity, fetal circulation and fetal lungs at birth are associated with the presence of bacterial organisms in the intrauterine environment

- That antenatal infection and the inflammatory and stress response to infection are significant factors in the development of CLD

Secondary hypothesis

- That reliable information regarding antenatal infection and inflammation may be obtained by applying molecular biology techniques to placental tissues.

Specific Aims

- To determine if intrauterine inflammation is consistently associated with the presence of bacterial organisms in the intrauterine environment. Bacterial involvement will be characterised by the detection of bacterial genomic material in placental tissues and amniotic and fetal compartments.

- To describe the patterns of intrauterine inflammation in a cohort of preterm deliveries. To determine if inflammation is prevalent in the fetal circulation and fetal lungs at delivery and whether this correlates with intrauterine infection.

- To examine the relationships between antenatal infection, the response to antenatal infection (inflammation and cortisol response) and the development of lung disease of prematurity (respiratory distress syndrome and chronic lung disease).

Chapter 2 : Methodological considerations

The first part of this chapter deals with the collection of specimens underpinning this investigation of intrauterine infection and inflammation. Analysis of these specimens is covered in the second section, focusing on molecular biology techniques for microbial detection and measuring inflammation. Data collection and the characteristics of the patients recruited are then described in the third section. Ethical considerations are an important component of any study, and so these are covered in the final section.

2.1 Perinatal Specimen collection

2.1.1 Overview of specimen collection

Amniotic fluid (AF) has been obtained in a number of studies for the analysis of cytokines (Section 1.1.4). Cells from amniotic fluid have been used for PCR detection of microorganisms (Section 1.4.2). Bronchoalveolar lavage (BAL) fluid, cord blood and amniotic fluid were collected and then centrifuged to separate a supernatant component suitable for the analysis of cytokines and a cellular fraction suitable for the PCR detection of bacterial organisms (Figure 2.1).

2.1.2 Amniotic fluid

2.1.2.1 Amniotic fluid collection

As discussed in the introduction, amniocentesis is not routinely performed in most centres in the UK as there is no evidence to show that analysis of amniotic fluid improves the outcomes of women in preterm labour. However it was possible to collect amniotic fluid at most caesarean section deliveries. The amniotic fluid was usually obtained by needle and syringe through the amniotic sac after operative entry into the uterus. This in practice was difficult to perform with the oligohydramnios often seen in pPROM and in emergency caesarean sections for fetal distress. In some cases, the amniotic fluid was obtained after delivery of the infant. The samples were collected in sterile universal containers and kept on ice until processing.

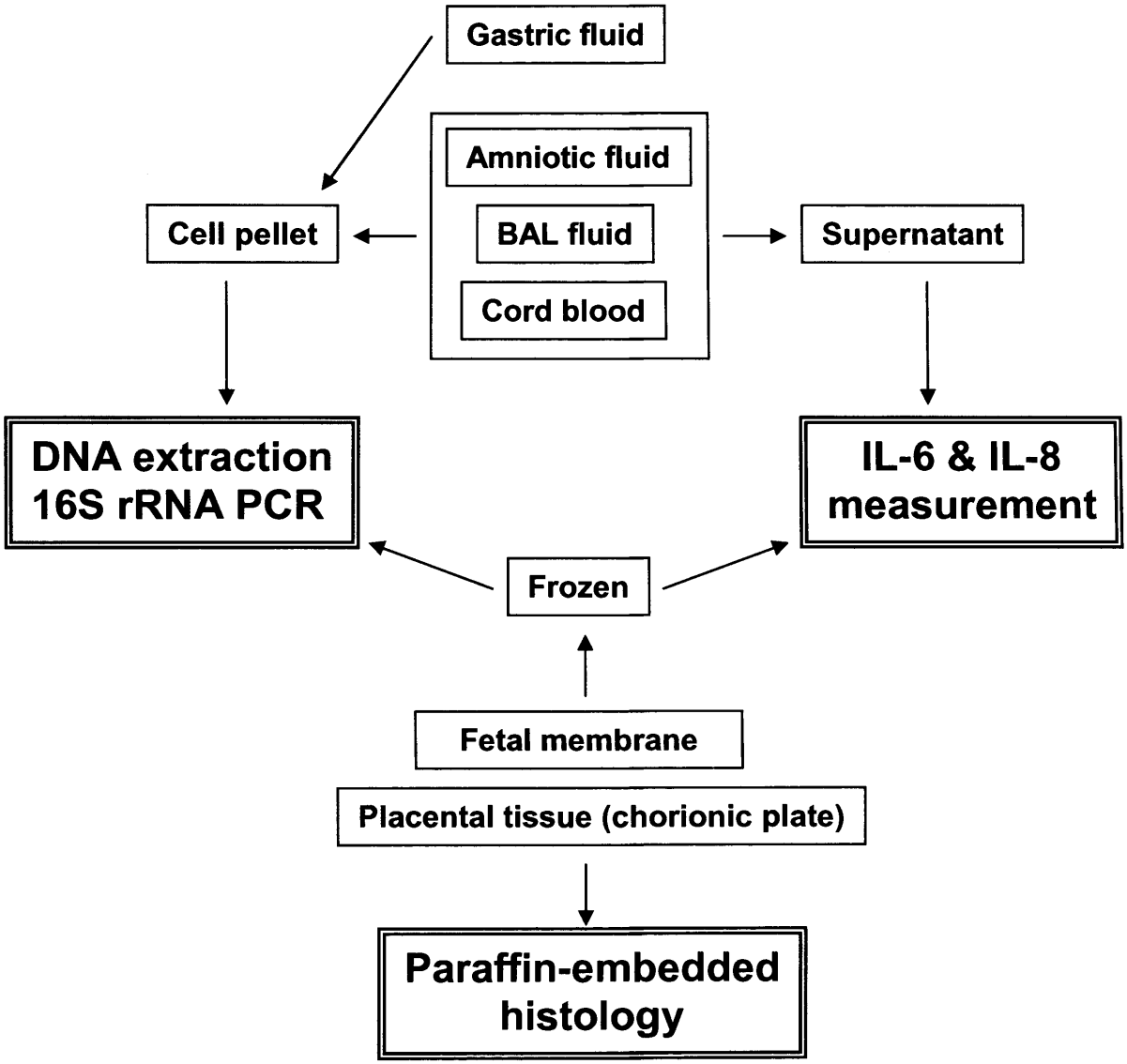


Figure 2.1 Overview of sample collection and processing

2.1.2.2 Sample collection at vaginal delivery

Amniotic fluid was not collected at vaginal deliveries in view of the difficulties gathering the fluid and the likely contamination from the lower genital tract. The possibility of collecting amniotic fluid using a dacron swab (fibronectin swab) at speculum examination was considered. However this did not prove feasible in practice due to the timing of the speculum examinations.

2.1.2.3 Amniotic fluid processing

The amniotic fluid was divided into two 2ml aliquots and then cooled and centrifuged at 2000g for 5 minutes. The supernatant was removed by pipette and frozen at –20°C. The cell pellets were frozen for later PCR investigation. A 1ml-2ml aliquot was sent for routine microbiology culture provided sufficient sample volume was available. Any remaining amniotic fluid was frozen without centrifugation.

2.1.3 Gastric Fluid

2.1.3.1 Gastric fluid sample collection and processing

A sample of gastric fluid was collected as soon as possible after delivery. In most cases this was collected immediately after delivery using a 6FG suction catheter. In cases where the parents did not consent to additional handling of their infant, the gastric fluid was obtained by syringe when a nasogastric tube was passed routinely for the first time on the neonatal unit. The gastric fluid was frozen without centrifugation and stored at –70°C. Centrifugation was performed during the thawing process just prior to DNA and RNA extraction from the cell pellet.

2.1.4 Bronchoalveolar lavage

2.1.4.1 Bronchoalveolar lavage introduction

Bronchoalveolar lavage (BAL) is a technique for retrieval of both cellular and acellular components of the respiratory tract. This can be achieved by administering sterile saline down a bronchoscope and then recovering the solution together with the epithelial lining fluid (Russi & Crystal, 1997). As the smallest bronchoscopes with a diameter of 2mm do

not have an adequate suction channel, a non-bronchoscopic method of BAL is usually performed in neonates (Koumbourlis *et al.*, 1993, Grigg *et al.*, 1992, Alpert *et al.*, 1992).

By obtaining cellular and non-cellular components from within the lung, BAL has provided a window into airway milieu of the lung. It has played an important role in the understanding of the pathogenesis of both respiratory distress syndrome and the development of chronic lung disease. This technique has been used previously by our group in a number of studies on newborn infants and aspects of safety have been explored (Vyas *et al.*, 1998). A disadvantage is that BAL can only be performed on intubated infants.

The method consisted of placing the infant supine with the head facing to the left. This position is thought to consistently facilitate entry of the BAL catheter into the right lower lobe. The catheter was inserted through a port in the ventilator circuit into the endotracheal tube until resistance was felt. One ml/kg of saline at room temperature was instilled. After three ventilator breaths, suction (5-10kPa) was applied to collect the fluid and the catheter was gradually withdrawn. The fluid was retrieved in a bronchoscopy sample collector. The procedure was then repeated. It is thought that the two aliquots retrieved represent different parts of the bronchoalveolar tree. The first aliquot represented primarily bronchial extracellular lung fluid (ELF) and subsequent lavages mainly represented alveolar ELF (de Blic *et al.*, 2000). For the purposes of this study the two aliquots were pooled. The volume of retrieved fluid was estimated by comparison with a standard amount of fluid and by pipette.

6FG catheters were used (or 5FG catheter if it was not possible to pass a 6FG catheter) with both an end hole and side hole. Sterile gloves were used and care was taken not to contaminate the tip of the suction catheter. The effect of using gauze to filter mucus from BAL fluid is not known so filtering of the samples was not performed (de Blic *et al.*, 2000).

The most common side-effects of the BAL procedure are vasovagal responses to catheter insertion and transient drops in oxygen saturation (Grigg *et al.*, 1992). In all cases the infants were on both ECG and pulse oximeter monitoring in an intensive care setting. Prior to the procedure, the inspired oxygen was increased by 10%. Further increases were not performed unless necessary in view of the risk of retinopathy of prematurity. In addition it was found that tolerance of the procedure was improved if the ventilation rate was set to a minimum of 30/min for the duration of the procedure.

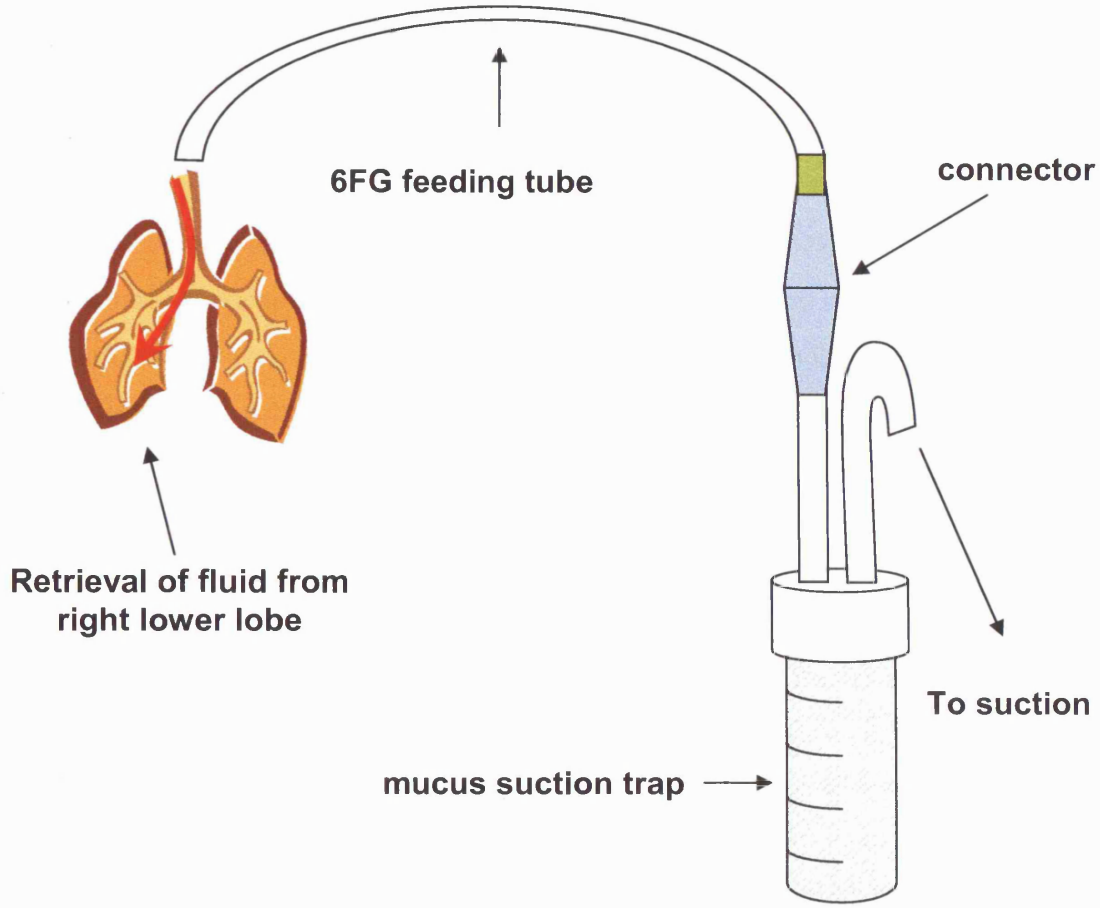


Figure 2.2 Equipment used for retrieval of bronchoalveolar lavage fluid

2.1.4.2 Cell count and differential

A small aliquot (50-100µl) was taken from the sample obtained to assess the cellularity and cell differential of the BAL fluid. Ten microlitres of the aliquot was diluted with 10 µl of trypan blue (0.4% solution) (Sigma-Aldrich Co.), a stain which is excluded by viable cells. Using a Neubauer haemocytometer, a viable cell count was made. In most cases the epithelial cells observed were non-viable and white cells were usually viable. In interpreting the cell counts, areas with mucus were avoided. Mucousy specimens or specimens with large numbers of cells were further diluted either 1 in 5 or 1 in 10 with trypan blue.

To obtain a differential count, a cytocentrifuge was used to spread the cell population onto a glass slide. Using the cell count, the remainder of the aliquot was diluted with normal saline to give a concentration of 2 to 5 x 10^5 cells/ml. 100µl of this diluted sample was place in the funnel. The cytocentrifuge was run at 450 rpm for 10 minutes at

room temperature. The resulting cytospin was allowed to dry before being fixed in methanol and stained with Diff-Quik (Dade Behring Inc). The differential cell count was then estimated by reviewing 10 random fields at 40x magnification.

2.1.4.3 BAL fluid processing

The remainder of the specimen was cooled and cells recovered by spinning at 500g for 10 minutes (Kotecha *et al.*, 1995, 1996). If a cell pellet was not seen the sample was spun again at 1000g. The supernatant was removed and frozen in 100µl aliquots for subsequent cytokine analysis.

2.1.4.4 Consideration of the Estimation of Extracellular lung fluid

The measurement of inflammatory cytokines and other non-cellular components is complicated by the dilution of extracellular lung fluid (ELF) by the saline solution used to retrieve it. Quantification of non-cellular components of the ELF can be performed. One method involves the measurement of urea in the retrieved fluid as ELF prior to retrieval is assumed to have a concentration of urea equal to that of plasma. By comparing the urea in the retrieved specimen with that in the plasma the dilution of the ELF may be estimated. A review of the methodology of BAL in children concluded that no current method was reliable (de Blic *et al.*, 2000). Therefore, although considered in this study, ELF dilution was not estimated.

2.1.5 Umbilical Cord blood

2.1.5.1 Umbilical cord blood collection

Samples of umbilical cord blood were collected by needle and syringe as soon as possible after the placenta is delivered, and placed in an Eppendorf tube. The blood was cooled prior to centrifugation.

2.1.5.2 Umbilical cord blood processing

The sample was spun at 7000g (10,000 rpm) for 4 minutes at room temperature to separate the cells from the supernatant. Because of the high haematocrit of the cord blood, it was often only possible to collect one or two aliquots of supernatant.

2.1.6 Infant blood

2.1.6.1 Infant blood collection

A sample of at least 200µl was collected into an Eppendorf tube during the initial cannula insertion or after insertion of an arterial line. The blood was cooled prior to centrifugation at 7000g for 4 minutes.

2.1.6.2 Processing

Again due to the high haematocrit of the blood, the viscosity of the serum, and the size of the specimen, only one aliquot of supernatant was usually obtained. After separation, the supernatant and cells were stored frozen.

2.1.7. Placental Specimens

2.1.7.1 Samples for histology

From each placenta, 3 samples of placental tissue (inner zone, mid zone and outer zone), three membrane rolls (3cm wide, 6-10cm length, rolled towards the placenta) and one sample of umbilical cord (0.5cm slice) were collected (Fig. 2.3). These samples were fixed in formalin for a minimum of 72 hours before further histological processing. Three 0.5cm slices were embedded into wax from each membrane roll. The membrane rolls were collected at the same time as the frozen specimens as soon as possible after delivery (30-60 mins). The chorionic plate and cord specimens were collected after allowing the outer region of the placenta to fix in formalin (10%) for around 1 hour.

2.1.7.2 Frozen specimens

From each placenta, small samples (approximately 100mg) of umbilical cord, placenta and membrane was snap frozen within 30-40 minutes of the delivery in screw-top sterilin tubes and stored at -70°C.

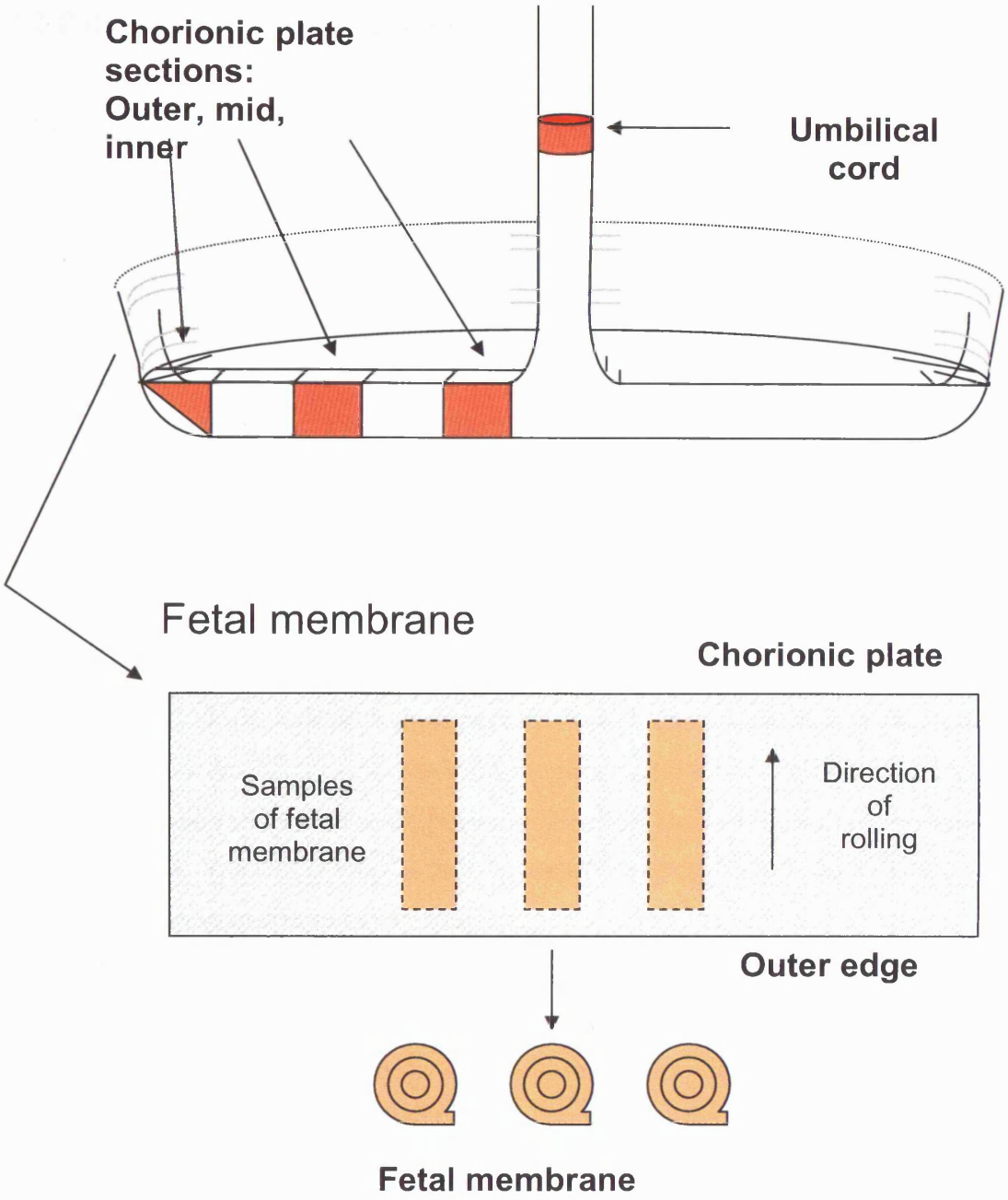


Figure 2.3 Diagrammatic representation of chorionic plate, fetal membrane and cord collection.

2.1.8 Chorioamnion swab and culture

A swab taken from between the fetal membranes (amnion and chorion) has been shown to provide useful information on *in utero* microbiology (Aquino *et al.*, 1984). A routine bacteriology swab was applied to the surface of the chorionic plate through a cut made in the membrane and sent for microbiology culture.

2.2 Polymerase Chain Reaction: Principles

2.2.1 Introduction to the Polymerase Chain Reaction

The polymerase chain reaction was developed in the 1980s and has enabled the rapid synthesis of a large number of copies of particular DNA sequences. The sequence copied is determined by the choice of primer. Primers are fragments of DNA, usually around 20 nucleotides in length, which bind to the DNA flanking the target DNA. One of the breakthroughs in developing PCR was the discovery of heat-stable DNA polymerase. The most common polymerase used is the thermostable *Taq* polymerase which is derived from the organism *Thermus aquaticas*, a bacteria capable of living in the hot springs of Yellowstone Park in Wyoming where it was first discovered. The high temperature required to separate the strands of DNA in the polymerase chain reaction does not denature this polymerase (Saiki *et al.*, 1988).

The PCR cycle consist of three steps that are repeated. The target DNA is heated usually to 94°C to separate the complementary strands. The temperature is then rapidly lowered to allow the DNA primers to anneal to the desired sites. The primers are usually in excess so that the DNA binds to the primers rather than back with the complementary strands of DNA. The temperature is then increased for the synthesis of new DNA copies, with nucleotides added from the 3′ ends.

After each complete cycle of three steps, the amount of DNA is doubled. Theoretically then 20 cycles will produce over 1 million copies of the target DNA and 30 cycles yield one billion. With the use of specialised PCR cycling equipment (thermal cyclers) these steps can be perfomed rapidly. Because the target concentration of DNA can be low this can be a very sensitive technique.

2.2.2 16S ribosomal RNA gene as a target for broad-spectrum bacterial detection

Bacteria have smaller ribosomes than eukaryotic cells. Their 70S ribosomes are constructed of 50S subunit and 30S subunit. Most work on bacterial detection has focused on the 16S (part of the 30S subunit) component (Clarridge 2004).

Analysis of the 16S rRNA gene has been used in the construction of phylogenetic trees. It is thought that closely related organisms have a greater similarity between the rRNA gene sequences (Fig. 2.4).

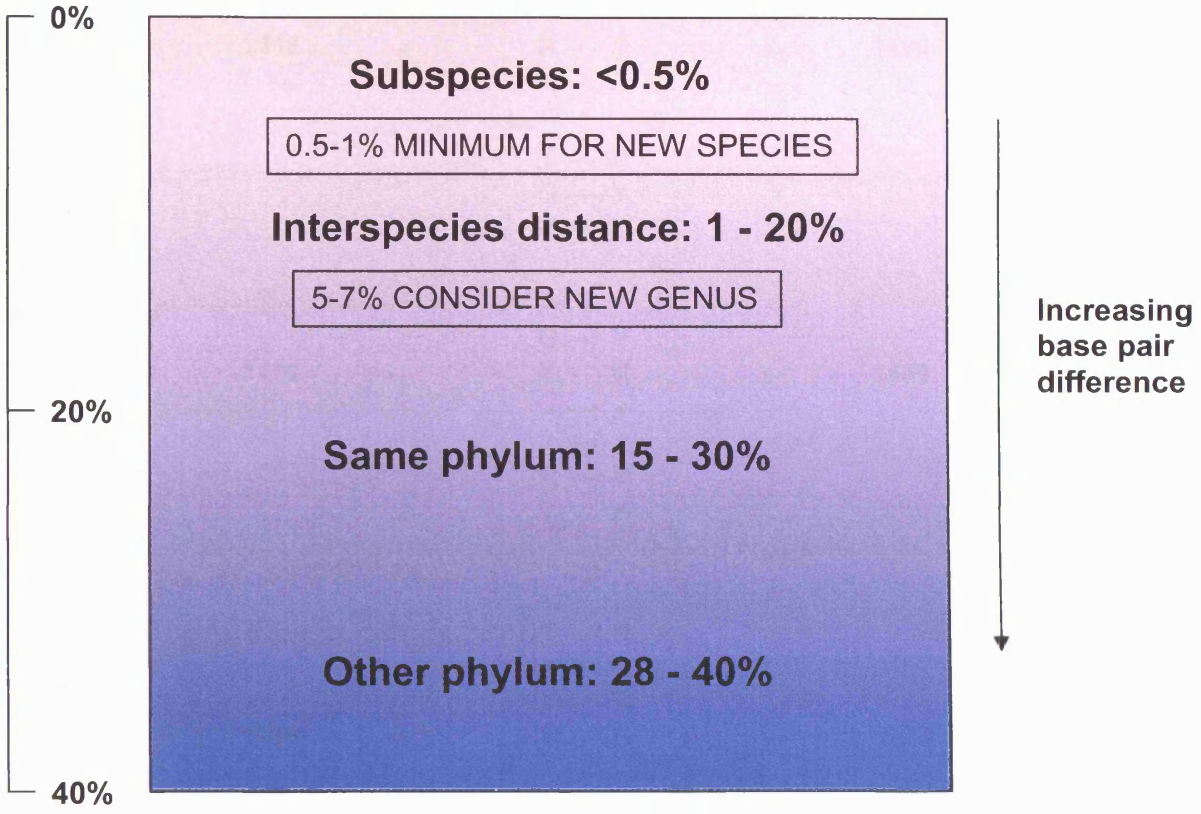


Figure 2.4 Diagrammatic representation of the relationship between bacteria and their 16S rRNA gene sequences. In general closely-related organisms have a greater similarity between the 16S rRNA gene sequences (data from Woese 1987, Clarridge 2004).

Sections of the 16S rRNA gene are conserved allowing universal bacterial primers to bind. Also it has been discovered that most groups of bacteria have characteristic nucleotide sequences termed oligonucleotide signatures within the variable portions of the 16S rRNA gene (Fig. 2.5). Sequencing of these portions of the DNA after amplification allows identification of the bacteria. Because eukaryotes have distinct ribosomal RNA genes, by targeting the 16S gene with bacterial primers, bacterial DNA can be amplified without interference from the presence of eukaryotic human DNA in clinical samples.

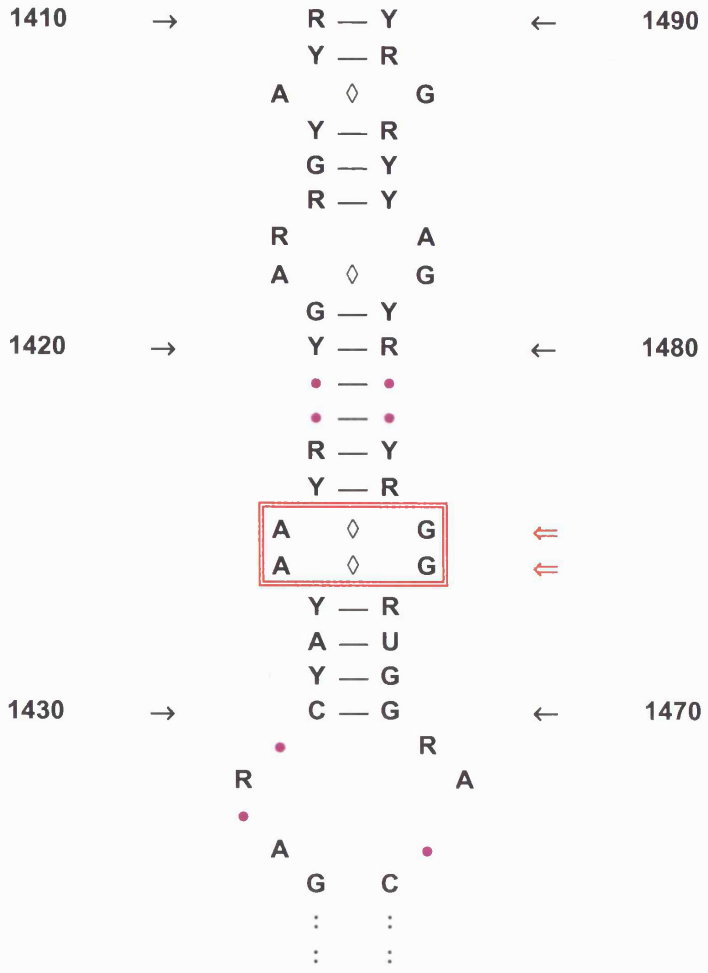


Figure 2.5 An example of distinctive bacterial characteristics observed in the nucleotide sequence of the 16S ribosome. The double A-G pairings at positions 1425-6 and 1474-5 are seen in most gram-positive cocci, but not in bacteria outside this group. (A: adenine, C: cytosine, G: guanine, U: uracil, R: purine, Y: pyrimadine, purple dots represent variable sites) (modified after Woese 1987).

Direct sequence analysis is usually achieved by a method related to that initially described by Sanger *et al.*, (1977). In synthesising new copies of DNA dideoxynucleoside phosphates are added to the reaction mixture. These are nucleotides that can be incorporated by the DNA polymerase into a growing chain of DNA. However once the dideoxynucleotide has been added further elongation of the chain is no longer possible. The initial manual sequencing method involved adding the polymerase, four deoxynucleotides

and one of the dideoxynucletides. The result was DNA fragments of different lengths that could be resolved and visualised on a gel.

The DNA sequences obtained in this study were produced by an automated system. In such a system, the four dideoxynucleotides are each labelled with a different fluorescent dye. The products for the four reactions are electrophoresed together. A scanner then reads the sequence of fluorescent colours on the gel, representing the DNA sequence (Fig. 2.6).

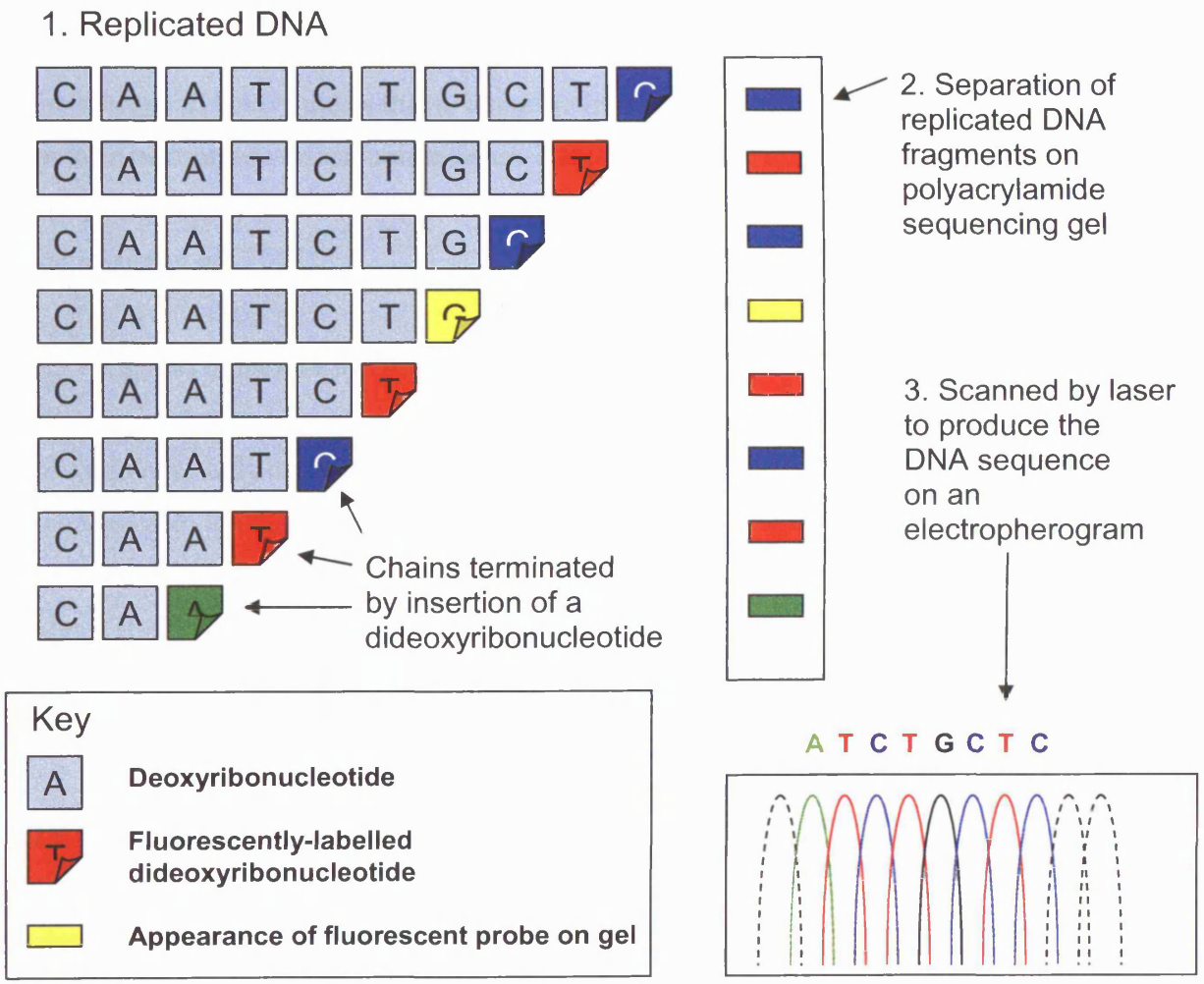


Figure 2.6 Diagrammatic representation of automated DNA sequencing analysis using fluorescently-labelled dideoxynucleotides.

2.2.3 Specific PCR for *Ureaplasma urealyticum*

The detection of *Uu* using specific primers has targeted several genes, the 16S rRNA gene, the urease gene and the multiple-banded gene sequences (Section 1.4.2). In a number of studies the detection of *Uu* by PCR has been compared to detection by culture. In all cases PCR based detection was as good if not better than detection by culture (Section 1.4.2).

2.2.4 PCR based detection of *Uu*: subtype analysis

An additional advantage of PCR is that by using primers to different genes, information can be obtained on different subspecies of *Uu*. This can be helpful in deciding whether detection of *Uu* is a contaminant or a genuine infecting agent. For instance if the *Uu* is the same subtype in BAL fluid as that in amniotic fluid or gastric fluid this would support genuine detection of vertical transmission. Examples include the biovar 1 (Parvo) and biovar 2 (T960) subdivisions identified by replication of the multiple-banded antigen gene (Domingues *et al.*, 2002).

2.2.5 PCR and detection of viruses

PCR can also be used for the detection of viruses. This has been the focus of two recent studies. In one study of 89 infants, the presence of adenovirus in tracheal aspirates was associated with the development of bronchopulmonary dysplasia (Couroucli *et al.*, 2000). A commentary accompanying this publication suggested that this could still be a chance finding (Hogg 2000). A further study demonstrated that the presence of viral genomes were frequently found in VLBW preterm infants but did not find any association between CLD and the presence of cytomegalovirus or adenovirus (Prosch *et al.*, 2002).

2.2.6 PCR detection of RNA

The PCR reaction can be applied to the detection of gene sequences in RNA, by the inclusion of a reverse transcriptase step to produce complementary DNA. This theoretically might improve the specificity of the PCR detection, as it would be expected that only viable replicating bacteria would have RNA present. In a previous study by our group, both universal and specific bacterial detection were applied to cDNA from RNA from neonatal BAL fluid (Kotecha *et al.*, 2004). To date this remains the only neonatal study to have targeted RNA for perinatal bacterial detection.

2.2.7 The application of polymerase chain reaction in this study

The following list summarises the choices available for the use of PCR in this study:

1. Universal bacterial detection or specific detection
2. Method of separating PCR products of different bacteria
3. In specific detection of organisms, which gene to target
4. Whether to investigate viral pathogens
5. Samples to use.
6. Targeting DNA or RNA

In applying PCR-based detection to the samples collected it was decided to use broad-spectrum universal bacterial primers directed at the 16S rRNA gene. This would enable detection of all bacteria, but might require the separation of DNA from different species of bacteria in the same sample. For example, by cloning the DNA product and sorting with tagged restriction fragment length polymorphism analysis (T-RFLP) prior to DNA sequencing of unique clones (Kotecha et al., 2004). However in the first instance we decided to directly sequence any 16S rRNA gene products and review the results. Because of the importance of *Uu* we used specific PCR primers directed at the urease gene as described previously by Blanchard (Blanchard *et al.,* 1993). The evidence currently for a role for viruses in preterm labour or in the development of CLD is not strong, so at this stage we did not use primers to viral sequences. DNA is known to be stable, while RNA is often rapidly degraded by ubiquitous ribonucleases. Although RNA could be prepared from the samples collected, DNA was chosen as the template for PCR, as in previous studies of intrauterine infection.

2.3 Polymerase Chain Reaction : Methods

2.3.1 DNA and RNA extraction

DNA and RNA were extracted using a Qiagen RNA/DNA kit (Qiagen Ltd, UK). The technique utilises an ion exchange resin in a small column ('Qiagen tip'). Alternative techniques involve phenol and chloroform extraction or caesium chloride step-gradient ultracentrifugation. The cell lysis buffer in the Qiagen kit contained guanidine isothiocyanate to eliminate RNAases, the ubiquitous contaminants which rapidly destroy

RNA. DNA remains bound to the resin (positively-charged DEAE, DiEthylAminoEthane groups) over a wide range of salt concentrations allowing RNA and other substances to be eluted from a column. The DNA remains bound until eluted with a high-salt buffer. The nucleic acids were separated from the salt by precipitation with isopropanol.

2.3.2 Detection of the presence of microbial 16S rRNA genes by PCR

The integrity of each DNA sample was confirmed by identifying the presence of β-actin as previously described (Kotecha *et al.*, 1996, Kotecha *et al.*, 2004) (Fig 2.7). Sequences for 16S ribosomal DNA genes were amplified by PCR using the primers FD1 (AGA GTT TGA TCC TGG CTC AG) and rP1 (ACG G(T/A/C)T ACC TTG TTA CGA CTT) at concentrations of 0.2 µM in the presence of *Taq* DNA Polymerase (Abgene, Epsom, UK) as previously described (Kotecha *et al.*, 2004).

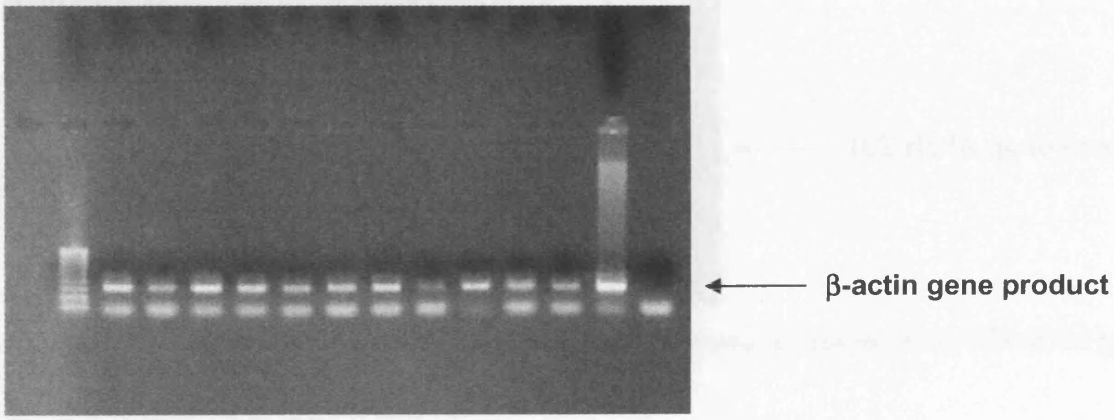


Figure 2.7 Electrophoresis gel demonstrating the presence of the β-actin gene product (315bp) in all samples (negative control on the far right well)

The DNA template was added to the PCR mixes including the primers described above and subjected to 95°C for 2 min followed by 30 cycles at 95°C for 30 sec, 55°C for 40 sec and 72°C for 2 min followed by a 10 minute extension cycle at 72°C. The reaction mixture is listed below:

Magnesium chloride	2.4µl	
10x reaction buffer	2.0 µl	
dNTP mixture 10mM	0.5 µl	(deoxyribonucleotide mixture)
FD1 primer	0.4 µl	

rP1 primer	0.4 µl
Taq polymerase	0.1 µl
DNA sample	1.0 µl
Water	12.7 µl
Mineral oil to cover reaction mixture	

Before addition of the DNA samples, the reaction mixture was treated with DNase I to remove any contaminating bacterial DNA as previously described (Hilali *et al.*, 1997, Kotecha *et al.*, 2004). The subsequent reaction products were separated by electrophoresis on 1.2% agarose gels stained with ethidium bromide and visualised using UV illumination (Figure 2.8).

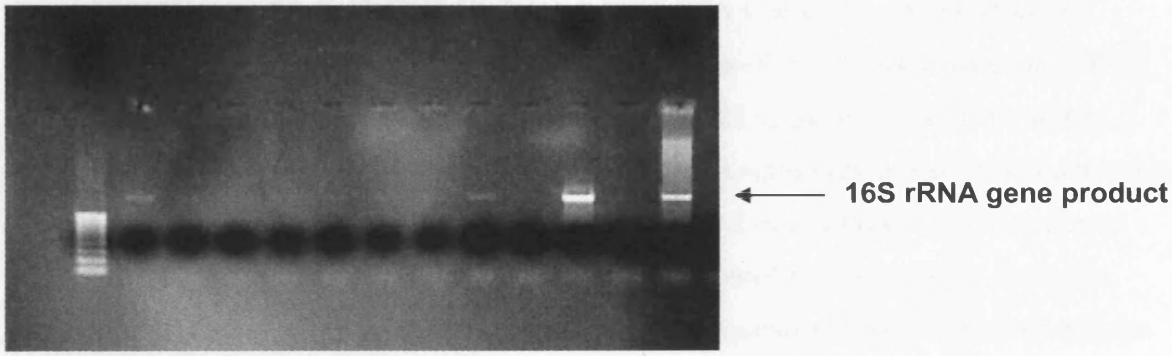


Figure 2.8 Visualisation of the DNA band representing amplification of the 16S rRNA gene.

The PCR DNA products were cut from the gel, purified using the QIAquick gel extraction system (Qiagen, Crawley, UK) and sequenced (Tables 3.2 & 3.3). In most cases organisms could then be identified. This was achieved by obtaining a 400 – 500 base pair sequence (by using Chromas software, version 1.45, Griffith University, Queensland, Australia) and finding the closest matches for the sequence using BLAST searches of databases posted at http://www.ncbi.nlm.nih.gov/.

The PCR method was based on that used to identify organisms in BAL fluid (Kotecha *et al.*, 2004). The method of PCR detection of infection is critical to the results obtained. Of particular importance is the number of PCR cycles required. In each cycle the amount of DNA is doubled. The sensitivity of PCR is such that if enough cycles are performed, then 16S rRNA genes will be observed in all samples. In trials with gastric fluid, it was found that if 20 cycles of PCR were performed, then only one sample was

positive for bacterial genes. However when 40 cycles were performed all samples appeared to have 16S rRNA gene product bands to some extent.

The number of cycles selected was thirty, as there appeared to be a good contrast between positive and negative results. Nested PCR can be used to increase the sensitivity of the PCR reactions. This involves performing a second round of PCR on the product of the first round of PCR. This technique was used for the detection of bacteria genes in cDNA derived from RNA extracted from BAL fluid in previous study (Kotecha *et al.*, 2004). However, the increased sensitivity was not required for the samples in this investigation.

2.3.3 Detection of *Uu* by using specific primers

PCR was applied to all samples to detect presence of *Uu* by using specific primers to the urease gene (U5 – CAA TCT GCT CGT GAA GTA TTA C and U4 – ACG ACG TCC ATA AGC AAC T) as previously described (Blanchard *et al.*, 1993, Kotecha *et al.*, 2004). Amplification by PCR with these primers resulted in a 428 bp product fragment of the components UreA and UreB of the *Uu* urease complex. Amplification was carried out using *Taq* DNA Polymerase (Abgene, Epsom, UK) at 95°C for 2 min followed by 30 cycles of 95°C for 30 sec, 55°C for 40 sec and 72°C for 1 min followed by a 10-minute extension cycle at 72°C. The reaction products were separated by agarose gel electrophoresis and the presence or absence of bands was again identified by examination of the gels under UV illumination (Fig. 2.9).

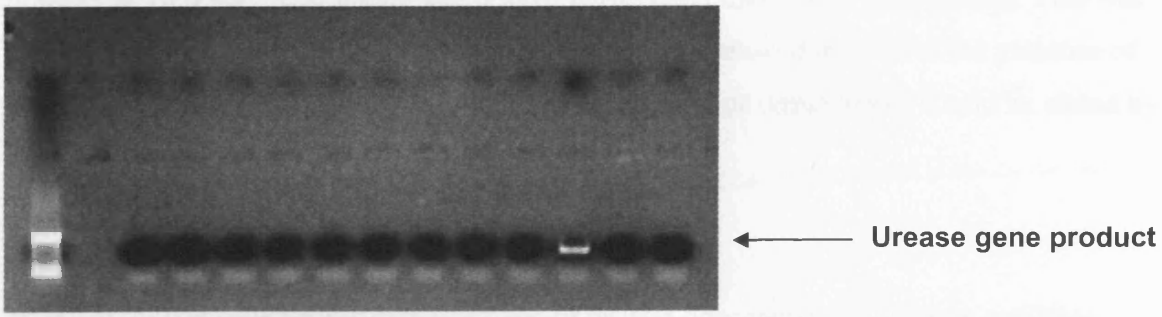


Figure 2.9 Specific PCR for Ureaplasma urealyticum. The appearance of the band represents the presence of Ureaplasma urealyticum in the sample.

2.4 Protein extraction and measurement of total protein and cytokines

2.4.1 Method of protein extraction

In order to determine the cytokine concentrations within the fetal membrane and chorionic plate, protein/peptides were first extracted from the tissue samples. Placental tissue and fetal membrane samples were homogenised in a solution containing HEPES buffer in an approximate ratio of 30μg sample to 300μl of buffer solution. Samples were homogenised individually. The solution used was that described previously (Sacks *et al.*, 1993, Crew *et al.*, 1999).

20 mmol/l HEPES (N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid)
750 μmol/l EDTA (ethylenediaminetetraacetic acid)
1 mmol/l benzamidine
500μmol/l phenylmethylsulphonyl-fluoride
1mg/μl oromucoid
pH 7.4 at 20°C.

HEPES is a biological buffer that is known not to interfere with testing by ELISA (enzyme-linked immunosorbent assay, Section 2.4.3). Other buffers can disrupt the assays by stripping away antibody that is bound to the ELISA microplates.

Cells were disrupted using a sonicating motor for 10 minutes and then spun for 10 minutes at 10,000g. Fetal membrane proved particularly difficult to homogenise. This was homogenised as far as possible and the soluble extract removed in spite of the presence of residue. The assumption was made that protein and peptide components would be eluted by the procedure in spite of the presence of a residue.

2.4.2 Review of methods of protein measurement

A variety of methods for the determination of protein concentrations are now available (Sapan *et al.*, 1999). Protein concentrations were measured in this study with the Biorad DC (detergent compatible) assay. This assay is a colormetric assay similar to the method described by Lowry (1951), but with a more rapid development of colour (maximum colour within 15 minutes) and improved colour stability. The assay involves a reaction between protein and copper in an alkaline medium (an alkaline copper tartrate solution) followed by

the reduction of the Folin agent by the copper-treated protein. The reduction of the Folin reagent gives rise to several possible compounds with a blue colour. Colour development is primarily due to the amino acids tyrosine and tryptophan and to a lesser extent cystine, cysteine and histidine. Therefore there is some variation in measurements depending on the protein present in the sample. Although the maximum absorbance of the compounds is at 750nm, the assay can also be read at 650nm. The microplate protocol with 5µl of samples or standards was carried out.

2.4.3 Introduction to Enzyme-Linked Immunosorbent Assay

The development of the Enzyme-Linked Immunosorbent Assay (ELISA) has allowed investigators to more easily measure cytokines. Prior to this development assays of bioactivity such as the hepatocyte stimulation assay and were required (Romero *et al.*, 1990). The principles of ELISAs are outlined below (Section 2.4.5).

2.4.4 Choice of Cytokines to be Measured

An increase in many cytokines, including IL-1, IL-6, IL-8, TNF-α is associated with chorioamnionitis (Lu and Goldenberg 2000) and significant increases in cytokines have been associated with infection in patients with preterm labour (El-Bastawissi *et al.,* 2000, Romero *et al.,* 1990).

The most frequently studied cytokine in amniotic fluid (AF) in relation to preterm delivery is IL-6 (El-Bastawissi *et al.,* 2000). IL-6 is a major mediator of the host response to tissue damage and infection. AF IL-6 is established as a marker of intra-amniotic inflammation (Romero *et al.*, 1990, Romero *et al.*, 1993a,b). It is strongly associated with preterm delivery and is a stronger determinant of preterm delivery than positive amniotic fluid cultures (Romero *et al.*, 1993a,b, Hillier *et al.*, 1993, El-Bastawissi *et al.*, 2000, Jacobsson *et al.*, 2003a,b). The change in AF IL-6 has consistently exceeded those of other cytokines (Hillier *et al.*, 1993, Saito *et al.*, 1993, Yoon *et al.*, 1998a). In one study AF IL-6 was increased in 88% of preterm deliveries before 34 weeks compared to 50%, 42%, 44% for IL-1α, IL1-β, and TNF-α respectively. In addition, the measurement of second trimester AF IL-6 has been found to predict preterm delivery (Wenstrom *et al.,* 1998) and pregnancy loss (Wenstrom *et al.,* 1996).

AF IL-8, a potent neutrophil chemoattractant, has also been measured in the amniotic fluid. Increased levels of AF IL-8 in the amniotic fluid have been detected in

association with infection in the context of preterm labour with and without intact membranes (Saito *et al.,* 1993, Jacobsson *et al.,* 2003a,b).

Concerning long-term outcome, both AF IL-6 and IL-8 have been associated with the development of BPD/CLD, PVL and cerebral palsy (Yoon *et al.*, 1997a,d, Ghezzi *et al.*, 1998, Yoon *et al.*, 2000a). As reviewed in the introduction, IL-6 and IL-8 have been measured in bronchoalveolar lavage (BAL) fluid and elevated concentrations have been associated with the subsequent development of chronic lung disease (Section 1.2.3). Elevated IL-6 in cord blood has been associated with neurological morbidity (Appendix 1.3).

In view of this evidence both IL-6 and IL-8 were measured in amniotic fluid, BAL fluid, cord blood, fetal membrane and placental tissue.

2.4.5 Measurement of IL-6 and IL-8 in supernatants

IL-6 and IL-8 were estimated in supernatants (amniotic fluid, BAL fluid and cord blood) using commercially available kits (R&D Systems Europe Ltd, Oxon, UK). The assays for both cytokines consisted of sandwich immunoassays (Fig. 2.10). A monoclonal antibody specific for IL-6 or IL-8 was pre-coated onto microplates. The standards and samples were added to the wells allowing IL-6/IL-8 to bind to the fixed antibody (Fig. 2.10A/B). After washing, an enzyme-linked polyclonal antibody for IL-6/IL-8 was added to the well. After a further wash, a substrate solution was added to the well and colour was observed in proportion to the concentration of cytokine in the sample (Fig. 2.10C). The results were obtained using a plate reader measuring absorbance at 450nm. A calibration curve was performed in duplicate using standards provided with the kit to allow calculation of the results. One hundred microlitres of sample was required for the IL-6 assay and fifty microlitres for the IL-8 assay. Because of the high haematocrit of cord blood, there was insufficient serum for testing in some cases and so sample dilution was required. The lowest detection limit for undiluted samples was 0.7pg/ml for IL-6 and 10pg/ml for IL-8 and neither assay was reported to have significant cross-reactivity. The results were expressed as pg per ml for amniotic fluid, BAL fluid and cord blood.

Protein was extracted from placenta and from fetal membranes by homogenising in HEPES buffer and sonication as previously outlined (Section 2.4.1). This method of extraction was known to be compatible with the ELISA cytokine assay. The IL-6 and IL-8 were estimated in the protein extracts using the same kit as described above. The total

protein in the extracts was measured and the tissue cytokine results expressed as pg per mg of protein.

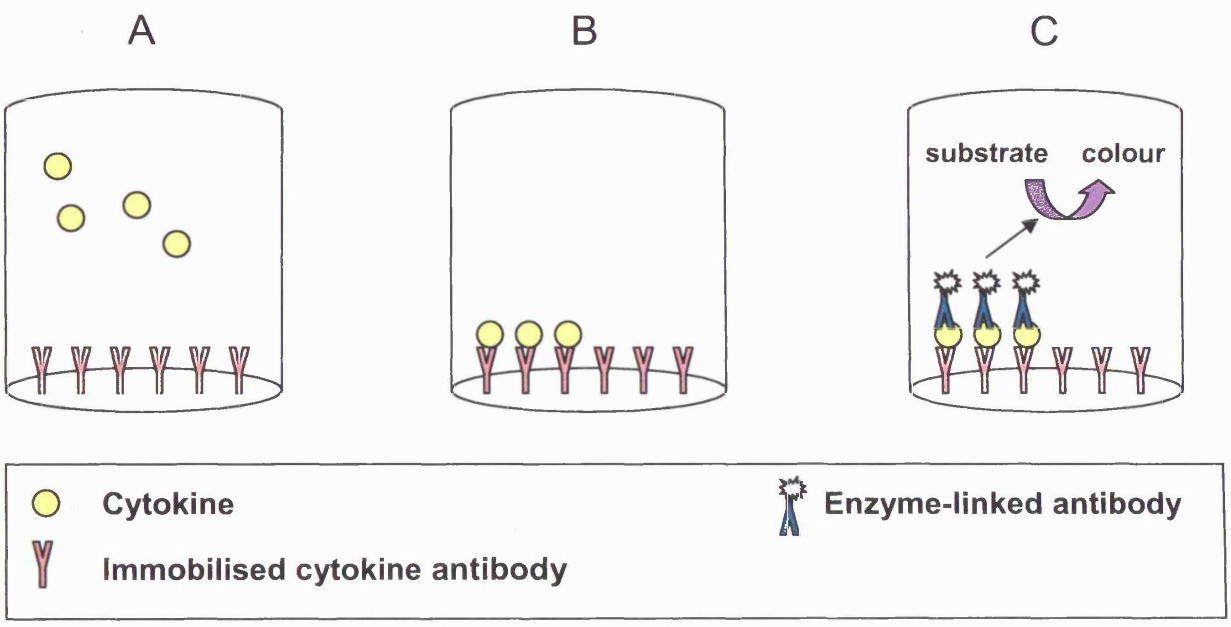


Figure 2.10 Diagrammatic representation of the ELISA for IL-6 and IL-8. See text for explanation (Section 2.4.5)

2.4.6. Measurement of cortisol

The rationale for the measurement of cortisol is discussed in Chapter 8 (Section 8.1.2). Cortisol measurements were determined with a cortisol ELISA (DRG Instruments GmbH, Germany) designed for the measurement of cortisol in serum or plasma. As with the cytokine kits described above, this kit came prepared as a microplate tray with 96 wells. The main advantage of this particular assay was the small volume of sample required (20µl).

The test was a competitive type immunoassay with competition between horseradish peroxidase-labelled cortisol and cortisol in the samples and standards (Fig. 2.11A). After allowing antibody binding to take place the chromogen/substrate solution was added and was converted to a blue compound (Fig. 2.11B). Being a competitive assay, the lower the cortisol concentration, the greater the colour.

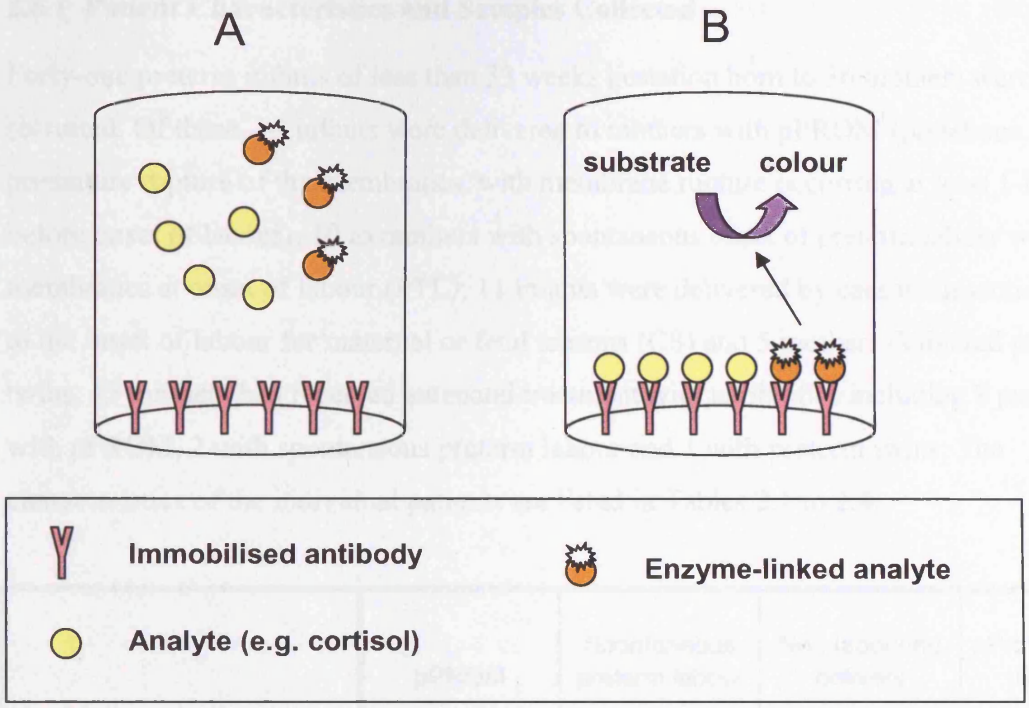


Figure 2.11 Diagrammatic representation of the Cortisol ELISA. See text for explanation (Section 2.4.6)

The lower limit of detection of the assay is 6.9nmol/l, well below the expected concentrations of cortisol. There was a 60% cross reactivity of the assay with prednisolone, but only 0.1% with dexamethasone. The cross-reactivity of the assay with dexamethasone was an important consideration in this study as some mothers received dexamethasone shortly before delivery (betamethasone was not used during this study period). Cortisol was measured only in samples of cord serum (but not in postnatal blood) because of the likely confounding effects of postnatal stress on the cortisol concentration.

2.5 Data collection

Data collection was based on details gathered for the Trent Neonatal Survey (Field 2000). Additional information on maximum ventilation, maximum oxygen and medication in each 24 hour period was also recorded.

2.5.1 Patient Characteristics and Samples Collected

Forty-one preterm infants of less than 33 weeks gestation born to 36 mothers were recruited. Of these, 10 infants were delivered to mothers with pPROM (prelabour premature rupture of the membranes, with membrane rupture occurring at least 1-hour before onset of labour); 10 to mothers with spontaneous onset of preterm labour with intact membranes at onset of labour (PTL); 11 infants were delivered by caesarean section prior to the onset of labour for maternal or fetal reasons (CS) and 5 mothers delivered preterm twins. 13 mothers had received antenatal treatment with antibiotics including 8 presenting with pPROM, 2 with spontaneous preterm labour and 1 with preterm twins. The characteristics of the individual patients are listed in Tables 2.1 to 2.4.

	pPROM	Spontaneous preterm labour	Non-labouring delivery	Preterm twin delivery
Number	10	10	11	10
Gestation	30 (24-32)	28 (25-32)	30 (29-32)	30 (26-32)
Birth weight	1470 (610-2050)	1220 (670-2070)	1090 (780-1942)	1175 (900-1560)
Antenatal dexamethasone	9/10 (90%)	9/10 (90%)	11/11 (100%)	10/10 (100%)
Caesarean section	3/10 (30%)	4/10 (40%)	11/11 (100%)	6/10 (60%)
Respiratory support	6/10 (60%)	9/10 (90%)	7/11 (64%)	7/10 (70%)
Surfactant	5/10 (50%)	8/10 (80%)	7/11 (64%)	6/10 (60%)
CLD at 28 d	2/9* (22%)	5/10 (50%)	4/11 (36%)	5/10 (50%)
CLD at 36 wk	2/9* (22%)	2/10 (20%)	4/11 (36%)	3/10 (30%)

CLD, chronic lung disease

* one infant death at 24 hours

Table 2.1 Summary table of the characteristics of the forty-one recruited mother-baby pairings listed by delivery category

Amniotic fluid was available from 14 deliveries. Gastric fluid was obtained soon after delivery from 29 infants. BAL fluid was obtained on the first day of life in 21 infants who required mechanical ventilation for respiratory disease. It was expected that the microbiology of gastric fluid and BAL fluid would reflect amniotic fluid microbiology as fetal lung fluid, gastric fluid and amniotic fluid are in continuity *in utero*. In this way, a fluid sample representative of the amniotic cavity was obtained in all but one delivery. Samples of chorionic plate and fetal membrane tissue were also obtained from most deliveries (Tables 3.2 to 3.3).

2.5.2 Statistical power

The number of mother-baby pairings to be recruited was estimated by assuming there would be a 70% concordance between the presence of antenatal infection (defined according to the presence of 16S rRNA genes in intrauterine samples) and the presence of inflammation, and then a 70% concordance between lack of 16S rRNA genes and inflammation. To achieve a power of 80% at $p<0.05$ level it was estimated that 36 mother-baby pairings would be needed.

Table 2.2: Individual characteristics of patients recruited

Study No	Gestation	Birth weight	IUGR	Delivery Category	Actual delivery
3	30	1040	Yes	CS	Emergency caesarean
4	30	1060	Yes	CS	Emergency caesarean
5	30	780	Yes	CS	Emergency caesarean
6	30	1090	Yes	CS	Emergency caesarean
17	32	1100	Yes	CS	Emergency caesarean
18	29	1080	Yes	CS	Emergency caesarean
19	32	1350	Yes	CS	Emergency caesarean
26	31	1350	Yes	CS	Emergency caesarean
49	29	990	Yes	CS	Emergency caesarean
59	32	1290	Yes	CS	Emergency caesarean
65	31	1942	No	CS	Emergency caesarean
7	28	1050	No	PPROM	Normal vaginal
9	32	1650	No	PPROM	Emergency caesarean
14	25	730	No	PPROM	Emergency caesarean
38	32	2050	No	PPROM	Normal vaginal
45	30	1490	No	PPROM	Emergency caesarean
47	32	1790	No	PPROM	Normal vaginal
54	24	610	No	PPROM	Normal vaginal
57	30	1450	No	PPROM	Normal vaginal
58	32	1842	No	PPROM	Normal vaginal
63	29	1090	Yes	PPROM	Normal vaginal
2	28	1110	No	PTL	Normal vaginal
10	25	670	No	PTL	Normal vaginal
41	31	2070	No	PTL	Normal vaginal
46	26	930	No	PTL	Emergency caesarean
51	29	1330	No	PTL	Emergency caesarean
52	32	1810	No	PTL	Normal vaginal
53	28	970	No	PTL	Emergency caesarean
61	28	1350	No	PTL	Emergency caesarean
62	25	740	No	PTL	Normal vaginal
64	30	1770	No	PTL	Normal vaginal
1	30	1250	Yes	TWIN	Emergency caesarean
1	30	1020	Yes	TWIN	Emergency caesarean
8	26	940	No	TWIN	Normal vaginal
8	26	900	No	TWIN	Normal vaginal
13	32	1389	Yes	TWIN	Emergency caesarean
13	32	1446	Yes	TWIN	Emergency caesarean
16	27	1150	No	TWIN	Emergency caesarean
16	27	1030	No	TWIN	Emergency caesarean
60	31	1560	No	TWIN	Normal vaginal
60	31	1200	Yes	TWIN	Normal vaginal

Abbreviations: CS: prelabour caesarean section, PTL: preterm labour,
PPROM: prelabour preterm rupture of membrane

Table 2.3: Individual patient characteristics - antenatal treatment

Study No	Delivery Category	Dex (doses)	Antibiotic 1	Duration	Antibiotic 2	Duration
3	CS	2				
4	CS	2				
5	CS	1	Amoxicillin IV	5 hours		
6	CS	2				
17	CS	2	Cephalexin PO	18 hours		
18	CS	2				
19	CS	2				
26	CS	2				
49	CS	1				
59	CS	2				
65	CS	2				
7	PPROM	4	Erythromycin PO	10 days		
9	PPROM	2				
14	PPROM	2	Cephalexin PO	2 days	Metronidazole PO	1 day
38	PPROM	1	*			
45	PPROM	2	Erythromycin PO	4 days		
47	PPROM	0	Cefuroxime IV	1 hour		
54	PPROM	2				
57	PPROM	2	Erythromycin PO	6 days	Clarithromycin IV	4 hours
58	PPROM	2	Erythromycin PO	2 days		
63	PPROM	4	Erythromycin PO	8 days	Metronidazole PO	8 days
2	PTL	3				
10	PTL	2				
41	PTL	2	*			
46	PTL	1				
51	PTL	2				
52	PTL	4	Cephalexin PO**	10 days	Cefuroxime IV	1 dose
53	PTL	1				
61	PTL	1	Cefuroxime IV	6 hours	Metronidazole IV	6 hours
62	PTL	1				
64	PTL	0				
1	TWIN	4				
1	TWIN	4				
8	TWIN	1				
8	TWIN	1				
13	TWIN	2				
13	TWIN	2				
16	TWIN	1				
16	TWIN	1				
60	TWIN	3	Erythromycin PO	15 hours		
60	TWIN	3	Erythromycin PO	15 hours		

* antibiotics during pregnancy for UTI PO: oral antibiotic Dex:dexamethasone

** course completed 2 weeks prior to delivery IV: intravenous antibiotic

Listing of antenatal treatment. All but two mothers received dexamethasone before delivery. Five of the pPROM mothers received erythromycin in accordance with the findings of the ORACLE I trial.

Table 2.4: Individual patient characteristics - Respiratory

Study No	Ventilator support	Surfactant given	Radiological RDS	CLD28	CLD36
3	VENT	Yes	Yes	Yes	Yes
4	VENT	Yes	Yes	Yes	Yes
5	VENT	Yes	Yes	No	No
6	VENT	Yes	No	No	No
17	NONE	No	Yes	No	No
18	VENT	Yes	Yes	Yes	Yes
19	NONE	No	No	No	No
26	CPAP	Yes	Yes	No	No
49	VENT	Yes	Yes	Yes	Yes
59	NONE	No	No	No	No
65	NONE	No	No	No	No
7	NONE	No	No	No	No
9	NONE	No	No	No	No
14	VENT	Yes	Yes	Died	Died
38	CPAP	Yes	Yes	No	No
45	VENT	Yes	No	No	No
47	NONE	No	Yes	No	No
54	VENT	Yes	No	Yes	Yes
57	VENT	Yes	Yes	Yes	Yes
58	NONE	No	No	No	No
63	CPAP	No	Yes	No	No
2	VENT	Yes	Yes	Yes	Yes
10	VENT	Yes	Yes	No	No
41	NONE	No	No	No	No
46	VENT	Yes	Yes	Yes	No
51	VENT	Yes	No	No	No
52	CPAP	No	No	No	No
53	VENT	Yes	Yes	Yes	Yes
61	VENT	Yes	No	Yes	No
62	VENT	Yes	Yes	Yes	No
64	VENT	Yes	Yes	No	No
1	VENT	Yes	Yes	Yes	Yes
1	VENT	Yes	Yes	Yes	Yes
8	VENT	Yes	Yes	No	No
8	VENT	Yes	Yes	Yes	No
13	NONE	No	No	No	No
13	NONE	No	No	No	No
16	VENT	Yes	Yes	Yes	No
16	VENT	Yes	Yes	Yes	Yes
60	NONE	No	No	No	No
60	CPAP	No	No	No	No

RDS: respiratory distress syndrome, CLD: chronic lung disease at 28 days/36 weeks

VENT: mechanical ventilation, CPAP: continuous positive airway pressure

Listing of ventilatory support and significant respiratory outcomes.

2.6 Ethical considerations

This study was approved by the Leicestershire Research Ethics Committee. Consent for the study was obtained as early as possible before delivery where possible. It was acknowledged that obtaining consent during threatened preterm labour was difficult, but that this was more acceptable than obtaining retrospective consent.

2.6.1 Ethics and Consent

The nature of the research undertaken here fulfilled the criteria required for 'non-benefit' research:

1. Information on the disease (i.e. antenatal infection) could only be obtained from newborn subjects and their mothers.
2. There would be minimal risk of harm to infants in the study.

Consent in neonatal research has been a subject of both debate and study (Allmark *et al.*, 2003). It is acknowledged that obtaining consent around the time of delivery of a sick premature infant is difficult. This issue was discussed with the local research ethics committee. In introducing the study to parents, they were issued with an information sheet as shown at the end of this chapter (Fig. 2.12). They were informed that an independent research ethics committee had approved the study and the right of the parents to withdraw from the study at any stage was emphasised. I obtained signed written consent in all cases, with a further member of staff as a witness. The consent form is shown in Fig. 2.13. Concerns have been expressed on the recall of parents who have allowed their infants to participate in research trials. I therefore continued discussions regarding the study with the parents after delivery.

2.7 Key points

1. In this chapter are details of the clinical and laboratory methodology including sample collection, PCR-based microbial detection and the cytokine analysis of fluids and tissues specimens. The rationale for the selecting the appropriate methods is discussed.

2. By analysing amniotic fluid proxies such as gastric fluid and bronchoalveolar lavage fluid, a fluid sample likely to be representative of the amniotic cavity was obtained in all but one delivery.

3. The PCR-based detection techniques included both broad-spectrum microbial detection, targeting the 16S rRNA gene, and specific PCR detection for *Ureaplasma urealyticum.*

4. The pro-inflammatory cytokines, IL-6 and IL-8 were selected for the measurement of intrauterine and fetal inflammation.

5. The recruited mother-baby pairings were divided into four main preterm delivery categories: prelabour caesarean section, prelabour preterm rupture of membranes (pPROM), spontaneous delivery with intact membranes and spontaneous twin deliveries.

6. The nature of the research undertaken here fulfilled the criteria required for 'non-benefit' research. Signed written consent was obtained in all cases.

Figure 2.12: Patient Information Sheet for parents

The search for intra-uterine factors in the pathogenesis of preterm labour and respiratory disease in newborn infants

Principal Investigator: **Dr Sailesh Kotecha, Consultant Neonatologist**

We would like to invite you to participate in the above study that is being carried out at the Leicester Royal Infirmary.

I am sorry to have to bother you at what I know must be a difficult time. However, I would be grateful if you would take the time to read this information sheet and consider participating in this study.

What do we know?
Whilst some women undergo a caesarean birth before full term because they or their baby are unwell, in most cases we do not know why women go into early labour. We know that in some women germs, which normally live on the skin and in the vagina, find their way into the womb and can trigger the process. This is not because the mother has done something wrong and it is not an infection she has caught. These are germs that have simply found their way into the wrong place. These same germs can sometimes make the baby ill.

What are we trying to find out?
However, we simply do not know how often infection may trigger premature birth and how or which specific germs affect the baby particularly their breathing. We are therefore trying to find out how often we can identify germs in women who undergo premature birth and we are also trying to identify the specific germs using sensitive techniques so we can develop targeted therapy against these germs.

What do we need for the study?
When your baby is born we will obtain a small piece of the placenta (after-birth), umbilical cord and blood (from the umbilical cord left behind). We will also use any fluid sucked from the baby's mouth at delivery. These samples will be frozen and used in our tests for infection.

We are also keen to know if any germs involved in setting off early delivery also get to the baby. For this reason we would like to suck a tiny sample of fluid from the baby's mouth (or breathing tube if he or she is requiring help with breathing) twice weekly whilst the baby is in hospital. This is often done as part of routine care and is quick and painless. However, it may need to be done as an extra sample not part of normal care. Again we would like to use this sample to test for the presence of germs.

Will you be able to get the results?
The tests for infection are very much more specialised than those we normally use and take a long time. Because of this we will not be able to use the results in the care of you or your baby. What we hope is that the results will help mothers and babies in the future.

Will information in the study be confidential?
The information collected during the study will be recorded in your medical notes and will be treated with the usual degree of confidentiality under the data protection act. You will not be identified in any way in any documents relating to the trial. Your GP will be informed of your participation in the trial.

What if I am harmed by the study?
Medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs.

What do you need to do next?
Once you have read the Information Leaflet and have had an opportunity to ask any questions that you may have, we will come back and ask you to sign a separate consent form. Whether you decide to participate in the study or not the care of you or your baby will not be affected. If wish you, you may join only part of the study. The doctors and midwives will be happy to answer any questions that you may have.

What happens if I do not wish to participate in this study or wish to withdraw from the study?

If you do not wish to participate in this study or if you wish to withdraw from the study at any time, you may do so without justifying your decision and the future treatment of you or your baby will not be affected.

Many thanks for taking the time to read this letter and I hope that you and your baby make rapid progress.

Figure 2.13: Consent form for parents delivering a preterm infant

The search for intra-uterine factors in the pathogenesis of preterm labour and respiratory disease in newborn infants

Principal Investigator: Dr Sailesh Kotecha,
Consultant Neonatologist
Neonatal Unit, Leicester Royal Infirmary
Tel: 0116 258 6464/5502

This form should be read in conjunction with the Patient Information Leaflet

I agree to take part in the above study as described in the Patient Information Leaflet, version 1 dated 15/01/01.

I agree to samples of placenta, amniotic fluid, cord and cord blood and fluid sucked from my baby's mouth or lungs being used in the study.

I understand that I may withdraw from the study at any time without justifying my decision and without affecting my normal care and medical management.

I understand that members of the research team may wish to view relevant sections of my medical records, but that all the information will be treated as confidential.

I understand medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs.

I have read the patient information leaflet on the above study and have had the opportunity to discuss the details with _____and ask any questions.

The nature and the purpose of the tests to be undertaken have been explained to me and I understand what will be required if I take part in the study.

Signature of Patient _____
Name _____
Date _____

I confirm I have explained the nature of the Study, as detailed in the Patient Information Leaflet, in terms which in my judgement are suited to the understanding of the patient.

Signature of Investigator _____
Date _____
Name in BLOCK LETTERS
_____

Signature of Witness_____
Date _____
Name in BLOCK LETTERS
_____

Chapter 3: The detection of intra-uterine and intra-amniotic infection using the polymerase chain reaction

3.1 Introduction

Although there is a clear association between intrauterine inflammation and infection (Hillier *et al.*, 1988), inflammation has been consistently observed more frequently than intrauterine infection detected by culture (Appendices 1.7, 1.8). Inability to culture organisms in the presence of inflammation could be due to low levels of bacteria in amniotic fluid, previous antibiotic therapy and the antibacterial effects of amniotic fluid. *Ureaplasma urealyticum* (*Uu*) and other mycoplasmas can be difficult to culture in view of their fastidious growth requirements. The detection of *Uu* is particularly important as it is implicated in both spontaneous preterm birth and the development of chronic lung disease.

The detection of microbial genomes using polymerase chain reaction (PCR) based techniques has now been applied to a range of clinical samples (Rantakokko-Jalava *et al.*, 2000). This molecular detection appears to be more sensitive than bacterial culture in the detection of infecting organisms.

3.2 Hypothesis

That reliable information regarding intrauterine infection may be obtained by applying PCR-based bacterial detection to gastric fluid, bronchoalveolar lavage (BAL) fluid and placental tissues.

3.3 Methods

3.3.1 Techniques used

DNA and RNA extraction (Section 2.3.1)

Detection of presence of microbial 16S rRNA genes by PCR (Section 2.3.2)

Detection of *U. urealyticum* by using specific primers (Section 2.3.3)

3.3.2 Statistical analysis

Chi-squared analysis was employed to compare rates of infection detection between the four preterm delivery groups.

3.4 Results

3.4.1 Detection of bacterial genes (16S rRNA and *Uu* urease gene)

The 16S rRNA gene or urease gene was detected in at least one sample in sixteen deliveries (Tables 3.1 to 3.3). The presence of 16S rRNA genes were detected mainly in the pPROM (7/10) and spontaneous preterm labour groups (8/10) with only one positive sample in the non-labouring group (unidentified bacterial presence in placenta) (Table 3.1). The results are presented in the four preterm delivery categories (Section 2.4.1).

	CS	PTL	PPROM	Twin	Significance
PCR positive (all tissues)	1/11	8/10	7/10	0/10	0.001
PCR positive (amniotic cavity)	0/11	5/10	7/10	0/9	0.005

Table 3.1 Numbers of organisms detected by 16SrRNA PCR or specific PCR for Uu by delivery category. All but one PCR positive sample was found in the PTL and pPROM groups. Amniotic cavity specimens included amniotic fluid, gastric fluid and bronchoalveolar lavage fluid.

Table 3.2: PCR results (amniotic cavity specimens)

Study No	Delivery Group	Amniotic fluid PCR	Gastric fluid PCR	BAL fluid PCR
3	CS			
4	CS			
5	CS			
6	CS			
17	CS			
18	CS			
19	CS			
26	CS			
49	CS			
59	CS			
65	CS			
7	PP		Uu	
9	PP	Not sequenced		
14	PP		Peptostreptococcus Uu	
38	PP			
45	PP			Staph aureus
47	PP		Strep pneumonia	
54	PP		Uu	Uu
57	PP			
58	PP		Leptotrichii amnionii Uu	
63	PP			
2	PTL			
10	PTL		Fusobacterium	Fusobacterium
41	PTL			
46	PTL			Mixed
51	PTL	Uu	Uu	Uu
52	PTL			
53	PTL			
61	PTL	GV		
62	PTL		GBS	
64	PTL			
1.1	TW			
1.2	TW			
8.1	TW			
8.2	TW			
13.1	TW			
13.2	TW			
16.1	TW			
16.2	TW			
60.1	TW			
60.2	TW			

GV: Gardnerella vaginalis, GBS: Streptococcus agalactiae, Uu: Ureaplasma urealyticum, Strep: Streptococcus Staph: Staphylococcus

Identification of bacterial organisms by PCR (targeting 16S rRNA and Uu) in samples thought to be representative of amniotic fluid. Shaded boxes represent samples that could not be obtained.

Table 3.3: PCR results (cord blood and tissue specimens)

Study No	Delivery Group	Cord / infant blood	Fetal membrane	Placental tissue
3	CS			
4	CS			
5	CS			
6	CS			NK
17	CS			
18	CS			
19	CS			
26	CS			
49	CS			
59	CS			
65	CS			
7	PP		Uu	
9	PP		mixed	
14	PP		Acinetobacter	
38	PP			
45	PP			
47	PP			NK
54	PP		Uu	
57	PP			
58	PP			NK
63	PP			
2	PTL			NK
10	PTL		Fusobacterium	NK
41	PTL		Uu	
46	PTL			
51	PTL		Uu	
52	PTL		Uu	
53	PTL			
61	PTL			
62	PTL		Acinetobacter	
64	PTL			
1.1	TW			
1.2	TW			
8.1	TW			
8.2	TW			
13.1	TW			
13.2	TW			
16.1	TW			
16.2	TW			
60.1	TW			
60.2	TW			

NK: Not known- sequence could not be identified, Uu: Ureaplasma urealyticum

Identification of bacterial organisms by PCR (targeting 16S rRNA and Uu) in cord blood, placenta (chorionic plate) and fetal membrane samples. Shaded boxes represent samples that could not obtained.

There were a variety of organisms identified from DNA sequencing of the positive samples (Figs. 3.1 & 3.2, Tables 3.2 & 3.3). *Uu* was the most common organism identified (7 deliveries) and often was detected in multiple samples from the same mother-baby pairings. The other organisms identified are shown in tables 3.2 and 3.3. Some of these have been previously shown to be common vaginal organisms (e.g. *Gardnerella vaginalis*). Organisms, such as *Fusobacterium nucleatum*, have previously been implicated in the pathogenesis of preterm labour (Altshuler *et al.*, 1988). In some instances the organism could not be identified from the DNA sequence because of frequent ambiguities, possibly due to the presence of multiple organisms (Fig. 3.3). Microbial 16S rRNA genes or urease genes were most frequently identified in fetal membranes (n = 9/37) and in gastric fluid (n = 8/29). There were missing samples mainly for amniotic fluid, as this could only be harvested at caesarean section, and BAL fluid which was only obtained if the infants were ventilated.

In one case, culture of amniotic fluid after intravenous antibiotics was negative although PCR was positive for *Gardnerella vaginalis*. Amniotic fluid obtained by amniocentesis prior to antibiotic therapy was also culture positive for the same organism. Bacteria were detected in a number of deliveries despite antibiotic therapy with erythromycin. In six deliveries erythromycin was given to the mothers in view of the results of the ORACLE I trial (Kenyon *et al.*, 2001a). The mean duration of treatment was 5 days (range 15 hours to 10 days). In three of these deliveries, bacteria were detected by PCR.

3.4.2 Culture Results

Only 4 samples were positive on bacterial culture of amniotic fluid and chorioamnion swabs, with 2 each in the pPROM and spontaneous preterm labour groups (Table 3.4). The placental swabs grew (a) *Streptococcus pneumoniae* and (b) *Enterococcus spp.* The two amniotic fluid samples grew (c) *Streptococcus milleri* and (d) *Gardnerella vaginalis*. The corresponding results by using PCR were (a) *Streptococcus pneumoniae* in gastric fluid but mixed species from placenta, (b) *Fusobacterium nucleatum*, (c) mixed species from fetal membranes, and (d) *Gardnerella vaginalis* in amniotic fluid. In addition to this, a growth of *Streptococcus agalactiae* was obtained from culture of an endotracheal tube from one infant. The same organism was identified by PCR from gastric fluid. Thus the detection of 16S rRNA genes usually identified those organisms cultured but also identified the presence of other microbial genes in several samples. The discrepancy in (b) is looked at further in the discussion.

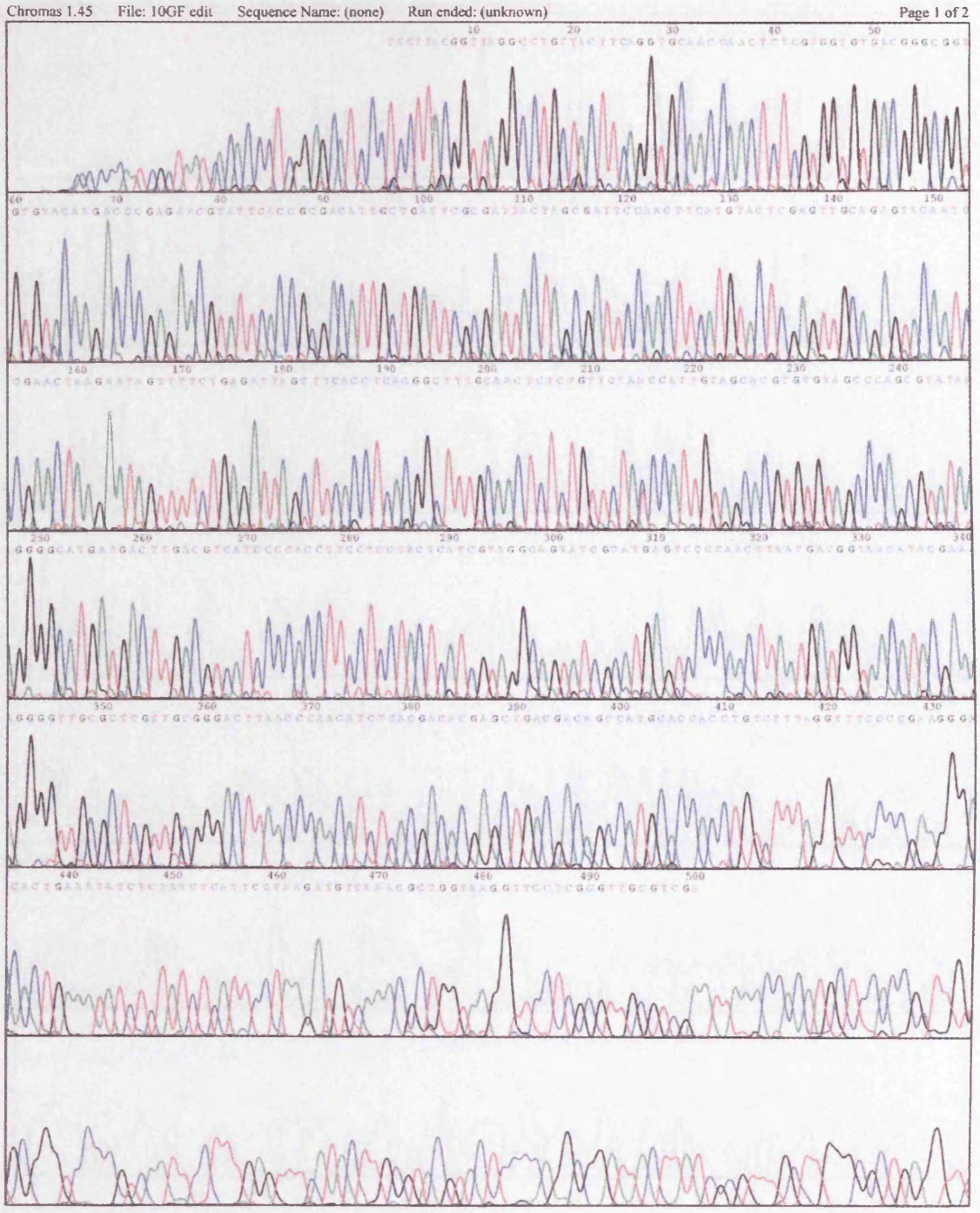


Figure 3.1: Example of DNA sequence obtained from gastric fluid (Fusobacterium nucleatum)

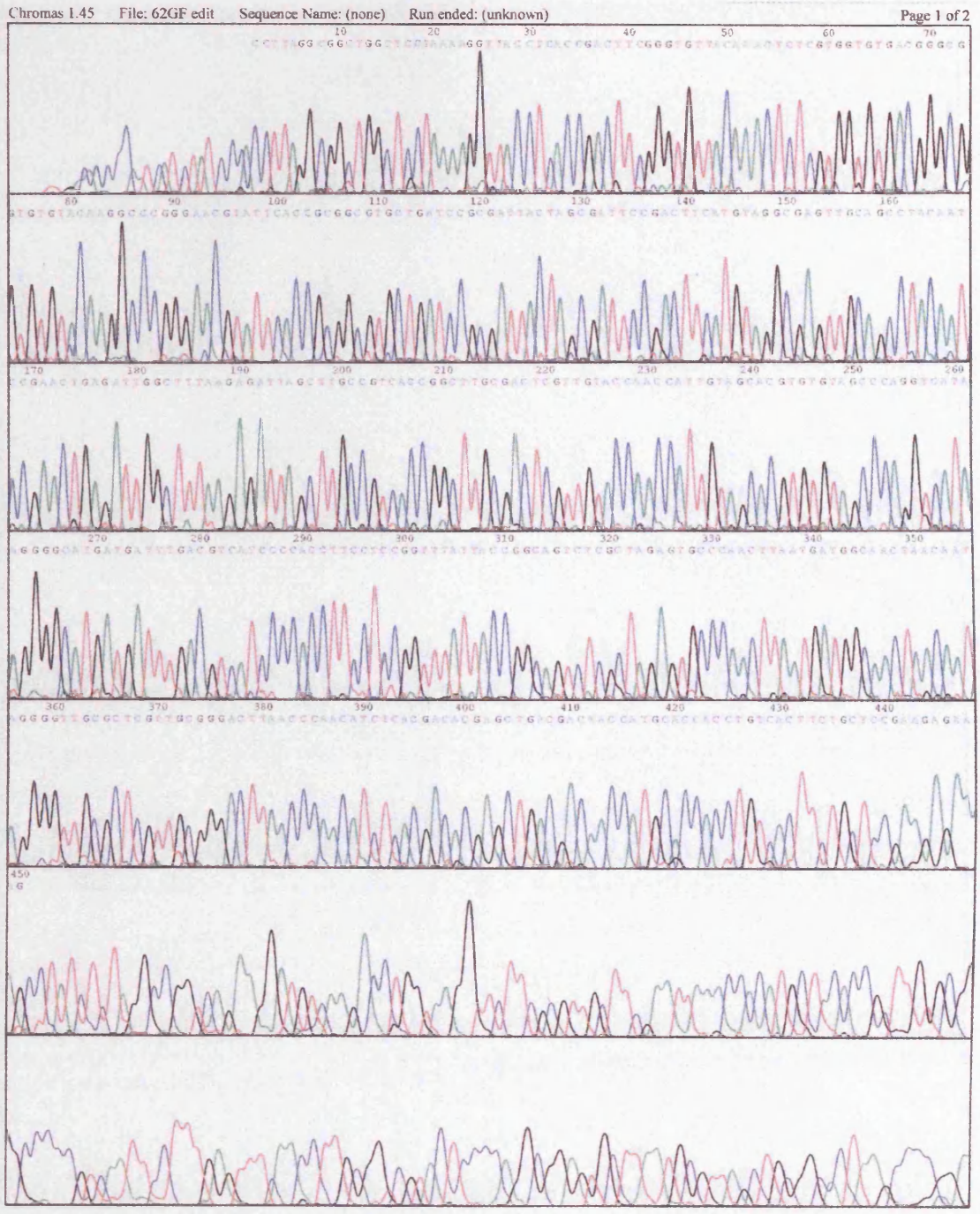


Figure 3.2: Example of DNA sequence obtained from gastric fluid (Streptococcus agalactiae)

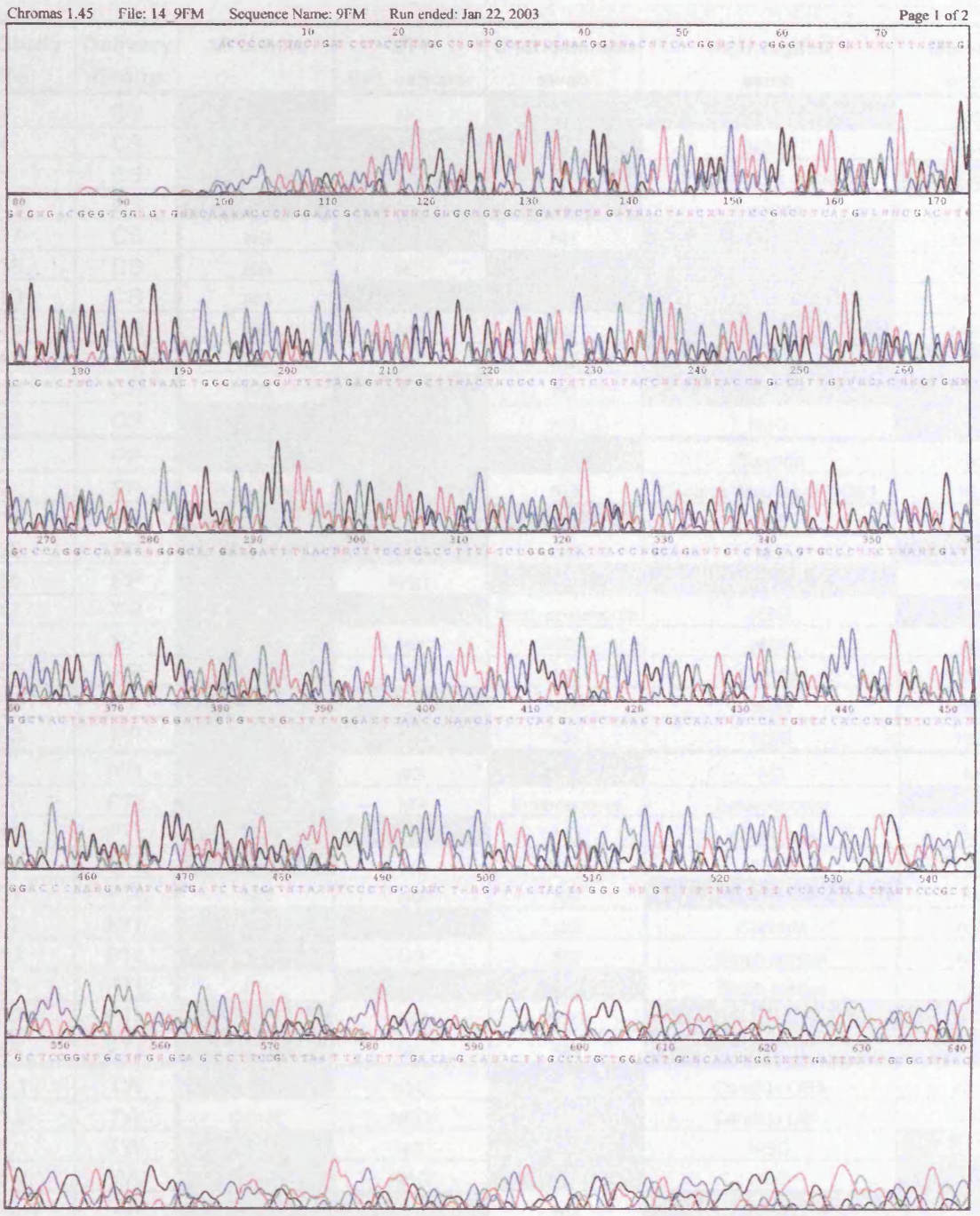


Figure 3.3: Example of a DNA sequence obtained from fetal membrane with too many ambiguities (N) to identify a single organism

Table 3.4: Perinatal culture results

Study No	Delivery Group	AF culture	ET / BAL catheter	Chorioamnion swab	High vaginal swab	Maternal urine
3	CS		NG			NG
4	CS				NSG	NSG
5	CS				Vaginal flora grade 3	
6	CS				Candida	NG
17	CS	NG		NG		NSG
18	CS	NG	NG			NSG
19	CS	NG				NSG
26	CS	NG	NG	NG		NSG
49	CS	NG	NSG	NG		
59	CS	NG		NG		
65	CS			NG	NSG	
7	PP				Candida	NG
9	PP	Strep milleri		NG	Candida Anaerobes ?GBS	NG
14	PP				NG	NG
38	PP			NG	NG	
45	PP		NSG			NSG
47	PP			Strep pneumonia	NSG	
54	PP		NG	NSG	NSG	NG
57	PP		NG	NG	Candida	NSG
58	PP			NSG	NSG	NSG
63	PP			NG	NSG	NSG
2	PTL		NG		NG	NG
10	PTL		NG	Enterococcus	Enterococcus	
41	PTL			NG	Anaerobes	NG
46	PTL		NG	NSG	NSG	NG
51	PTL	NG	NG	NG		NG
52	PTL			NG	Candida	NSG
53	PTL		NG	NG	Staph aureus	NSG
61	PTL	GV			Staph aureus	NSG
62	PTL		GBS	NSG		
64	PTL		NG	NG		
1.1	TW		NSG		Candida GBS	NSG
1.2	TW	CONS	NSG		Candida GBS	NSG
8.1	TW		NG		NSG	
8.2	TW		NSG		NSG	
13.1	TW			NG		
13.2	TW	NG		NG		
16.1	TW				NG	
16.2	TW	NG			NG	
60.1	TW			NG	NSG	NG
60.2	TW			NG	NSG	NG

Abbreviations: NG - no growth; NSG - no significant growth; Strep - Streptococcus; GV - Gardnerella vaginalis; CONS - coagulase negative staphylococci

Identification of bacterial organisms by laboratory culture of perinatal samples from both mother and infant. Shaded boxes represent samples that were not available.

3.5 Discussion

3.5.1. Bacterial gene detection in preterm deliveries

By using PCR to examine for evidence of microbes, we have confirmed a higher rate of detection of infection associated with spontaneous preterm delivery than detected by standard culture techniques. 70% of deliveries following pPROM and 80% of deliveries following preterm labour were associated with the presence of microbial genes in at least one tissue/fluid sample. Where multiple samples from the same mother-baby pair were present, good correspondence between microbes identified was observed. Some correspondence was also observed between microbes identified by standard culture techniques and those identified by PCR, suggesting that the technique is robust and that the hypothesis for this chapter is correct.

Our work, although on very small numbers, also supports previous work suggesting infection is less common as a mechanism of preterm labour in twin pregnancies (Romero *et al.*, 2002), and that a stretch mechanism may be the underlying cause of preterm delivery in multiple pregnancies.

We sequenced the PCR products of the amplified 16S rRNA genes and have identified the closest relatives of the sequences using published databases. Most of these close relatives we have identified have been associated with clinical infections. *U. urealyticum, Streptococcus agalactiae* (Group B *Streptococcus*) and *Streptococcus pneumoniae* are well known pathogens. *Streptococcus milleri*, also a beta-haemolytic, has been isolated from many infective sites and *Gardnerella spp.*, commensals of the vaginal tract, are associated with vaginosis and possibly preterm labour and intrauterine infection.

Recruitment to this study commenced soon after the publication of the ORACLE antibiotic trials (discussed in section 1.1.6.). Therefore mothers with pPROM were usually commenced on oral erythromycin. Despite this treatment, bacterial genes were still detected by PCR in the fluids and tissues studied (3 out of six deliveries). The reasons may include

1. Erythromycin is ineffective in eradicating organisms once established in the upper genital tact (although it might still prevent infection in some cases) – discussed in Section 1.1.6.
2. Bacterial products have remained *in situ* despite treatment

3.5.2 Individual organisms

3.5.2.1 Fusobacterium nucleatum

These are filamentous gram-negative organisms. The association between infection with fusobacteria and prematurity has been described. (Easterling *et al.,* 1985, Altshuler *et al.,* 1985, 1988). It has been found prenatally in amniotic fluid with intact membranes (Romero 1989). Chorioamniontis secondary to fusobacteria has been described and in a rat model, chorioamnionitis has been consistently produced (Altshuler *et al.,* 1985, 1988). The chorioamnionitis may be secondary to endotoxin which has been found in the amniotic fluid of a patient with fusobacterial infection.

3.5.2.2 Streptococcus agalactiae

Streptococcus agalactiae or group B streptococci is recognised as one of the most virulent organisms of the perinatal period. One case of group B infection was detected in this group associated with histological chorioamnionitis. In spite of its virulence, it has not always been associated with chorioamniontis (Bernischke & Kaufman, 2000).

3.5.2.3 Gardnerella vaginalis

This organism is a common organism in the vaginal flora. In the study by Hillier *et al (1988),* which recognised the importance of chorioamnionitis in prematurity, *Gardnerella vaginalis* was isolated in 26% of cases of premature delivery. Although usually thought of as only causing mild disease (Bernsichke & Kaufman, 2000), in this study the isolation of *Gardnerella vaginalis* was associated with both histological and clinical chorioamnionitis. Severe chorioamniontis and premature delivery has been previously described in association with *Gardnerella vaginalis* (Pinar *et al.,* 1988). Significantly, *Gardnerella vaginalis* was isolated on culture of amniotic fluid obtained by amniocentesis prior to antibiotic therapy but not from amniotic fluid obtained at delivery. However it was still possible to isolate this organism by PCR after antibiotic therapy underlining the utility of PCR for detection of organisms after such treatment.

3.5.2.4 Leptotrichii amnionii

A Medline search has indicated that there is only one previous case report of amniotic fluid infection with this recently described organism. Fetal demise was observed (Shukla *et al.,* 2002). In the cohort presented here it was isolated from the gastric fluid in a delivery in

association with *Uu* in pPROM delivery. Chorioamnionitis was present but because of co-infection (or co-colonisation), it is difficult to draw conclusions on the effect of this organism.

3.5.2.5 Ureaplasma urealyticum

This was the most frequently isolated organism in the samples collected. In a case control study of chorioammnionitis (Hillier *et al.,* 1988) *Uu* was found in 47% cases of prematurely delivered infants. Its presence was associated with chorioamnionitis. This finding is consistent with a previous report of the effects of *Uu* on both the amniotic and fetal compartments (Yoon *et al.,* 1998a). It was found in combination with other organisms in 3 deliveries and alone in a further 4 deliveries. Although isolated from fetal membrane, amniotic cavity, gastric fluid and BAL fluid, *Uu* was not isolated in any placental specimens from the same deliveries. The reason for this apparent compartmentalisation is not clear.

3.5.2.6 Peptostreptococcus

This organisms has been reported in association with preterm delivery (Hillier *et al.*, 1991)

3.5.2.7 Acinetobacter

This organism was isolated from two placental specimens. It is ubiquitous in the environment and it is said to colonise the skin of a large proportion of adults. The significance of *Acinetobacter* in this study is unclear (Mahon & Manuselis, 1995).

3.5.2 Discrepancies between PCR and culture results

In one patient, a discrepancy was noted between PCR results and culture results in multiple specimens. *Fusobacteria* were identified by PCR, while culture of both gastric fluid and placental swab showed enterococci (Tables 3.2 & 3.4). Such discrepancies between the results of PCR and laboratory culture have been described previously (Jalava *et al.*, 1996). In one case with fusobacteria detected on PCR, a peptostreptococcus sp. was isolated in culture. It may be that enterococcus (Group D streptococcus) may have been dominant in the routine culture medium, even if the fusobacterium may have been present in greater numbers in the specimen itself. Replication of certain organisms in preference to others has previously been put forward as a reason for differing results between PCR and culture.

3.6 Key points

1. PCR was successfully applied to gastric fluid, fetal membrane and placental tissue (hypothesis).

2. Bacterial genes were detected in 75% of spontaneous preterm deliveries. This is a higher number than those in studies using laboratory culture.

3. *Ureaplasma urealyticum* was the most commonly identified organism.

4. In most cases it was possible to identify organisms by direct sequencing of the gene product without the need for the separation of bacterial products.

5. It was not possible to identify bacteria from some sequences obtained from placental tissue. This could either be due to the presence of more than one bacterial species in the sample or because of bacterial degradation within the placenta. Identification of multiple species in a specimen would usually require the additional steps of cloning the DNA product and sorting the resultant samples by restriction fragment length polymorphism analysis before sequencing the unique clones.

6. Bacterial genes were detected even after maternal treatment with erythromycin.

Chapter 4 : The measurement of intraamniotic and intrauterine inflammation

4.1 Introduction

Previous studies investigating the effects of intraamniotic and intrauterine inflammation on fetal outcome have depended heavily either on the measurement of cytokines in amniotic fluid obtained by amniocentesis or on the histological examination of the placenta. Histological diagnosis of chorioamnionitis has been useful, but quantification has proved difficult.

Measurement of cytokines is attractive as cytokines do not just reflect levels of inflammation but appear themselves to play a central role in the mechanism of preterm labour (Goldenberg *et al.*, 2000, Romero *et al.*, 2002). In addition the impact of intrauterine and intra-amniotic inflammation on fetal outcome is thought to depend on the actions of cytokines as reviewed in the first chapter (sections 1.2.3 to 1.2.12).

However, as already discussed, amniocentesis of patients in preterm labour is not routinely performed in the UK. For the purposes of this project, it meant that amniotic fluid would only be available if the infant was being delivered by caesarean section, not usually the case after spontaneous preterm labour. It was therefore necessary to develop a technique for the measurement of inflammation that would be possible in all preterm deliveries. I hypothesised that the quantification of pro-inflammatory cytokines in placental tissue and fetal membrane could be used as a technique to measure intrauterine inflammation where amniotic fluid was not available.

4.2 Hypothesis

That fetal membrane and placental tissue IL-6 and IL-8 correlate well with (a) amniotic fluid IL-6 and IL-8 measurements where available and (b) histological evidence of inflammation.

4.3 Methods

4.3.1 Techniques

Measurement of IL-6 and IL-8 by ELISA (Section 2.4.5.)

Measurement of protein in placental tissue and fetal membrane (Section 2.4.2.)

Measurement of IL-6 and IL-8 in placental tissue and fetal membane (Sections 2.4.1. and 2.4.5.)

4.3.2 Statistical analysis

The non-parametric test Kruskal-Wallis test was used to compare cytokine concentrations in the four preterm delivery groups as the measurements were not normally distributed. A particular difficulty encountered in the measurement of fetal membrane cytokines was that in some cases, the cytokine concentrations exceeded the ceiling of the assay, even when samples were diluted. As this is a novel technique, previous guidance as to the dilutions required was not available from previous publications. The ceiling measurements were excluded from correlation calculations as they may have resulted in an inaccurate estimate of correlation. Ceiling values were included in determining whether fetal membrane cytokines were significantly elevated in PPROM and PTL deliveries as they represented a minimum possible cytokine concentration.

4.4 Results

4.4.1 Cytokine measurements on amniotic fluid, placental tissue and fetal membrane

The results of the ELISA analysis on amniotic fluid, fetal membrane and placental tissue and protein estimation on fetal membrane and placenta are shown in Table 4.1. The cytokine measurements in the amniotic fluid are shown as pg/ml and the measurements in the tissues are expressed as pg/mg of protein.

As anticipated, most amniotic fluid samples were obtained at caesarean section, limiting the samples available from spontaneous preterm deliveries. Figs. 4.1a and 4.1b show the amniotic fluid IL-6 and IL-8 concentrations by category of preterm delivery. The amniotic fluid cytokines are clearly elevated in the pPROM and PTL groups. However the pPROM and PTL groups are only represented by three patients in total. (Figures 4.1a & 4.1b)

Table 4.1: IL-6 & IL-8 measurements in amniotic fluid (AF), placenta and fetal membrane (FM)

Study No.	Delivery Group	IL-6 Measurements			IL-8 Measurements		
		AF	Placenta	FM	AF	Placenta	FM
3	CS	837	2.3	9.60	258	3.68	35.8
4	CS						
5	CS	7462	5.0	80.3	1227	1.25	26.9
6	CS	1975	5.8	3.3	1227	40.19	18.4
17	CS		8.9	8.5		23.57	22.5
18	CS	2751	6.4	2.0	58	5.83	7.1
19	CS	32	9.3	7.5	368	18.05	11.8
26	CS	1259	2.1	12.2	58	1.07	58.4
49	CS	1687	99.7	70.8	5007	100.08	605.1
59	CS	246	1.4	15.0	0	1.77	11.8
65	CS		6.6			1.67	
7	PPROM		28.5	607.8		54.13	3420.8
9	PPROM	27105	23.8	777.7	128913	42.70	5184.7
14	PPROM		17.0	874.2		133.43	4821.7
38	PPROM		3.0	6.7		.94	10.8
45	PPROM		20.8	616.6		18.48	4110.90
47	PPROM		106.7	724.4		101.26	4829.30
54	PPROM		58.4	1439.9		132.48	9599.0
57	PPROM		5.0	17.1		1.87	17.9
58	PPROM		49.4	556.6		60.80	3710.40
63	PPROM		7.9	199.6		46.05	918.1
2	PTL		65.1			152.35	
10	PTL		309.9	2648.9		814.37	17659.2
41	PTL		32.1	5904.5		125.88	39363.1
46	PTL		10.4	508.4		14.65	2152.8
51	PTL	19551	23.7	729.4	38605	50.76	4862.7
52	PTL		14.3	437.8		25.18	1667.5
53	PTL		15.6	543.7		8.96	760.1
61	PTL	30000	311.6	1316.0	46966	377.18	6908.8
62	PTL		201.7	686.7		370.97	4578.3
64	PTL		5.3	109.8		8.53	98.0
1	Twin						
1	Twin	759			313		
8	Twin		9.3	254.3		6.90	256.7
8	Twin		11.3	37.4		4.85	23.8
13	Twin		1.7	13.5		.82	12.7
13	Twin	1766	11.9	29.9	482	3.62	17.6
16	Twin		5.2	196.8		2.09	102.5
16	Twin	1369	10.8	26.6	368	5.74	137.6
60	Twin		5.1	47.4		.71	43.1
60	Twin		2.3	248.7		3.94	175.0

The result of cytokine measurements (IL-6 and IL-8) in amniotic fluid, placenta (chorionic plate) and fetal membrane. Amniotic fluid measurements were in ng/ml. Fetal membrane and placenta (tissue extract) measurements were in pg/mg protein.

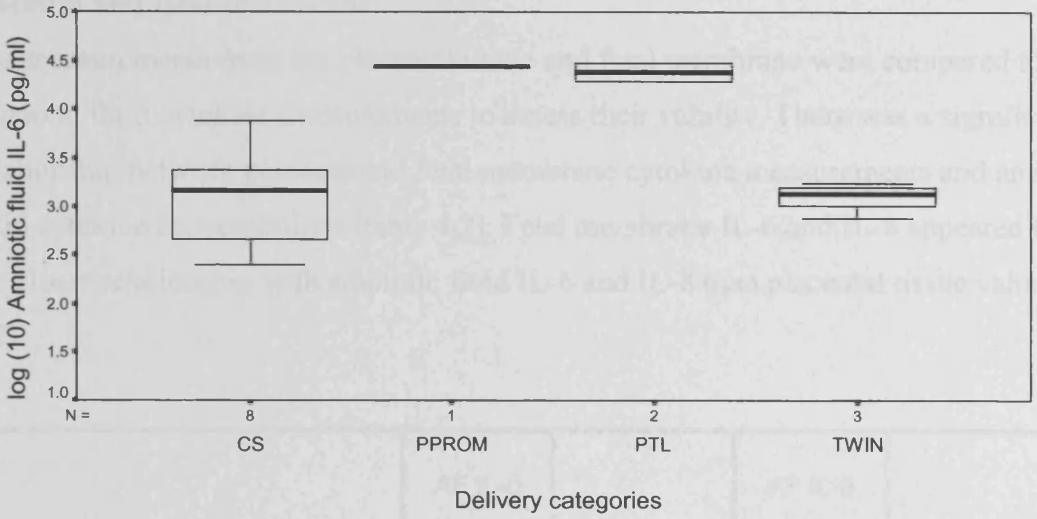


Figure 4.1a Comparison of amniotic fluid IL-6 concentrations in the four preterm delivery groups (CS, indicated caesarean section; PPROM, prelabour preterm rupture of membranes; PTL, preterm labour intact membranes; TWIN, twin delivery). Higher IL-6 concentrations are seen in the PPROM and PTL groups.

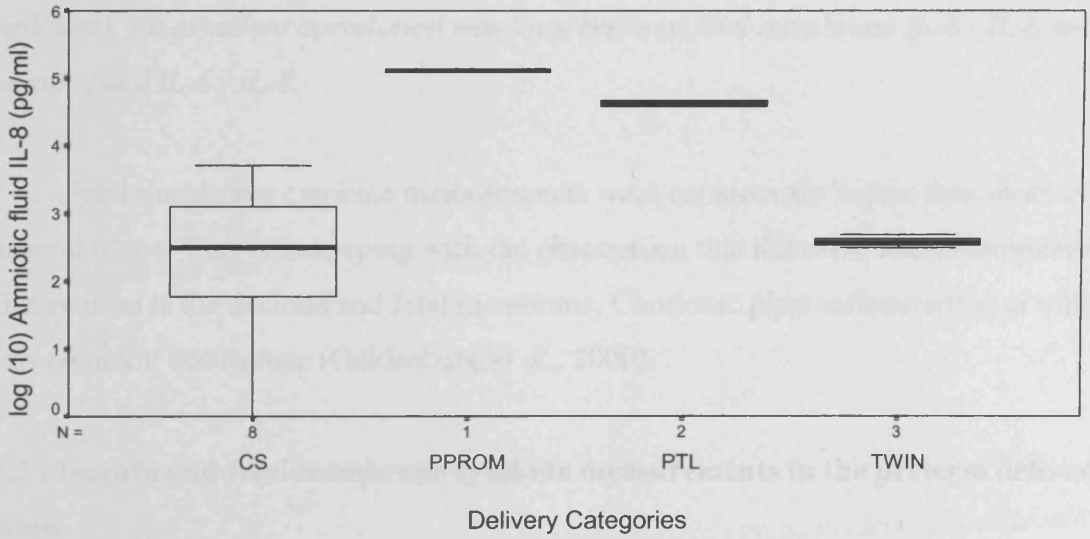


Figure 4.1b Comparison of amniotic fluid IL-8 concentrations in the four preterm delivery groups. Higher IL-8 concentrations are seen in the PPROM and PTL groups.

4.4.2 Correlation between amniotic fluid cytokines and cytokine concentrations in placenta and fetal membrane

The measurements from the placental tissue and fetal membrane were compared to amniotic fluid cytokine measurements to assess their validity. There was a significant relationship between placenta and fetal membrane cytokine measurements and amniotic fluid cytokine concentrations (table 4.2). Fetal membrane IL-6 and IL-8 appeared to have the closer relationship with amniotic fluid IL-6 and IL-8 than placental tissue values (table 4.2).

	AF IL-6 R	significance	AF IL-8 R	significance
Placental tissue IL-6 / IL-8	0.627	0.022	0.319	p<0.001
Fetal membrane IL-6 / IL-8	0.981	p<0.001	0.934	p<0.001

Table 4.2 Correlation between amniotic fluid IL-6 and IL-8 measurements with measurements made in placental tissue and fetal membranes. (R = Pearson correlation coefficient). An excellent correlation was seen between fetal membrane IL-6 / IL-8 and amniotic fluid IL-6 / IL-8.

Fetal membrane cytokine measurements were consistently higher than those in the placental tissue. This is in keeping with the observation that the usual site of intrauterine inflammation is the decidua and fetal membrane. Chorionic plate inflammation or villitis is a less common occurrence (Goldenberg *et al.*, 2000).

4.4.3 Placenta and fetal membrane cytokine measurements in the preterm delivery groups

Placental and fetal membrane IL-6 and IL-8 concentrations were both significantly elevated in pPROM and PTL deliveries compared to non-labouring deliveries and twin deliveries (Figure 4.2a, b, c, d $p<0.001$ Kruskal-Wallis test). There was no significant difference between pPROM and PTL deliveries, and no significant difference between non-labouring deliveries and twin deliveries.

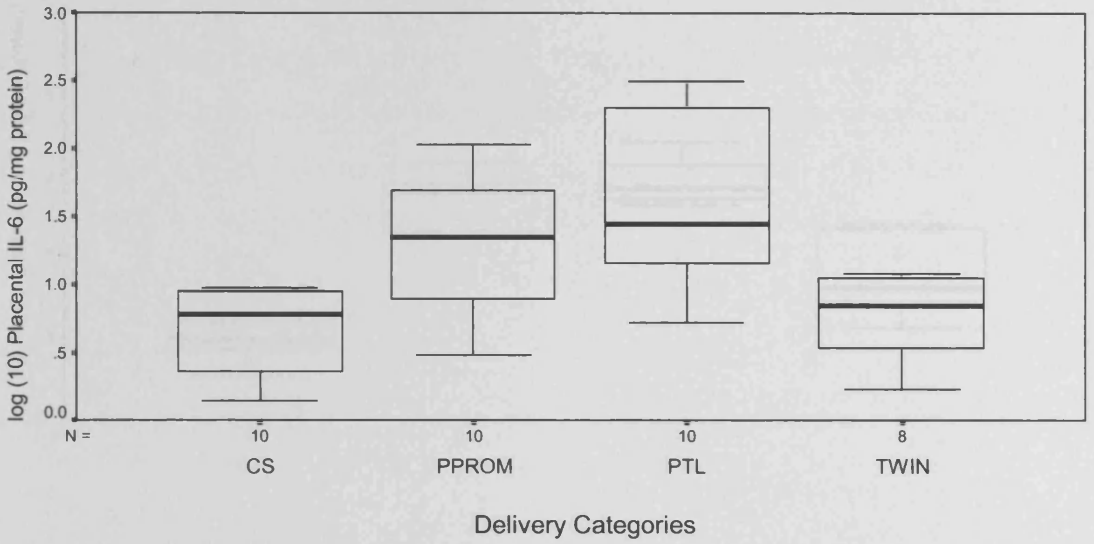


Figure 4.2a Comparison of placental tissue IL-6 concentrations in the four preterm delivery categories. Significantly higher IL-6 concentrations were observed in the PPROM and PTL groups ($p<0.001$ Kruskal-Wallis test).

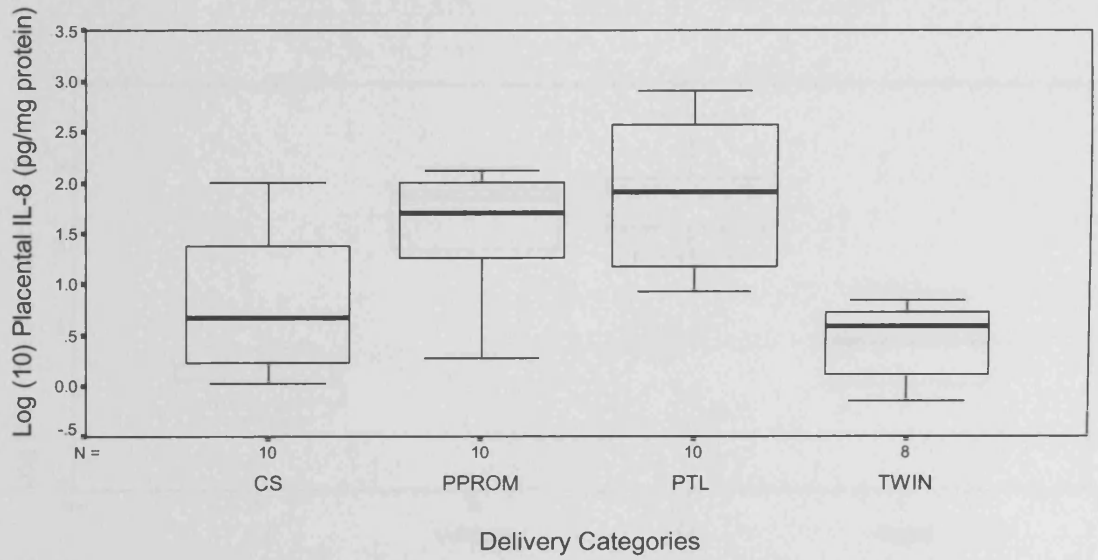


Figure 4.2b Comparison of placental tissue IL-8 concentrations in the four preterm delivery categories. Significantly elevated IL-8 concentrations were observed in the PPROM and PTL groups ($p<0.001$ Kruskal-Wallis test) .

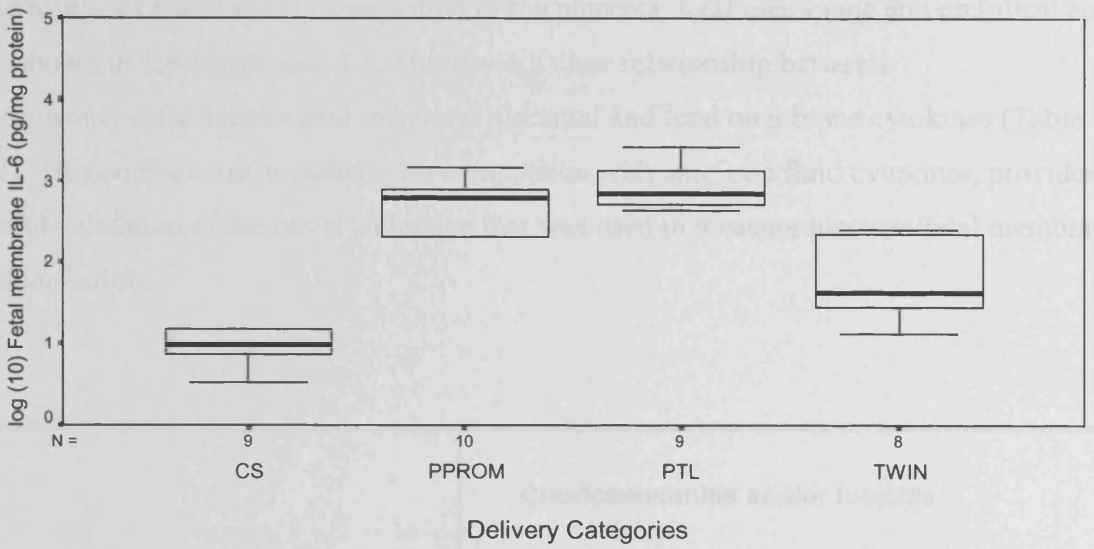


Figure 4.2c Comparison of fetal membrane IL-6 concentrations in the four preterm delivery categories demonstrating increased fetal membrane IL-6 in the PPROM and PTL categories ($p<0.001$ Kruskal-Wallis test).

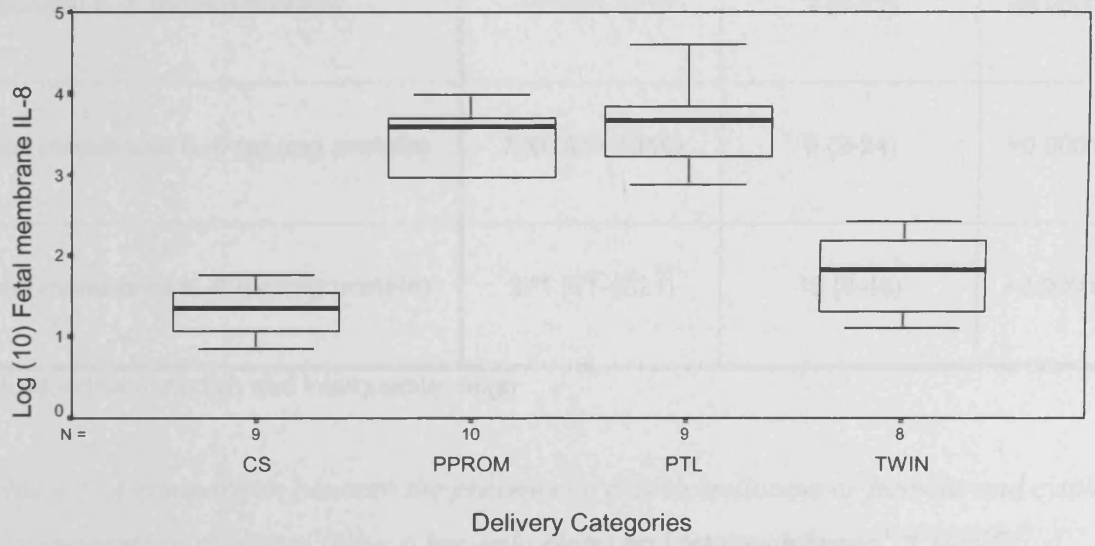


Figure 4.2d Comparison of fetal membrane IL-8 concentrations in the four preterm delivery categories with significantly higher IL-8 concentrations in the PPROM and PTL group ($p<0.001$ Kruskal-Wallis test).

4.4.4. Comparison of cytokine measurements with results of routine histology

The results of histological examination of the placenta, fetal membrane and umbilical cord are shown in Tables 4.4 and 4.5. There was a clear relationship between chorioamnionitis/funisitis and increased placental and fetal membrane cytokines (Table 4.3). This comparison, together with comparison with amniotic fluid cytokines, provides a useful validation of the novel technique that was used to measure placenta/fetal membrane inflammation.

	Chorioamnionitis and/or funisitis		
	Yes	**No**	**p value**
Placental IL-6 (pg/mg protein)	163 (94-221)	49 (24-202)	<0.0001
Placental IL-8 (pg/mg protein)	101 (51-371)	7 (5-11)	<0.0001
Fetal membrane IL-6 (pg/mg protein)	729 (617-1316)	6 (2-24)	<0.0001
Fetal membrane IL-8 (pg/mg protein)	371 (61-4821)	18 (8-49)	<0.0001

Values indicate median and interquartile range

Table 4.3 A comparison between the presence of chorioamnionitis or funisitis and cytokine measurements in placental tissue (chorionic plate) and fetal membrane. A significant elevation of both IL-6 and IL-8 was seen in both tissues.

Table 4.4: Histological evidence of inflammation

Study No	Type	Chorioamnionitis	Funisitis	Villitis	Deciduitis
3	Single	No	No	No	
4	Single	No	No	No	
5	Single	No	No	No	
6	Single	No	No	No	
17	Single	No	No	No	No
18	Single	No	No	No	No
19	Single	No	No	scattered villitis	No
26	Single	No	No	No	No
49	Single	No	No	No	No
59	Single	No	No	scattered villitis	
65	Single	No	No	No	No
7	Single	Present	Three vessels	No	
9	Single	Severe	Three vessels	No	
14	Single	Severe	One vessel	No	
38	Single	No	No	No	No
45	Single	No	Two vessels	No	
47	Single	Moderate	Three vessels	No	
54	Single	Present	Three vessels	No	No
57	Single	No	No	No	No
58	Single	Severe	Three vessels	No	
63	Single	No	No	No	No
2	Single	No	No	No	No
10	Single	Severe	Three vessels	No	
41	Single	No	No	No	Present
46	Single	No	No	No	No
51	Single	severe	No	No	Severe
52	Single	No	No	No	Present
53	Single	No	No	No	No
61	Single	Severe	One vessel	No	
62	Single	Severe	Two vessels	No	
64	Single	No	No	No	No
1.1	DiDi	No	No	No	
1.2	DiDi	No	No	No	
8.1	DiDi	No	No	No	
8.2	DiDi	No	No	No	
13.1	DiDi	No	No	No	No
13.2	DiDi	No	No	No	No
16.1	DiDi	No	No	extensive villitis	No
16.2	DiDi	No	No	extensive villitis	No
60.1	DiDi	No	No	No	No
60.1	DiDi	No	No	No	No

DiDi: Dichorionic diamniotic

The presence of chorioamionitis and funisitis on histological examination of the chorionic plate, fetal membrane and umbilical cord. The number of cord vessels involved in the funisitis is also listed. Inflammation was mainly seen in the preterm singleton deliveries.

Table 4.5: Other features on histological examination

Study No	Gram stain result	Syncitial Knots	Areas of necrosis or infarction
3	Not performed		
4	Not performed	Normal	Present
5	No organisms seen	Increased	Present
6	Not performed	Increased	Present
17	Not performed	Increased	Present
18	Not performed	Increased	Absent
19	Not performed	Normal	Present
26	Not performed	Normal	Present
49	Not performed	Increased	Present
59	Not performed	Normal	Absent
65	Not performed	Normal	Absent
7	No organisms seen	Normal	Absent
9	Gram positive cocci	Normal	Absent
14	No organisms seen	Normal	Present
38	Not performed	Normal	Absent
45	No organisms seen	Normal	Absent
47	No organisms seen	Normal	Absent
54	No organisms seen	Normal	Absent
57	Not performed	Normal	Absent
58	No organisms seen	Normal	Absent
63	Not performed	Normal	Absent
2	Not performed	Normal	Absent
10	No organisms seen	Normal	Absent
41	Not performed	Normal	Absent
46	Not performed	Normal	Absent
51	No organisms seen	Normal	Present
52	Not performed	Normal	Absent
53	Not performed	Increased	Absent
61	Not performed	Normal	Absent
62	Gram positive cocci	Normal	Absent
64	Not performed	Normal	Absent
1.1	Not performed	Normal	Absent
1.2	Not performed	Normal	Absent
8.1	Not peformed	Normal	Absent
8.2	Not performed	Normal	Absent
13.1	Not performed	Normal	Absent
13.2	Not performed	Normal	Absent
16.1	Not performed	Normal	Absent
16.2	Not performed	Normal	Absent
60.1	Not performed	Normal	Absent
60.2	Not performed	Normal	Absent

Gram staining was performed when inflammation was noted on histology (see table 4.4).The presence of syncitial knots (associated with pre-eclampsia) and areas of necrosis/infarction were also recorded.

4.4.5 Relationship between IL-6 and IL-8 measurements

A close relationship between IL-6 and IL-8 measurements was observed in amniotic fluid, fetal membrane and placental tissues (Table 4.3). This makes it likely that these cytokines are controlled by a common mechanism or stimulus.

	R	Significance
Amniotic fluid	0.845	p<0.001
Placenta	0.917	p<0.001
Fetal membrane	0.825	p<0.001

Table 4.6 Correlation between IL-6 concentrations and IL-8 concentrations in the samples collected showing the close correlation between the measurements of these cytokines in amniotic fluid and tissue samples.

4.5 Discussion

An excellent correlation between pro-inflammatory cytokine measurements in fetal membrane and measurements in amniotic fluid was observed. In addition, there was a clear relationship between elevated placenta and fetal membrane cytokine concentrations and chorioamnionitis/funisitis, also pointing to the validity of measuring the tissue cytokines. These fetal membrane measurements may therefore be useful in determining the exposure of the fetus to intrauterine and intra-amniotic inflammation, particularly where amniotic fluid is not available. The measurement of tissue cytokines could form the basis of the quantification of chorioamnionitis. There was a strong correlation between IL-6 and IL-8 in both amniotic fluid and tissue samples suggesting that their stimulus or control mechanism was the same.

The cytokine concentrations were markedly increased in placental tissue, fetal membranes from the pPROM and spontaneous preterm labour groups when compared to the twin deliveries and non-labouring groups. Few samples were available for amniotic

fluid from each group but there was a trend towards differences with the highest concentrations of IL-6 and IL-8 seen in the pPROM and spontaneous labour groups.

It was found that twins in active labour did not have significantly elevated cytokines in the fetal membranes or placenta when the comparison was made to caesarean deliveries without labour. This suggests that in most cases, twin deliveries may have a different mechanism for the onset of labour to that postulated in singleton preterm labour.

4.6 Key points

1. There was an excellent correlation between pro-inflammatory cytokine measurements in fetal membrane and measurements in amniotic fluid. Fetal membrane measurements are therefore likely to give a valid quantification of inflammation within the amniotic cavity (Hypothesis).

2. Increased placental and fetal membrane cytokine concentrations were seen in the presence of chorioamnionitis/funisitis. The measurement of tissue cytokines could form the basis of the quantification of chorioamnionitis (Hypothesis).

3. Fetal membrane cytokine measurements were consistently higher than those in the placental tissue, in keeping with the observation that the usual site of intrauterine inflammation is the decidua and fetal membrane.

4. The pro-inflammatory cytokines, IL-6 and IL-8 were significantly elevated in the singleton preterm labour groups (with or without membrane rupture) compared to the non-labouring and twin groups.

5. In most cases, twin deliveries are likely to have a different mechanism for the onset of labour compared to singleton preterm delivery. This was indicated by the difference in cytokine measurements between the singleton preterm delivery groups and the twin preterm delivery group.

Chapter 5: The Measurement of Fetal Systemic Inflammation

5.1 Introduction

5.1.1 Evidence of fetal inflammation

A link between placental inflammation and inflammation in the fetal circulation was observed when Kitajama *et al.* measured the levels of complement components in cord blood. It was found that, with the exception C1q, all the components were in higher concentrations in the presence of chorioamnionitis (Kitajama *et al.*, 1990). Further evidence of an inflammatory process came with measurement of the pro-inflammatory cytokines IL-6 and IL-8 in the cord blood of preterm infants. These cytokines were increased with histological chorioamnionitis and the concentrations correlated with the apparent severity of the inflammation (Kitajima *et al.*, 1992, Shimoya *et al.*, 1992). Cord IL-6 was also raised in preterm deliveries in the presence of clinical chorioamnionitis (Lencki *et al.*, 1994) while cord IL-1ß was increased in association with positive amniotic fluid cultures (Carroll *et al.*, 1995). Salafia noted that fetal IL-6, correlated with histological chorioamnionitis, but that maternal IL-6 did not (Salafia *et al.*, 1997).

5.1.2 Fetal systemic inflammation and funisitis

Further studies have looked at inflammation of the umbilical cord (funisitis). Funisitis may be graded according to the number of blood vessels involved in the inflammatory process. There may be inflammation of the umbilical vein alone (umbilical phlebitis) or inflammation of arteries in addition to the vein (umbilical arteritis). By comparing the cytokine concentrations with the severity of funisitis, including the number of inflamed vessels, it was determined that funisitis was in fact a histopathological correlate of the fetal systemic inflammation (Naccasha *et al.*, 2001, Kim *et al.*, 2001, Pacora *et al.*, 2002).

5.1.3 The impact of fetal inflammation

A number of studies have demonstrated that fetal inflammation may have an adverse effect on the fetus. The studies looking at outcomes following fetal inflammation fall into two categories:

- Outcomes associated with elevated cytokines
- Outcomes associated with funisitis.

Chronic lung disease (CLD), periventricular leukomalacia (PVL), cerebral white matter damage (WMD) (Yoon *et al.*, 1996), intracranial haemorrhage (Tauscher *et al.*, 2003) and neonatal sepsis (Yoon *et al.*, 2000b) have all been associated with increased concentrations of cytokines, in particular IL-6 (appendices 1.1, 1.3 and 1.5). In addition, two studies have shown an increase in combined serious neonatal morbidity associated with fetal inflammation (Weeks *et al.*, 1997, Gomez *et al.*, 1998). The risk of morbidity was doubled with an IL-6 > 11pg/ml (Gomez *et al.*, 1998). Preterm delivery within 48 hours of cordocentesis was also more likely with elevated fetal IL-6 (Romero *et al.*, 1998). However, the relationship between fetal cytokines and adverse outcome is complex; the results are statistically significant but the spread of measurements in the groups is wide with an area of overlap. Still, WMD is more strongly associated with fetal vasculitis than with chorioamnionitis (Leviton *et al.*, 1999, Damman & Leviton 2004). The term fetal systemic inflammatory syndrome (FIRS) was coined to encompass the idea that fetal inflammation was associated with preterm labour and serious adverse outcome (Romero *et al.*, 2002).

5.2 Hypothesis

Given that microbial genes and histological chorioamnionitis were more frequently detected in spontaneous singleton preterm deliveries, I hypothesised that the concentration of cytokines in the fetal circulation would be elevated in spontaneous premature deliveries.

5.3 Methods

5.3.1 Techniques used

Umbilical cord blood collection and processing (Section 2.1.5.1 and 2.1.5.2)
Measurement of IL-6 and IL-8 in supernatants (Section 2.4.5)

5.3.2 Statistical analysis

The Kruskal-Wallis test for non-parametric data was used to compare the cord cytokine concentrations in each of the four delivery groups.

5.4 Results

5.4.1. Relationship between serum IL-6, IL-8, funisitis and delivery groups

The individual cord and infant cytokine measurements are shown in Table 5.2. The IL-6 and IL-8 concentrations were grouped according to delivery group: prelabour caesarean section (CS), pPROM, preterm labour with intact membranes (PTL) and spontaneous twin deliveries (TWIN) (Figures 5.1a,b). The Kruskal-Wallis test identified a highly significant difference between the groups (IL-6 $p < 0.001$; IL-8 $p = 0.007$). Mann-Whitney tests performed between pairs of delivery groups identified that the concentrations of both cytokines were increased in the pPROM and PTL compared to the CS and twin groups (Table 5.1a,b). There were no significant differences between the CS and twin group and between the pPROM and PTL groups (Table 5.1).

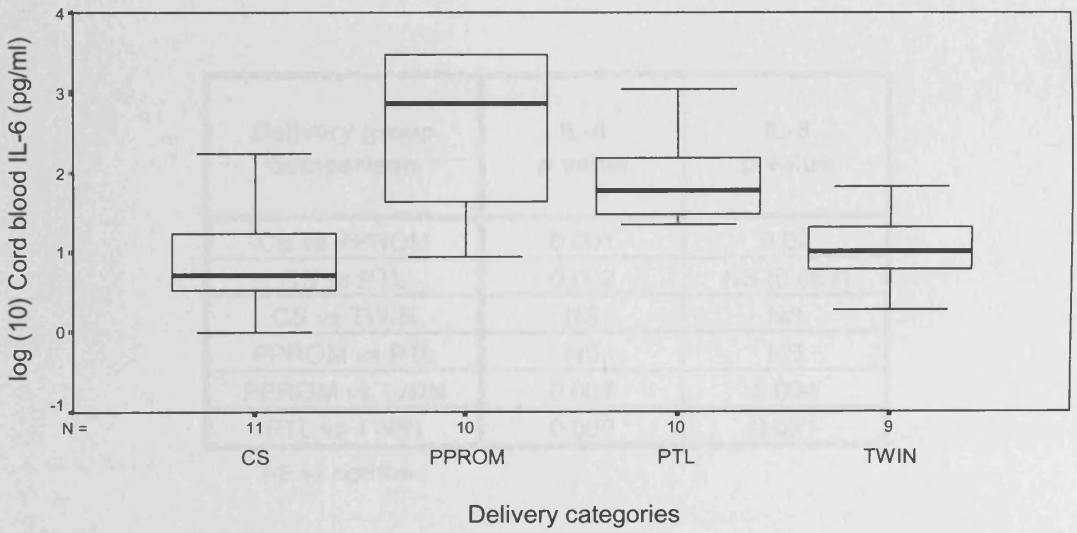


Figure 5.1a Cord blood IL-6 concentrations in the four preterm delivery groups with significant increases in the pPROM and spontaneous preterm labour categories ($p<0.001$).

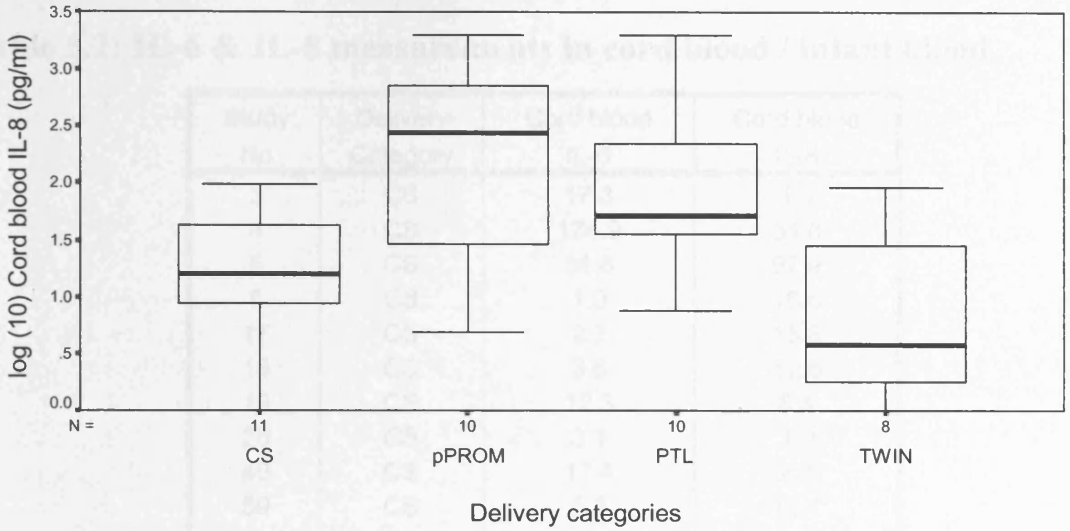


Figure 5.1b Cord blood IL-8 concentrations in the four preterm delivery groups with significant increases in the pPROM and spontaneous preterm labour categories (p=0.007).

Delivery group comparison	IL-6 p value	IL-8 p value
CS vs PPROM	0.001	0.02
CS vs PTL	0.002	NS (0.067)
CS vs TWIN	NS	NS
PPROM vs PTL	NS	NS
PPROM vs TWIN	0.007	0.004
PTL vs TWIN	0.009	0.021

NS not significant

Table 5.1 Results of Mann-Whitney comparisons between delivery groups for cord IL-6 and cord IL-8.

Table 5.2: IL-6 & IL-8 measurements in cord blood / infant blood

Study No	Delivery Category	Cord blood IL-6	Cord blood IL-8
3	CS	17.3	1.0
4	CS	174.9	34.8
5	CS	34.8	97.9
6	CS	1.0	16.6
17	CS	2.2	15.8
18	CS	3.6	52.6
19	CS	12.3	6.4
26	CS	3.1	1.0
49	CS	17.4	86.9
59	CS	5.2	12.1
65	CS	4.8	15.2
7	PPROM	767.0	348.8
9	PPROM	714.0	238.8
14	PPROM	98.2	146.8
38	PPROM	18.2	5.9
45	PPROM	3000**	2000**
47	PPROM	3000**	2000**
54	PPROM	3000**	305.2
57	PPROM	8.9	4.8
58	PPROM	1813.0	719.9
63	PPROM	43.1	29.4
2	PTL	82.8	222.2
10	PTL	908.0	2000**
41	PTL	151.9	54.8
46	PTL	47.6	35.8
51	PTL	22.9	48.1
52	PTL	29.8	11.0
53	PTL	42.7	55.6
61	PTL	73.9	47.4
62	PTL	1103.0	737.9
64	PTL	22.6	7.5
1	TWIN	20.5	1.0
1	TWIN	66.9	1.0
8	TWIN	496.8	
8	TWIN		
13	TWIN	4.9	3.8
13	TWIN	10.1	3.8
16	TWIN	10.2	3.2
16	TWIN	17.7	53.3
60	TWIN	6.0	14.6
60	TWIN	1.9	90.1

*measurement performed on blood from first venepuncture

**ceiling limit of assay

Increased cord IL-6 and IL-8 results were observed in the singleton preterm labour groups.

Funisitis was most frequently observed in the pPROM group (n=7/10), but was also seen in spontaneous preterm labour with intact membranes (n=3/10). Funisitis was not observed in either the prelabour caesarean section group or the twin group (Table 4.4).

5.4.2 Relationship between serum IL-6 and IL-8 measurements and chorioamnionitis / funisitis.

Consistent with the previous literature (discussed in section 5.1.2), both IL-6 and IL-8 were significantly elevated in the presence of funisitis (compared to no chorioamnionitis / no funisitis group $p < 0.001$ for both), chorioamnionitis ($p<0.001$) and chorioamnionitis/funisitis combined ($p<0.001$). In our sample, all but one of the placentas with chorioamnionitis had funisitis.

5.4.3 Relationship between cord IL-6 and IL-8 measurements.

As was the case with previous measurements performed on amniotic fluid, placenta and fetal membrane (Chapter 4), it was found that there was a strong significant association between IL-6 and IL-8 measured in cord serum or neonatal serum (before an hour of age) (Spearman's $\rho = 0.66$ $p<0.001$) (Figure 5.2).

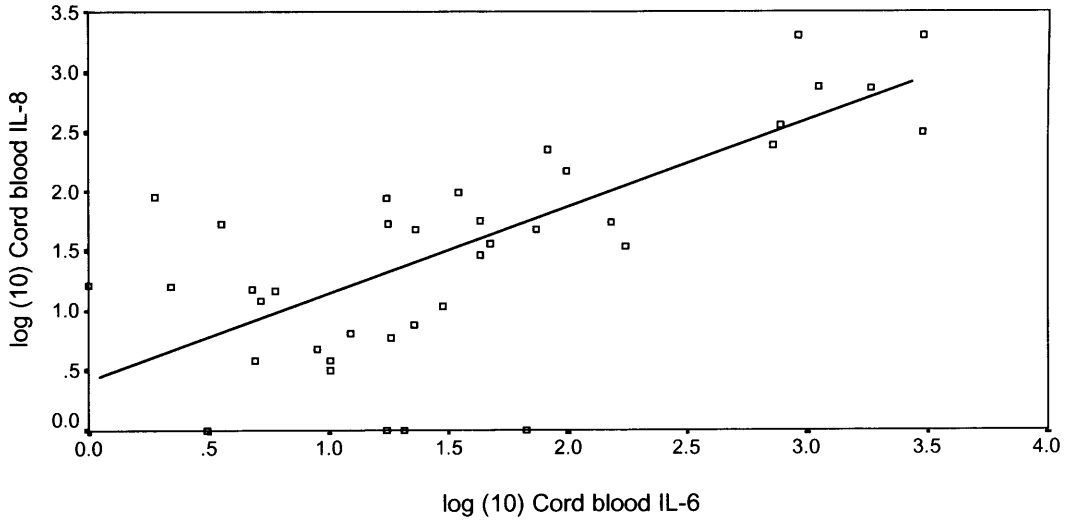


Figure 5.2 Correlation between cord blood IL-6 and IL-8 concentrations. A significant correlation was observed (Spearman's $\rho = 0.66$ $p<0.001$).

5.5 Discussion

Cord cytokine concentrations were significantly increased in both singleton preterm labour groups (pPROM and preterm labour with intact membranes) in accordance with the hypothesis for this chapter. There was a very strong relationship between the presence of funisitis and elevated cytokine concentrations in cord blood, as previously reported (section 5.1.2). In keeping with these results, funisitis, the histological correlate of fetal inflammation, was seen observed in 7/10 of the pPROM group and 3/10 in the preterm labour group with intact membranes.

5.6 Key points

1. Markedly increased cytokine concentrations were observed in the singleton spontaneous preterm labour groups (Hypothesis).

2. Funisitis was most frequently seen in the pPROM group. There were no cases of funisitis in either the prelabour caesarean section group or the twin group.

3. There was a very strong relationship between the presence of funisitis and elevated cytokine concentrations in cord blood, as previously reported.

Chapter 6: The Relationship between detection of bacterial genes with measures of intrauterine inflammation

6.1 Introduction

The association between the isolation of bacteria from the amniotic fluid or placenta and intrauterine inflammation has been demonstrated in a number of studies (Appendices 1.7 and 1.8). Studies have compared bacterial isolation with either increased amniotic fluid interleukin concentrations (Romero *et al.*, 1990, Romero *et al.*, 1993a,b, Hillier *et al.*, 1993, Jacobbson *et al.*, 2003a, Jacobsson *et al.*, 2003b, Yoon *et al.*, 2003c) or histological chorioamnionitis (Pankuch *et al.*, 1984, Hillier *et al.*, 1988, Romero *et al.*, 1992, Hillier *et al.*, 1993). However, a consistent finding is that bacteria are not always isolated from deliveries complicated by intrauterine inflammation. The rate of bacterial isolation from the amniotic fluid in the presence of increase IL-6 or histological chorioamnionitis has varied from 30% to 72%. The possible explanations include:

- that laboratory culture is not sufficiently sensitive to detect the relevant organisms. The inoculum may be smaller than that required by conventional microbiological techniques.
- The relevant organisms may be fastidious and require special laboratory techniques. PCR has been shown to increase the detection of *Uu* by 40% when compared to culture (Yoon *et al.*, 2000). The absence of cultures for Mycoplasma and Chlamydia may have explained some of the discrepancies between infection and inflammation in early studies.
- The organisms may be extraamniotic. Andrews *et al.* (1995) demonstrated that twice as many organisms could be cultured from the chorioamnion compared to amniotic fluid. In addition, these organisms were associated with high amniotic fluid IL-6 levels.
- That inflammation is not always due to bacterial invasion.

Specific detection of *Uu* using PCR, alone and in combination with culture results has previously been compared with measures of inflammation (Yoon *et al.,* 2000c, Jacobsson *et al.,* 2003a,b). However, the results of universal molecular bacterial detection (targeting 16S rRNA genes) have not been compared with measures of inflammation in any previous studies. I therefore compared the results of PCR detection in this cohort (Chapter 3) with the presence of inflammation as indicated by IL-6 and IL-8 measurements and histological analysis (Chapters 4 and 5). The measurement of fetal membrane and placental tissue interleukins allowed for the comparison of infection and inflammation where amniotic fluid was not available (Chapter 4).

6.2 Hypotheses

- That there is a strong link between intrauterine infection and inflammation.
- That the sensitivity of the polymerase chain reaction would allow microbial detection in all cases of intrauterine inflammation.

6.3 Methods

6.3.1 Statistical analysis

The Mann-Whitney test was performed to look for significant differences in the cytokine measurements with or without bacteria detected by PCR, while Chi-squared analysis was used to compare the presence of bacteria with histological evidence of inflammation.

6.4 Results

6.4.1 Comparison of the results of bacterial detection with cytokine measurements

The cytokine measurements are listed in Tables 4.1 and 5.1. Cytokine measurements were significantly higher in the bacterial PCR positive group. This was the case for measurements in all the different types of specimen collected (Fig. 6.1a,b,c, Table 6.1).

Fig. 6.1(a)

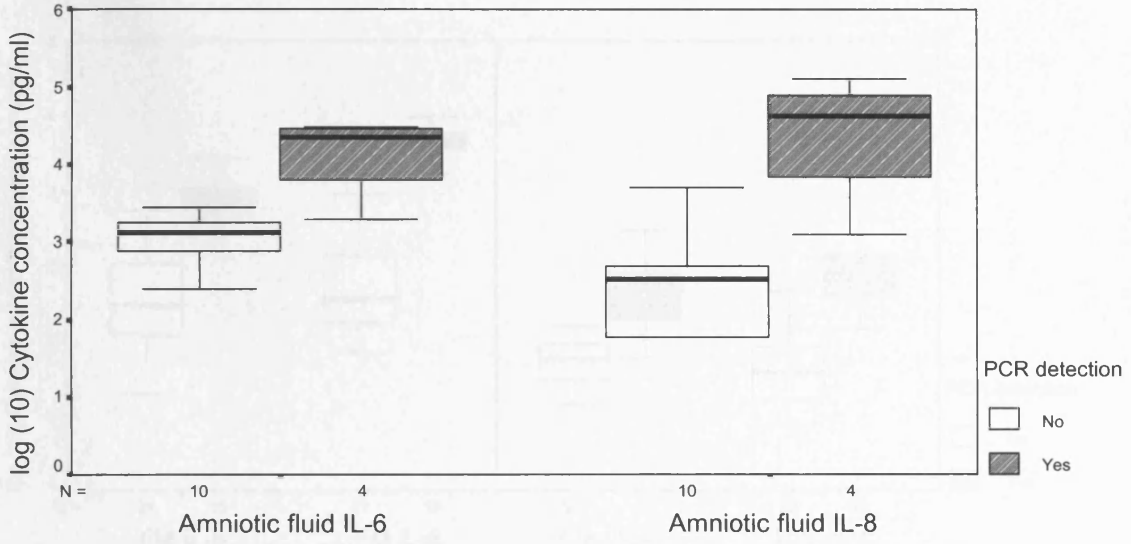


Fig 6.1(b)

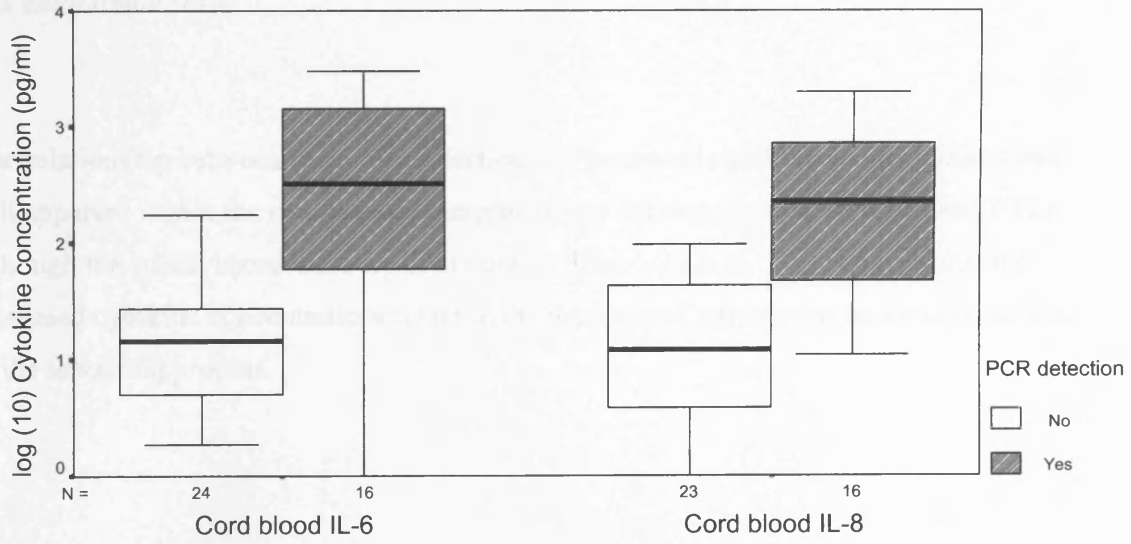


Fig. 6.1(c)

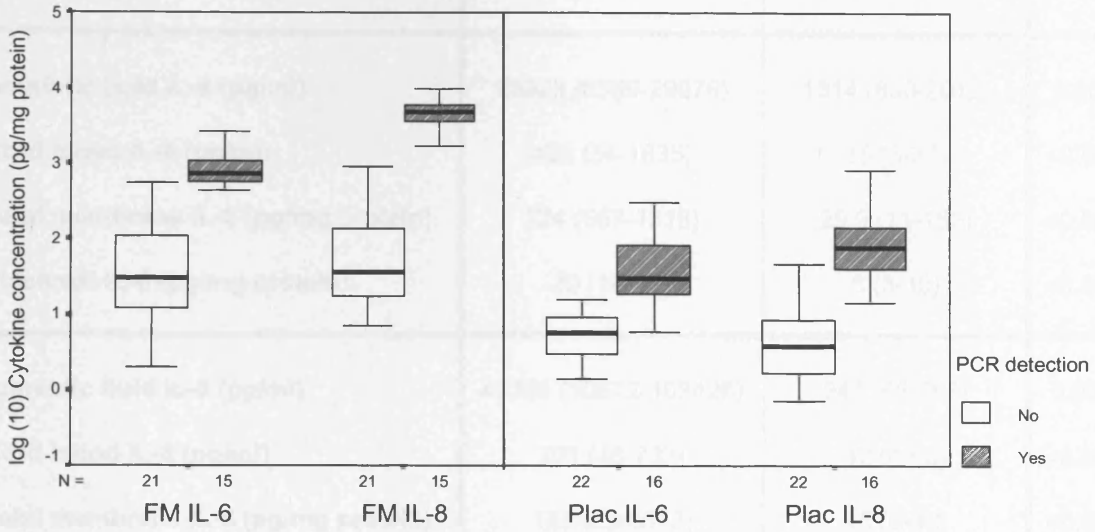


Figure 6.1 Cytokine measurements with and without the presence of intrauterine bacteria detected by PCR. These were higher in all tissues and fluid samples in the presence of intrauterine bacteria: (a) Amniotic Fluid, (b) Cord blood, (c) Fetal membrane (FM) and placental tissue (PL).

The relationship between microbial detection and increased cytokine concentrations was still apparent *within* the spontaneous preterm labour delivery groups (pPROM and PTL), although the subdivisions were small in number (Fig. 6.2 a,b,c). This suggests that the increased cytokine concentrations relate to the presence of intrauterine bacteria rather than to the labouring process.

Cytokine measurement	PCR positive	PCR negative	p
Amniotic fluid IL-6 (pg/ml)	23328 (6369-29276)	1314 (630-2012)	0.008
Cord blood IL-6 (pg/ml)	433 (54-1635)	15 (5-32)	<0.001
Fetal membrane IL-6 (pg/mg protein)	724 (557-1316)	29.9 (11-153)	<0.001
Placental IL-6 (pg/mg protein)	30 (18-96)	6 (3-10)	<0.001
Amniotic fluid IL-8 (pg/ml)	42786 (10572-108426)	341 (58-668)	0.004
Cord blood IL-8 (pg/ml)	231 (48-733)	12 (4-53)	<0.001
Fetal membrane IL-8 (pg/mg protein)	133 (25-4822)	15 (6-49)	<0.001
Placental IL-8 (pg/mg protein)	81 (41-148)	4 (2-9)	<0.001

Values indicate median and interquartile range

Table 6.1 Cytokine measurements with and without the presence of intrauterine bacteria detected by PCR. The cytokine concentrations were higher in all tissues and fluid samples in the presence of intrauterine bacteria.

Fig. 6.2(a)

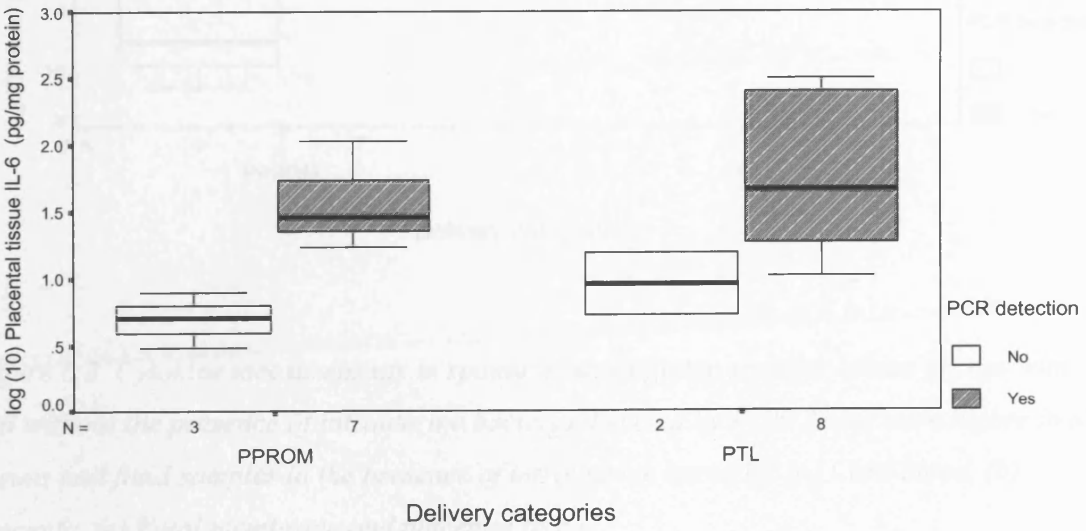
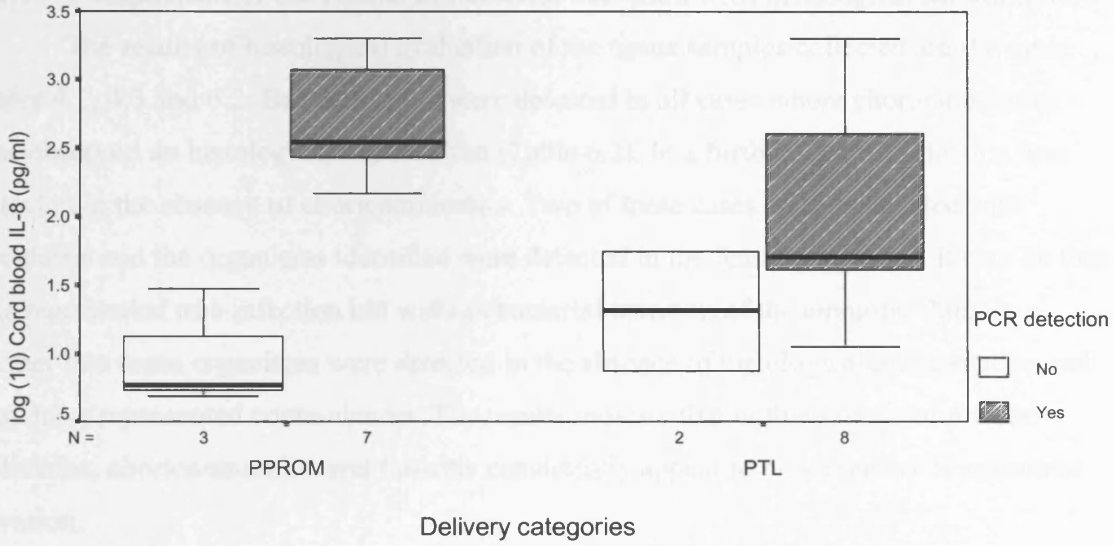


Fig. 6.2(b)



(c)

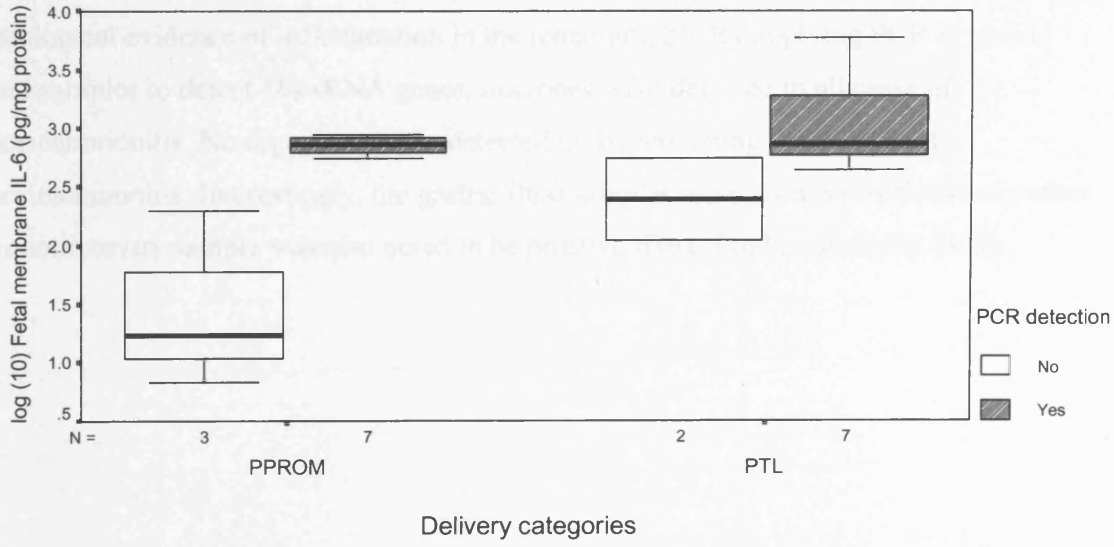


Figure 6.2 Cytokine measurements in spontaneous singleton preterm labour groups with and without the presence of intrauterine bacteria detected by PCR. These were higher in all tissues and fluid samples in the presence of intrauterine bacteria: (a) Cord blood, (b) Placenta, (c) Fetal membrane and placental tissue.

6.4.2 Comparison of the results of bacterial detection with histological inflammation

The results of histological evaluation of the tissue samples collected are shown in tables 4.2, 4.3 and 6.2. Bacteria or *Uu* were detected in all cases where chorioamnionitis was observed on histological examination (Table 6.2). In a further 4 cases, infection was detected in the absence of chorioamnionitis. Two of these cases were associated with deciduitis and the organisms identified were detected in the fetal membranes. It may be that this represented true infection but without bacterial invasion of the amniotic fluid. In a further two cases organisms were detected in the absence of histological inflammation and may have represented contaminants. The results indicate that in this sample of preterm deliveries, chorioamnionitis and funisitis consistently appear to be secondary to microbial invasion.

6.4.3 Identification of 16S rRNA genes in gastric fluid

Adequate samples of gastric fluid were obtained from 29 infants (71%). Within this subset, the delivery of 8 infants was associated with chorioamnionitis or funisitis. There was no histological evidence of inflammation in the remaining 21. By applying PCR to gastric fluid samples to detect 16s rRNA genes, microbes were detected in all cases of chorioamnionitis. No organisms were detected in the remaining cases without chorioamnionitis. Interestingly, the gastric fluid samples were always positive if any other amniotic cavity sample was also noted to be positive (BAL fluid or amniotic fluid).

Table 6.2 Organisms detected & inflammation on histology

Delivery	16S rRNA positive	Positive samples	Microbe identified	Chorio-amnionitis	Funisitis
pPROM-7	yes	GF, FM	Uu, Uu	yes	3bv
pPROM-9	yes	FM	Mix	yes	3bv
pPROM-14	yes	GF, FM	Ph/Uu, Asp	yes	1bv
pPROM-45	yes	BAL	Sau	yes	2bv
pPROM-47	yes	PL, GF	Mix, Sp	yes	3bv
pPROM-54	yes	BAL,GF,FM	Uu, Uu, Uu	yes	3bv
pPROM-58	yes	PL,GF	Mix, La/Uu	yes	3bv
pPROM-38	-			-	-
pPROM-57	-			-	-
pPROM-63	-			-	-
PTL-2	yes	PL	Mix	-	-
PTL-10	yes	PL,BAL, GF,FM	Fn, Fn, Fn, Fn	yes	3bv
PTL-41	yes	FM	Uu	deciduitis	-
PTL-46	yes	BAL	Mix	-	-
PTL-51	yes	BAL,GF,AF,FM	Uu, Uu, Uu, Uu	yes	-
PTL-52	yes	FM	Uu	deciduitis	-
PTL-61	yes	AF	Gv	yes	1bv
PTL-62	yes	GF, FM	Sag, Asp	yes	3bv
PTL-53	-			-	-
PTL-64	-			-	-
CS-6	yes	PL	Mix	-	-
CS-3	-			-	-
CS-4	-			-	-
CS-5	-			-	-
CS-17	-			-	-
CS-18	-			-	-
CS-19	-			-	-
CS-26	-			-	-
CS-49	-			-	-
CS-59	-			-	-
CS-65	-			-	-
TWIN-1,1	-			-	-
TWIN-1,2	-			-	-
TWIN-8,1	-			-	-
TWIN-8,2	-			-	-
TWIN-13,1	-			-	-
TWIN-13,2	-			-	-
TWIN-16,1	-			-	-
TWIN-16,2	-			-	-
TWIN-60,1	-			-	-
TWIN-60,2	-			-	-

Asp, *Acinetobacter* sp.; AF, amniotic fluid; bv, number of blood vessels involved; BAL, bronchoalveolar lavage fluid; Fn, *Fusobacterium nucleatum*; FM, fetal membrane; Gv, *Gardnerella vaginalis*; GF, gastric fluid; La, *Leptotrichia amnionii*; Ph, *Peptostreptococcus hareii*; PL, placenta; Sag, *Streptococcus agalactiae*; Sp, *Streptococcus pneumoniae/milleri*; Sau, *Staphylococcus aureus*; Uu, *Ureaplasma urealyticum*.

The predominance of PCR positive samples and chorioamnionitis/funisitis can be seen in the singleton preterm labour groups.

6.4.4 Cytokine measurements in predicting the presence of intrauterine bacteria

One of the difficulties with the measurement of cord blood cytokines has been deciding on a significant 'cut-off' concentration. 'Inflammation thresholds' for cord blood IL-6 have been calculated using receiver-operator characteristic (ROC) curves. For instance, using a ROC curve, Gomez *et al* (1998) found that the risk of serious neonatal morbidity was doubled for infants with a cord IL-6 >11pg/ml on cordocentesis. In a similar manner, ROC curves were plotted to define thresholds of fluid and tissue cytokines indicative of infection in this study. (Table 6.2, examples of ROC curves Fig. 6.3a,b). Equal weight was given to sensitivity and specificity.

	Threshold	Sensitivity	Specificity
Cord blood IL-6	45.4 pg/ml	81.3	87.5
Placental IL-6	13.1	87.5	90.9
Fetal membrane IL-6	346.1	93.3	95.2
Cord blood IL-8	35.3 pg/ml	87.5	73.9
Placental IL-8	18.3	93.8	86.4
Fetal membrane IL-8	48.4	73.3	77.3

Table 6.3 *Cut-off points for detection of intrauterine bacteria taking the maximum sum of specificity and sensitivity (derived from ROC curves)*

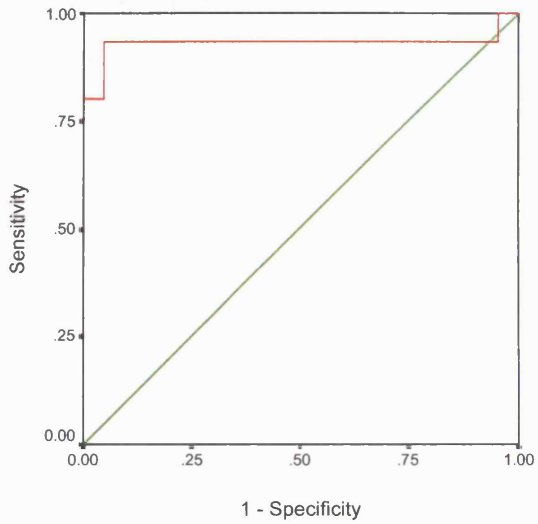


Figure 6.3a: Receiver operating characteristic curve for the detection of bacterial genes with fetal membrane IL-6

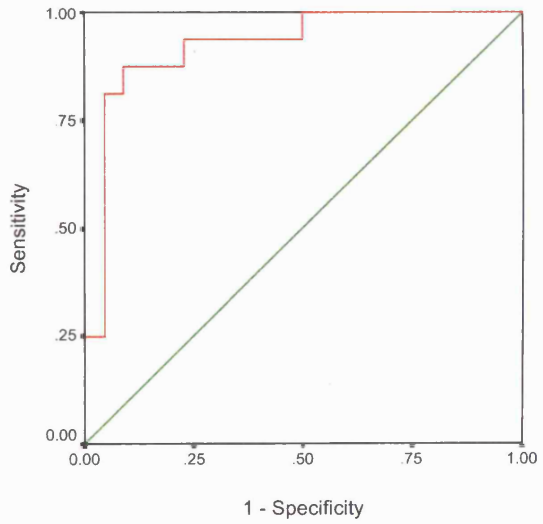


Figure 6.3b: Receiver operating characteristic curve for the detection of bacterial genes with fetal membrane IL-8

6.4.5 Early postnatal white cell count

All infants had full blood counts, and most had C-reactive protein measurements made within an hour of birth, as part of their routine clinical care (Table 6.5). Increase of both total white cell count and monocyte concentrations was observed following fetal exposure to intrauterine infection (Table 6.4). However, the CRP was not significantly elevated, and did not appear useful in either the diagnosis of intrauterine infection or fetal inflammation, although in other studies significant associations have been found (Watterberg *et al.*, 1997, Yoon *et al.*, 2003). This contrasts with cord IL-6 and IL-8, which were both sensitive and specific markers of microbial detection in this study (Table 6.3).

	Bacterial detection by PCR		
	Yes	**No**	**p value**
Total white cells (x $10^{9/l}$)	11.2 (6.8-13.7)	7.1 (5.6-9.7)	0.01
Neutrophils (x 10^9/l)	4.1 (2.8-5.3)	2.8 (1.6-4.2)	0.062
Lymphocytes (x 10^9/l)	4.5 (2.7-6.9)	3.5 (2.5-5.4)	NS
Monocytes (x 10^9/l)	0.95 (0.60-1.30)	0.39 (0.20-0.60)	<0.0001
C-reactive protein (mg/l)	5 (5-6)	5 (5-7)	NS

Values indicate median and interquartile range.
NS: not statistically significant

Table 6.4 Comparison of white cell counts and CRP with and without microbial detection by PCR. The total white cell count and monocyte count were elevated with positive PCR detection, but not the C-reactive protein.

Table 6.5 Routine full blood count data and CRP data at birth

Study No	Delivery group	Hb	WBC	*N*	*L*	*M*	*E*	*B*	NRBC	CRP
3	CS	18.9	3.7	1.78	1.48	0.37	0.04	0.04		5
4	CS	15.2	5.9							28
5	CS	17.6	3.2	0.61	2.53				1.55	
6	CS	18.5	5.6	3.47	1.62	0.45	0.06		3.41	6
17	CS	17.2	4.9	1.10	3.30	0.2	0.2			5
18	CS	17.4	7.1	0.5	5.61	0.57	0.43		15.81	5
19	CS	18.8	5.5	1.6	2.75	0.72	0.39	0.06	3.58	5
26	CS	19	11.8	8.2	2.8	0.6	0.1	0.1		56
49	CS	23.0	5.6	1.58	4.06				8.97	41
59	CS	17.6	6.6	2.96	3.22	0.20	0.13	0.07	0.72	5
65	CS	17.8	6.3	2.00	3.60	0.40	0.20	0.00		5
7	PP	17.6	10.6	4.77	4.35	0.95	0.53		0.53	5
9	PP	18.0	11.8	4.6	5.6	1.1	0.6			5
14	PP	8.1	27.7	15.24	6.93	1.66	0.55		37.46	5
38	PP	17.5	11.5	2.9	7.0	1.0	0.5			5
45	PP	17.2	8.4	2.8	4.5	0.9	0.3			5
47	PP	14.8	8.8	3.7	3.6	1.3	0.2			21
54	PP	13.0	5.4	3.10	1.77	0.32			4.55	5
57	PP	16.0	10.9	6.96	3.92				0.22	5
58	PP	17.4	6.3	2.7	2.7	0.5	0.3			5
63	PP	19.5	8.1	1.53	5.48	0.64	0.32	0.08	6.05	5
2	PTL	8.4	13.1	4.1	7.6	1.2	0.1	0.1		
10	PTL	12.9	17.8	11.93	2.85	1.96	0.53		4.8	62
41	PTL	14.4	16.8	5.3	10.5	0.6	0.3			5
46	PTL	17.6	12.8			1.2	0.2			5
51	PTL	18.6	13.4	6.4	5.9	0.7	0.4			5
52	PTL	18.7	13.8	5.2	7.0	0.9	0.7			5
53	PTL	14.0	6.5	3.29	2.58	0.58			3.94	5
61	PTL	19.7	8.7	1.50	5.60	1.00	0.40	0.10		5
62	PTL	13.6	5.3	0.70	2.40	1.80	0.40	0.10		15
64	PTL	20.2	7.8	2.11	5.39	0.31	0.00	0.00	3.98	5
1.1	TW	17.4	9.6	3.26	5.28	1.06				7
1.2	TW	17.7	4.8	1.44	3.22	.14				
8.1	TW	13.6	5.7	3.14	2.22	0.23	0.11		0.45	6
8.2	TW	17.3	4.4	3.17	0.84	0.4			0.92	7
13.1	TW	19.4	8.9	2.76	5.34	0.27	0.53		0.44	5
13.2	TW	20.8	9.8	4.7	4.02	0.49	0.59		1.57	5
16.1	TW	22.3	9.7	6.98	2.04	0.49	0.1	0.1	4.28	35
16.2	TW	14.5	8.0	5.76	2.0	0.16			2.71	36
60.1	TW	18.3	10.9	4.45	5.43	0.98	0.00	0.00	0.65	5
60.2	TW	17.9	9.5	2.00	6.95	0.38	0.19	0.00	2.48	5

6.5 Discussion

A comparison of microbial detection by PCR and intrauterine inflammation has confirmed a strong link between the two suggesting that the hypothesis for this chapter is correct. Infection was detected in all cases of funisitis or chorioamnionitis. The polymerase chain reaction can be used to detect small numbers of bacteria. Hence the technique used needs to be set to the appropriate sensitivity. The close concordance between histological inflammation and bacterial detection also indicates that the PCR assay as described had an appropriate sensitivity and specificity. It is likely that the estimates of sensitivity and specificity will be improved with studying greater numbers of infants.

6.6 Key points

1. There is strong link between intrauterine infection and inflammation. The findings presented support the contention that intrauterine inflammation is secondary to intrauterine inflammation. Bacteria or *Uu* were detected in all cases of funisitis or chorioamnionitis (hypothesis).

2. Gastric fluid may be useful in future studies of intrauterine infection especially in preterm deliveries when amniotic fluid from amniocentesis is not available.

3. Cytokine measurements in cord blood, fetal membrane and placenta are sensitive and specific in the detection of infection as detected by the polymerase chain reaction.

4. Total white cell count and monocyte count were increased in the presence of intrauterine bacteria detected by PCR. However, C-reactive protein within an hour of birth was not significantly altered in the presence of intrauterine infection. Therefore CRP did not appear to be useful in determining the presence of intrauterine infection in this sample of deliveries.

Chapter 7: Clinical consequences of antenatal infection and inflammation

7.1 Introduction

The association between antenatal infection and neonatal morbidity has been reviewed in the first chapter. Having shown that there is a tight link between intrauterine infection detected by PCR and inflammation, I sought to compare the clinical outcomes with and without infection and inflammation in the collected samples.

7.2 Hypothesis

- That intrauterine microbial detection by PCR is associated with a statistically significant effect on respiratory outcomes and other clinical outcomes

7.3 Methods used

7.3.1 Statistical analysis

Chi-squared analysis or Fisher's exact test were used to look at the occurrence of neonatal morbidity with or without the detection of intrauterine infection by PCR.

7.4 Results

As in previous chapters, intrauterine infection was said to have been detected if either 16S rRNA gene or *Uu* urease gene PCR reactions were positive in any of the samples. The comparison with clinical outcome measures is summarised in Table 7.1

	PCR +	PCR -	Significance	Test
Radiological RDS	6/16 (38%)	18/25 (72%)	**0.029**	Chi-sq
Ventilator support	11/16 (69%)	18/25 (72%)	NS	Fishers
Surfactant	10/16 (63%)	16/25 (64%)	NS	Chi-sq
Air leaks	3/16 (19%)	4/25 (16%)	NS	Fishers
CLD 28 days	5/15 (33%)*	11/25 (44%)	NS	Chi-sq
CLD 36 weeks	2/15 (13%)*	9/25 (36%)	NS	Fishers
Radiological NEC	1/16 (6%)	5/25 (20%)	NS	Fishers
IVH	4/12 (33%)	0/18 (0%)	**0.018**	Fishers

*one infant death at 24 hours
NS not significant; RDS respiratory distress syndrome; NEC necrotising enterocolitis

Table 7.1 Clinical outcomes with and without intrauterine infection detected by PCR. The significant associations with bacterial detection by PCR included a decrease in radiological RDS and an increased incidence of IVH. Ventilator support was defined as either full ventilatory support or CPAP.

7.4.1 Respiratory distress syndrome

Radiological RDS (respiratory distress syndrome) was taken as a report by a radiologist of RDS on routine chest radiology. It was assumed that infants that did not have a chest x-ray in the first 24 hours of life did not have RDS. A significant reduction in radiological RDS was observed in the infection group (OR 0.23, 95% CI 0.06 to 0.89). This finding is consistent with animal study observations which have shown short-term improvement in lung function following antenatal exposure to *E.coli* or endotoxin (appendix 1.2). It should be taken into account that this analysis is univariate, so confounding factors have not been taken into consideration.

Also recorded was the need for respiratory support and surfactant. There was no significant difference in these clinical measures between the PCR positive and negative groups (Table 7.1) and no significant difference in incidence of air leaks (Tables 7.1, 7.2).

Study No	Gestation (weeks)	Delivery category	Air leak	PCR +
1.2	30	TWIN	PIE	No
2	28	PTL	PIE	Yes
10	25	PPROM	Pneumothorax	Yes
14	25	PPROM	Pneumothorax	Yes
26	31	CS	Pneumothorax	No
57	30	CS	PIE on left Pneumothorax on right	No

Table 7.2 Infants with air leaks. There was no statistically significant difference between the PCR positive and negative groups.

7.4.2 Chronic lung disease

There was no statistically significant reduction in the incidence of chronic lung disease, associated with antenatal infection (Table 7.1). It may be that the lack of a significant difference, as shown in previous studies resulted, from the small sample size.

7.4.3 Intraventricular haemorrhage

Routine cranial ultrasounds were performed on thirty infants by radiologists independent to the study. In this sample of deliveries, all intraventricular haemorrhages occurred in the infection group (table 7.1 and 7.3). Although an interesting finding, consistent with previous reports (Tauscher *et al.*, 2003), all the infants with intraventricular haemorrhage were born after onset of labour, so this is a possible confounder. In addition, all infants were born at less than 27 weeks gestation. It has been suggested that the increased risk of IVH might occur because of blood pressure instability. However in this sample, none of the infants with IVH required inotropic support. Periventricular leukomalacia was not identified in any of the infants in this study.

Study No	Gestation (weeks)	Delivery category	Mode of delivery	Grade of IVH	PCR +
10	25	PTL	Vaginal	Grade 3 Left side	Yes
46	26	PTL	Emergency caesarean	Grade 2 Bilateral	Yes
54	24	PPROM	Vaginal	Grade 1 Left side	Yes
62	25	PTL	Vaginal	Grade 4 Right side Grade 1 Left side PHH	Yes

Table 7.3 Infants with intraventricular haemorrhage. All infants with IVH were in the 'infection detected group'. PHH: post-haemorrhagic hydrocephalus.

7.4.4 Necrotising enterocolitis

Most cases of radiological NEC occurred in the non-infection group (Tables 7.1, 7.4). Although this was not a statistically significant finding, one could hypothesise that either (a) antenatal infection could have a maturational effect on the gut in the same way that intrauterine infection and inflammation has a maturational effect on the lungs or (b) twins are at higher risk of NEC secondary to placental problems.

Study No	Gestation (weeks)	Delivery category	Surgery required	PCR +
1.2	30	TWIN	No	No
8.1	26	TWIN	No	No
8.2	26	TWIN	No	No
16.1	27	TWIN	No	No
46	26	PTL	Yes	Yes
49	29	CS	No	No

Table 7.4 Infants with necrotising enterocolitis

7.5 Discussion

The goal of studying intrauterine infection and inflammation is, in part, to accurately determine to what extent neonatal morbidity occurs as a result of intrauterine infection and inflammation. Because of the small numbers of infants in this cohort caution is needed in the interpretation of the findings. In particular there may be insufficient numbers of infants studied to detect some of the effects of intrauterine infection (e.g. an increase or decrease in CLD). The numbers studied are not sufficient to subject the data to multivariate analysis, which means that the statistical findings are subject to potential confounding factors. However, given these caveats, two statistically significant findings have arisen. The first is that decrease in radiological RDS in association with intrauterine infection. The strength of this clinical measure was that the x-rays were reported by a radiologist who was not involved in the direct care of the patient, and was not aware that the patient was in the study. However the weakness is that the same radiologist did not review the x-rays on each occasion. This finding is consistent with previous observations of the effects of chorioamnionitis in both clinical studies and animal studies. In view of the concordance of this finding with previous studies, we have chosen to explore this further by looking at potential mechanisms in the next two chapters: fetal exposure to increased cortisol and early pulmonary inflammation.

An increase in intraventricular haemorrhage was seen in the infection group. However all infants observed to have intraventricular haemorrhages were delivered before 27 weeks gestation and after spontaneous labour, potential confounding factors.

7.6 Key points

1. Radiological RDS was apparently decreased in the presence of intrauterine infection.

2. No statistically significant effect was seen on the incidence of CLD, but this may have been due to the limited number of infants it was possible to study. The results of this chapter need to be interpreted with this in mind.

3. An increase in intraventricular haemorrhage was seen in the infection group. However all infants observed to have intraventricular haemorrhage were delivered before 27 weeks gestation following spontaneous labour, potential confounding factors.

Chapter 8: Impact of antenatal inflammation and infection on umbilical cord blood cortisol

8.1 Introduction

8.1.1 Antenatal infection and lung function

A statistically significant reduction in radiological RDS was associated with fetal exposure to antenatal infection and inflammation (Section 7.4.1). These results are in keeping with the findings of both clinical and animal studies. Watterberg *et al.* (1996) found that fetuses exposed to chorioamnionitis had a lower incidence of RDS, although with an increased incidence of subsequent CLD. Exposure of animal fetuses to an intraamniotic inflammatory stimulus has improved the short-term function of preterm lungs (Appendix 1.2). The mechanisms by which the lung function might be modified require investigation. Both the stimulation of fetal cortisol (Watterberg et *al.*, 1997) and the direct effect of pro-inflammatory cytokines have been proposed (Jobe 1999).

To look at the potential mechanisms by which intrauterine infection might have an impact on lung development in the human fetus, I examined

1. Whether exposure of the fetus to infection was associated with increase in cortisol (this chapter).
2. Whether exposure of the fetus to infection was associated with an increase in pulmonary inflammation shortly after birth (Chapter 9).

There has been little work on the relationship between fetal cortisol and chorioamnionitis in human infants. In one study, a significant relationship was found between fetal cortisol and fetal IL-6 (Yoon *et al.*, 1998b) following pPROM.

8.2 Hypothesis

Antenatal infection and inflammation is associated with an increase in fetal cortisol (measured in cord blood immediately after delivery).

8.3 Methods

8.3.1 Techniques used

Collection of cord blood (Section 2.1.5)

Measurement of cortisol by ELISA (Section 2.4.6)

8.3.2 Statistical analysis

A comparison of the cortisol concentrations in the four delivery groups was made with the Kruskal-Wallis test. The Mann-Whitney test was used to look for a significant difference between the PCR positive and PCR negative groups. The relationship between cytokine concentrations and cortisol was characterised with Pearson's correlation coefficient and Spearman's rho after logarithmic transformation of the cytokine measurements.

8.4 Results

8.4.1 Cord serum cortisol concentrations in the presence of intrauterine bacteria detected by PCR

The cortisol measurements in cord blood (serum) samples are shown in Table 8.1. Measurements were possible in 31 infants. Values ranged from the baseline (55 nmol/l) to 790 nmol/l. Significantly higher cortisol concentrations were observed in the PCR positive group (Figure 8.1, Table 8.2). In keeping with this result, cortisol concentrations were also increased in the presence of chorioamnionitis and funisitis (Table 8.2).

Table 8.1 Cortisol measurements in cord blood

Study No	Delivery Category	Cortisol nmol/l
3	CS	83.4
4	CS	NA
5	CS	118.1
6	CS	90.0
17	CS	212.2
18	CS	165.3
19	CS	204.0
26	CS	161.2
49	CS	NA
59	CS	97.4
65	CS	175.3
7	PPROM	109.6
9	PPROM	417.0
14	PPROM	NA
38	PPROM	94.7
45	PPROM	87.2
47	PPROM	789.9
54	PPROM	350.8
57	PPROM	101.8
58	PPROM	700.2
63	PPROM	232.7
2	PTL	NA
10	PTL	NA
41	PTL	358.5
46	PTL	243.7
51	PTL	178.6
52	PTL	283.5
53	PTL	170.0
61	PTL	110.4
62	PTL	351.9
64	PTL	257.0
1	TWIN	NA
1	TWIN	NA
8	TWIN	NA
8	TWIN	NA
13	TWIN	145.2
13	TWIN	217.5
16	TWIN	55.2
16	TWIN	NA
60	TWIN	55.2
60	TWIN	55.2

NA: not available

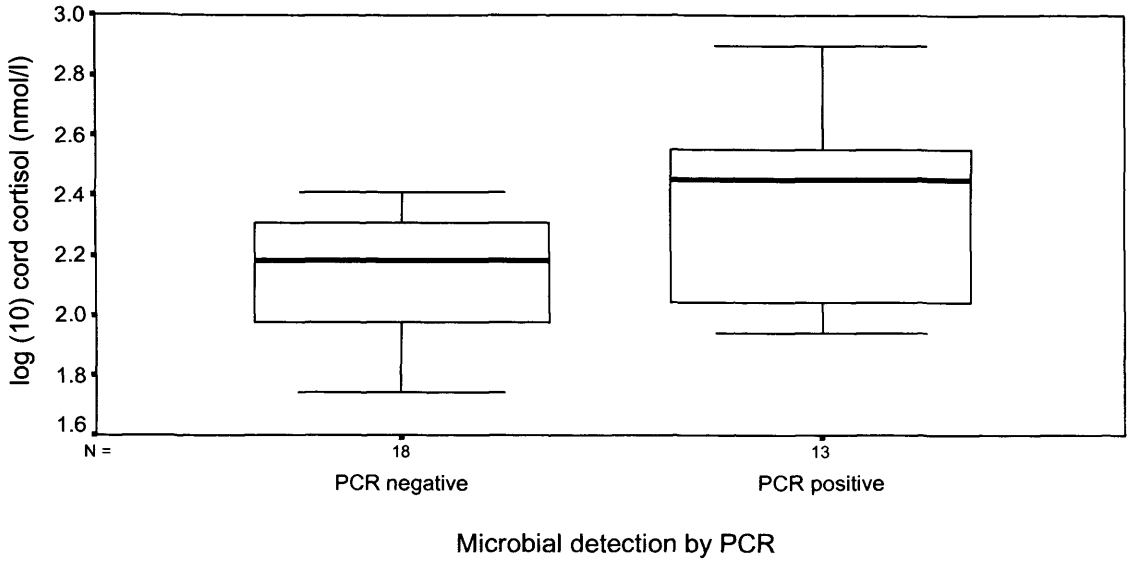


Figure 8.1 Significantly higher cord cortisol concentrations were associated with bacterial detection by polymerase chain reaction. ($p < 0.05$). Log_{10} Cortisol measurements displayed on the vertical axis

	Median cortisol (nmol/l)	**Range**	**p value**
Bacterial genes detected (n=13)	284	87-790	p<0.05
Bacterial genes absent (n=18)	153	55-257	
Funisitis (n=7)	352	87-790	p<0.05
No funisitis (n=24)	163	55-359	
Chorioamnionitis (n=8)	352	110-790	p<0.05
No chorioamnionitis (n=23)	262	55-359	

Table 8.2 Cord cortisol measurements with bacterial detection by PCR, funisitis and chorioamnionitis. Significant increases were observed in all three comparisons.

8.4.2 Relationship between fetal inflammation and the fetal stress response

A good correlation between the pro-inflammatory cytokines and cord cortisol was observed, with a stronger correlation for IL-6 (R = 0.64) than for IL-8 (R = 0.55). The closest relationship appeared to be after logarithmic transformation of the cytokine concentrations. (Figures 8.2a, b).

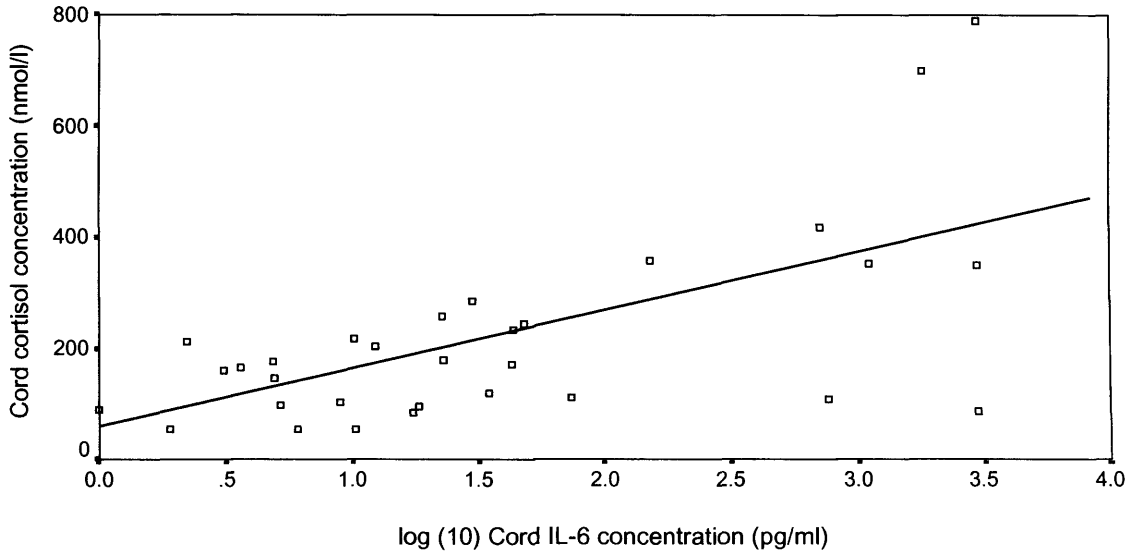


Figure 8.2a Correlation between cord interleukin-6 concentrations and cortisol (Spearman's ρ = 0.588, Pearson's R = 0.64 after logarithmic transformation of IL-6 measurements). Regression line: y = 105.6(log x) + 50.8, where y represents cortisol concentrations and x represents IL-6 measurements (p<0.001).

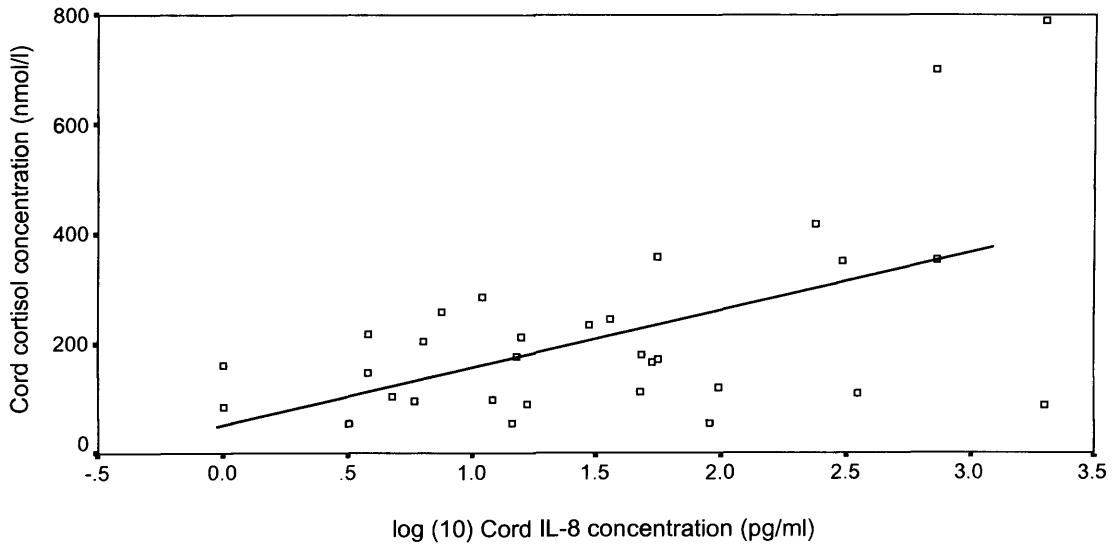


Figure 8.2b Correlation between cord interleukin-8 concentrations and cortisol (Spearmans ρ = 0.372, Pearsons R = 0.55 after logarithmic transformation of IL-8 measurements). Regression line: y = 105.4(log x) + 55.6, where y represents cortisol concentrations and x represents IL-8 measurements (p<0.001).

8.4.3 Cord serum cortisol concentrations in the preterm delivery categories

Cord cortisol values for the delivery groups were compared (Figure 8.3). The Kruskal-Wallis test identified a significant difference between the groups (p=0.032). Mann-Whitney tests between the groups are shown in Table 8.3.

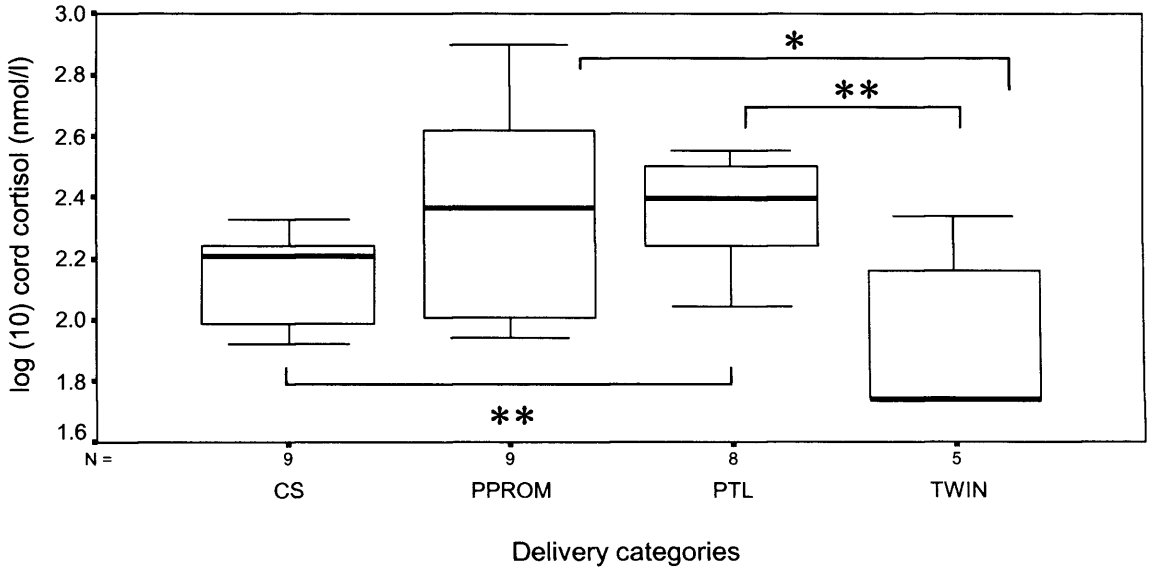


*Figure 8.3 Cortisol concentrations (vertical axis) in the four preterm delivery categories. *p=0.05, **p<0.02*

Delivery group Comparison	p value
CS vs PPROM	NS
CS vs PTL	0.016
CS vs TWIN	NS
PPROM vs PTL	NS
PPROM vs TWIN	0.05
PTL vs TWIN	0.019

Table 8.3 Results of Mann-Whitney comparisons of cortisol between delivery groups.

In term infants, vaginal delivery itself has been shown to increase fetal cortisol. Therefore the data was reanalysed including only vaginal deliveries. A significant correlation remained when the vaginal deliveries subgroup was analysed (Figure 8.4). The correlation did not remain when only caesarean section infants were included.

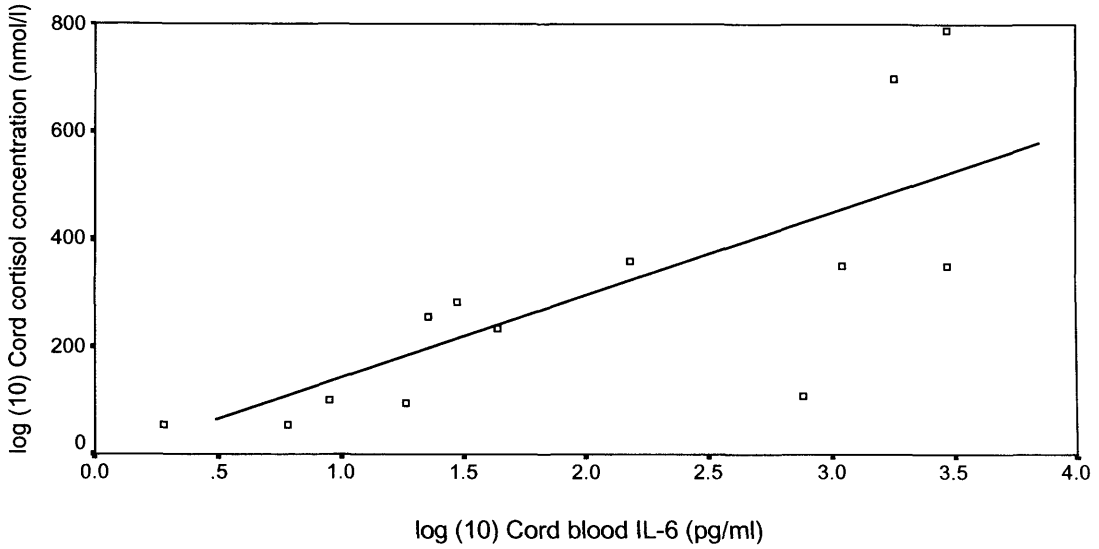


Figure 8.4 Cord serum cortisol concentrations showing a significant correlation with IL-6 measurements following preterm vaginal deliveries (n = 13, Pearson's R=0.754, p = 0.003).

8.5 Discussion

This is the first study to investigate cortisol levels immediately after birth in a cohort of preterm infants in whom the presence or absence of intrauterine bacteria has been determined using the polymerase chain reaction. Cortisol concentrations were significantly higher in deliveries complicated by intrauterine infection. Elevated cortisol measurements were also associated with funisitis, the histological counterpart of fetal systemic inflammation. The significantly increased cortisol concentrations in the spontaneous preterm labour groups may be due in part to the increased incidence of intrauterine infection in these groups compared to the prelabour caesarean and twin delivery groups. The lack of a difference between the pPROM group and prelabour caesarean groups may, in part, result from an increase in cortisol in the latter group secondary to perinatal stress (Parker *et al.*, 1993).

These results are consistent with previous findings. Watterberg *et al.* reported increased cortisol concentrations in very low birth weight infants on day 2 following exposure to chorioamnionitis (Watterberg *et al.,* 1997), which is closely related to intrauterine infection (Chapter 6, Miralles *et al.,* 2005). In addition, there was increased sensitivity of the hypothalamic-pituitary-adrenal (HPA) axis to stimulation in the first week of life following exposure to intrauterine inflammation (Watterberg *et al.,* 1997). Another study demonstrated increased fetal adrenal steroid production in response to maternal infection (Falkenberg *et al.,* 1999). Prior to these studies, a possible link between chorioamnionitis and the HPA axis had been noted when a series of post-mortem examinations revealed that infants exposed to chorioamnionitis had larger adrenal glands than those not exposed (Naeye *et al.,* 1971). More recently, Gravett et al. (2000) obtained amniotic fluid by amniocentesis from mothers in preterm labour. Increased amniotic fluid cortisol concentrations were observed in the presence of intrauterine infection.

A strong correlation between IL-6 and cortisol was observed in this study, a finding that persisted when vaginal deliveries alone were considered. A significant correlation has previously been reported in preterm infants born following pPROM (Yoon *et al.,* 1998b). I have shown that this relationship holds when the different categories of preterm delivery are included in the cohort. There is evidence that pro-inflammatory cytokines stimulate pituitary cells *in vitro* (Sapolsky *et al.,* 1987, Bernton *et al.,* 1987), and the HPA axis *in vivo* (Mastorakos *et al.,* 1993, Chrousos *et al.,* 1995), the likely reason for this observation.

Chorioamnionitis has previously been associated with improvements in short-term lung function (Watterberg *et al.,* 1996, Watterberg *et al.,* 1997, Shimoya *et al.,* 2000). It has been postulated that this effect results from an accompanying increase in cortisol (Watterberg *et al.,* 1997). Given the importance of steroids in lung development it would seem likely that increased cortisol concentrations secondary to intrauterine infection would contribute to an accelerated lung development. However it is also known that intra-amniotic inflammation in the preterm lamb model can induce lung development in the absence of a rise in cortisol, suggesting a possible role for the direct effect of cytokines (Jobe et al., 2000a). It may well be the case that that separate pathways exist.

To summarise, I have shown that intrauterine infection is associated with increased umbilical cord cortisol in cohort of preterm infants. In the human preterm infants, an increase in cortisol may play a role in modulating fetal lung development. Further studies on a larger cohort would be appropriate to relate antenatal infection and cortisol to clinical outcomes.

8.6 Key Points

1. Cortisol concentrations in cord blood were significantly higher in the presence of intrauterine bacteria detected by PCR when compared to the non-colonised group (hypothesis).

2. The cord blood cortisol concentrations were significantly higher in the presence of funisitis or chorioamnionitis (hypothesis).

3. There was a good correlation between cortisol and the concentrations of IL-6 and IL-8.

4. The cord blood cortisol concentrations were significantly higher in the spontaneous preterm labour group with intact membranes than the prelabour caesarean section group and twin group.

5. An increase in cortisol may play a role in modulating fetal lung development together with the direct effects of pro-inflammatory cytokines.

Chapter 9: Antenatal infection and pulmonary inflammation

9.1 Introduction

As the fetus is bathed in amniotic fluid, the fetal lung is likely to be exposed to the effects of inflammation or infection present in the amniotic cavity. Watterberg *et al.* (1996) found that infants born after chorioamnionitis had increased concentrations of IL-1β in their lower airway secretions and an increased risk of CLD. Increased concentrations of inflammatory mediators in amniotic fluid, indicative of chorioamnionitis, have also been identified as a risk factor for the development of CLD (Yoon *et al.,* 1997, Hitti *et al.,* 1997a). Post-mortem studies have also shown that chorioamnionitis can result in an antenatal inflammatory response in the fetal lung with inflammatory cell infiltration and increased cytokine expression (Schmidt *et al.*, 2001). In addition, chorioamnionitis can lead to a fetal systemic inflammatory response (with raised cytokines in umbilical cord blood) which is also associated with adverse respiratory outcomes (Yoon *et al.*, 1999). So although the pathogenesis of CLD is multifactorial, antenatal inflammation/infection appears to have a specific role in predisposing the fetal lung to develop CLD. In this study, bronchoalveolar (BAL) lavage was used to look at the following hypothesis.

9.2 Hypothesis

- Intrauterine infection as detected by PCR is associated with an increase in pulmonary inflammation on the day of delivery

9.3 Methods

9.3.1 Techniques used

Bronchoalveolar lavage (Section 2.1.4)

Measurement of cytokines by ELISA (Section 2.4.5)

9.3.2 Statistical analysis

The Mann-Whitney test was used to check for significant changes in BAL fluid neutrophils and cytokines in the presence of intrauterine bacteria.

9.4 Results

It was possible to retrieve BAL fluid from 21 infants. A summary of the cell count and cytokine data is shown in Table 9.1. The individual cell counts and cytokine measurements are listed in tables 9.2 and 9.3. Sufficient volume was retrieved to perform cell counts on 17 first-day BAL samples and cytokine analysis on 14 first-day BAL samples. Significant increases in pro-inflammatory cytokines were observed in association with intrauterine infection (Table 9.1 & Figures 9.1a, b). A significant increase in the number of neutrophils was also observed in association with bacterial detection in any of the intrauterine samples.

	Bacterial detection by PCR		
	Yes (n=6)	**No (n=11)**	**p value**
Neutrophils x 10^3/ml	367 (85-1098)	0 (0-42)	0.015
Alveolar macrophages x 10^3/ml	0 (0-43)	0	NS
Red cells x 10^3/ml	164 (3-15039)	111 (5-330)	NS
IL-6 (pg/ml)	2433 (1142-2925)	361 (216-1188)	0.029
IL-8 (pg/ml)	7785 (3870-16194)	1061 (241-1388)	0.013

Values indicate median and interquartile range

Table 9.1 Evidence of pulmonary inflammation in day 1 BAL samples with and without bacterial detection by PCR. Significant increases in BAL fluid neutrophils, IL-6 and IL-8 were observed.

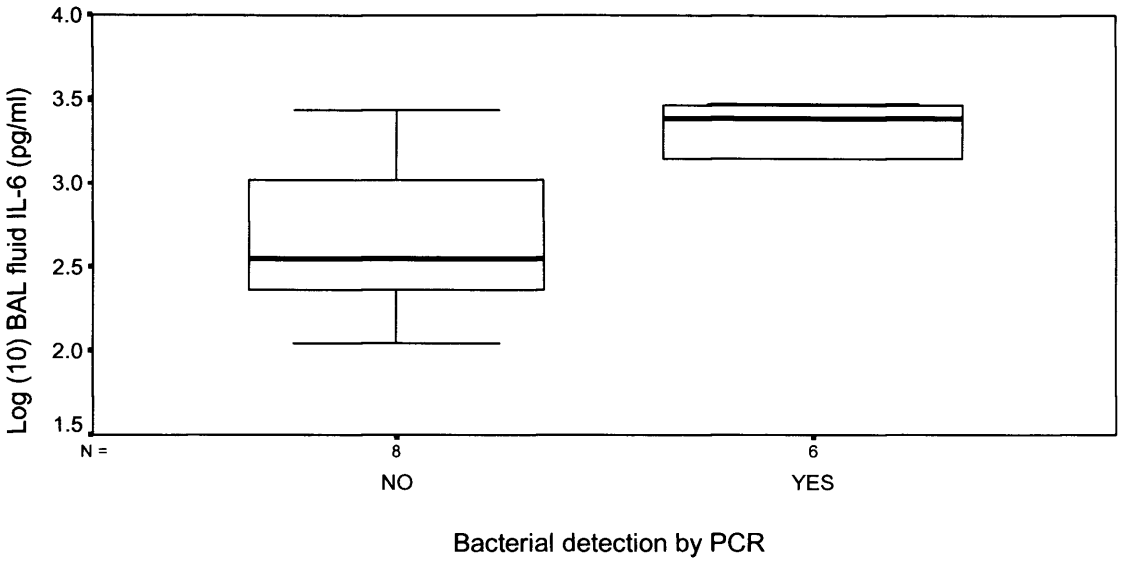


Figure 9.1a A significant increase in the IL-6 in BAL fluid on day 1 associated with intrauterine bacterial detection by PCR ($p<0.05$).

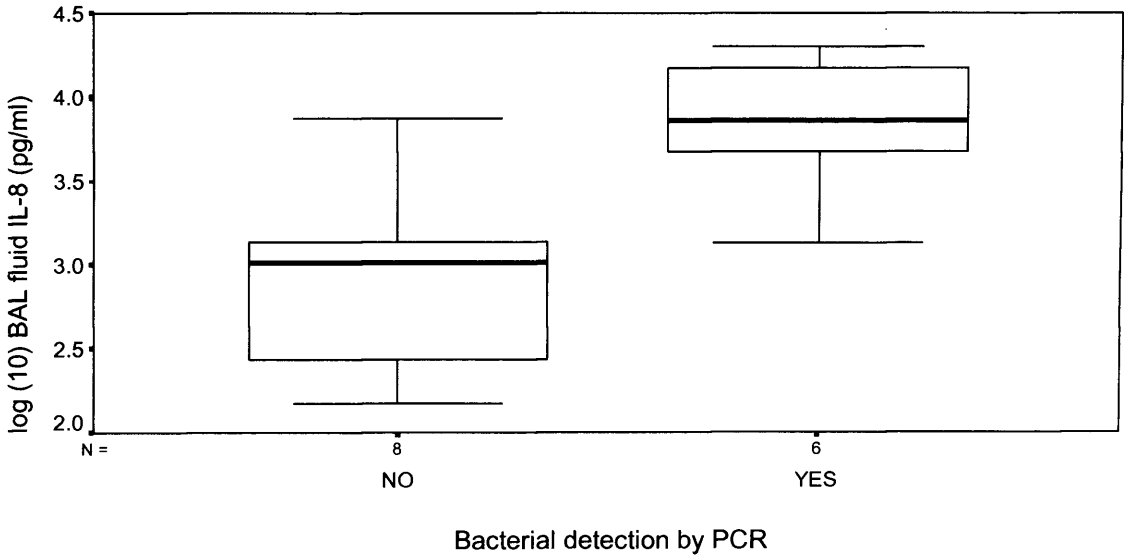


Figure 9.1b A significant increase in the IL-8 in BAL fluid on day 1 associated with intrauterine bacterial detection by PCR ($p<0.05$).

Table 9.2: Cell counts on day 1 BAL samples available from each patient

Study No	Delivery group	Day of BAL	Cell counts		
			Neut	Alv Macs	RBCs
3	CS	1		insufficient	
4	CS	1	0.0	0.0	22.8
5	CS	1	0.0	0.0	0.0
6	CS	1	4.0	0.0	0.0
17	CS				
18	CS	1	155.0	0.0	155.0
19	CS				
26	CS	1	0.0	0.0	790.0
49	CS	1	515.8	0.0	330.8
59	CS				
65	CS				
7	PPROM				
9	PPROM				
14	PPROM				
38	PPROM				
45	PPROM				
47	PPROM				
54	PPROM	1	111.8	0.0	284.2
57	PPROM	1	0.0	0.0	0.0
58	PPROM				
63	PPROM				
2	PTL	1	369.5	0.0	20250.0
10	PTL	1	1065.4	172.0	13302.5
41	PTL				
46	PTL	1	1196.6	0.0	3.4
51	PTL	1	364.1	0.0	42.6
52	PTL				
53	PTL	1	12.5	0.0	934.2
61	PTL				
62	PTL				
64	PTL				
1.1	TWIN				
1.2	TWIN	1	0.0	0.0	111.0
8.1	TWIN	1	16.7	0.0	23.3
8.2	TWIN	1	0.0	0.0	5.0
13.1	TWIN				
13.2	TWIN				
16.1	TWIN	1	41.8	0.0	153.2
16.2	TWIN				
60.1	TWIN				
60.2	TWIN				

BAL: bronchoalveolar lavage, Neut: neutrophil count, Alv Macs: alveolar macrophages, RBCs: red blood cells. Cell counts are x1000/ml BAL fluid

Cell counts on day 1 bronchoalveolar lavage sample (results available from 17 patients - one sample insufficient)

Table 9.3: Cytokine data on day 1 BAL samples available on each patient

Study No	Delivery group	Day of BAL	BAL IL-6	BAL IL-8
3	CS		insufficient	
4	CS	1	267	183
5	CS	1	112	416
6	CS	1	413	1328
17	CS			
18	CS	1	432	149
19	CS			
26	CS	1	199	1258
49	CS	1	2707	7498
59	CS			
65	CS			
7	PPROM			
9	PPROM			
14	PPROM			
38	PPROM			
45	PPROM			
47	PPROM			
54	PPROM	1	2900	14925
57	PPROM	1	290	1355
58	PPROM			
63	PPROM			
2	PTL	1	3000*	20000*
10	PTL	1	2441	10656
41	PTL			
46	PTL	1	1385	4914
51	PTL	1	2424	4717
52	PTL			
53	PTL	1	852	1399
61	PTL			
62	PTL			
64	PTL			
1.1	TWIN			
1.2	TWIN		insufficient	
8.1	TWIN		insufficient	
8.2	TWIN		insufficient	
13.1	TWIN			
13.2	TWIN			
16.1	TWIN	1	1301	864
16.2	TWIN			
60.1	TWIN			
60.2	TWIN			

* ceiling limit of assay Values pg/ml

Cytokine data on day 1 bronchoalveolar lavage samples (results available from 14 patients - four samples insufficient)

9.5 Discussion

The presence of intrauterine bacteria was associated with an increase in pulmonary inflammation on the day of delivery. Caution needs to be exercised in the interpretation of this result in view of the limited numbers studied. Bronchoalveolar lavage was only possible in the subset of infants who were ventilated and whose parents agreed for this sample to be collected. However this result is consistent with the previous observations by Groneck *et al.* (1996), who also found that microbial colonisation of the airways was associated with an inflammatory bronchopulmonary response on the first postnatal day. Therefore exposure of the fetal lung to increased concentrations of cytokines is a potential mechanism by which preterm lung disease may be modified by intrauterine infection.

9.6 Key points

1. The presence of pulmonary inflammation was assessed both by the measurement of cytokines and the assessment of BAL fluid cell counts.

2. Intrauterine infection detected by PCR is associated with pulmonary inflammation on the first day of life (hypothesis).

3. Exposure of the fetal lung to increased levels of cytokines may be part of the mechanism by which intrauterine infection and inflammation may influence lung disease of prematurity.

Chapter 10: Discussion

10.1 Significance of the Findings

10.1.1 Introduction

The work presented in the preceding chapters represents an investigation of intrauterine inflammation and infection in a cohort of premature deliveries. This cohort included mother–infant pairings from the four different subsets of preterm delivery – spontaneous preterm labour, prelabour preterm rupture of the membranes, spontaneous twin deliveries and preterm caesarean section for fetal or maternal compromise. The investigation was confined to deliveries of earlier gestation (less than 33 weeks) to increase the probability of infection-related deliveries.

The detection of intrauterine infection utilised the polymerase chain reaction to target bacterial genes, namely the 16S rRNA gene contained by all bacteria and the urease gene of *Ureaplasma urealyticum*. This technique was applied to fluids likely to be representative of the amniotic cavity (amniotic fluid, bronchoalveolar fluid, gastric fluid) as well as cord blood, placental tissue and fetal membranes.

Cytokine measurements were made in cord blood, bronchoalveolar lavage (BAL) fluid as well as placenta and fetal membrane. The presence of chorioamnionitis and funisitis was also assessed by histology. Although there are many studies which have compared the results of microbiological culture with chorioamnionitis (Pankuch *et al.*, 1984, Hillier *et al.*, 1988, Romero *et al.*, 1992, Hillier *et al.*, 1993), cytokines with culture techniques (Romero *et al.*, 1990, Romero *et al.*, 1993a,b, Jacobsson *et al.*, 2003a, Jacobsson *et al.*, 2003b, Yoon *et al.*, 2003), and cytokines with histological chorioamnionitis (Yoon *et al.*, 1995, Arntzen *et al.*, 1998, Tsuda *et al.*, 1998, Rogers *et al.*, 2002, Dollner *et al.*, 2002), this is the first study to include tissue from all subclassifications of preterm delivery and to compare all parameters using PCR-based microbial detection.

10.1.2 The detection of intrauterine infection in spontaneous preterm deliveries

Intrauterine microbes were detected by PCR in 70% of deliveries following pPROM and 80% of spontaneous singleton preterm deliveries. The frequency of detection was higher than in previous studies using laboratory culture (Lettieri *et al.*, 1993, Gonçalves *et al.*,

2002), but not as high as in studies employing fluorescent *in situ* hybridisation (FISH) to detect bacterial 16S rRNA genes (Sullivan *et al.*, 2004). The detection of bacterial genes in most spontaneous singleton deliveries is consistent with the role of antenatal infection in provoking preterm labour.

The PCR products of the amplified 16S rRNA genes were sequenced to identify the bacterial species. Frequently the same bacteria were identified in different tissues of fluid specimens from the same delivery, suggesting that the technique is robust. Most organisms that were identified have been associated with clinical infections or preterm labour. *Ureaplasma urealyticum* was the most commonly identified organism.

In one case there was a discrepancy between PCR results and culture results in multiple specimens. *Fusobacteria* were identified by PCR, while culture of both gastric fluid and placental swab showed enterococci (Tables 3.2 & 3.4). Similar discrepancies have been reported elsewhere. It may be that enterococcus (Group D streptococcus) was the dominant organism in the routine culture medium. The fusobacteria may then have been preferentially replicated by the PCR reaction. Replication of certain organisms in preference to others has previously been put forward as a reason for differing results between PCR and culture.

There was a low incidence of bacterial detection in twin deliveries compared to spontaneous singleton deliveries. In addition, twins in active labour did not have significantly elevated fetal membrane and placental cytokines. These findings support the contention that twin deliveries may have a different mechanism for the onset of labour to that postulated in singleton preterm labour, possibly a stretch mechanism.

Mothers with pPROM were usually commenced on oral erythromycin following the recommendations of the ORACLE I trial (Kenyon et al., 2001). Interestingly, bacterial genes were still detected by PCR in the fluids and tissues from three out of six deliveries in which erythromycin had been given to the mother. As suggested previously, it may be that erythromycin is ineffective in eradicating organisms once established in the upper genital tract, although it might still prevent infection in some cases.

In a minority of cases, spontaneous singleton preterm labour did occur in the absence of detectable bacterial genes or chorioamnionitis. This is an important finding as it suggests that intrauterine infection may only be one of a number of factors that lead to preterm delivery.

10.1.3 Comparison of microbial detection with measures of inflammation

In previous studies using laboratory culture to diagnose infection, bacteria have only been isolated in a proportion of those cases with evidence of intrauterine inflammation (Appendices 1.7 and 1.8). In view of these findings, it has been questioned whether chorioamnionitis may occur in the absence of infection (Hillier *et al.*, 1993). In the work presented here, bacteria were consistently detected by PCR in samples taken from deliveries with evidence of histological chorioamnionitis or funisitis. This, together with the increased rate of detection by PCR, suggests that most cases of chorioamnionitis in early preterm deliveries are secondary to intrauterine infection.

Further to this, very much higher levels of IL-6 and IL-8 in cord blood, fetal membrane, placental tissue and BAL fluid were associated with the detection of intrauterine organisms (Chapter 6). Increased concentrations of pro-inflammatory cytokines in cord blood suggest fetal inflammation which may be associated with both preterm labour and neonatal morbidity (Gomez *et al.*, 1998, Romero *et al.*, 1998).

Given the strong relationship between intrauterine infection and inflammation presented here, it is likely that the PCR assay was set at the correct sensitivity to detect bacterial infection. The increased frequency of detection using the FISH technique (*Sullivan et al.*, 2004) may have allowed the detection of additional contaminating or colonising bacteria. However the organisms detected may also have represented bacteria that were present in the fetal membranes but prevented from entering the amniotic cavity because of appropriate defences.

10.1.4 The development of a methodology for the molecular detection of intrauterine infection and inflammation

Also significant was that it proved possible to obtain information on the presence of infection in the amniotic cavity by analysing proxies for amniotic fluid, including gastric fluid and bronchoalveolar lavage fluid as well as placenta and fetal membrane.

The reason for such an approach was to develop a methodology for the detection of intrauterine infection that did not rely on amniocentesis. Of the samples collected, gastric fluid appeared particularly useful. Bacteria were detected in gastric fluid from all deliveries with chorioamnionitis on histology. Equally important, bacteria were not detected in gastric

fluid in the absence of chorioamnionitis. Therefore gastric fluid may prove useful as a research tool in the investigation of intrauterine infection. This needs to be confirmed in a larger study. The main disadvantage for clinical purposes would be that gastric fluid would not be available before birth.

In some cases organisms were also isolated from bronchoalveolar lavage (BAL) fluid. However, BAL is clearly more invasive than aspiration of gastric contents, and is only possible in ventilated infants.

Amniotic fluid was only obtained in a minority of patients and this prevented a direct comparison of gastric aspirate and amniotic fluid microbiology.

10.1.5 The development of a methodology to quantify inflammation in placenta and fetal membrane

An excellent correlation was observed between pro-inflammatory cytokine measurements made in fetal membrane and those made in amniotic fluid. Therefore, by quantifying pro-inflammatory cytokines in protein extracts from placenta and fetal membrane it was possible to make quantitative measurements of chorioamnionitis. The cytokine concentrations were significantly higher in deliveries associated with the detection of intrauterine bacteria. Moreover, it was shown that cytokine measurements in the fetal membranes and placenta could be both sensitive and specific at identifying those deliveries in which bacteria had been detected. Thus fetal membrane measurements may therefore be useful in determining the exposure of the fetus to intrauterine and intra-amniotic inflammation, particularly where amniotic fluid is not available.

10.1.6 Clinical outcomes

Univariate analysis demonstrated a reduction in radiological RDS with intrauterine infection. This finding is consistent with the previous report by Watterberg *et al.* (1996) and animal studies (Appendix 1.2). There was also an increased incidence of intraventricular haemorrhage (IVH) with infection. However it is not possible to say whether this was due to the infection itself or some other factor such as gestation or mode of delivery - all the infants with IVH had been delivered after the spontaneous onset of labour before 27 weeks gestation. The same issue is relevant to the conclusions reached by Tauscher et al. (2003). They observed significantly higher IL-6 concentrations in association with intracerebral

haemorrhage (Appendix 1.3). However the gestational age was lower in the intracerebral haemorrhage group and multivariate analysis was not performed.

Sufficient patients were recruited for this study to demonstrate an increase in inflammatory mediators in the presence of intrauterine infection. However, to adequately demonstrate changes in clinical outcomes such as chronic lung disease with multivariate analysis would require larger studies perhaps involving a number of centres as discussed below. Almost 300 infants were included in the study by Goepfert *et al.* (2004) allowing multivariate analysis to be performed. Also in a recent intervention trial, the estimated sample size to pick up a 10% reduction in survival without CLD at 36 weeks was 700 infants. This figure was based on a baseline incidence of CLD of 36%, similar to that in the cohort presented in this thesis (29%) (Watterberg et al., 2004).

10.1.7 Mechanisms by which lung disease may be modified

It had been postulated that increases in cortisol may lead to a decrease in RDS in preterm infants exposed to chorioamnionitis (Watterberg *et al.*, 1997). However, the need for elevations in cortisol was questioned by the findings of animal studies that found an improvement in lung function with intrauterine inflammation without any elevations in cortisol (Jobe et al., 2000a). The possibility of a direct effect of cytokines on fetal lung development has therefore been cited.

In this study it was found that intrauterine infection lead to both elevated fetal cortisol concentrations and increased pulmonary inflammation on the first day of life. These observations suggest that both pathways are potential mechanisms by which intrauterine infection may lead to changes in lung development and in neonatal lung diseases.

10.2 Further avenues of research arising from this work

10.2.1 Detection of intrauterine bacteria in a larger population

Intrauterine infection and chorioamnionitis are more common with decreasing gestation, as are the complications of prematurity such as PVL, IVH and CLD. Hence the question arises whether any increase in these complications of prematurity is due to intrauterine infection or whether it is due to prematurity *per se*.

It has been shown that gastric fluid may a useful proxy for amniotic fluid in the detection of intrauterine infection. No longer limited by the availability of amniotic fluid from amniocentesis, it should be possible to study larger number infants comparing different populations. This would be next logical step. While it is an invasive procedure to obtain gastric fluid, the procedure is certainly less invasive than both amniocentesis and bronchoalveolar lavage. A disadvantage of using gastric fluid is that it would only be available after delivery. However it may well be useful in any research looking at the effects of antenatal infection and inflammation on the fetus. A promising hypothesis to follow would be that of a 'two hit' model of chronic lung disease – that intrauterine infection and chorioamnionitis might prime the lung to be susceptible to injury, but that this injury only occurs if combined with significant postnatal ventilation and oxygen (Jobe 2005). Interestingly, a similar hypothesis has been proposed for brain injury. It has been put forward that infection may sensitise the fetus to a subsequent hypoxic-ishaemic insults by lowering the threshold for brain injury. (Kendall and Peebles, 2005).

10.2.2 Defences against invading bacteria and genetic variation in inflammatory response

Some bacteria detected in the fetal membrane may include those organisms failing to invade the amniotic cavity. The cases in which bacteria successfully invade the amniotic cavity may relate to inadequate defences, either due to genetic susceptibilities or nutritional deficiencies. In addition, functional cytokine polymorphisms have been identified which can affect the magnitude of an inflammatory response and may therefore have an impact on complications of prematurity such as CLD and cerebral white matter damage. These factors may be best investigated in conjunction with studies looking at the presence of intrauterine infection and inflammation as well as clinical outcomes.

10.2.3 Therapeutic strategies

Perhaps the most important challenge is to develop therapeutic strategies to determine if both preterm labour and neonatal morbidity can be decreased by appropriate antimicrobial treatment. As discussed in the introduction, erythromycin may fail to clear *Uu* from the genital tract and erythromycin may not consistently eradicate *Uu* from the preterm infant's respiratory tract (Baier *et al.*, 2003). In some of the cases studied here, there was evidence of chorioamnionitis together with infection with *Uu* and other organisms even after maternal treatment with the currently recommended oral erythromycin. It has been suggested that alternative antibiotic regimes may be more effective clinically, particularly if used in combination with initial intravenous antibiotics. The importance of using appropriate antibiotics was also illustrated well by the ORACLE studies (Kenyon *et al.*, 2001). Although outcome was improved by erythromycin in singleton pPROM, co-amoxiclav appeared to increase the incidence of NEC. If any screening regime is to be successful, then the antibiotic choice needs to be correct. The use of the techniques described, in particular the application of PCR detection to gastric fluid will enable the effectiveness of antibiotic regimes to be assessed in adequate numbers.

Another approach would be the use of treatments to reduce the effects of inflammation once infection had been eradicated, such as anti-inflammatory medications, anti-inflammatory cytokines or perhaps antibodies directed at pro-inflammatory cytokines. The most widely-used anti-inflammatory medication in the newborn is dexamethasone. However there are concerns about its side-effects, in particular possible long-term neurological consequences and there is a large variation in the dosage used. Bronchoalveolar lavage, with inflammatory cell counts and cytokine measurements, may be useful together with clinical outcomes in determining the optimum dosage.

10.2.4 Viral infections and neonatal morbidity

Recent studies have looked at the relationship between viral infection and the subsequent development of chronic lung disease. The concept that viral infections contribute to the development of chronic lung disease is plausible. In particular, the potential for both adenovirus and cytomegalovirus (CMV) to cause serious respiratory infections is well established.

In one study using PCR to detect a range of bacterial and viral organisms, adenovirus in the tracheal aspirates of preterm infants in the first week of life was associated with the development of CLD (Couroucli *et al.*, 2000). In another study CLD

was associated with an increased incidence of CMV infection (Sawyer *et al.*, 1997). However, a study examining the incidence of CMV and adenovirus in tracheal aspirates in the first months of life showed that these infections were common in preterm infants, but did not confirm an association with either CMV or adenovirus with the development of CLD (Prosch *et al.*, 2002).

It should be possible to detect viral organisms in the samples collected to confirm or refute the association with CLD by using PCR-based detection. In addition, while it is not anticipated that viral organisms contribute to chorioamnionitis, it is possible that they may contribute to villitis of unknown aetiology.

10.2.5 *In situ* hybridisation and the investigation of pPROM

Despite the high sensitivity of PCR in detecting bacteria, it does not give much information on the localisation of bacteria within the tissues. *In situ* hybridisation involves a hybridisation reaction between a labelled nucleotide probe and a target DNA in tissues. The reaction can be visualised allowing bacterial localisation. This technique has been recently applied to fetal membrane and placenta (Sullivan *et al.*, 2004).

The localisation of bacteria is probably not of critical importance in those cases where there is widespread inflammation in the amniotic fluid and fetal membrane. However, premature rupture of membranes was not associated with infection and inflammation in all cases presented here. It is conceivable that a localised area of bacteria may contribute to the process of rupture by weakening the fetal membrane without being present in the amniotic fluid and without provoking widespread chorioamnionitis. Despite the shortcomings discussed in Section 10.1.3 *in situ* hybridisation may well be useful in looking at this possibility.

10.2.6 Detailed study of subtypes of chorioamnionitis and the development of CLD

Knowing that elevated amniotic fluid and fetal cytokine concentrations are associated with the development of chronic lung disease and that increased cytokine levels are found in chorioamnionitis and funisitis it seems plausible that histological chorioamnionitis may be associated with the development of CLD. This was the finding of Watterberg *et al.* (1996).

Attempts have previously been made to relate subtypes of intrauterine inflammation to the development of chronic lung disease. In one study the development of chronic lung

disease was associated with a particular form of funisitis known as necrotising funisitis (Matsuda *et al.*, 1997). In another study, the authors reported that chorioamnionitis with amniotic epithelium necrosis was associated with the subsequent development of CLD (Ohyama *et al.*, 2002).

To look at patterns of chorioamnionitis in detail, paraffin embedded tissue was stained for the presence of neutrophil elastase. This was achieved using immunocytochemistry with an antibody directed at neutrophil elastase and visualisation with peroxidase reaction. In our samples, three basic patterns of fetal membrane inflammation were observed (Fig. 10.1):

1. Neutrophil invasion of the chorion.
2. Neutrophil invasion of both the chorion and amnion.
3. Destruction of the structure of the fetal membrane.

It is clear that in some samples the amnion basement membrane formed a barrier to the neutrophils. In some cases the inflammation was sufficiently damaging to destroy the structure of the fetal membrane so that amnion and chorion were not distinguishable. In addition in a number of cases, there were no viable cells in the amnionic membrane. It was also possible to easily identify the presence or absence of funisitis. Using this neutrophil elastase technique could be utilised to investigate the relationship between subtypes of chorioamnionitis and neonatal morbidity, including CLD. This work is at preliminary stage, and it is likely that if any correlation with clinical outcomes were to be achieved further samples would be required.

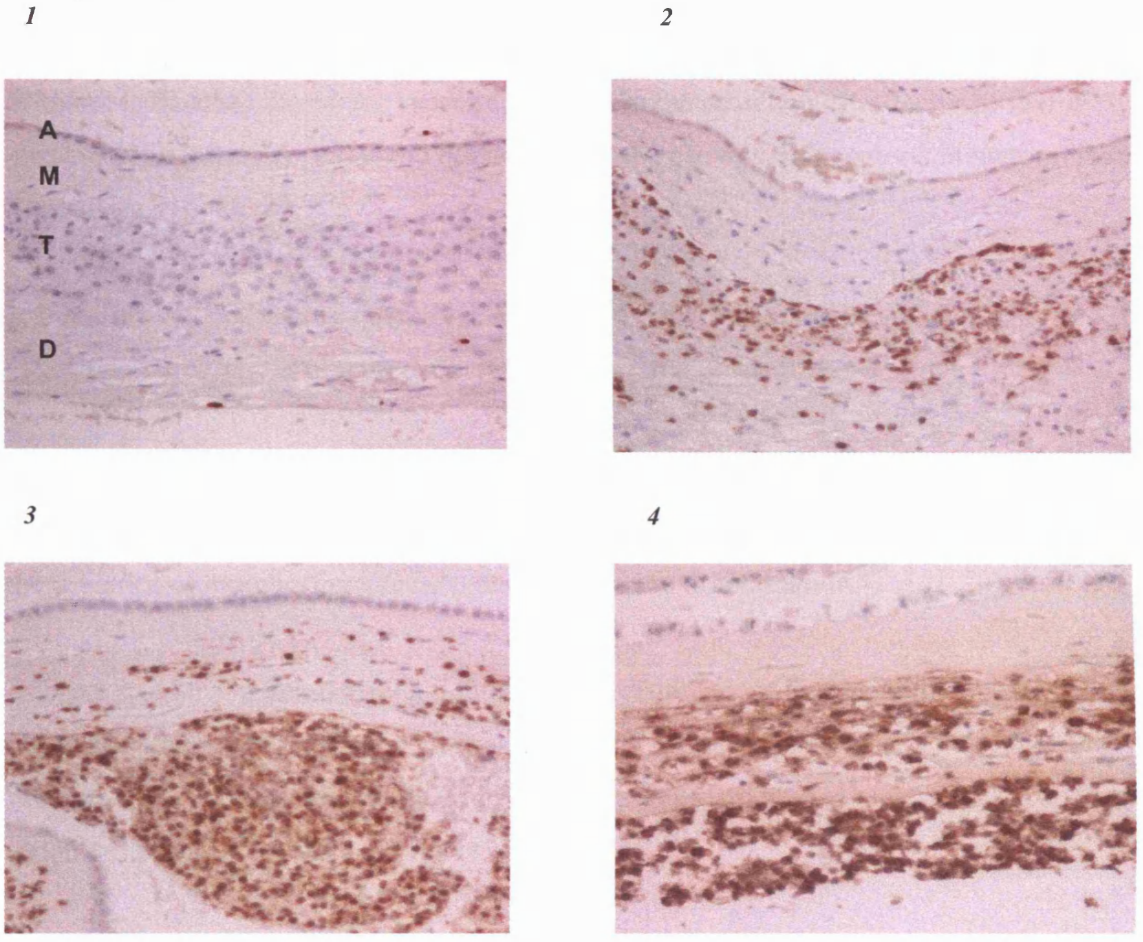


Figure 10.1 Patterns of fetal membrane inflammation visualised using neutrophil elastase immunocytochemistry:

1. Normal fetal membrane. (A: amniotic epithelium, M: mesoderm, T: trophoblast cell layer, D: decidua)

2. Invasion of the trophoblast layer by neutrophils, but without passage of neutrophils into the chorionic mesoderm.

3. Chorioamnionitis with neutrophil invasion into the trophoblast cell layer and mesoderm.

4. Destruction of fetal membrane structure by neutrophil invasion.

10.3 Key discussion points:

1. Novel techniques have been successfully used in the detection of intrauterine and intraamniotic infection. These did not depend on the use of amniocentesis in preterm labour (secondary hypothesis).

2. It has proved possible to quantify placental and fetal membrane inflammation by measuring cytokines in protein extracts (secondary hypothesis).

3. By applying PCR-based microbial detection (targeting 16S rRNA genes and the *Uu* urease gene) to a range of samples including gastric fluid, it has been possible to compare the presence of intrauterine infection and intrauterine inflammation.

4. There was strong link between intrauterine infection and inflammation. All cases of histological chorioamnionitis were associated with the positive microbial detection. The findings support the contention that intrauterine inflammation is in most cases secondary to intrauterine infection (Main Hypothesis).

5. The association between spontaneous singleton preterm deliveries and intrauterine infection and inflammation has been clearly shown.

6. Clinical findings included a reduction in radiological RDS and an increase in intraventricular haemorrhage. However only univariate analysis was possible in view of the number of infants studied.

7. Intrauterine infection may potentially modulate fetal lung development and neonatal lung disease in two ways: either by a direct effect of cytokines, or by provoking circulating cortisol in the fetus.

8. There are many avenues for research leading from this body of work. The development of a useful technique for the retrospective analysis of intrauterine infection and inflammation will allow study in greater numbers in different centres.

9. The exciting prospect of studying further the effects of intrauterine infection is that intervention trials may become possible in the future, either with effective antibiotics or with other anti-inflammatory strategies.

Appendix 1.1: Cord IL-6 and respiratory morbidity

Study	Patients	n	Clinical feature & Incidence	IL-6 Feature present	IL-6 Feature absent
An 2004*	Preterm less than 29 weeks	30	CLD at 28 days (n=18 case-control)	median 103 range 0-17100	median 7.1 range 0-1400 (p<0.05)
Goepfert 2004*	Preterm 24-32 weeks	294	CLD at 28 days n=42 (14%)	median 13.95 IQR 7.5 to 176.5	median 11.3 IQR 4.1 to 44.1 (p=0.03) Not significant when controlling for gestation
			CLD at 36 weeks n=16 (5%)	median 9.7 IQR 8.4 to 56.6	median 11.4 IQR 4.2 to 54.2 (p=0.32)
Yoon 1999	Preterm 25-34 weeks	203	CLD 34/203 (17%)	median 68.3 range 0.3 to 6150	median 6.9 range 0-19230 (p<0.001)

*published during completion of this thesis
Abbreviations: CLD: chronic lung disease, IQR: interquartile range.

Published studies investigating the relationship between cord IL-6 and the subsequent development of chronic lung disease. The two columns on the right of the table illustrate the higher IL-6 concentrations in cord blood taken from those infants who subsequently developed chronic lung disease compared to IL-6 levels from infants who did not develop chronic lung disease. Importantly, the study by Goepfert et al. indicated that this difference might not be significant when taking confounding factors (e.g. gestational age) into account.

Appendix 1.2: Respiratory effects of experimentally induced inflammation in animal models

Study	Model	Stimulus	Effects
Bry 1997	Preterm rabbit	Intra-amniotic recombinant IL-1alpha	Increased mRNA levels for surfactant proteins Improved compliance
Emerson 1997	Preterm lamb	Intra-amniotic IL-1 alpha	Improved lung function No elevation of cortisol, catecholamine levels
Jobe 2000a	Preterm lamb	Intra-amniotic E.coli endotoxin	Increased compliance (2-fold) Increased lung gas volumes (4 to 5-fold) Increased saturated phosphatidylcholine No increase in fetal plasma cortisol

Published studies investigating the respiratory effects of experimentally induced inflammation in animal models. An improvement in lung function and compliance was noted after short term exposure to an inflammatory stimulus. In two studies there was no observed increase in fetal plasma cortisol.

Appendix 1.3: Cord IL-6 and neurological morbidity

Study	Patients	n	Clinical feature & Incidence	IL-6 Feature present	IL-6 Feature absent
Goepfert 2004*	Preterm 24-32 weeks (PTL 193 indicated 116)	294	Periventricular leukomalacia 9/294 (3.1%)	median 535.2 IQR 107.8 to 1517.6	median 11.1 IQR 4.3 to 41.4 (p=0.002)
Tauscher 2003*	Preterm less than 32 weeks	106	Intracerebral haemorrhage 20/106 (18.9%)	89.2 IQR 11.5 - 1223	2.7 IQR 1.2 - 16.7 (p<0.001)
Yoon 1996	Preterm 25 to 36 weeks	172	Periventricular leukomalacia 25/172 (14.5%)	median 718 range 226-32000	range 226-43670

*published during completion of this thesis
Abbreviations: PTL: preterm labour, IQR: interquartile range.

Published studies investigating the relationship between cord IL-6 and neurological pathology identified by cranial ultrasound. The two columns on the right of the table illustrate the higher IL-6 concentrations in cord blood associated with either periventricular white matter damage or intracerebral haemorrhage.

Appendix 1.4: Neurological effects of experimentally induced inflammation in animal models

Study	Model	Stimulus	Effects
Gilles 1976	Newborn kitten	Intraperitoneal Bacterial endotoxin	White matter damge in telencephalon: Gliosis and necrosis
Young 1983	Newborn dogs	Subcutaneous E.coli endotoxin	Cerebral damage with a marked inflammatory response
Ornoy 1976	Rats	Maternal bacterial infection (E.coli endotoxin)	Neuronal necrosis (10 times that seen in controls)
Yoon 1997c	Rabbit	Maternal bacterial infection Inoculation E. coli into amniotic sac	White matter damage including apoptosis
Debillon 2000	Rabbit	Maternal bacterial infection Inoculation into amniotic fluid	Programmed cell death in white matter

Published studies of experimentally induced inflammation in animal models. The effects of exposure to an inflammatory stimulus included white matter damage and apoptosis.

Appendix 1.5: Cord IL-6 and other morbidity

Study	Patients	n	Clinical feature & Incidence	IL-6 Feature present	IL-6 Feature absent
Goepfert 2004*	Preterm 24-32 weeks (PTL 193 indicated 116)	294	NEC	median 21.7 IQR 6.9 to 162.9	median 9.8 IQR 3.9 to 38.7 (univariate p=0.003)
Goepfert 2004*	Preterm 24-32 weeks (PTL 193 indicated 116)	309	Combined neonatal morbidity	median 13.4 IQR 4.4 to 66.8	median 7.2 IQR 3.8 to 26.3 (univariate p= 0.02) not significant when controlling for GA
Gomez 1998	Preterm (pPROM 52 PTL 105)	157	Severe neonatal morbidity	median 14 range 0.5 to 900	median 5.2 range 0.3 to 900 ($p < 0.005$)
Weeks 1997	Preterm	133	Neonatal complications 16/133	median 145 range 0 to 2628	median 0 range 0 to 2024 (p=0.002)

*published during completion of this thesis

Abbreviations: PTL: preterm labour, pPROM: preterm prelabour rupture of membranes, NEC: necrotising enterocolitis, IQR, interquartile range, GA: gestational age.

Published studies investigating the relationship between cord IL-6 and neonatal morbidity (further to Appendices 1.1 and 1.3). The two columns on the right of the table illustrate the higher IL-6 concentrations in cord blood taken from those infants who subsequently developed neonatal complications. The study by Goepfert et al. indicates that a positive result with univariate analysis may not be statistically significant when controlling for confounding factors.

Appendix 1.6: Cord IL-6 study characteristics

Study	Patients	n	Clinical feature & Incidence	Correction for gestational age
An 2004*	Preterm less than 29 weeks	30	CLD at 28 days	Univariate analysis No correction. Lower gestation in CLD group although not statistically significant
Goepfert 2004*	Preterm 24-32 weeks (PTL 193 indicated 116)	309	BPD, CLD PVL Combined neonatal morbidity	Controlled for gestational age Multivariate analysis
Tauscher 2003*	Preterm less than 32 weeks	106	Intracerebral haemorrhage	Univariate analysis No correction Significantly lower gestation infants in ICH group
Yoon 1999	Preterm 25-34 weeks	203	CLD at 28 days	Correction for gestational age and logistic regression
Duggan 2001	Preterm 23-29 weeks	50	Cerebral lesions on early MRI	Univariate No correction
Yoon 1996	Preterm 25 to 36 weeks	172	Periventricular leukomalacia	Multivariate analysis
Gomez 1998	Preterm (pPROM 52 PTL 105)	157	Severe neonatal morbidity	Multiple logistic regression
Weeks 1997	Preterm	133	Neonatal complications	Multiple logistic regression

Abbreviations: PTL: preterm labour, PPROM: preterm prelabour rupture of membranes, CLD: chronic lung disease, BPD: bronchopulmonary dysplasia, PVL: periventricular leukomalacia, ICH: intracerebral haemorrhage

The characteristics of the studies investigating the association between neonatal morbidity and IL-6. These are presented here in particular to illustrate that even some recent studies have only performed univariate analysis and have not controlled for gestational age.

Appendix 1.7: Relationship between bacterial isolation and histological chorioamnionitis in preterm deliveries in previous studies

Study	Patients	n	Histological CAM (Chorio-amnionitis)	Bacterial isolation Amniotic fluid	Bacterial isolation Chorioamnion	Bacteria isolated when inflammation present
Pankuch 1984	PTL PPROM Term Term PROM Overall	24 23 19 2 68	25/68 (37%)			3/3 (100%) 6/8 (75%) 18/25 (72%)
Hillier 1988	Preterm less than 37 weeks	38	20/38 (53%)		23/38 (61%)	15/20 (75%)
Zlatnik 1990	16-26 weeks 27-34 weeks Overall	50 45 95	51/95 (54%)			33/51 (65%)
Hillier 1991	PTL less than 34 weeks	112	66/112 (59%)		36/112 (32%)	22/66 (33%)
Romero 1992	PTL delivery within 48 hours of amniocentesis	92		35/92 (38%)		Amnionitis cases 71.1% Funisitis cases 78.7%
Hillier 1993	PTL less than 34 weeks (afebrile)	50	22/50 (44%)	9/50 (18%)	16/50 (32%)	

The right hand column details the number of cases of chorioamnionitis from which it was possible to isolate bacteria by culture. The overall percentages have varied from 33% to 75%, leaving at least 25% of cases of chorioamnionitis in which no bacteria were identified. In the study by Hillier et al. (1993) no percentage is available in the publication but the rate of bacterial isolation is clearly lower than the detection of chorioamnionitis.

Appendix 1.8: Relationship between elevated amniotic fluid IL-6 and detection of bacteria by culture in previous studies

Study	n	Patients	Elevated IL-6	Bacterial Isolation Amniotic fluid	Bacterial Isolation Chorioamnion	Bacteria isolated when IL-6 raised
Hillier 1993	50	PTL before than 34 weeks gestation	30/49 (61%)	9/50 (18%)	16/50 (32%)	9/30 (30%)
Coultrip 1994	89	PTL	25/89 (28%)*	12/89 (13%)		9/25 (36%)*
Yoon 2001	206	PTL	44/206 (21%)	21/206 (10%)		19/44 (43%)
Jacobsson 2003a	61	PTL	39% (46% with IL-8)	10/61 (16%)		
Jacobsson 2003b	58	PPROM before 34 weeks gestation	47% (57% with IL-8)	25%		10/27 (37%)

* calculated figure
Abbreviations: PTL: preterm labour, PPROM: preterm prelabour rupture of membranes

A comparison between increased amniotic fluid IL-6 concentraions and the isolation of bacteria from amniotic fluid/chorioamnion in preterm labour. The right hand column details the number of cases of chorioamnionitis (as diagnosed by an elevated amniotic fluid IL-6 concentration) from which it was possible to isolate bacteria by culture. The percentages varied from 30% to 43% indicating a significant number of cases of chorioamnionitis in which no bacteria are identified.

Appendix 1.9: Characteristics of previous studies investigating amniotic fluid inflammation and infection

Study	n	Patients	Study Information	Amniotic fluid IL-6 threshold
Hillier 1993	50	PTL before 34 weeks gestation		1500pg/ml
Coultrip 1994	89	PTL		6170pg/ml ROC for a positive AF culture
Yoon 2001	206	PTL		2600pg/ml ROC for the a positive AF culture
Jacobsson 2003a	61	PTL	included specific PCR for Uu and Myc hominis	1500pg/l ROC for the identification of preterm delivery within 7 days
Jacobsson 2003b	58	PPROM before 34 weeks gestation	included specific PCR for Uu and Myc hominis	800pg/ml ROC for the identification of preterm delivery within 7 days

Abbreviations: PTL: preterm labour, PPROM: prelabour preterm rupture of membranes, *Uu*: Ureaplasma urealyticum, *Myc*: Mycoplasma, ROC: receiver operating characteristic, AF: amniotic fluid.

The characteristics of the studies investigating the association between amniotic fluid inflammation (IL-6) and infection. Receiver operator characteristic curves have been used to determine the threshold of a significantly elevated IL-6 concentration. This has lead to different thresholds in each of the studies.

References

Allmark P, Mason S, Gill AB, Megone C. Obtaining consent for neonatal research. Arch Dis Child Fetal Neonatal Ed. 2003;88:F166-7.

Alpert BE, O'Sullivan BP, Panitch HB. Nonbronchoscopic approach to bronchoalveolar lavage in children with artificial airways. Pediatr Pulmonol 1992;13:38-41.

Altshuler G, Hyde S. *Fusobacteria.* An important cause of chorioamnionitis. Arch Pathol Lab Med 1985;109:739-43.

Altshuler G, Hyde S. Clinicopathologic considerations of *fusobacteria* chorioamnionitis. Acta Obstet Gynecol Scand 1988;67:513-7.

An H, Nishimaki S, Ohyama M, Haruki A, Naruto T, Kobayashi N, Sugai T, Kobayashi Y, Mori M, Seki K, Yokota S. Interleukin-6, interleukin-8, and soluble tumor necrosis factor receptor-I in the cord blood as predictors of chronic lung disease in premature infants. Am J Obstet Gynecol 2004;191:1649-54.

Andrews WW, Hauth JC, Goldenberg RL, Gomez R, Romero R, Cassell GH. Amniotic fluid interleukin-6: correlation with upper genital tract microbial colonization and gestational age in women delivered after spontaneous labor versus indicated delivery. Am J Obstet Gynecol 1995;173:606-12.

Andrews WW, Hauth JC, Goldenberg RL. Infection and preterm birth. Am J Perinatol 2000;17:357-65.

Aquino TI, Zhang J, Kraus FT, Knefel R, Taff T. Subchorionic fibrin cultures for bacteriologic study of the placenta. Am J Clin Pathol. 1984;81:482-6.

Arntzen KJ, Kjollesdal AM, Halgunset J, Vatten L, Austgulen R. TNF, IL-1, IL-6, IL-8 and soluble TNF receptors in relation to chorioamnionitis and premature labor. J Perinat Med 1998;26:17-26.

Asrat T. Intra-amniotic infection in patients with preterm prelabor rupture of membranes. Pathophysiology, detection, and management. Clin Perinatol 2001;28:735-51.

Attar MA, Donn SM. Mechanisms of ventilator-induced lung injury in premature infants. Semin Neonatol 2002;7:353-60.

Bagchi A, Viscardi RM, Taciak V, Ensor JE, McCrea KA, Hasday JD. Increased activity of interleukin-6 but not tumor necrosis factor-alpha in lung lavage of premature infants is associated with the development of bronchopulmonary dysplasia. Pediatr Res 1994;36:244-52.

Baier RJ, Loggins J, Kruger TE. Failure of erythromycin to eliminate airway colonization with *Ureaplasma urealyticum* in very low birth weight infants. BMC Pediatr 2003;3:10.

Bearfield C, Davenport ES, Sivapathasundaram V, Allaker RP. Possible association between amniotic fluid micro-organism infection and microflora in the mouth. BJOG 2002;109:527-33.

Bernton EW, Beach JE, Holaday JW, Smallridge RC, Fein HG. Release of multiple hormones by a direct action of interleukin-1 on pituitary cells. Science 1987:238:519-521.

Bernischke K, Kaufman P. Pathology of the Placenta, 4th Ed. Springer-Verlag, New York, pp 591-684.

Berry SM, Romero R, Gomez R, Puder KS, Ghezzi F, Cotton DB, Bianchi DW. Premature parturition is characterized by in utero activation of the fetal immune system. Am J Obstet Gynecol 1995;173:1315-20.

Berry SM, Gomez R, Athayde N, Ghezzi F, Mazor M, Yoon BH. The role of granulocyte colony stimulating factor in the neutrophilia observed in the fetal inflammatory response syndrome. Am J Obstet Gynecol 1998;178:S202

Blanc WA. Amniotic sac infection syndrome: pathogenesis, morphology and significance in circumnatal mortality. Clin Obstet Gynecol 1959;2:705-734.

Blanchard A, Hentschel J, Duffy L, Baldus K, Cassell GH. Detection of *Ureaplasma urealyticum* by polymerase chain reaction in the urogenital tract of adults, in amniotic fluid, and in the respiratory tract of newborns. Clin Infect Dis 1993;17:S148-S153

Bobitt JR, Ledger WJ. Unrecognized amnionitis and prematurity: a preliminary report. J Reprod Med 1977;19:8-12.

Bobitt JR, Hayslip CC, Damato JD. Amniotic fluid infection as determined by transabdominal amniocentesis in patients with intact membranes in premature labor. Am J Obstet Gynecol 1981;140:947-52.

Botting N, Powls A, Cooke RW, Marlow N. Attention deficit hyperactivity disorders and other psychiatric outcomes in very low birthweight children at 12 years. J Child Psychol Psychiatry. 1997;38:931-41.

Botting N, Powls A, Cooke RW, Marlow N. Cognitive and educational outcome of very-low-birthweight children in early adolescence. Dev Med Child Neurol. 1998;40:652-60.

Bowman ED, Dharmalingam A, Fan WQ, Brown F, Garland SM. Impact of erythromycin on respiratory colonization of *Ureaplasma urealyticum* and the development of chronic lung disease in extremely low birth weight infants. Pediatr Infect Dis J 1998;17:615-20.

Bry K, Lappalainen U, Hallman M. Intraamniotic interleukin-1 accelerates surfactant protein synthesis in fetal rabbits and improves lung stability after premature birth. J Clin Invest 1997;99:2992-9.

Carroll SG, Abbas A, Ville Y, Meher-Homji N, Nicolaides KH. Concentration of fetal plasma and amniotic fluid interleukin-1 in pregnancies complicated by preterm prelabour amniorrhexis. J Clin Pathol 1995;48:368-71.

Carroll SG, Papaioannou S, Ntumazah IL, Philpott-Howard J, Nicolaides KH. Lower genital tract swabs in the prediction of intrauterine infection in preterm prelabour rupture of the membranes. Br J Obstet Gynaecol 1996;103:54-9.

Cassell GH, Davis RO, Waites KB, Brown MB, Marriott PA, Stagno S, Davis JK. Isolation of *Mycoplasma hominis* and *Ureaplasma urealyticum* from amniotic fluid at 16 to 20 weeks of gestation: potential effect on outcome of pregnancy. Sex Transm Dis 1983;10(4 Suppl):294-302.

Cassell GH, Waites KB, Crouse DT, Rudd PT, Canupp KC, Stagno S, Cutter GR. Association of *Ureaplasma urealyticum* infection of the lower respiratory tract with chronic lung disease and death in very-low-birth-weight infants. Lancet 1988;2:240-5.

Chrousos GP.The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. N Engl J Med 1995;332:1351-62.

Clarridge JE 3rd. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases.Clin Microbiol Rev 2004;17:840-62.

Cole TJ, Blendy JA, Monaghan AP, Krieglstein K, Schmid W, Aguzzi A, Fantuzzi G, Hummler E, Unsicker K, Schutz G. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev 1995;9:1608-21.

Couroucli XI, Welty SE, Ramsay PL, Wearden ME, Fuentes-Garcia FJ, Ni J, Jacobs TN, Towbin JA, Bowles NE. Detection of microorganisms in the tracheal aspirates of preterm infants by polymerase chain reaction: association of adenovirus infection with bronchopulmonary dysplasia. Pediatr Res 2000;47:225-32.

Crew JP, O'Brien T, Bicknell R, Fuggle S, Cranston D, Harris AL. Urinary vascular endothelial growth factor and its correlation with bladder cancer recurrence rates. J Urol 1999;161:799-804.

Crowley P. Prophylactic corticosteroids for preterm birth. Cochrane Database Syst Rev. 2000.

Cummins SK, Nelson KB, Grether JK, Velie EM. Cerebral palsy in four northern California counties, births 1983 through 1985. J Pediatr 1993;123:230-7.

Dammann O, Leviton A. Inflammatory brain damage in preterm newborns--dry numbers, wet lab, and causal inferences. Early Hum Dev 2004;79:1-15.

de Blic J, Midulla F, Barbato A, Clement A, Dab I, Eber E, Green C, Grigg J, Kotecha S, Kurland G, Pohunek P, Ratjen F, Rossi G. Bronchoalveolar lavage in children. ERS Task Force on bronchoalveolar lavage in children. European Respiratory Society. Eur Respir J 2000;15:217-31.

Debillon T, Gras-Leguen C, Verielle V, Winer N, Caillon J, Roze JC, Gressens P. Intrauterine infection induces programmed cell death in rabbit periventricular white matter. Pediatr Res 2000;47:736-42.

Deguchi K, Oguchi K, Takashima S. Characteristic neuropathology of leukomalacia in extremely low birth weight infants. Pediatr Neurol 1997;16:296-300.

Dollner H, Vatten L, Halgunset J, Rahimipoor S, Austgulen R. Histologic chorioamnionitis and umbilical serum levels of pro-inflammatory cytokines and cytokine inhibitors. BJOG 2002;109:534-9.

Dombroski RA, Woodard DS, Harper MJ, Gibbs RS. A rabbit model for bacteria-induced preterm pregnancy loss. Am J Obstet Gynecol 1990;163:1938-43.

Domingues D, Tavira LT, Duarte A, Sanca A, Prieto E, Exposto F. *Ureaplasma urealyticum* biovar determination in women attending a family planning clinic in Guine-Bissau, using polymerase chain reaction of the multiple-banded antigen gene. J Clin Lab Anal 2002;16:71-5.

Draper ES, Manktelow B, Field DJ, James D. Prediction of survival for preterm births by weight and gestational age: retrospective population based study. BMJ 1999;319:1093-7.

Duggan PJ, Maalouf EF, Watts TL, Sullivan MH, Counsell SJ, Allsop J, Al-Nakib L, Rutherford MA, Battin M, Roberts I, Edwards AD. Intrauterine T-cell activation and increased proinflammatory cytokine concentrations in preterm infants with cerebral lesions. Lancet 2001;358:1699-700.

Easterling TR, Garite TJ. *Fusobacterium*: anaerobic occult amnionitis and premature labor. Obstet Gynecol 1985;66:825-8.

Ehrenberg HM, Mercer BM. Antibiotics and the management of preterm premature rupture of the fetal membranes. Clin Perinatol 2001;28:807-18.

El-Bastawissi AY, Williams MA, Riley DE, Hitti J, Krieger JN. Amniotic fluid interleukin-6 and preterm delivery: a review. Obstet Gynecol 2000;95:1056-64.

Elder HA, Santamarina BA, Smith S, Kass EH. The natural history of asymptomatic bacteriuria during pregnancy: the effect of tetracycline on the clinical course and the outcome of pregnancy. Am J Obstet Gynecol 1971;111:441-62.

Elliman AM, Bryan EM, Elliman AD, Walker J, Harvey DR. Coordination of low birthweight seven-year-olds. Acta Paediatr Scand 1991;80:316-22.

Emerson GA, Bry K, Hallman M, Jobe AH, Wada N, Ervin MG, Ikegami M. Intra-amniotic interleukin-1 alpha treatment alters postnatal adaptation in premature lambs. Biol Neonate 1997;72:370-9.

Falkenberg ER, Davis RO, DuBard M *et al.* Effects of maternal infections on fetal adrenal steroid production. Endocr Res 1999;25:239-249.

Fidel PI Jr, Romero R, Maymon E, Hertelendy F. Bacteria-induced or bacterial product-induced preterm parturition in mice and rabbits is preceded by a significant fall in serum progesterone concentrations. J Matern Fetal Med 1998;7:222-6.

Field D. Trent Neonatal Survey 1999 Annual report. University of Leicester.

Fowlie PW, Schmidt B. Diagnostic tests for bacterial infection from birth to 90 days -a systematic review. Arch Dis Child Fetal Neonatal Ed 1999;80:F78-9.

Friese K. The role of infection in preterm labour. BJOG 2003;110 (Suppl 20):52-4.

Galle PC, Meis PJ. Complications of amniocentesis: a review. J Reprod Med 1982;27:149-155.

Garland SM, Bowman ED. Reappraisal of C-reactive protein as a screening tool for neonatal sepsis. Pathology 2003;35:240-3.

Garite TJ, Freeman RK, Linzey EM, Braly P. The use of amniocentesis in patients with premature rupture of membranes. Obstet Gynecol 1979;54:226-30.

Ghezzi F, Gomez R, Romero R, Yoon BH, Edwin SS, David C, Janisse J, Mazor M. Elevated interleukin-8 concentrations in amniotic fluid of mothers whose neonates subsequently develop bronchopulmonary dysplasia. Eur J Obstet Gynecol Reprod Biol 1998;78:5-10.

Gibbs RS, Romero R, Hillier SL, Eschenbach DA, Sweet RL. A review of premature birth and subclinical infection. Am J Obstet Gynecol 1992;166:1515-28.

Gilles FH, Leviton A, Kerr CS. Endotoxin leucoencephalopathy in the telencephalon of the newborn kitten. J Neurol Sci 1976;27:183-91.

Goepfert AR, Andrews WW, Carlo W, Ramsey PS, Cliver SP, Goldenberg RL, Hauth JC. Umbilical cord plasma interleukin-6 concentrations in preterm infants and risk of neonatal morbidity. Am J Obstet Gynecol 2004;191:1375-81.

Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. N Engl J Med. 2000;342:1500-7.

Gomez R, Romero R, Ghezzi F, Yoon BH, Mazor M, Berry SM. The fetal inflammatory response syndrome. Am J Obstet Gynecol 1998;179:194-202.

Gonçalves LF, Chaiworapongsa T, Romero R. Intrauterine infection and prematurity. Ment Retard Dev Disabil Res Rev 2002;8:3-13.

Gravett MG, Hummel D, Eschenbach DA, Holmes KK. Preterm labor associated with subclinical amniotic fluid infection and with bacterial vaginosis. Obstet Gynecol 1986;67:229-37.

Gravett MG, Haluska GJ, Cook MJ, Novy MJ. Fetal and maternal endocrine responses to experimental intrauterine infection in rhesus monkeys. Am J Obstet Gynecol. 1996;174:1725-31.

Gravett MG, Hitti J, Hess DL, Eschenbach DA. Intrauterine infection and preterm delivery: evidence for activation of the fetal hypothalamic-pituitary-adrenal axis. Am J Obstet Gynecol 2000;182:1404-13.

Gray DJ, Robinson HB, Malone J, Thomson RB Jr. Adverse outcome in pregnancy following amniotic fluid isolation of *Ureaplasma urealyticum*. Prenat Diagn 1992;12:111-117.

Grether JK, Nelson KB, Walsh E, Willoughby RE, Redline RW. Intrauterine exposure to infection and risk of cerebral palsy in very preterm infants. Arch Pediatr Adolesc Med 2003;157:26-32.

Grigg J, Arnon S, Silverman M. Fractional processing of sequential bronchoalveolar lavage fluid from intubated babies. Eur Respir J 1992;5:727-32.

Groneck P, Gotze-Speer B, Oppermann M, Eiffert H, Speer CP. Association of pulmonary inflammation and increased microvascular permeability during the development of bronchopulmonary dysplasia: a sequential analysis of inflammatory mediators in respiratory fluids of high-risk preterm neonates. Pediatrics 1994;93:712-718.

Groneck P, Goetze-Speer B, Speer CP. Inflammatory bronchopulmonary response of preterm infants with microbial colonisation of the airways at birth. Arch Dis Child Fetal Neonatal Ed 1996;74:F51-5.

Guzzetta F, Shackelford GD, Volpe S, Perlman JM, Volpe JJ. Periventricular intraparenchymal echodensities in the premature newborn: critical determinant of neurologic outcome. Pediatrics 1986;78:995-1006.

Hack M, Taylor HG, Klein N, Eiben R, Schatschneider C, Mercuri-Minich N. School-age outcomes in children with birth weights under 750 g. N Engl J Med 1994;331(12):753-9.

Hannaford K, Todd DA, Jeffery H, John E, Blyth K, Gilbert GL. Role of *Ureaplasma urealyticum* in lung disease of prematurity. Arch Dis Child Fetal Neonatal Ed 1999;81:F162-7.

Held HD, Boettcher S, Hamann L, Uhlig S. Ventilation-induced chemokine and cytokine release is associated with activation of nuclear factor-kappaB and is blocked by steroids. Am J Respir Crit Care Med 2001;163:711-6.

Hilali F, Saulnier P, Chachaty E, Andremont A. Decontamination of polymerase chain reaction reagents for detection of low concentrations of 16S rRNA genes. Mol Biotechnol 1997;7:207-16.

Hillier SL, Martius J, Krohn M, Kiviat N, Holmes KK, Eschenbach DA. A case-control study of chorioamnionic infection and histologic chorioamnionitis in prematurity. N Engl J Med 1988;319:972-8.

Hillier SL, Krohn MA, Kiviat NB, Watts DH, Eschenbach DA. Microbiologic causes and neonatal outcomes associated with chorioamnion infection. Am J Obstet Gynecol 1991;165:955-61.

Hillier SL, Witkin SS, Krohn MA, Watts DH, Kiviat NB, Eschenbach DA. The relationship of amniotic fluid cytokines and preterm delivery, amniotic fluid infection, histologic chorioamnionitis, and chorioamnion infection. Obstet Gynecol. 1993;81:941-8.

Hirsch E, Saotome I, Hirsh D. A model of intrauterine infection and preterm delivery in mice. Am J Obstet Gynecol 1995;172:1598-603.

Hitti J, Krohn MA, Patton DL, Tarczy-Hornoch P, Hillier SL, Cassen EM, Eschenbach DA. Amniotic fluid tumor necrosis factor-alpha and the risk of respiratory distress syndrome among preterm infants. Am J Obstet Gynecol 1997;177:50-6.

Hitti J, Riley DE, Krohn MA, Hillier SL, Agnew KJ, Krieger JN, Eschenbach DA. Broad-spectrum bacterial rDNA polymerase chain reaction assay for detecting amniotic fluid infection among women in premature labor. Clin Infect Dis 1997;24:1228-32.

Holling EE, Leviton A. Characteristics of cranial ultrasound white-matter echolucencies that predict disability: a review. Dev Med Child Neurol 1999;41:136-9.

Hogg JC. The adenovirus and bronchopulmonary dysplasia: an association that could be causal or coincidental. Pediatr Res 2000;47:225-32.

Horowitz S, Mazor M, Romero R, Horowitz J, Glezerman M. Infection of the amniotic cavity with *Ureaplasma urealyticum* in the midtrimester of pregnancy. J Reprod Med. 1995;40:375-9.

Husain AN, Siddiqui NH, Stocker JT. Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. Hum Pathol 1998;29:710-7.

Jacobsson B, Mattsby-Baltzer I, Andersch B, Bokstrom H, Holst RM, Wennerholm UB, Hagberg H. Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women in preterm labor. Acta Obstet Gynecol Scand 2003;82:120-8.

Jacobsson B, Mattsby-Baltzer I, Andersch B, Bokstrom H, Holst RM, Nikolaitchouk N, Wennerholm UB, Hagberg H. Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women with preterm prelabor rupture of membranes. Acta Obstet Gynecol Scand 2003;82:423-31.

Jalava J, Mantymaa ML, Ekblad U, Toivanen P, Skurnik M, Lassila O, Alanen A. Bacterial 16S rDNA polymerase chain reaction in the detection of intra-amniotic infection. Br J Obstet Gynaecol 1996;103:664-9.

Jobe AJ. The new BPD: an arrest of lung development. Pediatr Res 1999;46:641-3.

Jobe AH, Newnham JP, Willet KE, Moss TJ, Gore Ervin M, Padbury JF, Sly P, Ikegami M. Endotoxin-induced lung maturation in preterm lambs is not mediated by cortisol. Am J Respir Crit Care Med 2000;162:1656-61.

Jobe AH, Newnham JP, Willet KE, Sly P, Ervin MG, Bachurski C, Possmayer F, Hallman M, Ikegami M. Effects of antenatal endotoxin and glucocorticoids on the lungs of preterm lambs. Am J Obstet Gynecol 2000;182:401-8.

Jobe AH, Ikegami M. Antenatal infection/inflammation and postnatal lung maturation and injury. Respir Res 2001;2:27-32.

Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med 2001;163:1723-9.

Jobe AH. Antenatal associations with lung maturation and infection.
J Perinatol 2005;25 Suppl 2:S31-5.

Jonsson B, Rylander M, Faxelius G. *Ureaplasma urealyticum*, erythromycin and respiratory morbidity in high-risk preterm neonates. Acta Paediatr 1998;87:1079-84.

Kendall G, Peebles D. Acute fetal hypoxia: the modulating effect of infection. Early Hum Dev 2005;81:27-34.

Kenyon SL, Taylor DJ, Tarnow-Mordi W; ORACLE Collaborative Group. Broad-spectrum antibiotics for preterm, prelabour rupture of fetal membranes: the ORACLE I randomised trial. ORACLE Collaborative Group. Lancet 2001;357:979-88.

Kenyon SL, Taylor DJ, Tarnow-Mordi W; ORACLE Collaborative Group. Broad-spectrum antibiotics for spontaneous preterm labour: the ORACLE II randomised trial. ORACLE Collaborative Group. Lancet 2001;357:989-94.

Kilbride HW, Thibeault DW. Neonatal complications of preterm premature rupture of membranes. Pathophysiology and management. Clin Perinatol. 2001;28:761-85.

Kim CJ, Yoon BH, Romero R, Moon JB, Kim M, Park SS, Chi JG. Umbilical arteritis and phlebitis mark different stages of the fetal inflammatory response. Am J Obstetric Cynical 2001;185:496-500.

Kitajima H, Fujimura M, Takeuchi T, Miyano A, Nakayama M, Fujita T, Imai S, Shimizu A. Effect of amnionitis on the complement system of preterm infants. Early Hum Dev 1990;21:59-69

Kitajima H, Nakayama M, Miyano A, Shimizu A, Taniguchi T, Shimoya K, Matsuzaki N, Fujimura M. Significance of chorioamnionitis. Early Hum Dev 1992;29:125-30.

Kotecha S, Chan B, Azam N, Silverman M, Shaw RJ. Increase in interleukin-8 and soluble intercellular adhesion molecule-1 in bronchoalveolar lavage fluid from premature infants who develop chronic lung disease. Arch Dis Child Fetal Neonatal Ed 1995;72:F90-6.

Kotecha S, Wilson L, Wangoo A, Silverman M, Shaw RJ. Increase in interleukin (IL)-1 beta and IL-6 in bronchoalveolar lavage fluid obtained from infants with chronic lung disease of prematurity. Pediatr Res 1996;40:250-6.

Kotecha S. Lung growth: implications for the newborn infant. Arch Dis Child Fetal Neonatal Ed 2000;82:F69-74.

Kotecha S, Hodge R, Schaber JA, Miralles R, Silverman M, Grant WD. Presence of pulmonary *Ureaplasma urealyticum* is associated with acute pulmonary inflammation in preterm infants. Pediatric Research 2004;55:61-68.

Koumbourlis AC, Kurland G. Nonbronchoscopic bronchoalveolar lavage in mechanically ventilated infants: technique, efficacy, and applications. Pediatr Pulmonol 1993;15:257-262.

Lamont RF. Infection in the prediction and antibiotics in the prevention of spontaneous preterm labour and preterm birth. BJOG 2003;110 (Suppl 20):71-5.

Lencki SG, Maciulla MB, Eglinton GS. Maternal and umbilical cord serum interleukin levels in preterm labor with clinical chorioamnionitis. Am J Obstet Gynecol 1994;170:1345-51.

Lettieri L, Vintzileos AM, Rodis JF, Albini SM, Salafia CM. Does "idiopathic" preterm labor resulting in preterm birth exist? Am J Obstet Gynecol 1993;168:1480-5.

Leviton A, Paneth N, Reuss ML, Susser M, Allred EN, Dammann O, Kuban K, Van Marter LJ, Pagano M, Hegyi T, Hiatt M, Sanocka U, Shahrivar F, Abiri M, Disalvo D, Doubilet P, Kairam R, Kazam E, Kirpekar M, Rosenfeld D, Schonfeld S, Share J, Collins M, Genest D, Shen-Schwarz S, et al. Maternal infection, fetal inflammatory response, and brain damage in very low birth weight infants. Developmental Epidemiology Network Investigators. Pediatr Res 1999;46:566-75.

Lodish H et al. Molecular cell biology. Fourth Edition: W.H. Freeman, New York & Basingstoke 1999.

Lowry, OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193: 265-275.

Lu GC, Goldenberg RL. Current concepts on the pathogenesis and markers of preterm births. Clin Perinatol 2000;27:263-83.

Lyon AJ, McColm J, Middlemist L, Fergusson S, McIntosh N, Ross PW. Randomised trial of erythromycin on the development of chronic lung disease in preterm infants. Arch Dis Child Fetal Neonatal Ed 1998;78:F10-4.

Lyon A. Chronic lung disease of prematurity. The role of intra-uterine infection. Eur J Pediatr 2000;159:798-802.

Mabanta CG, Pryhuber GS, Weinberg GA, Phelps DL. Erythromycin for the prevention of chronic lung disease in intubated preterm infants at risk for, or colonized or infected with *Ureaplasma urealyticum*. Cochrane Database Syst Rev 2003.

Mahon C, Manuselis G *eds.* Textbook of diagnostic microbiology. Saunders, Philadelphia 1995.

Manktelow BN, Draper ES, Annamalai S, Field D. Factors affecting the incidence of chronic lung disease of prematurity in 1987, 1992, and 1997. Arch Dis Child Fetal Neonatal Ed 2001;85:F33-5.

Martinez E, Figueroa R, Garry D, Visintainer P, Patel K, Verma U, Sehgal PB, Tejani N. Elevated Amniotic Fluid Interleukin-6 as a Predictor of Neonatal Periventricular Leukomalacia and Intraventricular Hemorrhage. J Matern Fetal Investig. 1998;8:101-107.

Markenson GR, Martin RK, Tillotson-Criss M, Foley KS, Stewart RS Jr, Yancey M. The use of the polymerase chain reaction to detect bacteria in amniotic fluid in pregnancies complicated by preterm labor. Am J Obstet Gynecol 1997;177:1471-7.

Mastorakos G, Chrousos GP, Weber JS. Recombinant interleukin-6 activates the hypothalamic-pituitary-adrenal axis in humans. J Clin Endocrinol Metab 1993;77:1690-4

Matsuda T, Nakajima T, Hattori S, Hanatani K, Fukazawa Y, Kobayashi K, Fujimoto S. Necrotizing funisitis: clinical significance and association with chronic lung disease in premature infants. Am J Obstet Gynecol 1997;177:1402-7.

Miller JM Jr, Pupkin MJ, Hill GB. Bacterial colonization of amniotic fluid from intact fetal membranes. Am J Obstet Gynecol 1980;136:796-804.

Mueller-Heubach E, Rubinstein DN, Schwarz SS. Histologic chorioamnionitis and preterm delivery in different patient populations. Obstet Gynecol 1990;75:622-6.

Miralles R, Hodge R, McParland PC, Field DJ, Bell SC, Taylor DJ, Grant WD, Kotecha S. Relationship between antenatal inflammation and antenatal infection identified by detection of microbial genes by polymerase chain reaction. Pediatr Res 2005;57:473-4.

Muglia LJ, Bae DS, Brown TT, Vogt SK, Alvarez JG, Sunday ME, Majzoub JA. Proliferation and differentiation defects during lung development in corticotropin-releasing hormone-deficient mice. Am J Respir Cell Mol Biol 1999;20:181-188.

Naccasha N, Hinson R, Montag A, Ismail M, Bentz L, Mittendorf R. Association between funisitis and elevated interleukin-6 in cord blood.Obstet Gynecol 2001;97:220-4.

Naeye RL, Harcke HT Jr, Blanc WA. Adrenal gland structure and the development of hyaline membrane disease. Pediatrics. 1971;47:650-7

Nelson KB, Ellenberg JH. Antecedents of cerebral palsy. Multivariate analysis of risk. N Engl J Med 1986;315:81-6.

Nelson KB, Grether JK, Dambrosia JM, Walsh E, Kohler S, Satyanarayana G, Nelson PG, Dickens BF, Phillips TM. Neonatal cytokines and cerebral palsy in very preterm infants. Pediatr Res 2003;53:600-7.

Nelson S, Matlow A, Johnson G, Th'ng C, Dunn M, Quinn P. Detection of *Ureaplasma urealyticum* in endotracheal tube aspirates from neonates by PCR. J Clin Microbiol 1998;36:1236-9.

Newnham JP, Moss TJ, Padbury JF, Willet KE, Ikegami M, Ervin MG, Sly P, Jobe A. The interactive effects of endotoxin with prenatal glucocorticoids on short-term lung function in sheep. Am J Obstet Gynecol 2001;185:190-197.

Northway WH Jr, Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. N Engl J Med 1967;276:357-68.

Northway WH Jr, Moss RB, Carlisle KB, Parker BR, Popp RL, Pitlick PT, Eichler I, Lamm RL, Brown BW Jr. Late pulmonary sequelae of bronchopulmonary dysplasia. N Engl J Med 1990;323:1793-9.

O'Brodovich HM, Mellins RB. Bronchopulmonary dysplasia. Unresolved neonatal acute lung injury. Am Rev Respir Dis 1985;132:694-709.

Odibo AO, Rodis JF, Sanders MM, Borgida AF, Wilson M, Egan JF, Campbell WA. Relationship of amniotic fluid markers of intra-amniotic infection with histopathology in cases of preterm labor with intact membranes. J Perinatol 1999;19:407-12.

Ohyama M, Itani Y, Yamanaka M, Goto A, Kato K, Ijiri R, Tanaka Y. Re-evaluation of chorioamnionitis and funisitis with a special reference to subacute chorioamnionitis. Hum Pathol 2002;33:183-90.

Ornoy A, Altshuler G. Maternal endotoxemia, fetal anomalies, and central nervous system damage: a rat model of a human problem. Am J Obstet Gynecol 1976;124:196-204.

O'Shea TM, Klinepeter KL, Meis PJ, Dillard RG. Intrauterine infection and the risk of cerebral palsy in very low-birthweight infants. Paediatr Perinat Epidemiol 1998;12:72-83.

Pacora P, Chaiworapongsa T, Maymon E, Kim YM, Gomez R, Yoon BH, Ghezzi F, Berry SM, Qureshi F, Jacques SM, Kim JC, Kadar N, Romero R. Funisitis and chorionic vasculitis: the histological counterpart of the fetal inflammatory response syndrome. J Matern Fetal Neonatal Med 2002;11:18-25.

Pankuch GA, Appelbaum PC, Lorenz RP, Botti JJ, Schachter J, Naeye RL. Placental microbiology and histology and the pathogenesis of chorioamnionitis. Obstet Gynecol 1984;64:802-6.

Parker CR Jr, Favor JK, Carden LG, Brown CH. Effects of intrapartum stress on fetal adrenal function. Am J Obstet Gynecol 1993;169:1407-11.

Patterson AM, Taciak V, Lovchik J, Fox RE, Campbell AB, Viscardi RM. *Ureaplasma urealyticum* respiratory tract colonization is associated with an increase in interleukin 1-beta and tumor necrosis factor alpha relative to interleukin 6 in tracheal aspirates of preterm infants. Pediatr Infect Dis J 1998;17:321-8.

Peebles DM. Intrauterine infection and perinatal brain injury. Scientific Advisory Committee RCOG 2002.

Pharoah PO, Stevenson CJ, Cooke RW, Stevenson RC. Prevalence of behaviour disorders in low birthweight infants. Arch Dis Child 1994;70:271-4.

Pinar H, Sotomayor E, Singer DB. Pathological case of the month. Turquoise discoloration of the umbilical cord and membranes after intra-amniotic injection of indigo carmine dye for premature rupture of membranes. Arch Pediatr Adolesc Med 1998;152:199-200.

Pinto-Martin JA, Riolo S, Cnaan A, Holzman C, Susser MW, Paneth N. Cranial ultrasound prediction of disabling and nondisabling cerebral palsy at age two in a low birth weight population. Pediatrics 1995;95:249-54.

Powls A, Botting N, Cooke RW, Marlow N. Motor impairment in children 12 to 13 years old with a birthweight of less than 1250 g. Arch Dis Child Fetal Neonatal Ed 1995;73:F62-66.

Prosch S, Lienicke U, Priemer C, Flunker G, Seidel WF, Kruger DH, Wauer RR. Human adenovirus and human cytomegalovirus infections in preterm newborns: no association with bronchopulmonary dysplasia. Pediatr Res 2002;52:219-24.

Raivich G, Bohatschek M, Kloss CU, Werner A, Jones LL, Kreutzberg GW. Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. Brain Res Brain Res Rev 1999;30:77-105.

Rantakokko-Jalava K, Nikkari S, Jalava J, Eerola E, Skurnik M, Meurman O,Ruuskanen O, Alanen A, Kotilainen E, Toivanen P, Kotilainen P. Direct amplification of rRNA genes in diagnosis of bacterial infections. J Clin Microbiol 2000;38:32-9.

Relman DA, Loutit JS, Schmidt TM, Falkow S, Tompkins LS. The agent of bacillary angiomatosis. An approach to the identification of uncultured pathogens. N Engl J Med 1990;323:1573-80.

Relman DA, Schmidt TM, MacDermott RP, Falkow S. Identification of the uncultured bacillus of Whipple's disease. N Engl J Med 1992;327:293-301.

Relman DA. The search for unrecognized pathogens. Science 1999;284:1308-10.

Rogers BB, Alexander JM, Head J, McIntire D, Leveno KJ. Umbilical vein interleukin-6 levels correlate with the severity of placental inflammation and gestational age. Hum Pathol 2002;33:335-40.

Rojas MA, Gonzalez A, Bancalari E, Claure N, Poole C, Silva-Neto G. Changing trends in the epidemiology and pathogenesis of neonatal chronic lung disease. J Pediatr 1995;126:605-10.

Romero R, Oyarzun E, Mazor M, Sirtori M, Hobbins JC, Bracken M. Meta-analysis of the relationship between asymptomatic bacteriuria and preterm delivery/low birth weight. Obstet Gynecol 1989;73:576-82.

Romero R, Avila C, Santhanam U, Sehgal PB. Amniotic fluid interleukin 6 in preterm labor. Association with infection. J Clin Invest 1990;85:1392-400.

Romero R, Mazor M, Tartakovsky B. Systemic administration of interleukin-1 induces preterm parturition in mice. Am J Obstet Gynecol 1991;165:969-71.

Romero R, Salafia CM, Athanassiadis AP, Hanaoka S, Mazor M, Sepulveda W, Bracken MB. The relationship between acute inflammatory lesions of the preterm placenta and amniotic fluid microbiology. Am J Obstet Gynecol 1992;166:1382-8.

Romero R, Yoon BH, Mazor M, Gomez R, Diamond MP, Kenney JS, Ramirez M, Fidel PL, Sorokin Y, Cotton D, *et al.* The diagnostic and prognostic value of amniotic fluid white blood cell count, glucose, interleukin-6, and gram stain in patients with preterm labor and intact membranes. Am J Obstet Gynecol 1993;169:805-16.

Romero R, Yoon BH, Mazor M, Gomez R, Gonzalez R, Diamond MP, Baumann P, Araneda H, Kenney JS, Cotton DB, *et al.* A comparative study of the diagnostic performance of amniotic fluid glucose, white blood cell count, interleukin-6, and gram stain in the detection of microbial invasion in patients with preterm premature rupture of membranes. Am J Obstet Gynecol 1993;169:839-51.

Romero R, Gomez R, Ghezzi F, Yoon BH, Mazor M, Edwin SS, Berry SM. A fetal systemic inflammatory response is followed by the spontaneous onset of preterm parturition. Am J Obstet Gynecol 1998;179:186-93.

Romero R, Maymon E, Pacora P, Gomez R, Mazor M, Yoon BH, Berry SM. Further observations on the fetal inflammatory response syndrome: a potential homeostatic role for the soluble receptors of tumor necrosis factor alpha. Am J Obstet Gynecol 2000;183:1070-1077.

Romero R, Espinoza J, Chaiworapongsa T, Kalache K. Infection and prematurity and the role of preventive strategies. Semin Neonatol 2002;7:259-74.

Rudd PT, Cassell GH, Waites KB, Davis JK, Duffy LB. *Ureaplasma urealyticum* pneumonia: experimental production and demonstration of age-related susceptibility. Infect Immun 1989;57:918-25.

Russi TJ, Crystal RG. Use of bronchoalveolar lavage and airway brushing to investigate the human lung in The Lung: Scientific Foundations. Second edition. ed Crystal RG et al. Lippincott-Raven Publishers, Philadelphia 1997.

Sacks NP, Smith K, Norman AP, Greenall M, LeJeune S, Harris AL. Cathepsin D levels in primary breast cancers: relationship with epidermal growth factor receptor, oestrogen receptor and axillary nodal status. Eur J Cancer 1993;29A:426-8.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA. Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase. Science 1988;239:487-491.

Saito S, Kasahara T, Kato Y, Ishihara Y, Ichijo M. Elevation of amniotic fluid interleukin 6 (IL-6), IL-8 and granulocyte colony stimulating factor (G-CSF) in term and preterm parturition. Cytokine 1993;5:81-8.

Salafia CM, Sherer DM, Spong CY, Lencki S, Eglinton GS, Parkash V, Marley E, Lage JM. Fetal but not maternal serum cytokine levels correlate with histologic acute placental inflammation. Am J Perinatol 1997;14:419-22.

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. 1977. Biotechnology 1992;24:104-8.

Sapan CV, Lundblad RL, Price NC. Colorimetric protein assay techniques. Biotechnol Appl Biochem 1999;29:99-108.

Sapolsky R, Rivier C, Yamamoto G. Interleukin-1 stimulates the secretion of hypothalamic corticotrophin releasing factor, Science 1987;238:522-524

Sawyer MH, Edwards DK, Spector SA. Cytomegalovirus infection and bronchopulmonary dysplasia in premature infants. Am J Dis Child 1987;141:303-5.

Schmidt B, Cao L, Mackensen-Haen S, Kendziorra H, Klingel K, Speer CP. Chorioamnionitis and inflammation of the fetal lung. Am J Obstet Gynecol 2001;185:173-177.

Shennan AT, Dunn MS, Ohlsson A, Lennox K, Hoskins EM. Abnormal pulmonary outcomes in premature infants: prediction from oxygen requirement in the neonatal period. Pediatrics 1988;82:527-32.

Shimoya K, Matsuzaki N, Taniguchi T, Jo T, Saji F, Kitajima H, Fujimura M, Nakayama M, Tanizawa O. Interleukin-8 in cord sera: a sensitive and specific marker for the detection of preterm chorioamnionitis. J Infect Dis 1992;165:957-60.

Shimoya K, Taniguchi T, Matsuzaki N, Moriyama A, Murata Y, Kitajima H, Fujimura M, Nakayama M. Chorioamnionitis decreased incidence of respiratory distress syndrome by elevating fetal interleukin-6 serum concentration. Hum Reprod 2000;15:2234-40.

Shukla SK, Meier PR, Mitchell PD, Frank DN, Reed KD. *Leptotrichia amnionii sp.* nov., a novel bacterium isolated from the amniotic fluid of a woman after intrauterine fetal demise. J Clin Microbiol 2002;40:3346-9.

Stark CM, Smith RS, Lagrandeur RM, Batton DG, Lorenz RP. Need for urgent delivery after third-trimester amniocentesis. Obstet Gynecol 2000;95:48-50.

Stewart AL, Reynolds EO, Hope PL, Hamilton PA, Baudin J, Costello AM, Bradford BC, Wyatt JS. Probability of neurodevelopmental disorders estimated from ultrasound appearance of brains of very preterm infants. Dev Med Child Neurol 1987;29:3-11.

Sullivan MH, Steel J, Kennea N, Feldman RG, Edwards AD. The role of intrauterine bacteria in brain injury. Acta Paediatr Suppl. 2004;93:4-5.

Tauscher MK, Berg D, Brockmann M, Seidenspinner S, Speer CP, Groneck P. Association of histologic chorioamnionitis, increased levels of cord blood cytokines, and intracerebral hemorrhage in preterm neonates. Biol Neonate 2003;83:166-70.

Thibeault DW, Mabry SM, Ekekezie II, Truog WE. Lung elastic tissue maturation and perturbations during the evolution of chronic lung disease. Pediatrics 2000;106:1452-9.

Tin W, Wariyar U, Hey E. Changing prognosis for babies of less than 28 weeks' gestation in the north of England between 1983 and 1994. Northern Neonatal Network. BMJ 1997;314:107-11.

Toews GB. Cellular alterations in fibroproliferative lung disease. Chest 1999;116(1 Suppl):112S-116S.

Tsuda A, Ikegami T, Hirano H, Sanada H, Ogawa M, Sasaki M, Tanaka T. The relationship between amniotic fluid interleukin-6 concentration and histologic evidence of chorioamnionitis. Acta Obstet Gynecol Scand 1998;77:515-20.

van Waarde WM, Brus F, Okken A, Kimpen JL. *Ureaplasma urealyticum* colonization, prematurity and bronchopulmonary dysplasia. Eur Respir J 1997;10:886-90.

The Victorian Infant Collaborative Study Group. Outcome at 2 years of children 23-27 weeks' gestation born in Victoria in 1991-92. The Victorian Infant Collaborative Study Group. J Paediatr Child Health 1997;33:161-5.

Volpe JJ. Neurobiology of periventricular leukomalacia in the premature infant. Pediatr Res 2001;50:553-562.

Vyas R, Tudor G, Rickett A, Kotecha S. Serial bronchoalveolar lavage of preterm infants is not associated with chest radiological changes. Eur J Pediatr 2002;161:313-318.

Walsh WF, Butler J, Coalson J, Hensley D, Cassell GH, deLemos RA. A primate model of *Ureaplasma urealyticum* infection in the premature infant with hyaline membrane disease. Clin Infect Dis 1993;17 (Suppl 1):S158-62.

Wang EE, Frayha H, Watts J, Hammerberg O, Chernesky MA, Mahony JB, Cassell GH. Role of *Ureaplasma urealyticum* and other pathogens in the development of chronic lung disease of prematurity. Pediatr Infect Dis J 1988;7:547-51.

Wang EE, Ohlsson A, Kellner JD. Association of *Ureaplasma urealyticum* colonization with chronic lung disease of prematurity: results of a metaanalysis. J Pediatr 1995;127:640-644.

Wang EE, Matlow AG, Ohlsson A, Nelson SC. *Ureaplasma urealyticum* infections in the perinatal period. Clin Perinatol 1997;24:91-105.

Ward RM, Beachy JC. Neonatal complications following preterm birth. BJOG 2003;110 (Suppl 20):8-16.

Watterberg KL, Demers LM, Scott SM, Murphy S. Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops. Pediatrics 1996;97:210-5.

Watterberg KL, Scott SM, Naeye RL. Chorioamnionitis, cortisol, and acute lung disease in very low birth weight infants.
Pediatrics 1997;99:E6

Watterberg KL, Gerdes JS, Cole CH, Aucott SW, Thilo EH, Mammel MC, Couser RJ, Garland JS, Rozycki HJ, Leach CL, Backstrom C, Shaffer ML. Prophylaxis of early adrenal insufficiency to prevent bronchopulmonary dysplasia: a multicenter trial. Pediatrics 2004;114:1649-57.

Watts DH, Krohn MA, Hillier SL, Eschenbach DA. The association of occult amniotic fluid infection with gestational age and neonatal outcome among women in preterm labor. Obstet Gynecol 1992;79:351-7.

Weeks JW, Reynolds L, Taylor D, Lewis J, Wan T, Gall SA. Umbilical cord blood interleukin-6 levels and neonatal morbidity. Obstet Gynecol 1997;90:815-8.

Weiss SJ. Mechanisms of disease: Tissue destruction by neutrophils. New Engl J Med 1989;365-376

Wenstrom KD, Andrews WW, Tamura T, DuBard MB, Johnston KE, Hemstreet GP. Elevated amniotic fluid interleukin-6 levels at genetic amniocentesis predict subsequent pregnancy loss. Am J Obstet Gynecol 1996;175:830-3.

Wenstrom KD, Andrews WW, Hauth JC, Goldenberg RL, DuBard MB, Cliver SP. Elevated second-trimester amniotic fluid interleukin-6 levels predict preterm delivery. Am J Obstet Gynecol 1998;178:546-50.

Woese CR. Bacterial evolution. Microbiol Rev 1987;51:221-71.

Wood NS, Marlow N, Costeloe K, Gibson AT, Wilkinson AR. Neurologic and developmental disability after extremely preterm birth. EPICure Study Group. N Engl J Med 2000;343:378-84.

Yoon BH, Romero R, Kim CJ, Jun JK, Gomez R, Choi JH, Syn HC. Amniotic fluid interleukin-6: a sensitive test for antenatal diagnosis of acute inflammatory lesions of preterm placenta and prediction of perinatal morbidity. Am J Obstet Gynecol 1995;172:960-70.

Yoon BH, Romero R, Yang SH, Jun JK, Kim IO, Choi JH, Syn HC. Interleukin-6 concentrations in umbilical cord plasma are elevated in neonates with white matter lesions associated with periventricular leukomalacia. Am J Obstet Gynecol 1996;174:1433-40.

Yoon BH, Jun JK, Romero R, Park KH, Gomez R, Choi JH, Kim IO. Amniotic fluid inflammatory cytokines (interleukin-6, interleukin-1beta, and tumor necrosis factor-alpha), neonatal brain white matter lesions, and cerebral palsy. Am J Obstet Gynecol 1997;177:19-26.

Yoon BH, Romero R, Kim CJ, Koo JN, Choe G, Syn HC, Chi JG. High expression of tumor necrosis factor-alpha and interleukin-6 in periventricular leukomalacia. Am J Obstet Gynecol 1997;177:406-11.

Yoon BH, Kim CJ, Romero R, Jun JK, Park KH, Choi ST, Chi JG. Experimentally induced intrauterine infection causes fetal brain white matter lesions in rabbits. Am J Obstet Gynecol 1997;177:797-802.

Yoon BH, Romero R, Jun JK, Park KH, Park JD, Ghezzi F, Kim BI. Amniotic fluid cytokines (interleukin-6, tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia. Am J Obstet Gynecol 1997;177:825-30.

Yoon BH, Romero R, Park JS, Chang JW, Kim YA, Kim JC, Kim KS. Microbial invasion of the amniotic cavity with *Ureaplasma urealyticum* is associated with a robust host response in fetal, amniotic, and maternal compartments. Am J Obstet Gynecol 1998;179:1254-60.

Yoon BH, Romero R, Jun JK, Maymon E, Gomez R, Mazor M, Park JS.An increase in fetal plasma cortisol but not dehydroepiandrosterone sulfate is followed by the onset of preterm labor in patients with preterm premature rupture of the membranes. Am J Obstet Gynecol 1998;179:1107-14.

Yoon BH, Romero R, Kim KS, Park JS, Ki SH, Kim BI, Jun JK. A systemic fetal inflammatory response and the development of bronchopulmonary dysplasia. Am J Obstet Gynecol 1999;181:773-9.

Yoon BH, Romero R, Park JS, Kim CJ, Kim SH, Choi JH, Han TR. Fetal exposure to an intra-amniotic inflammation and the development of cerebral palsy at the age of three years. Am J Obstet Gynecol 2000;182:675-81.

Yoon BH, Romero R, Park JS, Kim M, Oh SY, Kim CJ, Jun JK. The relationship among inflammatory lesions of the umbilical cord (funisitis), umbilical cord plasma interleukin 6 concentration, amniotic fluid infection, and neonatal sepsis. Am J Obstet Gynecol 2000;183:1124-9.

Yoon BH, Romero R, Kim M, Kim EC, Kim T, Park JS, Jun JK. Clinical implications of detection of *Ureaplasma urealyticum* in the amniotic cavity with the polymerase chain reaction. Am J Obstet Gynecol 2000;183:1130-7.

Yoon BH, Romero R, Moon JB, Shim SS, Kim M, Kim G, Jun JK. Clinical significance of intra-amniotic inflammation in patients with preterm labor and intact membranes. Am J Obstet Gynecol 2001;185:1130-6.

Yoon BH. Intrauterine infection and the development of cerebral palsy. BJOG 2003;110 (Suppl 20):124-7.

Yoon BH, Romero R, Lim JH, Shim SS, Hong JS, Shim JY, Jun JK. The clinical significance of detecting *Ureaplasma urealyticum* by the polymerase chain reaction in the amniotic fluid of patients with preterm labor. Am J Obstet Gynecol 2003;189:919-24.

Yoon BH, Romero R, Shim J-Y, Shim SS, Kim CJ, Jun JK. C-reactive protein in umbilical cord blood: a simple and widely available clinical method to assess the risk of amniotic fluid infection and funisitis. J Mat Neonat Med 2003;14:85-90

Young RS, Yagel SK, Towfighi J. Systemic and neuropathologic effects of E. coli endotoxin in neonatal dogs. Pediatr Res 1983;17:349-53.

Acknowledgements

I owe a debt of gratitude to the parents who supported this research, often at a time of great worry about how their newborn infants would fare.

There are many people who have helped along the way. I would like to thank Rachel Hodge for her technical support and laboratory work; the Preterm Birth Group in Leicester, in particular Professor Stephen Bell, for his support and regular discussion, Dr. Penny McParland and Susan Spurling; Professor William Grant and his team, who shared their expertise in the field of PCR-based bacterial detection; Dr. Pat McKeever, who provided the routine histology results; Professor David Field, for his constructive approach and encouragement; Professor Michael Silverman and Professor David Taylor, heads of the divisions of Child Health and Obstetrics and Gynaecology; the medical and nursing staff on the neonatal unit at the Leicester Royal Infirmary; and, of course, my supervisor Professor Sailesh Kotecha who has guided this work through to its final stages.

Finally, I would like to thank my wife, Bríd. She has patiently supported my training and research for many years now. Without her there would have been no study and no thesis. Go raimh maith agat. Bail ó Dhia ort.

Meallan muilte Dé go mall ach meallan siad go mion.

Publications from this thesis

Relationship between antenatal infection and antenatal inflammation identified by detection of microbial genes by polymerase chain reaction

Miralles RE, Hodge R, McParland P, Field DJ, Bell S, Taylor DJ, Grant WD, Kotecha S.

Pediatric Research 2005; 57:570-577

Antenatal Inflammation and Infection in Chronic Lung Disease of Prematurity

Miralles RE, Hodge R, Kotecha S.

Child: Care, Health and Development 2002;28 (Suppl 1);11-15

Fetal cortisol response to intrauterine microbial colonisation identified by the polymerase chain reaction and fetal inflammation

Miralles RE, Hodge R, Kotecha S.

Archives of Disease in Childhood 2008;93:F51–F54

0031-3998/05/5704-0570
PEDIATRIC RESEARCH

Vol. 57, No. 4, 2005
Printed in U.S.A.

Relationship between Antenatal Inflammation and Antenatal Infection Identified by Detection of Microbial Genes by Polymerase Chain Reaction

ROBIN MIRALLES, RACHEL HODGE, PENNY C. MCPARLAND, DAVID J. FIELD, STEPHEN C. BELL, DAVID J. TAYLOR, WILLIAM D. GRANT, AND SAILESH KOTECHA

Reproductive Sciences Section [R.M., R.H., P.C.M., S.C.B., D.J.T., S.K.], Department of Cancer Studies and Molecular Medicine, Leicester LE2 7LX, UK, Department of Epidemiology and Public Health [D.J.F.], Division of Microbiology and Immunology [W.D.G.], Department of Infection, Immunity and Inflammation, University of Leicester, Leicester LE1 9HN, UK

ABSTRACT

Although antenatal infection is thought to play an important role in the pathogenesis of preterm labor and neonatal diseases, the exact mechanisms are largely unknown. We sought to clarify the relationship between antenatal infection and intrauterine and neonatal inflammation. Samples were obtained from 41 preterm infants of <33 wk gestation delivered to 36 mothers and analyzed for the presence of 16s ribosomal RNA (16s rRNA) genes using PCR and for the proinflammatory cytokines IL-6 and IL-8. In 16 (44%) mother-baby pairings, at least one sample was found to be positive for the presence of 16s rRNA genes. All but one of the positive samples were from mothers presenting with preterm prelabor rupture of membranes (pPROM) or in spontaneous idiopathic preterm labor. A strong association was found between the presence of 16s rRNA genes and chorioamnionitis and with funisitis. A marked increase in IL-6 and IL-8 was noted in all tissues positive for 16s rRNA genes, including placenta, fetal membranes, cord blood serum, and, where samples were available, in bronchoalveolar lavage fluid (BAL) and in amniotic fluid. Interestingly, gastric fluid was always positive for 16s rRNA genes if any other intrauterine or BAL sample was positive, suggesting that this sample may provide an alternative to amniotic fluid to identify antenatal infection. In conclusion, we have found that microbial genes are particularly prevalent in pPROM and spontaneous preterm labor groups and that their presence is strongly associated with a marked intrauterine inflammatory response. (***Pediatr Res* 57: 570–577, 2005**)

Abbreviations

BAL, bronchoalveolar lavage
CLD, chronic lung disease
pPROM, preterm prelabor rupture of membranes
rRNA, ribosomal RNA

Preterm birth is the most common cause of neonatal death (1) and is associated with increased neonatal morbidity and childhood disability (2,3). Evidence from studies over the past two decades suggests that subclinical infection and inflammation of the amnion/chorion/decidua is implicated in the pathogenesis of pPROM and "spontaneous" preterm labor (4,5). More recently, intrauterine infection/inflammation has been associated with neonatal morbidity, including white matter cerebral lesions (6–8) and CLD (9–11). It has been estimated that as many as 40% of spontaneous preterm births may be attributed to antenatal infection (12). Furthermore, because many infections are subclinical and the women have often been treated with antibiotics, culture results may underestimate the "true" infection rates.

Many studies have used amniotic fluid to identify antenatal intrauterine infection. The organisms commonly identified are vaginal commensals, suggesting ascending infection as a route of entry to the uterine cavity (13). Few studies have investigated other components of the uterine cavity to accurately identify the presence of infection and the nature of the relationship between such infection and inflammation. A clear association has been demonstrated between chorioamnionitis and intrauterine infection (14–16). Often, intrauterine inflammation, including chorioamnionitis, is considered synonymous with intrauterine infection (4,17). However, in many cases of chorioamnionitis it has not been possible to culture bacteria from a significant proportion of placentas, fetal membranes, and amniotic fluid samples (14–16).

The identification of conserved microbial genes (*e.g.* ribosomal genes) to detect bacteria is a technique that has been applied to amniotic fluid successfully to improve this yield (18–21). We have applied such technology to lung lavage fluid from preterm infants. By detecting microbial 16s ribosomal

Received January 27, 2004; accepted August 10, 2004.
Correspondence: Sailesh Kotecha, FRCPCH Ph.D., Department of Child Health, Cardiff University, Cardiff, CF14 4XN, UK; e-mail: sk43@le.ac.uk
DOI: 10.1203/01.PDR.0000155944.48195.97

RNA genes using PCR with subsequent cloning and sequencing, we have identified a variety of organisms in infants who are chronically ventilated (22). In addition, we used specific PCR primers to demonstrate the relationship between *Ureaplasma urealyticum* (*U. urealyticum*) and pulmonary inflammation (22).

To clarify the relationship between antenatal infection, inflammation, and the pathogenesis of preterm labor, we had three aims: a) to detect microbes by identifying the presence of 16s rRNA genes by applying PCR to placenta, fetal membranes, and, wherever possible, amniotic fluid, cord blood serum, gastric fluid, and BAL fluid obtained from preterm deliveries of <33 wk gestation; b) to relate the presence of microbes with the proinflammatory cytokine IL-6 and potent neutrophil chemotactic factor IL-8 , which have both previously been shown to be increased in preterm labor (23–25); and c) to compare the prevalence of infection in the subgroups of preterm delivery—*i.e.* i) pPROM, ii) spontaneous preterm labor, iii) preterm deliveries due to maternal or fetal reasons, and iv) preterm twin deliveries.

METHODS

Patient population. All mothers likely to deliver at <33 wk gestation were invited to join the study over a 15-mo period from January 2001. Only one mother refused consent and samples were obtained whenever the research fellow was informed of imminent preterm delivery. The study was approved by the Leicestershire Research Ethics Committee and written informed consent was obtained from all mothers. A placental and fetal membrane sample was obtained from almost all deliveries and, wherever possible, serum from cord blood and a sample of cord, amniotic fluid (mainly at cesarean section), gastric fluid, and BAL fluid from infants requiring mechanical ventilation were obtained. Three fetal membrane rolls from placental edge to rupture line (each approximately 2 cm ×10 cm) with the edge of the chorionic plate innermost, three sections of placenta (inner, mid-section, and outer chorionic plate), and three sections through the umbilical cord were collected. Both formalin-fixed, wax-embedded and snap liquid nitrogen frozen tissues samples were obtained. BAL fluid was obtained immediately after birth, wherever possible, from infants requiring mechanical ventilation, as previously described (26–28). Gastric fluid was obtained after routine passage of FG 6–8 nasogastric tube as soon after delivery as possible. All gastric fluid samples were obtained within 1 h of delivery and before any milk feeds. All fluid samples were centrifuged and separated and the cell pellet and supernatant were stored at −70°C for later analysis.

Placental and umbilical cord histology. It is our routine practice for all placentas and membranes to be examined by our pediatric pathologist, who was blinded as to the sample origins. Chorioamnionitis was diagnosed in the presence of an acute inflammatory infiltrate in the fetal membrane roll or chorionic plate (29). Funisitis was defined as presence of neutrophil infiltration into the umbilical walls or Wharton's jelly and graded as one-, two-, or three-vessel funisitis (30).

Detection of presence of microbial 16s RNA genes by PCR. DNA was extracted from all tissues, including placenta, fetal membranes, and cell pellets from fluid samples using a commercially available RNA/DNA extraction kit (QIAGEN, Dorking, Surrey, UK). The integrity of each DNA sample was confirmed by identifying the presence of β-actin, as previously described (22,27). Sequences for 16s rRNA genes were amplified by PCR using the primers FD1 (AGA GTT TGA TCC TGG CTC AG) and rP1 (ACG G(T/A/C)T ACC TTG TTA CGA CTT) at concentrations of 0.2 μM in the presence of *Taq*DNA Polymerase (Abgene, Epsom, Surrey, UK), as previously described (22). The DNA template was then added to the PCR mixes including the primers described above and subjected to 95°C for 2 min followed by 30 cycles at 95°C for 30 s, 55°C for 40 s, and 72°C for 2 min followed by a 10-min extension cycle at 72°C. The reaction products were separated by electrophoresis on 1.2% agarose gels stained with ethidium bromide and visualized using UV illumination. The PCR DNA products were cut from the gel, purified using the QIAquick gel extraction system (QIAGEN), and sequenced. In most cases, single organisms were identified by identifying 400–500 base pairs (using Chromas software, version 1.45, Griffith University, Queensland, Australia) and the closest relatives for the sequence by BLAST searches of databases posted at http://www.ncbi.nlm.nih.gov/ to ensure the same results were obtained on each occasion. The repeatability of the technique was assessed by reanalyzing all positive and a selection of negative samples. Repeatability of the results was also ensured in the development phase and in a previous study (22).

Detection of* U. urealyticum *by using specific primers. PCR was applied to all samples to detect presence of *U. urealyticum* by using specific primers to the urease gene (U5–CAA TCT GCT CGT GAA GTA TTA C and U4–ACG ACG TCC ATA AGC AAC T) as previously described (22,31). Amplification by PCR with these primers resulted in a 428 bp product fragment of the components UreA and UreB of the *U. urealyticum* urease complex. Amplification was carried out using *Taq*DNA Polymerase (Abgene) at 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 55°C for 40 s, and 72°C for 1 min followed by a 10-min extension cycle at 72°C. The reaction products were separated by agarose gel electrophoresis and the presence or absence of bands was identified by examination of the gels under UV illumination.

Measurement of IL-6 and IL-8 in tissues and fluid samples. IL-6 and IL-8 were estimated in supernatants using commercially available kits as per manufacturer's instructions (R & D Systems Europe, Oxford, UK). The lowest detection limit for undiluted samples was 0.7 pg/mL for IL-6 and 10 pg/mL for IL-8. Total protein was extracted from placenta and from fetal membranes after homogenizing in HEPES buffer and sonication as previously described (32). IL-6 and IL-8 were measured by ELISA as above and the results expressed as picograms per milligram of protein.

Statistical analysis. The number of mother-baby dyads that needed to be studied was estimated by assuming that there would be a 70% concordance between the presence of antenatal infection (defined as presence of 16s rRNA genes in intrauterine samples) and the presence of inflammation (defined as IL-8 >20 pg/mL) and a 70% concordance between lack of 16s rRNA genes and inflammation. To achieve a power of 80% at $p < 0.05$ level, it was estimated that 36 mother-baby pairings would need to be studied. Data are expressed as medians ± interquartile ranges (IQR). The data for IL-6 and IL-8 are given as picograms per milliliter of fluid and for solid tissues as picograms per milligram of protein. The nonparametric tests Kruskal-Wallis test was used to compare multiple groups and the Mann-Whitney *U* test to compare two unrelated groups. Fisher's exact test was used to compare associations between the presence of microbial material and the presence of funisitis or chorioamnionitis or the development of CLD of prematurity, defined as oxygen dependency at 28 d of age using a two-by-two or four-by-two table as appropriate.

RESULTS

Patient characteristics and samples collected. Forty-one preterm infants of <33 wk gestation born to 36 mothers were recruited. Of these, 10 infants were delivered to mothers with pPROM with membrane rupture occurring at least 1 h before onset of labor; 10 to mothers with spontaneous onset of preterm labor with intact membranes at onset of labor; 11 infants were delivered by cesarean section before the onset of labor for maternal (severe preeclampsia) or fetal reasons (compromised fetal growth or umbilical doppler flow abnormalities); and 5 mothers delivered preterm twins. The membranes were intact or had ruptured for <12 h before onset of labor in the twin pregnancies. Thirteen mothers had received antenatal treatment with antibiotics, including 8 presenting with pPROM, 2 with spontaneous preterm labor, and 1 with preterm twins.

Amniotic fluid was available from 14 deliveries. Gastric fluid was obtained soon after delivery from 29 infants. BAL fluid was obtained on the first day of life in 21 infants who required mechanical ventilation for respiratory disease. It was expected that the microbiology of gastric fluid and BAL fluid would reflect amniotic fluid microbiology as fetal lung fluid, gastric fluid, and amniotic fluid may be in continuity *in utero*. In this way, a fluid sample representative of the amniotic cavity was obtained in all but one delivery. Samples of chorionic plate and fetal membrane tissue were also obtained from most deliveries.

Detection of 16s rRNA genes. From all deliveries, 16 had at least one sample positive for the presence of 16s rRNA (Table 1). It will be noted that, except for one sample showing a mixture of microbes in the placenta from the nonlaboring group delivered due to maternal or fetal reasons, all the remaining positive samples were either in the pPROM or spontaneous preterm labor groups (Table 1). There were a variety of organisms identified from DNA sequencing of the positive samples. *U. urealyticum* was the most common organism identified and often was identified in multiple samples from the same mother-baby pairings. The other organisms identified are shown in Table 1. Some of these have been previously shown to be common vaginal organisms (*e.g. Gardnerella vaginalis*). Organisms, such as *Fusobacterium nucleatum*, have previously been implicated in the pathogenesis of preterm labor (33). In some instances, we were only able to state that a variety of organisms were present. Microbial 16s rRNA genes were most frequently identified in fetal membranes ($n = 9$) and in gastric fluid ($n = 8$), but as there were missing samples, especially for amniotic fluid and BAL fluid (which was only obtained if the infants was ventilated), only a few samples were positive (Table 1).

Evidence of infection was detected in all cases where there was evidence of chorioamnionitis or funisitis. There were four cases in which infection was detected but there was no evidence of chorioamnionitis or funisitis. Interestingly, all of these were from the spontaneous preterm labor groups (Table 1). In two of these, significant deciduitis was noted, whereas in the other two there was no histologic evidence of inflammation.

Identification of 16s rRNA genes in gastric fluid. Adequate samples of gastric fluid were obtained from 29 infants. In this subset, the delivery of eight infants was associated with cho-

Table 1. *Presence of 16s RNA genes shown by applying PCR to samples obtained from women with preterm deliveries*

Delivery	16S rRNA positive	Positive samples	Microbe identified	Chorionoamnionitis	Funisitis
PPROM	Yes	GF, FM	Uu, Uu	Yes	3 bv
PPROM	Yes	FM	Mix	Yes	3 bv
PPROM	Yes	GF, FM	Ph/Uu, Asp	Yes	1 bv
PPROM	Yes	BAL	Sau	Yes	2 bv
pPROM	Yes	PL, GF	Mix, Sp	Yes	3 bv
pPROM	Yes	BAL, GF, FM	Uu, Uu, Uu	Yes	3 bv
pPROM	Yes	PL, GF	Mix, La/Uu	Yes	3 bv
pPROM	No			No	No
pPROM	No			No	No
pPROM	No			No	No
Spontaneous preterm labor	Yes	PL	Mix	No	No
Spontaneous preterm labor	Yes	PL, BAL, GF, FM	Fn, Fn, Fn, Fn	Yes	3 bv
Spontaneous preterm labor	Yes	FM	Uu	No	No
Spontaneous preterm labor	Yes	BAL	Mix	No	No
Spontaneous preterm labor	Yes	BAL, GF, AF, FM	Uu, Uu, Uu, Uu	Yes	No
Spontaneous preterm labor	Yes	FM	Uu	Deciduitis	No
Spontaneous preterm labor	Yes	AF	Gv	Yes	1 bv
Spontaneous preterm labor	Yes	GF, FM	Sag, Asp	Yes	3 bv
Spontaneous preterm labor	No			No	No
Spontaneous preterm labor	No			No	No
Nonlaboring delivery	Yes	PL	Mix	No	No
Nonlaboring delivery	No			No	No
Nonlaboring delivery	No			No	No
Nonlaboring delivery	No			No	No
Nonlaboring delivery	No			No	No
Nonlaboring delivery	No			No	No
Nonlaboring delivery	No			No	No
Nonlaboring delivery	No			No	No
Nonlaboring delivery	No			No	No
Nonlaboring delivery	No			No	No
Nonlaboring delivery	No			No	No
Preterm twin delivery	No			No	No
Preterm twin delivery	No			No	No
Preterm twin delivery	No			No	No
Preterm twin delivery	No			No	No
Preterm twin delivery	No			No	No
Preterm twin delivery	No			No	No
Preterm twin delivery	No			No	No
Preterm twin delivery	No			No	No
Preterm twin delivery	No			No	No

Asp, *Acinetobacter* sp.; AF, amniotic fluid; bv, number of blood vessels involved; BAL, bronchoalveolar lavage fluid; Fn, *Fusobacterium nucleatum*; FM, fetal membrane; Gv, *Gardnerella vaginalis*; GF, gastric fluid; La, *Leptotrichia amnionii*; Ph, *Peptostreptococcus hareii*; PL, placenta; Sag, *Streptococcus agalactiae*; Sp, *Streptococcus pneumoniae/milleri*; Sau, *Staphylococcus aureus*; Uu, *Ureaplasma urealyticum*.

rioamnionitis or funisitis. There was no histologic evidence of inflammation in the remaining 21. By applying PCR to gastric fluid samples to detect 16s rRNA genes, microbes were detected in all cases of chorioamnionitis. No organisms were detected in the remaining cases without chorioamnionitis. Interestingly, the gastric fluid samples were always positive if any other intrauterine sample was also noted to be positive.

Culture results. Only four women were positive on routine bacterial cultures, with two each in the pPROM and spontaneous preterm labor groups. Two placental swabs grew *Streptococcus pneumoniae* and *Enterococcus* spp. and two amniotic fluid samples grew *Streptococcus milleri* and *G. vaginalis*. The corresponding results by using 16s rRNA were *S. pneumoniae* in gastric fluid but mixed species from placenta; *F. nucleatum* (an *Enterococcus* spp.) 16s rRNA genes isolated from placenta, fetal membranes, BAL fluid, and gastric fluid; mixed species from fetal membranes; and *G. vaginalis* in amniotic fluid. Thus, the detection of 16s rRNA genes identified those organisms cultured and also identified the presence of other microbial genes in several samples (Table 1).

Relationship of clinical characteristics and inflammation to detection of 16s rRNA genes. When we compared the clinical characteristics of the women-baby pairings who had at least one positive sample for 16s rRNA genes, we were unable to find any differences in the use of antenatal dexamethasone, mode of delivery, need for mechanical ventilation, use of surfactant, or development of respiratory distress syndrome or CLD of prematurity (Table 2). Although there appears to be lower incidence of CLD in the 16s RNA genes–positive group, this difference is not significant and is likely to be due to the small numbers studied. One infant died from respiratory failure at 24 h (gestation 25 wk, birth weight 730 g) and *U. urealyticum* and *Peptostreptococcus* were identified in gastric fluid and *Acinetobacter* in fetal membrane. There were marked differences in inflammatory responses between the 16S rRNA group positive and negative groups. Marked differences in inflammatory responses between the two groups with highly significant differences were noted for IL-6 in placenta ($p < 0.0001$), fetal membranes ($p < 0.0001$), cord blood serum ($p < 0.0001$), amniotic fluid ($p < 0.01$), and d-1 BAL fluid ($p < 0.05$) (Figs. 1–3). The results were very similar for IL-8 in the same samples in that marked statistical differences were noted between the two groups in placenta ($p < 0.0001$), fetal membranes ($p < 0.0001$), cord blood serum ($p < 0.0001$), amniotic fluid ($p < 0.01$), and d-1 BAL fluid ($p < 0.05$) (Figs. 1–3). Because twin pregnancies may have a different mechanism for initiating labor, we reanalyzed the data excluding these infants and found that significant differences remained for all samples for both IL-6 and IL-8.

Table 2. *Patient characteristics of preterm deliveries with at least one sample positive for 16s rRNA genes*

	Positive for 16s rRNA genes	Negative for 16s rRNA genes
Number	16	25
Gestation (wk)	29 (24–32)	30 (26–32)
Birth weight (g)	1220 (610–2070)	1150 (780–2050)
Antenatal dexamethasone	15/16 (94%)	24/25 (96%)
Surfactant	10/16 (63%)	16/25 (64%)
Cesarean section	7/16 (44%)	17/25 (68%)
Infant respiratory support	11/16 (69%)	18/25 (72%)
RDS	10/16 (63%)	16/25 (64%)
CLD at 28 d	5/15** (33%)	11/25 (44%)
CLD at 36 wk	2/15** (13%)	9/25 (36%)
Chorioamnionitis*	11/16 (69%)	0/25 (0%)
Funisitis*	10/16 (63%)	0/25 (0%)

RDS, respiratory distress syndrome.
* p Value < 0.0001.
** One infant death at 24 h.

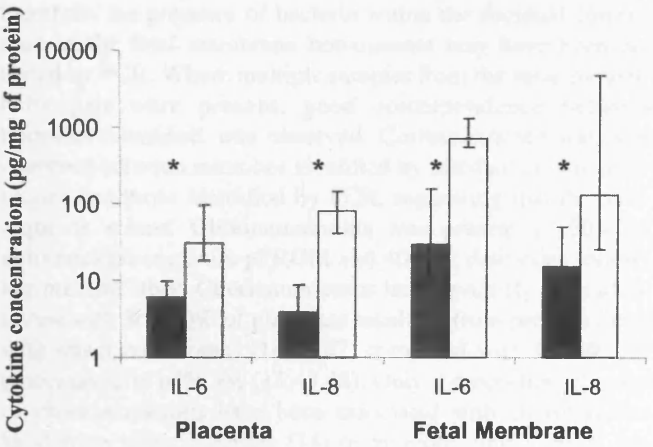


Figure 1. Concentration of IL-6 and IL-8 in placenta and fetal membrane in the group negative (*shaded bars*) and positive (*open bars*) for 16s rRNA genes. Concentration of cytokine is given per milligram of protein. *$p < 0.0001$.

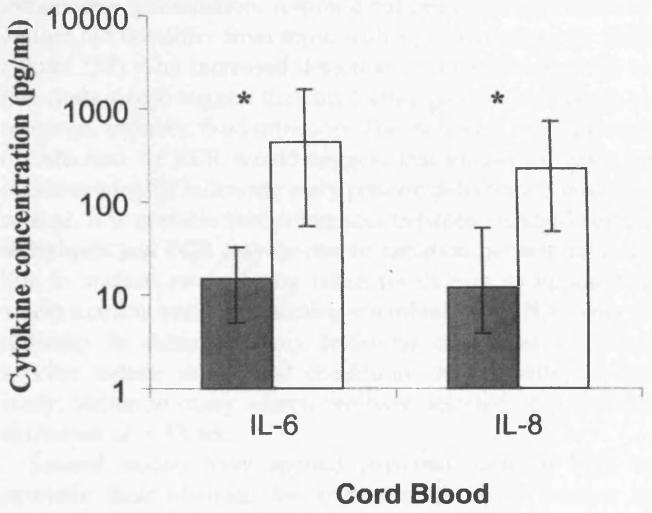


Figure 2. Concentration of IL-6 and IL-8 in cord blood in the group negative (*shaded bars*) and positive (*open bars*) for 16s rRNA genes. *$p < 0.0001$.

Preterm labor groups. We divided the mother-baby pairings into four delivery groups, namely i) pPROM, ii) spontaneous preterm labor group, iii) preterm delivery by cesarean section for maternal or fetal reasons, and iv) preterm twin deliveries. The patient characteristics are shown in Table 3. There were no significant differences between the groups for gestational age, birth weight, antenatal dexamethasone use, ventilation, neonatal respiratory distress syndrome, need for mechanical ventilation, or CLD of prematurity defined as oxygen dependency at either 28 d or 36 wk postconceptual age. However, when we

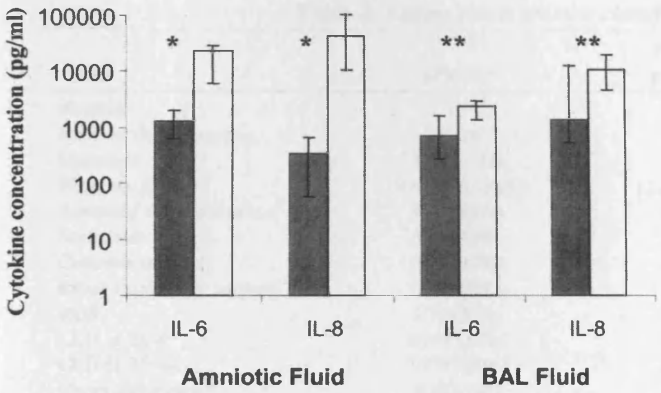


Figure 3. Concentration of IL-6 and IL-8 in amniotic fluid and BAL fluid in the group negative (*shaded bars*) and positive (*open bars*) for 16s rRNA genes. $*p < 0.01$, $**p < 0.05$.

searched for antenatal infection by identifying the presence of 16s rRNA genes, the positive samples were found in the pPROM (7/10) and spontaneous preterm labors groups (8/10), with only one positive sample found in the nonlaboring group (which identified mixed growth in placenta). Furthermore, the cytokines IL-6 and IL-8 were markedly increased in placental tissue, fetal membranes, and cord blood serum from the pPROM and spontaneous preterm labor groups when compared with the twin deliveries and nonlaboring groups (all $p < 0.05$) (Figs. 4–6). Few samples were available for amniotic fluid and BAL fluid from each group but there was a trend toward differences, with the highest concentrations of IL-6 and IL-8 seen in the pPROM and spontaneous labor groups.

DISCUSSION

This study examined the detection of microbes associated with preterm delivery using PCR detection of genes for 16S rRNA. It compared the results with the histologic presence of chorioamnionitis, and the levels of IL-6 and IL-8 in placental tissue, fetal membranes, and cord blood serum in deliveries at <33 wk gestation classified according to type of labor (pPROM, preterm labor, or elective delivery). By confining the work to deliveries at earlier gestation, we increased the likelihood of including infection-related deliveries (34–35). Although numerous studies have compared standard microbial culture techniques with histologic chorioamnionitis (14–16,36), cytokines with culture techniques (37–41), and cytokines with histologic chorioamnionitis (42–46), none has compared all parameters and included both fetal and maternal tissues from all subclassifications of preterm delivery.

By using PCR to examine for evidence of microbes, we have confirmed a higher rate of detection of infection associated with spontaneous preterm delivery than detected by standard culture techniques. Seventy percent of deliveries following pPROM and 80% of deliveries following preterm labor were associated with the presence of microbial rRNA in at least one tissue/fluid sample. Microbes were identified in samples from all deliveries with histologic chorioamnionitis, and in samples from four deliveries with no evidence of chorioamnionitis. However, two of these cases were associated with deciduitis, therefore, the presence of bacteria within the decidual component of the fetal membrane homogenate may have been detected by PCR. Where multiple samples from the same mother-baby pair were present, good correspondence between microbes identified was observed. Correspondence was also observed between microbes identified by standard culture techniques and those identified by PCR, suggesting that the technique is robust. Chorioamnionitis was present in 60% of deliveries arising from pPROM and 40% of deliveries following preterm labor. Chorioamnionitis has previously been associated with 36–60% of placentas resulting from preterm labor with intact membranes (14,16,47) compared with 42–80% of placentas after pPROM (15,45,48). Only a proportion of cases of chorioamnionitis have been associated with microbes isolated from either placenta (14) or amniotic fluid (16). It has therefore been suggested that an inflammatory response and chorioamnionitis may occur in the absence of infection (16). However, the outcomes of pregnancies in women with an intrauterine inflammatory response but negative amniotic fluid culture did not differ from those with a positive amniotic fluid culture (29). The increased detection of microbes by PCR in this study would suggest that this former group is also likely to represent amniotic fluid infection. The increased detection rate of infection by PCR would suggest that almost all cases of chorioamnionitis following early preterm delivery are infection related. It is possible that differences between standard culture techniques and PCR may be due to variation in bacterial load, due to surface swabs being taken for culture as opposed to whole extracts used for detecting microbial 16s rRNA genes or difficulty in culturing many fastidious organisms that need specific culture media and conditions. Additionally, in this study, unlike in many others, we have selected only preterm deliveries of <33 wk.

Several studies have applied universal bacterial PCR to amniotic fluid obtained by amniocentesis from women in preterm labor (19,20) or following premature rupture of membranes (18). In one study, the sensitivity for the detection of infection by PCR was 95% compared with 76% by bacterial culture (19) and in another PCR detected infection in 55.5% and culture in 9.2% (20). These studies are not directly comparable to ours as they applied PCR techniques to amniotic fluid to detect infection, whereas ours relied on both fluid samples and solid tissues, including those where a histologic inflammatory response, namely chorioamnionitis and funisitis, was observed.

This work suggests similar infection rates in preterm labor and pPROM, although the group sizes are very small. Previous work has suggested a higher rate of both chorioamnionitis and intrauterine infection associated with pPROM than with spontaneous preterm labor. However, some of the work on pPROM involves examination of tissues obtained after labor and delivery, and following a latency period after membrane rupture, and thus may by considered to reflect infection acquired after membrane rupture. Ghidini *et al.* (49), however, found that the duration of the latency period in pPROM did not increase the rate of inflammation in placenta, fetal membranes, or cord examined after delivery. Our work, although having very small numbers, also supports previous work suggesting infection is

Table 3. *Patient characteristics classified according to type of preterm delivery*

	pPROM	Spontaneous preterm labor	Nonlaboring delivery	Preterm twin delivery
Number	10	10	11	10
No. 16s rRNA positive	7 (70%)	8 (80%)	1 (9%)	0 (0%)
Gestation (wk)	30 (24–32)	28 (25–32)	30 (29–32)	30 (26–32)
Birth weight (g)	1470 (610–2050)	1220 (670–2070)	1090 (780–1942)	1175 (900–1560)
Antenatal dexamethasone	9/10 (90%)	9/10 (90%)	11/11 (100%)	10/10 (100%)
Surfactant	5/10 (50%)	8/10 (80%)	7/11 (64%)	6/10 (60%)
Cesarean section	3/10 (30%)	4/10 (40%)	11/11 (100%)	6/10 (60%)
Infant respiratory support	6/10 (60%)	9/10 (90%)	7/11 (64%)	7/10 (70%)
RDS	5/10 (50%)	8/10 (80%)	7/11 (64%)	6/10 (60%)
CLD at 28 d	2/9** (22%)	5/10 (50%)	4/11 (36%)	5/10 (50%)
CLD at 36 wk	2/9** (22%)	2/10 (20%)	4/11 (36%)	3/10 (30%)
Chorioamnionitis*	6/10 (60%)	4/10 (40%)	0/11 (0%)	0/10 (0%)
Funisitis*	7/10 (70%)	3/10 (30%)	0/10 (0%)	0/7 (0%)

RDS, respiratory distress syndrome.
* p Value < 0.005.
** One infant death at 24 h.

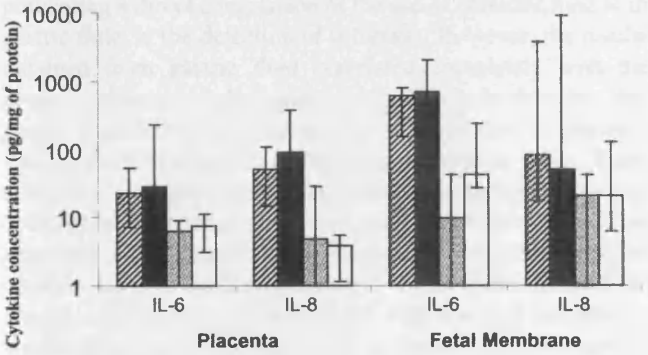


Figure 4. Concentration of IL-6 and IL-8 in placenta and fetal membrane from mother/baby from the pPROM (*hatched bars*), spontaneous preterm labor (*shaded bars*), nonlaboring preterm (*dotted bars*), and preterm twin groups (*open bars*). Concentration of cytokine is given per milligram of protein.

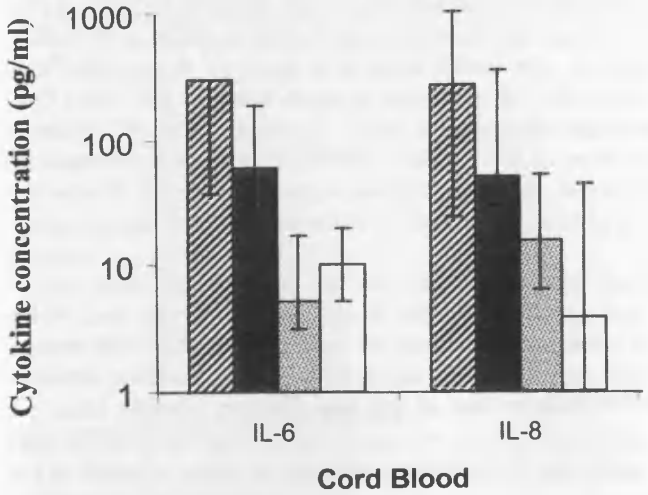


Figure 5. Concentration of IL-6 and IL-8 in cord blood from mother/baby from the pPROM (*hatched bars*), spontaneous preterm labor (*shaded bars*), nonlaboring preterm (*dotted bars*), and preterm twin groups (*open bars*).

less common as a mechanism of preterm labor in twin pregnancies (5), and that a stretch mechanism may be the underlying cause of preterm delivery in multiple pregnancies.

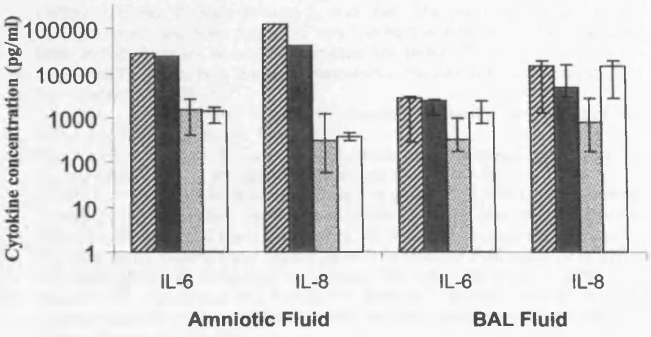


Figure 6. Concentration of IL-6 and IL-8 in amniotic fluid and BAL fluid from mother/baby from the pPROM (*hatched bars*), spontaneous preterm labor (*shaded bars*), nonlaboring preterm (*dotted bars*), and preterm twin groups (*open bars*).

We have sequenced the PCR products of the amplified 16s rRNA genes and have identified the closest relatives of the sequences using published databases. Most of these close relatives we have identified have been associated with clinical infections. *U. urealyticum, Streptococcus agalactiae* (Group B *Streptococcus*), and *S. pneumoniae* are well-known pathogens. Inasmuch as periodontal disease may be associated with preterm labor, the identification of the normal oral inhabitants *Acinetobacter* spp., *Peptostreptoccus* spp., and *Leptotrichia* spp. in intrauterine samples is an interesting observation (50). *Streptococcus milleri*, also a beta-haemolytic, has been isolated from many infective sites and *Gardnerella* spp., commensals of the vaginal tract, are associated with vaginosis and possibly preterm labor and intrauterine infection.

Increased concentrations of inflammatory markers IL-6 and IL-8 in amniotic fluid have been previously related to preterm delivery (23,24). More specifically, elevations in both IL-8 and IL-6 have been associated with microbial invasion of the amniotic cavity in both pPROM (40) and preterm labor (39). Increased IL-6 concentration in the fetus has suggested the presence of a fetal inflammatory response, and a role for the fetus in preterm labor (25). We have identified higher levels of IL-8 and IL-6 associated with microbial presence in placenta, fetal membranes, cord blood serum, and BAL fluid, suggesting

that both mother and baby are exhibiting an inflammatory response. The association of increased cytokines with preterm labor and pPROM is consistent with the high rate of detection of microbes among these groups.

Among the tissue and fluid samples used in this study were BAL fluid and neonatal gastric fluid wherever possible. Previous work examining the role of infection in preterm labor has almost exclusively examined amniotic fluid. This involves amniocentesis to obtain amniotic fluid, a procedure not routinely used in the management of preterm labor in the UK. As the amniotic fluid could be considered in continuum with fetal lung fluid and gastric fluid, microbes present within amniotic fluid could also theoretically be isolated from these latter sources. Fetal lung fluid can only be obtained by BAL from neonates who are ventilated, whereas gastric fluid is readily obtained from all newborn infants. Amniotic fluid was not obtained from the majority of patients in this study, thus preventing a direct comparison of the use of amniotic fluid with gastric fluid in the detection of infection. However, the results obtained from gastric fluid correlated completely with the presence/absence of chorioamnionitis. This is the first time that gastric fluid has been suggested as an alternative to amniotic fluid in the investigation of infection in preterm labor. There remains a need to examine the relationship between infection detected in gastric fluid and subsequent neonatal outcome. This approach would not have any clinical utility antenatally but could be used in the management of the newborn infant if the results were found to be associated with neonatal outcome. It would be useful as a research tool in future studies relating to infection and preterm delivery, to act as a surrogate for amniotic fluid and avoid amniocentesis.

There is increasing evidence implicating antenatal intrauterine infection in the pathogenesis of neonatal disease, particularly cerebral white matter lesions (51) and the development of CLD (11). In addition, it has been postulated that the pulmonary inflammation observed in preterm infants who develop CLD (26,27,52) in many instances may arise from antenatal infection (53). In this study, CLD was not associated with the development of respiratory distress syndrome, CLD, need for mechanical ventilation, or other neonatal outcomes, although to demonstrate such an association would require much larger numbers.

This work combines the use of PCR and a novel fluid, gastric fluid, to improve detection of infection associated with preterm labor. Although this may not have any direct utility in antenatal treatment of preterm labor and pPROM, it may have use in the neonatal treatment and may be used in research in place of amniotic fluid. Larger studies to confirm this work, and to relate the results to neonatal outcomes, will help define the utility of this approach.

Acknowledgments. The authors thank the mothers, babies, and staff from the maternity and neonatal units at the Leicester Royal Infirmary, Leicester. We would also like to thank Dr. Pat McEveer, pediatric pathologist at the Leicester Royal Infirmary, for histologic reporting on the placentas, fetal membranes, and umbilical cords.

REFERENCES

1. Goldenberg RL 2002 The management of preterm labour. Obstet Gynecol 100:1020–1037
2. Manktelow BN, Draper ES, Annamalai S, Field D 2001 Factors affecting the incidence of chronic lung disease of prematurity in 1987, 1992, and 1997. Arch Dis Child Fetal Neonatal Ed 85:F33–F35
3. Wood NS, Marlow N, Costeloe K, Gibson AT, Wilkinson AR 2000 Neurologic and developmental disability after extremely preterm birth. EPICure Study Group. N Engl J Med 343:378–384
4. Goldenberg RL, Hauth JC, Andrews WW Intrauterine infection and preterm delivery. N Engl J Med 342:1500–1507, 2000
5. Romero R, Espinoza J, Chaiworapongsa T, Kalache K 2002 Infection and prematurity and the role of preventive strategies. Semin Neonatol 7:259–274
6. Yoon BH, Romero R, Yang SH, Jun JK, Kim IO, Choi JH, Syn HC 1996 Interleukin-6 concentrations in umbilical cord plasma are elevated in neonates with white matter lesions associated with periventricular leukomalacia. Am J Obstet Gynecol 174:1433–1440
7. Yoon BH, Jun JK, Romero R, Park KH, Gomez R, Choi JH, Kim IO 1997 Amniotic fluid inflammatory cytokines (interleukin-6, interleukin- 1beta, and tumor necrosis factor-alpha), neonatal brain white matter lesions, and cerebral palsy. Am J Obstet Gynecol 177:19–26
8. Leviton A, Paneth N, Reuss ML, Susser M, Allred EN, Dammann O, Kuban K, Van Marter LJ, Pagano M, Hegyi T, Hiatt M, Sanocka U, Shahrivar F, Abiri M, Disalvo D, Doubilet P, Kairam R, Kazam E, Kirpekar M, Rosenfeld D, Schonfeld S, Share J, Collins M, Genest D, Shen-Schwarz S, *et al.* 1999 Maternal infection, fetal inflammatory response and brain damage in very low birth weight infants. Developmental Epidemiology Network Investigators. Pediatr Res 46:566–575
9. Lyon A 2000 Chronic lung disease of prematurity. The role of intra-uterine infection. Eur J Pediatr 159:798–802
10. Jobe AH, Ikegami M 2001 Antenatal infection/inflammation and postnatal lung maturation and injury. Respir Res 2:27–32
11. Miralles RE, Hodge R, Kotecha S 2002 Antenatal inflammation and infection in chronic lung disease of prematurity. Child Care Health Dev 28:11–15
12. Lettieri L, Vintzileos AM, Rodis JF, Albini SM, Salafia CM 1993 Does "idiopathic" preterm labor resulting in preterm birth exist? Am J Obstet Gynecol 168:1480–1485
13. Hillier SL, Krohn MA, Cassen E, Easterling TR, Rabe LK, Eschenbach DA 1995 The role of bacterial vaginosis and vaginal bacteria in amniotic fluid infection in women in preterm labor with intact fetal membranes. Clin Infect Dis 20:S276–S278
14. Pankuch GA, Appelbaum PC, Lorenz RP, Botti JJ, Schachter J, Naeye RL 1984 Placental microbiology and histology and the pathogenesis of chorioamnionitis. Obstet Gynecol 64:802–806
15. Hillier SL, Martius J, Krohn M, Kiviat N, Holmes KK, Eschenbach DA 1988 A case-control study of chorioamnionic infection and histologic chorioamnionitis in prematurity. N Engl J Med 319:972–978
16. Hillier SL, Witkin SS, Krohn MA, Watts DH, Kiviat NB, Eschenbach DA 1993 The relationship of amniotic fluid cytokines and preterm delivery, amniotic fluid infection, histologic chorioamnionitis, and chorioamnion infection. Obstet Gynecol 81:941–948
17. Bernischke K, Kaufman P. 2000 Pathology of the Placenta, 4th Ed. Springer-Verlag, New York, pp 591–684
18. Jalava J, Mantymaa ML, Ekblad U, Toivanen P, Skurnik M, Lassila O, Alanen A 1996 Bacterial 16S rDNA polymerase chain reaction in the detection of intra-amniotic infection. Br J Obstet Gynaecol 103:664–669
19. Hitti J, Riley DE, Krohn MA, Hillier SL, Agnew KJ, Krieger JN, Eschenbach DA 1997 Broad-spectrum bacterial rDNA polymerase chain reaction assay for detecting amniotic fluid infection among women in premature labor. Clin Infect Dis 24:1228–1232
20. Markenson GR, Martin RK, Tillotson-Criss M, Foley KS, Stewart RS Jr, Yancey M 1997 The use of the polymerase chain reaction to detect bacteria in amniotic fluid in pregnancies complicated by preterm labor. Am J Obstet Gynecol 177:1471–1477
21. Oyarzun E, Yamamoto M, Kato S, Gomez R, Lizama L, Moenne A 1998 Specific detection of 16 micro-organisms in amniotic fluid by polymerase chain reaction and its correlation with preterm delivery occurrence. Am J Obstet Gynecol 179:1115–1119
22. Kotecha S, Hodge R, Schaber JA, Miralles R, Silverman M, Grant WD 2004 Pulmonary ureaplasma urealyticum is associated with the development of acute lung inflammation and chronic lung disease in preterm infants. Pediatr Res 55:61–68
23. El-Bastawissi AY, Williams MA, Riley DE, Hitti J, Krieger JN 2000 Amniotic fluid interleukin-6 and preterm delivery: a review. Obstet Gynecol 95:1056–1064
24. Romero R, Ceska M, Avila C, Mazor M, Behnke E, Lindley I 1991 Neutrophil attractant/activating peptide-1/interleukin-8 in term and preterm parturition. Am J Obstet Gynecol 165:813–820
25. Romero R, Gomez R, Ghezzi F, Yoon BH, Mazor M, Edwin SS, Berry SM 1998 A fetal systemic inflammatory response is followed by the spontaneous onset of preterm parturition. Am J Obstet Gynecol 179:186–193
26. Kotecha S, Wangoo A, Silverman M, Shaw RJ 1996 Increase in the concentration of transforming growth factor beta-1 in bronchoalveolar lavage fluid before development of chronic lung disease of prematurity. J Pediatr 128:464–469
27. Kotecha S, Wangoo A, Silverman M, Shaw RJ 1996 Increase in interleukin (IL)-1 beta and IL-6 in bronchoalveolar lavage fluid obtained from infants with chronic lung disease of prematurity. Pediatr Res 40:250–256
28. de Blic J, Midulla F, Barbato A, Clement A, Dab I, Eber E, Green C, Grigg J, Kotecha S, Kurland G, Pohunek P, Ratjen F, Rossi G 2000 Bronchoalveolar lavage in children. ERS Task Force on bronchoalveolar lavage in children. European Respiratory Society. Eur Respir J 15:217–231

29. Yoon BH, Romero R, Moon JB, Shim SS, Kim M, Kim G, Jun JK 2001 Clinical significance of intra-amniotic inflammation in patients with preterm labor and intact membranes. Am J Obstet Gynecol 185:1130–1136
30. Yoon BH, Romero R, Park JS, Kim M, Oh SY, Kim CJ, Jun JK 2000 The relationship among inflammatory lesions of the umbilical cord (funisitis), umbilical cord plasma interleukin-6 concentration, amniotic fluid infection, and neonatal sepsis. Am J Obstet Gynecol 183:1124–1129
31. Blanchard A, Hentschel J, Duffy L, Baldus K, Cassell GH 1993 Detection of Ureaplasma urealyticum by polymerase chain reaction in the urogenital tract of adults, in amniotic fluid, and in the respiratory tract of newborns. Clin Infect Dis 17:S148–S153
32. Sacks NP, Smith K, Norman AP, Greenall M, LeJeune S, Harris AL 1993 Cathepsin D levels in primary breast cancers: relationship with epidermal growth factor receptor, oestrogen receptor and axillary nodal status. Eur J Cancer 29A:426–428
33. Altshuler G, Hyde S 1988 Clinicopathologic considerations of fusobacteria chorioamnionitis. Acta Obstet Gynecol Scand 67:513–517
34. Watts DH, Krohn MA, Hillier SL, Eschenbach DA 1992 The association of occult amniotic fluid infection with gestational age and neonatal outcome among women in preterm labor. Obstet Gynecol 79:351–357
35. Andrews WW, Hauth JC, Goldenberg RL, Gomez R, Romero R, Cassell GH 1995 Amniotic fluid interleukin-6: correlation with upper genital tract microbial colonization and gestational age in women delivered after spontaneous labor versus indicated delivery. Am J Obstet Gynecol 173:606–612
36. Romero R, Salafia CM, Athanassiadis AP, Hanaoka S, Mazor M, Sepulveda W, Bracken MB 1992 The relationship between acute inflammatory lesions of the preterm placenta and amniotic fluid microbiology. Am J Obstet Gynecol 166:1382–1388
37. Romero R, Avila C, Santhanam U, Sehgal PB 1990 Amniotic fluid interleukin 6 in preterm labor. Association with infection. J Clin Invest 85:1392–1400
38. Romero R, Yoon BH, Mazor M, Gomez R, Gonzalez R, Diamond MP, Baumann P, Araneda H, Kenney JS, Cotton DB, Sehgal P 1993 A comparative study of the diagnostic performance of amniotic fluid glucose, white blood cell count, interleukin-6, and Gram stain in the detection of microbial invasion in patients with preterm premature rupture of membranes. Am J Obstet Gynecol 169:839–851
39. Jacobsson B, Mattsby-Baltzer I, Andersch B, Bokstrom H, Holst RM, Wennerholm UB, Hagberg H 2003 Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women in preterm labor. Acta Obstet Gynecol Scand 82:120–128
40. Jacobsson B, Mattsby-Baltzer I, Andersch B, Bokstrom H, Holst RM, Nikolaitchouk N, Wennerholm UB, Hagberg H 2003 Microbial invasion and cytokine response in amniotic fluid in a Swedish population of women with preterm prelabor rupture of membranes. Acta Obstet Gynecol Scand 82:423–431
41. Yoon BH, Romero R, Moon J, Chaiworapongsa T, Espinoza J, Kim YM, Edwin S, Kim JC, Camacho N, Bujold E, Gomez R 2003 Differences in the fetal interleukin-6 response to microbial invasion of the amniotic cavity between term and preterm gestation. J Matern Fetal Neonatal Med 13:32–38
42. Yoon BH, Romero R, Kim CJ, Jun JK, Gomez R, Choi JH, Syn HC 1995 Amniotic fluid interleukin-6: a sensitive test for antenatal diagnosis of acute inflammatory lesions of preterm placenta and prediction of perinatal morbidity. Am J Obstet Gynecol 172:960–970
43. Arntzen KJ, Kjollesdal AM, Halgunset J, Vatten L, Austgulen R 1998 TNF, IL-1, IL-6, IL-8 and soluble TNF receptors in relation to chorioamnionitis and premature labor. J Perinat Med 26:17–26
44. Tsuda A, Ikegami T, Hirano H, Sanada H, Ogawa M, Sasaki M, Tanaka T 1998 The relationship between amniotic fluid interleukin-6 concentration and histologic evidence of chorioamnionitis. Acta Obstet Gynecol Scand 77:515–520
45. Rogers BB, Alexander JM, Head J, McIntire D, Leveno KJ 2002 Umbilical vein interleukin-6 levels correlate with severity of placental inflammation and gestational age. Hum Pathol 33:335–340
46. Dollner H, Vatten L, Halgunset J, Rahimipoor S, Austgulen R 2002 Histologic chorioamnionitis and umbilical serum levels of pro-inflammatory cytokines and cytokine inhibitors. BJOG 109:534–539
47. Odibo AO, Rodis JF, Sanders MM, Borgida AF, Wilson M, Egan JF, Campbell WA 1999 Relationship of amniotic fluid markers of intra-amniotic infection with histopathology in cases of preterm labor with intact membranes. J Perinatol 19:407–412
48. Mueller-Heubach E, Rubinstein DN, Schwarz SS 1990 Histologic chorioamnionitis and preterm delivery in different patient populations. Obstet Gynecol 75:622–626
49. Ghidini A, Salafia CM, Minior VK 1998 Lack of relationship between histologic chorioamnionitis and duration of the latency period in preterm rupture of membranes. J Matern Fetal Med 7:238–242
50. Lopez NJ, Smith PC, Gutierrez J 2002 Higher risk of preterm birth and low birth weight in women with periodontal disease. J Dent Res 81:58–63
51. Yoon BH, Park CW, Chaiworapongsa T 2003 Intrauterine infection and the development of cerebral palsy. BJOG 110:S124–S127
52. Kotecha S, Chan B, Azam N, Silverman M, Shaw RJ 1995 Increase in interleukin-8 and soluble intercellular adhesion molecule-1 in bronchoalveolar lavage fluid from premature infants who develop chronic lung disease. Arch Dis Child Fetal Neonatal Ed 72:F90–F96
53. Speer CP 2001 New insights into the pathogenesis of pulmonary inflammation in preterm infants. Biol Neonate 79:205–209

Antenatal inflammation and infection in chronic lung disease of prematurity

R. E. Miralles, R. Hodge and S. Kotecha

Department of Child Health, University of Leicester, Leicester, UK

Abstract

In spite of improved neonatal care, chronic lung disease of prematurity (CLD) remains a major cause of morbidity and mortality in extremely preterm infants. Our current understanding is that antenatal infection can trigger intra-uterine inflammation which then promotes preterm labour. Recent studies suggest that antenatal infection and inflammation can also increase the preterm infant's susceptibility to develop CLD. It may be that exposure of the fetal lung to high concentrations of pro-inflammatory cytokines is the cause of this increased susceptibility. One candidate for initiating intra-uterine inflammation is ascending infection by the vaginal commensal *Ureaplasma urealyticum* (Uu). Antibiotics administered to mothers prior to delivery appear to improve the neonatal outcome in cases of preterm prolonged rupture of membranes, but not in cases of preterm labour with intact membranes. Uu can be transmitted vertically to the airways of the preterm infant, but the role of Uu in causing CLD remains uncertain. Small trials of antibiotics given to preterm infants after delivery have not shown any consistent benefit in reducing CLD. Although CLD remains a significant problem for the extremely preterm infant, it is likely that molecular biology techniques, such as the polymerase chain reaction, will enhance the detection of antenatal infection and further our understanding of the pathogenesis of CLD.

Keywords
chronic lung disease of prematurity, intra-uterine infection, pro-inflammatory cytokines, preterm labour

Introduction

Preterm delivery remains a leading cause of perinatal mortality and morbidity (Goldenberg *et al.* 2000). With improvements in obstetric and neonatal care, such as antenatal steroids, surfactant therapy and newer modes of ventilation, the survival of preterm neonates has improved considerably. This is particularly the case for extremely low birth weight infants (below 1000 g birth weight). However the incidence of chronic lung disease of prematurity (CLD), defined either as oxygen dependency at 28 days of life or at 36 weeks postconceptual age, has not declined (Field 2000). Much work has therefore been devoted to understanding the mechanisms that lead to the development of CLD.

Correspondence:
Sailesh Kotecha MA PhD FRCPCH, Department of Child Health, University of Leicester LE2 7LX, UK
E-mail: sk43@le.ac.uk

The changing pathology of CLD

Prior to the introduction of antenatal steroids and exogenous surfactant, the main risk factors for the development of CLD were immaturity of the preterm lung, barotrauma or volutrauma, and oxygen therapy. Pathological studies demonstrated inflammation, fibrosis and smooth muscle hypertrophy (originally termed 'bronchopulmonary dysplasia' or BPD). With improvements in neonatal care, CLD is now largely confined to smaller, very

immature infants (typically at 24–28 weeks gestation and weighing less than 1000 g). These infants often have mild respiratory disease early on but remain oxygen-dependent for prolonged periods. Decreased alveolization and abnormal airway development are observed with far less lung fibrosis noted than previously when CLD affected larger more mature infants (Jobe 1999).

Inflammation in the pathogenesis of CLD

Studies on lung fluid obtained by saline lavage of ventilated preterm infants have provided valuable information on the pathogenesis of CLD in surviving infants (Kotecha *et al.* 1996a). Increased levels of inflammatory cells and proinflammatory cytokines have been reported in bronchoalveolar lavage fluid from infants who develop CLD when compared to lavage fluid from infants who develop respiratory distress syndrome (RDS) and then recover. Particularly high concentrations of cytokines may be due to on-going injury, e.g. barotrauma or oxygen therapy, due to antenatal or postnatal infection, or a failure to down-regulate inflammation due to immaturity. It is likely that the resultant environment enriched in proinflammatory cytokines may adversely affect lung growth (Kotecha 2000).

The contribution of antenatal inflammation and infection to CLD

Infection is an underestimated cause of mortality and morbidity in the preterm population. *Postnatal* systemic infection can certainly contribute to the development of CLD (Rojas *et al.* 1995). In addition, infection or bacterial colonization of lung tissue is a potent stimulus for lung inflammation. A number of observations now also support a role for *antenatal* infection in the development of CLD in some infants.

Chorioamnionitis is an inflammation of the upper genital tract affecting the fetal membranes (Goldenberg *et al.* 2000) and is usually due to ascending infection from the lower genital tract. In one study, infants born after chorioamnionitis had a decreased risk of RDS but an increased risk of CLD when compared to control infants (Watterberg *et al.* 1996). In the same study, those born after chorioamnionitis also had higher levels of IL-1β in their endotracheal secretions (Watterberg *et al.* 1996). Increased levels of IL-6 in amniotic fluid (obtained by amniocentesis), indicative of chorioamnionitis, have also been found to be a risk factor for the development of CLD (Yoon *et al.* 1997). Chorioamnionitis can lead to a *fetal* systemic inflammatory response (with raised cytokines in umbilical cord blood) and this is associated with both adverse respiratory and neurological outcomes (Gomez *et al.* 1998). Post-mortem studies have shown that chorioamnionitis can result in an antenatal inflammatory response in the fetal lung with inflammatory cell infiltration and increased expression of IL8 mRNA (Schmidt *et al.* 2001). Finally, transforming growth factor-β (TGF-β), a profibrotic factor, is increased soon after delivery in those infants who subsequently develop CLD. As fibrosis usually follows inflammation, this finding would be consistent with antenatal inflammatory process (Kotecha *et al.* 1996b). Thus, although the pathogenesis of CLD is multifactorial, antenatal infection appears to have a specific role in triggering in an inflammatory response in the fetal lung.

Antenatal Infection as a possible cause of preterm labour and CLD

Intrauterine infection has an important part to play in preterm labour (Goldenberg *et al.* 2000). Increased concentrations of cytokines have been found in amniotic fluid from women in preterm labour. It is thought that ascending bacterial infection leads to inflammation of the fetal membranes which in turn produce proinflammatory cytokines, even with subclinical infection. These cytokines lead to neutrophil infiltration and activation. This in turn leads to weakening of the fetal membranes and to prostaglandin release which promotes uterine contractions (Goldenberg *et al.* 2000). Thus both CLD and preterm labour may have a common trigger, i.e. antenatal inflammation. Antenatal infection has also been associated with an adverse neurological outcome in the newborn preterm infant (Damman & Leviton 1997).

The role of *Ureaplasma urealyticum* in preterm labour and CLD

Ureaplasma urealyticum (Uu) is the most commonly isolated organism in amniotic fluid from women in preterm labour or with preterm rupture of membrane. It is also the organism most frequently associated with chorioamnionitis. This organism can be vertically transmitted to the infant and can cause significant disease. An imbalance of pro- and anti-inflammatory cytokines has been demonstrated in the airways of infants colonized with Uu (Patterson *et al.* 1998). Whether Uu, which is regularly isolated from the lungs of infants at risk of developing CLD, is incidental or associated with the development of CLD is unknown. There is a clear association between Uu isolated from the lungs of infants who develop CLD and with decreasing gestation but whether the association is greater with the latter or former is unclear from published reports (Van Waarde *et al.* 1997). This debate is further complicated by current microbiological techniques which are insensitive at best. Better methods are necessary to accurately identify the presence of organisms including Uu.

Improving techniques for detecting infection

Detecting bacteria in clinical specimens by standard laboratory culture has limitations and there are a number of reasons why it may not be possible to culture an organism. The bacteria may be present in insufficient numbers or may have been destroyed by antibiotic therapy. Some organisms, such as Uu, are particularly fastidious. Newer methods utilize the polymerase chain reaction (PCR) (Rantokkoko-Jalava *et al.* 2000) and also the published genomic sequences of organisms including Uu. PCR will greatly amplify the organism's DNA isolated from small clinical samples and the nature of the organism can be determined either by using specific primers to organisms or by using universal primers which identify the presence of bacterial genome. The latter is followed by DNA sequencing and identification of the organism from published databases of bacterial genomes.

As only a small amount of DNA or RNA is required, PCR-based methods can identify bacteria with a greater sensitivity than laboratory culture. A recent study demonstrated that preterm infants were at greater risk of significant neonatal morbidity (including RDS, CLD, congenital infection, intraventricular haemorrhage and necrotizing enterocolitis) if Uu was detected in the amniotic fluid (Yoon *et al.* 2000). The infants were also at increased risk if Uu was detected by PCR even when culture results for Uu were negative suggesting that sensitive PCR-based methods may provide better information on infection (Yoon *et al.* 2000). Viruses can also be detected by PCR and in one group of infants, adenovirus was associated with the development of CLD (Couroucli *et al.* 2000).

Antibiotics for the prevention of preterm delivery and CLD

As infection leading to inflammation may cause CLD and preterm labour, then some degree of prevention should be possible with antibiotics. The use of antibiotics in preterm labour and preterm prolonged rupture of membranes (pPROM) has been recently reviewed (Lamont *et al.* 2000).

The results of two large multicentre randomised trials have now been published (Kenyon *et al.* 2001a; Kenyon *et al.* 2001b). In the ORACLE I trial, women presenting with pPROM were randomized to receive antibiotics (erythromycin or coamoxiclav or both) or placebo. Among infants born to women allocated to the erythromycin group, there was a non-significant decreased incidence of the composite neonatal outcome (neonatal death, CLD or major cerebral abnormality) when compared to the placebo group (12.7% erythromycin group, 15.2% placebo group, $P = 0.08$). However, when only singletons were analysed statistical significance was reached for the same composite for erythromycin ($P = 0.02$) but not for coamoxiclav (Kenyon *et al.* 2001a). In the ORACLE II trial, mothers with suspected preterm labour with intact membranes were randomized to receive the same antibiotics or placebo. No significant benefits in outcomes for either the mother or infant were noted by the use of antibiotics (Kenyon *et al.* 2001b). One likely explanation for this observation is the use of antibi-

otics may be too late if preterm labour has already established. Benefits may only be possible if antibiotics are administered once infection is present but before the effects of antenatal inflammation to promote preterm labour occur.

Small randomized trials of postnatal antibiotics administered to the infants to reduce chronic lung disease have not demonstrated any consistent benefit. Better larger randomized trials using appropriate antibiotics (e.g. including a macrolide) are required to demonstrate whether or not antibiotics given postnatally to infants at the greatest risk of developing CLD are beneficial.

Conclusions

Although the aetiology of CLD is multifactorial, there is now a significant body of evidence linking both CLD and preterm labour to antenatal inflammatory processes triggered by infection. This raises hopes for possible treatment of antenatal infection with antibiotics although to date benefits shown have been modest in most studies. To aid future studies, better methods of diagnosing infection are necessary and newer methods in molecular biology may have a role to play in both understanding the pathogenesis of CLD and in more accurately diagnosing infection — antenatally and postnatally — in infants at risk of developing CLD.

References

Couroudi, X.I., Welty, S.E., Ramsay, P.L., Wearden, M.E., Fuentes-Garcia, F.J., Jacobs, T.N., Towbin, J.A., Bowles, N.E. (2000) Detection of microorganisms in the tracheal aspirates of preterm infants by polymerase chain reaction: association of adenovirus infection with bronchopulmonary dysplasia. *Pediatric Research*, **47**, 225–232.

Damman, O. & Leviton, A. (1997) Maternal intrauterine infection, cytokines and brain damage in the preterm newborn. *Pediatric Research*, **42**, 1–8.

Field, D. (2000) *Trent Neonatal Survey 1999 Annual Report*, University of Leicester, Leicester, UK.

Goldenberg, R.L., Hauth, J.C. & Andrews, W.W. (2000) Intrauterine infection and preterm delivery. *New England Journal of Medicine*, **342**, 1500–1507.

Gomez, R., Romero, R., Ghezzi, F., Yoon, B.H., Mazor, M. & Berry, S.M. (1998) The fetal inflammatory response syndrome. *American Journal of Obstetrics and Gynecology*, **179**, 194–202.

Jobe, A. J. (1999) The new BPD: an arrest of lung development. *Pediatric Research*, **46**, 641–643.

Kenyon, S.L., Taylor, D.J. & Tarnow-Mordi, W. (2001a) Broad-spectrum antibiotics for preterm, prelabour rupture of fetal membranes: the ORACLE I randomised trial. ORACLE Collaborative Group. *Lancet*, **357**, 979–988.

Kenyon, S.L., Taylor, D.J. & Tarnow-Mordi, W. (2001b) Broad-spectrum antibiotics for spontaneous preterm labour: the ORACLE II randomised trial. ORACLE Collaborative Group. *Lancet*, **357**, 989–994.

Kotecha, S. (2000) Lung growth: implications for the newborn infant. *Archives of Disease in Childhood*, **82**, F69–F74.

Kotecha, S., Wangoo, A., Silverman, M. & Shaw, R.J. (1996) Increase in the concentration of transforming growth factor beta-1 in bronchoalveolar lavage fluid before development of chronic lung disease of prematurity. *Journal of Pediatrics*, **128**, 464–469.

Kotecha, S., Wilson, L., Wangoo, A., Silverman, M. & Shaw, R. J. (1996) Increase in interleukin (IL)-1 beta and IL-6 in bronchoalveolar lavage fluid obtained from infants with chronic lung disease of prematurity. *Pediatric Research*, **40**, 250–256.

Lamont, R.F., Mason, M.R. & Adinkra, P.E. (2000) Advances in the use of antibiotics in the prevention of preterm birth. *Recent Advances in Obstetrics and Gynaecology*, **21**, 35–44.

Patterson, A.M., Taciak, V., Lovchik, J., Fox, R.E., Campbell, A.B. & Viscardi, R.M. (1998) Ureaplasma urealyticum respiratory tract colonization is associated with an increase in interleukin 1-beta and tumor necrosis factor alpha relative to interleukin 6 in tracheal aspirates of preterm infants. *Pediatric Infectious Disease Journal*, **17**, 321–328.

Rantokkoko-Jalava, K., Nikkari, S., Jalava, J., Eerola, E., Skurnik, M., Meurman, O., Ruuskanen, O., Alanen, A. *et al.* (2000) Direct amplification of rRNA genes in diagnosis of bacterial infections. *Journal of Clinical Microbiology*, **38**, 32–39.

Rojas, A., Gonzalez, A., Bancalari, E., Claure, N., Poole, C. & Silva-Neto, G. (1995) Changing trends in the epidemiology and pathogenesis of neonatal chronic lung disease. *Journal of Pediatrics*, **126**, 605–610.

Schmidt, B., Cao, L., Mackensen-Haen, S., Kendziorra, H., Klingel, K. & Speer, C. P. (2001) Chorioamnionitis and inflammation of the fetal lung. *American Journal of Obstetrics and Gynecology*, **185**, 173–177.

Van Waarde, W.M., Brus, F., Okken, A. & Kimpen, J.L. (1997) Ureaplasma urealyticum colonization, prematurity and bronchopulmonary dysplasia. *European Respiratory Journal*, **10**, 886–890.

Watterberg, K.L., Demers, L.M., Scott, S.M. & Murphy, S. (1996) Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops. *Pediatrics*, **97**, 210–215.

Yoon, B.H., Romero, R., Jun, J.K., Park, K.H., Park, J.D., Ghezzi, F. & Kin, B.I. (1997) Amniotic fluid cytokines (interleukin-6, tumour necrosis factor-α, interleukin-8) and the risk for the development of bronchopulmonary dysplasia. *American Journal of Obstetrics and Gynecology*, **177**, 825–828.

Yoon, B.H., Romero, R., Kim, M., Kim, E.C., Kim, T., Park, J.S. *et al.* (2000) Clinical implications of detection of *Ureaplasma urealyticum* in the amniotic cavity with the polymerase chain reaction. *American Journal of Obstetrics and Gynecology*, **183**, 1130–1137.

Fetal cortisol response to intrauterine microbial colonisation identified by the polymerase chain reaction and fetal inflammation

R Miralles, R Hodge, S Kotecha

Department of Child Health, University of Leicester, Leicester, UK

Correspondence to: Professor S Kotecha, Department of Child Health, Cardiff University, Heath Park, Cardiff CF14 4XN; KotechaS@cardiff.ac.uk

Accepted 21 March 2007
Published Online First 3 April 2007

ABSTRACT

Objective: To determine the fetal cortisol response to intrauterine infection.

Study design: 16s ribosomal RNA genes or the urease genes of *Ureaplasma* spp were identified by the polymerase chain reaction in intrauterine samples. Cord blood cortisol, interleukin 6 (IL6) and IL8 were measured in samples from 31 infants born at <32 weeks gestation.

Results: 13 infants (median gestation 30 weeks, birth weight 1350 g) had at least one positive intrauterine sample for microbial genes and 18 infants (31 weeks, 1320 g) did not. The cord blood cortisol concentration was significantly higher in fetuses exposed to intrauterine infection and significantly increased in fetuses/mothers presenting in preterm labour with intact membranes compared with infants delivered by elective prelabour caesarean section ($p<0.05$). The cord blood cortisol concentration was increased in the mothers with prelabour premature rupture of membranes but this was not significant compared with the caesarean section group. The cord blood cortisol concentration was significantly increased in the presence of chorioamnionitis or funisitis and was moderately correlated with cord blood IL6 ($r = 0.64$, $p<0.01$) and IL8 ($r = 0.52$, $p<0.01$).

Conclusions: In this study, cord blood cortisol was increased in the colonised group compared with non-colonised infants. It is unclear if infants born following prelabour premature rupture of the membranes mount an adequate anti-inflammatory response.

Intrauterine infection and inflammation are frequent findings in spontaneous preterm deliveries of less than 33 weeks.[1 2] A decrease in acute lung disease in premature infants has been observed in infants exposed to intrauterine infection and chorioamnionitis.[3–5] Chorioamnionitis also appears to improve early survival after preterm birth.[6] Despite the possible short-term beneficial effects, chorioamnionitis may have deleterious effects, such as pulmonary inflammation with subsequent chronic lung disease (CLD)[3 7–10] and neurological injury.[9–12] The fetal responses to intrauterine infection and inflammation need to be further characterised if we are to prevent the pulmonary inflammation that is so often observed in infants who progress to develop CLD of prematurity.[13 14]

An increase in glucocorticoids near to term in most animals is thought to contribute to maturation of the lungs. In mice, the absence of corticotrophin-releasing hormone or glucocorticoid receptors results in delayed maturation of distal lung structures.[15 16] Exogenous glucocorticoids induce the maturation of premature lungs in animal models[17] and humans.[18] Based on this evidence, it has been proposed that chorioamnionitis might induce accelerated lung maturation by increasing endogenous cortisol secretion.[19] However, this observation was not confirmed in a model of intra-amniotic inflammation in preterm lambs in which improvements in lung function were seen in the absence of a rise in cord blood cortisol concentrations.[20]

We extended our previous study[21] by investigating the fetal cortisol responses to the presence of intrauterine microbial genes detected by using the polymerase chain reaction (PCR) and to intrauterine inflammation identified by presence of interleukin 6 (IL6) and IL8.

METHODS

Our previous study, which was approved by the local ethics committee, had included 41 preterm infants of less than 33 weeks, and the presence of intrauterine infection was identified by detecting the presence of microbial genes (16s ribosomal RNA (rRNA) and the urease gene for *Ureaplasma* spp) by PCR and intrauterine inflammation by the presence of IL6 and IL8 in cord blood and other intrauterine samples.[21] Cord blood was available in a subset of 31 infants in whom cortisol in umbilical cord blood was determined by ELISA (DRG Instruments, Germany). The assay was based on a monoclonal antibody directed at a unique site on the cortisol molecule. There was 45% cross-reactivity with corticosterone, less than 9% cross-reactivity with progesterone, less than 2% cross-reactivity with deoxycortisol and dexamethasone, and no remarkable cross-reactivity with oestriol, oestrone and testosterone. The lowest sensitivity was 6.9 nmol/l.

Methods used for detecting the presence of 16s rRNA genes and the urease gene for *Ureaplasma* spp, measurement of IL6 and IL8 in intrauterine samples and presence of chorioamnionitis and funisitis are described in more detail in our previous paper.[21] The results for blood counts and C-reactive protein (CRP) were obtained by routine venepuncture within an hour of birth for all infants. Chest *x* rays were obtained in infants who had clinical signs of respiratory distress beyond 4 h of age and independently reported by radiologists who were unaware of other study results.

Statistical analysis

Data are given as median (interquartile (IQR)) ranges. Two-group comparisons were made by using the non-parametric Mann–Whitney U test and multiple groups were compared by using the

Kruskal–Wallis test. Correlation between cord blood IL6, IL8 and cortisol concentrations was assessed using the Spearman rank correlation test for non-parametric data. The receiver operating characteristics curves were plotted with SPSS version 9.

RESULTS

Patient characteristics

Cortisol and cytokine concentrations in cord blood were available from 31 preterm infants. Of these infants, 9 were delivered after prelabour premature rupture of the membranes (pPROM), 8 were delivered after spontaneous onset of preterm labour with intact membranes, 5 were spontaneous preterm twin deliveries and 9 were delivered by caesarean section or maternal or fetal indications without onset of labour. All but two mothers had received antenatal steroid therapy (dexamethasone). Median time of administration of the first dose of dexamethasone was 4 days prior to delivery (IQR 0.6–13.6 days). For the different groups the medians (IQRs) were: caesarean section 3.1 days (0.8–7.2), pPROM 5.3 days (2.2–11.9), preterm labour with intact membranes 0.4 days (0.1–8.1) and twin group 22 days (11.1–26.0). Twelve mothers had received antibiotics prior to delivery with the greatest use in the pPROM group: caesarean section 2/9 (22%), pPROM 7/9 (78%), preterm labour with intact membranes 1/9 (11%) and twin 2/5 (40%). However, none of the infants had positive blood cultures during the first week following delivery. Microbial genes were detected in intrauterine samples from 13 (42%) infants (6 in the pPROM group, 6 in the spontaneous onset of preterm labour with intact membranes group, 1 in the caesarean section group and none in the preterm twin group).

Table 1 shows the patient characteristics. Histological chorioamnionitis was noted in 8 (62%) and funisitis in 7 (54%) of the 13 infants with microbial colonisation but no chorioamnionitis or funisitis was noted in any of the 18 infants in the non-colonised group. When the complete study group was considered, radiological evidence of respiratory distress syndrome (RDS) was less frequent in the colonised group than in the uncolonised group although this difference was not reflected clinically (table 1).

Measurement of umbilical cord blood cortisol

Cortisol concentrations in cord blood were significantly higher in the colonised group than in the non-colonised group (fig 1, table 2). As previously reported in this same group of babies, the current subset of preterm infants had increased cord blood IL6 and IL8 in the presence of intrauterine microbial colonisation.[21] Although there were no significant differences between the two groups for

Table 1 Patient subgroup characteristics

	Microbial genes present	Microbial genes absent
Number	13	18
Gestation (weeks)*	30 (24–32)	31 (27–32)
Birth weight (g)*	1350 (610–2070)	1320 (780–2050)
Antenatal dexamethasone	12 (92%)	17 (94%)
Caesarean delivery	6 (46%)	12 (67%)
Surfactant treatment	7 (54%)	9 (50%)
Infant respiratory support	8 (62%)	11(61%)
CLD at 28 days	4 (31%)	5 (28%)
CLD at 36 weeks	1 (8%)	4 (22%)
Chorioamnionitis***	8 (62%)	0 (0%)
Funisitis***	7 (54%)	0 (0%)

*Median values displayed. Values in parenthesis indicate total ranges.
***p<0.001.
CLD, chronic lung disease.

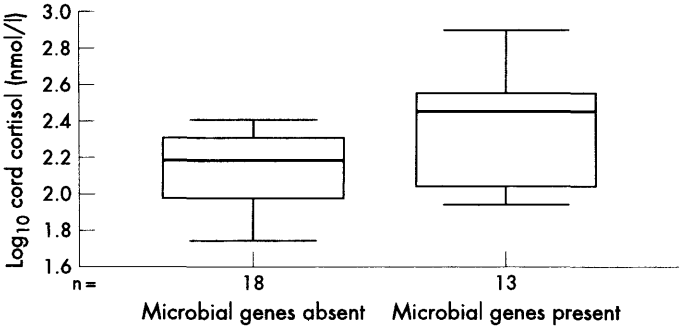


Figure 1 Significantly higher cord blood cortisol was associated with presence of microbial genes detected by polymerase chain reaction ($p<0.05$).

CRP or total white cell counts, monocytes were increased in the colonised group compared with uncolonised infants (table 3). The cord blood cortisol levels were significantly higher if funisitis (the histological correlate of fetal systemic inflammation) or chorioamnionitis were present (table 4). There was a moderate correlation between cortisol and IL6 ($r = 0.64$ after $\log_{10}$ transformation of IL6, $p<0.01$) and IL8 concentrations ($r = 0.52$ after $\log_{10}$ transformation of IL8, $p<0.01$) in cord blood (fig 2). This relationship persisted when only vaginal deliveries were considered. Since there may be a relationship between gestation and cord blood cortisol, we repeated the analyses controlling for gestational age: $r = 0.71$, $p<0.001$ between cortisol and IL8, and $r = 0.60$, $p<0.001$ between cortisol and IL8.

Table 5 shows the sensitivity and specificity obtained by a receiver operator curve for cortisol, IL6, IL8 and monocytes to identify the presence of microbial genes as detected by PCR. Cord blood IL6 and IL8 seemed to show the greatest sensitivity and specificity at a concentration of 45.4 pg/ml and 35.3 pg/ml, respectively.

Table 2 Umbilical cord cortisol and interleukin measurements

	Bacterial genes detected	Bacterial genes absent
Number	13	18
Cord cortisol* (nmol/l)	284 (87–790)	153 (55–257)
Cord IL6 (pg/ml)***	714 (1–3000)	10 (2–43)
Cord IL8 (pg/ml)***	239 (11–2000)	10 (1–98)

Median values shown. Values in parenthesis indicate total range.
*p<0.05, **p = 0.01, ***<0.001.

Table 3 Comparison of routine laboratory parameters with the detection of infection by polymerase chain reaction (complete study group n = 41)

	Bacterial genes detected	Bacterial genes absent
Number	16	25
C-reactive protein (mg/l)	5 (5–62)	5 (5–56)
Total white cell count ($\times10^9$/l)**	11.2 (5.3–27.7)	7.1 (3.2–11.8)
Neutrophils ($\times10^9$/l)	4.1 (0.7–15.2)	2.8 (0.5–8.2)
Lymphocytes ($\times10^9$/l)	4.5 (2.7–6.9)	3.5 (0.8–7.0)
Monocytes ($\times10^9$/l)***	0.95 (0.60–1.30)	0.39 (0.2–0.60)

Median values shown. Values in parenthesis indicate total range.
*p<0.05, **p = 0.01, ***p<0.001.

Table 4 Cord cortisol measurements with bacterial detection, funisitis and chorioamnionitis

	Median cortisol (nmol/l)	Range	p Value
Bacterial genes detected (n = 13)	284	87–790	p<0.05
Bacterial genes absent (n = 18)	153	55–257	
Funisitis (n = 7)	352	87–790	p<0.05
No funisitis (n = 24)	163	55–359	
Chorioamnionitis (n = 8)	352	110–790	p<0.05
No chorioamnionitis (n = 23)	262	55–359	

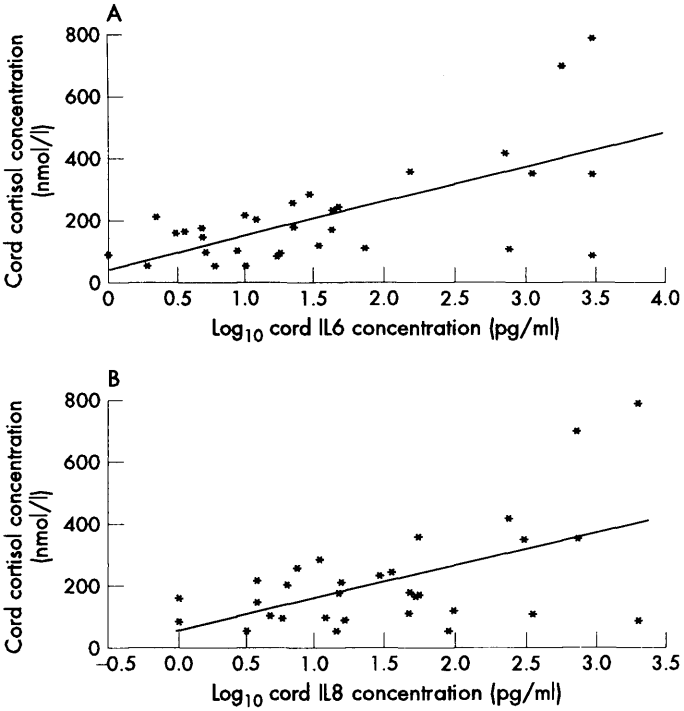


Figure 2 Correlation between cord blood cortisol and cord blood (A) interleukin 6 (IL6, r = 0.64, p<0.01) and (B) IL8, (r = 0.55, p<0.01). Note the cytokines are $\log_{10}$ transformed.

When the results in the four preterm delivery categories were compared, the cord blood cortisol concentrations were significantly higher in the spontaneous onset of preterm labour with intact membranes group than in the caesarean section or twin groups (p<0.02) (fig 3). Concentrations of cord blood IL6 and IL8 are also shown.[21] Interestingly, in the pPROM group the cortisol was increased when compared to the caesarean section group but this difference did not reach statistical significance (p = 0.23).

DISCUSSION

Our study showed that cortisol was increased in umbilical cord blood from preterm infants in whom microbial genes were identified in intrauterine samples by using PCR. Furthermore, the cortisol concentrations were increased significantly in the preterm labour with intact membranes group. Such a significant increase was not seen in the pPROM group although the numbers that we studied in each group were low. Cord blood cortisol was also increased in the infants who had funisitis or histological chorioamnionitis. The cortisol concentration correlated significantly with both IL6 and IL8. We also found that IL6 and IL8 had reasonable sensitivity and specificity for predicting presence of microbial genes in the intrauterine environment, but cortisol was only moderately predictive.

Table 5 Sensitivity and specificity of laboratory parameters in predicting presence of intrauterine infection (with equal weighting given to sensitivity and specificity)

	n	Sensitivity (%)	Specificity (%)	Threshold
IL6	41	81.3	87.5	45.4 pg/ml
IL8	41	87.5	73.9	35.3 pg/ml
Total white blood cell count	41	75.0	64.0	8.25 ($\times 10^9$/l)
Monocytes	39	73.3	83.3	0.67 ($\times 10^9$/l)
Cortisol	31	69.2	72.2	177 nmol/l

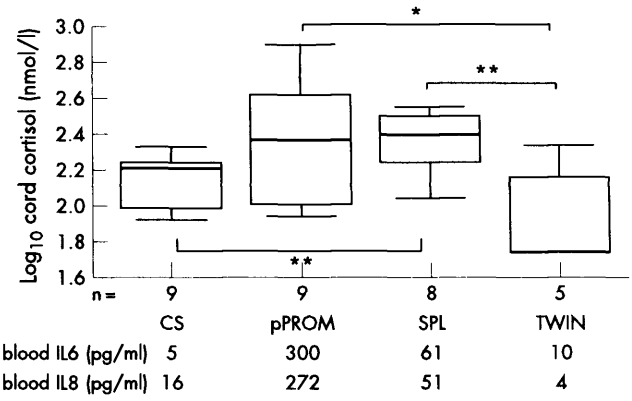


Figure 3 Cord blood cortisol concentrations in four preterm delivery categories (preterm infants of <32 weeks born after prelabour caesarean section (CS), prelabour premature rupture of membranes (pPROM), spontaneous onset of preterm labour with intact membranes (SPL) and preterm twin deliveries with intact membranes (TWIN). *p = 0.05, **p<0.02. Median cord blood IL6 and IL8 concentrations have been previously.[21]

These results are consistent with previous findings. Watterberg *et al* reported increased cortisol concentrations in very low birthweight infants on day 2 following exposure to chorioamnionitis,[19] which is closely related to intrauterine infection.[21] In addition, there was increased sensitivity of the hypothalamic–pituitary–adrenal (HPA) axis to stimulation in the first week of life following exposure to intrauterine inflammation.[19] Another study demonstrated increased fetal adrenal steroid production in response to maternal infection.[22] Prior to these studies, a possible link between chorioamnionitis and the HPA axis had been noted when a series of post-mortem examinations revealed that infants exposed to chorioamnionitis had larger adrenal glands than those not exposed.[23]

In the present study, the finding of a moderate correlation between cortisol and both IL6 and IL8 persisted when vaginal deliveries alone were considered. Such a correlation has previously been reported in preterm infants born following pPROM.[24] Presumably the increase in cortisol is partly due to the stresses of labour but greater increases were seen in the colonised group suggesting a potential increase due to the inflammatory processes occurring in the intrauterine cavity. There is evidence that proinflammatory cytokines stimulate pituitary cells in vitro,[25 26] and the HPA axis in vivo,[27] the likely mechanisms for our observation.

Chorioamnionitis has previously been associated with improvements in short-term lung function.[3 5 19] It has been postulated that this effect results from an accompanying increase in cortisol.[19] Given the importance of corticosteroids

What is already known on this topic

- Intrauterine infection and inflammation are frequent findings in spontaneous preterm deliveries of less than 33 weeks.
- Fetal cortisol is important in responding to antenatal infection.

What this study adds

- Cord blood cortisol is increased in colonised preterm infants compared with non-colonised infants.
- The increase in cord blood cortisol may be inadequate in infants with prelabour premature rupture of the membranes.

in lung development, it would seem likely that increased cortisol concentrations secondary to intrauterine infection would contribute to accelerated lung development. However, it is also known that intra-amniotic inflammation in the preterm lamb model can induce lung development in the absence of a rise in cortisol, suggesting a role for the direct effect of cytokines.[20] Whether two separate pathways exist is currently unclear.

Two recent trials of low-dose hydrocortisone to prevent CLD have been discontinued because of adverse effects, in particular an increased incidence of intestinal perforation.[28 29] As infants with cortisol concentrations above the median seem more susceptible to side effects, a more selective approach to steroid supplementation has been suggested.[30] Infants exposed to intrauterine infection are likely to have higher concentrations of cortisol immediately after birth, so this may be a group in whom prophylactic steroid treatment should be avoided. However, in one trial, the infants exposed to chorioamnionitis seemed to benefit most from hydrocortisone supplementation in preventing CLD. Our data suggest that the infants delivered to mothers with pPROM had a high inflammatory response but did not have a marked increase in cord blood cortisol compared with those preterm infants delivered for maternal or fetal reasons. Our hypothesis that there is an inadequate anti-inflammatory response of cortisol in infants born to mothers with pPROM is consistent with other published data but needs to be formally confirmed in an adequately powered study.

The most sensitive and specific markers of intrauterine infection in our study were the proinflammatory cytokines, IL6 and IL8. An increase of monocyte concentration was also observed. However, total white cell count and CRP did not seem useful in the diagnosis of intrauterine infection or fetal inflammation, although in other studies appreciable associations have been found.[19 31] The numbers of mother–infant pairings studied here do not allow firm conclusions to be drawn although the colonised group did have decreased radiological evidence of RDS.

SUMMARY

We have shown that intrauterine infection was associated with increased umbilical cord blood cortisol in cohort of preterm infants. In the human preterm infants, an increase in cortisol may have a role in modulating fetal lung development along with the direct effects of proinflammatory cytokines. The most useful markers of antenatal infection were IL6 and IL8 rather than CRP or total white cell count. Further studies on a larger cohort would be appropriate to confirm if specific groups of infants would benefit from cortisol supplementation.

Competing interests: None.
Ethics approval: The study was approved by the local research ethics committee.

REFERENCES

1. **Goldenberg RL,** Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. *N Engl J Med* 2000;**342**:1500–7.
2. **Goncalves LF,** Chaiwaorapongsa T, Romero R. Intrauterine infection and Prematurity. *Ment Retard Dev Disabil Res Rev* 2002;**8**:3–13.
3. **Watterberg KL,** Demers LM, Scott SM, *et al*. Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops. *Pediatrics* 1996;**97**:210–5.
4. **Hannaford K,** Todd DA, Jeffery H, *et al*. Role of ureaplasma urealyticum in lung disease of prematurity. *Arch Dis Child Fetal Neonatal Ed* 1999;**81**:F162–7.
5. **Shimoya K,** Taniguchi T, Matsuzaki N, *et al*. Chorioamnionitis decreased incidence of respiratory distress syndrome by elevating fetal interleukin-6 serum concentration. *Hum Reprod* 2000;**15**:2234–40.
6. **Lahra MM,** Jeffery HE. A fetal response to chorioamnionitis is associated with early survival after preterm birth. *Am J Obstet Gynecol* 2004;**190**:147–51.
7. **Jobe AH,** Ikegami M. Antenatal infection/inflammation and postnatal lung maturation and injury. *Respir Res* 2001;**2**:27–32.
8. **Miralles RE,** Hodge R, Kotecha S. Antenatal inflammation and infection in chronic lung disease of prematurity. *Child Care Health Dev* 2002;**28**(Suppl 1):11–5.
9. **Maxwell N,** Davies PL, Kotecha S. Review of the role of antenatal infection in the pathogenesis of chronic lung disease of prematurity. *Curr Opin Infect Dis* 2006;**19**:253–8.
10. **Davies PL,** Maxwell N, Kotecha S. The role of inflammation and infection in the development of chronic lung disease of prematurity. *Adv Exp Med Biol* 2006;**582**:101–10.
11. **Yoon BH,** Jun JK, Romero R, *et al*. Amniotic fluid inflammatory cytokines (interleukin-6, interleukin- 1beta, and tumor necrosis factor-alpha), neonatal brain white matter lesions, and cerebral palsy. *Am J Obstet Gynecol* 1997;**177**:19–26.
12. **Leviton A,** Paneth N, Reuss ML, *et al*. Maternal infection, fetal inflammatory response and brain damage in very low birth weight infants. *Pediatr Res* 1999;**46**:566–75.
13. **Kotecha S,** Wilson L, Wangoo A, *et al*. Increase in interleukin (IL)-1 beta and IL-6 in bronchoalveolar lavage fluid obtained from infants with chronic lung disease of prematurity. *Pediatr Res* 1996;**40**:250–6.
14. **Kotecha S,** Chan B, Azam N, *et al*. Increase in interleukin-8 and soluble intercellular adhesion molecule-1 in bronchoalveolar lavage fluid from preterm infants who develop chronic lung disease. *Arch Dis Child Fetal Neonatal Ed* 1995;**72**:F90–6.
15. **Cole TJ,** Blendy JA, Monaghan AP, *et al*. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes Dev* 1995;**9**:1608–21.
16. **Muglia LJ,** Bae DS, Brown TT, *et al*. Proliferation and differentiation defects during lung development in corticotropin-releasing hormone-deficient mice. *Am J Respir Cell Mol Biol* 1999;**20**:181–8.
17. **Newnham JP,** Moss TJ, Padbury JF, *et al*. The interactive effects of endotoxin with prenatal glucocorticoids on short-term lung function in sheep. *Am J Obstet Gynecol* 2001;**185**:190–7.
18. **Vyas JR,** Kotecha S. The effects of antenatal and postnatal corticosteroids on the preterm lung. *Arch Dis Child Fetal Neonatal Ed* 1997;**77**:F147–50.
19. **Watterberg KL,** Scott SM, Naeye RL. Chorioamnionitis, cortisol, and acute lung disease in very low birth weight infants. *Pediatrics* 1997;**99**:E6.
20. **Jobe AH,** Newnham JP, Willet KE, *et al*. Endotoxin induced lung maturation in preterm lambs is not mediated by cortisol. *Am J Respir Crit Care Med* 2000;**162**:1656–61.
21. **Miralles R,** Hodge R, McParland PC, *et al*. Relationship between antenatal inflammation and antenatal infection identified by detection of microbial genes by polymerase chain reaction. *Pediatr Res* 2005;**57**:570–7.
22. **Falkenberg ER,** Davis RO, DuBard M, *et al*. Effects of maternal infections on fetal adrenal steroid production. *Endocr Res* 1999;**25**:239–49.
23. **Naeye RL,** Harcke HT Jr, Blanc WA. Adrenal gland structure and the development of hyaline membrane disease. *Pediatrics* 1971;**47**:650–7.
24. **Yoon BH,** Romero R, Jun JK, *et al*. An increase in fetal plasma cortisol but not dehydroepiandrosterone sulfate is followed by the onset of preterm labor in patients with preterm premature rupture of the membranes. *Am J Obstet Gynecol* 1998;**179**:1107–14.
25. **Sapolsky R,** Rivier C, Yamamoto G. Interleukin-1 stimulates the secretion of hypothalamiccorticotrophin releasing factor, *Science* 1987;**238**:522–4.
26. **Bernton EW,** Beach JE, Holaday JW, *et al*. Release of multiple hormones by a direct action of interleukin-1on pituitary cells. *Science* 1987;**238**:519–21.
27. **Mastorakos G,** Chrousos GP, Weber JS. Recombinant interleukin-6 activates the hypothalamic-pituitary-adrenal axis in humans. *J Clin Endocrinol Metab* 1993;**77**:1690–4.
28. **Watterberg KL,** Gerdes JS, Cole CH, *et al*. Prophylaxis of early adrenal insufficiency to prevent bronchopulmonary dysplasia: a multicenter trial. *Pediatrics* 2004;**114**:1649–57.
29. **Peltoniemi O,** Kari MA, Heinonen K, *et al*. Pretreatment cortisol values may predict responses to hydrocortisone administration for the prevention of bronchopulmonary dysplasia in high-risk infants. *J Pediatr* 2005;**146**:632–7.
30. **Jobe AH.** Steroids and GI perforations in preterms—again. *J Pediatr* 2005;**146**:A2.
31. **Yoon BH,** Romero R, Shim J-Y, *et al*. C-reactive protein in umbilical cord blood: a simple and widely available clinical method to assess the risk of amniotic fluid infection and funisitis. *J Matern Fetal Neonatal Med* 2003;**14**:85–90.