The multiple effects of exposure to antenatal inflammation on the fetal lung

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THE MULTIPLE EFFECTS OF
EXPOSURE TO ANTENATAL INFLAMMATION
ON THE FETAL LUNG

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THE MULTIPLE EFFECTS OF EXPOSURE TO ANTENATAL INFLAMMATION ON THE FETAL LUNG

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
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Contents

Chapter 1	Introduction	7
Chapter 2	Animal model of chorioamnionitis	23
Chapter 3	Dose and time response after intraamniotic endotoxin in preterm lambs. Kramer, B.W., Moss, T.J., Willet, K.E., Newnham, J.P., Sly, P.D., Kallapur, S.G., Ikegami, M., Jobe, A.H. <i>Am. J. Respir. Crit. Care Med.</i> 2001; 164: 982–988	29
Chapter 4	Injury, inflammation and remodeling in fetal sheep lung after intraamniotic endotoxin. Kramer, B.W., Kramer, S., Ikegami, M., Jobe, A.H. <i>Am. J. Physiol.</i> 2002; 283: L452-L459	39
Chapter 5	Antenatal inflammation induced TGF-beta1 but suppressed CTGF in preterm lungs. Kunzmann S., Speer C.P., Jobe A.H., Kramer B.W. <i>Am. J. Physiol.</i> 2007; 292: L223–231	49
Chapter 6	Surfactant protein A recruits neutrophil granulocytes into the lungs of ventilated preterm sheep. Kramer, B.W., Jobe, A.H., Bachurski, C.J., Ikegami, M. <i>Am. J. Respir. Crit. Care Med.</i> 2001; 163: 158–165	61
Chapter 7	The clever fetus: responding to inflammation to minimize lung injury. Kramer B.W., Jobe A.H. <i>Biol. Neonate.</i> 2005; 88: 202–207	71
Chapter 8	Intratracheal endotoxin induces systemic inflammation in ventilated preterm lambs. Kramer, B.W., Ikegami, M., Jobe, A.H. <i>Am. J. Respir. Crit. Care Med.</i> 2002; 165:463–469	79
Chapter 9	Monocyte function in preterm, near term and adult sheep. Kramer, B.W., Jobe, A.H., Ikegami, M. <i>Pediatr. Res.</i> 2003; 54: 52–57	89
Chapter 10	Antenatal betamethasone changes cord blood monocyte responses to endotoxin in preterm lambs. Kramer, B.W., Ikegami, M., Moss, T.J.M, Nitsos, I., Newnham, J.P., Jobe, A.H. <i>Pediatr. Res.</i> 2004; 55:764–768	97

Chapter 11	Endotoxin induced chorioamnionitis modulates innate immunity of monocytes in preterm sheep. Kramer B.W., Ikegami M., Moss T.J.M., Nitsos I., Newnham, J.P., Jobe, A.H. <i>Am. J. Respir. Crit. Care Med.</i> 2005; 171: 73–77	105
Chapter 12	Summary and Outlook	113
	Samenvatting	123
	Acknowledgement	131
	Curriculum vitae	133
	List of Publications	134

CHAPTER 1

Introduction

Preterm birth is the major problem in reproductive medicine. Preterm birth causes problems for the preterm baby, the mother, the family and the health care system despite the increased survival rate of preterm infants in recent years (85, 87). On a population basis preterm birth is the major variable that results in neonatal death and adversely affects the developmental potential of children and adults (55, 77, 93). The progress in the treatment of preterm babies has not been matched in the reduction of preterm delivery which in fact has increased in the past decades (11, 86). After reviewing the epidemiology and the multifactorial causes for preterm births, the role of infections of the membranes and amniotic fluid (chorioamnionitis) will be discussed. An animal model will be introduced to study effects of chorioamnionitis on lung function, lung development and the immune system. Chorioamnionitis will be discussed as a disease with multiple effects on the fetal lung.

A. Epidemiology of preterm birth

The correct incidence of preterm birth can only be assessed with a consistent definition of preterm birth and a population-based approach (7, 11, 21). The reported incidence of preterm births are therefore affected by the method of assessment of gestational age and by differing definitions of viability and thus the registration of the preterm delivery as a live-born (49). Preterm birth occurs in an extremely heterogeneous group of patients since preterm delivery may occur close to 37 weeks of gestation with no pathological cause and few clinical problems or it may occur at the extremes of prematurity at 24 weeks of gestation with low survival rates and a high risk for morbidity in the survivors (11). There is agreement that prematurity is defined by a gestational age below 37 weeks (see below), however there is little agreement on the minimal gestational age and on the subdivision of the preterm period into intervals defined by outcomes (113). In addition, the determination of gestational age can be quite inaccurate unless early ultrasound scanning is performed (8). This holds especially true in women of low socioeconomic status where the lack of prenatal healthcare is one of the risk factors for preterm birth. These limitations render the comparison of the incidence and outcomes of preterm birth in different countries particularly problematic.

The definition of preterm birth according to the World Health Organisation (WHO) is birth before the com-

pletion of 37 weeks of gestation (259 days) from the first day of the last menstrual period (11). Earlier WHO guidelines were by birthweight since it is more accurately ascertained. However, gestational age is much more predictive of outcome than birthweight (16). The formerly used terms of „low birth weight“ and „extremely low birth weight“ fail to reflect prematurity adequately (75). These definitions include babies of relatively late gestations with severe in utero growth restriction (IUGR). The term of *extreme low gestational age newborn* (ELGAN) states the importance of gestational age (26) but does not provide a lower limit for gestational age either. The lower limit of preterm birth is therefore defined by legislation in most countries. The legal definition often takes into account the availability of neonatal intensive care units, local definitions of viability and varies considerably between countries. The United Kingdom has the legal definition of 24 weeks and above for viability. Scotland however includes all births greater than 20 weeks in official statistics on the assumption that the causes for pregnancy lost between 20 and 24 weeks are likely similar to those causing preterm birth at 24 to 26 weeks gestation (15).

The date of conception is usually unknown unless the pregnancy resulted from assisted fertilization. The last day of the menstrual period is used to estimate gestational age. This estimation is based on the correct recall of the last day of menstruation, a 28 day menstrual cycle and on the assumption that conception occurred mid-cycle (49). Irregularity of the menstrual cycle due to individual differences or hormonal drugs, amenorrhea because of breastfeeding or failure to recall the last day can affect this method. Ultrasound examination in early pregnancy is very accurate since there is little biological variation in fetal size during this time of pregnancy. The use of early ultra-sound examinations is however limited to the developed world. Even in developed countries women of low socio-economic status or educational backgrounds are likely to attend antenatal care so late in the antenatal period that ultrasound estimated gestational age are no longer reliable (31).

The incidence of preterm birth is reported to be between 5 and 13 % in the developed world (7, 11, 49). In the developed world the rate has dropped in very few countries but has been static or on the rise in the majority of all countries for the past 20 years. In New Zealand the rate of single-

Table 1 Conditions resulting in preterm delivery

Maternal	
Severe pre-eclampsia or eclampsia	
Major antepartum haemorrhage	
Chorioamnionitis with maternal symptoms	
Maternal disease:	cardiac
	respiratory
	renal
	psychiatric
Multiple pregnancy	
Fetal	
Rhesus (or other) isoimmunisation	
Severe intrauterine growth restriction	
Fetal compromise:	anaemia
	hypoxia/acidosis
	cardiac/other organ failure
Cord entanglement (monoamniotic twins)	

ton preterm birth has risen from 4.3 % in 1980 to 5.8 % in 1999 which is an increase of 37 % overall (22). The increase was only 3.5 % in the lower socio-economic group but 72 % in high socio-economic group (22). Delay in child bearing among affluent career women and an increase in assisted reproduction have been suggested as explanations. Similar trends were shown in Canada (107, 108). The proportion of births to women over 35 years increased from 8.4 % in 1990 to 12.6 % in 1996. The preterm delivery rate increased among those mothers to 14 %. The increase in assisted reproduction also resulted in an increase in multiple births which added to the increase in preterm birth rate. The rate of twin pregnancies in the United Kingdom increased from 0.9% in 1980 to 1.2% in 1993 to 2% in 2002 (12). The rate of triplet and higher order births in the UK and in France has increased from 10 per 100,000 pregnancies before 1975 to 35 per 100,000 in 1993. Estimates range from 15 to 20% in twin pregnancies and up to 69 % in higher order pregnancies result from assisted reproduction (12).

B. Causes for preterm birth

Maternal and fetal indications

Among all preterm deliveries 30% are indicated for maternal reasons in the developed world (83). Clinicians will consider preterm delivery if the continuation of the pregnancy will be a greater risk for the life or the well being of mother and/or fetus (56). Maternal conditions include pre-eclampsia or eclampsia,

haemorrhage, chorioamnionitis with maternal symptoms such as fever, or other severe maternal organ dysfunction (table 1) (83). Detection of intra-uterine growth restriction (IUGR) or other fetal problems resulting in prompt delivery include rhesus isoimmunisation, fetal anemia, hypoxia/acidosis or organ failure (table 1). The underlying idea is to remove the fetus from an adverse environment or to treat the underlying disease ex-utero (9).

The decision for continuing the pregnancy is complicated by the fact that every day the pregnancy is continued is associated with an increase in still-birth (107). Moreover, the clinical diagnosis of preterm labour may not necessarily prompt preterm delivery. In randomized controlled trials of tocolytics many women with apparent preterm labour were given placebo and did not deliver before term (105). Expectant management of preterm labour, the threshold for caeserean section and breech presentation may affect the rate of preterm deliveries in such a situation (105).

Previous preterm birth

A previous preterm delivery is the most important risk factor for subsequent preterm delivery (4, 5, 51). The risk factor has been estimated to be 4 fold higher (14, 51). A further subgroup analysis identified women with chorioamnionitis to be at a more than 50% risk to deliver preterm in the following pregnancy with subsequent diagnosis of chorioamnionitis (81). However, the population group that suffer most preterm births have usually more than one risk factor contributing to the increased risk of preterm birth (22, 91). Genetic factors have been indentified to be part of the multifactorial contributors to preterm birth (27).

Cervical incompetence

The diagnosis is made by the appearance of a cervix dilatation in the absence of labour. The true incidence of cervix incompetence is difficult to determine but risk factors include previous surgery and congenital malformations (74). Ascending infections may cause increases in prostaglandins and thus soften the cervix. In a randomized controlled trial only 1 in 25 women thought to have cervix incompetence profited from cervix cerclage (74).

Infection

Considerable evidence suggests a link between infection and preterm birth (92). Infections associated with preterm delivery may be in lower genital tract, intrauterine or extra-uterine (generalized maternal infection). A causative link is plausible in some cases and is supported by the efficiency of antibiotic therapy (38).

Lower genital tract infections

Bacterial vaginosis (BV) occurs in 10 to 25% of pregnant women (62). The normal vaginal microflora of predominantly lactobacilli is replaced by other organisms, especially by anaerobic bacteria, *Gardnerella vaginalis* and mycoplasmas. Women with BV are at risk to give preterm birth with an odds ratio of 2.19 (95% confidence interval 1.54 – 3.12) (72). Genetic polymorphism in women is associated with this kind of microflora and preterm birth (91). A systemic review of randomized controlled trials identified the subgroup of women with a previous preterm birth to benefit from antibiotic treatment (76).

Asymptomatic bacteriuria

The presence of asymptomatic bacteriuria is an indication for antibiotic treatment since untreated bacteriuria is associated with a 30% incidence of pyelonephritis later in pregnancy and a 30% to 50% increase in preterm birth (98). Antibiotic treatment reduces the risk of pyelonephritis (odds ratio 0.24, 95% confidence interval 0.19-0.32) and preterm birth (odds ratio 0.60, 95% confidence interval 0.45-0.85) (98).

Group B streptococcus

Group B streptococcus (GBS) is a common vaginal pathogen but it is not clear that it is a pathogen for non-pregnant women. GBS has had a major impact on perinatal care (82). The early onset sepsis (within the first 3 days of life) by GBS occurs in 0.5 to 3 per 1000 live births in the USA (81). The pathogen is transmitted during birth to the baby. Therefore, maternal colonization, a previous infant who had GBS sepsis, antenatal GBS asymptomatic bacteriuria, rupture of membranes greater than 12 hours, intrapartum fever, and gestational age less than 37 weeks are established risk factors for early onset GBS sepsis (50). GBS sepsis is very dangerous since the case-fatality rate is very high especially in

preterm infants (50). Many survivors will have long-term neurodevelopmental handicaps. Intrapartum antibiotic treatment will reduce the incidence of early onset GBS sepsis but has no effect on the incidence of late onset GBS sepsis (81). Conflicting data exist about GBS colonization as a cause of preterm delivery. The prevalence of GBS in women delivering preterm is not increased (69) but GBS is more frequently detected in women with preterm labor and preterm pre-labour rupture of membranes (81).

Chlamydia trachomatis

Genital chlamydial infection is detected in 5% to 15% of all women in child-bearing age, making *Chlamydia trachomatis* one of the most commonly sexually transmitted diseases in developed countries, especially among young women (36, 63). The associated risk for preterm delivery is 3 fold higher after controlling for confounding variables (89).

C. Chorioamnionitis

Inflammation of the amniotic fluid and membranes is called chorioamnionitis. Chorioamnionitis is caused by a variety of bacterial species. The clinical course can be distinguished between a systemic inflammatory response of the mother with increased leukocyte counts and fever (amnionitis inflammatory syndrome) which is treated by delivery of the fetus and antibiotic therapy, and a clinically silent chorioamnionitis with no symptoms in the mother (38). The predominant form at early gestational age is clinically silent which is usually diagnosed postnatally by pathological review of the placenta (61, 70). If the umbilical cord vessels have inflammatory changes the chorioamnionitis is thought to have progressed to fetal inflammation or funisitis (23). The incidence of clinically silent, histological chorioamnionitis has been reported to be up to 60% in extremely preterm infants throughout different populations (figure 1) (70). The diagnosis of chorioamnionitis is however a major challenge. Steel et al., 2005, showed with bacterial 16S ribosomal RNA probes to detect DNA from bacteria that the bacterial invasion of the membranes and placenta did not necessarily induce an inflammatory response (104). They reported that 25 of 29

Histologic Diagnosis Chorioamnionitis [%]

Figure 1: Incidence of histologic chorioamnionitis in preterm deliveries at the University of Sydney, Australia. Numbers of samples are given per gestational age. Modified according to Lahra und Jeffrey, *Am. J. Ob. Gyn*, 190: 147 pp., 2004.

fetuses that delivered prematurely had bacteria that were deep within intact fetal membranes and in 25% of all cases there was no associated inflammation. The majority of preterm infants delivered without ruptured membranes had evidence of colonized membranes using this bacterial probe.

The possible explanations for this finding are numerous. First of all, there is little to no information on the time course of chorioamnionitis relating the invasion of microbes with the time necessary for an inflammatory response (3, 104). Secondly, the interaction of the microbes with the immune system of the host is not well understood. In the case of chorioamnionitis the host is the mother and the fetus and mechanism that might prevent or control inflammatory response have not been studied (38). Thirdly, the function of the immune system may be primed or modulated by previous exposures to proinflammatory agonists (116). The initial exposure to a non-lethal dose of endotoxin for example reduced the response of the immune cells to a second usually lethal dose of endotoxin in experimental animal models (116). This phenomenon, called endotoxin tolerance, is considered as a major cause for morbidity in adult intensive care units. Previous exposures to proinflammatory stimuli renders the immune system of patients tolerant. A secondary infection with living bacteria is therefore not addressed adequately by the immune

systems which gives the bacteria a competitive advantage (116). Little information is available on the effects of the indolent chronic infection of chorion and amniotic fluid on the function of the immune cells (65).

Bacterial invasion of the amniotic cavity

Until recently, the placenta, membranes and fetus were considered to be sterile in most cases and during much of the pregnancy (38). This general understanding has been challenged by a number of clinical studies. Sophisticated sampling of the endometrium and subsequent microbiological analysis yielded in most cases viable bacteria in the endometrium 3 months after delivery (3). There was no difference in the incidence of preterm delivery with respect to bacterial infection (table 2).

The amniotic cavity thus appears to be more frequently exposed to bacterial invasion without problems in conception or resulting necessarily in preterm birth (3). This view was previously suggested by results from Perni et al. (84). Samples of amniotic fluid for genetic analysis were analysed for *Ureaplasma urealyticum* and *Mycoplasma hominis*. *Ureaplasma urealyticum* were

Table 2: Endometrial microbial colonization 3 months after pregnancy

	Delivery		
	Normal term	Spontaneous preterm	Indicated preterm
Number of patients	303	375	142
Gestational age at delivery (weeks)	39.6	26.4	27.2
Positive endometrial cultures	81 %	85 %	79 %

According to Andrews et al., Am. J. Obstet. Gynecol. 193, pp. 739–745, 2005

Table 3: Detection by polymerase chain reaction of *Ureaplasma urealyticum* or *Mycoplasma hominis* in amniotic fluids collected at 15 to 19 weeks from 179 asymptomatic women

	<i>Ureaplasma urealyticum</i>	<i>Mycoplasma hominis</i>
Amnionitic fluid positive	22 (13 %)	11 (6 %)
Preterm premature rupture of membranes & preterm birth	3 (14 %)	2 (18 %)
Term birth	19 (86 %)	9 (82 %)

According to Perni et al., Am. J. Obstet. Gynecol. 191, pp. 1382-1386, 2004

found in 13 % of all samples (table 3). Preterm premature rupture of membranes and subsequent preterm birth occurred only in 3 of the 22 (14 %) women that tested positive for *Ureaplasma urealyticum*. Thus the majority (19 of 22; 86 %) of the women who tested positive for *Ureaplasma urealyticum* delivered at term. Similar results were obtained for *Mycoplasma hominis* (table 3). Therefore, the presence of microbacteria in the amniotic cavity may not necessarily result in preterm birth (35). Taken together these reports suggest that fetal coexistence with commensal organisms may be the rule and not the exception.

D. Importance of Chorioamnionitis to Neonatal Mortality and Morbidity

The importance of antenatal exposure to inflammation for preterm infants is a complex issue (100). Clinical trials suggest that chorioamnionitis is beneficial for the survival of extremely preterm infants probably by induction of early lung maturation (20, 114). In contrast, exposure to funisitis or a systemic inflammatory response has been associated with bad neurodevelopment and pulmonary outcomes (78, 79, 112). Among the possible mechanisms suggested by animal experiments are an increased susceptibility to hypoxia and ischemia after expo-

sure to antenatal inflammation (45). There also may be direct effects on the vasculature (23, 67).

Lung outcomes after very preterm birth

Lung immaturity and lung injury are major contributors to the morbidity and mortality of premature infants less than 30 weeks of gestation (52). The two major adverse outcomes are respiratory distress syndrome (RDS) caused by surfactant deficiency and structural lung immaturity resulting in early death, and bronchopulmonary dysplasia (BPD). The mortality of RDS has been estimated to be 40% (48, 122). RDS has been the most common cause of mortality in preterm infants until the introduction of surfactant replacement therapy and the wide-spread use of antenatal maternal corticosteroid therapy (48). The cause of RDS is the immaturity of the lung in preterm infants. Capillaries and air conducting structures come close to each other at the beginning of the saccular phase of lung development at around 22-24 weeks of post menstrual age (64). The diffusion of gases from air saccules to the capillaries and vice versa is not possible at an earlier stage of lung development. Therefore, all efforts of oxygenation and carbon dioxide elimination via diffusion are restricted by this gestational age or more precisely by this stage of lung development. From the second trimester alveolar type II cells produce a complex mixture of lipids and proteins, which is secreted into the air space (40, 68). This mixture

Figure 2: Incidence of respiratory distress syndrome (RDS) is inversely correlated with gestational age and increase in surfactant pool size. Surfactant pool size was quantified by saturated dipalmitoylphosphatidylcholine (DPPC) the major component of surfactant. Modified from Jobe AH. Pathophysiology of RDS. In: *Fetal and Neonatal Physiology*, edited by Polin and Fox. Philadelphia: Saunders, 1998, p. 1299-1314.

reduces the surface tension and is therefore called surfactant which stands for surface active agent(s) (122). Surfactant maintains the alveoli open during the cyclic changes in surface area and tension during inspiration and expiration. The incidence of RDS decreases with gestational age as the lung structure matures and the surfactant pool increases (figure 2) (122). Preterm infants have little surfactant in both the air space and in the alveolar type II cells. The barrier between air and water in the alveoli mandates the presence of surfactant to remain intact. Delayed or impaired surfactant synthesis is at the beginning of RDS.

Bronchopulmonary Dysplasia

BPD is a chronic lung injury and inflammation syndrome in very preterm infants that results in disrupted development of the saccular lung intra- and extra-uterinely (1). The lung development is in a very immature state at 24 weeks gestation and is referred to as the saccular phase (64). Air containing structures and blood vessels come close to each other for the first time in gestation. Babies that have BPD have a lower number of alveoli than normal (53). In addition, the individual size of the alveoli is bigger in babies with BPD (53). The changes induced in the post-natal lung development with a chronic inflammation in the airways are shown in figure 3. Alveolar septation

and microvasculature development are reduced in babies with BPD. Therefore, the lung development is severely impaired resulting in less surface area for gas exchange and impaired gas exchange (10, 18, 53, 71). BPD is therefore defined clinically by the need for supplemental oxygen (58). A workshop of the National Institute of Child Health and Human Development/National Heart, Lung, and Blood Institute proposed a severity-based definition of BPD for infants <32 weeks' gestational age (GA) in 2000 (58). Mild BPD was defined as a need for supplemental oxygen (O_2) until or for over 28 days but not at 36 weeks' postmenstrual age (PMA) or discharge. Moderate BPD was considered as oxygen dependency until or for over 28 days plus treatment with below 30% O_2 at 36 weeks' PMA, and severe BPD as O_2 for oxygen dependency for until or for over 28 days plus more than 30% O_2 and/or positive airway pressure at 36 weeks' PMA. The impaired lung development represents the most common adverse outcome of preterm birth. The categorization of BPD by this definition is predictive for adverse pulmonary and neurodevelopmental outcomes in early infancy (28). Infants with BPD are significantly more likely than their gestational age counterparts without BPD to be rehospitalized within the first year of life, to develop long-term neurologic disability, and they consume twenty

Normal lung development

Figure 3: The normal development of the lung is depicted on the upper part of the cartoon. During late pregnancy and early childhood alveoli are formed which provide the increase in area for gas exchange to meet the demand by growth. Alveoli do not form in the same pattern in infants suffering from BPD which is depicted on the lower part. Chronic exposure to proinflammatory cytokines in the airways impairs development of alveoli and microvasculature thus reducing the area for gas exchange and making supplementary oxygen necessary. Modified from Husain et al., *Hum. Pathol.*, 29: 710–727, 1998 and from Groneck et al., *Pediatrics*, 93: 712–718, 1994.

times more annualized health care resources than unaffected children in the USA (55, 77, 93, 99). Approximately 5.9% of all resources spent for children from 0 to 18 years of age are consumed by infants with BPD in the USA (55, 93, 99).

BPD is associated with a chronic inflammatory response in the airways of preterm babies. This inflammatory response may be initiated antenatally by chorioamnionitis and/or sustained by postnatal interventions such as resuscitation, oxygen toxicity, and mechanical ventilation with baro- or volutrauma (41, 42, 94). The oxygen concentration of preterm infants is depicted in figure 4 during the first weeks of life. Analyses of antenatal risk factors associated for example low gestational age, absence of maternal steroids and chorioamnionitis with the development of BPD. Postnatal risk factors such as mechanical ventilation, high oxygen concentrations and a systemic inflammatory response have been identified to sustain the chronic inflammation in the airways (figure 4) (17, 19, 39, 95, 110–120).

Few large studies have correlated chorioamnionitis with lung outcomes. Analysis of outcomes for all infants born before 26 weeks in the United Kingdom and Ireland in 1995 showed that the major cause of death was pulmonary insufficiency and survival was increased with antenatal corticosteroids and when clinical chorioamnionitis was diagnosed (20). In con-

trast, histologic chorioamnionitis was not associated with decreased death in a recent trial of cortisol to prevent BPD or in a retrospective analysis (61, 115). Remarkably, although chorioamnionitis is frequent, only about 2% of extremely preterm infants have positive blood cultures at birth (106). Despite the clinical bias that infection should severely compromise the fetus, preterm infants exposed to chorioamnionitis seldom are septic and may have decreased mortality (20).

Watterberg et al. correlated histological chorioamnionitis with the incidence of respiratory distress syndrome (RDS) and bronchopulmonary dysplasia (BPD) in ventilated preterm babies (114). They found in patients from the 1990's that preterm infants had a lower incidence of RDS if they were antenatally exposed to chorioamnionitis. However, the incidence of BPD increased in the very same infants (table 4).

Recent clinical studies did not confirm the results that Watterberg et al. obtained a decade before in ventilated preterm babies and what van Marter et al. confirmed in patients with sepsis or mechanical ventilation (111). Chorioamnionitis was not associated with the development of BPD in a large cohort in Australia or in the USA (2, 61, 115). Several practices in perinatal medicine have changed during the past decade. The venti-

Figure 4: Oxygen needs of infants with BPD in the first three weeks of life. A subpopulation will develop BPD in the context of a very immature lung with RDS after birth and subsequent lung injury and inflammation. However, some babies develop BPD although they were born without RDS but chronic inflammation in the airways is sustained or initiated by postnatal events such as mechanical ventilation or systemic inflammation. Modified from Charafeddine et al., *Pediatrics*, 103: 759–765, 1999

Table 4: Incidence of RDS and BPD in preterm infants exposed to chorioamnionitis

	Chorioamnionitis	No Chorioamnionitis
RDS	33 %	67 %
BPD	63 %	37 %

According to Watterberg et al., *Pediatrics* 97, pp. 210-215, 1996

lation style has moved to favour continuous positive airway pressure (CPAP) which is a less invasive way to support ventilation. Experimental models showed that CPAP induced less injury and inflammation in the lung of preterm lambs than state-of-the-art mechanical ventilation (59). Surfactant replacement therapy is nowadays readily available and is given soon after birth to minimize lung injury (102). In vivo and in vitro studies have shown anti-inflammatory properties of surfactant proteins (SP)-B and -C and phospholipids in commercial surfactant preparations (30, 54, 109). SP-B, SP-C and phospholipids reduced the transcription of tumor necrosis factor alpha in monocytes (6, 101). In addition, surfactant preparations of SP-B and phospholipids enhanced the phagocytosis of apoptotic cells as part of the resolution of inflammation (66). Therefore, surfactant therapy not only has effects on the compliance of the lung and subsequent injury but also has anti-inflammatory properties to modulate the pulmonary inflammatory response.

Currently commensal bacteria such as *Ureaplasma urealyticum* in the vagina are considered to be the source of the bacterial invasion of the amniotic cavity (38). However, other possible origins have been identified. Periodontal disease has been linked with the onset of preterm labour and preterm birth. The local infection in the gums with bacteria that produce potent endotoxins may result in a systemic exposure to endotoxins which may trigger preterm delivery (80). Intervention trials are currently underway to assess the option of prevention of preterm birth by periodontal treatment (37, 97).

Systemic effects of chorioamnionitis

Hemodynamic disturbances in premature infants have been reported after exposure to chorioamnionitis (117). In addition, the concentration of proinflammatory cytokines in the cord blood have been associated with poor neurodevelopmental outcome (60) but also with a reduction in the incidence of RDS (96). In a large retrospective study the association of systemic inflammation with intraventricular haemorrhage and white matter disease was detected which has been later confirmed by other studies (24, 25, 78, 79, 121).

The susceptibility of brain tissue to perinatal injury is considered to be an increased susceptibility to hypoxic-ischemic injury after exposure to pro-in-

inflammatory cytokines (33, 45, 47). This hypothesis is based on animal experiments in which a cerebral inflammation was induced with subsequent hypoxic injury (32, 34, 46). Animals previously exposed to inflammation had more cerebral lesions than control animals (44, 73). Clinical studies confirmed this association (25). The individual contribution of these factors associated with poor perinatal outcome have not yet been delineated (29, 90).

Summary

Clinical and experimental data suggest that the antenatal exposure to inflammation is a double-edged sword for the fetus. On the one hand, the antenatal inflammation induces changes that improve the postnatal adaptation and increase survival. On the other hand, the later development of organs, namely the lung, are impaired. New therapeutic options should exploit the beneficial effects induced by antenatal inflammation and limit the damage.

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CHAPTER 2

Aim of the Thesis and Animal Model of
Chorioamnionitis

Although the effects of antenatal infection on prematurity have been explored in many animal experiments, the primary outcomes of interest have usually been the pathogenesis of premature labor and adverse effects on the fetal brain (6). The first report of an antenatal inflammatory effect other than pneumonia on the fetal lung was the observation that intra-amniotic injection of the pro-inflammatory cytokine IL-1 α to pregnant rabbits improved lung compliance after preterm delivery (4). The mRNA for the surfactant proteins SP-A and SP-B in the fetal lungs and surfactant lipids also increased as indicators of induced lung maturation. This initial experiment, together with clinical observations linking chorioamnionitis to fetal lung development (1), prompted to explore how inflammation can alter lung development.

Lung development in human and sheep

The embryonic development of the lung is a well defined sequence of proliferation and differentiation which have been histologically characterized (figure 1) (13). The tracheal bud which forms during the fourth week soon divides into the primary bronchial buds (embryonal phase) (5). Together with the surrounding mesenchyme, the bronchial buds differentiate into the main bronchi and lungs. The main bronchi subdivide into secondary bronchi that form lobar, segmental, and intrasegmental bronchi. By 18 weeks in the human fetus, about 17 orders of airway branches have formed. The developing lung resembles histologically an exocrine gland during the pseudoglandular phase from 6 to 16 weeks. By 16 weeks all major elements of the lung have formed except those involved in gas exchange. Respiration is therefore not possible. The canalicular phase overlaps in the upper parts of the lung with the pseudoglandular phase since cranial segments of the lung develop faster than caudal ones. Respiration is possible at the end of the canalicular phase since thin-walled terminal saccules have developed at the ends of the respiratory bronchioles. The whole lung is well vascularized, making gas exchange via diffusion possible. Although a fetus born at the end of this phase may survive, the incidence of severe respiratory distress syndrome is extremely high and makes sophisticated neonatal intensive care mandatory. During the saccular phase from 26 weeks of gestation till close to term more terminal saccules develop with an increase in the gas exchange surface. Alveoli are not formed until 32 weeks of gestation. Capillaries begin to bulge around these new alveoli

and some 4 million orders of airway branches have developed. The intimate contact between epithelial and endothelial cells establishes the blood-air barrier, which permits adequate gas exchange for survival. The alveolar phase starts at approximately 32 weeks and lasts probably until 2 years of age. About 20% of the alveoli form prenatally. The major mechanism for increasing the number of alveoli is the formation of secondary connective tissue septa that subdivide existing alveoli (2, 3). Initially the septa are relatively thick and marked by a concentrated deposition of elastin fibers, but they are soon transformed into mature thin septa that are capable of gas exchange. The exponential growth of alveoli postnatally is opening a window of opportunity for catch-up growth in infants with BPD.

The timing of lung development during gestation and after birth varies from species to species. Rodents for example do not form alveoli until postnatally (13). Among all species sheep and monkeys are closest to the human situation. Much of the pioneering research in neonatal medicine has been done in sheep (figure 2).

Intra-Amniotic Endotoxin as a Model of Chorioamnionitis

Endotoxin (lipopolysaccharide from gram negative organisms) is used widely as a potent pro-inflammatory agonist to study shock and inflammatory responses. Endotoxin initiates inflammation in many cell types by signaling through toll-like receptor 4 (TLR4) to activate nuclear factor (NF)-kappa B and to initiate the inflammatory cascade. Empirically, endotoxin can induce chorioamnionitis and inflammatory responses in human pregnancies and preterm infants and TLR4 is expressed in the human chorioamnion (8). Using ultrasound guidance, intra-amniotic injections of *E. coli* endotoxin in sheep induce chorioamnionitis indicated by increased inflammatory cells in amniotic fluid, histologic inflammation of the chorioamnion, and increased IL-1 β , IL-6 and IL-8 mRNA in cells from amniotic fluid (7).

All animals were of the same gestational age at delivery and evaluation which allows the comparison of control animals and animals that were exposed to chorioamnionitis. As an example the study design of the first three papers in Chapter 3, 4 and 5 is shown in figure 4.

Figure 1: Schematic development of the lung in human. The different phases are depicted over gestational age in the upper part of the figure. Lung structures are depicted on the lower part with the number of airway generations listed beneath and the predominantly formed structures during the individual phase of lung development (modified from www.embryology.ch and 5).

Figure 2: Schematic development of the lung in human and sheep. The correlation of individual histologic lung development phases to gestation are very similar in human and sheep. Modified according to (13).

Figure 3: Study design (9, 10, 11). All fetuses were analyzed after operative delivery (c-section) at the same gestational age of 125 days. Term gestation in the sheep is 150 days. Endotoxin-induced chorioamnionitis was induced by intra-amniotic (IA) injection at 5 h, 24 h, 72 h and 7 days before delivery.

Aim and outline of the thesis

The aim of this thesis was to explore chorioamnionitis in an animal model suitable for the study of lung development with respect to human development. In **chapter 3** the minimal dose of endotoxin inducing lung maturation was determined. In addition, the time course of events induced in the chorioamnion and in the fetal lung and airways was studied. The results of **chapter 3** suggested that chorioamnionitis caused by intra-amniotic injection of endotoxin induced a cascade of lung injury, pulmonary inflammation and remodelling in the fetal lung, similar to lung injury previously described in adult animal models. This hypothesis was tested and confirmed in **chapter 4**. The structural changes in the fetal lung after chorioamnionitis show little to no fibrosis. Cytokine networks and regulators were identified in **chapter 5** that may explain the absence of fibrosis after intra-uterine inflammation induced alveolar simplification similar to BPD.

The role of fetal immune regulation in the chronicity or resolution of pulmonary inflammation was investigated in **chapter 7**. Postnatal immune modulation by addition of surfactant protein (SP)-A to standard surfactant preparations was tested in ventilated preterm lambs (**chapter 6**). In **chapter 8** we tested the hypothesis that the ventilated preterm lung may be both the source and the target of systemic inflammation. The immaturity of the preterm lung allowed

translocation of endotoxin from the lung into the systemic circulation during ventilation. The function of the monocytes in the developing lung were assessed since they initiate and terminate the inflammatory reaction. In **chapter 9** we hypothesized that both pro- and anti-inflammatory functions of monocytes from preterm lambs are impaired. The immaturity of blood-derived monocytes was tested at two different gestational ages. Tests of how the function of monocytes were modulated were explored in **chapter 10** where blood derived monocytes after previous maternal corticosteroid therapy were compared to untreated monocytes of the same gestational age. The effects of repetitive endotoxin injections into the amniotic fluid were tested in **chapter 11** which resulted in a phenotype of endotoxin tolerance.

The thesis comprises a line of research in an animal model of chorioamnionitis to understand the sites of injury, the time course of cellular responses and the effects on organ and cellular function after intra-uterine exposure to inflammation with respect to the fetal lung and systemic monocytes. The mechanisms of antenatal inflammation in the fetal lung were analysed to identify mediators of inflammation-associated adverse lung development and immune function which may result in potential new targets for therapy.

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CHAPTER 3

Dose and time response after intraamniotic endotoxin in preterm lambs

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Effect and Time Response after Intraamniotic Endotoxin in Preterm Lambs

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Intraamniotic endotoxin causes chorioamnionitis, which is followed by improved fetal lung function after 4 d in fetal sheep. We administered 0.1 mg, 1 mg, 4 mg, and 10 mg endotoxin for inflammatory maturation effects after 7 d. Four and 10 mg endotoxin enhanced alveolar lung maturation and inflammation in the lung and pleural space. The number of neutrophils in cord blood and the number of macrophages in alveolar lavage and fetal lung tissue increased in a dose-dependent manner. Lower endotoxin doses are indicators of chorioamnionitis, lung and systemic inflammation without inducing lung maturation. Therefore, some degree of inflammation can occur without subsequent lung maturation. The airway changes caused by 4 mg endotoxin were assessed at 5 h, 72 h, and 7 d to discern local versus systemic inflammatory responses to intraamniotic endotoxin. At 5 h active inflammation in the airways producing hydrogen peroxide, and interleukin-8 were increased in the cord blood indicating both systemic responses. Cells recruited into the amniotic cavity produced proinflammatory cytokine mRNA for 7 d with no change in mRNA in chorioamnion, lung, or spleen after 72 h. The amniotic fluid may be a source of prolonged fetal exposure to inflammatory cytokines.

Keywords: respiratory distress syndrome; bronchopulmonary dysplasia; leukocyte recruitment; surfactant; cytokines

Exposure to inflammation/infection has been associated with numerous adverse outcomes that include preterm delivery, increased risks of bronchopulmonary dysplasia (BPD), cerebral palsy, and neonatal sepsis (1–3). How biological chorioamnionitis and fetal colonization with organisms such as *Ureaplasma urealyticum* also have been associated with a decreased incidence of respiratory distress syndrome (RDS) (4, 5), and in one epidemiological study chorioamnionitis predicted increased survival of infants born at gestational age < 26 wk (6). There is very little information about how the fetus responds to intraamniotic infection/inflammation. We used intraamniotic injections of endotoxin to characterize chorioamnionitis and fetal lung responses to an inflammatory stimulus (7–10). Using a dose of 20 mg endotoxin,

we found that inflammatory cells were recruited to the chorioamnion and the chorioamnion produced interleukin (IL)-1 β , IL-6, and IL-8 mRNA within 5 h (10). The expression of the mRNAs for the proinflammatory cytokines returned to control values within 2 d but inflammatory cells were increased in the chorioamnion for 25 d after the endotoxin exposure (10). The lung tissue expressed the same cytokine mRNAs within 5 h of the intraamniotic endotoxin injection but expression was higher at later time points and inflammatory cells also persisted in the lungs (10). The mRNAs for the surfactant proteins increased within 24 h of endotoxin exposure and remained increased for 15 d, although lung mechanics did not improve until 4 to 7 d after the endotoxin exposure (9). We found a minimal systemic response to the intraamniotic endotoxin. The cord blood granulocytes initially decreased and then increased moderately (10), fetal cortisol levels did not increase (11), and there was no increased cytokine expression in liver, gut, or placental tissue (10). In this study we asked whether systemic or local inflammatory changes are required for subsequent lung maturation by evaluating various endotoxin doses ranging from 0.1 mg to 10 mg, and by evaluating both the systemic and local inflammatory changes at different times after intraamniotic endotoxin injection.

METHODS

Animals, Endotoxin, Delivery, and Postnatal Assessment

The animal component of the studies was performed in Western Australia, as approved by the animal care and use committees from Children's Hospital Medical Center, Cincinnati, OH, the Ethics Committee of the Western Australian Department of Agriculture, and the University of Western Australia. Date bred Merino ewes with a singleton fetus were randomized for the two protocols (Table 1). To evaluate the response to endotoxin dose, animals were randomized to saline, 0.1, 1, 4, or 10 mg *Escherichia coli* 055:B5 endotoxin (Sigma, St. Louis, MO), given 7 d before preterm delivery at 125 d gestation. To evaluate the inflammatory responses by time interval from endotoxin exposure other animals were randomized to receive 4 mg endotoxin at 5 h, 24 h, or 72 h before delivery. The endotoxin was solubilized in saline and filtered. The intraamniotic injection was given into the amniotic cavity with ultrasound guidance (8, 11). Preterm lambs were delivered at 125 d gestational age by cesarean section (12). For evaluating lung function 7 d after endotoxin exposure, the preterm lambs were ventilated for 40 min with time-cycled, pressure-limited infant ventilators (11). Peak inspiratory pressures were limited to 40 cm H₂O. A pressure transducer (model 8507C-2; Endevco, San Juan Capistrano, CA) and pneumotachograph (model 35-597; Hans Rudolph, Kansas City, MO) were placed between the tracheostomy tube and the ventilator to measure tracheal pressure and flow, respectively. Volume was obtained by integrating flow (12).

Measurements on Lungs, Spleen, Plasma, and Cells from Amniotic Fluid and Alveolar Lavage Fluid

Lungs were removed and processed as described (13). The deflation limb of the pressure–volume curve was measured. Saturated phosphatidylcholine (Sat PC) (14, 15), total protein (13, 16), and surfactant proteins (Western blot) were measured (9). The mRNA for the surfactant proteins and for selected cytokines were quantified using sheep-

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TABLE 1. ENDOTOXIN DOSE AND TIME INTERVAL

Dose	Number of Animals	Birth Weight (kg)	Cord Blood pH
Endotoxin dose given 7 d before delivery			
Saline	7	2.47 ± 0.09	7.29 ± 0.02
0.1 mg	7	2.55 ± 0.07	7.33 ± 0.01
1 mg	7	2.76 ± 0.10	7.28 ± 0.01
4 mg	7	2.62 ± 0.11	7.30 ± 0.02
10 mg	7	2.73 ± 0.07	7.27 ± 0.01
Time interval after 4 mg endotoxin			
5 h	5	2.53 ± 0.08	7.28 ± 0.07
24 h	5	2.60 ± 0.06	7.31 ± 0.05
72 h	5	2.55 ± 0.06	7.29 ± 0.03
7 d	7	2.73 ± 0.07	7.27 ± 0.01

specific probes (9, 10, 17–19). To reduce the high viscosity, amniotic fluid was incubated at 37° C with *N*-acetyl-L-cysteine, neuraminidase, and hyaluronidase. Cells from amniotic fluid and alveolar lavage fluid were isolated by centrifugation.

The right upper lobe was inflation fixed at 30 cm H₂O. After hematoxylin and eosin staining the tissue was assessed for inflammatory changes (8). Hydrogen peroxide was measured in cells recovered from alveolar lavage fluid of the left lung. Flow cytometry was used to measure CD11b (αM subunit of integrin CR3) and CD44 (proteoglycan link protein) and the percentage of apoptotic cells was determined by annexin V and propidium iodide staining (20). Enzyme-linked immunosorbent assays (ELISA) were developed for IL-6 and IL-8 with antibodies from Chemicon (Temecula, CA) and recombinant proteins as standards from CSIRO (Parkville, Australia). Interferon-gamma (IFN-γ) was measured by ELISA (CSL, Parkville, Australia; recombinant protein was provided by Dr. S. L. Jones, CSL).

Data Analysis

Results are given as means ± SEM. Inflammatory scores are given as medians with interquartile, 5th and 95th percentile ranges. Comparisons between endotoxin-treated and untreated lambs were by analysis of variance with Student–Newman–Keuls tests used for post-hoc analyses. Tissue scores were compared using the Mann–Whitney nonparametric tests. Significance was accepted at *p* < 0.05.

RESULTS

Inflammation and Lung Maturation

There were no differences between the groups in body weights or pH in cord blood (Table 1). Lung function was improved

after intraamniotic injection of 4 mg and 10 mg endotoxin given 7 d prior to delivery (Figure 1). Lung gas volumes measured by deflation pressure–volume curves were increased for the 4 and 10 mg dose groups. At 40 cm H₂O pressure, volumes were increased from a mean value of 11 ml/kg in control animals to 32–38 ml/kg after endotoxin exposure of 4 mg and 10 mg (Figure 1A). The compliances also were about 2-fold higher after 4 mg or 10 mg endotoxin than in control lambs (Figure 1B). The alveolar surfactant pool size was significantly increased from 0.5 μmol/kg (control) to values over 4 μmol/kg after 4 mg and 10 mg endotoxin (Figure 1C). The mRNAs for surfactant proteins A and C remained elevated 7 d after injection of 4 mg and 10 mg endotoxin (Figure 1D). The dose of 1 mg endotoxin significantly increased SP-C mRNA but not other surfactant proteins.

Indicators of Inflammation with Endotoxin Doses

The inflammatory scores in the fetal lung were increased 7 d after the intraamniotic injection of 1, 4, or 10 mg endotoxin in a dose-dependent manner (Figure 2A). The number of inflammatory cells recovered from the alveolar lavage also increased with dose (Figure 2B). The number of granulocytes and monocytes increased significantly after the 0.1 mg and higher doses of endotoxin. Therefore, inflammatory scores and inflammatory cells in alveolar lavages increased for doses of endotoxin that did not induce lung maturation. The increases were dose dependent. After 1, 4, and 10 mg endotoxin there was an increase in inflammatory cells in chorioamniotic membranes (Figure 3A). In all endotoxin-treated animals there were increased numbers of monocytes and lymphocytes in the amniotic fluid (Figure 3B). After doses of 4 mg and 10 mg there was an influx of neutrophils. The absolute number of cells increased in a dose-dependent fashion.

The white blood cells increased in cord blood 7 d after the 4 mg and 10 mg endotoxin doses (Figure 4A). The neutrophils were the cell type that accounted for most of the increase in cord blood white cells, and the neutrophils were also increased in the animals treated with a dose of 1 mg after 7 d (Figure 4B). The increases in white blood cells and neutrophils were dose dependent. The number of platelets increased in all groups after the 7 day exposure interval, including in the animals that received 0.1 mg endotoxin (Figure 4C).

Figure 1. Lung maturation and function. (A) Pressure–volume curves. After 40 min of ventilation the deflation limbs of the pressure volume curves were improved after 4 and 10 mg endotoxin. (B) Compliance after 40 min ventilation. Compliance increased after 4 mg and 10 mg endotoxin by about 2-fold. (C) Sat PC in alveolar lavage fluid increased after 4 mg and 10 mg endotoxin. (D) mRNAs for the surfactant proteins SP-A, B, and C. The mRNAs were increased 7 d after instillation of 10 mg endotoxin, and SP-A and SP-C mRNAs were increased after 4 mg endotoxin, SP-C mRNA was increased after 1 mg endotoxin (**p* < 0.05 versus control).

Figure 2. Inflammation in the fetal lungs. (A) Inflammatory scores in the fetal lungs (airspace + tissue). Plots show median, 25th, and 75th percentile ranges as boxes and 5th to 95th percentile ranges as error bars. The numbers of inflammatory cells were ranked from few inflammatory cells (score = 1), moderate cell infiltration (score = 2), to extensive influx of cells (score = 3). Endotoxin doses of 1 mg, 4 mg, and 10 mg increased the inflammation scores. (B) Number of cells recovered from alveolar lavage. All doses of endotoxin increased monocytes and neutrophils in alveolar lavage. The number of lymphocytes was increased after doses of 4 mg and 10 mg (* $p < 0.05$ versus control).

Time Interval for Inflammatory Response

For the animals studied at different time intervals after 4 mg endotoxin the number of total white blood cells in cord blood decreased at 24 h because the lymphocytes decreased and subsequently increased at 7 d due to increased granulocytes (Figure 4D and 4E). The increase in platelets occurred only 7 d after the 4 mg dose of endotoxin (Figure 4F). The increases in cord blood white cells and platelets were late events after endotoxin exposure.

In contrast, inflammatory cytokine mRNAs for IL-1 β , IL-6, IL-8, and tumor necrosis factor- α (TNF- α) increased in the chorioamnion within 5 h after intraamniotic endotoxin (Figure 5A). The 3- to 7-fold increases were the highest at 5 h. The increase in expression of TNF- α mRNA was not large at any time, and the expression of IL-10 mRNA in chorioamnion did not change at any time. An increase in inflammatory cells in the amniotic fluid occurred by 5 h after the intraamniotic endotoxin and was highest at 72 h (Figure 5C). The cells recovered from the amniotic fluid had a different time pattern of cytokine mRNA expression than did the chorioamnion (Figure 5B). The expression of IL-1 β mRNA increased at 24 h and remained elevated at 7 d, IL-8 mRNA was increased at 72 h and 7 d, and IL-6 mRNA did not increase.

Inflammatory cells were recruited into the alveolar lavage by 5 h, and peaked at 72 h (Figure 6A). IL-1 β , IL-8, and TNF- α mRNAs were increased in lung tissue only 24 h after endotoxin exposure (Figure 6B). The mRNAs for IL-1 β and IL-8 were increased in the alveolar cells recruited to the airways at 24 h and 72 h (Figure 6C). Surfactant protein mRNAs for SP-D was increased 5 h after 4 mg endotoxin and at all later time

Figure 3. Inflammatory cells in fetal membranes and amniotic fluid. (A) Plots show median, 25th, and 75th percentile ranges as boxes and 5th to 95th percentile ranges as error bars. Membranes of control animals had few inflammatory cells and animals treated with 1 mg or higher endotoxin doses had an extensive influx of cells (score = 3). Most of the inflammatory cells were neutrophils. (B) Number and type of inflammatory cells in amniotic fluid. The number of inflammatory cells increased after all doses of endotoxin (* $p < 0.05$ versus control).

points (Figure 6D). SP-A, B, and C mRNA were increased 24 h after endotoxin exposure. Activation of cells recruited to the airways was assessed by measuring the expression of the adhesion molecules (CD11b and CD44) (Figure 7, A and B). The percentage of positive cells increased at 5 h and was highest at 72 h for both cell surface markers. The numbers of apoptotic and necrotic cells per body weight in the alveolar lavage also were the highest at 72 h after endotoxin (Figure 7, C and D). The cells recruited to the airways at 5 h were highly active based on the amount of hydrogen peroxide per cell (Figure 7E). However, relative to the number of cells recruited to the airways, the total amount of hydrogen peroxide normalized to body weight was highest at 24 h (Figure 7F).

Septemic Inflammation

Several variables were measured to evaluate the systemic inflammation. In cord blood plasma IFN- γ was increased 12-fold at 24 h (Figure 8A). IL-6 was increased significantly at 5 h (Figure 8A). IL-8 was increased from 5 h to 7 d by about 3-fold, and IL-8 was the only cytokine that was elevated at all time points measured. The expression of TNF- α mRNA increased 2-fold at 24 h in spleen tissue (Figure 8B). However, the overall cytokine expression pattern indicates minimal cytokine production by the spleen.

DISCUSSION

We have shown that there was no lung maturation—as indicated by increased lung gas volume, improved compliance, and increased surfactant Sat PC and increased SP mRNAs—without preceding inflammation in chorioamnion and fetal lung using various doses of endotoxin. On the other hand, mild inflammation was not followed by lung maturation. The lower doses of 0.1 mg and 1 mg endotoxin induced an influx of inflammatory cells into the chorioamnion, the amniotic fluid, lung

Kramer, Moss, Willet, *et al.*: Endotoxin and Lung Maturation in Lambs

Figure 4. Effect of intraamniotic endotoxin on white blood cells and platelets in cord blood. (A) Doses of 4 mg and 10 mg endotoxin caused an increase in white blood cells after 7 d. (B) The number of circulating neutrophils increased in a dose-dependent manner after 1 mg and higher doses of endotoxin. (C) The number of platelets increased in every group of animals treated with endotoxin. (D) The number of white blood cells decreased after 24 h and increased at 7 d. (E) The circulating neutrophils increased at 7 d. (F) The number of platelets was elevated only 7 d after endotoxin (* $p < 0.05$ versus control).

tissue, and alveolar lavages that persisted 7 d after endotoxin exposure but lung function was not improved. The increases in white blood cells and inflammatory cells in fetal lung and alveolar lavage were dose dependent. Even the lowest dose of endotoxin induced an increase in cells in amniotic fluid and in platelets indicating a subtle systemic response to the inflammatory stimulus given into the amniotic fluid.

The inflammatory response in the fetal lungs and cord blood increased with endotoxin dose, suggesting that a threshold amount of inflammation was needed to induce the improved lung function. A distinction between local and systemic inflammatory response could not be made after 7 d. We previously found endotoxin doses of 1, 4, 20, or 100 mg caused equivalent maturational exposure without increases in fetal cortisol (11). The variability of response to 1 mg endotoxin may be attributed to the variation in endotoxin preparations. In the fetal sheep model, a dose of 1 or 4 mg given by intraamniotic injection is the minimal stimulus needed to enhance lung function after preterm delivery. There was a clear dose-response relationship between inflammation and the amount of endotoxin. However there were no clearly graded or partial lung maturation responses.

In a previous study we found that 20 mg endotoxin given by intraamniotic injection increased IL-1 β , IL-6, and IL-8 mRNA in the chorioamnion at 5 h with a parallel response in the lungs beginning at 5 h that increased for about 2 d (10). For this study we used the minimal effective dose of 4 mg endotoxin for inducing lung maturation and addressed the question whether

Figure 5. Cytokine mRNA in fetal membranes and cells from amniotic fluid. (A) IL-1 β , IL-6, IL-8, and TNF- α mRNAs were increased chorioamnion at 5 h and 24 h. IL-10 mRNA was not increased at any time. (B) IL-1 β and IL-8 mRNA increased at 24 h, and 72 h and cells from amniotic fluid. (C) The number of monocytes, lymphocytes and neutrophils in amniotic fluid increased at 5 h and remained increased at all time points (* $p < 0.05$ versus control).

systemic versus local inflammation in the fetus was induced by intraamniotic endotoxin and which compartment was producing proinflammatory cytokines at later time points. mRNAs for IL-1 β , IL-6, IL-8, and TNF- α were most increased at 5 h in the chorioamnion. Inflammatory cells in the amniotic fluid were increased at 5 h, but the 40-fold increase in granulocytes was at 24–72 h, and the up to 35-fold increases in monocytes and IL-8 mRNA occurred over the 24 h to 7 day interval. No increase in IL-6 mRNA. The distinct differences between the chorioamnion and cells in amniotic fluid suggest that inflammation in amniotic fluid was regulated differently from the response of the chorioamnion. The cytokine response continued in amniotic fluid after it was extinguished in the chorioamnion and fetal lungs.

The mRNAs for the proinflammatory cytokines were increased in lung and alveolar lavage by 24 h, but not at 5 h as noted previously with the higher endotoxin dose (10). The first detectable effect of inflammation in the lungs was a large increase in H₂O₂ production by the few cells recruited at 5 h. At the same time the mRNA for SP-D was increased. A large number of cells in alveolar lavages at 72 h expressed surface adhesion molecules CD11b and CD44, which may indicate cell recruitment to sites of inflammation. Although the surface antigens indicate that many cells evaluated at 72 h were quickly recruited to the airways, they were no longer producing H₂O₂. We used the measurements of annexin V and propidium iodide to evaluate the percentage of apoptotic and necrotic cells in the cell population. Because total cell numbers increased, we calculated the number of necrotic and apoptotic cells per kilogram body weight,

Figure 6. Cells in alveolar lavage, cytokine mRNAs in cells from alveolar lavage and lung, and surfactant protein mRNAs in lung tissues. (A) The number of monocytes and neutrophils in alveolar lavage increased after 5 h and remained increased to 7 d. (B) At 24 h IL-1 β , IL-8, and TNF- α mRNA were elevated in mRNA from lung tissue. (C) The IL-1 β and IL-8 mRNA from cells recovered from alveolar lavage increased at 24 h and 72 h. (D) Time course of induction of surfactant protein mRNAs increased after 4 mg endotoxin. The SP-D mRNA was induced after 5 h, the mRNAs of all were increased after 24 h (* $p < 0.05$ versus control).

should best reflect the timing of resolution of inflammation in the lungs. The high numbers of apoptotic and necrotic cells at 72 h indicated resolution of inflammation at 72 h (21). However, complete resolution had not occurred by 7 d, and we found residual inflammatory cells in alveolar lavage as long as 25 d after intraamniotic endotoxin (10).

None of the indicators of inflammation that were detected at 7 days correlated very well with the improved lung function

after preterm delivery. Early indicators such as H₂O₂ and cytokine production in the chorioamnion and the lung cannot be evaluated because those indicators have been extinguished before the physiological maturation response occurs. In mature lungs, endotoxin and proinflammatory cytokines initially suppress the mRNAs for surfactant proteins and the surfactant proteins can rebound to higher levels than normal with healing (17, 22). Although detectable, the mRNAs for SP-A and SP-B are very low at 125 d gestation in the fetal sheep lung, and no decreases are seen after intraamniotic endotoxin. SP-C mRNA should be the most sensitive indicator because it is relatively high at 125 d gestation, and it is unchanged 5 h

Figure 7. Markers of activation of cells from alveolar lavage. (A) Percent of CD11b (α M subunit of integrin CR3) positive cells. The number of CD11b positive cells increased from 5 h to 72 h. (B) The percentage of CD44 (proteoglycan link protein) positive cells also increased from 5 h to 72 h. (C, D) The number of apoptotic cells in the alveolar lavage was determined by annexin V staining and counterstaining with propidium iodide to detect necrotic cells. The numbers of apoptotic and necrotic cells were highest at 72 h. (E) The production of hydrogen peroxide per 10⁶ cells was maximal after 5 h. (F) The production of hydrogen peroxide per kilogram body weight was highest at 24 h when the total cell number in the alveolar lavage was also most elevated (* $p < 0.05$ versus control).

Figure 8. Systemic inflammation. (A) Interferon gamma (IFN- γ) was increased in cord plasma at 24 h. Interleukin-6 was increased at 5 h and interleukin-8 was increased from 5 h to 7 d. (B) The amount of IL-1 β , IL-6, IL-8, and IL-10 mRNA in the spleen did not increase. The amount of TNF- α increased by 2-fold at 24 h only (* $p < 0.05$ versus control).

and increased 24 h after intraamniotic endotoxin. Inflammatory cells producing H_2O_2 and cytokines were accumulating in the lungs over 3 d, providing a source for direct lung injury. The wet-to-dry weights did not change from control values nor did the total protein in the alveolar lavages (data not shown). However, the antioxidant enzymes catalase, glutathione peroxidase, and superoxide dismutase are induced after endotoxin exposure, indicating activation of oxidant-mediated pathways in the fetal lung (23). SP-A and SP-D may function as antioxidants (24). SP-D mRNA was increased within 5 h. A hypothesis for the events that result in lung maturation after intraamniotic endotoxin is that neutrophils producing oxidants cause a primary injury and that the fetal lung responds with lung maturation as a protective response.

We previously found that 20 mg intraamniotic endotoxin did not increase proinflammatory cytokine mRNA in the placenta, fetal liver, or fetal gut. We used the lower dose of 4 mg endotoxin to search for other indicators of a systemic inflammatory response. The TNF- α mRNA increased by about 2-fold at 24 h only in the spleen, with no increase in IL-1 β , IL-6, or IL-8, indicating a very minimal response from the fetal spleen. In preterm lambs that were treated with intratracheal endotoxin the spleen produced high amounts of proinflammatory cytokines within 6 h demonstrating that the preterm spleen can respond to a proinflammatory stimulus (25). Nevertheless, IL-6 and IL-8 were increased in cord plasma over the extended interval from 24 h to 7 d. Interferon- γ also was increased in plasma 12-fold at 24 h. Using IL-8 as a representative proinflammatory cytokine, we found no increases in mRNA at 72 h or 7 d in chorioamnion, lung, or spleen. However, the mRNA for IL-8 was elevated in cells in amniotic fluid at 72 h and 7 d, suggesting that the amniotic fluid is the source of the increased cytokine in the cord plasma. As with the other components of the inflammatory response, we cannot specifically link the increased plasma cytokines at 5 h and 24 h to the lung maturation response.

The interpretation of this *in vivo* model relative to clinical settings is complex. The preterm human fetus at risk of delivering before 30 wk gestational age may be continuously exposed to live organisms, their products, and inflammatory mediators and the exposure may be prolonged (1). The kinetics and dynamics of chorioamnionitis caused by live organisms are probably quite different from a single dose of endotoxin from gram-negative bacteria. The most common microorganism associated with chorioamnionitis is *Ureaplasma urealyticum*, which does not have a cell wall but induces IL-1 β , IL-6, and TNF- α in amniotic fluid (26). In the clinical literature cord plasma elevations of IL-6 have been used to diagnose systemic inflammation in the fetus (27, 28). We found that there were subtle indications of systemic inflammation after intraamniotic injection of endotoxin. The increase of IL-6 in cord plasma within 5 h after intraamniotic endotoxin showed that the systemic inflammation was present at the same time as the local inflammation in chorioamnion and fetal lung. However, the animals were not injured in any way that was evident by the endotoxin exposure (8, 9, 11). The antiinflammatory cytokine protein IL-10 was not detectable but sustained increases in IL-8 were in airway samples from infants that developed BPD (29). We found no significant induction of IL-10 mRNA in membranes, in lung, or in spleen. IL-8 was increased in plasma at all time points after intraamniotic endotoxin in the fetal sheep. Increased cytokine proteins in cord blood were reported in clinical studies to be associated with subsequent BPD or cerebral palsy (3, 28) and interferons can cause cerebral palsy when given to infants (30). The pattern of cytokine responses in the fetal sheep—elevated IL-8 and IFN- γ with no induction of the antiinflamma-

tory cytokine IL-10—parallels the clinical observations that have been associated with poor lung and neurodevelopmental outcomes (2, 31). In clinical studies oxidative injury has been implicated in the development of BPD after preterm birth (3). In our animal model the response of the fetal lung to intraamniotic endotoxin may be different because the oxidant stress is of short duration.

In summary, we found that intraamniotic doses of 0.1 mg and 1 mg endotoxin induced inflammatory cell recruitment to the chorioamnion and the lungs. However, this inflammation was not accompanied by physiological improvements in lung function. Higher doses of endotoxin sufficient to induce lung maturation were accompanied by systemic inflammation in the fetus within 5 h. Activated cells in the airways producing hydrogen peroxide within 5 h might cause an initial lung injury. Fetal exposure to sufficient inflammation can therefore induce lung maturation, but the precise link between the components and the intensity of the inflammatory response and lung maturation remains elusive.

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CHAPTER 4

Injury, inflammation and remodeling in fetal sheep lung after intraamniotic endotoxin

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Injury, inflammation, and remodeling in fetal sheep lung after intra-amniotic endotoxin

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Kramer, Boris W., Susanne Kramer, Machiko Ikegami, and Alan H. Jobe. Injury, inflammation, and remodeling in fetal sheep lung after intra-amniotic endotoxin. *Am J Physiol Lung Cell Mol Physiol* 283: L452–L459, 2002. First published March 22, 2002; 10.1152/ajplung.00407.2001.—Chorioamnionitis is frequent in preterm labor and increases the risk of bronchopulmonary dysplasia. We hypothesized that intra-amniotic endotoxin injures the lung in utero, causing a sequence of inflammation and tissue injury similar to that which occurs in the injured adult lung. Preterm lamb lungs at 125 days gestational age were evaluated for indicators of inflammation, injury, and repair 5 h, 24 h, 72 h, and 7 days after 4 mg of intra-amniotic endotoxin injection. At 5 h, the epithelial cells in large airways expressed heat shock protein 70, and alveolar interleukin-8 was increased. Surfactant protein B (SP-B) decreased in alveolar type II cells at 5 h, and SP-B in lung tissue and alveolar lavage fluid increased by 72 h. By 24 h, neutrophils were recruited into the large airways, and cell death was the highest. Alveolar type II cells decreased by 25% at 24 h, and proliferation was highest at 72 h, consistent with tissue remodeling. Intra-amniotic endotoxin caused surfactant secretion, inflammation, cell death, and remodeling as indications of lung injury. The recovery phase was accompanied by maturational changes in the fetal lung.

surfactant; lung maturation; alveolar type II cells; respiratory distress syndrome; chorioamnionitis

CHORIOAMNIONITIS is frequently associated with preterm birth, with indicators of systemic and pulmonary inflammation in the newborn at birth and with increased risks of bronchopulmonary dysplasia and brain injury (cerebral palsy) (9, 17, 20). In contrast, histological chorioamnionitis is also associated with a decreased risk of respiratory distress syndrome and may predict increased survival of preterm infants (6, 25). With the use of a lamb model to study fetal lung responses to chorioamnionitis induced by intra-amniotic endotoxin, we found that the lung responded by recruiting proinflammatory cells that produced hydrogen peroxide (15, 16). Proinflammatory cytokine mRNAs were expressed in the lung 5 and 24 h after intra-amniotic endotoxin. Subsequently, there was a striking maturational effect characterized by improved lung mechanics and gas exchange and large increases in surfactant pool sizes

within 7 days of the intra-amniotic endotoxin (1, 13, 14). The maturational effects on the fetal lung were accompanied by an arrest in alveolarization (26). Inflammatory cells that were not producing hydrogen peroxide or cytokine mRNA persisted in the lungs for several weeks, indicating ineffective clearing of the inflammatory process (15, 16). There was a minimal systemic response to the intra-amniotic endotoxin with no increase in fetal cortisol levels or in cytokine expression in liver, gut, or placental tissue (13, 15). Low doses of intra-amniotic endotoxin that did not induce a maturational response also did not increase indicators of inflammation in the fetal lung (16). We hypothesize that intra-amniotic endotoxin initially induces a lung injury response in fetal sheep lung. We evaluated injury responses to endotoxin characteristic of lung injury in the adult. We assessed induction of heat shock protein 70 (HSP70) as an indicator of oxidant injury (19, 28), interleukin (IL)-8 as a mediator of neutrophil recruitment (17), and the loss of surfactant protein B (SP-B) from alveolar type II cells as an indicator of injury to alveolar type II cells (29). Apoptosis, proliferation, and alveolar type II cell numbers were evaluated as indicators of remodeling of the fetal lung.

MATERIALS AND METHODS

Animals, study design, and endotoxin. The animal studies were performed in Western Australia, as approved by the Animal Care and Use Committees from the Cincinnati Children's Hospital and the Western Australian Department of Agriculture. Date-bred Merino ewes were randomized to receive 4 mg of endotoxin at 5 h, 24 h, 72 h, or 7 days before delivery at 125 days gestational age (Fig. 1). The 4-mg dose was the lowest dose that gave consistent maturational responses (16). Endotoxin (*Escherichia coli* 055:B5; Sigma Chemical, St. Louis, MO) was solubilized in saline and filtered through a 0.45- μ m filter. Intra-amniotic saline or endotoxin injections were given with 20-gauge spinal needles with ultrasound guidance (13, 14). The inflammatory responses in the chorioamnion, amniotic fluid, and lung tissue were recently reported using samples from the same animals (16).

Delivery and postnatal assessment. Preterm lambs were delivered at 125 days gestational age by cesarean section (7, 12). The thorax was opened, and the top right lobe of the lung was inflated fixed with 10% formalin at 30 cmH₂O pressure.

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Fig. 1. Study design: intra-amniotic injections of 4 mg of endotoxin were given 5 h, 24 h, 72 h, and 7 days before delivery at 125 days (d) gestational age (GA). Intra-amniotic injections are marked with arrows. The numbers of animals/group are given in parentheses.

The left lung was lavaged as previously described (24). Five alveolar lavages of the left lung with 0.9% NaCl at 4°C were pooled, and aliquots were saved for determination of saturated phosphatidylcholine (Sat PC), IL-8, and SP-B (7, 24). A piece of the lavaged left lung was frozen.

Immunohistochemistry and histological analyses. Lung tissue was formalin fixed, embedded in paraffin, and cut in 5- μ m sections. Endogenous peroxidase activity was removed by incubation with hydrogen peroxide. Nonspecific binding sites were blocked with serum. Sections were incubated with a rabbit polyclonal antibody for HSP70 (Santa Cruz Biotechnology, Santa Cruz, CA). After being washed with PBS to remove unbound antibody, the slides were incubated with the secondary biotinylated antibody against rabbit IgG (Vector Laboratories, Burlingame, CA). After adding avidin and horseradish peroxidase (Vector Laboratories), staining of HSP70-positive cells was developed with diaminobenzidine and cobalt with a nuclear fast red counterstain (21).

Fig. 3. Interleukin (IL)-8. IL-8 concentration was measured in alveolar lavage fluid by ELISA and standardized per kilogram of body wt. IL-8 increased at 5 h and at all later time points vs. control. At 7 days, IL-8 increased further (* P < 0.05 vs. control, ** P < 0.05 vs. 72 h).

Alveolar type II cells were stained after antigen retrieval with a mouse monoclonal antibody specific for thyroid transcription factor-1 (TTF-1) as previously described (11, 21, 22). The antibody was kindly provided by Dr. J. A. Whitsett. The SP-B in the alveolar type II cells was evaluated by immunohistochemistry using a rabbit anti-SP-B that reacts with both completely processed and unprocessed forms of SP-B (1). Sections were graded for the number of SP-B-positive cells using a scale from 0 to 4. For each triplicate section from each animal, 12 areas were randomly selected and evaluated. Sections stained with hematoxylin and eosin were evaluated

Fig. 2. Heat shock protein 70 (HSP70) and inflammatory cells. A: HSP70 was expressed at 5 h in the epithelial cells of the fetal airways but not in the parenchyma. B: at 24 h, no HSP70 was detectable. C: hematoxylin and eosin staining of fetal lung after 5 h of endotoxin exposure. D: inflammatory cells in epithelium of large airways at 24 h after intra-amniotic endotoxin are indicated by arrow. The cells were predominantly neutrophils. Magnification $\times 320$.

for inflammatory changes, such as neutrophil influx, in the airways.

IL-8. The potent neutrophil chemoattractant IL-8 was quantified by enzyme-linked immunosorbent assay using antibodies from Chemicon (Temecula, CA) (16). Recombinant protein as a standard was kindly provided by CSIRO (Parkville, Victoria, Australia). The level of detection was 0.5 ng/ml.

Quantification of SP-B and Sat PC. SP-B was measured in alveolar lavage fluid and in lung tissue after alveolar lavage. Lung tissue was homogenized in 10 mM Tris, pH 7.3, and 2 M sucrose (vol/vol 7:1) to which protease inhibitors were added (Protease Cocktail; Roche Molecular Diagnostics, Indianapolis, IN). Homogenates were centrifuged for 10 min at 500 *g* at 4°C, and the supernatant was used for SP-B measurement by Western blots as previously described (1). SDS-PAGE for SP-B was carried out using 10–20% gradient tricine gels (Novex, San Diego, CA). Samples containing 3

nmol of Sat PC were electrophoresed along with an ovine SP-B standard under nonreducing conditions (1). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Schleicher & Schuell, Keene, NH) for immunoblot analysis using a rabbit anti-SP-B that reacts with both completely processed and unprocessed forms of SP-B (1). The PVDF membrane was first blocked with 5% low-fat milk (wt/vol) in Tris-buffered saline, pH 7.4, containing 0.1% (vol/vol) Tween 20. The antibody was diluted and incubated overnight. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Calbiochem, La Jolla, CA) was used as secondary antibody, and enhanced chemiluminescence substrates (Amersham, Arlington Heights, IL) were used to develop the immunoblots. Separate estimates of the amount of fully processed and partially processed SP-B were made by calculation of the relative band densities using Alpha-Imager 2000 documentation and Analysis software (Alpha Innotech, San Leandro, CA). Mature SP-B was calculated per kilogram

Fig. 4. Surfactant protein B (SP-B) in alveolar type II cells and in the lung. **A:** sections were stained with an antibody that reacts with processed and unprocessed SP-B. Representative sections for each time point are shown. Magnification $\times 320$. **B:** immunostaining for SP-B was graded from 0 to 4. Intra-amniotic endotoxin reduced the SP-B content of type II cells within 5 h. At 72 h and at 7 days after intra-amniotic injection of endotoxin, the score for SP-B staining was higher than in controls. **C:** quantification of total (fully processed and partially processed) SP-B in total lung by Western blot expressed relative to a normalized value of 1.0 for the control group. At 72 h and at 7 days, the amount of total SP-B increased (* $P < 0.05$ vs. control).

of body wt in the alveolar lavage fluid and in the lung homogenate. Percent secretion of mature SP-B was calculated by dividing the amount in the alveolar lavage fluid by the sum of mature SP-B in alveolar lavage fluid and lung homogenate.

Sat PC was measured in alveolar lavage and lung tissue after lavage. Sat PC was isolated from chloroform-methanol (2:1) extracts by neutral alumina column chromatography after exposure of lipid extracts to osmium tetroxide (18). Sat PC was quantified by phosphorus assay (3).

Apoptosis and alveolar type II cell quantification. Apoptotic cells were detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling using biotin-dUTP (TACS 2 TdT-DAB kit; Trevigen, Gaithersburg, MD). Alveolar type II cells were identified by staining with a monoclonal antibody against TTF-1 (see *Immunohistochemistry and histological analyses*). The percentages of apoptotic cells and alveolar type II cells were quantified relative to all cells of the lung parenchyma. Intra-amniotic endotoxin injection induced both lung maturation and an arrest of alveolar septation, which reduced the volume of the parenchyma and increased the lung gas volume within 7 days (14, 26). The number of alveolar type II cells was not quantified after intra-amniotic endotoxin exposure. The quantification of alveolar type II cells relative to lung volume will be influenced by changes in the parenchyma to air space volume. Therefore, we quanti-

fied the apoptotic cells and alveolar type II cells relative to all cells by counting nuclei. Five sections for each animal were quantified.

Proliferation. Proliferating cell nuclear antigen (PCNA) is a nonhistone protein found in nuclei in the S, G2, and M phases of cell mitosis (8). Staining for PCNA was performed with a biotinylated monoclonal antibody (Zymed Laboratories, San Francisco, CA) and was developed with diaminobenzidine and cobalt with a nuclear fast red counterstain. The percentage of proliferating cells was quantified relative to all cells of the lung parenchyma.

Data analysis. Results are given as means $\pm$ SE. Comparisons between endotoxin-treated and untreated lambs were by analyses of variance with Student-Newman-Keuls tests used for post hoc analyses. Significance was accepted at $P < 0.05$.

RESULTS

HSP70, inflammatory cells, and IL-8 as indicators of lung injury. HSP70, an indicator of oxidant-stress mediated lung injury, was not expressed in control animals. In endotoxin-treated animals, HSP70 stained intensely in the epithelium of the conducting airways but not in the alveolar or vascular structures at 5 h

Fig. 5. Alveolar type II cells. A: alveolar type II cells were stained for thyroid transcription factor-1. Representative sections are shown for controls and lungs 24 h and 7 days after endotoxin exposure. Magnification $\times 320$. B: number of alveolar type II cells was determined relative to all cells in the lung parenchyma by counting nuclei. The percentage of alveolar type II cells was reduced at 24 h. At other time points, the numbers were not different (* $P < 0.05$ vs. control).

INJURY AFTER INTRA-AMNIOTIC ENDOTOXIN

A). By 24 h, there was no expression of HSP70 in epithelial cells lining the large airways or distal lung parenchyma (Fig. 2B), and no HSP70 expression was detectable at 72 h and at 7 days. At 5 h, there were no inflammatory changes in the large airways or in the distal lung parenchyma (Fig. 2C). By 24 h, the large airways were infiltrated with inflammatory cells, most of which were neutrophils based on appearance at high magnification (Fig. 2D). Neutrophils were also present in the alveoli. The inflammatory cells persisted at 72 h and at 7 days. There were no inflammatory cells in the lungs of control animals. SP-B, a potent neutrophil chemoattractant, was increased approximately threefold in alveolar washes from endotoxin-exposed lambs at 5 h and remained increased 7 days after endotoxin exposure (Fig. 3).

SP-B and alveolar type II cell numbers. Immunohistochemistry demonstrated that SP-B was lost from alveolar type II cells 5 h after intra-amniotic endotoxin exposure (Fig. 4, A and B). This depletion was not present at 24 h, and more SP-B containing type II cells were identified at 72 h and at 7 days. The total amount of SP-B in the alveolar wash and lung tissue decreased qualitatively at 5 h and then increased 4.6-fold by 7 days (Fig. 4C). The ratio of secreted mature SP-B in the airspace to total SP-B increased from 2.0% in controls to $26.1 \pm 5.2\%$ at 5 h ($P < 0.05$). The percent of SP-B in the air spaces increased to

$62.7 \pm 8.5\%$ at 7 days ($P < 0.05$ vs. control and 5 h). The loss of SP-B from the tissue is consistent with the decreased immunostaining for SP-B.

The number of alveolar type II cells identified by TTF-1 staining as a proportion of total cells was not changed 5 h after intra-amniotic endotoxin exposure (Fig. 5). At 24 h, the percent of alveolar type II cells was reduced by ~25%, and at 72 h and at 7 days, the number of type II cells was not different from control animals.

Tissue repair by apoptosis and proliferation. The percent of apoptotic cells and the cell populations undergoing apoptosis changed over time. In controls and at 5 h, few cells were apoptotic (Fig. 6A). At 24 h, the highest percent of apoptotic cells was identified (Fig. 6B). Inflammatory cells in both the air spaces and epithelial cells were apoptotic. By 72 h and 7 days, fewer parenchymal cells were apoptotic, and the apoptotic cells were primarily inflammatory cells.

To assess proliferation, lung sections were stained for PCNA, a nonhistone protein elevated in the S, G2, and M phases of mitosis, and the cells were quantified (Fig. 7). There was no increase in proliferating cells as indicated by PCNA staining 5 or 24 h after intra-amniotic endotoxin exposure. At 72 h and at 7 days, the number of proliferating epithelial cells was increased.

Apoptotic cells. A: at 5 h, few apoptotic cells are detected. B: at 24 h, parenchymal and inflammatory cells in the air spaces are positive. Arrows indicate apoptotic cells. Magnification $\times 640$ in A and B. C: quantification of apoptotic cells per high power field as percent of total cells (* $P < 0.05$ vs. control).

Fig. 7. Cell proliferation in fetal lung. A: lung sections were stained for proliferating cell nuclear antigen (PCNA), a nonhistone protein elevated in the S, G2, and M phases of cell mitosis. Magnification $\times 320$. B: percentage of proliferating cells. At 5 and 24 h after intra-amniotic injection of endotoxin, the number of proliferating cells did not change. At 72 h and at 7 days, more cells were positive for PCNA (* $P < 0.05$ vs. controls).

DISCUSSION

We found that intra-amniotic endotoxin induces injury, inflammation, apoptosis, and remodeling of the fetal lung. A lung injury response was indicated by the expression of HSP70 in the epithelial cells of the large airways and depletion of SP-B from alveolar type II cells at 5 h. The percent of alveolar type II cells decreased at 24 h but was not different from controls at 7 days. Apoptotic cell death was maximal at 24 h, and cell proliferation was maximal at 72 h. We previously reported that intra-amniotic endotoxin caused recruitment of hydrogen peroxide-producing neutrophils to the fetal lungs and proinflammatory cytokine expression by cells recovered by alveolar lavage (15, 16). The identification of HSP70 in the large airway lining cells at 5 h indicates an oxidant injury response of the tissue (19, 28). Although hydrogen peroxide production was maximal at 24 h (16), the HSP70 response occurred at 5 h and was localized to the airways. Cells in the amniotic fluid contained increased IL-8 mRNA within 5 h of endotoxin exposure, and IL-8 protein was elevated in amniotic fluid at 72 h and at 7 days (16). The inflammatory cells recruited to the lungs were activated based on hydrogen peroxide production only at 5 and 24 h, and cell numbers were decreasing by 7 days.

The acute inflammatory responses in the fetal lung resolved quickly, and the remodeling and maturation effects then became evident. However, IL-8 remained elevated in alveolar washes and was increased at 7 days, and inflammatory cells persisted. We previously found that cells in amniotic fluid continued to express proinflammatory cytokines for 7 days after a single dose of intra-amniotic endotoxin, and repeated doses of endotoxin did not cause increased inflammation (13, 16). Therefore, the brief inflammatory response of the fetal lung may result from a downregulation of the response of the fetal lung to the inflammatory stimulus rather than disappearance of the stimulus.

In the fetal lung, the alveolar type II cells are not fully developed, and SP-B is only partially processed (1, 10). In these animals, more cells stained for TTF-1 than for SP-B, indicating that some of the immature type II cells were not making enough SP-B to immunostain. We found that the immature alveolar type II cells that contained SP-B responded to intra-amniotic endotoxin with loss of SP-B. In adult mice, the secretion of SP-B after intratracheal adenovirus instillation was a marker of alveolar type II cell injury (29). Two events related to SP-B occurred after intra-amniotic endotoxin. At 5 h, the lung injury caused a loss of

mature SP-B from alveolar type II cells, followed by a lung maturation response with large increases in SP-B in the tissue and the alveoli at 7 days. Therefore, the initial injury response of the immature alveolar type II cells was followed by a maturation of the alveolar type II cells.

The transient decrease of alveolar type II cells at 24 h coincided with the maximal percent of apoptotic cells. Alveolar type II cells can undergo apoptosis in vivo after injury (2). Because the lung parenchyma-to-air space volume ratio changes after intra-amniotic endotoxin exposure, lung structure and cell numbers also change at each time point. The quantification of alveolar type II cells relative to all cells is independent of the thinning of the parenchyma and should give a reasonable estimate of alveolar type II cell numbers. The general conclusion was that alveolar type II cells did not change much because of a balance between cell loss and proliferation.

We have better characterized the early phase of lung injury that occurs in our model of endotoxin-induced chorioamnionitis. The chorioamnion and the fetal lung are inflamed very soon after exposure to intra-amniotic endotoxin, and the inflammation persists for several days. Elements characteristic of lung injury in adult animals occurred in the preterm lung soon after endotoxin exposure. There was a sequence of injury and inflammatory changes in the fetal lung within hours of endotoxin exposure that was followed by apoptotic cell death and remodeling within days. The injury resolves with thinning of the mesenchyme, increases in surfactant lipids and proteins, and an arrest of septation (14–16, 26). These changes appear physiologically to be maturation because lung mechanics and gas exchange improve (13). However, the resolution of injury resulted in altered alveolar development, also characteristic of oxidant- or ventilation-induced lung injury in the preterm (4, 5, 23). We recently reported that IL-1 given by intra-amniotic injection can also induce the same alterations in lung development that result from endotoxin exposure (27). A question that remains to be resolved is whether injury to the fetal lung invariably triggers alterations in subsequent lung development.

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INJURY AFTER INTRA-AMNIOTIC ENDOTOXIN

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CHAPTER 5

Antenatal inflammation induced TGF-beta1 but suppressed CTGF in preterm lungs

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Antenatal inflammation induced TGF- β 1 but suppressed CTGF in preterm lungs

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Kunzmann, Steffen, Christian P. Speer, Alan H. Jobe, and Boris W. Kramer. Antenatal inflammation induced TGF- β 1 but suppressed CTGF in preterm lungs. *Am J Physiol Lung Cell Mol Physiol* 292: L223–L231, 2007. First published August 26, 2006; doi:10.1152/ajplung.00159.2006.—Chorioamnionitis is frequently associated with preterm birth and increases the risk of adverse outcomes such as bronchopulmonary dysplasia (BPD). Transforming growth factor (TGF)- β 1 is a key regulator of lung development, airway remodeling, lung fibrosis, and regulation of inflammation, and all these processes contribute to the development of BPD. Connective tissue growth factor (CTGF) is a downstream mediator of some of the profibrotic effects of TGF- β 1, vascular remodeling, and angiogenesis. TGF- β 1-induced CTGF expression can be blocked by TNF- α . We asked whether chorioamnionitis-associated antenatal inflammation would regulate TGF- β 1, the TGF- β 1 signaling pathway, and CTGF in preterm lamb lungs. Fetal sheep were exposed to 4 mg of intra-amniotic endotoxin or saline for 5 h, 24 h, 72 h, or 7 days before preterm delivery at 125 days gestation (full term = 150 days). Intra-amniotic endotoxin increased lung TGF- β 1 mRNA and protein expression. Elevated TGF- β 1 levels were associated with TGF- β 1-induced phosphorylation of Smad2. CTGF was selectively expressed in lung endothelial cells in control lungs, and intra-amniotic endotoxin caused CTGF expression to decrease to 30% of control values and TNF- α protein to increase. The antenatal inflammation-induced TGF- β 1 expression and Smad signaling in the fetal lamb lung may contribute to impaired lung alveolarization and reduced lung inflammation. Decreased CTGF expression may inhibit vascular development or remodeling and limit lung fibrosis during remodeling. These effects may contribute to the impaired alveolar and pulmonary vascular development that is the hallmark of the new form of BPD.

chorioamnionitis; bronchopulmonary dysplasia; Smad signaling; fibrosis

LUNG INJURY IN THE MATURE lung often resolves with fibrosis, which is prominent in larger infants who developed bronchopulmonary dysplasia (BPD) after prolonged exposure to oxygen and mechanical ventilation (1, 22, 25, 27). In contrast, the lungs of preterm infants who most frequently develop BPD have less fibrosis but striking inhibition of alveolar septation and microvascular development (14, 22). This altered lung development occurs in preterm ventilated baboons and sheep in association with persistent inflammation.

The alterations in lung development resulting in BPD also have been associated with chorioamnionitis in very low-birth-weight infants (48). Chorioamnionitis results in increased concentrations of proinflammatory cytokines in human amniotic fluid and fetal cord blood, presumably in response to bacterial

products and injury (18, 55). These proinflammatory cytokines may be important mediators in the early inflammatory response that recruits activated inflammatory cells to the fetal lung (12). Inflammation-induced tissue injury is normally followed by a phase of repair (39, 40, 44). The endotoxin-induced chorioamnionitis in preterm lambs initiated a sequence of lung injury, inflammation, apoptosis, and remodeling that resulted in a phenotype of clinical lung maturation with increased surfactant and improved gas exchange, but with microvascular injury and decreased alveolar septation (27, 30, 36). Within 7 days of intra-amniotic endotoxin exposure, the histological changes were similar to those in fetal sheep with mild BPD (52). The fetal lungs did not develop fibrosis, even with prolonged exposure to intra-amniotic endotoxin (30).

Lung injury in adult animals induces transforming growth factor (TGF)- β 1 as part of the tissue repair-and-healing process ("airway remodeling"). TGF- β 1 is present in a latent state, which prevents interaction with its receptor (32). The biological effects of TGF- β 1 may be controlled at the level of conversion of latent TGF- β 1 to active TGF- β 1 (33). If the repair processes are repeatedly triggered and not adequately controlled, fibrosis will ensue (4). TGF- β 1 also contributes to normal lung development. Overexpression of TGF- β 1 during the critical period of lung alveolarization in mice caused morphological changes similar to those in human BPD (16, 50). Downstream of TGF- β 1, connective tissue growth factor (CTGF) and other proteins modulate the cellular response to TGF- β 1 and can prolong wound healing and promote the fibrotic response. CTGF mediates the profibrotic effects of TGF- β 1 by stimulating fibroblast growth and extracellular matrix synthesis (20). In addition, CTGF increases the affinity of TGF- β 1 for its receptors (3) and influences the regulation of endothelial cell function, angiogenesis, and vascular remodeling (7, 41). TGF- β 1 normally induces CTGF expression in fibroblasts and endothelial cells (6), and CTGF expression can be inhibited by proinflammatory cytokines such as TNF- α (2). Because of its involvement in airway and vascular remodeling processes, CTGF might have a role in mediating the pathogenesis of BPD. However, there is no information about CTGF in BPD or in preterm animals. Therefore, we used lung tissue from preterm fetal sheep exposed to endotoxin-induced chorioamnionitis to measure TGF- β 1 signaling and CTGF expression. Our hypothesis was that chorioamnionitis induced by endotoxin would increase TGF- β 1 in the fetal lung but that CTGF might not be induced.

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MATERIALS AND METHODS

Tissue for study. The animal studies were performed in Western Australia, as approved by the Animal Care and Use Committees of the Cincinnati Children's Hospital and the Western Australian Department of Agriculture. Date-bred Merino ewes were randomized to receive 4 mg of endotoxin (*Escherichia coli*, serotype 055:B5; Sigma Chemical, St. Louis, MO) at 5 h, 24 h, 72 h, or 7 days before delivery at 125 days gestational age. Control animals received an intra-amniotic saline injection under ultrasound guidance at the same time points. Preterm lambs were delivered by cesarean section, and the lungs were taken for multiple assessments. The tissues used for the measurements reported here were from previous studies (35, 36).

RNA extraction and RT-PCR. Total RNA was isolated from tissue pieces from the right lower lung lobe by guanidinium thiocyanate-phenol-chloroform extraction, as described elsewhere (31). RT-PCR were performed with *Taq* polymerase (TaqPCR Master Mix Kit, Qiagen, Hilden, Germany). Primers and optimized PCR conditions for all the molecules have been reported previously (Table 1) (17, 31, 54). A negative control of non-reverse-transcribed total RNA did not yield a PCR product for any of the primers, indicating there was no detectable genomic DNA contamination. The PCR product for CTGF was confirmed by sequencing. PCR products were loaded to a standard and analyzed on 1% agarose gels. Image analysis was performed using a fluorescence image analyzer (model FLA 3000, Fuji, Dielsdorf, Switzerland) and quantified using AIDA software (Raytest, Urdorf, Switzerland).

Immunohistochemistry and histological analyses. The immunostaining methods were performed as previously described (35). Lung tissue was fixed in formalin, embedded in paraffin, and cut into 5- μ m sections. Endogenous peroxidase activity was removed by incubation with hydrogen peroxide. Nonspecific binding sites were blocked with serum. Sections were incubated at 4°C overnight with a rabbit polyclonal antibody for bioactive TGF- β 1 (catalog no. sc-146, Santa Cruz Biotechnology, Santa Cruz, CA), a goat polyclonal antibody for CTGF (catalog no. sc-14939, Santa Cruz Biotechnology), or a rabbit anti-phosphorylated Smad2 (generous gift from Dr. C.-H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden). Unbound antibody was removed with PBS, and the slides were incubated with the secondary biotinylated antibody against rabbit or goat IgG (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Immunostaining was visualized by the Vectastain ABC peroxidase Elite kit for detection of the antigen-antibody complexes (Vector Laboratories). For analysis of immunohistochemistry, we used a previously described five-step semiquantitative scale (15). Two independent observers who were unaware of the identity of the samples performed the analysis at $\times 20$ and $\times 40$ magnification. Four to five tissue sections of each lung from different regions were analyzed. The average count of all results was calculated for each lung.

Western blot. Frozen lung samples were homogenized in ice-cold buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM

EDTA, and protease inhibitor cocktail (complete minitabets; Roche, Mannheim, Germany) supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical). The samples were sonicated and then centrifuged at 500 g for 20 min at 4°C for removal of cellular debris. Protein content in the supernatant was determined by the bicinchoninic acid method, with BSA as the standard. Protein samples (50 μ g/lane) were boiled and loaded onto a molecular weight marker (Invitrogen, Karlsruhe, Germany) on NuPAGE 4–12% Bis-Tris gel (Invitrogen) under nonreducing conditions. The proteins were electrophoretically transferred onto a Hybond-P polyvinylidene difluoride membrane (Amersham Life Science, Freiburg, Germany), and the blots were blocked for 1 h in 5% nonfat dry milk in TBS with 0.1% Tween 20. Activated TGF- β 1 was detected with an anti-TGF- β 1 antibody (catalog no. TS05, BD PharMingen, Heidelberg, Germany) and with a secondary antibody (goat anti-mouse IgG1 horseradish peroxidase-conjugated antibody; Amersham Life Science). The reaction was visualized on X-ray medical film (Konica Minolta, Unterföhring, Germany) after incubation of membranes with luminol-based chemiluminescence reagent (Pierce Biotechnology, Rockford, IL) for 1 min. For normalization of the experiments, membranes were stripped, as recommended by the manufacturer (Pierce Biotechnology), and re-probed with an antibody against β -actin (Santa Cruz Biotechnology). The autoradiographs were scanned at high resolution, and the images were acquired using Adobe Photoshop software. The densitometric quantitation was performed using AIDA software.

Measurement of TGF- β 1 and TNF- α protein in lung tissue by ELISA. Pieces of the right lower lobe were sonicated as described above. Supernatants were diluted 1:10. Levels of bioactive TGF- β 1 were determined using a specific TGF- β 1 enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems, Minneapolis, MN) that cross-reacts with porcine, rodent, and sheep TGF- β 1, but not with TGF- β 2 or TGF- β 3. Total active TGF- β 1 levels were measured using acid-activating samples, as recommended by the manufacturer (32). TNF- α levels were measured with an ELISA kit to detect ovine TNF- α (ENDOGEN, Rockford, IL). TGF- β 1 and TNF- α protein concentrations were calculated per kilogram of body weight.

Statistical analysis. Values are means $\pm$ SE. Analysis of variance was used for comparisons between endotoxin groups at each gestational age; Student-Newman-Keuls tests were used for post hoc analyses. Significance was accepted at $P < 0.05$.

RESULTS

TGF- β 1 expression in response to endotoxin-induced chorioamnionitis. We evaluated the expression of TGF- β 1 mRNA in fetal lung tissue by RT-PCR. Signal intensity of TGF- β 1 mRNA was expressed relative to that of the ribosomal gene L32. The ratio of TGF- β 1 mRNA to L32 mRNA increased with time in the endotoxin-exposed groups compared with the control group (Fig. 1). To confirm that increased TGF- β 1

Table 1. Primer sequences and PCR conditions

Primer	Sequence	Temperature/Time			No. of Cycles	PCR Product, bp	Ref.
		Denaturation	Annealing	Extention			
TGF-β1							
Forward	5' GGC GAC CCA CAG AGA GGA AAT AG 3'	94°C/40 s	58°C/40 s	72°C/40 s	30	349	17
Reverse	5' AGG CAG AAA TTG GCG TGG TAG C 3'						
CTGF							
Forward	5' ACG GCG AGG TCA TGA AGA AGA ACA-3'	94°C/45 s	61°C/45 s	72°C/60 s	40	521	54
Reverse	5' TGG GGC TAC AGG CAG GTC AGT G 3'						
L32							
Forward	5' AAG TTC ATC CGG CAT CAG TC 3'	94°C/30 s	55°C/30 s	72°C/30 s	20	203	31
Reverse	5' TCA AGC TCC TTG ACG TTG TG 3'						

TGF- β 1, transforming growth factor- β 1; CTGF, connective tissue growth factor.

ANTENATAL INFLAMMATION INDUCED TGF- β 1 IN PRETERM LUNGS

L225

Fig. 1. Intra-amniotic endotoxin increases steady-state levels of transforming growth factor (TGF)- β 1 mRNA in the lung. **A**: representative RT-PCR for TGF- β 1 and L32 from lung of control animals and animals exposed to endotoxin for 5 h, 24 h, 72 h, and 7 days. **B**: densitometric analysis of PCR results. TGF- β 1 was quantitated at 5 h, 24 h, 72 h, and 7 days after normalization to L32 (ribosomal protein mRNA). Mean RNA value in control animals was set to 1, and levels at each time point are expressed relative to the mean control value. Values are means $\pm$ SE; $n = 4$ animals in each group at each time point. * $P < 0.05$ vs. control.

mRNA corresponded with increased protein levels, bioactive TGF- β 1 was quantified by Western blot and ELISA analysis of whole lung homogenates and by immunohistochemistry in lung tissue. Bioactive TGF- β 1 protein in lung tissue increased by 300% in Western blot analysis (Fig. 2A) and by 350% in ELISA analysis (Fig. 2B). TGF- β 1 immunostaining was weakly detected in the control lungs. Staining for TGF- β 1 was more apparent in lung stroma of the endotoxin-exposed group (Fig. 3). Immunostaining for bioactive TGF- β 1 was increased about twofold at all times after endotoxin exposure.

Induction of the Smad signaling pathway. TGF- β 1 signaling from the cell membrane to the nucleus is mediated through Smad proteins (47). Early events for TGF- β 1 signal transduction are the phosphorylation of TGF- β type I and type II receptors and the subsequent phosphorylation and translocation of the intracellular effectors Smad2 and Smad3 to the nucleus, where they regulate gene transcription. Therefore, the effect of intra-amniotic endotoxin on the subcellular distribution of phosphorylated Smad2 was evaluated in the lung of preterm lambs. Smad2 phosphorylation was detected by immunostaining with anti-phosphorylated Smad2-specific antibodies. Cytoplasmic staining for phosphorylated Smad 2 was weak in bronchial epithelial cells in the control lungs (Fig. 4A). In contrast, endotoxin exposure resulted in intense staining of phosphorylated Smad2 in almost all bronchial epithelial cells and some staining in vascular endothelial and fibroblast-like cells (Fig. 4B). The Smad2 staining increased 5 h after intra-amniotic exposure to endotoxin (Fig. 4C). The majority of the cells had nuclear staining, consistent with TGF- β 1 signaling and translocation of phosphorylated Smad2 to the nucleus.

CTGF expression in response to endotoxin-induced chorioamnionitis. We evaluated the steady-state levels of mRNA of CTGF mRNA in sheep lung homogenates by RT-PCR. The ratio of CTGF mRNA to L32 mRNA decreased to <33% of control levels in the endotoxin-exposed groups (Fig. 5). Expression of CTGF protein was evaluated by immunohisto-

chemistry (Fig. 6). CTGF was exclusively detected in lung endothelial cells in control lungs, with decreased immunostaining in endotoxin-exposed animals (Fig. 6, A and B). A semi-quantitative analysis of the immunohistochemistry demonstrated that CTGF protein was reduced to <50% of control values by 5 h of endotoxin exposure and remained low at later times (Fig. 6).

Intra-amniotic endotoxin induced TNF- α expression in the lung. We measured the TNF- α concentration in whole lung homogenates by ELISA, because TNF- α is a known inhibitor of TGF- β 1-induced CTGF expression (2). TNF- α protein increased in lung tissue of the endotoxin-exposed groups >20-fold relative to the control group (Fig. 7).

DISCUSSION

Chorioamnionitis is a complex disease that has multiple and contrasting effects on preterm babies. Although the incidence of respiratory distress syndrome may be decreased, the incidence of BPD is increased (51). We and others have developed models to study this disease, which occurs in the context of a developing fetus (8). Bry and colleagues (10) were the first to describe an increase in surfactant pool size and compliance after intrauterine exposure to the proinflammatory cytokine IL-1 in the preterm rabbit lung. This phenotypic lung maturation, however, was different from lung maturation induced by maternal corticosteroid treatment, since the systemic cortisol levels were unchanged (26). The phenotypic lung maturation was induced in fetal sheep within 7 days of intrauterine exposure to inflammation; however, an injury response resulting in interference with alveolar and microvascular development also occurred (52). Moreover, the response of the fetal lung to a secondary inflammatory stimulus, such as mechanical ventilation, also was increased by intrauterine exposure to inflammation (23).

Pulmonary inflammation could be induced in utero with IL-1 α , IL-1 β , endotoxin from periodontal organisms, or endo-

Fig. 2. Intra-amniotic endotoxin exposure increases TGF- β 1 protein concentration in the lung. **A**: Western blot measurement of TGF- β 1 protein concentration in whole lung homogenates. Total bioactive TGF- β 1 protein was analyzed by Western blotting with an anti-TGF- β 1 antibody. The same membrane was analyzed with anti- β -actin antibody. TGF- β 1 and β -actin protein levels were quantified by densitometry. Optical density of the TGF- β 1 protein band was corrected for β -actin, and results are expressed as ratio (%) of endotoxin-exposed to control animals. **B**: ELISA measurement of TGF- β 1 protein concentration in whole lung homogenates. TGF- β 1 protein concentration was standardized per kilogram of body weight. Values are means $\pm$ SE; $n = 4$ animals in each group at each time point. * $P < 0.05$ vs. control.

toxin from *E. coli*, all of which resulted in a phenotype of maturation of the fetal lung with increased surfactant pool sizes in sheep (26, 42, 53). The injury and developmental responses of the fetal lung seem to be generalizable. Perinatal expression of IL-1 β in a transgenic mouse model resulted in a phenotype similar to BPD, with alveolar simplification and impaired microvascular development (9).

We have identified increased expression of TGF- β 1 and decreased expression of CTGF in fetal sheep exposed to endotoxin-induced chorioamnionitis. The BPD phenotype of

alveolar simplification and microvascular injury resulting from intra-amniotic endotoxin exposure is not mediated by mechanical ventilation and oxygen exposure, which are the traditional causes of BPD, but by inflammatory responses of the fetal lung (26, 52). The increase in TGF- β 1 was accompanied by an activation of the Smad signaling pathway. Generally, CTGF expression increases if TGF- β 1 expression increases (6). However, the elevated TGF- β 1 expression was associated with reduced CTGF expression in endotoxin-exposed lungs in association with increased TNF- α protein concentration. We speculate that the CTGF downregulation may be mediated by an endotoxin-induced induction of TNF- α expression (Fig. 8).

In 1967, Northway and colleagues (43) described BPD as severe lung injury resulting from the mechanical ventilation and oxygen exposure of preterm infants. The oxidant injury and mechanical ventilation resulted in inflammation, fibrosis, and hypertrophy of airway smooth muscle. With progress in neonatal care, which includes antenatal glucocorticoids, surfactant treatment, and more gentle ventilation strategies, larger preterm infants now seldom develop BPD. However, the disease remains the most common lung complication for extremely immature babies with gestations <28 wk (46), a patient group that generally did not survive when BPD was first described. BPD is defined as supplemental oxygen dependency of preterm babies at a postmenstrual age of 36 wk (25). The "new" BPD is frequently diagnosed in very preterm infants who did not initially have severe respiratory distress syndrome (13, 27). In clinical series and animal models, chorioamnionitis resulted in a lung maturation response, because surfactant increased and the volume of the potential gas space in the fetal lung increased. However, prenatal exposure to chorioamnionitis was associated with the development of BPD in these very preterm infants (19, 49, 51). The inflammation already present in the lungs may be sustained or aggravated by therapeutic interventions, such as resuscitation, oxygen, mechanical ventilation, or postnatal infection (45). Angiogenesis occurs with alveolarization during normal lung development, and injury to the developing pulmonary circulation during a critical period of growth can also contribute to development of BPD (1). Decreased concentrations of vascular endothelial growth factor and its receptors were measured in lung tissue of preterm babies who died of BPD and in the lungs of preterm lambs exposed to chorioamnionitis (5, 29, 37).

The activation of latent TGF- β 1 to active TGF- β 1 has been proposed to contribute to the pathogenesis of acute respiratory distress syndrome, causing a disruption of the alveolar epithelial barrier function (21). However, the role of TGF- β 1 in the normal development and injury response of the lung in preterm infants remains unclear. The TGF- β family has been associated with the pathophysiology of BPD, because the levels of TGF- β 1 protein increased in airway samples from preterm infants developing BPD (28, 34, 38). However, it is not known whether an elevated TGF- β 1 concentration in the fetal lung induced activation of the Smad signaling pathway in vivo. In fetal sheep exposed to chorioamnionitis, the Smad signaling pathway was activated during the inflammation-triggered lung injury and remodeling processes.

TGF- β 1 is important for wound healing and in the pathogenesis of fibrosis, but it is also involved in other critical biological activities, including suppression of immune reactions. Because of its multifunctional role, inhibition of TGF- β 1

ANTENATAL INFLAMMATION INDUCED TGF- β 1 IN PRETERM LUNGS

L227

Fig. 3. Immunohistochemical evaluation of TGF- β 1 protein in lung tissue. *A*: representative sections from a control animal and an animal exposed to endotoxin-induced chorioamnionitis for 7 days stained with an antibody that detects TGF- β 1. Magnification $\times 320$. *B*: immunohistochemical quantification of TGF- β 1 in lung sections. Immunostaining for TGF- β 1 was graded on a scale from 0 to 5. Values are means $\pm$ SE; $n = 4$ animals in each group at each time point. * $P < 0.05$ vs. control.

Fig. 4. Intra-amniotic endotoxin exposure induces the Smad signaling pathway in lungs of preterm lambs. Smad2 phosphorylation was evaluated in lung tissue by immunohistochemistry. *A*: representative sections from a control animal and an animal exposed to endotoxin-induced chorioamnionitis for 7 days stained with an antibody that reacts specifically with phosphorylated Smad2. Magnification $\times 320$. *B*: immunohistochemical quantification of phosphorylated Smad2 in lung sections. Immunostaining for phosphorylated Smad2 was graded on a scale from 0 to 5. Values are means $\pm$ SE; $n = 4$ animals in each group at each time point. * $P < 0.05$ vs. control.

Fig. 5. Intra-amniotic endotoxin exposure decreased steady-state levels of connective tissue growth factor (CTGF) mRNA in the lung. **A**: representative RT-PCR for CTGF and L32 from lung of control animal and animals exposed to endotoxin for 5 h, 24 h, 72 h, and 7 days. **B**: densitometric analysis of PCR results. CTGF was quantified at 5 h, 24 h, 72 h, and 7 days after normalization to L32 (ribosomal protein mRNA). Mean RNA value in control animals was given a value of 1, and levels at each time point are expressed relative to the mean control value. Values are means $\pm$ SE; $n = 4$ animals in each group at each time point. * $P < 0.05$ vs. control.

would be expected to impact numerous other important functions, many in an adverse way. CTGF might play a role in targeting fibrotic processes. The exposure of fibroblasts and endothelial cells to TGF- β 1 is sufficient to induce CTGF expression, which mediated some of the profibrotic effect of TGF- β 1 (6). Recent reports suggest that CTGF may anchor vascular endothelial growth factor to the extracellular matrix to

promote angiogenesis (11). We found that the CTGF production was reduced in endotoxin-exposed animals. Furthermore, CTGF expression was limited to endothelial cells of blood vessels in the lung. The limited expression of CTGF in endothelial lung cells and the downregulation of CTGF in these cells in the endotoxin-exposed animals support the vascular hypothesis of BPD. The selective expression of

Fig. 6. Immunohistochemical evaluation of CTGF protein in lung tissue. Intra-amniotic endotoxin exposure decreased CTGF protein in the lung. **A**: representative sections from a control animal and an animal exposed to endotoxin-induced chorioamnionitis for 7 days stained with an antibody that reacts with CTGF. Arrow, brownish-stained endothelial cell layer. Magnification $\times 320$. **B**: immunohistochemical quantification of CTGF in lung sections. Immunostaining for CTGF was graded on a scale from 0 to 5. Values are means $\pm$ SE; $n = 4$ animals in each group at each time point. * $P < 0.05$ vs. control.

ANTENATAL INFLAMMATION INDUCED TGF- β 1 IN PRETERM LUNGS

L229

Fig. 7. Intra-amniotic endotoxin exposure increased TNF- α protein in the fetal lung. TNF- α concentration was measured in whole lung homogenates by ELISA and standardized per kilogram of body weight. Values are means $\pm$ SE; $n = 4$ animals in each group at each time point. * $P < 0.05$ vs. control.

TGF- β 1 in endothelial cells could play an important role during normal lung development and for the homeostasis of endothelial cells (7).

The regulation of the CTGF promoter is well understood. TNF- α was found to suppress the TGF- β 1-induced expression of CTGF protein in cultured normal fibroblasts at the transcriptional level (2). Interestingly, the sequences between -244 and -166 of the CTGF promoter were necessary for TGF- β 1 and TNF- α modulation of CTGF expression. Taken together, TNF- α could influence two steps in the pathogenesis of BPD:

1) TNF- α initiates the inflammatory responses, and 2) TNF- α seems to inhibit the generation of fibrosis by suppressing TGF- β 1 synthesis (2). Increased TNF- α concentrations in preterm babies have been associated with the development of BPD (5). Surprisingly, fetal sheep do not respond to bioactive recombinant ovine TNF- α , irrespective of the route of admin-

istration (24). Intra-amniotic or intratracheal administration did not result in an inflammatory response by the pulmonary immune system. However, mechanical ventilation and chorioamnionitis induced some TNF- α mRNA (24). Although TNF- α may not be an important mediator of inflammation in the sheep fetus, it may modulate other signaling pathways.

Increased TGF- β 1 concentration did not result in increased CTGF concentration in the fetal lung that was exposed to inflammation. The changed ratio of TGF- β 1 to CTGF may explain, in part, the lack of prominent fibrosis in the new BPD. We summarized our results and interpretations with respect to the clinical findings in new BPD in Fig. 8. The first aspect is the physiological role of TGF- β 1 in lung development. Increased concentrations of TGF- β 1 reduce alveolarization (50). Furthermore, the anti-inflammatory effects of TGF- β 1 reduce lung inflammation (4). TGF- β 1 is also essential for the "wound remodeling or healing" after lung injury. These effects are mediated in part by TGF- β 1 itself, but the fibrosis is particularly mediated by CTGF. We found that endotoxin-mediated chorioamnionitis induced TGF- β 1 and TNF- α but decreased CTGF. The downregulation of CTGF could be mediated by TNF- α . Therefore, fibrosis was not initiated, which is typical for the new BPD. The reduced concentrations of CTGF might increase the impaired development of the microvasculature, since CTGF is involved in normal vascular development (7). Our findings support the notion that increased TGF- β 1 and decreased CTGF concentrations in the fetal lung impaired alveologenesis (50). In addition, in this sheep model, chronic chorioamnionitis induced by 28 days of exposure to endotoxin did not induce lung fibrosis (30), demonstrating the resistance of the fetal lung to the development of fibrosis, despite the prolonged presence of proinflammatory agonists.

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Fig. 8. Model of interaction between endotoxin, TNF- α , TGF- β 1, and CTGF in the pathophysiology of "new" BPD. Some of the hallmarks of the new BPD may be explained by the cytokine profiles. TGF- β 1 is essential for the "wound remodeling or healing" after lung injury, but increased TGF- β 1 concentration impairs alveologenesis. These effects are mediated in part by TGF- β 1 itself, but the fibrosis is primarily mediated by CTGF. Furthermore, TGF- β 1 reduces lung inflammation. TNF- α and TGF- β 1 were induced, but CTGF was decreased, in this model of endotoxin-induced chorioamnionitis. Downregulation of CTGF might be mediated by TNF- α . Therefore, the reduced concentrations of CTGF might have limited fibrosis but also impaired microvascular development, since CTGF is involved in normal vascular development. Taken together, the cytokine profile may explain, in an overly simplified way, the findings of new BPD.

L230

ANTENATAL INFLAMMATION INDUCED TGF- β 1 IN PRETERM LUNGS

GRANTS

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ANTENATAL INFLAMMATION INDUCED TGF- β 1 IN PRETERM LUNGS

L231

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CHAPTER 6

Surfactant protein A recruits neutrophil granulocytes into the lungs of ventilated preterm sheep

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Surfactant Protein A Recruits Neutrophils into the Lungs of Ventilated Preterm Lambs

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We tested the effects of surfactant protein A (SP-A) on inflammation and surfactant function in ventilated preterm lungs. Preterm lambs of 131 d gestation were ventilated for 15 min to initiate a mild inflammatory response, and were then treated with 100 mg/kg recombinant human SP-C surfactant or with the same surfactant supplemented with 3 mg/kg ovine SP-A. Addition of SP-A to the SP-C surfactant did not change lung function. After 6 h of ventilation, cell numbers in the alveolar wash were 4.9 times higher in SP-A + SP-C-surfactant-treated animals. Cellular infiltrates consisted of neutrophils that produced less hydrogen peroxide than did cells from SP-C-surfactant-treated animals. Expression of adhesion molecules CD11b and CD44 was significantly greater after SP-A treatment, whereas the expression of CD14 was unchanged. Messenger RNAs (mRNAs) for the proinflammatory cytokines interleukin (IL)-1 β , IL-6, and IL-8, but not tumor necrosis factor- α , were increased in SP-A-treated lungs. Surfactant protein mRNAs and protein leakage into alveolar washes were not altered by SP-A, indicating that SP-A treatment produces no evidence of lung injury. The present study identifies an unanticipated role of SP-A in neutrophil recruitment in the lungs of preterm lambs.

Surfactant treatment of infants with respiratory distress syndrome (RDS) decreases morbidity and mortality and is now the standard of care. Nevertheless, many infants of very low birth weight progress to having bronchopulmonary dysplasia (BPD), and airway samples from infants destined to have BPD have increased numbers of proinflammatory cells and cytokines within the first days after birth and initiation of ventilation (1). The surfactants used to treat preterm infants with RDS are isolated from animal lungs, and the organic solvent extraction and purification steps used in their isolation remove the innate host-defense protein surfactant protein A (SP-A) while leaving variable amounts of SP-B and SP-C. SP-A is a member of the collectin family of proteins that bind carbohydrates and lipopolysaccharides (2). Although SP-A binds to phospholipids and is essential for tubular myelin formation, SP-A-ablated mice have normal surfactant function, metabolism, and tolerance to exercise and hyperoxia (3–5). However, SP-A-deficient mice are more susceptible to bacterial and viral infections (6, 7). SP-A enhances the efficiency of clearance and killing of bacteria, fungi, and viruses *in vitro* and in SP-A-deficient mice that are treated with SP-A (7, 8). Immunomodulatory functions of SP-A in the airways include binding and

modulation of processing of endotoxin, modulation of cytokine production by alveolar macrophages (AM) (9, 10), and interaction with the endotoxin receptor CD14 (11).

SP-A has been proposed as being either pro- or antiinflammatory on the basis of its properties *in vitro* (12–14). There is no information about host-defense effects of exogenous SP-A in the preterm lung, in which SP-A is normally present in very small amounts. The initiation of ventilation with mechanical ventilation induces transcription of proinflammatory cytokines in preterm lamb lungs (15, 16). This cytokine induction is presumed to be injurious, because preterm fetuses exposed to chorioamnionitis and increased levels of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and IL-8 have an increased risk of developing BPD, as do infants with increased levels of the same cytokines in samples of bronchoalveolar lavage fluid (BALF) taken within the first days of life (1, 17). In preterm infants, low concentrations of SP-A in the airways correlated with increased mortality and with a higher rate of BPD (18). In the preterm baboon model of BPD, SP-A was present in lung tissue, but was present at very low levels in BALF, demonstrating an abnormality of secretion of SP-A (19).

The host-defense functions of the preterm fetal lung differ from those of the adult lung because the fetal lung contains virtually no granulocytes or mature macrophages (1). Multiple immune defects have been described for white blood cells from infants, and levels of innate host-defense molecules such as lysozyme and defensins also are low (20). We hypothesized that SP-A would alter the proinflammatory response of the preterm lung to mechanical ventilation. To test this hypothesis, we treated preterm lambs with an effective synthetic surfactant that contained human recombinant SP-C, or with the same surfactant supplemented with 3% sheep SP-A, and evaluated lung function, surfactant components, and multiple local and systemic indicators of inflammation.

METHODS

SP-A and SP-C Surfactant

SP-A was isolated from alveolar washes of healthy adult sheep by butanol extraction (21). After extraction of whole surfactant with butanol, insoluble proteins were resuspended in octylglucopyranoside, and SP-A was solubilized in 5 mM Tris-HCl in water (pH 7.4). SP-A was dialyzed for 48 h against the same buffer to remove residual octylglucopyranoside, and the purification was verified by sodium dodecyl-sulfate–polyacrylamide gel (SDS–PAGE) analysis. The SP-A used to treat the lambs was tested for bacterial endotoxin contamination with the *Limulus* amoebocyte lysate assay (Sigma Chemical Co., St. Louis, MO), and no endotoxin was detected. The treatment dose of 3 mg/kg SP-A in 100 mg/kg of recombinant human SP-C surfactant (Byk Gulden, Konstanz, Germany) was similar to the amount of SP-A and surfactant recovered from alveolar lavage of full-term animals. The SP-C surfactant contained 2% recombinant human SP-C in phospholipids (dipalmitoylphosphatidylcholine and palmitoyloleoylphosphatidylglycerol in a 70:30 [wt/wt] ratio) and 5% palmitic acid (22). This SP-C surfactant was very effective when tested in preterm lambs and in models

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of lung injury (22, 23). Currently, SP-C surfactant is being tested in clinical trials for the treatment of acute respiratory distress syndrome (ARDS) (24). The recombinant SP-C consists of the 34-amino-acid human SP-C peptide, altered by the replacement of cysteine in positions 4 and 5 with phenylalanine, and of methionine in position 32 by isoleucine. The altered sequence of recombinant SP-C improves solubility and prevents the aggregation of native SP-C, but maintains the functions of the dipalmitoylated form of SP-C (25). This recombinant protein has clearance kinetics from the airways and lungs of rabbits and preterm lambs that are similar to those of native SP-C (22). The SP-C surfactant was resuspended in 0.9% NaCl to a final concentration of 25 mg/ml.

Delivery and Ventilation of Preterm Lambs

Preterm lambs were delivered at 130 d to 132 d gestational age by cesarean section, as previously described (term is 150 d) (22, 26). Each pregnant ewe was preanesthetized with ketamine (20 mg/kg intramuscularly) and acepromazine (0.3 mg/kg), and then given spinal-epidural anesthesia (10 ml of 2% lidocaine and 0.5% bupivacaine [1:1, vol/vol]). After exposure of the head, the preterm lamb was given ketamine (10 mg/kg intramuscularly) and acepromazine (0.1 mg/kg intramuscularly). A tracheal tube of 4.5-mm I.D. was inserted and tied into the trachea after local anesthesia of the anterior neck with 2% lidocaine. Fluid from fetal airways was removed with a syringe. After the umbilical cord was cut, the newborn lamb was delivered and weighed. Ventilation was started with the following settings: fraction of inspired oxygen ($F_{I_{O_2}}$) = 1.0, respiratory rate (RR) = 60 breaths/min, inspiratory time (Ti) = 0.4 s, positive end-expiratory pressure (PEEP) = 4 cm H₂O, and a peak inspiratory pressure ($P_{I_{max}}$) sufficient to yield a tidal volume (Vt) of 6 to 8 ml/kg, but with $P_{I_{max}}$ limited to 40 cm H₂O. Vt was monitored continuously (CP-100; Bicore Monitoring Systems, Anaheim, CA). A size 5 French catheter was advanced into the aorta via an umbilical artery, and a 10 ml/kg transfusion of filtered fetal blood collected from the placenta was administered within 10 min of delivery. After 15 min of ventilation, each preterm lamb was randomly assigned to treatment with 100 mg/kg SP-C surfactant ($n = 7$; SP-C surfactant group) or 100 mg/kg SP-C surfactant plus 3 mg/kg SP-A ($n = 7$; SP-A + SP-C-surfactant group). The surfactant treatment was given in two equal aliquots, with the lamb in the left lateral and right lateral positions, respectively (26). The initial ventilation for 15 min without surfactant treatment was used to initiate the injury process in the preterm lungs, and to mimic the clinical sequence for resuscitation followed by surfactant treatment of preterm infants. After surfactant treatment, the ventilation pressure was adjusted to achieve an arterial carbon dioxide tension (P_{aCO_2}) of 45 to 60 mm Hg and an arterial oxygen tension of 150 to 200 mm Hg with a Vt of 6 to 8 ml/kg. Vt was measured at 15 min, 45 min, 2 h, 4 h, and 6 h with a pneumotachometer, and dynamic compliance (Cdyn) was calculated as Vt normalized to body weight and divided by the ventilatory pressure ($P_{I_{max}} - PEEP$) (22, 26). The ventilatory efficiency index (VEI) was calculated as $VEI = 3,800 / (\text{respiratory rate} \times [P_{I_{max}} - PEEP] \times P_{aCO_2})$, where 3,800 is a CO₂ production constant ($[\text{ml} \times \text{mm Hg}] / [\text{kg} \times \text{min}]$) (25). Complete blood counts and differentials were done on from cord blood and on peripheral blood at 6 h. The arterial catheter was used for blood gas analysis, pH measurement, and blood pressure recording, and to infuse 10% dextrose (100 ml/kg/d). Rectal temperature was monitored and kept at 38° to 39° C with heating pads and radiant heat. Supplemental ketamine (10 mg/kg intramuscularly) and acepromazine (0.1 mg/kg intramuscularly) were administered to suppress spontaneous breathing. At 2 h of afterbirth, a sample of BALF was obtained by wedging a feeding tube in an airway and infusing 1 ml of saline, followed by aspiration. After 6 h, each animal was deeply anesthetized with 25 mg/kg pentobarbital given intravenously, and was ventilated briefly with 100% oxygen. The endotracheal tube was clamped for 3 min to permit oxygen absorption. The animal was exsanguinated by cutting the abdominal aorta.

Pressure-Volume Curve and Lung Processing

The thorax of the lamb was opened, the lungs were inflated with 40 cm H₂O pressure for 1 min, and maximal lung volume was recorded (26). The pressure was sequentially lowered to 20, 15, 10, 5, and 0 cm H₂O, and lung volumes were recorded 30 s after each pressure was reached.

Volumes were corrected for the compliance of the system. Lungs were then removed from the thorax. Pieces of the right lower lobe were immediately frozen in liquid nitrogen for RNA isolation. Alveolar washing was done on the left lung by filling the lung with 0.9% NaCl at 4° C until it was visually distended, and was repeated five times (22, 26). The washes were pooled and aliquots were saved for measurement of saturated phosphatidylcholine (SatPC), total protein, cell number and differential count, surfactant protein quantification, and flow cytometric analysis. Cell pellets were used for hydrogen peroxide assay and RNA isolation. The lung was homogenized in 0.9% NaCl and SatPC was measured in aliquots of homogenate.

Measurement of SatPC and Total Protein

SatPC was isolated from chloroform-methanol (2:1) extracts by neutral alumina column chromatography after exposure to osmium tetroxide, and was quantified by phosphorus assay (25). Total protein in alveolar washes was measured as previously described (26).

Alveolar Cells

Collected alveolar washings were centrifuged at $500 \times g$ for 10 min and the pellet was resuspended in phosphate-buffered saline (PBS). Total cells were stained with trypan blue and counted. Differential cell counts were performed on cytopsin preparations after staining with Diff-Quik (American Scientific Products, San Diego, CA). In order to assess the activation state of the cells recruited to the airways, hydrogen peroxide was measured with an assay based on the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) by hydrogen peroxide under acidic conditions (Bioxytech H₂O₂-560 Assay; OXIS International, Portland, OR).

Aliquots of alveolar cells were incubated on ice with monoclonal antibodies (primary antibody) against ovine CD11b (αM subunit of integrin CR3), CD14 (receptor for complex of lipopolysaccharide and lipopolysaccharide binding protein), and CD44 (proteoglycan link protein). The cell pellet was washed twice with PBS to remove unbound antibody, and was incubated with phycoerythrin (PE)-labeled F(ab')₂ anti-IgG fragments (secondary antibody) in the dark on ice. Control staining was performed with isotype antibodies and with secondary antibody alone to obtain background fluorescence. Cells were washed twice, resuspended in PBS, kept on ice, and immediately analyzed on a fluorescence-activated cell sorter (FACS) (Calibur; Becton-Dickinson, Inc., Mountain View, CA). All antibodies were purchased from Serotec (Raleigh, NC).

Western Blots for SP-B and SP-D

SDS-PAGE electrophoresis was done with 8 to 16% gradient Tris/glycine gels (Novex, San Diego, CA). Samples containing 3 nmol of SatPC were electrophoresed along with ovine SP-B or murine SP-D standards. Proteins were transferred to nitrocellulose paper (Schleicher & Schuell, Keene, NH) for immunoblot analysis with rabbit anti-SP-B or anti-SP-D serum. The nitrocellulose membrane was first blocked with 5% bovine serum albumin (wt/vol) (Sigma Chemicals) in Tris-buffered saline, pH 7.4, containing 0.1% (vol/vol) Tween-20 (5). The antibody was diluted and incubated overnight. Horseradish peroxidase-conjugated goat antirabbit immunoglobulin (Calbiochem, La Jolla, CA) was used as secondary antibody, and enhanced chemiluminescence substrates (Amersham, Arlington Heights, IL) were used to develop the immunoblots. Estimates of the amount of SP-B and SP-D were made by calculation of the relative band densities, using Alpha-Imager 2000 Documentation and Analysis Software (Alpha Innotech, San Leandro, CA).

Cytokine Messenger RNA

Total RNA was isolated from a portion of the right lower lobe and from cell pellets of the tracheal aspirate and alveolar washings by guanidium thiocyanate-phenol-chloroform extraction (27). Ribonuclease (RNase) protection assays were performed with total RNA from lung tissue and cell pellets (28). Briefly, RNA transcripts of ovine interleukin (IL)-1 β , IL-6, IL-8, IL-10, tumor necrosis factor (TNF)- α , and ovine ribosomal protein L32 as a reference RNA were synthesized with [³²P]uridine triphosphate (Life Sciences Products, Boston, MA), using SP6 or T7 polymerase (Ribonuclease Protection Assay III; Ambion, Austin, TX). Aliquots of 10 μ g RNA were incu-

bated with excess radiolabeled probes for cytokines and L32 at 55° C for 18 h. Remaining single-stranded RNA was digested with RNase A/RNase T1 (Ambion). After inactivation and precipitation, protected fragments were electrophoresed on a 6% polyacrylamide-urea (8 mol/L) sequencing gel and visualized by autoradiography. Densities of the protected bands were quantified on a Phosphorimager (Molecular Dynamics Inc., Sunnyvale, CA), using ImageQuant software (Molecular Dynamics).

Surfactant Protein mRNA

The relative abundance of surfactant protein mRNA was measured with the S1 nuclease protection assay as previously described (27). Briefly, an excess of linearized probes for SP-A, SP-B, SP-C, and L32, all 5' end-labeled with [³²P], were hybridized at 56° C with 3 µg of total RNA from lung tissue. SP-D was detected in a separate hybridization using 10 µg RNA. The SP-D probe was a kind gift of Dr. M. Hallman of the University of Oulu, Finland. After incubation with S1 nuclease, the protected fragments were resolved on a 6% polyacrylamide-urea (8 mol/L) sequencing gel, visualized by autoradiography, and quantified on a Phosphorimager with ImageQuant software.

Lung Morphology/Inflammation Score

The right upper lobe was inflated fixed with 10% formalin at 30 cm H₂O pressure. Paraffin tissue sections of 5 µm thickness, stained with hematoxylin and eosin, were graded for the degree of inflammation in a blinded fashion by scoring three sections from each animal as zero if they had no inflammatory cells in tissue or airspaces, 1 if they had a few cells, 2 if they exhibited moderate cell infiltration, and 3 if they had a large number of inflammatory cells in airspaces and tissue (27). Airspaces and tissue were assessed separately. Average scores were calculated for each animal.

Data Analysis

Results are given as mean ± SEM. Comparisons between SP-A-treated lambs and SP-C-surfactant-treated animals were made with two-tailed *t* tests. Significance was accepted at *p* < 0.05.

RESULTS

Lung Function

The seven lambs randomized to SP-C surfactant treatment weighed 3.4 ± 0.2 (mean ± SEM) kg, and the seven lambs randomized to SP-A + SP-C-surfactant treatment weighed 3.3 ± 0.3 kg. There were no differences between the groups in cord blood gas or pH values. Values of C_{dyn} were similar for the two groups of lambs at 15 min of age (Figure 1A). After surfactant treatment, C_{dyn} increased in both groups. A V_T of 7.8 ± 0.2 ml/kg were used to maintain target P_{CO}₂ values of 45 to 60 mm Hg for both groups. Over the 6-h period of ventilation, there were no significant differences in ventilation between the SP-A + SP-C-surfactant-treated group and the SP-C-surfactant-treated group, as evaluated with the ventilation efficiency index, a measure of the efficiency of gas exchange (Figure 1B). Sequential blood gas and pH values also were not different. Lung volumes for the deflation limbs of the pressure-volume curves of the two groups of animals were similar after 6 h of ventilation (Figure 1C). Therefore, the presence of SP-A had no effect on lung function.

Surfactant Measurements

The amounts of SatPC in alveolar washes and in homogenized total lungs, measured after 6 h of ventilation, were not different in the SP-A + SP-C-surfactant and the SP-C-surfactant-treated groups (Table 1). The relative amounts of SP-B and SP-D were measured by Western blotting. There was no difference between SP-A + SP-C-surfactant-treated and SP-C-surfactant-treated animals after 6 h. The steady-state levels of mRNAs for SP-A, SP-B, SP-C, and SP-D were not affected by treatment with SP-A (Figure 2), although the increase in

Figure 1. Sequential measurements of lung function in premature lambs treated with SP-C surfactant alone or with ovine SP-A (SP-A + SP-C surfactant). (A) C_{dyn}. Initial C_{dyn} was similar for both groups. After surfactant treatment, C_{dyn} increased in both groups. (B) The ventilation efficiency index (VEI) was similar in the two study groups over the ventilation period of 6 h. (C) Deflation limbs of pressure-volume curves. After 6 h of ventilation there were no significant differences in the maximal lung volumes of the SP-A + SP-C-surfactant-treated and the SP-C-surfactant-treated animals.

mRNA for SP-D in SP-A-treated animals approached significance (*p* = 0.07).

Indicators of Inflammation

The total protein in alveolar washes was low in both study groups, and was not typical of lung injury (26) (Table 1). The total cell number in alveolar washes of the SP-A + SP-C-surfactant-treated group was increased by 4.9-fold above the cell numbers for SP-C-surfactant-treated animals (Figure 3A). The recruited cells consisted predominantly of neutrophils and macrophages/monocytes (Figure 3B). Hydrogen peroxide was measured in aliquots of 1 × 10⁶ cells, to evaluate metabolic activation. In cells from SP-A + SP-C-surfactant-treated

TABLE 1
SATURATED PHOSPHATIDYLCHOLINE, SURFACTANT PROTEIN B, SURFACTANT PROTEIN D, AND TOTAL PROTEIN IN ALVEOLAR WASHES AND WHOLE LUNGS OF VENTILATED PRETERM SHEEP

	SP-C Surfactant	SP-A + SP-C Surfactant
Sat PC, µmol/kg		
Alveolar	19.5 ± 1.4	19.6 ± 2.4
Total lung	82.0 ± 5.4	78.6 ± 6.1
Surfactant proteins (Intensity relative to control)		
SP-B	1.0 ± 0.2	1.0 ± 0.2
SP-D	1.0 ± 0.2	1.1 ± 0.1
Protein, mg/kg		
Alveolar	41.2 ± 8.3	49.4 ± 7.5

Definition of abbreviations: SatPC = saturated phosphatidylcholine; SP = surfactant protein.

Figure 2. (A) Representative S1 nuclease protection assays for SP-A, SP-B, SP-C, and SP-D mRNAs and ribosomal protein L32 mRNA. (B) Quantification for surfactant protein mRNA. Intensity of hybridization of mRNA was related to that of L32 mRNA a ribosomal protein mRNA for each reaction. SP-A treatment did not significantly change mRNAs of surfactant proteins.

lambs, the average hydrogen peroxide production was 15 μM per 10^6 cells, as compared with 42 μM per 10^6 cells in SP-C-surfactant-treated animals (Figure 3C). Stimulation with phorbol myristate acetate did not significantly increase hydrogen peroxide production. Therefore, cells from the alveolar washes of SP-A + SP-C-surfactant-treated animals produced significantly less hydrogen peroxide than did those from SP-C-surfactant-treated animals.

Cells from alveolar washes were analyzed for the expression of intercellular adhesion molecule-1 receptor (CD11b) and proteoglycan link protein (CD44), both of which molecules are involved in the vascular-to-tissue migration of neutrophils and monocytes to sites of inflammation (29). The cells from SP-A-treated animals expressed significantly higher levels of both proteins as measured by the percentages of CD11b- and CD44-positive cells (representative samples are shown in Figure 4A) and by mean fluorescence intensity (Figure 4B). The expression of endotoxin receptor CD14 was low and not different between the two study groups.

The mRNAs for IL-1 β , IL-6, IL-8, TNF- α , and IL-10 were measured in total RNA from cells recovered from BALF at 2 h, from cells recovered from alveolar washes, and from total lung homogenates after 6 h of ventilation. These mRNAs were virtually undetectable in fetal lungs at this gestational age (16). Low levels of proinflammatory cytokine mRNAs were detected in cell pellets from BALF samples after 2 h of ventilation, and no differences were observed between the two study groups of animals (Figure 5A). After 6 h of ventilation, there were significant differences between SP-A + SP-C-surfactant-treated and SP-C-surfactant-treated lambs in terms of cytokine expression in cells from alveolar washes (Figure 5B).

Figure 3. Cell numbers in alveolar washes, differential counts, and hydrogen peroxide production. (A) The total number of nucleated cells recovered from alveolar washes per unit body weight was 4.9-fold greater in SP-A + SP-C-surfactant-treated than in SP-C-surfactant-treated animals. (B) Absolute number of lymphocytes, neutrophils, and monocytes in alveolar washes. The recruited cells in SP-A + SP-C-surfactant-treated animals consisted mainly of neutrophil granulocytes. The numbers of lymphocytes and monocytes recruited to the airway of SP-A + SP-C-surfactant-treated animals were slightly higher than for SP-C-surfactant-treated animals. (C) Quantification of hydrogen peroxide in alveolar washes. Hydrogen peroxide was measured in aliquots of 1×10^6 cells with and without stimulation with phorbol myristate acetate (PMA, 5 nM). Cells of SP-A-treated animals produced significantly less hydrogen peroxide both before and after stimulation with PMA than did cells of SP-C-treated animals. * $p < 0.05$ versus SP-C surfactant.

Levels of IL-1 β and IL-8 mRNAs were significantly higher in SP-A + SP-C-surfactant-treated animals than in SP-C-surfactant-treated animals. In lung tissue, the staining intensities of IL-1 β , IL-6, and IL-8 mRNA relative to that of ribosomal protein L32 mRNA were higher in SP-A + SP-C-surfactant-treated animals than in the SP-C-surfactant-treated group (Figure 5C). The level of IL-10 mRNA did not differ in the groups, and TNF- α mRNA was not detected in either group. The pattern of mRNA levels for IL-1 β , IL-6, and IL-8 in alveolar cells at 6 h differed from the pattern found in lung tissue at 6 h. The ratio of IL-1 β mRNA in SP-A + SP-C-surfactant-treated animals to that in SP-C-surfactant-treated animals increased from 3.2 in tissue to about 6.0 in the cells from alveolar washes (Figures 5B and 5C). For IL-8, the ratio of tissue mRNA of SP-A + SP-C-surfactant-treated to that of SP-C-surfactant-treated animals was about 3.2, and this ratio rose to 8.5 for cells from the respective two groups' alveolar washes. The level of IL-6 mRNA was approximately 2.6-fold greater in the lung tissue of SP-A + SP-C-surfactant-treated animals than in that of SP-C-surfactant-treated animals. In contrast, in cells from alveolar washes the level of IL-6 mRNA was lower in SP-A + SP-C-surfactant-treated than in SP-C-surfactant-treated animals (ratio 0.5).

Lung pathology. Representative sections of lung tissue illustrate the increased numbers of inflammatory cells in SP-A + SP-C-surfactant-treated animals but show no other signs of

Figure 4. Flow-cytometric analysis of cells from alveolar washes. Cells from SP-A + SP-C-surfactant-treated animals expressed greater amounts of CD11b and CD44. The expression of CD14 was low in both groups and unchanged after SP-A + SP-C surfactant treatment * $p < 0.05$ versus SP-C surfactant.

injury (Figure 6). Inflammatory cells were scored in lung tissue and airspaces (Figure 7). There were significantly higher scores in SP-A + SP-C-surfactant-treated animals.

White blood cell count. Systemic effects of SP-A treatment were evaluated by white blood cell and differential counting of cells from cord blood and after 6 h of ventilation (Table 2). The cord blood had the typical lymphocytosis of fetal blood for both study groups. After 6 h of ventilation the number of leukocytes changed in both groups, without significant differences between the groups.

DISCUSSION

We treated preterm lambs with recombinant SP-C surfactant containing 3% ovine SP-A in order to evaluate the responses of the immature lung to SP-A treatment. This experiment was designed to initiate a proinflammatory response through a 15-min period of ventilation before surfactant treatment, so as to test whether SP-A would modulate the proinflammatory injury response of the preterm lung to ventilation. The presence of SP-A resulted in a 4.9-fold increase in cells in alveolar washes after 6 h of ventilation. The increased cells were predominantly granulocytes, expressing CD44 and CD11b adhesion molecules, that were recruited to the lungs. However, these granulocytes and the monocytes that accompanied them were not activated, because expression of the endotoxin receptor CD14 was not increased, and because hydrogen peroxide production was 3-fold lower for alveolar cells from SP-A + SP-C-surfactant-treated than for alveolar cells from SP-C-surfactant-treated lambs. Nevertheless, SP-A treatment was associated with increased mRNA levels for the proinflammatory cytokines IL-1 β , IL-6, and IL-8 in total lung, with no change in IL-10 or TNF- α mRNA levels. SP-A treatment had no effect on lung function or measured concentrations of selected surfactant proteins, or on mRNA levels for the surfactant proteins. These results demonstrate unanticipated responses of the preterm lung to SP-A.

In our experiment, we used species-specific SP-A from healthy sheep. Butanol extraction was used for purification of

Figure 5. Quantification of cytokine mRNAs. (A) Proinflammatory cytokine mRNA in cells recovered at 2 h from BALF. After 2 h of ventilation, there was only moderate expression of proinflammatory cytokine mRNAs, with no differences between the SP-A + SP-C-surfactant-treated and SP-C-surfactant-treated groups. (B) Proinflammatory cytokine mRNA levels in cells from alveolar washes after 6 h of ventilation. After 6 h there was a much higher level of proinflammatory cytokine mRNA in cells from the airways. In SP-A + SP-C-surfactant-treated animals, levels of IL-1 β and IL-6 were significantly higher than in SP-C-surfactant-treated animals. In SP-C-surfactant-treated animals the level of IL-6 mRNA was significantly higher than in SP-A + SP-C-surfactant-treated animals. (C) Expression of mRNA for IL-1 β , IL-6, IL-8, IL-10, and TNF- α in 10 μ g of total lung RNA relative to mRNA for ribosomal protein L32. In SP-A + SP-C-surfactant-treated animals the level of proinflammatory cytokines IL-1 β , IL-6, and IL-8 was significantly higher than in SP-C-surfactant-treated animals. The level of IL-10 mRNA did not differ in the SP-A + SP-C-surfactant-treated and SP-C-surfactant-treated groups. TNF- α was not detectable in either group * $p < 0.05$ versus SP-C surfactant.

the SP-A to maintain its protein function (21). On the basis of *in vitro* studies, SP-A has been labeled as either proinflammatory or antiinflammatory (11–13). Disparity in results with the protein may be partly explained by the use of SP-A from different sources and different purification methods. In some experiments, proinflammatory effects of SP-A were attributed to possible endotoxin contamination because SP-A binds endotoxin. Because the response of the immature lung to SP-A was predominantly proinflammatory, we carefully evaluated the possibility of endotoxin contamination of the SP-A used in our study. The SP-A tested negatively for endotoxin in the sensitive *Limulus* lysate assay, and we verified the earlier report that SP-A did not mask endotoxin in the assay (10). We treated three other lambs with the highest amount of *Escherichia coli* O55/B5 endotoxin that could be present in SP-A with-

Figure 6. Histology of lungs. Upper panel shows a representative section from SP-C-surfactant-treated group. Lower panel shows a section from SP-A + SP-C-surfactant-treated group. The difference was the presence of more inflammatory cells in lung tissue and airspace.

out being detected by the *Limulus* lysate assay. Lambs were also treated with the same SP-C surfactant as used in our study and which contained 0.1 ng/kg *E. coli* O55/B5 endotoxin. After 6 h of ventilation, results for most variables were similar to those for SP-C-surfactant-treated animals: the total number of cells per unit weight in alveolar washes was $1.4 \pm 0.4 \times 10^7$ cells/kg after endotoxin treatment, in comparison to $1.6 \pm 0.3 \times 10^7$ cells/kg in SP-C-surfactant-treated animals. The numbers of granulocytes were similar, at $6.5 \pm 1.0 \times 10^6$ /kg after endotoxin treatment versus $6.8 \pm 2.0 \times 10^6$ /kg in SP-C-surfactant-treated animals. The level of CD11b was 187 ± 33 mean fluorescence units (FU) in endotoxin-treated animals, versus 128 ± 43 FU in SP-C-surfactant-treated animals, and that of CD44 was 112 ± 21 FU in endotoxin-treated animals, versus 89 ± 16 FU in SP-C-surfactant-treated animals. Hydrogen peroxide production was similar, at 45 ± 4 μ M per 1×10^6 cells for endotoxin-treated animals, versus 42 ± 9 μ M per 1×10^6 cells for SP-C-surfactant-treated animals. However, the endotoxin receptor CD14 was increased on cells in the alveolar washes of endotoxin-treated animals, at 92 ± 11 FU, versus 59 ± 7 FU in SP-C-surfactant-treated animals ($p < 0.05$), and

Figure 7. Inflammatory scores in airspace and lung tissue. Treatment with SP-A + SP-C surfactant increased the inflammatory score in both lung tissue and airspace * $p < 0.05$ versus control.

the mRNA level for proinflammatory cytokines was 10-fold higher in the total lung homogenates of endotoxin treated animals than in those of SP-C-surfactant-treated animals. The responses of the preterm lung to treatment with SP-A were distinct from the responses to endotoxin.

In adult animals, SP-A functions as a potent host-defense molecule and modulator of the inflammatory responses to pathogens. Mice that are SP-A deficient are unable to successfully phagocytose and kill bacteria and viruses (6, 7). The lungs become more inflamed in response to group B streptococcus, with greater recruitment of activated granulocytes that produce higher levels of proinflammatory cytokines, including TNF- α , than is the case for the lungs of normal SP-A-sufficient mice (6). Treatment of adult SP-A-deficient mice with SP-A corrects the phagocytosis and killing defects and modulates the proinflammatory cytokine response (7).

In vitro studies have given conflicting results for the pro- and antiinflammatory properties of SP-A. Kremlev and colleagues found proinflammatory cytokine production after incubation of a monocytic cell line with SP-A (13, 30). Blau and coworkers reported that both SP-A and endotoxin stimulated the production of nitrite by AM (12). On the other hand, McIntosh and associates reported a downregulation of production of the proinflammatory cytokine TNF- α by SP-A (14), and Borron and colleagues reported that SP-A had antiinflammatory effects on lymphocytes (31). In all of these experiments, mature AM and monocytic and lymphocytic cell lines were used. There is no information about interactions of SP-A *in-vitro* with immature cells from animals.

On the basis of the effects of SP-A on mature macrophages and granulocytes, and the *in vivo* results with SP-A-deficient mice, we anticipated that SP-A treatment would suppress the

TABLE 2
WHITE BLOOD CELL COUNT AND DIFFERENTIAL COUNT AT BIRTH AND AFTER 6 h OF VENTILATION OF PRETERM SHEEP

	SP-C Surfactant	SP-A + SP-C Surfactant
Cord blood at birth, $10^3/\mu$ l		
Leukocytes	2.8 ± 0.5	2.9 ± 0.3
Lymphocytes	2.4 ± 0.2	2.3 ± 0.3
Monocytes	0.1 ± 0.1	0.2 ± 0.1
Neutrophils	0.3 ± 0.1	0.4 ± 0.2
At 6 h, $10^3/\mu$ l		
Leukocytes	2.4 ± 0.4	1.7 ± 0.3
Lymphocytes	1.4 ± 0.1	0.7 ± 0.1
Monocytes	0.4 ± 0.1	0.3 ± 0.1
Neutrophils	0.6 ± 0.2	0.7 ± 0.1

Definition of abbreviation: SP = surfactant protein.

granulocyte recruitment and decrease the proinflammatory cytokine transcription that accompanies mechanical ventilation after preterm birth. We chose to measure the cytokines IL-1 β , IL-6, IL-8, IL-10, and TNF- α because these cytokines increase in SP-A-deficient mice (7), increase in the airways of infants developing bronchopulmonary dysplasia (BPD) (1), are increased in the amniotic fluid with chorioamnionitis (17), and contribute to lung injury in adult-lung models of lung injury (32). We found that SP-A induced granulocyte recruitment and increased the mRNAs of IL-1 β , IL-6, and IL-8, but did not increase the mRNA of IL-10 or TNF- α . The hydrogen peroxide assay indicated that the granulocytes in alveolar washes were not activated. This unusual pattern of response is probably explained by the immaturity of the host-defense response of the preterm newborn (33). Granulocytes in term and preterm newborns have deficiencies in adherence, deformability, and locomotion in comparison with mature granulocytes (33), and chemotaxis of granulocytes from preterm infants to sites of inflammation is impaired irrespective of gestational age (34). Phagocytosis of opsonized particles is normal in term newborns, but the respiratory burst may be impaired (33). In most types of immunologic cell the second-messenger responses to exogenous stimuli are different from the responses measured in adult cells, partly explaining the immaturity of the host-defense response (20, 33). The present study suggests that SP-A is a potent neutrophil chemoattractant in the preterm lung. In our study a high dose of SP-A was locally administered to an immature lung with an immature immune system, in which other immunomodulatory molecules are present in low concentrations (33). SP-A functioned in the immature immune system as a potent neutrophil chemoattractant, with upregulation of the cell-surface molecules CD11b and CD44, which are involved in the recruitment of cells to sites of inflammation. The chemoattraction of neutrophils (35) and AM (36) by SP-A has previously been described *in vitro*. The present study shows that *in vivo*, SP-A is a potent neutrophil chemoattractant that efficiently recruits immature neutrophils and partially overcomes deficiencies of immature granulocytes. Our study evaluated a unique situation: the initiation of ventilation in a preterm, surfactant-deficient lung, and there is no comparable information for full-term or adult lungs.

The simplest interpretation of the findings in our study is that SP-A treatment of the preterm lung is predominantly proinflammatory and therefore harmful. However, this interpretation may be incorrect. The preterm lung has major defects in both immune and innate host defenses (1, 18). SP-A may promote host defenses as a chemoattractant by recruiting unactivated granulocytes to an alveolar environment without significant numbers of mature macrophages. Although low levels of SP-A were correlated with subsequent lung injury in preterm infants (18), there is no information about whether or how exogenous SP-A alters host defenses in the preterm newborn, or how SP-A might improve outcomes. Although supplementation of surfactant with SP-A can improve the lung function of preterm animals to a greater extent than can surfactant that lacks SP-A, these effects are modest and are not apparent in animals ventilated with sufficient PEEP (25). SP-A also can minimize inhibition of surfactant by proteinaceous pulmonary edema, but this effect will be of benefit only after severe lung injury has occurred, and injury in general suppresses SP-A synthesis (18, 37). Treatment with SP-A in our study was not associated with decreased mRNA levels for any of the surfactant proteins investigated, and TNF- α mRNA did not increase.

The function of SP-A as a neutrophil chemoattractant in a preterm human ventilated lung is of particular interest with

regard to ventilation of preterm human newborns. In these patients bacterial infections of the airways have been associated with BPD (38). Therefore, adding SP-A to surfactant treatment may overcome deficiencies in recruitment and adhesion of neutrophil granulocytes in clearing bacteria from the airways in preterm infants. SP-A treatment of the preterm lung may be beneficial because of induction of protective responses.

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CHAPTER 7

The clever fetus: responding to inflammation to minimize lung injury

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Review

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The Clever Fetus: Responding to Inflammation to Minimize Lung Injury

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Key Words

Innate immunity · Respiratory distress syndrome ·
Bronchopulmonary dysplasia · Chorioamnionitis

Abstract

Clinical and experimental information indicate that fetal exposure to inflammation can induce lung maturation. This inflammation may be chronic and indolent. We present clinical and experimental information that challenge assumptions about chorioamnionitis associated infection/inflammatory exposures to the fetus. We question the assumption that the fetal compartment is sterile, and that delivery is inevitable if chronic infection/inflammation is present. We demonstrate that the preterm fetus can develop a brisk and adequate inflammatory response, and the fetus also can quickly modulate and downregulate inflammation to prevent injury. The fetus at risk of early preterm delivery may be exposed to both chorioamnionitis and glucocorticoids. Both exposures can either increase or decrease fetal inflammatory responses depending on the timing of the exposures. The immunomodulatory ability of the fetus to fetal exposures remains an unexplored research field.

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Our goals are to challenge four assumptions about how the fetus and fetal lung cope with infection/ inflammation during development. Perhaps the most central assumption is that the fetal compartment (fetus, amniotic fluid, membranes) is sterile in the normal fetus. A second assumption is that if infection/inflammation occurs, then preterm labor and delivery is inevitable. A third assumption is that the fetal inflammatory response may be poor or inadequate because of immaturity, and a fourth assumption is that inflammation, once initiated, is likely to be progressive and to injure the fetus. The field of fetal immunology is dominated by the general concepts that the fetus is immunologically immature and will respond poorly to an immune challenge [1]. The fetus is in a protected and unique environment because the mother is 'tolerant' to the fetus as foreign and there is some maternal immune suppression (e.g. increased susceptibility to varicella infection). Fetuses in general do not make antibodies either because they are not exposed to foreign antigen or because acquired immune response mechanisms are immature or suppressed. The innate immune system also is very immature based on the levels of many modulators of innate immunity (table 1). Although quantitative levels are not available for all these factors and many others that are not listed, the levels are undetectable earlier in gestation and very low during late fetal life. Therefore, the concept that the fetus should be extremely susceptible to infection is reasonable.

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Table 1. Components of innate immune defenses in the lung: fetus relative to adult

Component of innate immunity	Preterm vs. adult
Cellular	
Alveolar macrophages	immature and decreased
Lung tissue neutrophils	decreased
Dendritic cells	probably decreased
Molecular	
Complement	decreased
Fibronectin	decreased
SP-A, SP-D	decreased
Surfactant lipids	decreased
Defensins	decreased
Lysozyme	decreased
Iron binding proteins	decreased
Mechanical	
Mucocilliary function	decreased
Cough/forced expiration	decreased

Assumption 1: The Fetal Compartment Is Sterile

The uterine cavity is directly connected to the vagina and is unlikely to be truly sterile at the time of conception. There must be very effective innate host defenses that protect the mother from ascending infection with differential expression of Toll-like receptors in the female reproductive tract [2]. Few organisms other than *Neisseria gonorrhea* frequently cause an ascending infection. After conception a mucus plug isolates the fetal compartment from the lower urogenital track, but is it really sterile and if not, how is overt infection suppressed? There is information to suggest that 'silent' colonization of amniotic fluid can occur. Two reports using PCR techniques indicate that about 10% of fluids collected from normal pregnancies at about 18 weeks of gestation for genetic amniocentesis are positive for *Ureaplasma* and other low-pathogenicity organisms [3, 4]. Using a fluorescently labeled probe for bacterial 16s ribosomal RNA, Steel et al. [5] recently reported that 25 of 29 fetuses that delivered prematurely had bacteria deep within intact fetal membranes and in some cases there was no associated inflammation. The majority of infants delivered without preterm labor or ruptured membranes had evidence of colonized membranes using this bacterial probe. Bacteria in membranes also were identified in the majority of term infants delivered by C-section without ruptured membranes. This report suggests that fetal coexistence with commensal organisms may be the rule and not the exception. More

research is needed to define if organisms are present and if they influence pregnancy outcomes. An interesting hypothesis is that the fetus may contribute to suppressing colonization/infection. One fact is for certain – the majority of infants delivering before 30 weeks gestational age have been exposed to low pathogenicity organisms and histologic chorioamnionitis [6, 7]. There presently is no way to evaluate the duration or intensity of fetal exposure to inflammation prior to preterm birth.

Assumption 2: If Infection/Inflammation of the Fetal Compartment Occurs, then Delivery Is Inevitable

When highly pathogenic organisms such as *Escherichia coli* and *Listeria monocytogenes* cause infection, rapid delivery generally occurs. However, colonization/infection of the fetal compartment with organisms of low pathogenicity may not uniformly cause preterm labor. Women that were culture positive at the time of genetic amniocentesis (and were not treated with antibiotics) did not deliver for weeks [3, 4]. It is not known for how long the organisms identified using the bacterial ribosomal RNA probe had been in the membranes or even if the inflammation was in response to the organisms identified by the probe [5]. We injected live *Ureaplasma parvum* into the amniotic fluid of fetal sheep as early as 60 days of gestation (term = 150 days) and demonstrated inflammation and culture positivity in the amniotic fluid and fetal lungs 65 days later [8]. Low-dose *U. parvum* given to fetal sheep at 80 days gestation colonized the amniotic fluid and fetal lungs and caused inflammation at term. There was no associated preterm labor or signs of fetal injury. While the sheep may tolerate infection/inflammation better than the human, these results demonstrate in principle that chronic colonization of the fetus with organisms of low pathogenicity is possible.

Assumption 3: The Fetus Will Have an Inadequate Initial Inflammatory Response to Infection

An inflammatory response depends on the fetus recognizing something as foreign. Innate immune signaling results from a family of receptors recognizing antigenetic motifs common to gram-positive, gram-negative, viral and fungal organisms – the toll like receptors or TLRs [9]. TLR signaling to initiate an inflammatory response is

Fig. 1. Responses of monocytes recovered from adult ewe and preterm fetal blood to challenge in culture with different TLR receptor agonists. The amount of hydrogen peroxide produced by 10^6 monocytes from adult sheep blood was similar for all TLR agonists. The monocytes from the preterm fetus produced less hydrogen peroxide (* $p < 0.05$ preterm vs. adult).

modulated by other components of the innate immune system that promote clearance or inactivation of organisms (table 1) and which are quantitatively deficient in the fetus. We tested the TLR signaling responses of fetal blood monocytes (fig. 1). In comparison to blood monocytes from adult sheep, the fetal monocytes produced much less peroxide when stimulated with the TLR agonists. Fetal sheep also do not respond to ovine recombinant TNF- α , which is another demonstration of a decreased inflammatory response [10]. These results are consistent with a depressed inflammatory potential.

In contrast, intra-amniotic injections of the TLR4 agonist *E. coli* endotoxin (lipopolysaccharide) induce chorioamnionitis (increased granulocytes and IL-8 in amniotic fluid and inflammatory cell accumulation in the chorioamnion) [11]. Within hours of intra-amniotic injection granulocytes appear in the lung tissue and fetal lung fluid and pro-inflammatory cytokine expression increases to high levels for about 2 days. The intra-amniotic endotoxin initiates the lung inflammation by direct contact with the airway epithelium, which propagates an injury sequence (inflammation and apoptosis followed by cell proliferation) that evolves in 5–7 days to striking lung maturation [12–14]. The fetus responds similarly to intra-amniotic IL-1, a prototypic pro-inflammatory cytokine that shares receptor homology and signaling pathways with TLR4 [15]. Therefore, the fetus can develop a prompt inflammatory response.

The severity/intensity of the inflammatory response of the preterm fetus relative to the response of the term or adult sheep has not been formally studied. However, fetal

sheep given only 10 μ g LPS have a high mortality which is similar to the lethal dose in adult sheep [16]. Following delivery, preterm and term lambs, given intratracheal endotoxin, recruited similar numbers of neutrophils and expressed similar amounts of pro-inflammatory cytokines in the lungs [17]. Therefore, the preterm certainly can mount a prompt and potentially lethal inflammatory response if endotoxin enters the systemic circulation.

The dose-response relationship for the effects of intra-amniotic endotoxin on the fetal lung tells a different story. Low intra-amniotic doses of 0.1 mg endotoxin cause some lung inflammation, increase blood platelet counts but do not induce lung maturation [18]. Doses from 1 to 10 mg endotoxin cause much more inflammation, but the amount of inflammation does not increase further with doses up to 100 mg [14]. Endotoxin has a biological half-life in amniotic fluid of about 35 h [19]. Therefore, despite very high doses and prolonged exposures, lung inflammation does not increase with time beyond about 2 days or with dose. Very large exposures of 100 mg that are 1,000 times higher than the amount needed to cause some lung inflammation do not cause enough inflammation to interfere with the lung maturation response [14]. Chronic colonization or infection of the fetal lung with *U. parvum* for many weeks also causes inflammation and lung maturation [8]. To date, fetal responses to other TLR agonists have not been evaluated. These results demonstrate that the fetus can quickly develop an inflammatory response to intra-amniotic inflammation that is not very dose sensitive. The lung inflammatory response to intra-amniotic endotoxin is not severe.

Fig. 2. Effects of chronic intra-amniotic endotoxin on fetal sheep lungs at late gestational age. Fetal sheep were exposed to 1 mg intra-amniotic endotoxin infused each day for 28 days from 80 to 108 days of gestation using an osmotic pump. The fetal lungs were assessed at 138 days (term is 150 days). The measurements are expressed relative to a normalized value of 1 for controls implanted with saline filled pumps. The lungs had increased inflammation and surfactant but no changes in lung gas volumes or alveolar numbers. Data redrawn from Kalapur et al. [22].

Assumption 4: Once Fetal Inflammation Has Begun It Will Progress and Injure the Fetus

A single dose of intra-amniotic endotoxin causes acute inflammation that resolves over several weeks. The residual effects on the fetal lung are microvascular injury, decreased alveolar septation and functional lung maturation [20]. The anatomic abnormalities are not progressive because alveolar number is normal at term or at 2 months postnatal age [21]. However, a prolonged exposure to chronic indolent chorioamnionitis is probably the more common clinical situation [6]. Therefore, we modeled prolonged fetal endotoxin exposure using either Alzet osmotic pumps to deliver 1 mg/day of endotoxin into the amniotic fluid for 28 days (gestational age 80–108 days) or 4 intra-amniotic injections of 10 mg LPS given at 100, 107, 114 and 121 days [22]. Fetal lungs were assessed between 130 and 145 days of gestation. Our primary question was if chronic exposure to endotoxin injured the fetus or caused progressive lung injury. We found no effects on cord blood gas values, birth weights or plasma cortisol values, indicating no obvious effects on the fetus. Although a single dose of endotoxin or a 28-day intra-amniotic exposure inhibit alveolar septation following preterm delivery [23], the fetal lungs had healed and had no significant decreases in alveolar numbers and no markers of vascular injury when delivery occurred close to term [22]. The surfactant pool sizes were greatly increased and low-grade lung inflammation (increased inflammatory cells, IL-8 mRNA and IL-1 β mRNA) was present until term (fig. 2). These experiments demon-

strate that the fetus did not develop progressive inflammation/injury in response to chronic exposures to large amounts of endotoxin. Similarly, chronic colonization of the fetal sheep lung with *U. parvum* for periods as long as 65 days did not cause much injury [8]. If the fetus were truly immune naïve and immune deficient, then these exposures would be anticipated to cause progressive injury. Clinical experience is consistent with the response we find in sheep. Tracheal aspirates collected soon after birth from many preterm infants contain multiple indicators of inflammation, presumably in response to chronic indolent chorioamnionitis [24]. However, these preterm infants seldom have pneumonia. More commonly they have less RDS and lung maturation despite the inflammation. These experiments demonstrate that the fetus can 'control' or 'downregulate' inflammation. The fourth assumption that fetal inflammation will be progressive and injure the fetus may not be true in many cases – except for the small number of preterm babies with sepsis [25].

How Does a Fetus Control Inflammation?

There is very little information about how a fetus responds to infection/inflammation other than clinical-pathologic descriptions, which focus on invasive organisms known to cause fetal injury and death (e.g. cytomegalovirus, *Treponema pallidum*, *Listeria monocytogenes*). The much more common occurrence of preterm delivery after chronic, indolent chorioamnionitis caused by low

Fig. 3. Hydrogen peroxide production by blood monocytes challenged in vitro with endotoxin. The amount of hydrogen peroxide production by 10^6 blood monocytes from preterm fetal sheep was less than for adult sheep. Maternal treatment with betamethasone initially suppressed and then stimulated the response to endotoxin. Intra-amniotic endotoxin also suppressed and then stimulated monocyte responses to a second challenge to endotoxin in vitro response to endotoxin. * $p < 0.05$. Data redrawn from Kramer et al. [29] and Kramer et al. [27].

pathogenic organisms, remains to be studied. The clinical context for adaptation to inflammation is the concept of innate immune tolerance, often referred to as endotoxin tolerance. Mice and man exposed to an agonist such as endotoxin have brisk inflammatory responses, but second or subsequent exposures soon after the first exposure cause less or no systemic responses (fever, cytokine expression, granulocyte responses) by mechanisms that are not well understood [26]. Duncan et al. [16] demonstrated that fetal sheep become tolerant to an intravascular dose of 1 μ g endotoxin. We asked if chorioamnionitis caused by intra-amniotic injections induced an endotoxin tolerance-type response in blood monocytes of fetal sheep. After intra-amniotic injections of endotoxin, we recovered blood monocytes 3, 7 and 14 days later [27]. One group of fetal sheep received intra-amniotic endotoxin injections 14 and 7 days before blood monocytes were isolated, placed in culture and challenged again with endotoxin. A single exposure to intra-amniotic LPS suppressed monocyte production of peroxide and IL-6 at 3 days, demonstrating an endotoxin tolerance response. However, by 14 days, the blood monocyte had increased responsiveness to a second endotoxin challenge in vitro. The immature preterm monocyte seems to have matured to function more like an adult monocyte (fig. 3). However, a second fetal exposure 7 days after the first exposure again caused an endotoxin tolerance-type response. These results demonstrate that the fetus has the capacity to down regulate inflammation.

In clinical practice, the fetus at risk of preterm delivery is likely to be exposed to inflammation and to maternal corticosteroids. Maternal corticosteroids suppressed the initial inflammatory responses in fetal sheep lungs to intra-amniotic endotoxin [28]. However, 5 or 15 days after the simultaneous exposure to maternal corticosteroids and intra-amniotic endotoxin, the lung inflammatory response was increased. We interpret this increased response to the maturation of inflammatory cell function by 7 days [29]. There is no information about how corticosteroids might modulate inflammatory responses in chronic chorioamnionitis.

New clinical observations about fetal exposure to infection and inflammation together with our initial studies of fetal lung and blood monocyte responses to acute and chronic chorioamnionitis indicate that common assumptions about fetal responses to inflammation may be wrong. Far more needs to be learned about how the fetus copes with a chronically inflamed environment.

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CHAPTER 8

Intratracheal endotoxin induces systemic inflammation in ventilated preterm lambs

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Intratracheal Endotoxin Causes Systemic Inflammation in Ventilated Preterm Lambs

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Intratracheal endotoxin causes acute inflammation in the adult lung, and injurious styles of mechanical ventilation can result in systemic inflammation derived from the lungs. We asked how ventilated premature and near-term lungs responded to intratracheal endotoxin and if systemic inflammation occurred. Lambs delivered at 130 d gestational age (GA) were treated with surfactant or surfactant plus endotoxin (0.1 mg/kg or 10 mg/kg) (*Escherichia coli*, serotype O55:B5) and were ventilated for 6 h. Both endotoxin doses resulted in impaired gas exchange and systemic inflammation in the preterm lambs. Lambs at 141 d GA (term 146 d) were given either 10 mg/kg intratracheal endotoxin, 10 mg/kg endotoxin plus high tidal volume ventilation for the first 30 min of life, or 5 μ g/kg endotoxin given intravenously. Endotoxin alone (10 mg/kg) caused lung inflammation but no systemic effects after 6 h of ventilation. Lambs given 10 mg/kg endotoxin plus high tidal volume ventilation or 5 μ g/kg endotoxin intravenously had decreased gas exchange and systemic inflammation. Endotoxin was detected in the plasma of lambs at 130 d GA but not at 141 d GA. Inflammation in the lungs was more severe in preterm animals. Mechanical ventilation of the endotoxin-exposed preterm lung resulted in systemic effects at a low endotoxin dose and without high tidal volume ventilation.

Keywords: lung injury; bronchopulmonary dysplasia; neutrophils; cytokines

Endotoxin administered intratracheally causes an inflammatory lung injury in adult animals. Intratracheal endotoxin results in the rapid recruitment of activated white cells to the lungs and the production of tumor necrosis factor- α (TNF- α) and other proinflammatory cytokines (1). In adult animals responses to intratracheal endotoxin are localized to the lungs unless the lungs are ventilated with injurious ventilation styles (2). A leak of endotoxin from the airspaces to the systemic circulation and increased plasma cytokines were demonstrated in adult rabbits ventilated with high tidal volumes and no positive end-expiratory pressure (PEEP) (3).

Little is known of the responses of newborn lungs to intratracheal endotoxin. The term or preterm newborn lung may be exposed to endotoxin or other proinflammatory products as a result of chorioamnionitis and microorganisms in amniotic fluid (4). The term lung differs from the adult lung because it is structurally immature and has very few resident macrophages (5). The preterm lung has more structural immaturity and surfactant deficiency and is more easily injured by mechanical ventilation (6–8). The preterm fetal lung does not have increased

permeability to protein relative to the mature lung (9). Therefore, the increased permeability that occurs in ventilated preterm lungs is thought to result from the effects of nonuniform inflation on a structurally immature and surfactant-deficient lung that contains insufficient collagen and elastin (10, 11). The immature lung also has decreased host defense surfactant protein (SP)-A and SP-D and has immature immune systems with depressed second messenger responses making it potentially more susceptible to endotoxin-induced injury (12, 13). Therefore, we hypothesized that intratracheal endotoxin would injure 130-d gestational age (GA) newborn lambs more than near-term lambs. We assessed the effects of mechanical ventilation on the endotoxin-induced inflammation in the immature and near-term lung and the implications for systemic inflammation.

METHODS

Study Design

We evaluated the effects of intratracheal endotoxin on the lungs and systemic responses in lambs with two protocols. We delivered preterm lambs at 130 d GA and near-term lambs at 141 d GA from Suffolk ewes bred to Dorset rams (term gestation is 146 ± 1 d) (Figure 1). The protocols were approved by the Animal Care and Use Committee of the Children's Hospital Research Foundation. Based on our previous experiences with ventilation-induced lung injury in the preterm at 130 d GA (14–16), we gave surfactant before initiating ventilation and used a PEEP of 4 cm H₂O and continuous tidal volume monitoring and an initial target Pco₂ of 50 mm Hg to minimize lung injury. We maintained tidal volume (V_T) at about 10 ml/kg even if the Pco₂ increased during the 6 h of ventilation. We randomized the 130-d GA lambs to a high dose of 10 mg/kg endotoxin or a lower dose of 0.1 mg/kg endotoxin based on our observations that doses as high as 100 mg of endotoxin given by intraamniotic injection did not harm the sheep fetus and doses less than 1 mg/kg did not consistently induce lung maturation in the fetal sheep (17, 18).

The near-term animals were randomized to receive a saline placebo or the same high dose of 10 mg/kg of endotracheal endotoxin at birth as given to the preterm lambs. Other term lambs received this dose together with high V_T ventilation for 30 min after birth to evaluate if a brief period of increased lung stretch would alter the response to endotoxin. A separate group of near-term lambs was given intravascular endotoxin (5 μ g/kg) to characterize the systemic response to the endotoxin. The near-term lambs were not surfactant treated.

Delivery, Endotoxin, and Ventilation

All animals were delivered by Cesarean section, and after exposure of the fetal head and neck, an endotracheal tube was tied into the trachea (16). The fetal lung fluid that could be easily aspirated by syringe was removed, and the lambs were delivered and weighed. The lambs then received either the endotracheal saline placebo or endotoxin before the initiation of mechanical ventilation. The 130-d GA lambs received the endotoxin mixed with 100 mg/kg surfactant (Venticute; Byk Gulden, Konstanz, Germany) containing 2% recombinant human SP-C in phospholipids (19). All animals were ventilated with time-cycled and pressure-limited infant ventilators (Sechrist Industries, Anaheim, CA) with the following initial ventilator setting: fraction of inspired oxygen (F_IO₂, 1.0; rate: 40 breaths/min; inspiratory time: 0.6 s; PEEP: 4 cm H₂O; peak inspiratory pressure (PIP) sufficient to yield a target V_T of 10 ml/kg but with pressure limited to 35 cm H₂O. V_T was monitored continuously (CP-100; Bicore Monitoring

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Figure 1. Study design for preterm lambs delivered at 130 d GA and near-term lambs delivered at 141 d GA. Number of animals per group is given in parentheses. All animals were ventilated for 6 h. Interventions are indicated at birth and in the first 30 min of life by arrows.

Systems, Anaheim, CA). Ventilation pressure and FiO_2 were adjusted to achieve a target $PaCO_2$ of 45–55 mm Hg and a PaO_2 of 150–200 mm Hg. For the 141-d GA lambs that were ventilated to a V_T target of 25 ml/kg (high V_T group) for 30 min, supplemental CO_2 was added to the ventilatory circuit to normalize the $PaCO_2$. A 5 F catheter was advanced into the aorta via an umbilical artery, and a 10 ml/kg transfusion of filtered fetal blood collected from the placenta was administered within 10 min of delivery.

Dynamic compliances were calculated from V_T measured with a pneumotachometer that was normalized to body weight and divided by the ventilatory pressure ($PIP - PEEP$) (14, 19). The ventilatory efficiency index (VEI) was calculated as $VEI = 3,800 \div (\text{respiratory rate} \times [PIP - PEEP] \times PaCO_2)$, where 3,800 is a CO_2 production constant ($[ml \times mm\ Hg] \div [kg \times min]$) (20). Complete blood cell counts and differentials were performed for cord blood and for peripheral arterial blood at 2, 4, and 6 h. The arterial catheter was used for blood gas, pH, heart rate, and blood pressure recording and was infused with 10% dextrose (100 ml/kg/d). Rectal temperature was monitored and kept at 38–39° C with heating pads and radiant heat. Supplemental ketamine (10 mg/kg intramuscularly) and acepromazine (0.1 mg/kg intramuscularly) suppressed spontaneous breathing. After 6 h each animal was deeply anesthetized with 25 mg/kg pentobarbital intravenously and ventilated briefly with 100% oxygen. The endotracheal tube was clamped for 3 min to permit oxygen absorption.

Lung Gas Volume at 40 cm H_2O and Lung Processing

The thorax was opened, the lungs were inflated with air to 40 cm H_2O pressure for 1 min, and maximal lung volume was recorded (14). Lung tissue of the right lower lobe was frozen in liquid nitrogen for RNA isolation. Bronchoalveolar lavage was performed on the left lung by filling with 0.9% NaCl at 4° C until visually distended, and the lavage was repeated five times (14, 19). The bronchoalveolar lavage fluids (BALF) were pooled and aliquots saved for determination of total protein, cell number, and cell differential. Cell pellets were used for hydrogen peroxide assay and RNA isolation. Total protein in BALF was measured (21).

Alveolar Cells

BALF was centrifuged at $500 \times g$ for 10 min, and the cells in the pellets were counted using trypan blue. Differential cell counts were performed on stained cytopsin preparations (Diff-Quick; Scientific Products, McGaw Park, IN). Activation of the cells recruited to the airways was assessed by measuring hydrogen peroxide using an assay based on the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) by hydrogen peroxide under acidic conditions (Bioxytech H_2O_2 -560 assay; OXIS International, Portland, OR) (18).

Cytokine Messenger RNA

Total RNA was isolated from the right lower lung lobe, from BALF cells, and from the spleen by guanidinium thiocyanate-phenol-chlo-

roform extraction (22). Spleen tissue was used to evaluate if the endotoxin induced a systemic inflammation. RNase protection assays were performed using RNA transcripts of ovine interleukin (IL)-1 β , IL-6, IL-8, IL-10, and tumor necrosis factor- α (TNF- α) that was described previously (18, 22). Ovine ribosomal protein L32 was the reference RNA. Densities of the protected bands were quantified on a phosphor imager using ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA).

Plasma, Endotoxin, and Cytokines

Endotoxin was quantified in plasma after 6 h ventilation with the Limulus amoebocyte lysate assay (Bio Whittaker, Walkersville, MD). Enzyme-linked immunosorbent assays (ELISA) were run for IL-6 and IL-8 with antibodies from Chemicon (Temecula, CA), and recombinant proteins were kindly provided by CSIRO (Parkville, Australia) (16).

Data Analysis

Results are given as means $\pm$ SEM. Comparisons between endotoxin groups at each gestational age were by analysis of variance with Student-Newman-Keuls tests used for post hoc analyses. Comparison between 141 d GA and 130 d GA was by two-tailed t tests for control and 10 mg/kg endotoxin groups. Significance was accepted at $p < 0.05$.

RESULTS

130-d GA Lambs

The 130-d GA lambs averaged 3.5 ± 0.3 kg and mean weights were similar for the lambs in each group. In comparison with the control lambs, the lambs that received 0.1 or 10 mg/kg endotoxin and were ventilated with similar V_T had higher P_{CO_2} values and decreased oxygenation after 6 h ventilation (Table 1). Compliance and lung gas volumes were not changed by endotoxin exposure. Gas exchange as measured by the VEI and blood pressure decreased in the 10 mg/kg endotoxin group (Figure 2). Heart rate increased for both endotoxin-exposed groups. Protein in the BALF was increased from 69.6 ± 8.4 mg/kg for control lungs to 86.3 ± 5.1 mg/kg for the 10 mg/kg endotoxin group ($p < 0.05$). Therefore, the endotoxin resulted in systemic effects without a large effect on lung mechanics.

The low and high doses of endotoxin caused inflammation in the lungs of the 130-d GA lambs. Neutrophils and hydrogen peroxide production by cells in the BALF were greatly increased (Figure 3), although there were no consistent changes in lymphocytes or macrophage numbers in the BALF (data not shown). Cytokine mRNA for IL-1 β , IL-6, and IL-8 increased in both cell pellets and lung tissue, with qualitatively higher mRNA expression for the higher dose of endotoxin (Figure 4).

Endotoxin could be quantified in the plasma of these ventilated preterm lambs (Figure 5). The intratracheal endotoxin at either dose also decreased peripheral neutrophils to very low numbers. Large increases in mRNA for IL-1 β , IL-6, and IL-8 were detected in the spleens of the lambs exposed to 10 mg/kg endotoxin (Figure 6). Plasma IL-6 and IL-8 levels also were increased in response to either dose of endotracheal endotoxin.

141-d GA Lambs

There were no differences in the mean birth weight of 4.4 ± 0.2 kg between the groups of lambs. Lung mechanics and gas exchange after 6 h of mechanical ventilation were similar for control lambs and lambs given 10 mg/kg endotracheal endotoxin (Table 1). These lambs had a mean V_T of 9.4 ml/kg at 30 min of age. In contrast the lambs that were ventilated with a V_T of 24 ± 2 ml/kg at 30 min of age and were subsequently ventilated with a mean V_T of 10 ml/kg had respiratory failure at 6 h of age. These lambs had higher ventilatory pressure requirements, decreased oxygenation, decreased gas exchange as indicated by the VEI, and decreased lung compliance and

TABLE 1. PULMONARY FUNCTION AFTER 6 h OF MECHANICAL VENTILATION

	Lambs at 130 d GA			Lambs at 141 d GA			
	Control	0.1 mg/kg	10 mg/kg	Control	10 mg/kg	10 mg/kg + High V _T	5 µg/kg Intravenously
PIP - PEEP, cm H ₂ O	19.3 ± 1.1*	21.3 ± 1.7	25.0 ± 1.3*†	13.2 ± 1.2	12.5 ± 2.2	21.5 ± 4.6†	21.3 ± 2.6†
V _T , ml/kg	9.1 ± 0.9	11.1 ± 1.2	11.1 ± 1.2	9.9 ± 0.7	11.5 ± 0.3	10.1 ± 0.8	11.7 ± 1.4
P _{CO} ₂ , mm Hg	53.1 ± 2.0*	70.5 ± 9.3†	75.8 ± 12.1*†	37.8 ± 2.6	34.5 ± 3.5	47.4 ± 2.5†	44.4 ± 3.3
pH	7.28 ± 0.02*	7.08 ± 0.03†	7.08 ± 0.06*†	7.37 ± 0.01	7.39 ± 0.01	7.26 ± 0.04†	7.34 ± 0.03
P _O ₂ /F _I O ₂	297 ± 36	78 ± 34†	31 ± 6*†	370 ± 62	408 ± 16	128 ± 70†	177 ± 31†
Compliance, ml/kg · cm H ₂ O	0.46 ± 0.03*	0.52 ± 0.05	0.46 ± 0.03	0.78 ± 0.08	0.98 ± 0.12	0.51 ± 0.07†	0.53 ± 0.14
VEI	0.097 ± 0.009*	0.070 ± 0.014	0.055 ± 0.009*†	0.215 ± 0.022	0.252 ± 0.051	0.102 ± 0.027†	0.103 ± 0.014†
Lung volume at 40 cm H ₂ O, ml/kg	38.0 ± 2.0*	36.1 ± 4.8	37.4 ± 3.9*	66.6 ± 4.3	84.3 ± 6.3†	53.8 ± 5.3†	44.2 ± 9.0†

Definition of abbreviations: F_IO₂ = fraction of inspired oxygen; GA = gestational age; PIP - PEEP = ventilatory pressure (peak inspiratory pressure - positive end-expiratory pressure); VEI = ventilatory efficiency index; V_T = tidal volume.

* p < 0.05 versus corresponding group of lambs at 141 d GA.

† p < 0.05 versus control.

lung volumes. The magnitude of the effect on lung function was similar to the lambs that received the intravascular endotoxin. The only lambs with decreased blood pressures were those that were endotoxin exposed and ventilated with high V_T and those exposed to intravascular endotoxin (Figure 2).

The inflammatory responses in the lungs were similar for the three endotoxin-exposed groups. There were similar increases in neutrophils and hydrogen peroxide in the BALF (Figure 3), and cytokine mRNA for IL-1β, IL-6, and IL-8 from cells in the BALF and lung tissue was increased (Figure 4). Total protein in BALF was 26 ± 1 mg/kg for the control lambs, 58 ± 5 for the lambs that received the intravenous endotoxin, 72 ± 17 for the 10 mg/kg endotoxin group, and 88 ± 20 mg/kg for the endotoxin plus high V_T group (all values greater than control, p < 0.05). Therefore, a dose of 5 µg/kg of intravascular endotoxin caused the same inflammatory response in the lungs as did 10 mg endotoxin given by endotra-

cheal injection, and the high V_T ventilation for 30 min did not alter the inflammatory response in the lungs.

The three endotoxin-exposed groups had decreased neutrophils in blood, with lower values for the animals given the high V_T for the first 30 min after birth and for the animals exposed to intravascular endotoxin (Figure 5). Endotoxin was not detected in the plasma of these 141-d GA lambs. Only the groups ventilated with the high V_T or given intravascular endotoxin had increases in mRNA for IL-1β, IL-6, or IL-8 in the spleen and had elevated cytokine levels in plasma at 6 h (Figure 6). The plasma levels of IL-6 and interferon-γ were increased in these two groups of lambs by 4 h, and no increases were detected in control or the 10 mg endotoxin groups (Figure 7). Therefore, 10 mg/kg of endotracheal endotoxin induced lung inflammation but minimal systemic effects in ventilated near-term lambs.

Figure 2. Heart rates (A) and mean blood pressures (B) after 6 h of ventilation. The control lambs at 130 d GA had lower mean blood pressures than the control lambs at 141 d GA. Endotoxin doses are indicated on the horizontal axis. Endotoxin increased heart rate and decreased blood pressure in the 130-d GA lambs. Endotoxin decreased blood pressure in the 141-d GA lambs when given by intravenous injection or when accompanied by high tidal volume (V_T) ventilation for the first 30 min of life. *p < 0.05 versus control at each GA; †p < 0.05 versus corresponding group at 141 d GA.

Figure 3. Neutrophils and production of hydrogen peroxide by cells in BALF. (A) Number of neutrophils in BALF/kg body weight. The number of neutrophils increased after endotoxin administration and high tidal volume (V_T) ventilation did not further increase the neutrophils in 141-d gestation lambs. Neutrophils were higher in lambs at 130 d GA than in lambs at 141 d GA given 10 mg/kg endotoxin. (B) Hydrogen peroxide production by cells in BALF increased after intratracheal endotoxin in 130-d and 141-d GA lambs. Cells from lambs at 130 d GA produced more hydrogen peroxide than cells from lambs at 141 d GA. *p < 0.05 versus control; †p < 0.05 versus corresponding group at 141 d GA.

Figure 4. Proinflammatory cytokine mRNA in cells from BALF and in lung tissue. (A) Cytokine mRNA in cells from BALF. Results are shown as fold increase relative to value of 1 for control groups. IL-1 β , IL-6, and IL-8 mRNA were higher after intratracheal endotoxin in all groups. High tidal volume (V_T) ventilation did not further increase the amount of mRNA in lambs at 141 d GA. (B) Cytokine mRNA in lung tissue. The mRNA for IL-1 β , IL-6, and IL-8 were increased for endotoxin-exposed groups relative to their controls. * $p < 0.05$ versus control. □ IL-1 β ; ▨ IL-6; ■ IL-8.

Comparison of Control and 10 mg Endotoxin Groups at 130 d and 141 d GA

In comparison with the control 141-d GA lambs, the control 130-d GA lambs required higher ventilatory pressures to achieve equivalent V_T , had higher P_{CO_2} values, and had lower lung gas volumes (Table 1). Therefore, the 130-d GA lungs were more immature functionally despite surfactant treatment. The 10 mg/kg dose of endotoxin increased neutrophils, increased hydrogen peroxide, and tended to increase cytokine mRNA in BALF cells and lung tissue in the 130-d GA lambs more than in the 141-d GA lambs (Figures 3 and 4). At the equivalent high dose of 10 mg/kg, the preterm lungs demonstrated more acute inflammation than did the near-term lungs. The 130-d GA lambs given 10 mg/kg intratracheal endotoxin had decreased mean blood pressures, a larger decrease in peripheral neutrophils, and endotoxin detected in the plasma, and these effects did not occur in the 141-d lambs (Figures 1 and 5). There were no increases in cytokine mRNA or in plasma cytokines in the 10 mg/kg endotoxin group at 141 d GA, whereas large increases were measured for the corresponding group at 130 d GA (Figure 6). These systemic effects of the endotracheal endotoxin were similar for doses of 0.1 mg/kg at 130 d GA. The pattern of responses indicates larger lung and systemic inflammatory responses of the ventilated preterm than the near-term lambs.

DISCUSSION

Preterm and term infants are susceptible to sepsis/pneumonia and infection acquired before birth (23). The majority of infants born before 30 wk GA have been exposed to chorioamnionitis and aspiration of infected amniotic fluid or amniotic fluid containing proinflammatory mediators is common (4). Airway samples from these infants contain increased numbers of neutrophils and proinflammatory mediators (24). To simulate the clinical situation we used intratracheal endotoxin as a proinflammatory stimulus and assessed the effect of the initiation of ventilation at birth on the lung and systemic inflammation.

Figure 5. Endotoxin in plasma and blood neutrophils. (A) Quantification of endotoxin in plasma. Endotoxin was detectable in plasma from lambs at 130 d but not in lambs at 141 d GA. (B) Neutrophils in peripheral blood at 6 h. The number of neutrophils decreased in all endotoxin-exposed groups. * $p < 0.05$ versus control; ^t $p < 0.05$ versus corresponding group at 141 d GA.

We demonstrated that the near-term lamb lung at 141 d GA can contain a very large dose of 10 mg/kg endotoxin for the 6-h study period without a significant systemic response. We found that the low dose of 5 µg/kg endotoxin given by intravascular injection caused lung inflammation similar to the 10 mg/kg endotracheal dose of endotoxin. The systemic response was similar for the low intravascular dose or the high endotracheal dose of endotoxin in the lambs ventilated with a high V_T for the first 30 min of life. The newborn lung at 141 d lost the ability to localize the inflammatory response if the lungs were stretched with the high V_T for 30 min. High tidal volumes are frequently used inadvertently with resuscitation and the initiation of mechanical ventilation in infants (11, 25). High tidal volumes injure adult animal lungs and have resulted in systemic responses to intratracheal infection or inflammatory mediators (3, 26, 27). In a recent report, ventilation of adult rats with a V_T of 42 ml/kg did not increase systemic cytokines unless the lungs were pretreated with intratracheal endotoxin (28). The high V_T ventilation did not increase the endotoxin-induced inflammation in the lamb lung at 141 d GA when compared with the normal V_T ventilation and intratracheal endotoxin group. However, the high V_T group had increased proinflammatory mRNAs in spleen and cytokines in the plasma. We did not allow the P_{CO_2} to decrease in the high V_T group because of the new information about the effects of CO_2 on lung injury (29). The near-term ventilated newborn lung can protect the systemic circulation from endotoxin unless that lung is stretched with a high V_T .

In contrast, ventilation with V_T monitoring and avoidance of hyperventilation by targeting the P_{CO_2} to 50 mm Hg for the surfactant-treated 130-d GA preterm lung resulted in a similar systemic inflammatory response to endotracheal endotoxin doses of 10 and 0.1 mg/kg. We mixed the endotoxin with the surfactant to avoid giving endotoxin to regions of the lung that did not receive surfactant and might be more easily injured with mechanical ventilation (30). Endotoxin was detected in the plasma of the preterm lambs given either dose of endotracheal endotoxin. The lung at 130 d GA did not prevent loss of endotoxin to the circulation at a dose 100 times less than the lung at 141 d GA. The clinically relevant point is that ventilation of the infected or endotoxin/inflammatory mediator-exposed preterm lung may cause

Figure 6. Proinflammatory cytokine mRNAs in spleen and cytokine concentrations in plasma. (A) Proinflammatory cytokine mRNAs in spleen were increased in 130-d lambs after either dose of endotoxin. IL-1 β , IL-6, and IL-8 mRNA were increased in the spleens of 141-d lambs after high tidal volume (V_T) ventilation plus intratracheal endotoxin or intravascular endotoxin. (B) IL-6 and IL-8 concentrations in plasma were increased in lambs at 130 d GA after either endotoxin dose and in 141-d lambs after high V_T ventilation plus intratracheal endotoxin and after intravascular endotoxin. *p < 0.05 versus control; †p < 0.05 versus corresponding group at 141 d GA. □ IL-1 β ; ▨ IL-6; ■ IL-8.

showering of the systemic circulation with inflammatory mediators and result in a systemic inflammatory response. This finding helps explain the common occurrence of a sepsis syndrome with pneumonia or chorioamnionitis in sick preterm newborns (23). A caveat to our result is that the endotoxin was given with surfactant to the preterm lambs and the endotoxin was mixed with fetal lung fluid for the term lambs that were not surfactant treated. Either technique should result in a good distribution of the endotoxin (31).

The lung is susceptible to injury during the initiation of ventilation (11, 16). The airspaces are fluid filled and the newborn must clear fluid, establish a surfactant film at the rapidly expanding surface area of the air–fluid interface, and establish the appropriate functional residual capacity. We used mechanical ventilation for this transition but limited peak inspiratory pressures to less than 35 cm H₂O and V_T to about 10 ml/kg, using a strategy that we found caused minimal lung injury with the initiation of ventilation in surfactant-treated preterm lambs (15, 16). Despite the adaptations required for transition from the fetal state to air breathing, the only systemic effect of exposure of the lamb lung at 141 d to the very high dose of 10 mg/kg endotoxin was lung inflammation that did not alter lung function and a decrease in blood neutrophils. This decrease in neutrophils probably resulted from neutrophil recruitment to the lungs (32, 33).

The lamb lung begins to alveolarize after about 110 d GA and lung volumes increase approximately linearly to term (6, 34). The preterm sheep lung at 130 d GA also is very surfactant deficient, and ventilation of the surfactant-deficient preterm lung causes nonuniform inflammation characterized by overinflation adjacent to regions of atelectasis (10). We treated the lambs with surfactant to minimize regional overdistention and to permit ventilation with lower pressures. Such a strategy decreases the bidirectional movement of protein between the alveolar and vascular spaces and decreases the protein content of lung lymph (7, 14, 35). Nevertheless, total protein was higher in BALF of the preterm than the near-term lambs

demonstrating more vascular to alveolar leak of protein. The surfactant treatment did not prevent the systemic effects of the low dose of endotracheal endotoxin probably because the surfactant treatment did not prevent some regional overdistention of the preterm lungs with an incompletely developed collagen and elastin matrix. The functional residual capacity was not measured in this experiment. However, a 10 ml/kg V_T

Figure 7. IL-6 and interferon- γ in plasma of 141-d GA lambs. (A) IL-6 concentration increased at 4 h and remained high at 6 h after intratracheal endotoxin plus high tidal volume (V_T) ventilation and after intravenous endotoxin. (B) Interferon- γ increased in the same groups at 4 and 6 h. *p < 0.05 versus values in cord blood at 0 time. ○ Control; ▽ 10 mg/kg IT; ◇ 10 mg/kg IT + High V_T; □ 5 μ g/kg IV.

applied to lung with an average total gas volume of 38 ml/kg is more likely to cause injury than the same V_T applied to the 141-d GA lungs with a total gas volume of 67 ml/kg that have uniformly distributed endogenous surfactant.

The design of this study also allows us to compare pulmonary function and lung inflammation responses of the 130-d GA and 141-d GA lambs to 10 mg/kg endotoxin given intratracheally. The 10 mg/kg dose of intratracheal endotoxin did not alter lung mechanics in the 130-d GA lungs but gas exchange deteriorated. Although we did not measure cardiac output, heart rate increased and blood pressure fell, indicating that the likely explanation for the poor gas exchange was the sepsis syndrome. The 141-d GA lambs exposed to 10 mg/kg endotracheal endotoxin maintained lung mechanics and gas exchange. This result is consistent with the lack of a sepsis syndrome and the modest inflammation in the lungs by 6 h of age. We have no explanation for the increased lung volumes after the endotoxin in the 141-d GA lambs other than the possibility that the animals randomized to that group were somewhat more mature. The lungs at 130 d had a larger inflammatory response than did the lungs at 141 d GA. The preterms recruited more neutrophils to the lungs, the cells in the BALFs produced more H_2O_2 , and more cytokine mRNA was expressed in alveolar cells and lung tissue. We had anticipated less inflammation in the preterm because of the immaturity of the immune system (36). A possible explanation is that the preterm is very deficient in the host defense proteins SP-A and SP-D (37). These proteins bind endotoxin and are known to modulate inflammatory responses in the lung (38). The surfactant that we used to treat the preterm lamb did not contain SP-A or SP-D.

The systemic inflammatory response in the lambs at 141 d GA initially ventilated with a high V_T was similar to that resulting from the low dose of 5 μ g/kg endotoxin given intravenously. However, the intravenous endotoxin resulted in a lung injury response similar to that measured with high V_T ventilation plus intratracheal endotoxin. This result in lambs at 141 d GA is similar to the observation of Wiener-Kronisch and co-workers that the adult sheep lung is more sensitive to the endothelial damage from systemic endotoxin than from intratracheal endotoxin (39). This finding suggests how systemic inflammation and lung injury can occur in preterm newborns exposed to proinflammatory mediators *in utero*. Initiation of mechanical ventilation may transmit proinflammatory mediators from the lungs into the systemic circulation, initiating a systemic inflammation that injures the lung endothelium. The lung can be both the source and the target of the systemic inflammation (40). This study demonstrates that the surfactant-treated preterm lung that is not intentionally stretched or hyperventilated can transmit proinflammatory mediators from the lung to the systemic circulation.

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CHAPTER 9

Monocyte function in preterm, near term and adult sheep

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Monocyte Function in Preterm, Term, and Adult Sheep

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ABSTRACT

The preterm infant has functionally immature monocytes. The effects of common clinical interventions and exposures that might modulate inflammation were evaluated using monocytes isolated from blood of preterm lambs [130 d gestational age (GA)], near-term lambs (141 d GA), and adult sheep. Endotoxin stimulated hydrogen peroxide production by adult monocytes, but monocytes from 130-d and 141-d GA lambs had a reduced and delayed hydrogen peroxide production. Endotoxin did not decrease apoptosis of monocytes from 130-d and 141-d GA lambs but decreased apoptosis of adult monocytes. Dexamethasone increased the phagocytosis of bacteria and apoptotic cells by adult monocytes by 35% but not by monocytes from 130-d and 141-d GA lambs. Synthetic and natural surfactants and dipalmitoylphosphatidylcholine increased phagocytosis of apoptotic cells by monocytes from preterm, term, and adult sheep.

Monocytes from preterm and term lambs differ from adult monocytes in tests of both the initiation and the resolution of inflammation. The reduced phagocytosis of apoptotic cells by monocytes from the preterm may contribute to prolonged inflammation in diseases such as bronchopulmonary dysplasia. (*Pediatr Res* 54: 52–57, 2003)

Abbreviations:

BPD, bronchopulmonary dysplasia
RDS, respiratory distress syndrome
SP, surfactant protein
GA, gestational age
FITC, fluorescein isothiocyanate
DPPC, dipalmitoylphosphatidylcholine
DMEM, Dulbecco's modified Eagle medium

Multiple immune defects have been described for monocytes from preterm and term newborn infants and animals (1–3). Monocytes from newborns have a reduced oxidative burst after phagocytosis of bacteria, and the second messenger responses to exogenous stimuli are different from the responses measured in adult cells (3, 4). Few monocytes have differentiated to macrophages in the preterm fetus, resulting in altered acute responses to inflammation and depressed immune responses (5). The normal fetus is in an environment that is normally protected from inflammation and the need to mount immune responses. Immature monocyte function may alter injury responses of fetal and newborn organs when the fetus or preterm newborn is challenged to mount an inflammatory response (5). As an example, the lung is where the preterm fetus often first encounters inflammation. Chronic indolent chorioamnionitis is frequently associated with preterm labor and increased cytokine and inflammatory cells in the lungs at birth (6–8). Chorioamnionitis and the subsequent inflammation induced by

mechanical ventilation, oxygen, and nosocomial infection promote a persistent inflammatory response that often leads to the chronic lung disease called bronchopulmonary dysplasia (BPD) (9, 10).

Bone marrow–derived monocytes enter the fetal lung from the circulation in the last trimester of the pregnancy and are probably the major population of innate immune cells in the fetal lung because the majority of monocytes do not differentiate into macrophages until after term birth (11–14). Although the total number of monocyte-derived cells in the fetal lungs are not known, very few monocytes or macrophages can be recovered by alveolar lavage. Therefore, as a model system for *in vitro* testing, we used blood monocytes from preterm, near-term, and adult sheep to characterize monocyte responses to clinically relevant stimuli that occur in preterm infants who develop BPD. Because nosocomial infection is a risk factor for BPD (15), monocyte responses to endotoxin and phagocytosis of bacteria were evaluated. Phagocytosis of apoptotic cells was tested as a functional assay for the resolution of inflammation, because apoptotic cells that are not phagocytosed become necrotic and proinflammatory (16, 17). Dexamethasone and surfactant have anti-inflammatory properties that might modulate monocyte function (18, 19). Therefore, we evaluated the

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effects of dexamethasone and surfactant on phagocytosis of apoptotic cells.

METHODS

Cell isolation from alveolar lavage fluid. The protocols were approved by the animal use committee of the Cincinnati Children's Hospital Research Foundation. Cells were isolated from fetuses or newborn lambs from the mating of Suffolk sheep with Dorset rams. The preterm [110 d gestational age (GA), 125 d GA, 130d GA] and near-term (141 d GA) lambs were delivered by cesarean section. Term newborn lambs delivered spontaneously at a gestational age of 146 ± 1 d and were studied at 3 d of age. The adult sheep were approximately 2 y of age. Each age group consisted of four animals. For estimating macrophage numbers, left and right lungs were weighed and the left lungs were lavaged five times with 4°C saline and all lavages were pooled (20). Alveolar lavage fluid was centrifuged at $500 \times g$ for 10 min, and the pellet was resuspended in PBS. Total cells were stained with trypan blue and counted. Differential cell counts were performed on cyto-spin preparations after staining with Diff-Quick (Scientific Products, McGaw Park, IN, U.S.A.).

Alveolar monocytes were distinguished from macrophages by measuring autofluorescence with an argon laser emitting at 430 nm using a FACS caliber flow cytometer (Becton Dickinson, Mountain View, CA, U.S.A.) (21). Autofluorescence measured at 530 nm was high in macrophages but low in monocytes (21). We used alveolar macrophages and blood-derived monocytes from adult ewes to establish a gate and analyze the alveolar cells from preterm lambs.

Monocyte and thymocyte isolation. Monocytes were the predominant cell population in preterm and term newborn lungs. Because sufficient numbers of cells for study could not be collected from alveolar washes, we isolated monocytes from cord blood or peripheral blood. Cord blood was collected from the umbilical arteries from animals at 130 d GA and 141 d GA after cesarean section. Blood from adult sheep was obtained by phlebotomy. The blood was diluted with PBS and layered onto a Percoll gradient (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) (22). After centrifugation for 40 min at $400 \times g$ at 20°C, the mononuclear cells were recovered, washed twice with PBS at 4°C, centrifuged for 5 min at $400 \times g$ at 4°C, and resuspended in culture media [Dulbecco's modified Eagle medium (DMEM)] supplemented with 10% heat-inactivated sheep serum (Sigma Chemical Co., St. Louis, MO, U.S.A.). Cells were counted with trypan blue used to evaluate viability and plated in culture dishes. After incubation at 37°C for 2h, nonadherent cells were removed and plates were washed twice with PBS. For estimating the monocyte number, the cells were scraped from the culture dish and differential cell counts were performed after staining with Diff-Quick. The adherent cell population was $91 \pm 2\%$ monocytes for all age groups. Thymic tissue was dissociated with a metal mesh and filtered through a cell strainer (nylon, 70 μ m; Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.) (23). Single thymocytes were cultured as described above.

Reagents. Endotoxin from *Escherichia coli*, serotype O55:B5 (Sigma Chemical Co.), was resuspended in saline and filtered through a 0.45- μ m filter. A concentration of 100 ng/mL yielded a robust response in alveolar macrophages. Dexamethasone (Sigma Chemical Co.) was solubilized in ethanol for a stock solution of 1 mmol. Dexamethasone was tested in cell culture at concentrations from 10^{-6} to 10^{-9} mol, which stimulated surfactant protein synthesis *in vitro* (24). Dipalmitoylphosphatidylcholine (DPPC; Sigma Chemical Co.) was resuspended in saline with glass beads (25). Surfactant (Ventecute; Byk Gulden, Konstanz, Germany) contains 2% recombinant human SP-C in DPPC and palmitoyloleoylphosphatidylglycerol in at a 70:30 (wt/wt) ratio and 5% palmitic acid (26). This surfactant was very effective when tested in preterm lambs and in lung injury models (27, 28). Natural sheep surfactant containing SP-A, SP-B, and SP-C was recovered from alveolar lavages of adult sheep by centrifugation (29). Lipid extracted surfactant containing SP-B and SP-C but not SP-A was made by extraction of natural sheep surfactant with chloroform-methanol (2:1). Extracts were dried by rotary evaporation and resuspended in 0.9% NaCl using glass beads (25). All surfactant and DPPC preparations were used at final concentrations of 100 μ g/mL phospholipids based on previous studies of surfactant catabolism by alveolar macrophages *in vitro* (30). All surfactant preparations tested negative for endotoxin contamination with the *Limulus* amoebocyte lysate assay (Sigma Chemical Co.).

Hydrogen peroxide production. Hydrogen peroxide was measured for 1×10^6 monocytes using an assay based on the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) by hydrogen peroxide under acidic conditions (Bioxytech H_2O_2 -560 assay, OXIS International, Portland, OR, U.S.A.). Control samples kept under identical incubation conditions were evaluated in all experiments (31).

Apoptotic cells and phagocytosis of apoptotic cells. Monocytes isolated as described were separated into aliquots containing the same cell numbers (2×10^6 /mL) for culture in six-well plates for 24 h. Endotoxin was added to separate wells to achieve a concentration of 100 ng/mL 6 h and 1 h before harvest. The control cells were cultured for 24 h in a separate well of the plate. The cells were scraped from the plates and centrifuged along with the medium at $400 \times g$ for 5 min at 4°C. The cells were washed twice with PBS and resuspended in PBS, apoptotic cells were stained with fluorescent labeled annexin V, and necrotic cells were counterstained with propidium iodide (32). The apoptotic and necrotic cells were counted by flow cytometry and expressed as a percentage of the total cell population recovered from the plates.

Apoptosis of thymocytes was induced by 10 min of UV radiation followed by 3–4 h of culture (33). Phagocytosis of apoptotic thymocytes was tested by adding 2×10^6 apoptotic thymocytes to the adherent monocytes that had been cultured for 12 h (33). Surfactant preparations at final concentrations of 100 μ g/mL phospholipids were added 15 min before apoptotic thymocytes were added to the cell cultures. The monocytes and apoptotic thymocytes were incubated for 6 h at 37°C, washed with cold PBS containing 0.5 mM EDTA, and stained with hematoxylin and eosin. Phagocytosis was evaluated by count-

ing 300 monocytes per well. Results were expressed as percentage of monocytes containing at least one ingested apoptotic cell.

Phagocytosis of *E. coli*. Monocytes were incubated with FITC-labeled *E. coli* (Sigma Chemical Co.) in the absence or presence of dexamethasone for 4 h. Monocytes were resuspended, washed three times with PBS, and divided into two equal aliquots. The total bound and ingested bacteria were measured by flow cytometry. Trypan blue (1.25 mg/mL) was added to the second aliquot to measure ingested bacteria only. Trypan blue effectively quenches fluorescence of FITC labeled *E. coli* that are surface-bound but not ingested (34).

Data analysis. Results are given as means $\pm$ SEM. Comparisons between treated groups and untreated controls were by ANOVA with Student-Newman-Keuls tests used for post hoc analyses. Significance was accepted at $p < 0.05$.

RESULTS

Numbers of alveolar monocytes and macrophages. The numbers of alveolar macrophages and monocytes identified by autofluorescence were lower in preterm and term animals than in adult animals (Fig. 1). Alveolar macrophages and monocytes increased 4-fold between 141 d GA and 3 d of age after spontaneous birth. Monocytes were the predominant cell population in the preterm and term newborn lung. In the adult lung, alveolar cells were predominantly macrophages with cell numbers almost 4-fold higher per kilogram of body weight than in 3-d-old lambs.

Monocyte responses to endotoxin. Hydrogen peroxide production by monocytes from 130-d GA lambs did not increase significantly until 24 h after endotoxin exposure (Fig. 2A). The hydrogen peroxide was increased for monocytes from 141-d GA lambs by 6 h. In contrast, endotoxin induced a large increase in hydrogen peroxide production by monocytes from adult sheep at 1 h. Production of hydrogen peroxide by monocytes from 130-d and 141-d GA lambs was reduced and delayed relative to the adult monocytes.

The percentage of cells that were apoptotic after 24 h in culture were similar for monocytes from 130-d GA and 141-d GA lambs and adult animals without endotoxin exposure (Fig. 2B). The percentage of adult monocytes that were apoptotic were reduced after incubation with endotoxin for 1 h and 6 h. This decreased apoptosis was not significant in monocytes from animals at 130 d GA and 141 d GA, although there was a trend toward decreased apoptosis for monocytes from the 141-d GA lambs. Total number of cells recovered after 24 h of culture and the percentage of necrotic cells did not change.

Dexamethasone effects on phagocytosis. Fluorescent-labeled *E. coli* were added to the cultured monocytes containing different concentrations of dexamethasone (Fig. 3A). In the absence of dexamethasone, there was no difference in the phagocytosis of fluorescent-labeled bacteria for monocytes from all age groups. Dexamethasone increased the phagocytosis of bacteria in monocytes from adult animals by 35% and did not increase phagocytosis in monocytes from 130-d and 141-d GA lambs.

Figure 1. The numbers of alveolar macrophages and monocytes per kilogram body weight were measured in lambs at the indicated gestational ages and in 3-d-old newborn and adult sheep. Monocytes were distinguished from macrophages by autofluorescence. The number of alveolar macrophages and monocytes were lower in preterm and term animals than in newborn and adult animals. * $p < 0.05$ versus adult

Figure 2. Hydrogen peroxide (H_2O_2) production and percentage of apoptotic cells after stimulation of monocytes with endotoxin. *A*, Hydrogen peroxide was measured in aliquots of 1×10^6 monocytes after stimulation with 100 ng/mL endotoxin. Monocytes from lambs at 130 d GA and 141 d GA produced less hydrogen peroxide and at later times than did monocytes from adult sheep. *B*, The percentage of apoptotic cells was determined as percentage of total cells recovered from plates after 24 h of culture and after exposure to endotoxin for 1 h or 6 h. The percentage of apoptotic cells in monocytes from adult animals decreased after stimulation with endotoxin. * $p < 0.05$ versus control

Monocytes were incubated with apoptotic thymocytes as a model of T lymphocytes for the delayed hypersensitivity reaction. The phagocytosis of apoptotic thymocytes by monocytes from 130-d GA and 141-d GA lambs was reduced relative to monocytes from adult sheep (Fig. 3B). Incubation with dexamethasone did not increase the phagocytosis of apoptotic thymocytes by monocytes from lambs at 130 d GA and 141 d GA. Dexamethasone increased phagocytosis of apoptotic thymocytes by adult monocytes.

Surfactant effects on phagocytosis of apoptotic cells. Evaluations of phagocytosis were done in the presence of natural sheep surfactant, lipid-extracted sheep surfactant, recombinant human SP-C surfactant, and DPPC. All surfactants and DPPC increased the phagocytosis of apoptotic thymocytes in the monocyte cultures (Fig. 4). There were qualitative differences in the phagocytosis with the different surfactants, probably as a result of the surfactant proteins. Natural surfactant containing SP-A, SP-B, and SP-C increased the phagocytosis more than DPPC alone in monocytes from adults and from lambs at 130 d GA and 141 d GA. Lipid-extracted surfactant containing SP-B and SP-C also increased the phagocytosis by monocytes from adult animals more than DPPC alone.

DISCUSSION

Alveolar macrophages initiate the innate inflammatory response and clear inflammation (17). We evaluated both functions in a model system using peripheral monocytes from

Figure 3. Effect of dexamethasone on phagocytosis. *A*, Monocytes were incubated with FITC-labeled *E. coli* in the presence or absence of dexamethasone. Phagocytosis of fluorescent-labeled bacteria is shown relative to the control value of 1. Dexamethasone increased the phagocytosis of fluorescent-labeled bacteria in monocytes from adult animals but not in monocytes from 130-d and 141-d GA animals (* $p < 0.05$ versus control). *B*, Results are given as percentage of monocytes that contained at least one apoptotic thymocyte. Phagocytosis of apoptotic thymocytes was lower in monocytes from lambs at 130 d GA and 141 d GA than from adult monocytes under control conditions. Incubation with dexamethasone did not increase the phagocytosis in monocytes from lambs at 130 d GA and 141 d GA but increased phagocytosis by monocytes from adult animals. * $p < 0.05$ versus control; $t p < 0.05$ versus adult

Figure 4. Effect of surfactants and DPPC on phagocytosis of apoptotic thymocytes. Phagocytosis of apoptotic cells are shown as increase over controls, which had a normalized value of 1. The addition of different surfactant preparations and DPPC at 100 μg phospholipid per milliliter of culture media increased the percentage of monocytes that phagocytosed apoptotic cells. Natural surfactant caused the largest increase. Natural surfactant increased the phagocytosis more than DPPC alone. * $p < 0.05$ versus all other groups; $t p < 0.05$ versus DPPC

preterm, near-term, and adult sheep. The rationale for using monocytes from blood was that most of the cells recovered by alveolar lavage of preterm and term lambs were monocytes rather than macrophages, as has also been reported for other species (11, 12, 35, 36). A limitation to this study is the use of blood monocytes, which may differ from the monocyte pool in the fetal lung. Approximately 10% of the blood monocyte pool

express homing receptors for the lung, which are up-regulated with infection or lung injury (13). Although pulmonary intravascular macrophages contribute to lung inflammation in adult ruminants and pigs, there are no pulmonary intravascular macrophages in newborn pigs and the number is low in term lambs (37). There is no information about pulmonary intravascular macrophage populations in preterm sheep. The differences between premature, newborn, and adult immune cells are mechanistically very complex. For example, phagocytosis of opsonized particles is normal in term human newborn neutrophils; however, the respiratory burst may be impaired (1). The second messenger responses to exogenous stimuli are different for immune cells from newborn and adult cells (4).

This study was designed to evaluate monocyte functions that might be relevant to clinical exposures and interventions for BPD. BPD develops after prolonged inflammation in the lung (38). This inflammation can be initiated prenatally by chorioamnionitis or can be initiated and sustained by postnatal treatments that have proinflammatory effects such as oxygen exposure, nosocomial infection, and mechanical ventilation (31, 39, 40). The hydrogen peroxide responses of preterm and near-term monocytes to endotoxin were reduced and delayed. Endotoxin exposure did not reduce the percentage of apoptotic monocytes from lambs at 130 d GA and 141 d GA. Dexamethasone increased the phagocytosis of bacteria and apoptotic thymocytes by monocytes from adult animals but not by monocytes from lambs at 130 d GA and 141 d GA. Monocytes from lambs at 130 d GA and 141 d GA have a reduced capacity to clear inflammation by phagocytosis of apoptotic inflammatory cells. However, phagocytosis of apoptotic cells was increased by surfactant and DPPC. Our results suggest that once an inflammatory response is present, the clearance of inflammation may be prolonged as a result of a defect in the phagocytosis of apoptotic cells. Apoptotic cells become necrotic if they are not phagocytosed (16, 41), and necrotic cells release toxic contents and proinflammatory mediators that may sustain inflammation (42).

A new finding is that surfactant increased phagocytosis by monocytes from all ages of sheep. The enhanced phagocytosis of apoptotic cells may contribute to the anti-inflammatory properties of surfactant treatment (1). The phagocytosis of apoptotic cells by alveolar macrophages was previously reported to be enhanced by SP-A (43). There was a qualitative tendency for natural surfactant to enhance more effectively the phagocytosis of apoptotic cells compared with DPPC alone. The enhancement of phagocytosis by DPPC may result from the interaction of phospholipids with the phosphatidylserine receptor (44). Phosphatidylserine is exposed in the outer membrane of cells undergoing apoptosis (32), and the phosphatidylserine receptor mediates the phagocytosis of apoptotic cells (45). The enhanced phagocytosis by surfactant may be compromised in BPD because the surfactant from baboons developing BPD is deficient in SP-A and SP-D (46, 47). Increased apoptosis or necrosis of monocytes was not the reason for the reduced phagocytosis of apoptotic thymocytes.

Infants with BPD have been treated with dexamethasone to reduce inflammation and the duration of mechanical ventilation (48). We found that dexamethasone did not reduce the phago-

cytosis of bacteria by monocytes from lambs at 130 d GA and 141 d GA. In contrast, dexamethasone increased the phagocytosis of both bacteria and apoptotic cells by adult monocytes, a response similar to that described previously for alveolar macrophages from newborn rat lungs (49). The effect of dexamethasone on phagocytosis by monocytes from preterm and near-term lambs will not augment the immature immune system. The significance of this effect is unknown for humans who are being treated postnatally with dexamethasone for BPD.

We found that monocytes from preterm lambs are different from monocytes from adult sheep in both the initiation and the resolution of inflammation. There was no difference between the cells from 131- and 141-d lambs, indicating that the maturation of function is a postterm event. Inflammatory cells persisted in the lungs of preterm fetal lambs for weeks after a single exposure to intra-amniotic endotoxin (39). The reduced phagocytosis in preterm monocytes may contribute to the persistent lung inflammation after chorioamnionitis. Dexamethasone may not increase the clearance of bacteria or apoptotic cells in preterm lungs. The low amounts of surfactant and SP-A in preterm lungs and lungs developing BPD may also adversely affect phagocytosis (46, 47).

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PRETERM AND TERM MONOCYTES

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CHAPTER 10

Antenatal betamethasone changes cord blood monocyte responses to endotoxin in preterm lambs

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Antenatal Betamethasone Changes Cord Blood Monocyte Responses to Endotoxin in Preterm Lambs

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ABSTRACT

Corticosteroids are routinely administered to women at risk for preterm delivery to induce fetal lung maturation. Antenatal corticosteroids have immunomodulatory effects on fetal immune cells that are poorly understood. We hypothesized that maternal betamethasone would alter in fetal monocytes both the initiation of inflammation in response to pro-inflammatory stimuli and the resolution of inflammation by phagocytosis of apoptotic neutrophils. Preterm lambs at 124 d gestation were delivered 15 h, 1 d, 2 d, or 7 d after 0.5 mg/kg maternal intramuscular betamethasone. Monocytes from cord blood were isolated and cultured and results were compared with monocytes from preterm lambs exposed to maternal saline or monocytes from adult sheep. Phagocytosis of *Escherichia coli* was not changed, however, phagocytosis of apoptotic neutrophils was low in fetal monocytes but increased after 7 d exposure to maternal betamethasone to the level found in adult monocytes. Hydrogen peroxide production after endotoxin stimulus was significantly reduced to 7.1 ± 2.2

μmol at 5 h, $8.7 \pm 2.9 \mu\text{mol}$ at 24 h, and $4.1 \pm 1.9 \mu\text{mol}$ at 48 h versus $16.4 \pm 3.6 \mu\text{mol}$ in control animals; at 7 d, the hydrogen peroxide production increased to $74.3 \pm 19.7 \mu\text{mol}$ ($p < 0.05$, per 10^6 monocytes). IL-6 production was reduced at 15 h after maternal betamethasone but at no other time point. Maternal betamethasone initially suppressed several fetal monocyte functions, however, at 7 d, measurements of initiation and resolution of inflammation were increased to levels similar to monocytes from adult sheep. The time-dependent changes in maternal betamethasone modulation of the responses of fetal monocytes may influence immune function of the preterm lamb after delivery. (*Pediatr Res* 55: 764–768, 2004)

Abbreviations

BPD, bronchopulmonary dysplasia

GA, gestational age

Corticosteroids are potent modulators of fetal development, and antenatal corticosteroids are routinely used to decrease morbidity and mortality after preterm birth by inducing lung maturation (1). However, corticosteroids also are potent acute suppressors of immune/inflammatory responses (2). Fetal plasma corticosteroid levels are about 30% of the maternal levels after antenatal treatment with betamethasone (3). Fetal plasma corticosteroid concentrations decline rapidly within 4 h after maternal treatment with betamethasone (4). The effects of corticosteroids and their metabolites on fetal immune cells *in vivo* are poorly understood. *In vitro* exposure of fetal immune cells to corticosteroids acutely alters functions such as cell proliferation, apoptosis, phagocytosis, production of colony-

stimulating factors, and anti-inflammatory cytokine production (5–9). We have previously used cord blood–derived monocytes to study agents *in vitro* that are frequently used in the treatment of pulmonary inflammation in preterm infants (10). Similar suppressive effects on fetal immune cell function also occur after maternal corticosteroid treatments *in vivo*, and examples are decreased granulocyte chemokines and decreased lymphocyte numbers (11, 12). In contrast, Kavelaars *et al.* (11), also found enhanced natural killer cell activity in cord blood after antenatal corticosteroid exposure. The few evaluations of immune cell responses after maternal corticosteroid treatments did not control for the time interval from corticosteroid exposure to delivery. Because corticosteroids cause both immunosuppression and maturation, we hypothesized that the responses of fetal cord monocytes would change with time after maternal corticosteroid treatment. An initial suppression might be followed by a functional change that could represent reprogramming that might alter subsequent immune responses.

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Therefore, we exposed fetal sheep to a maternal corticosteroid treatment for time intervals from 15 h to 7 d. We evaluated fetal blood monocytes for phagocytosis of *Escherichia coli*, responses to endotoxin exposure by hydrogen peroxide production, and IL-6 production as the marker for fetal inflammatory response syndrome (13). The resolution of inflammation was tested by phagocytosis of apoptotic neutrophils.

METHODS

Animals. The animal component of the study was performed in Western Australia, as approved by the animal care and use committees of Cincinnati Children's Hospital Medical Center (Cincinnati, OH, U.S.A.) and the Western Australian Department of Agriculture. Lung development in sheep at 124 d GA is in the saccular phase corresponding to human fetuses at 26–29 wk of gestation (14). A single dose of 0.5 mg/kg betamethasone induces lung maturation in preterm sheep (15). Injections were given at different gestational ages to deliver all animals at 124 d GA. Date-bred Merino ewes with singleton fetuses were randomly assigned in groups of five to nine animals. A single dose of maternal betamethasone (0.5 mg/kg intramuscularly) (Celestone Chronodose, Schering-Plough Pty. Ltd., Baulkham Hills, NSW, Australia) was given 15 h, 1 d, 2 d, or 7 d before preterm delivery at 124 d GA. Control animals were concurrently given 0.9% saline injections at 15 h, 1 d, 2 d, or 7 d before delivery at 124 d GA.

Cell isolation and culture. Cord blood was collected from an umbilical artery from each fetus at the time of cesarean section. Jugular venous blood from healthy adult ewes also was collected ($n = 10$). Complete white blood counts were performed on umbilical cord blood samples. Fetal plasma cortisol concentrations were determined with a commercial RIA (Valeant Pharmaceuticals International, Costa Mesa, CA, U.S.A.). The blood was diluted with PBS and layered onto a Percoll gradient (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, U.S.A.) (16). After centrifugation for 40 min at $400 \times g$ at 20°C , the mononuclear cells and granulocytes were recovered, washed twice with PBS at 4°C , centrifuged for 5 min at $400 \times g$ at 4°C , and resuspended in culture media (Dulbecco's modified minimal media) supplemented with 10% heat-inactivated FCS (Sigma Chemical, St. Louis, MO, U.S.A.). Cells were counted using trypan blue to evaluate viability and then plated in culture dishes. After incubation at 37°C for 2 h, nonadherent cells were removed and plates were washed twice with PBS. To estimate the number of monocytes, cells were scraped from the culture dishes, and differential cell counts were performed on cytospin preparations stained with DiffQuick (Baxter Healthcare Corp., McGraw Park, IL, U.S.A.). The adherent cell population was $90 \pm 3\%$ monocytes for all treatment and control groups. Monocytes in other culture dishes were cultured overnight, and the experiments were done the next day after about 16 h in culture.

Phagocytosis of *E. coli*. After overnight incubation, monocytes were washed with PBS and new culture media was added. Monocytes were exposed to FITC-labeled *E. coli* (Sigma Chemical) for 6 h. The monocytes were resuspended, washed three times with PBS, and divided into two equal

aliquots. The total bound and ingested bacteria were measured by flow cytometry. Trypan blue (1.25 mg/mL) was added to one aliquot to measure ingested bacteria only. Trypan blue quenches fluorescence of FITC-labeled *E. coli* that are surface bound but not ingested (17).

Hydrogen peroxide production and IL-6 concentration. After overnight incubation, monocytes were washed with PBS and new culture media was added. After 6 h incubation with endotoxin (100 ng/mL, *E. coli*, serotype 055:B5, Sigma Chemical) [a dose that induces a robust response in preterm monocytes (10)], monocytes were scraped from the culture dish, counted, and assessed for viability with trypan blue. Production of hydrogen peroxide by 1×10^6 monocytes was measured with an assay based on the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) by hydrogen peroxide under acidic conditions (Bioxytech H_2O_2 -560 assay, OXIS International, Portland, OR, U.S.A.). Control samples were exposed to saline instead of endotoxin and were included in all experiments (10). Concentrations of IL-6 were determined in the supernatant of endotoxin-stimulated monocytes by an ovine-specific ELISA as previously described (18).

Induction of apoptosis and phagocytosis of apoptotic neutrophils. Neutrophils were isolated from adult animals. Contaminating erythrocytes were removed by osmotic lysis (16). Apoptosis in neutrophils was induced by 10 min of UV radiation followed by 3 h of culture (19). Apoptosis and necrosis were quantified by flow cytometry. Apoptotic cells were stained with fluorescent-labeled annexin V. Necrotic cells were stained with propidium iodide and the relative cell populations counted by flow cytometry (20). After overnight incubation, monocytes were washed with PBS and new culture media was added. Phagocytosis of apoptotic neutrophils was tested by adding $2 \times 10^6/\text{mL}$ apoptotic neutrophils to the adherent monocytes in culture dishes (19). The monocytes and apoptotic neutrophils were incubated for 6 h at 37°C and washed with ice-cold PBS containing 0.5 mM EDTA. Cells in the culture dishes were fixed and stained with hematoxylin and eosin. Phagocytosis was evaluated by counting apoptotic vesicles in 300 monocytes per well (10).

Data analysis. Results are presented as mean $\pm$ SEM. Comparisons between betamethasone-treated groups and untreated controls were by ANOVA with Student-Newman-Keuls tests used for posthoc analyses. Results also were compared with monocytes from healthy sheep. Statistical significance was accepted at $p < 0.05$.

RESULTS

Monocyte counts in complete white blood counts and plasma cortisol concentrations. Maternal betamethasone did not change the number of monocytes in umbilical cord blood in preterm lambs (Table 1). The fetal plasma cortisol concentrations were not different after maternal treatment with betamethasone (Table 1).

Phagocytosis. Although phagocytosis of *E. coli* by blood monocytes from adult sheep tended to be higher than for fetal sheep at 124 d gestation, the difference was not significant (Fig. 1A). Maternal betamethasone treatments had no effect on the

Table 1. Number of monocytes in cord blood and cortisol concentration in cord plasma

	Control	Time after maternal betamethasone treatment			
		15 h	1 d	2 d	7 d
Monocytes (per μL)	60 ± 15	35 ± 25	95 ± 35	120 ± 40	70 ± 20
Cortisol ($\mu\text{g/dL}$)	0.5 ± 0.2	0.7 ± 0.3	0.3 ± 0.1	0.4 ± 0.1	0.7 ± 0.2

Figure 1. (A) Phagocytosis of fluorescent-labeled bacteria was not different between monocytes from preterm lambs ($n = 9$) and monocytes from adult sheep ($n = 10$). Phagocytosis was not changed by exposure to maternal betamethasone. The number of measurements were at 15 h $n = 5$, at 1 d $n = 6$, at 2 d $n = 6$, and 7 d $n = 6$. (B) Phagocytosis of apoptotic neutrophils was quantified by counting apoptotic vesicles in monocytes. Results are shown as fold increase over control animals, which were set to 1. The low phagocytosis in monocytes from preterm lambs increased 7 d after maternal betamethasone to a value similar to the phagocytosis of monocytes from adult sheep ($p < 0.05$ vs control).

phagocytosis of *E. coli* by monocytes from the preterm lamb. However, phagocytosis of apoptotic neutrophils was increased in blood monocytes from adult sheep relative to the preterm lamb (Fig. 1B). The maternal betamethasone treatment did not change apoptotic neutrophil phagocytosis at 15 h, 1 d, or 2 d. However, phagocytosis increased 3-fold to be similar to the value in the adult monocytes 7 d after maternal betamethasone treatment.

Endotoxin-stimulated H_2O_2 and IL-6 production. Monocyte activation was assessed after exposure of monocytes in culture to endotoxin. Blood monocytes from adult sheep produced about three times more H_2O_2 in response to endotoxin than did the monocytes from the preterm lambs (Fig. 2).

Figure 2. Production of hydrogen peroxide after stimulation with 100 ng/mL endotoxin. Production of hydrogen peroxide was lower in monocytes from preterm animals than in monocytes from adult sheep. Exposure to maternal betamethasone decreased production of hydrogen peroxide at 15 h, 1 d, and 2 d. At 7 d after maternal betamethasone exposure, hydrogen peroxide production was increased in monocytes from preterm animals and not different from monocytes from adult sheep ($p < 0.05$ vs control).

Maternal betamethasone treatment suppressed H_2O_2 production at 15 h, 1 d, and 2 d. However, 7 d after the maternal treatment, the H_2O_2 production was similar to that of adult monocytes. IL-6 secretion by the cultured monocytes was about 2.5 times higher for adult monocytes than for preterm monocytes (Fig. 3). Maternal betamethasone treatment initially

Figure 3. Concentrations of IL-6 in culture media after stimulation with endotoxin. Concentrations were determined by ELISA. IL-6 concentrations were lower in monocytes from preterm sheep than in monocytes from adult sheep. Exposure to maternal betamethasone reduced IL-6 concentrations after 15 h ($p < 0.05$ vs control).

suppressed IL-6 after a 15 h exposure, and IL-6 was qualitatively increased to a value similar to the adult cell response at 7 d.

DISCUSSION

We used a sheep model that allowed us to evaluate monocyte responses to antenatal corticosteroid exposures that are similar in dose and duration to the clinical use of maternal corticosteroid treatments. We and others have demonstrated that monocytes and macrophages from preterm subjects have multiple differences in responses from the adult blood monocytes (10, 21–25). The effects of antenatal corticosteroids on human monocytes have not been evaluated in detail, although corticosteroids can acutely modulate inflammatory responses when cord blood monocytes are exposed to corticosteroids in culture (7, 26, 27). As would be anticipated from anti-inflammatory effects, antenatal betamethasone suppressed H_2O_2 production for 2 d and IL-6 production at 15 h. The interesting responses were the augmented phagocytosis of apoptotic neutrophils, the increased H_2O_2 , and the trend to increase IL-6 secretion by the monocytes from the 7 d corticosteroid group. These monocytes have responses that are similar to monocytes from the adult animal. With the limited information that we have, it is not clear what these changes in responses are. It is too simplistic to say that the monocytes were initially suppressed and then matured by the corticosteroid because we do not know the fate or persistence of the changed responses. However, at least for an interval of 7 d, a single corticosteroid exposure reprogrammed the responses of the fetal monocytes.

This finding adds to other effects of maternal corticosteroids on fetal immune cells. Several deficits in immune function have been reported after maternal corticosteroid treatment, such as decreased neutrophil chemotaxis (28) and decreased absolute numbers and proliferation of fetal lymphocytes with a reduced production of IL-2 (12, 29). This effect was not limited to the fetus but affected maternal lymphocytes as well (29). However, maternal corticosteroid effects are not exclusively immunosuppressive inasmuch as maternal treatment increased the activity of natural killer cells in preterm infants (11).

The effects of altered monocyte function in the fetus will depend on how other components of the immune/inflammatory system are altered by the antenatal corticosteroid exposure. There are several target organs for adverse immune/inflammatory effects in the preterm infant. The brain may be injured as a consequence of a systemic inflammatory response (30). BPD is associated with chorioamnionitis and fetal infection and antenatal corticosteroids suppress alveolar septation in the developing lung (31). Assuming that blood monocytes populate the fetal lung and differentiate to become alveolar macrophages (10, 32, 33), altering monocyte function to be more pro-inflammatory by increasing the capacity for H_2O_2 and IL-6 production could be detrimental. In a previous study, we used cord blood-derived monocytes to study agents *in vitro* that are used in the treatment of pulmonary disease in preterm infants (10). The phagocytosis of apoptotic cells by monocytes was increased in the presence of both surfactant proteins and

phospholipids. As an example of interactive phenomena resulting in the potential for increased lung injury, we reported that fetuses exposed to intra-amniotic endotoxin 30 d before preterm delivery had increased numbers of monocytes in alveolar washes and more pro-inflammatory cytokine mRNA after mechanical ventilation than did control lambs (34). This experiment demonstrates that a fetal exposure can change a postnatal immune/inflammatory response. Antenatal corticosteroids decrease respiratory distress syndrome but do not decrease BPD (1). In the meta-analysis of Crowley (1), the relative risk of BPD after antenatal steroids was 1.38 (95% confidence interval, 0.90–2.11) which was a trend toward increasing the risk of BPD. This unanticipated outcome has been explained by survival of the smaller and more marginal infants at highest risk for BPD. Banks *et al.* observed severe respiratory failure in some preterm infants after repetitive courses of antenatal corticosteroids (35). Perhaps antenatal corticosteroids can augment an inflammatory response to a second stimulus after birth and contribute to the development of BPD in some infants.

This concept of interacting signals amplifying a fetal response also has been demonstrated by the simultaneous exposure of fetal sheep to maternal corticosteroids and intra-amniotic endotoxin. Intra-amniotic endotoxin is a model of the chronic chorioamnionitis that is frequently associated with preterm birth in humans (36). Intra-amniotic endotoxin causes chorioamnionitis and lung inflammation in fetal sheep (15, 18, 37). Maternal corticosteroids suppress the acute inflammation for about 3 d after the intra-amniotic endotoxin, but there is a late inflammatory response that persists for 5–15 d (38). The late inflammation that follows the initial corticosteroid-induced suppression of inflammation may result in part from priming of the immature inflammatory response by corticosteroids.

We found that maternal corticosteroid had a biphasic immunomodulatory effect on blood monocytes in the fetal sheep—suppression of function followed by augmented function. We speculate that this augmented function represents reprogramming that could have long-term effects on subsequent immune responses in fetal and later life.

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CHAPTER 11

Endotoxin induced chorioamnionitis modulates innate immunity of monocytes in preterm sheep

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Endotoxin-induced Chorioamnionitis Modulates Innate Immunity of Monocytes in Preterm Sheep

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The preterm fetus is immune naïve and has immature innate immune function. Although the preterm fetus is frequently exposed to chorioamnionitis, the effects of exposure of the fetal lung to inflammation on innate immune responses are unknown. Using the fetal sheep model of chorioamnionitis, cord blood monocytes were isolated from preterm lambs 1 to 14 days after intra-amniotic endotoxin injection, cultured for approximately 16 hours, and challenged with endotoxin *in vitro*. Compared with monocytes from adult sheep, the preterm monocytes produced less H₂O₂ and interleukin-6, and toll-like receptor 4 expression was decreased. Three days after intra-amniotic endotoxin exposure, preterm monocyte responses to *in vitro* endotoxin challenge demonstrated decreased H₂O₂ and interleukin-6 production and decreased CD14 and major histocompatibility complex class II expression. Preterm monocyte responses 7 to 14 days after endotoxin tended to exceed those of adults and preterm control animals indicating augmented function. In contrast, a second intra-amniotic endotoxin injection 7 days after the initial endotoxin exposure suppressed monocyte function at 14 days. The fetal monocytes demonstrated patterns of responses consistent with endotoxin tolerance (immune paralysis) as well as maturation of function. Modulation of fetal innate immune responses by exposure to inflammation may alter subsequent immune adaptation after birth.

Keywords: endotoxin tolerance; fetal immunity; nosocomial infection; priming

The fetus is generally recognized as immunologically naïve, but chronic indolent chorioamnionitis caused by commensal or low pathogenic organisms occurs in the majority of pregnancies complicated by preterm labor and delivery before 30 weeks gestation (1–3). Fetal exposure to inflammation/infection or a fetal systemic inflammatory response increases neonatal morbidity and mortality and neurodevelopmental or lung injury (4–7). Approximately 30% of preterm infants with a very low birth weight also develop nosocomial infections that often have no identifiable source (8, 9). The innate immune system of the preterm is considered to be immature because multiple host defense proteins are low, immune cell numbers are low, and immune cell functions are low relative to cells from older children or adults (10).

Very little information is available about innate immune responses and their modulation in the fetus, although such responses may be central to a number of the injuries associated with preterm birth (7). Fetal exposure to inflammation might

either increase or suppress subsequent immune responses (1). Immune responsiveness could be decreased by exposure to inflammatory products resulting in the fetal equivalent of endotoxin tolerance or innate immune paralysis. For example, plasma interleukin (IL)-6 levels are strikingly decreased in fetal sheep after repetitive injections of low dose *Escherichia coli* endotoxin (11). An example of increased responsiveness is the augmented production of H₂O₂ and phagocytosis by blood monocytes in fetal sheep 7 days after maternal betamethasone exposure (12).

Chorioamnionitis induced by intra-amniotic endotoxin is primarily a pulmonary exposure for the fetus and results in lung inflammation and striking lung maturation as a result of direct contact of endotoxin or IL-1 with the fetal lung (13–15). Fetal inflammation occurs mainly in the lung with a modest systemic inflammatory response (lower granulocytes, modest plasma elevations of IL-6) (16, 17). We hypothesized that a result of the modest systemic inflammatory response to intra-amniotic endotoxin-induced lung inflammation would be a modulation of the responses of blood monocytes to a later exposure to endotoxin. Altered monocyte function could affect subsequent immune function in the fetus and newborn. To test this hypothesis, we gave fetal sheep injections of intra-amniotic endotoxin for intervals from 1 to 14 days before preterm delivery. Monocyte function was evaluated by measuring phagocytosis of *E. coli*, hydrogen peroxide production, and IL-6 production in response to endotoxin stimulation *in vitro*. Several aspects of endotoxin binding and signal transduction were explored by quantification of endotoxin receptor (CD14) expression, expression of endotoxin signal transducing receptor (toll-like receptor 4 [TLR4]) and major histocompatibility complex (MHC) class II on the monocytes.

METHODS

Animals

The animals were studied in Western Australia with approved protocols by the Cincinnati Children's Hospital (Cincinnati, OH) and the Western Australian Department of Agriculture. Time-mated Merino ewes with singleton fetuses were randomly assigned in groups of five to seven animals to receive a single dose of 10 mg endotoxin (*E. coli* 055:B5; Sigma, St. Louis, MO) by intra-amniotic injection at 1, 3, 7, or 14 days or at 7 and 14 days before cesarean delivery at 124 days gestational age (18).

Monocyte Isolation and Culture

Fetal cord blood drawn at delivery and blood from healthy pregnant ewes was used to isolate monocytes using Percoll gradients as described before (12, 19). Cells were counted using trypan blue to evaluate viability, and the cells were plated on culture dishes in RPMI 1640 media supplemented by heat-inactivated fetal calf serum. After incubation at 37°C for 2 hours, nonadherent cells were removed by washing. The adherent cell population was 89 ± 4% monocytes for all treatment and control groups. Monocytes in other culture dishes were cultured overnight, and the experiments were done after approximately 16 hours in culture.

Phagocytosis of *E. coli*

After overnight incubation, monocytes were exposed to fluorescein isothiocyanate-labeled *E. coli* (Sigma Chemicals) for 6 hours. Monocytes

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were suspended, and trypan blue (1.25 mg/ml) was added to one aliquot to measure ingested bacteria only. The total bound and ingested bacteria were measured by flow cytometry (12, 19).

Hydrogen Peroxide Production and IL-6 Concentration

After overnight incubation, monocytes were exposed to 10 and 100 ng/ml *E. coli* endotoxin for 6 hours (20). Production of hydrogen peroxide by 10^6 monocytes was measured with an assay kit (OXIS International, Portland, OR). Control samples were exposed to saline instead of endotoxin and were included in all experiments. IL-6 was measured in the cell culture medium of endotoxin-stimulated monocytes with an ovine-specific ELISA (17).

Expression of CD14, TLR4, and MHC II on Monocytes

Sheep-specific CD14 endotoxin receptor and MHC II antibodies (VMRD Inc., Pullman, WA) and a species cross-reacting anti-TLR4 antibody (BD Biosciences, San Jose, CA) were used to measure these surface receptors by fluorescence-activated cell sorter analysis using appropriate secondary antibodies. Control staining was performed with isotype antibodies and with secondary antibody alone to evaluate background fluorescence (17).

Data Analysis

Results are presented as mean $\pm$ SEM. Comparisons between endotoxin-treated groups and untreated control animals were by analyses of variance with Student-Newman-Keuls tests used for *post hoc* analyses. Results were compared with monocytes from healthy adult sheep. Statistical significance was accepted at $p < 0.05$.

RESULTS

Description of Animals

The birth weights of the animals in the control and intra-amniotic endotoxin-exposed groups were similar (Table 1). Intra-amniotic endotoxin increased total circulating white blood cells for the groups exposed 7 days or 7 and 14 days before delivery but not significantly at 14 days. Neutrophils accounted for the increase in total cell number, whereas blood monocyte numbers did not change with the *in vivo* endotoxin exposure.

Phagocytosis

Phagocytosis of *E. coli* by blood monocytes from adult sheep tended to be higher than for fetal sheep at 124 days gestation, but this difference was not statistically significant (Table 1). Intra-amniotic endotoxin had no effect on the phagocytosis of *E. coli* by cord blood-derived monocytes from the preterm lambs.

H₂O₂ Production

Endotoxin-stimulated blood monocytes from adult sheep produced more H₂O₂ than did cord blood monocytes from the control preterm lambs after stimulation with 10 or 100 ng/ml

endotoxin (Figure 1). Monocytes from adult or fetal sheep produced very little H₂O₂. Monocytes from lambs exposed to intra-amniotic endotoxin for 1 day and 3 days had suppressed H₂O₂ production when compared with monocytes from the preterm control animals. However, 14 days after the intra-amniotic endotoxin, the endotoxin-induced H₂O₂ production exceeded considerably that of both the control preterm and adult monocytes. In striking contrast to the 14-days endotoxin exposed group, H₂O₂ production was very low after fetal exposure to intra-amniotic endotoxin 7 and 14 days before the *in vitro* endotoxin challenge.

IL-6 Concentration

Endotoxin concentrations of 10 or 100 ng/ml increased IL-6 concentration in the media of cultured monocytes from adult and preterm control sheep relative to the low IL-6 concentration without endotoxin stimulation (Figure 2). A prior exposure to intra-amniotic endotoxin 3 days before delivery reduced the concentration of IL-6 in culture media after re-exposure to endotoxin *in vitro*. The IL-6 concentrations in the media of monocytes exposed to 10 or 100 ng/ml endotoxin after the fetal endotoxin exposure were similar to the concentrations of IL-6 from monocytes from adult sheep. The 7- and 14-day repeated exposures of fetuses to intra-amniotic endotoxin had no effects on endotoxin-induced IL-6 concentration compared with preterm control animals.

CD14, TLR4, and MHC II Expression

CD14 expression was not different between monocytes from control preterm lambs and monocytes from adult animals after stimulation with 100 ng/ml endotoxin (Figure 3A). Similar results were obtained with 10 ng/ml endotoxin (data not shown). CD14 expression was decreased qualitatively 1 and 7 days after intra-amniotic endotoxin exposure and significantly after 3 days. The signal-transducing molecule for endotoxin mediated cell activation, TLR4, had a different pattern of expression. TLR4 expression was higher by approximately twofold on monocytes from adult animals than those from preterm control animals (Figure 3B). The expression was increased in cord blood-derived monocytes 14 days after intra-amniotic endotoxin. The expression was not different at the other time points. MHC II expression was similar for adult and preterm control monocytes (Figure 3C). Expression was strikingly decreased 3 days after intra-amniotic endotoxin.

DISCUSSION

The temporary suppression of innate immune cell responses by endotoxin has been referred to as "endotoxin tolerance" (21, 22).

TABLE 1. DESCRIPTION OF ANIMALS

Groups	Animal No.	Birth Weight (kg)	WBCs in Cord Blood ($\times 10^9/L$)	Monocytes Cord Blood ($\times 10^6/L$)	Phagocytosis by Monocytes*
Adult	8	—	—	—	104 $\pm$ 19
Preterm control	7	2.7 $\pm$ 0.1	3.4 $\pm$ 0.3	95 $\pm$ 30	78 $\pm$ 8
1 d	7	2.8 $\pm$ 0.2	2.4 $\pm$ 0.4	300 $\pm$ 100	83 $\pm$ 9
3 d	6	2.9 $\pm$ 0.1	4.3 $\pm$ 0.6	230 $\pm$ 40	73 $\pm$ 13
7 d	6	2.7 $\pm$ 0.1	7.1 $\pm$ 0.9 [†]	150 $\pm$ 20	85 $\pm$ 14
14 d	6	2.9 $\pm$ 0.1	5.1 $\pm$ 0.5	160 $\pm$ 60	93 $\pm$ 15
7 and 14 d	5	2.9 $\pm$ 0.1	5.9 $\pm$ 0.7 [†]	280 $\pm$ 40	73 $\pm$ 12

Definition of abbreviations: WBCs = white blood cells.

* Mean fluorescent units of phagocytosed fluorescent *E. coli* by monocytes.

[†] $p < 0.05$ versus preterm control.

Figure 1. Production of hydrogen peroxide by monocytes after stimulation with 10 or 100 ng/ml endotoxin. Intra-amniotic endotoxin suppressed H_2O_2 production at 1 days, 3 days, and 7 + 14 days when compared with preterm control animals. However, at 14 days after intra-amniotic endotoxin, the H_2O_2 production exceeded that of both preterm and adult monocytes. In comparison to the group after 14 days intra-amniotic exposure, H_2O_2 production was lower after a second dose of intra-amniotic endotoxin at 7 days. Monocytes in culture media alone produced very little H_2O_2 (* $p < 0.05$ vs. endotoxin-stimulated preterm control animals; † $p < 0.05$ vs. preterm and adult endotoxin-stimulated control animals).

Endotoxin tolerance is used to describe *in vivo* models in which a normally lethal endotoxin dose can be tolerated by animals that were previously exposed to sublethal dose of endotoxin (21). Chronically catheterized preterm fetal sheep respond to intravascular endotoxin with a tolerance type response (11). Endotoxin tolerance/immune paralysis is a risk factor for multiorgan failure or sepsis in hospitalized patients (23). Preterm infants are extremely susceptible to nosocomial infections several weeks after preterm birth, at a time when they are often doing well (24). This clinical susceptibility to infection is consis-

Figure 2. Concentrations of interleukin (IL)-6 in culture media after stimulation of monocytes with 10 or 100 ng/ml endotoxin. The IL-6 concentration was approximately three times higher for endotoxin-stimulated adult monocytes than for endotoxin-stimulated preterm monocytes. Intra-amniotic endotoxin reduced the concentration of IL-6 in culture media 3 days after re-exposure to endotoxin. After 14 days of intra-amniotic endotoxin, the concentration of IL-6 increased to levels similar to monocytes from adult animals. The IL-6 concentration in culture media of monocytes from control animals was very low (* $p < 0.05$ vs. endotoxin-stimulated preterm control animals).

Figure 3. Expression of CD14, toll-like receptor 4 (TLR4), and major histocompatibility complex (MHC) II in mean fluorescent units (MFU) after stimulation with 100 ng/ml endotoxin. (A) Flow cytometry analysis of expression of receptor for complex of endotoxin and lipopolysaccharide binding protein (CD14) on cord blood monocytes after incubation with endotoxin *in vitro*. The signal was lower than in preterm control animals 3 days after intra-amniotic endotoxin exposure. (B) Expression of TLR4, the signal transduction molecule for endotoxin, on cord blood monocytes after incubation with endotoxin *in vitro*. TLR4 was higher on monocytes from adult animals than preterm control animals. The expression was increased 14 days after intra-amniotic endotoxin. (C) MHC II was similarly expressed on monocytes from preterm and adult control animals. The expression was reduced 3 days after endotoxin exposure (* $p < 0.05$ vs. preterm control animals).

tent with immune paralysis, but we are not aware of studies that address this possibility.

The responses of monocytes from fetal sheep to endotoxin are complex. The initial suppression of H_2O_2 and IL-6 and decreased CD14 and MHC II receptors are typical of endotoxin tolerance. In contrast, the increased production of H_2O_2 and IL-6 and increased TLR4 expression 14 days after endotoxin exposure indicate an augmented innate host response relative to the control fetal monocytes. However, the repeated exposure at a 7-day interval resulted in profound suppression of H_2O_2 production. A similar pattern of response occurred with the different endotoxin concentrations tested *in vitro*. Clinically, chorioamnionitis is often indolent and persists for weeks or months (2). Our results suggest that chronic fetal exposure to inflammation could cause a profound and persistent tolerance-type response and thus put these infants at risk of nosocomial infection (25).

These experiments are a first evaluation of how fetal exposure to inflammation might modulate or affect innate immune responses. The concepts of endotoxin tolerance have been developed primarily in mice and the adult human. In adult mice, endotoxin exposure decreases tumor necrosis factor- α and in-

creases IL-1 secretion by monocytes (21). The mechanisms proposed for endotoxin tolerance in humans and mice are controversial but may include downregulation of endotoxin receptors, lipopolysaccharide-binding protein, or CD14 and alterations in the signaling pathways to decrease nuclear factor- κ B nuclear translocation as well as changes in the cytokine expression profile of the cells (21).

We did not use catheterized animals to avoid the stress of fetal surgery and the potential for infection in catheterized animals. These studies of the fetal innate immune system are unique because the immune system is naive and immature. An example of differences between the preterm and the adult is the observation that the fetal sheep does not respond to ovine tumor necrosis factor- α but has systemic and lung inflammatory responses to IL-1 (13, 26). Fetal responses to IL-6 have not been evaluated. The decrease in monocyte surface receptors and suppressed function with single or repetitive fetal endotoxin exposure are consistent with an immune paralysis-type response by immature monocytes (22). The enhanced response 14 days after intra-amniotic endotoxin exposure may represent recovery from immune paralysis together with a maturation of monocyte function. A similar pattern of suppression followed by increased function occurs in response to maternal glucocorticoid exposure (12, 18). Fetal monocytes have inhibited function that resolves to resemble the function of adult monocytes by 7 days after maternal betamethasone exposure (12).

Another unique aspect of these studies is that the intra-amniotic exposure to endotoxin both induces lung maturation and impairs lung development (27–29). Lung maturation was induced in the fetal sheep by doses of endotoxin from 1 to 100 mg. Similar amounts of intra-amniotic endotoxin were used in murine models of fetal exposure to inflammation (18, 30). In contrast, intravascular endotoxin at a much smaller dose of 5 μ g/kg causes either fetal death or tolerance to repeated intravascular doses of 1 μ g/kg in fetal sheep (11). Despite the high amounts of intra-amniotic endotoxin given to fetal sheep, fetal well-being or growth were not impaired (18). The lung inflammation and maturation responses require the direct contact of endotoxin with the fetal lung, presumably by the mixing of fetal lung fluid with amniotic fluid as a result of fetal breathing (15). The intra-amniotic endotoxin caused subtle changes in fetal circulating white blood cells, but the response was not characteristic of a systemic inflammatory response (17). Nevertheless, systemic monocytes were modified to have an immune paralysis-type phenotype by fetal lung inflammation. We previously found that fetal exposure to intra-amniotic endotoxin given 30 days before preterm delivery resulted in increased monocytes and lymphocytes in the alveolar lavages of preterm lambs (31). Therefore, intra-amniotic endotoxin can alter the subsequent immune function of the lung and the systemic monocytes. A cautionary note is that monocytes/macrophages from different tissue locations can respond differently after exposure to inflammation (32). The integrated fetal responses to inflammation remain to be explored.

The long-term implications of alterations in fetal immune responses are just beginning to be explored. Recently, increased cord blood levels of IL-4 and interferon- γ were associated with less atopy and asthma (33). The indirect evidence that increased maternal antibiotic use during pregnancy increases the risk of asthma in children supports the concept that fetal immune responses may change subsequent immune responses (34, 35). This fetal sheep model will be useful for future studies of long-term effects of lung and systemic innate immune modulation after preterm and term birth.

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CHAPTER 12

Summary and Outlook

Samenvatting

Summary

Lung injury following chorioamnionitis

In **chapter 3 and 4** a cascade of lung injury, lung inflammation, lung repair with apoptosis and subsequent cell proliferation and morphologic changes following intra-amniotic endotoxin injection is described. Findings are summarized in figure 1. Within 5 hours of injection of intra-amniotic endotoxin, the fetal airways expressed heat shock protein-70 and inflammatory cells were increased in bronchoalveolar lavage fluid (**chapter 4**) (33). Within 24 hours, monocytes and granulocytes were increased many fold in the bronchoalveolar lavage, and mRNA for pro-inflammatory cytokines IL-1 β , IL-6 and IL-8 were strikingly increased in lung tissue (**chapter 3**) (34). Apoptosis of lung cells increased 24 hours after intra-amniotic endotoxin and lung cell proliferation was higher after 72 hours. The acute inflammatory responses of the fetal lung had resolved by 7 days; however, residual inflammatory cells remained in the bronchoalveolar lavage (**chapter 3**) (34).

Inhibition of microvascular development is striking in the lungs of infants with BPD (1) and placentas with chorioamnionitis (32), and parallel responses occur in the fetal sheep lungs. IP-10 and MIG, chemokines associated with inflammation and angiostasis, are expressed highly by inflammatory cells and the bronchioles (21). The lungs have decreased expression of multiple markers of vascular development (endothelial nitric oxide synthase, vascular endothelial growth factor, platelet endothelial cell adhesion molecule, and VEGF receptor 2) for 1 to 4 days after exposure to intra-amniotic endotoxin (20). By 7 days the medial thickness of arteriolar walls has increased, alveolar size has increased and alveolar number has decreased relative to controls (52).

In chapter 5, the role of transforming growth factor β -1 (TGF- β 1) was studied in the chorioamnionitis model after intra-amniotic endotoxin injection. TGF- β 1 is a key regulator of lung development, lung inflammation, wound healing and remodeling, which all contribute to the development of BPD (**chapter 5**) (35). Downstream of TGF- β 1, connective tissue growth factor (CTGF) modulates fibrosis as a result of TGF- β 1 activity and promotes in addition vascular development in mouse embryos (8). Endotoxin-in-

duced chorioamnionitis induced a considerable expression of TGF- β 1 and activated Smad signaling in the fetal lung. However, CTGF expression was markedly reduced, probably mediated by an increase in TNF- α expression. This cytokine profile may contribute to the impaired alveolar and pulmonary vascular development, absence of fibrosis and limited lung inflammation that are hallmarks of BPD (figure 2) (35, 37). Therefore, intra-amniotic endotoxin caused chorioamnionitis and altered the fetal lung anatomy similarly to the pathology of the lungs of infants with BPD (5).

Intra-amniotic endotoxin causes lung maturation

Despite fetal lung injury, the functional outcome after intra-amniotic endotoxin was lung maturation (2). The mRNA for surfactant proteins A, B, C and D were increased within 24 hours and the amounts of the surfactant proteins were increased by about 100-fold in bronchoalveolar lavages by 7 days (**chapter 3**; figure 1). In parallel with the increases in surfactant, lung function of ventilated preterm lambs improved, with large increases in lung compliance, gas exchange and oxygenation (18, 19). Of note, cord plasma cortisol did not increase in response to intra-amniotic endotoxin suggesting a mechanism of lung maturation distinct from that induced by glucocorticoids (18).

Endotoxin-induced increases in surfactant lipids and proteins are much greater than the increases achieved with corticosteroid treatments (19). The clinically relevant response of the fetal lung to chorioamnionitis is induced lung maturation, which will result in a decreased incidence and severity of RDS. In this experimental sheep model intra-amniotic doses of 1 to 100 mg *E. coli* endotoxin induced similar inflammatory and lung maturation responses (19). No lung inflammation and no subsequent lung maturation was seen with lower doses (**chapter 3**) (34). In contrast, intra-allantoic endotoxin caused no chorioamnionitis or lung responses (40). No significant amount of intra-amniotic endotoxin enters the circulation of the fetus because intravascular doses as low as 1 μ g can kill the fetus (7). Thus, the fetal response to the inflammatory mediator

Figure 1: Summary of cascade that was induced by a single dose of endotoxin from *E. coli*, injected into the amniotic fluid at 117d gestational. Lung injury with subsequent inflammation and an aberrant repair reaction was induced which resulted within 7 days in a mature lung phenotype with increased surfactant pool size but also with injury to alveologenesis and microvasculature development. This animal model yields within the 7 days of intra-uterine exposure to inflammation a mild phenotype of BPD (Summarized from chapter 3 & 4 (2, 18–20, 25, 33, 34, 52))

Figure 2: Schematic summary of cytokine effects after intra-amniotic endotoxin. Pulmonary inflammation results in increased TGF- β 1 concentrations in the fetal lung (chapter 5). TGF- β 1 is essential for the „wound remodeling or healing“ after lung injury but increased TGF- β 1 concentration impairs alveologenesis. In other models these effects are mediated in part by TGF- β 1 itself but the fibrosis is primarily mediated by CTGF. Furthermore, TGF- β 1 reduces lung inflammation. TGF- β 1 and TNF- α were induced but CTGF was decreased in this model of endotoxin-induced chorioamnionitis. The downregulation of CTGF might be mediated by TNF- α . Therefore, the reduced concentrations of CTGF might have limited fibrosis but also impaired microvasculature development since CTGF is involved in normal vascular development. Taken together, the cytokine profile may explain in an overly simplified way the findings of BPD in the fetal sheep lung.

Figure 3: Hypothesis for the impaired regulation of a fetal inflammatory response. After the initiation of inflammation by a proinflammatory stimulus neutrophils are recruited into the tissue from the blood circulation. Neutrophils undergo apoptotic cell death. The apoptotic vesicles are usually readily phagocytosed by neighbouring cells, monocytes and macrophages. If apoptotic vesicles are not taken up in a timely fashion they become necrotic which means that they burst open and spill their contents which are a pro-inflammatory stimulus on their own. The fetal lung is deficient in monocytes and macrophages and those cells are deficient in their efficiency to phagocytose apoptotic vesicles which may explain the persistence of an inflammation in a fetal or prematurely born organism (chapter 9) (31).

was dependent on the compartment with the inflammation.

How endotoxin signals lung maturation

Endotoxin induced a cascade of pro-inflammatory mediators and recruited activated inflammatory cells to the chorioamnion (**chapter 3**; figure 1) (25, 34, 39). Diffuse inflammation of the chorioamnion could release effector molecules into the fetal systemic circulation and indirectly induce lung inflammation and maturation. However, intra-amniotic endotoxin has very modest effects on circulating white blood cells and platelets (chapter 3) (18, 34). Pro-inflammatory cytokine mRNA in the fetal liver is inconsistently and minimally increased and plasma $\text{IFN}\gamma$, IL-6 and IL-8 levels are minimally increased (25, 34, 54). Also, cytokine mRNA levels in the placenta and gut do not increase appreciably (25). Therefore a diffuse fetal inflammatory response does not explain the local lung inflammation.

To identify the relationships between chorioamnionitis and the fetal lung, the fetal lungs were surgically separated from the amniotic fluid using tracheotomy and a silastic bag to collect lung fluid (39). Endotoxin in the amniotic fluid caused chorio-

amnionitis but no lung inflammation or lung maturation (39). In contrast, a 24-hour infusion of endotoxin into the fetal trachea caused lung inflammation and subsequent lung maturation without chorioamnionitis. This result demonstrated that endotoxin can cause the inflammation/maturation sequence by direct contact with the fetal lung. Therefore, tidal fetal breathing must cause mixing of fetal lung fluid with amniotic fluid, which then signals the inflammation and maturation responses in the fetal lung (39). In addition, the clearance of endotoxin in the amniotic fluid is very slow. Measurements of endotoxin activity estimated the half-life to be approximately 41 h (41).

In the clinical scenario, the mediator of inflammatory effects on the lungs could be the TLR4 agonist endotoxin, some other TLR agonist, or an inflammatory product of chorioamnionitis (**chapter 7**) (29). The mRNA for IL-1 β is the cytokine which is most increased in the chorioamnion in sheep after intra-amniotic endotoxin similar to the findings in babies exposed to chorioamnionitis (6). The original description of lung maturation responses to intra-amniotic IL-1 α in rabbits did not include an evaluation for chorioamnionitis (4).

Intra-amniotic injections of sheep recombinant IL-1 α or IL-1 β to ewes induced chorioamnionitis followed by striking lung maturation (47, 53). However, the fetus did not respond to sheep recombinant TNF α with chorioamnionitis or lung inflammation/lung maturation (16). Thus the fetal compartment seems to be exquisitely sensitive to IL-1 β , the endogenously produced IL-1, and quite unresponsive to other normally potent inflammatory cytokines.

In other experiments, an anti-CD-18 antibody blocked intra-amniotic endotoxin-mediated inflammatory cell recruitment to the fetal lungs and prevented lung maturation (23). Our current understanding is that the fetal lung responds to pro-inflammatory mediators in amniotic fluid with a recruitment of inflammatory cells, which may be the major source of IL-1 β , which in turn initiates the maturational signals. Recently, TGF- β was reported to bind SP-A which may partially explain the controversial data that were obtained with different SP-A preparations (**chapter 6**) (30,36). The link between surfactant and immunomodulation should be further studied because the immature lung is highly vulnerable by inflammation. In a set of experiments, lambs of differential gestational ages were intubated and endotoxin was given intratracheally. In very immature lambs the lung did not contain endotoxin but the endotoxin leaked into the systemic blood circulation causing a septic shock syndrome (**chapter 8**) (26). The same dose of endotoxin did not cause a systemic response in lambs close to term. The immaturity of the immune system is an essential component in this sheep model. Alveolar macrophages are not present in the fetal lung until close to term (**chapter 9**) (31). Blood-derived monocytes enter the fetal lung late in gestation, proliferate and differentiate into alveolar macrophages under the control of granulocyte-macrophage-colony stimulating factor (GM-CSF) and its downstream mediator transcription factor PU.1 (46). The monocytes from cord blood of preterm lambs are functionally less mature than monocytes from near-term lambs or adult sheep in response to proinflammatory stimuli such as endotoxin (**chapter 9**).

Glucocorticoids and chorioamnionitis

The majority of women delivering before 30 weeks of gestation have clinically silent chorioamnionitis (10), and most of these women receive antenatal corticosteroids to induce lung maturation (11). The practice of corticosteroid treatment is supported by numerous clinical reports and a meta-analysis dem-

onstrating decreased RDS, intra-ventricular hemorrhage (IVH) and death when corticosteroids are given to women with preterm rupture of membranes, a surrogate clinical indicator of chorioamnionitis (11). In preterm fetal sheep, maternal corticosteroids given 7 days before delivery do not improve lung function as effectively as intra-amniotic endotoxin. The major difference is the much larger increase in surfactant induced by endotoxin (18, 52). Maternal betamethasone causes fetal growth restriction and fetal thymic involution but intra-amniotic endotoxin does not (42). Surprisingly, intra-amniotic endotoxin prevents betamethasone-induced fetal growth restriction and thymic involution when these interventions are combined (42). Betamethasone suppressed the lung inflammation induced by endotoxin for about 2 days (22). However, 5 and 15 days after both exposures, inflammatory cells and pro-inflammatory mediators were higher in the lungs exposed to both endotoxin and betamethasone than to endotoxin alone (22). A possible explanation for this result was suggested by the observation that maternal betamethasone treatments initially suppressed the inflammatory responses of fetal monocytes but those responses were strikingly augmented 7 days after maternal betamethasone treatment (**chapter 10**) (27). Thus maternal betamethasone may augment fetal lung inflammation induced by endotoxin by "maturing" the inflammatory potential of fetal inflammatory cells (**chapter 10**). There is no experimental information about how the fetal lung will respond to betamethasone superimposed on chronic chorioamnionitis, the more usual clinical scenario.

We also evaluated the function of fetal monocytes after repetitive exposures to proinflammatory agonists (**chapter 11**) (28). The initial exposure to a non-lethal dose endotoxin for example reduced the response of the immune cells to a second usually lethal dose of endotoxin in experimental animal models (51). This phenomenon is called endotoxin tolerance and is considered as a major cause for morbidity in adult intensive care units. We found that repetitive intra-amniotic injections of endotoxin did not increase lung maturation but rendered the fetal monocytes non-responsive or inert to a second exposure to the same endotoxin (**chapter 11**) (29).

Figure 4: Multifactorial pathogenesis of BPD. Inflammation of the fetal airways may be induced in utero by chorioamnionitis. Antenatal glucocorticoids given to the mother may have anti-inflammatory effects on the pulmonary inflammation in the airways. Postnatal management and ventilation strategies may sustain the inflammation. If the preterm baby manages to control or resolve inflammation and is given the necessary conditions for repair and lung growth the symptoms of BPD may not develop or resolve (48).

Outlook - The clever fetus - controlling inflammation

Clinical experience and experimental results indicate that fetal exposure to antenatal inflammation has both good and bad effects on the fetus. Chorioamnionitis induces lung maturation but also induces a simplification of the lung development. Many organs are affected by the exposure to antenatal inflammation, which suggests that chorioamnionitis induces "a multi-organ disease of the fetus". The unifying link between all affected organs is however the immune response. Increased survival after exposure to chorioamnionitis/antenatal steroids may in part be attributed to effects on the immune system. In addition, no lung maturation was seen after initiation of chorioamnionitis if the inflammatory response was small or blocked (**chapter 3**) (23, 34). Structural changes of the fetal airways and lungs after antenatal inflammation were not apparent in later gestation, although the pulmonary inflammation persisted nevertheless (24, 38). The fetal lung is able to "heal" the inflammation induced injury if given enough time.

Inflammation is marked by the recruitment of neutrophils into the tissue (figure 3) (12). Neutrophils undergo apoptotic cell death and form apoptotic vesi-

cles within a short period of time (14). Apoptotic vesicles are usually phagocytosed quickly by neighbouring cells, monocytes or macrophages (50). If apoptotic neutrophils are not phagocytosed, they become necrotic which means that they burst open and spill their contents (13). The contents of these apoptotic neutrophils are a pro-inflammatory stimulus and may reinitiate the cascade of inflammation (figure 3).

The fetal lung is deficient in monocytes and macrophages which reduces the number of possible cells to phagocytose apoptotic neutrophils (**chapter 9**) (31, 43). The monocytes from preterm lambs are however less effective in phagocytosing apoptotic vesicles than monocytes from adult animals (**chapter 9**) (31). These two factors may explain why the fetal inflammation is resolved so poorly following intra-amniotic endotoxin. Commercial surfactant preparations seem to have anti-inflammatory properties (3, 49). Surfactant protein A was previously shown to enhance the phagocytosis of apoptotic vesicles (44, 45). Surfactant protein A and other components of surfactant increased the phagocytosis of

monocytes from preterm lambs were tested (**chapter 9**) (31). This is a new finding in the anti-inflammatory properties of surfactant and surfactant preparations.

BPD clearly is a multifactorial disease with the primary risk factors being delivery at early gestation, mechanical ventilation, supplemental oxygen exposure, and postnatal sepsis. Other than gestational age, these risk factors are pro-inflammatory (figure 4). Antenatal lung inflammation may initiate and potentiate a progressive inflammatory process that results in BPD (15). The preterm baby may recover from the initial hit by the absence from further aggravation of the existing inflammation (17). With favorable conditions for repair and lung growth the symptoms of BPD may not develop or resolve (48). There may be survival advantages by the inflammation-induced lung maturation for the fetus. The fetus must however control inflammation and avoid systemic inflammation which may be lethal (chapter 4) (26). Many factors come into play for the modulation of the inflammatory response (figure 4). Along with the immaturity of the preterm immune system (9), antenatal and postnatal factors may condition and modulate the pulmonary inflammation (48). Future research must aim at differentiating the beneficial and detrimental effects of antenatal inflammation to make prevention and therapy more effective.

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Samenvatting

Bronchopulmonaire dysplasie (BPD) is een ziektebeeld van vroeg geboren baby's, waarbij de ontwikkeling van de longen nog niet voltooid is. Vroeg geboren baby's hebben na de geboorte vaak toegenomen zuurstofbehoefte omdat het oppervlak voor de gasuitwisseling niet toereikend is om voldoende zuurstof op te nemen. De ontwikkeling van BPD is geassocieerd met een chronische ontsteking in de luchtwegen die gekenmerkt wordt door een afwijkende longanatomie zonder fibrosevorming. De oorzaak voor deze ontsteking kan antenataal zijn, bijvoorbeeld een ontsteking van de vliezen en het amnionvocht (chorioamnionitis) of postnataal, bijvoorbeeld reanimatie kort na de geboorte, zuurstoftoxiciteit, mechanische beademing of infectie.

In dit proefschrift zijn de effecten van chorioamnionitis in een proefdiermodel onderzocht. De gebruikte methode wordt beschreven in **hoofdstuk 2**. Chorioamnionitis wordt tijdens een echogeleide vruchtwaterpunctie bij zwangere schapen geïnduceerd door endotoxine van de *E. coli* bacterie in de amnionholte te injecteren. De foetale schapen aspireren de endotoxine, hetgeen een pulmonale ontsteking induceert.

In de **hoofdstukken 3 en 4** wordt de cascade beschreven die veroorzaakt wordt door intra-amniotische toediening van endotoxine: longschade, ontsteking in de longen en herstel van de longen met apoptose en vervolgens celproliferatie met morfologische veranderingen. De bevindingen zijn samengevat in figuur 1: Binnen 5 uur na toediening van het endotoxine in het amnionvocht kwam in de foetale luchtwegen heat shock protein-70 tot expressie en in de bronchoalveolaire vloeistof namen de ontstekingscellen toe (**hoofdstuk 4**) (33). Binnen 24 uur was er een veelvoudige verhoging van het aantal monocytten en granulocyten in de bronchoalveolaire vloeistof en ook van het mRNA in het longweefsel voor de pro-inflammatoire cytokines IL-1 β , IL-6 en IL-8 (**hoofdstuk 3**) (34). Apoptose van longcellen was verhoogd na 24 uur en na 72 uur was er sprake van een toegenomen proliferatie van longcellen. Na 7 dagen was de acute ontstekingsreactie van de long uitgedoofd; echter, er bleven nog ontstekingscellen in de bronchoalveolaire vloeistof (**hoofdstuk 3**) (34).

In de longen van kinderen met BPD wordt een duidelijke simplificatie van de microvasculaire ontwikke-

ling gezien (1). Dit wordt ook gezien in placenta's met chorioamnionitis (32). Analoge veranderingen zijn ook in foetale schapenlongen gevonden. Na intra-amniotische blootstelling aan endotoxine hadden de longen gedurende 1 tot 4 dagen een verminderde expressie van verschillende markers van vasculaire ontwikkeling (endotheliale NO synthetase, vasculaire endotheliale groeifactor (VEGF), plaatjes endotheliale cel adhesie molecuul, en VEGF receptor 2) (20). Na 7 dagen was de media van de arteriolaire wanden verdikt, waren de alveolen groter en was het aantal alveolen afgenomen ten opzichte van controles (52).

In **hoofdstuk 5** wordt de rol van transforming growth factor β 1 (TGF- β 1) beschreven in het chorioamnionitis model. TGF- β 1 is een belangrijke factor in de regulatie van de ontwikkeling van de longen, van ontsteking in de longen en van longherstel. Al deze processen spelen een belangrijke rol in de ontwikkeling van de BPD (**hoofdstuk 5**) (35). Ten gevolge van TGF- β 1 activiteit wordt de fibrosevorming veroorzaakt via connective tissue growth factor (CTGF), bovendien is hiervan bekend dat het de vasculaire ontwikkeling bevordert in muizenembryo's (8). Chorioamnionitis door toediening van endotoxine induceerde een duidelijke expressie van TGF- β 1 en signalering in de foetale long via geactiveerd Smad. Echter, de expressie van CTGF was duidelijk verlaagd, mogelijk gemedieerd door de verhoogde expressie van TNF- α . Dit profiel van cytokines kan een rol spelen bij de gestoorde alveolaire en vasculaire ontwikkeling in de long, de afwezigheid van fibrose en de geringe inflammatie in de long, welke kenmerkend zijn voor BPD (figuur 2) (35, 37). Concluderend veroorzaakte een intra-amniotische toediening van endotoxine een chorioamnionitis die de anatomie van de foetale long veranderde vergelijkbaar met de pathologie van de longen van kinderen met BPD (5).

Intra-amniotische endotoxine veroorzaakt longrijping

Ondanks het ontstaan van foetale longschade was de functionele uitkomst na intra-amniotische endotoxine toediening een toegenomen longrijping (2, 21, 24). Het mRNA voor surfactant proteï-

Figuur 1: Samenvatting van de cascade die geïnduceerd werd door toediening van een enkele dosis endotoxine van *E. coli* in het amnionvocht bij 117 dagen zwangere schapen. Longschade werd geïnduceerd met daaropvolgend ontsteking en een abnormale herstelreactie, resulterend na 7 dagen in een fenotype met mature longen en een verhoogde surfactant hoeveelheid, maar echter ook met abnormale ontwikkeling van de alveolen en het microvasculair vaatbed. Dit diersmodel geeft binnen 7 dagen na intra-uteriene blootstelling aan ontsteking een mild fenotype van BPD (Samengevat van hoofdstuk 3 & 4 (2, 18-20, 25, 33, 34, 52))

Figuur 2: Schematische samenvatting van de effecten van cytokines na het toedienen van endotoxine in het amnionvocht. Ontsteking in de longen resulteerde in verhoogde TGF- β 1 concentraties in de foetale long (hoofdstuk 5). TGF- β 1 is essentieel voor "wondherstel" na longschade maar verhoogde TGF- β 1 concentraties verstoren de alveolaire ontwikkeling. In andere modellen worden deze effecten gedeeltelijk gemedieerd door TGF- β 1 zelf maar de fibrose is vooral gemedieerd door CTGF. Bovendien verlaagt TGF- β 1 de ontsteking in de longen. In dit model van chorioamnionitis door endotoxine toediening werden TGF- β 1 en TNF- α geïnduceerd maar was CTGF verlaagd. De verlaging van CTGF was mogelijk het gevolg van het verhoogde TNF- α . De verlaagde concentratie van CTGF limeerde mogelijk de fibrosering, maar was wel de oorzaak voor de verstoorde ontwikkeling van het microvasculair vaatbed omdat het duidelijk is dat CTGF een rol speelt in de normale vasculaire ontwikkeling. Samengevat kunnen dus de cytokine profielen in dit sterk vereenvoudigd model de bevindingen van BPD in het foetale longmodel verklaren.

ne A, B, C en D was binnen 24 uur verhoogd en na 7 dagen waren de hoeveelheden surfactant eiwitten in bronchoalveolaire lavage vloeistof honderdvoudig toegenomen (**hoofdstuk 3**; figuur 1). Parallel aan de toename van surfactant verbeterde de longfunctie van de premature lammeren met een sterke toename van de longcompliantie en verbetering van de gasuitwisseling en oxygenatie (18, 19). Op te merken valt dat het plasma cortisol in navelstrengbloed niet verhoogd was na intra-amniotische endotoxine toediening, hetgeen suggereert dat er een ander mechanisme verantwoordelijk was voor de longrijping dan dat bij inductie door glucocorticoïden (18). De toename van zowel surfactant lipiden als eiwitten was veel groter na de endotoxine toediening dan na behandeling met corticosteroïden (19).

De klinisch relevante respons van de foetale long op chorioamnionitis is een toename in longrijping. Dit laatste zal resulteren in een verlaagde incidentie en ernst van RDS. In het gebruikte experimentele schapenmodel induceerden intra-amniotische doses van 1 tot 100 mg *E. coli* endotoxine gelijkwaardige ontstekingsreacties en longrijping (19). Met lagere doses werd geen longinflammatie en daaropvolgende longrijping gezien (**hoofdstuk 3**) (34). Vanuit de amnionholte kwam geen noemenswaardige hoeveelheid endotoxine in de circulatie van de foetus aanzien zelfs een zeer lage intravasculaire dosis van 1 µg de foetus kan doden (7). Toediening van endotoxine in het allantoïs veroorzaakte geen chorioamnionitis en geen verandering in longstructuur (40). Dus kan men concluderen dat de foetale respons op de ontsteking afhankelijk is van het compartiment waarin de ontsteking optreedt.

Hoe endotoxine longrijping signaleert

Endotoxine toediening induceerde een cascade van ontstekingsmediatoren en trok geactiveerde ontstekingscellen naar het chorioamnion (**hoofdstuk 3**; figuur 1) (25, 34, 39). De diffuse ontsteking in het chorioamnion kan effector moleculen afgeven in de foetale circulatie en zo indirect ontsteking in de longen veroorzaken met longrijping als gevolg. Echter, intra-amniotische endotoxine toediening had slechts weinig effect op witte bloedcellen en bloedplaatjes in de circulatie (**hoofdstuk 3**) (18, 34). Messenger RNA's van pro-inflammatoire cytokines waren slechts minimaal en niet consistent verhoogd in de foetale lever en de concentraties van INFγ, IL-6 en IL-8 in plasma waren slechts minimaal verhoogd (15, 17, 25, 26, 34, 54). Ook de mRNA's van cytokines in de placenta en

de darm waren niet duidelijk verhoogd (25). Dit alles wijst erop dat de locale ontsteking in de longen niet verklaard kan worden door een systemische ontstekingsreactie in de foetus.

Om de invloed van chorioamnionitis op de foetale long nader te kunnen bestuderen werden in een volgend model de foetale longen chirurgisch gescheiden van het amnionvocht door een tracheotomie te verrichten en het longvocht via een beademingstube in een plastic zak te verzamelen (39). Endotoxine toegediend in het amnionvocht veroorzaakte nu een chorioamnionitis zonder dat er een ontsteking optrad in de longen (39). Daarentegen veroorzaakte een 24-uur infuus van endotoxine in de foetale trachea wel ontsteking in de longen met daarna longrijping maar nu echter zonder chorioamnionitis. Deze resultaten tonen aan dat endotoxine de sequentie longontsteking – longrijping veroorzaakt door direct contact met de foetale long. Hiervoor is het dus noodzakelijk dat amnionvocht met het foetaal longvocht wordt gemengd door middel van foetale ademhalingsbewegingen. Bovendien is de klaring van endotoxine uit het amnionvocht zeer traag. Metingen van de endotoxine activiteit geven een geschatte half-waarde tijd van 41 uur (41).

In het klinische scenario zouden de inflammatoire effecten op de foetale longen veroorzaakt kunnen worden door het effect van het endotoxine op TLR4 receptoren, door stimulatie van andere TLR receptoren of door nog andere ontstekingsproducten van de chorioamnionitis (**hoofdstuk 7**) (29). Na intra-amniotische toediening van endotoxine was van alle gemeten cytokines het mRNA voor IL-1β het meest verhoogd in het chorioamnion van het schaap. Dit is vergelijkbaar met de bevindingen bij baby's die intrauterien bloot stonden aan chorioamnionitis (6). De originele beschrijving van longrijping na intra-amniotische toediening van IL-1α in konijnen gaf geen omschrijving van de chorioamnionitis zelf (4). Intra-amniotische injecties met schapen recombinant IL-1α of IL-1α bij zwangere schapen induceerde een chorioamnionitis gevolgd door een duidelijk rijping (47, 53). Echter, injecties met schapen recombinant TNFα gaf geen foetale respons met chorioamnionitis noch longontsteking, noch longrijping (16). Dus het foetale compartiment lijkt speciaal gevoelig te zijn voor IL-1 en relatief ongevoelig

voor andere ontstekingscytokines die normaal gesproken zeer potent zijn.

In andere proeven werd de influx van ontstekingscellen in de foetale longen na intra-amniotische toediening van endotoxine geblokkeerd door een anti-CD-18 antistof. Hierdoor werd ook de longrijping geblokkeerd (23). Onze huidige opvatting is dat de foetale long reageert op ontstekingsmediatoren in het amnionvocht met een influx van ontstekingscellen die vervolgens de belangrijkste bron zijn van IL-1 α en de longrijping in gang zetten.

Recent werd aangetoond dat TGF- α gebonden wordt aan Surfactant Proteïne-A (SP-A) (**hoofdstuk 6**) (30,36, 43-45). De invloed van de surfactant eiwitten op het immuunsysteem dient verder bestudeerd te worden aangezien de onrijpe long heel gevoelig is voor ontsteking (3, 9, 12-14, 48-50). In enkele experimenten werden lammeren van verschillende zwangerschapsleeftijden geïntubeerd en vervolgens werd endotoxine intra-tracheaal gegeven. Bij erg premature lammeren (80% zwangerschapsduur) bleef het endotoxine niet alleen in de longen maar lekte het ook in de systemische bloedcirculatie en veroorzaakte een septische shock syndroom (**hoofdstuk 8**) (26). Bij lammeren die meer (95% zwangerschapsduur) tegen de $\grave{a}$ terme leeftijd waren veroorzaakte dezelfde dosis endotoxine geen systemische reactie meer. De rijpheid van het immuunsysteem lijkt dus een essentiële component in dit schapenmodel. In ons model konden in de foetale long geen alveolaire macrofagen aangetoond worden tot laat in de zwangerschap (**hoofdstuk 9**) (31). Laat in de zwangerschap migreren monocytten uit het bloed naar de longen, prolifereren en differentiëren vervolgens tot alveolaire macrofagen onder invloed van granulocyte-macrophage-colony stimulating factor (GM-CSF) en zijn transcriptie factor PU.1 (46).

Ook bleek dat in respons op endotoxine, monocytten uit navelstrengbloed van premature lammeren functioneel minder rijp waren in vergelijking met monocytten van lammeren die meer tegen de $\grave{a}$ terme leeftijd waren of die van volwassen schapen (**hoofdstuk 9**).

Glucocorticoiden en chorioamnionitis

Bij de meerderheid van de vrouwen die voor 30 weken zwangerschap bevalt, is er sprake van een subklinische chorioamnionitis (10). De meeste van deze vrouwen krijgen antenataal corticosteroïden toegediend om longrijping bij de foetus te induceren (11). De antenatale behandeling met corticosteroïden

wordt ondersteund door vele klinische studies en systematische reviews. Indien antenataal corticosteroïden gegeven worden aan vrouwen met vroegtijdig breken van de vliezen, hetgeen als een surrogaat klinische marker voor chorioamnionitis beschouwd kan worden, is er een verlaagde incidentie van RDS, intraventriculaire bloedingen en overlijden bij de pasgeborene in vergelijking met placebo behandeling (11).

Maternale behandeling met corticosteroïden, 7 dagen voor de bevalling, verbeterde de longfunctie van de foetale schapen niet zo goed als een intra-amniotische toediening van endotoxine. Het grote verschil was een veel grotere toename van de hoeveelheid surfactant na intra-amniotische endotoxine toediening (18, 52). Maternale antenatale behandeling met het glucocorticoïd betamethason, veroorzaakte tevens foetale groeivertraging en involutie van de thymus wat niet optrad na intra-amniotische toediening van endotoxine (42). Wanneer beide interventies werden gecombineerd, werd de groeirestrictie veroorzaakt door betamethason evenals de involutie van de thymus tot onze verbazing zelfs voorkomen (42). Betamethason onderdrukte de ontsteking in de longen, geïnduceerd door endotoxine, gedurende 2 dagen (22). Echter na 5 en 15 dagen waren de ontstekingscellen en ontstekingsmediatoren hoger in de longen blootgesteld aan zowel endotoxine als betamethason dan in de longen blootgesteld aan endotoxine alleen (22).

Een mogelijke verklaring voor dit resultaat wordt gesuggereerd door onze bevinding dat maternale behandeling met betamethason aanvankelijk de ontstekingsrespons van de foetale monocytten onderdrukte, maar dat deze respons juist versterkt was na 7 dagen (**hoofdstuk 10**) (27). Dus maternale behandeling met betamethason kan wellicht de foetale ontsteking in de longen, veroorzaakt door endotoxine, versterken door de inflammatoire potentie van foetale ontstekingscellen te "rijpen". (**hoofdstuk 10**). Er zijn echter op dit moment geen experimentele gegevens beschikbaar over hoe de foetale long zal reageren op betamethason behandeling bovenop een chronische amnionitis, wat klinisch een gebruikelijker scenario is.

Ook werd de functie van foetale monocytten geëvalueerd na herhaalde blootstelling aan endotoxine (**hoofdstuk 11**) (28). De initiële blootstelling aan een niet-lethale dosis endotoxine vermindert de respons van immuuncellen op een tweede, wel lethale dosis endotoxine in dierexpe-

rimentele modellen (51). Dit fenomeen wordt endotoxine tolerantie genoemd en het wordt beschouwd als een belangrijke oorzaak van morbiditeit op volwassen intensive care units. Herhaalde intra-amnionische toediening van endotoxine had geen toegevoegd effect op de longrijping maar maakte de foetale monocytten niet-responsief of zelfs inert aan een volgende blootstelling aan hetzelfde endotoxine (hoofdstuk 11) (28, 29).

Conclusie

Concluderend kan gezegd worden dat onze experimentele chorioamnionitis in het schapenmodel zowel positieve als negatieve effecten had voor de foetus. De longen rijpen versneld wat de kansen op overleving bij premature partus verhoogd. Daarentegen is de longstructuur sterk vereenvoudigd wat op de langere termijn BPD kan veroorzaken. Vervolgonderzoek zal de mechanismen van deze goede en schadelijke effecten nog verder moeten differentiëren om therapeutische en preventieve interventies mogelijk te maken.

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Curriculum vitae

Boris W. Kramer was born on October 8th, 1970, in Böblingen, Baden-Württemberg, Germany. He grew up in Gäufelden-Öschelbronn, a village close to the Black Forest („Schwarzwald“), where he attended primary school. After secondary school education in the Schickhardt-Gymnasium in Herrenberg he fulfilled his general service obligation with the German-French Brigade in Böblingen and the University Hospital Tübingen, Germany. He enrolled in medical school at the Eberhardts-Karl-Universität Tübingen, Germany from which he graduated in 1997 after internships at the Kantonsspital Luzern, Switzerland, the Mayo Medical School, Rochester, USA, the University of Sydney, Australia, and the Kinderspital Zürich, Switzerland. He did a medical thesis with summa cum laude entitled „Functional characterisation of a binding protein for coxsackievirus B3“ with Prof. Dr. R. Kandolf, Molecular Pathology, in

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AWARDS & SCHOLARSHIPS

Scholarship of the Studienstiftung des deutschen Volkes (National Fund to support the top 1% students in Germany, 1991–1997)

Scholarship of the Alumnies of the University of Tübingen, Germany, 1992–1997

Research Fellowship, Deutsche Forschungsgemeinschaft (National Research Fund of Germany, 1999–2000)

Honorary Research Fellow of the Women and Infants Research Foundation of Western Australia, Perth, Australia (since 2001)

Scientific Award of the German-Austrian Society for Neonatology and Pediatric Intensive Care Medicine (2003)

Young Investigator Award of the European Society for Pediatric Research (2003)

Travel Award for the Scientific Meeting of the European Society for Pediatric Research 2003 in Bilbao/Spain

Scientific Award of the German Society for Perinatal Medicine (2003)

List of Publications

a) Original papers

- 1) Raab de Verdugo, U., Selinka, H.-C., Huber, M., Kramer, B., Kellermann, J., Hofschneider, P. H., Kandolf, R. (1995). Characterization of a 100-kilodalton binding protein for the six serotypes of coxsackie B viruses. *J. Virol.* 69: 6751–6757.
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