THE EFFECT OF MECONIUM AND AMNIOTIC FLUID ON ALVEOLAR MACROPHAGE FUNCTION

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ABSTRACT

The meconium aspiration syndrome (MAS) is an important cause of respiratory distress in newborn infants. The pathophysiology of the syndrome is complex, involving airway obstruction, surfactant dysfunction, and a pulmonary inflammatory response. Alveolar macrophages are the cells that provide first line defense in the lower respiratory tract against inhaled pathogens and particles such as meconium. These phagocytic cells also orchestrate inflammatory responses in the lung. Although numerous studies have examined prophylactic and therapeutic strategies for MAS, relatively little is known about the cellular pathophysiology of this syndrome, particularly in regard to alveolar macrophages. Since the meconium aspirated by neonates is necessarily diluted with amniotic fluid, the aim of this study was to examine the effect of both meconium and amniotic fluid on phagocytosis, respiratory burst, and production of proinflammatory cytokines in rat NR8383 alveolar macrophages.

Meconium was obtained from both human and equine neonates, and similar effects on the phagocytic and respiratory burst activity were observed following exposure of alveolar macrophages to meconium from either species. Incubation of alveolar macrophages with meconium (0.25 to 25 mg/ml) produced a dose dependant decrease in phagocytosis of fluorescent latex beads that was due to a reduction in both the percentage of actively phagocytosing cells and in the average number of particles phagocytosed per cell ($p<0.01$). Filtration of the meconium to remove particles larger than 0.2 μm attenuated, but did not eliminate the effect.

The effect of meconium-exposure on the respiratory burst response in alveolar macrophages was quantified using flow cytometry to measure oxidation of dichlorofluorescin diacetate. A very robust respiratory burst was triggered by 1 h incubation of macrophages with 12 or 24 mg/ml of meconium ($p<0.01$). Again, this effect was attenuated but not eliminated by 0.2 μm filtration of the meconium ($p<0.01$). However, meconium-exposed alveolar macrophages demonstrated a significantly reduced respiratory burst in response to subsequent stimulation with phorbol myristate acetate ($p<0.01$).

The observed effects of meconium on these alveolar macrophage functions were not due to decreased cell viability, as measured by trypan blue exclusion, nor to an elevation of intracellular cAMP.

Amniotic fluid (10%) induced a small but significant increase in alveolar macrophage phagocytosis ($p<0.05$), as well as a significant increase in the magnitude of the respiratory burst response ($p<0.01$). Neither meconium nor amniotic fluid induced alveolar macrophages to increase mRNA expression for interleukin-1β or tumor necrosis factor-α, as determined by RT-PCR.

Our results suggest human neonates with meconium aspiration syndrome probably have impaired alveolar macrophage function, which could make them more susceptible to pulmonary injury from subsequent exposure to inhaled pathogens or toxicants. Furthermore, excessive production of reactive oxygen intermediates generated by the meconium-stimulated alveolar macrophages could be a contributing factor in the initial inflammation and pulmonary injury seen in MAS. A better understanding of the effect of meconium on all aspects of alveolar macrophage function is necessary to aid in the development of appropriate treatment and preventative strategies for babies with MAS.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. GENERAL INTRODUCTION

1.1. Meconium

1.1.1. Definition

Meconium is the material contained in the digestive tract of the fetus, and is composed of secretions from the intestinal glands, along with bile, pancreatic juice, mucous, cellular debris, lanugo, vernix caseosa, and amniotic fluid [1]. Meconium begins to accumulate in the distal small intestine at about 16 weeks of human gestation, and then gradually moves to the colon. Meconium is viscous, sticky and greenish black in color. Water constitutes about 75% of this complex substance, with mucopolysaccharides comprising most of the dry matter. The protein content is relatively low, but includes glycoproteins and proteolytic enzymes. During the first few days after birth, the neonate begins ingesting food, and the composition of the feces gradually changes [1].

1.1.2. Meconium Passage

The first passage of meconium usually occurs within 12 hours after birth, and 90% of infants pass meconium within their first 24 hours [1]. Meconium is not normally expelled *in utero* due to tonic contraction of the fetal anal sphincter, lack of strong peristaltic contractions, and a particularly viscous terminal cap [2]. However, *in utero* passage of meconium is associated with increasing gestational age, and there is a direct relationship between birth weight and meconium passage [3]. Indeed, up to 50% of post-mature fetuses (older than 40 weeks) may expel meconium *in utero* [2]. Some

investigators consider meconium passage to be a normal physiological event for the term or postmature fetus [3].

Although *in utero* meconium passage in a pre-term human fetus does not normally occur, certain fetal stressors such as hypoxia, acidemia or infection may increase peristalsis and relaxation of the anal sphincter and subsequent meconium release [4].

When meconium is passed *in utero*, it contaminates the amniotic fluid (AF) and results in yellow staining of the neonate's skin. About 10-15% of human infants are born through meconium contaminated AF [5]. The amount of meconium present in the AF is quite variable, and is often categorized by its visible appearance. Thin meconium is sometimes defined as a solution through which it is possible to read newsprint. Moderate meconium is more opaque, and thick meconium has a consistency similar to pea-soup, containing visible particles [6].

The clinical significance of meconium staining in a newborn is a matter of debate. Some investigators do not believe the presence of meconium is necessarily associated with a poor outcome unless there are accompanying signs of distress, such as respiratory compromise [7]. However, infants born through meconium-stained AF are 100 times more likely to develop respiratory distress, including meconium aspiration syndrome [8].

1.2. Meconium Aspiration Syndrome

1.2.1. Definition

Meconium aspiration syndrome (MAS) is an important cause of respiratory distress in newborn infants [4]. MAS has been defined as respiratory distress in an infant who was meconium-stained at birth, who has compatible roentgenographic findings, and whose symptoms cannot be otherwise explained [4]. The radiographic findings in MAS are classically described as diffuse patchy infiltrates [5], but can also include consolidation, hyperinflation, and segmental or lobar atelectasis [6]. Consolidation and atelectasis seen radiographically are usually indicative of poor outcome, although the severity of the radiographic findings do not correlate well with the severity of the clinical disease [7].

1.2.2. Etiology

In order for MAS to occur, the fetus must first expel meconium from the intestine into the surrounding AF, and then inhale it. Aspiration may occur before birth, or, it may occur immediately after parturition [4]. Although the normal fetus does have respiratory movement while *in utero*, these shallow breaths do not draw AF into the distal airway, and the net flow of fluid is out of the lungs. However, stressors such as hypoxia can trigger gasping, which can result in aspiration of AF and also meconium if present in this fluid [2]. In the immediate postnatal period, if meconium is present in the mouth or upper airway, it can be inhaled when the infant takes its first breaths [2]. For any particular infant with MAS, it can be difficult to determine whether meconium aspiration was pre or postnatal. Some investigators argue that most aspiration occurs *in utero*, and is secondary to chronic hypoxia [8,9]. However, others point out that many

MAS babies are vigorous at the time of birth [6], and that current perinatal management strategies have reduced the incidence of MAS, both of which seem to indicate that a significant number of aspirations occur immediately after birth [4].

1.2.3. Risk Factors

When meconium is present in the AF, 1.7 to 35.8% of neonates will develop MAS [4,5]. Several risk factors have been associated with MAS, including the amount and thickness of the meconium. Aspiration of meconium is most likely to occur when the meconium-stained amniotic fluid (MSAF) is of a thick consistency [6], and the thicker the MSAF, the more respiratory support the infant is likely to require. However, it can also occur when the consistency is thin [2,3]. In fact, thin MSAF may be more likely to travel to the distal alveoli, where it cannot be removed by suctioning [5]. Other risk factors for the development of MAS include one and five minute Apgar scores of less than 7, black race, male gender, few or no prenatal visits, abnormal fetal heart rate tracing, oligohydramnios, cesarean delivery, fetal acidemia, and no intrapartum oropharyngeal suctioning [2,3,10] .

1.2.4. Clinical Presentation

Meconium aspiration syndrome represents a broad spectrum of disease severity. Some infants have only transient respiratory distress, while others develop severe hypoxemia or die, even with treatment and ventilatory support [7]. About 30% of infants who develop MAS require mechanical ventilation [4], and 5% of these subsequently develop chronic lung disease [2]. Up to 25% of infants with MAS develop pneumothorax, and 5% or more die [2].

1.2.5. Prevention and Intrapartum Management

Anecdotal and observational reports in the 1970's suggested that suctioning the trachea and pharynx of infants born with meconium staining could reduce neonatal fatality by preventing the development of MAS [6]. Most hospitals subsequently adopted a policy of routinely suctioning the oropharynx of meconium-stained infants as soon as the head and neck are delivered, even before delivery of the trunk [6]. However, there is controversy regarding whether all meconium-stained infants require intubation and tracheal suctioning. Previous recommendations emphasized aggressive suctioning of all babies born through thick meconium-stained AF, but recent studies have shown that intubation and tracheal suctioning in vigorous meconium-stained infants does not improve outcome [6]. About 74% of infants born through MSAF are apparently vigorous [6]. Current resuscitation guidelines recommend tracheal suctioning primarily for meconium-stained infants who experience apnea or respiratory distress [11]. Although the incidence of MAS has declined with the advent of routine suctioning of the oropharynx and trachea of newborns, there is no evidence to support the contention that MAS and subsequent infant mortality are completely preventable [12].

Another intrapartum management strategy to prevent MAS involves infusing saline solution into the amniotic cavity. This procedure is known as amnioinfusion, and has been recommended as a means of diluting MSAF. Numerous studies have reported an improvement in perinatal outcome following amnioinfusion, with or without a resulting decrease in the incidence of MAS [13]. However, this prophylactic procedure

remains controversial, since it may also increase the risk of complications such as chorioamnionitis, and some studies have shown no benefit from this treatment [14,15].

1.2.6. Treatment of Neonates

Various mechanical ventilator strategies are used to treat MAS infants with respiratory distress, but the optimal management approach has not yet been agreed upon [4]. Positive end expiratory pressure has been found to improve ventilation by some investigators, while others have found that it exacerbates air trapping [4]. Some have advocated hyperventilation in order to cause alkalosis and subsequent pulmonary vasodilation. The sickest babies are usually treated with extracorporeal membrane oxygenation (ECMO) when other management strategies have failed. This temporary form of lung bypass is used to treat babies with reversible pulmonary lesions, in the hope that the lungs will improve over a period of several days [16]. Several experimental treatments have been examined. For example, saline lavage of affected neonatal lungs has been used, on the theory that this treatment could help to dilute and wash out meconium, neutrophils, inflammatory mediators and exudates [5]. Some investigators have reported success using surfactant lavage to treat infants with MAS [17-20]. This is a reasonable approach because meconium aspiration is associated with surfactant dysfunction, displacement and/or inactivation [21-23], but there is a definite need for randomized clinical trials to properly evaluate this therapy [5].

Inhaled nitric oxide treatment is used to enhance vasodilation in cases of MAS complicated by persistent pulmonary hypertension of the newborn. There have been conflicting reports on the efficacy of this treatment. Some have reported improved oxygenation following nitric oxide therapy, but others have found no improvement in

outcome [5,24,25]. Administration of corticosteroids to control lung inflammation has shown promise in animal models of MAS [26,27], although no therapeutic effect was seen in an earlier clinical trial in human neonates [28]. These authors theorized that steroids might depress the function of alveolar macrophages, thus preventing clearance of meconium from the alveoli.

1.2.7. Pathophysiology

The pathophysiology of MAS is complex, and involves acute airway obstruction, surfactant dysfunction, as well as chemical injury to the respiratory membrane and inflammation. The airway obstruction is caused by the meconium itself, or by large aggregates of edema fluid and exudates [5]. Meconium can completely obstruct some airways, causing atelectasis, with resulting hypoxia, hypercapnea and acidosis. Partial obstruction may result in a ball-valve effect, where air enters on inspiration, but cannot exit on expiration [4]. This partial obstruction can cause ventilation-perfusion mismatching, alveolar distention or rupture, interstitial emphysema, or even pneumothorax [29].

Chemical pneumonitis due to meconium has been reported in experimental models, although there is controversy regarding the relative roles of hypoxia and meconium toxicity in the pathogenesis of MAS. For example, Jovanovic and Nguyen (1989) reported that meconium did not cause pneumonitis in their neonatal guinea pig model, and that histological changes thought to be characteristic of MAS were in fact due to asphyxia [30]. Nonetheless, when meconium is instilled into the lungs of rabbits and rats, there is a loss of cilia from airway epithelium and necrosis of bronchial and bronchiolar epithelium [29,31]. The bile salts found in meconium are thought to be the

component most likely causing chemical pneumonitis [32], although proteolytic enzymes could be another source [29]. There is also injury to type II pneumocytes with resulting changes in surfactant production and function [5,21,23]. Decreased levels of surfactant proteins A and B have been reported [5].

A profound pulmonary inflammatory response is apparent within several hours of meconium aspiration, with a peak influx of inflammatory cells and serum proteins at 16-24 hours [23]. Neutrophils are found diffusely throughout the lungs and release various chemical mediators that can exacerbate the inflammation and damage lung parenchyma leading to vascular leakage [4,5]. Meconium and meconium-stained AF have been shown to possess chemotactic activity for neutrophils and to contain interleukin-8, which is a potent neutrophil chemoattractant [33-36]. Meconium has also been found to negatively affect neutrophil function by inhibiting phagocytosis and respiratory burst [37]. Vasoactive mediators such as eicosanoids and endothelin-1 are released from inflammatory cells and can cause vasoconstriction [5,38]. One study showed that intratracheal administration of meconium to neonatal piglets resulted in increased levels of the eicosanoids leukotriene B_4 and D_4 in intratracheal aspirates [39]. Pulmonary vasoconstriction can lead to persistent pulmonary hypertension of the newborn (PPHN), which can be almost impossible to treat successfully [4]. In fact, PPHN is a major contributing factor in most of the fatal cases of MAS. However, it has also been suggested that PPHN is actually due to persistent *in utero* hypoxia rather than to direct effects of meconium. It may be that persistent *in utero* hypoxia is the underlying event that typically results in poor outcome [7], and that it often causes incidental meconium passage and subsequent aspiration.

Some children who suffered from MAS at birth have long-term pulmonary function test abnormalities [2]. Spontaneous wheezing, coughing and exercise induced bronchospasm have been documented in these children up to 11 years after birth [40,41], although some of these long-term sequelae may be due to oxygen therapy and barotrauma from the ventilatory support [4]. Survivors of MAS are at increased risk for chronic seizures, mental retardation and cerebral palsy [5], though certainly not all cases of MAS cause sufficient asphyxia to result in neurologic damage [4].

Meconium also reduces host resistance to bacterial infection. Bryan (1967) found that when sterile meconium was injected intraperitoneally into mice, or instilled intratracheally in rats, the LD_{50} for *E. coli* bacteria administered concurrently was significantly reduced [42]. Clinical findings in humans demonstrate that meconium-stained AF is associated with intra-amniotic infection [43-46]. However, babies born through MSAF are not more likely to have systemic infections [5].

1.3. Meconium Aspiration in Animals

Respiratory disease is very common in neonatal animals, although the existence of MAS in species other than humans is controversial. There is some evidence to suggest that meconium passage *in utero* may be a physiological event, at least in some species. When contrast medium was instilled intranasally to fetal goats, the material could be detected radiographically in the AF within 16-22 hours, even though central venous blood gas values indicated that the animals were not experiencing hypoxic distress [47]. A similar study in fetal rabbits injected intramuscularly with radionuclides showed that radioactivity could later be detected in AF [48].

Evidence of meconium aspiration is commonly seen in aborted calves and is usually considered to be a terminal event related to fetal distress and hypoxia. Lopez and Bildfell (1992) examined necropsy submissions and found that 44% of calves that died in the first 2 weeks of life had evidence of AF aspiration, with or without meconium [49]. These calves were 1.5 times more likely to have pulmonary inflammation than those without evidence of aspiration. In a later study designed to determine if aspiration of AF or meconium is associated with failure of passive transfer (FPT), neonatal calves were fed colostrum with a known amount of immunoglobulin. Evidence of AF and meconium aspiration was found in 71% and 29% of these calves respectively, although it was not associated with FPT or respiratory acidosis. They concluded that aspiration of AF is common event in calves at the time of parturition [50].

Meconium staining, respiratory distress, and radiographic findings compatible with aspiration pneumonia, have also been reported in a neonatal foal [51]. In this case there was a history of probable *in utero* hypoxia, which may have precipitated meconium passage and aspiration. Perinatal asphyxia is relatively common in foals, and meconium aspiration has been recognized as an associated factor [52].

Interestingly, a genetically altered mouse model of MAS has been described. Mouse null mutants for the transcription factor ATF-2 reportedly demonstrate a placental defect that causes *in utero* hypoxia. These mice live for only a few minutes after birth, and display signs of severe respiratory distress, along with meconium-filled lungs. The authors speculate that hypoxia in the mutant embryos induces respiratory gasping and subsequent meconium aspiration [53].

1.4 Amniotic Fluid

The fetus *in utero* is enclosed in a fluid filled cavity. The cells of the amnion, which are fetal in origin, produce amniotic fluid. This fluid serves as a buffer to protect the fetus from trauma and ascending infection, and it also allows fetal movement, swallowing, urination, and respiratory movements [54]. The composition of AF is initially very similar to that of extracellular fluid, but as gestation progresses, the composition changes as a result of fetal micturition. Amniotic fluid is about 98% water, with 1-2% organic and inorganic solids [55]. It also contains exfoliated particulate matter from the fetus, such as hair, cells, keratin and fat from sebaceous glands [54], as well as phospholipids locally produced in the lung and subsequently transported into the AF.

The meconium aspirated by neonates with MAS is necessarily diluted with AF. Few studies have investigated the effect of administration or inhalation of AF alone, without meconium contamination. In the neonatal bovine lung, AF typically invokes only a mild inflammatory reaction, and aspiration of AF appears to be a common event at the time of parturition [50]. In neonatal rats, meconium instilled intratracheally induced an acute and robust inflammatory reaction characterized by a significant influx of neutrophils, while AF produced only mild inflammation [31]. This lack of significant inflammation is remarkable given that AF contains keratin and squamous epithelial cells, which would be expected to trigger a vigorous foreign body inflammatory response in the lung. The failure of AF to induce an inflammatory response could be due to the presence of anti-inflammatory substances, a number of which have been identified, including interleukin-1 receptor antagonist [56], secretory

leukocyte protease inhibitor [57], Clara cell protein [58], and interleukin-10 [59-61]. Since the meconium aspirated by neonates with MAS is diluted with AF, the presence of anti-inflammatory substances in AF could potentially over-ride the presumed pro-inflammatory effects of meconium. It is therefore important to examine the role of AF as well as meconium in the pathogenesis of MAS.

1.5 Alveolar Macrophages

The alveolar macrophage is a specialized phagocytic cell that resides within the alveoli of the lung, and plays a crucial role in clearing inhaled particles and protecting the host against inhaled pathogens. The respiratory tract is protected from foreign material by a number of mechanisms. Particles larger than 10 μm are prevented from reaching the lower respiratory tract by mechanical defenses, including nasal hairs, sneeze reflexes, and tortuous nasopharyngeal channels [62,63]. Particles in the size range of 5-10 μm can escape these defenses and gain access to the tracheobronchial tree where they usually become trapped in a layer of mucous lining the airways. These particles are cleared by the mucociliary escalator, which is the term used to describe the process whereby the ciliated cells of the conducting airways continuously propel a layer of mucous up the respiratory tract, to be removed by swallowing or expectoration [64,65]. Only particles smaller than 5 μm normally reach the alveoli, where they are rapidly phagocytosed by alveolar macrophages [64]. These phagocytic cells provide the primary means of defense against particles that reach the lower respiratory tract. Inhaled pathogens that have been phagocytosed by alveolar macrophages can be killed by a variety of microbicidal mechanisms within the cell, including the production of

reactive oxygen intermediates generated by the respiratory burst. Non-infectious particles generally remain sequestered within alveolar macrophages, and are eventually cleared from the alveoli as particle-laden macrophages migrate to the airways, to be removed from the respiratory tract via the mucociliary escalator [66]. Some of the alveolar macrophages may also migrate through the interstitium to the regional lymph nodes, where they can present antigen to T cells [66,67].

Although phagocytosis is usually considered the primary function of alveolar macrophages, these cells also play an essential role in the generation and orchestration of immune responses in the lung, through production of numerous pro- and anti-inflammatory molecules [68]. Compared to other macrophages, alveolar macrophages are relatively poor in their ability to process and present antigen [69], but are particularly good at phagocytosing unopsonized, inert particles [70]. The response of alveolar macrophages to inhaled particles is variable, and can range from simple ingestion and clearance, to release of abundant inflammatory mediators [70-72]. This discrimination is adaptive and necessary, because the lung is constantly exposed to inhaled particles, and inflammation in response to all encounters would be potentially damaging to host tissues.

The effects of meconium on neutrophil function and activation have been examined *in vitro* [37]. Although influx of neutrophils is a readily apparent aspect of MAS pathology, it is a secondary event. Aspirated meconium is initially phagocytosed by alveolar macrophages, and these cells are capable of increasing production of neutrophil chemoattractants in response to inflammatory stimuli [73]. Recently, meconium has been shown to increase production of nitric oxide and the transcription

factor NF-κB in alveolar macrophages [74]. Although alveolar macrophages play a pivotal role in lung inflammation, the effect of meconium on other functions of these cells remains uncertain. A better understanding of the specific effects of meconium on various functions of alveolar macrophages could aid in the development of therapeutic interventions aimed at preventing or ameliorating the inflammatory component of MAS. This in turn could have significant implications in terms of improving outcome for babies with this syndrome.

1.6 Objectives

The general objective of this study was to examine the effect of meconium and amniotic fluid on various functions of alveolar macrophages. Based on the results previously reported with neutrophils [37], we hypothesized that incubation of alveolar macrophages with meconium would cause a decrease in phagocytic and respiratory burst activity. Since significant pulmonary inflammation is seen in infants with MAS, we also hypothesized that meconium would stimulate alveolar macrophages to increase production of proinflammatory cytokines. On the other hand, since amniotic fluid aspiration is not associated with a significant pulmonary inflammatory response [31,50], we hypothesized that amniotic fluid would neither induce the production of proinflammatory cytokines, nor inhibit other alveolar macrophage functions.

Specific objectives:

1. To examine the effect of meconium on phagocytic activity in alveolar macrophages.
2. To examine the effect of meconium on respiratory burst activity in alveolar macrophages.
3. To examine the effect of meconium on production of the inflammatory cytokines IL-1βand TNF-α in alveolar macrophages.
4. To examine the effect of AF on phagocytosis, respiratory burst and cytokine production in alveolar macrophages.

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2. MECONIUM INHIBITS THE PHAGOCYTIC ACTIVITY OF ALVEOLAR MACROPHAGES

2.1 Introduction

Meconium aspiration syndrome (MAS) is an important cause of respiratory distress in newborn infants [1,2]. Meconium is the material contained in the digestive tract of the fetus, and is composed of secretions from the intestinal glands, along with bile, pancreatic juice, mucous, cellular debris, lanugo, vernix caseosa, and amniotic fluid [3]. In order for MAS to occur, the fetus must first expel meconium from the intestine into the surrounding amniotic fluid, and then inhale it. The passage of meconium *in utero* is associated with increasing gestational age, and with fetal hypoxia or other stresses that cause increased peristalsis and relaxation of the anal sphincter [4]. Aspiration may occur either before birth if the fetus gasps *in utero*, or it may occur immediately after parturition when the infant takes its first breaths. About 10-15% of human infants are born through meconium contaminated amniotic fluid [5], and these infants are 100 times more likely to develop respiratory distress [6]. When meconium is present in the amniotic fluid, 1.7 to 35.8% of neonates will develop MAS [5], and about 5% of these will die [4].

Meconium inhalation is associated with respiratory distress, atelectasis, and respiratory acidosis [5]. The pathophysiology of this syndrome is complex and involves airway obstruction, surfactant dysfunction, and pulmonary inflammation [5]. Alveolar macrophages are important components of the cellular immune defense mechanism in the lungs and play an essential role in defending the alveolar space against inhaled

pathogens and particles [7]. One of the key functions of alveolar macrophages is phagocytosis. Phagocytosis is a complex process initiated by the interaction of specific receptors on the cell surface with specific ligands on the particle [8]. The number and type of phagocytic receptors expressed by macrophages can change depending on conditions such as the activation state of the cell [8], and the cytokine milieu [9]. Other immune cells, particularly lymphocytes, produce cytokines that can regulate receptor expression by macrophages [7]. Furthermore, actively phagocytosing macrophages can alter receptor expression of neighboring macrophages by a paracrine mechanism involving cytokine secretion [10].

Phagocytic receptors can be divided into two main categories. The first category includes receptors that recognize opsonins such as complement and IgG. Bacteria or other particles coated in opsonins bind to specific complement or Fc receptors on the macrophage surface and are phagocytosed with optimal efficiency [11]. However, macrophages also have receptors for binding non-opsonized particles, and alveolar macrophages, in contrast to other phagocytes, can phagocytose very efficiently via these mechanisms [12]. These receptors generally recognize conserved molecular patterns on the surface of pathogens [8]. Scavenger receptors are an example of these pattern recognition receptors. These trans-membrane receptors have different structures but generally function to allow internalization and clearance of effete components, including apoptotic cells and modified lipoproteins, as well as exogenous particles, including microbial organisms [13]. Alveolar macrophages evidently phagocytose meconium, since the characteristic pigments can be readily seen within the cells

following exposure. Nonetheless, the receptors involved in attachment and ingestion of meconium are not known.

The intracellular signaling events that occur subsequent to receptor binding are only partially understood. Protein kinase C (PKC) is an important regulator of phagocytosis, and inhibitors of PKC are known to inhibit the internalization of particles attached to various types of receptors [14]. Phorbol myristate acetate (PMA) directly activates PKC, and can be used to stimulate macrophages and other cells to increase their phagocytic activity [15].

Since the phagocytic activity of alveolar macrophages is a crucial component of host defense, it would be important to know what effect meconium has on this function. Meconium has been shown to decrease phagocytosis by neutrophils *in vitro* [16] , so it is reasonable to postulate that alveolar macrophage phagocytic activity may be adversely affected as well. Indeed, various inhaled particles have been shown to decrease phagocytic activity of macrophages [17,18]. In addition, Shabarek *et al* (1996, 1997) reported that meconium stimulates murine macrophage procoagulant activity and increases production of prostaglandin E2 [19,20]. Since prostaglandin E2 elevates intracellular cAMP, which can inhibit phagocytosis [21-23], meconium may indirectly inhibit phagocytic activity of alveolar macrophages.

The main objective of this study was to determine the effect of meconium on the phagocytic activity of alveolar macrophages. A secondary objective was to determine the effect of meconium on the intracellular production of cAMP by alveolar macrophages.

2.2 Materials and Methods

2.2.1. Cells

A continuous rat alveolar macrophage cell line, NR8383, (American Type Culture Collection, Rockville, MD) was used as a source of cells for all experiments. Cells were cultured in Ham's F12 nutrient mixture with glutamate, supplemented with 15% heat inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all components purchased from Sigma-Aldrich, St. Louis, MO). Cultures were maintained in a humidified, 5% CO_2 incubator at 37° C. This spontaneously transformed cell line grows as a mixture of adherent and non-adherent cells [24]. On the day prior to each experiment, a cell scraper was used to dislodge adherent cells from the culture flask, and all cells were then transferred to a new flask. On the day of the experiment, non-adherent cells were collected for use in assays by pelleting.

2.2.2. Meconium

Human meconium samples were collected from soiled diapers of 5 healthy full term newborns within 8 h of delivery. The samples were cultured (Diagnostic Services, Atlantic Veterinary College, Charlottetown, PE, Canada) to determine sterility, then pooled and diluted with supplemented Ham's media to make a 20% solution. Aliquots were frozen at –70° C. After desiccation, the dry matter of the 20% meconium was determined to be 47.5 mg/ml.

Equine meconium was collected aseptically from the distal colon of a newborn foal submitted for necropsy at the Atlantic Veterinary College. A portion of it was cultured and examined microscopically to confirm absence of bacterial growth. The equine meconium was lyophilized and stored at –70° C. When required, lyophilized

meconium was solubilized in complete Ham's media at a concentration of 50 mg/ml. For some experiments, filtered meconium was obtained by centrifuging the meconium solution at 360 *g* for 10 minutes, and then passing the supernatant through a 0.2 μm filter.

2.2.3. Viability Assay

The viability of NR8383 cells following exposure to meconium was assessed by the trypan blue exclusion test [25]. The test is based on the principle that live cells with intact membranes exclude the dye, whereas dead cells take up the dye and stain blue. Macrophages were incubated at 2 x 10^6 cells/ml in Ham's media with various concentrations of meconium for 6 hours at 37° C. Some cells were incubated with 10 ng/ml PMA for the same time period. Aliquots of at least 100 cells were then stained with 0.4% trypan blue (Sigma-Aldrich), and the number of stained and unstained cells was counted separately on a hemocytometer. The percentage of live cells following meconium exposure was compared to control macrophages that were incubated for a similar length of time without meconium exposure.

2.2.4. Phagocytic Assay

Phagocytic activity of meconium-exposed alveolar macrophages was assessed and compared to control macrophages by quantifying the uptake of fluorescent latex beads using flow cytometry [26-28]. For these assays, NR8383 cells were suspended in Ham's media at a concentration of 2 x 10^6 cells per ml. Aliquots of 500 μl (1 million cells) were placed in 5 ml round-bottom polystyrene tubes. Various concentrations of meconium were added to experimental samples prior to incubation. Control samples contained only macrophages with additional media to bring the final volume in the tube

to that of the experimental tubes (1 ml). In some experiments, 10 ng/ml of PMA was added to some of the tubes. Carboxylated fluorescent latex beads (Sigma-Aldrich) with an average diameter of 2 μm were added at a ratio of 50 beads per cell to half of the tubes. The beads were thoroughly vortexed before use, and flow cytometer analysis showed that less than 7% existed as doublets or larger aggregates. An equal volume (10 μl) of sterile phosphate buffered saline was added to the remaining tubes. The tubes were then capped, vortexed briefly, placed in an opaque container to protect them from light, and incubated for 90 min in a shaking incubator at 37° C. After incubation, 2 ml of ice-cold PBS was added to stop phagocytosis. The cells were centrifuged at 360 *g* for 5 minutes, washed with 1 ml PBS, and centrifuged again. The pellet was resuspended in 400 μl of PBS, and the cells were kept on ice until analysis by flow cytometry (less than 1 h).

2.2.5. Flow Cytometry

The fluorescent intensity of 10,000 cells from each sample was measured using a Becton Dickinson FACSCalibur cytometer, equipped with an argon ion laser operating at 350 mW, 488 nm. Data was acquired in list mode using CellQuest software (Becton Dickinson, San Jose, CA), and analyzed for red and green fluorescence using FCS Express software (De Novo Software). Since the fluorescent beads were much smaller than the cells, forward scatter threshold was set to eliminate free beads and meconium debris from analysis. Propidium iodide (3 μl, Sigma-Aldrich) was added to each tube just prior to analysis. This dye is excluded from viable cells, but penetrates cell membranes of dead cells and intercalates into double-stranded nucleic acids, where it is excited by laser light and fluoresces red. Electronic gating

was used to eliminate from analysis those cells that had taken up propidium iodide and consequently had elevated FL2 (red) fluorescence. Fluorescence data was collected on log scale. Green fluorescence from the FITC labeled beads was measured at 530 ± 30 nm, and red fluorescence from the propidium iodide was measured at 585 ± 42 nm. Electronic compensation was used to minimize spectral overlap between the two fluorochromes. Measurement of phagocytosis included assessment of two parameters—the percentage of cells that were actively phagocytic, and the net median fluorescent intensity of the sample population. The percentage of phagocytic cells was determined by comparison of the fluorescent profile of cells exposed to latex beads to the background autofluorescence of cells not exposed to beads, using Overton histogram subtraction [29]. The cells with a fluorescent intensity greater than background were considered to be actively phagocytic, meaning that they were associated with one or more fluorescent beads. The net median fluorescent intensity (MFI) was calculated by subtracting the background MFI from the MFI of the sample population after exposure to latex beads. Since the fluorescent intensity of each cell is directly proportional to the number of associated fluorescent beads [27], the net MFI is directly related to the average number of beads phagocytosed per cell.

2.2.6. cAMP ELISA

The intracellular level of cAMP in alveolar macrophages was measured using an ELISA kit (Amersham Pharmacia Biotech Inc, Piscataway NJ). NR8383 cells ($1x10^6$ cells/ml) were incubated for 2 h with various concentrations of equine meconium or filtered equine meconium. The competitive binding assay was then performed according to the manufacturer's instructions. Briefly, the cells were lysed, centrifuged,

and the supernatant transferred to an antibody-coated 96 well microtitre plate. Anti-cAMP antibody was added, and the plate was incubated for 2 h. cAMP peroxidase conjugate was then added, the plate was incubated for 1 h, and all wells were washed four times before adding enzyme substrate. Sulfuric acid was added to the wells after 20 min to terminate the reaction, and optical density was read at 450 nm in a model EL311 Microplate Autoreader (Bio-Tek Instruments Inc., Winooski, VT). A standard curve was generated using serial dilutions of a known concentration of cAMP provided in the kit. The concentration of cAMP in fmol/well was determined for the experimental samples by comparison to this standard curve. The assays were performed in quadruplicate for all unknowns, and results are presented as means ± standard deviation.

2.2.7. Data Analysis

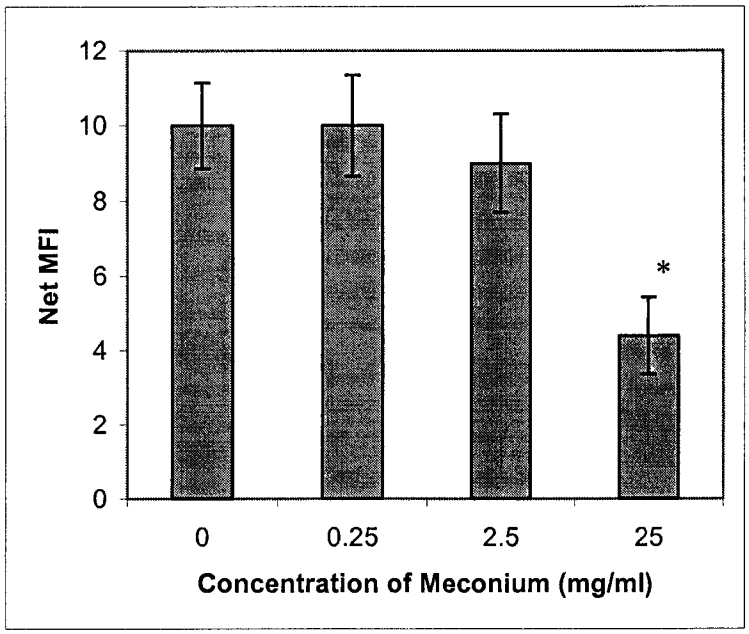
Statistical analysis was performed using InStat2 software (Graphpad Software Inc, CA). Results are reported as means ± SEM unless otherwise stated. Analysis of variance followed by Dunnett's multiple comparison test was used to compare control values to various concentrations of meconium. Significance was accepted as $p<0.05$.

2.3 RESULTS

2.3.1. Effect of Equine and Human Meconium on Phagocytosis

Figure 1a shows that meconium exposure, at a concentration of 25 mg/ml, caused a significant decrease in the net MFI of alveolar macrophages phagocytosing latex beads. This indicates that the meconium-exposed cells phagocytosed fewer latex

(a)



(b)

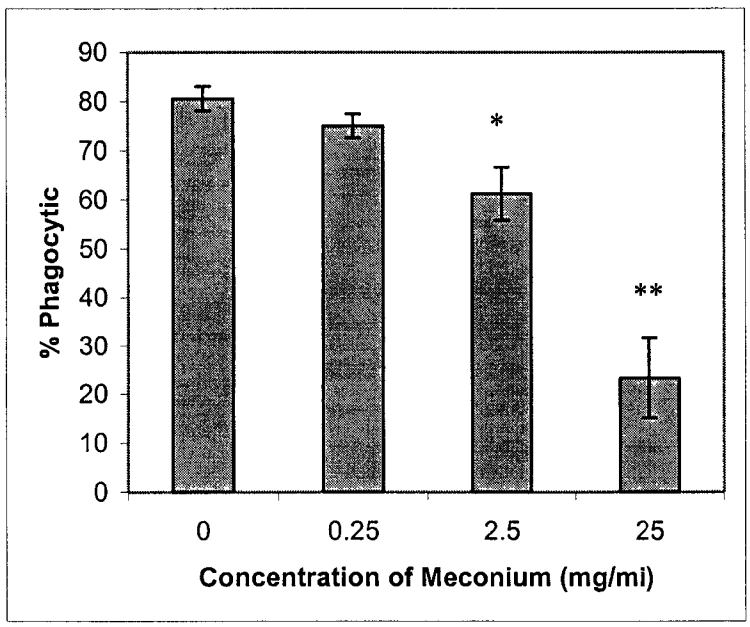


Figure 1. Equine Meconium Inhibits Phagocytosis in Alveolar Macrophages. Alveolar macrophages were incubated with various concentration of equine meconium, and the net MFI (a) and percentage of actively phagocytosing cells (b) was determined after exposure to fluorescent latex beads. Results are shown as means ± SEM (n=5; *, $p<0.05$ **, $p<0.01$).

beads on average than the control cells. Figure 1b shows a dose-dependant decrease in the percentage of macrophages that actively phagocytosed latex beads after meconium exposure. A mean of 81% of the control cells were actively phagocytic, whereas only 23% of the cells exposed to the highest meconium concentration phagocytosed one or more latex beads. This represents a reduction of 70% in phagocytic activity. The percent of phagocytosing cells was significantly different from control cells at a meconium concentration of 2.5 mg/ml ($p<0.05$) and 25 mg/ml ($p<0.01$).

These results were not unique to equine meconium since similar results were obtained using human meconium. These results are shown in Fig. 2.

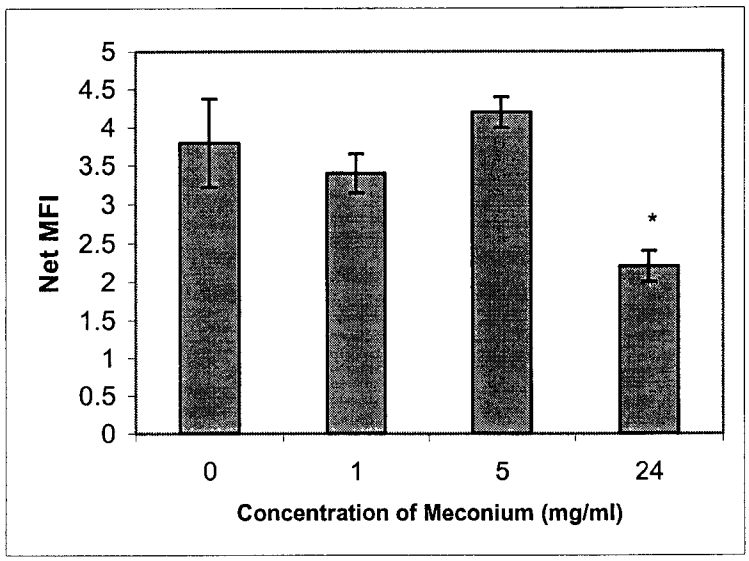
2.3.2 Effect of Meconium Combined with Phorbol Myristate Acetate on Phagocytosis

In order to demonstrate that decreased phagocytosis by macrophages exposed to meconium was not entirely due to saturation by uptake of meconium particles, the experiments were repeated, exposing some cells to PMA as well as meconium. Although the differences were not statistically significant, Fig. 3a shows that PMA stimulation caused an increase in net MFI, indicating that macrophages were capable of phagocytosing more latex beads even after meconium exposure. The percentage of actively phagocytosing cells however, did not appear to increase following PMA exposure (Figure 3b).

2.3.3. Effect of Filtered Meconium on Phagocytosis

To further characterize its effect on phagocytosis, some meconium was passed through a 0.2 μm filter to remove particulate matter. This filtered meconium was then incubated with macrophages and the experiments repeated. The results in Fig. 4b show that filtered meconium significantly decreased the percentage of actively phagocytosing

(a)



(b)

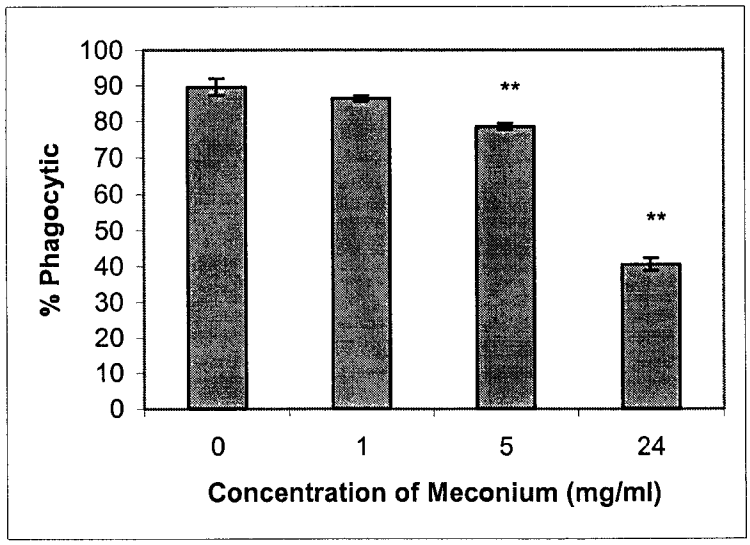
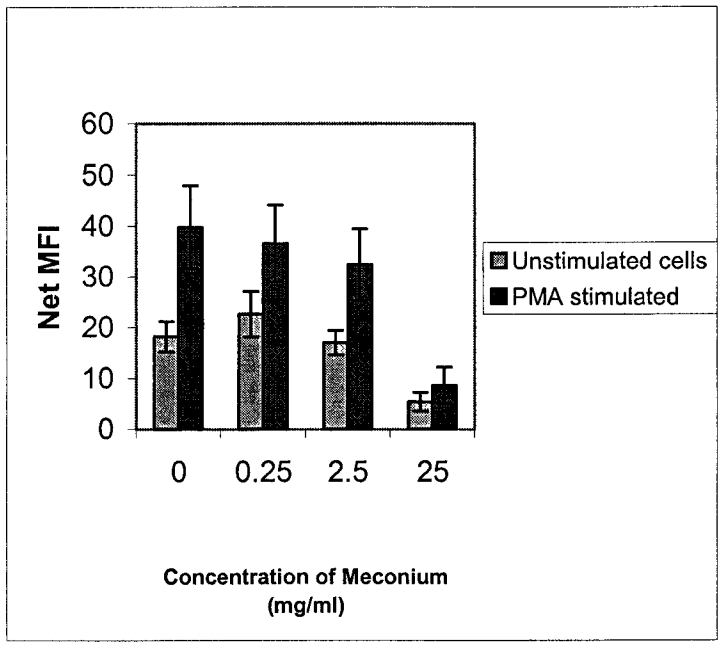


Figure 2. Human Meconium Inhibits Phagocytosis in Alveolar Macrophages. Alveolar macrophages were incubated with various concentration of human meconium, and the net MFI (a) and percentage of actively phagocytosing cells (b) was determined after exposure to fluorescent latex beads. Results are shown as means ± SEM (n=5; *, $p<0.05$ **, $p<0.01$).

(a)



(b)

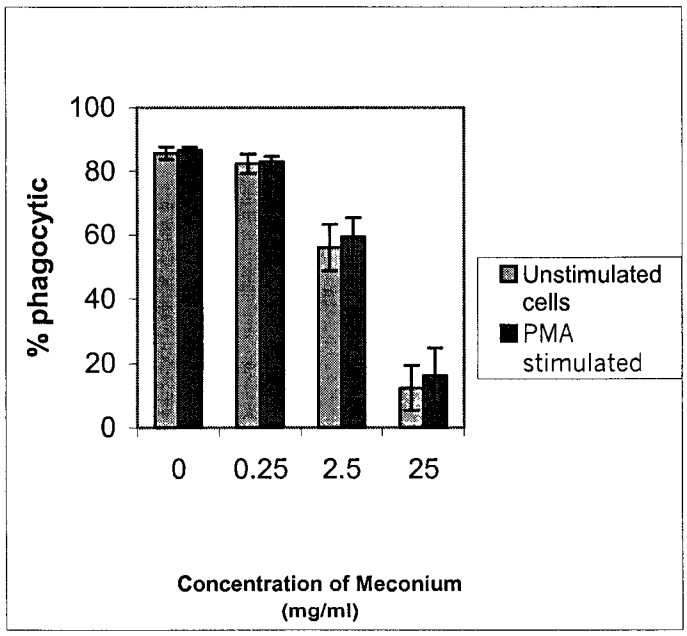
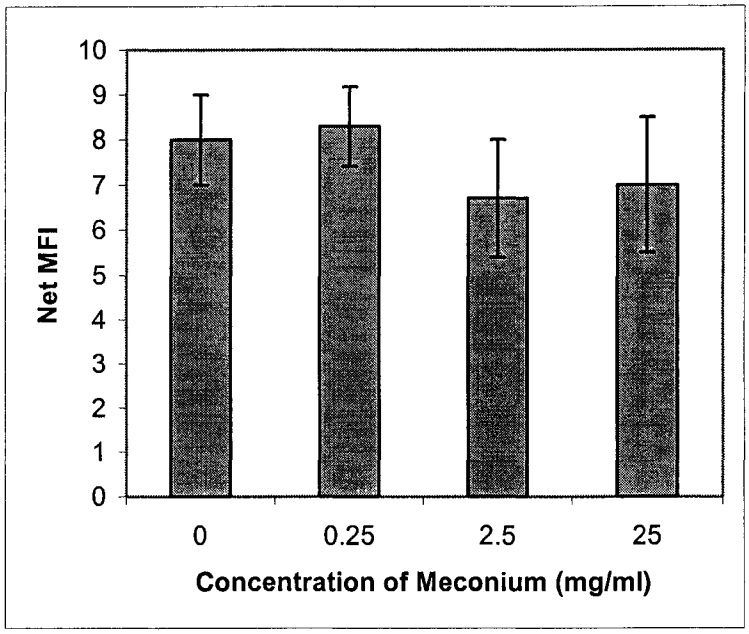


Figure 3. Phorbol Myristate Acetate Stimulates Additional Phagocytosis in Meconium-Exposed Alveolar Macrophages. Alveolar macrophages were incubated with various concentration of equine meconium with or without PMA, and the net MFI (a) and percentage of actively phagocytosing cells (b) was determined after exposure to fluorescent latex beads. Results are shown as means ± SEM (n=5).

(a)



(b)

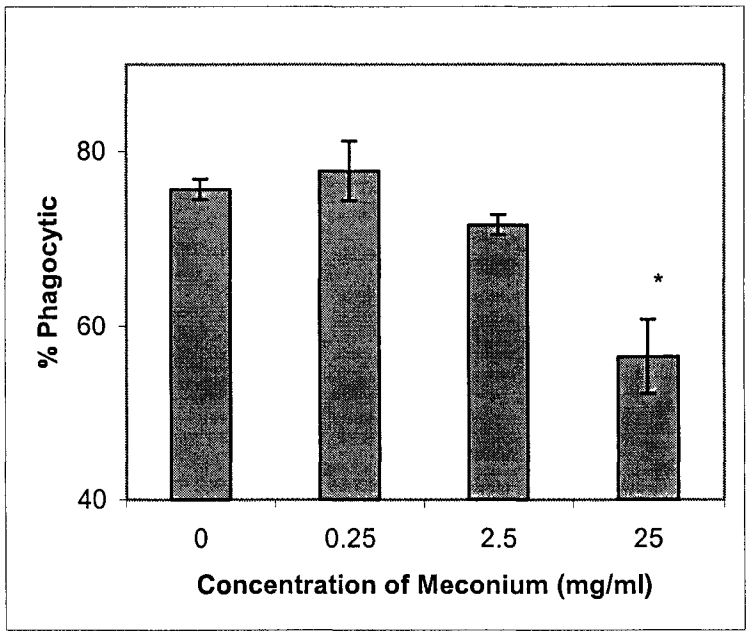


Figure 4. The Effect of Filtered Equine Meconium on Phagocytosis in Alveolar Macrophages. Alveolar macrophages were incubated with various concentrations of 0.2μm filtered equine meconium, and the net MFI (a) and percentage of actively phagocytosing cells (b) was determined after exposure to fluorescent latex beads. Results are shown as means ± SEM (n=3; *, $p<0.05$).

cells at a concentration of 25 mg/ml. The percentage of actively phagocytosing cells decreased from a mean of 75% in the control samples, to 56% in the filtered meconium samples, a reduction of about 25%. The filtered meconium did not cause a significant reduction in net MFI. In general, the effect of filtered meconium on phagocytic activity was attenuated compared to that of the unfiltered meconium shown in Figures 1 and 2.

2.3.4. Effect of Meconium on Cell Viability

To determine if meconium caused cell death at the concentrations used in these assays, NR8383 cell viability was assessed following incubation with meconium or PMA. Tables 1 and 2 show the percentage of viable cells after a 6 h incubation with either equine (Table I) or human (Table II) meconium. There were no significant differences in viability of meconium-treated compared to similarly incubated control cells.

2.3.5. Effect of Meconium on Intracellular cAMP Production

The role of cAMP in producing the inhibition of phagocytic activity seen in meconium-exposed macrophages was investigated by measuring intracellular cAMP using an ELISA kit. Figure 5 shows that the intracellular production of cAMP within macrophages was not significantly altered by incubation with various concentrations of filtered or unfiltered meconium, suggesting that alternate inhibitory pathways might be involved.

Table I.

Alveolar Macrophage Viability is Not Reduced Following Six Hours of Incubation With Equine Meconium

Treatment	**% Live Cells[a]**
Control (media)	93.7 ± 1.9
Equine Meconium (25mg/ml)	92.3 ± 3.4
Filtered Equine meconium (25mg/ml)	93.5 ± 3.2
Phorbol Myristate Acetate (10ng/ml)	92.2 ± 2.5

[a] Cell viability after 6 hours of incubation with equine meconium was measured using trypan blue exclusion. Results are shown as means ± SEM ($n \geq 4$).

Table II.

Alveolar Macrophage Viability is Not Reduced Following Six Hours of Incubation With Human Meconium

Treatment	% Live Cells[a]
Control (media)	83.2 ± 3.5
Human Meconium (10%)	82.6 ± 1.8

[a] Cell viability after 6 hours of incubation with human meconium was measured using trypan blue exclusion. Results are shown as means ± SEM ($n \geq 4$).

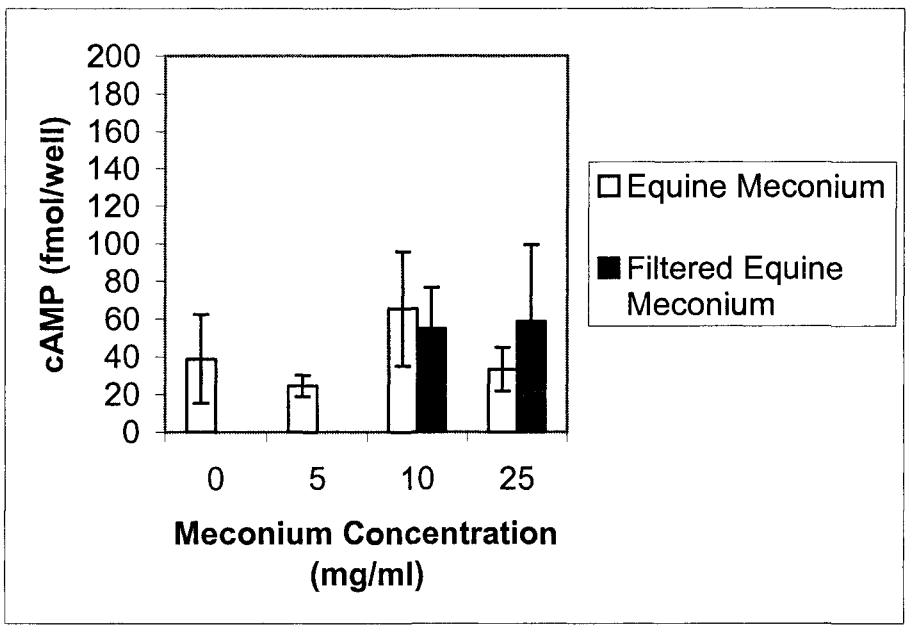


Figure 5. Incubation with Equine Meconium Does Not Increase Intracellular cAMP Levels in Alveolar Macrophages. The effect of meconium exposure on macrophage production of cAMP was determined using ELISA. Three concentrations of equine meconium were used (25, 10 and 5 mg/ml) and two concentrations of filtered equine meconium (25 and 10 mg/ml). Results are shown as means ± standard deviation (n=4).

2.4. DISCUSSION

2.4.1. Effect of Meconium on Phagocytosis

This study demonstrates that exposure to meconium can inhibit the phagocytic activity of alveolar macrophages. Incubation of macrophages with either equine or human meconium caused a significant decrease in the percentage of cells that subsequently phagocytosed latex beads, and this effect appeared to be dose related. The effect of meconium on the net MFI was more variable, but nonetheless was significant at the highest concentrations (25 mg/ml for equine, 24 mg/ml for human).

A decrease in alveolar macrophage phagocytic activity following meconium exposure was not entirely unexpected, since others have reported decreases in phagocytic activity following exposure of alveolar macrophages to various inhaled particulates, including cotton smoke [30], carbon particles [31,32], silica [33], diesel exhaust [34], titanium oxide [35] and other air pollution particles [36].

All experiments in this study were performed using a rat alveolar macrophage cell line. Although considerable differences are seen in phagocytic activity of alveolar macrophages depending on the cell source and experimental conditions [37-39], the NR8383 line has been shown to be a good model for alveolar macrophage phagocytosis, and to produce levels of activity similar to that of freshly derived rat alveolar macrophages [37,40]. The NR8383 macrophages used in this study were probably not physiologically equivalent to neonatal macrophages, since some studies have reported that neonatal macrophages have reduced phagocytic ability compared to mature macrophages [41,42]. Nonetheless, neonatal alveolar macrophages are functionally capable of phagocytosis, since surfactant can be identified microscopically in alveolar

macrophages both pre- and postnatally [43], and meconium can be observed in alveolar macrophages in neonatal rats following experimental instillation [44]. Extrapolation of the results of this study to the clinical problem of MAS in neonates is not without risk however. Alveolar macrophages in human neonates exist in an exceedingly complex environment, and are exposed to a whole host of influences that could alter their phagocytic activity. For example, various neuropeptides and hormones have been identified in bronchoalveolar lavage fluid, including substance P [45], calcitonin [46], adrenocorticotropic hormone, and corticotropin-releasing hormone [47]. Many of these are known to modulate immune responses, including macrophage phagocytic activity [48,49]. In addition, the presence of surfactant in neonatal lungs is likely to affect phagocytic uptake of particles [50,51]. Furthermore, aspirated meconium is necessarily diluted with amniotic fluid, which may contain anti-inflammatory substances, a number of which have been identified, including IL-1 receptor antagonist [52], secretory leukocyte protease inhibitor [53], alpha-fetoprotein [54], Clara cell protein [55], and interleukin-10 [56,57]. Some of these, including alpha-fetoprotein and IL-10, have been linked to impairment of phagocytic activity [58,59]. While the goal of this study was to specifically determine the effect of meconium on phagocytosis in alveolar macrophages, an *in vivo* analysis would be useful to delineate the combined effect of these and other factors.

2.4.2. Comparison Between Human and Equine Meconium

It is noteworthy that both human and equine meconium had similar effects on rat alveolar macrophages. The preparation of the two types of meconium differed, since the equine meconium was lyophilized before use and then reconstituted at 50 mg/ml,

while the human meconium was stored as a 20% solution. However the dry matter of the human meconium was calculated later, and the 20% solution was found to be equivalent to 47.5 mg/ml. Although the diets of these two mammals are quite different, the newborn digestive tract does not contain ingesta, so the meconium composition might in fact be quite similar. To date most studies have used human meconium, however, substitution of equine meconium for human in *in vitro* experiments has practical significance, since large quantities of animal meconium can be collected easily from necropsy specimens without the requirement for human ethics approval.

2.4.3. Effect of Meconium and PMA on Phagocytosis

The experiments using PMA demonstrate that the inhibitory effect of meconium on phagocytosis is not merely due to saturation of the macrophages with meconium particles prior to latex bead exposure, since the cells could be stimulated to phagocytose more particles when PMA was added. PMA stimulates phagocytes by directly activating protein kinase C, and therefore bypasses receptor-mediated signaling mechanisms. This suggests the involvement of other possible mechanisms for the meconium-induced decrease in phagocytic activity, such as alteration of surface receptor expression, distribution, or availability [60], inactivation of some component of receptor-mediated intracellular signaling pathways, or activation of an inhibitory pathway.

2.4.4. Effect of Filtered Meconium

A possible explanation for the decrease in phagocytic activity following meconium-exposure could be competition between meconium particles and latex beads for phagocytic receptor binding sites. This mechanism was suggested by Clark and

Duff (1995) when they reported that meconium decreased the phagocytic uptake of fluorescent bacteria by neutrophils [16]. Scavenger receptors are believed to mediate phagocytosis of unopsonized latex beads [61,62]. Although the specific receptors mediating phagocytosis of meconium are unknown, scavenger receptors are likely to be involved given that meconium particles in this study were also unopsonized. However, filtration of the meconium to remove the particulate matter did not prevent the inhibitory effect on phagocytosis, since there was still a significant decrease in the percentage of phagocytosing cells. Nonetheless, at the same concentration filtered meconium reduced the percentage of phagocytosing cells by only 25%, while unfiltered meconium reduced it by 70%. Competition for binding sites may therefore be responsible for at least some of the meconium-induced reduction in phagocytic activity. Altering the experimental design to use an opsonized particle as a target for the phagocytic assay could assess the magnitude of this contribution. Then the meconium and the assay targets would be phagocytosed by different receptors, and simple competition for binding sites would not be an issue.

2.4.5. Effect of Meconium on Viability

Another possible explanation for the observed decrease in phagocytic activity could be that meconium is directly toxic to macrophages. This is the likely mechanism for the inhibitory effect of silica, since this particle is considered a macrophage poison, and cell viability is decreased in a dose-dependant way following exposure [63]. In the present study, this possibility was investigated using trypan blue exclusion. Since no significant difference was seen in the number of live cells following 6 h of meconium exposure, it does not seem that direct cell death can account for the difference in

phagocytosis seen after the 90 min exposure to meconium. However, the trypan blue exclusion test does not preclude the possibility that meconium may have more subtle toxic effects on macrophages. It is possible that cell viability may be compromised even though membrane integrity is unaffected. Soukup and Becker (2001) reported that alveolar macrophages exposed to air pollution particles showed little toxicity by trypan blue but increased apoptosis [36]. Increased apoptosis of macrophages following exposure to various ultrafine particles was also reported by Moller *et al* (2002), although the dominant fraction of nonviable cells were necrotic [35]. The possibility of meconium-induced apoptosis could be investigated using a technique such as the JAM test, which is a simple and sensitive assay for DNA fragmentation and cell death [64].

Toxicity to the macrophage cytoskeleton is another possible mechanism for the observed effect of meconium on phagocytosis. Moller *et al* (2002) studied the effect of various fine and ultrafine particles on the cytoskeleton of alveolar macrophages using cytomagnetometry [35]. They reported that high concentrations of particles caused cytoskeletal dysfunction, including retarded intracellular transport processes and increased cytoskeletal stiffness. Although the particles also caused impairment in phagocytosis, the magnitude of these defects was not correlated.

2.4.6. Effect of Meconium on Intracellular cAMP Production

Meconium could produce its effect on phagocytic activity by activating an inhibitory pathway. A known inhibitory pathway for phagocytosis involves production of cAMP and activation of protein kinase A [21-23]. Meconium has been found to increase production of prostaglandin E_2 (PGE_2) in murine macrophages [19,20]. Since PGE_2 elevates intracellular cAMP, indirect inhibition of phagocytic activity via this

pathway seemed likely. However, incubation of macrophages with equine meconium did not cause a significant increase in cAMP. This finding does not rule out the possible involvement of an inhibitory pathway, since meconium could act downstream of cAMP. Other, less well characterized inhibitory molecules or pathways might also be involved. For example, Ichinose [48] reported that adrenocorticotropic hormone caused suppression of macrophage phagocytosis by a mechanism that was not mediated by cAMP. In addition, meconium has been shown to induce production of nitric oxide (NO) in alveolar macrophages [65], and one of the many effects of this molecule is to inhibit phagocytosis [66].

Rather than activating an inhibitory pathway, meconium could deactivate some component of the particle-induced intracellular signaling pathways required for phagocytosis. Signal transduction pathways involved in phagocytosis are exceedingly complex, and although they remain poorly characterized, they offer many potential sites for interference by deactivation. Some candidate molecules known to be involved in signaling pathways triggered by various phagocytic receptors include protein kinase C [8,67-69], phospholipase D [69,70], and protein tyrosine kinases. Protein tyrosine phosphorylation of receptors is one of the first events triggered by phagocytosis, and tyrosine kinases play a crucial role in signal transduction in Fcγ-mediated [71], CR-mediated [69], and opsonin-independent phagocytosis [72]. Furthermore, tyrosine kinase inhibitors such as herbimycin A reduce phagocytic activity [69]. Phagocytosis and production of proinflammatory cytokines by macrophages have been shown to coincide with increased tyrosine phosphorylation of distinct proteins following exposure to various stimuli including the microbial products zymosan and LPS, as well

as titanium particles [69,73]. Some pathogens are known to secrete products that cause tyrosine dephosphorylation and thus inhibit phagocytosis [74,75]. Further investigation into the mechanism involved in meconium-induced inhibition of phagocytosis should include examination of the role of protein tyrosine phosphorylation.

2.4.7. Technical Issues

Flow cytometry is advantageous for the assessment of phagocytosis because it allows large numbers of cells to be analyzed and offers major improvements in speed and accuracy over manual counting methods [26,27]. In addition, it is not necessary to employ washing to physically separate cells from free particles prior to analysis [76].

It was not possible to distinguish between beads that had been ingested and those that were merely bound to the surface of macrophages using the technique described in this study. Two-color staining [77] or fluorescence quenching [78-80] techniques involving a different type of target particle can be used when this information is required. Quenching techniques cannot be used with fluorescent beads because the fluorescence is not confined to the surface of the target. Initially, confocal microscopy was used in an effort to distinguish between ingested and attached beads. However, this method was found to be extremely imprecise and time-consuming, requiring the examination of individual cells. These limitations negated many of the advantages of the flow cytometric technique, since only a small number of cells could be analyzed by confocal microscopy. Some investigators have reported that this distinction may not be necessary, since the majority of cell-associated targets are eventually internalized by alveolar macrophages [81-83].

The addition of meconium to cells caused a directly proportional increase in fluorescent intensity, even when no latex beads were present. This meant that the fluorescent intensity after latex bead exposure could not be directly compared between control and meconium-exposed cells. To overcome this problem, the net MFI was calculated by subtracting the MFI of cells alone from the MFI of cells with beads. The net MFI could then be compared between control and meconium-exposed cells.

One of the difficulties with achieving significant results was that there was considerable day-to-day variation in the net MFI of macrophages, even in the control groups. Since the phagocytic activity of macrophages depends in part on their activation state, it seems likely that macrophages used in some experiments were more or less activated than others. Macrophages *in vitro* can be activated by a variety of stimuli, including temperature changes, changes in oxygen tension [84], and adherence to plastic [85] or glassware. Furthermore, serum contains various proteins, lipids and electrolytes that might affect uptake of particles [86], and more than one batch of heat-inactivated serum was used for these experiments. Variations in experimental procedures such as incubation time, particle to cell ratio, and mixing are known to have a major influence on phagocytic activity [86]. Although these factors were standardized in the present series of experiments, it is possible that slight variations in unmeasured environmental factors may have influenced the activation state of the cells.

2.4.8. Biological Significance

The findings reported in this study are likely to have biological significance. Human neonates with meconium aspiration syndrome probably have impaired alveolar macrophage phagocytic function. Since these phagocytic cells are critical in defending

the host against inhaled pathogens, such neonates might be at increased risk for pulmonary infections. Alveolar macrophages also defend against other inhaled particles that could cause damage to more sensitive cells if not cleared [31]. Meconium-induced inhibition of phagocytosis could therefore render the infant more susceptible to further lung damage. This study investigated only the effect of meconium on phagocytosis, but other macrophage functions might be affected as well, including respiratory burst and cytokine production. Since the alveolar macrophage is the most important regulator of the immune response in the lung, a better understanding of the effect of meconium on these cells could aid in the development of therapeutic interventions aimed at preventing or ameliorating the inflammatory component of MAS. This in turn could have significant implications in terms of improving outcome for babies with this syndrome.

2.5. CONCLUSIONS

Meconium (equine or human) caused a dose dependant decrease in the phagocytic activity of alveolar macrophages. The effect was due to a reduction in both the percentage of actively phagocytosing cells, and in the average number of particles phagocytosed per cell. Filtration of the meconium (0.2 μm) only partially eliminated this effect. Exposure of alveolar macrophages to meconium did not decrease cell viability as measured by trypan blue exclusion, and did not produce an elevation of intracellular cAMP. The mechanism by which meconium effects this inhibition of phagocytosis remains to be elucidated. Since alveolar macrophages are essential in

clearing inhaled foreign particulates from the lungs, decreased phagocytic activity following exposure of macrophages to meconium could have important implications for neonates with MAS, such as increased susceptibility to infections.

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3. MECONIUM STIMULATES REPIRATORY BURST ACTIVITY IN ALVEOLAR MACROPHAGES, BUT ATTENUATES OXIDATIVE RESPONSE TO SECONDARY CHALLENGE

3.1 Introduction

Meconium aspiration syndrome occurs when a fetus or newborn infant inhales meconium. In the majority of normal infants, meconium is expelled in the first 24 hours after parturition [1]. However, in about 12-15% of infants, meconium is expelled while the infant is still *in utero* [2]. This occurrence is more common in post mature infants, and in those stressed by fetal hypoxia [3,4]. Meconium aspiration syndrome (MAS) is seen in about 5% of infants born through meconium-stained amniotic fluid, and is an important cause of respiratory distress in newborns [5].

The pathophysiology of this syndrome is complex, involving airway obstruction, with subsequent ventilation-perfusion mismatching, hypoxia, hypercapnia, and respiratory acidosis [4]. Inflammation is also an important part of the pathophysiology. A profound pulmonary inflammatory response is apparent within several hours of meconium aspiration, with a peak influx of neutrophils, macrophages and serum proteins at 16-24 hours [6]. Bile salts and other substances in meconium are thought to irritate and injure blood vessels and other cells in the lung, particularly the type II pneumocytes, with resulting changes in surfactant production and function [6,7]. Meconium has been shown to reduce host resistance to bacterial infection in rats and

mice [8]. Furthermore, clinical findings in humans demonstrate that meconium-stained amniotic fluid is associated with intra-amniotic infection [9-11].

Pulmonary alveolar macrophages are specialized phagocytic cells found in the alveoli of the lung. Their primary role is phagocytosis and clearance of inhaled particles, but they are also important in initiating inflammatory responses in the lung [12]. Phagocytosis of aspirated meconium by alveolar macrophages is a primary event in MAS, and the response of these cells is likely to play a pivotal role in the ensuing pathology.

Once phagocytosis has occurred, macrophages can undergo an oxidative metabolic process called respiratory burst, which generates microbicidal products. Although primarily a function of macrophages and neutrophils, respiratory burst is seen in other types of cells as well, including B lymphocytes, fibroblasts, and endothelial cells [13]. Respiratory burst is a series of interconnected events involving consumption of oxygen, catabolism of glucose through the hexose monophosphate shunt, and the generation of reduced, chemically reactive oxygen species, including superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl radicals. These reactive oxygen species (ROS) comprise an effective microbicidal system against a large variety of invading organisms. In addition to their role in killing and inflammation, there has recently been increasing evidence that ROS may serve as second messengers for cell signaling. Reactive oxygen species, particularly hydrogen peroxide, have been shown to activate signaling pathways and modulate physiological responses in macrophages [14]. Indeed, some have speculated that signaling, rather than microbial killing, may be the most important role of ROS in these cells [14].

Phagocytosis of particulate material is a known trigger of respiratory burst. In fact, respiratory burst was first identified when it was noticed that oxygen consumption increased following phagocytosis. It was later determined that particle binding to phagocytic receptors was sufficient stimulus to trigger respiratory burst, and that phagocytosis *per se* was not necessary. Some soluble stimuli, such as phorbol myristate acetate (PMA), complement components, chemotactic peptides or calcium ionophores can also trigger a strong respiratory burst response [15,16]. PMA is one of the most potent activators of respiratory burst [17] and is a commonly used stimulus for respiratory burst assays. However, because PMA directly activates protein kinase C (PKC), it bypasses receptor-mediated signaling mechanisms that are necessary for combating disease [18] and may therefore not represent a physiologic stimulus [19].

Exposure of phagocytes to various types of particulates has been shown to stimulate respiratory burst. Bacteria such as *Staphylococcus aureus* [20] and *Pneumocystis carinii* [21], and other particles including zymosan [22,23], asbestos fibers [24], ceramic biomaterials [25], and various environmental air pollution particles [26,27] have all been shown to stimulate a respiratory burst response in macrophages. However, the magnitude and duration of the oxidative response following ingestion of particles is variable, and may depend partly on the specific receptors ligated. Some particles, particularly Fc-opsonized ones, consistently trigger a strong oxidative response [28]. However, particles which are phagocytosed via other receptors may not always trigger respiratory burst [29,30]. Kobzik *et al* [28] investigated this phenomenon in alveolar macrophages and found that whereas opsonized particles triggered a respiratory burst that was correlated with the number of particles ingested,

phagocytosis of unopsonized particles led to inhibition of respiratory burst. This inhibitory effect was most evident when only small numbers of unopsonized particles (1-3 per cell) were phagocytosed. Alveolar macrophages that had ingested unopsonized particles also showed inhibition of oxidative metabolism after subsequent ingestion of opsonized particles. Unopsonized particles are phagocytosed mainly via scavenger receptors [31], one of which, MARCO, has been implicated in binding environmental particles such as titanium oxide and iron oxide, as well as latex beads [32]. Particle binding to scavenger receptors does not appear to trigger a significant respiratory burst in alveolar macrophages [31].

The primary reaction of the respiratory burst is catalyzed by an enzyme called NADPH oxidase and involves the reduction of oxygen to form superoxide anion. NADPH serves as an electron donor for this reaction. The oxidase enzyme is made up of several components. The core component is flavocytochrome b_{558}, which is located within the plasma membrane of the cell. It is a heterodimer made up of a glycoprotein, $gp91^{phox}$, and a protein, $p22^{phox}$. The other components are water-soluble cytosolic proteins named $p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and a small G protein, Rac. When phagocytes are stimulated, these cytosolic components are translocated to the membrane-bound flavocytochrome, the enzyme becomes activated, and superoxide anion is produced. Binding of ligands to specific receptors on the surface of the phagocyte is the usual stimulus, although soluble stimuli such as PMA can also effect this stimulation. Some parts of this activation pathway are beginning to be understood. PKC phosphorylates $p47^{phox}$, which then undergoes a conformational change, allowing it to bind to other cytosolic phox proteins [33]. This triad of cytosolic phox proteins then translocates to

the membrane-bound flavocytochrome to assemble the active oxidase [13]. Phosphorylation of membrane-bound $p22^{phox}$ also coincides with NADPH oxidase activation, but seems to occur by two different mechanisms, one of which is phospholipase D-dependant [34]. Lipid second messengers also appear to be involved in regulation of this process [35].

The product of this initial reaction, superoxide anion, is an extremely reactive and unstable molecule containing two oxygen atoms with an extra electron. The superoxide anion is rapidly converted to hydrogen peroxide within the cell through the action of superoxide dismutase. Superoxide and hydrogen peroxide can damage and kill pathogens directly and can also combine with iron to form hydroxyl radicals which can cause breaks in strands of DNA [36]. The importance of the respiratory burst in host defense is illustrated by an inherited disorder called chronic granulomatous disease. People suffering from this condition have neutrophils and macrophages that are unable to mount a respiratory burst, due to one of several defects in the oxidase enzyme complex. These people are susceptible to severe and recurring infections [37]. Macrophages also produce reactive nitrogen intermediates, including nitric oxide, through the action of inducible nitric oxide synthase [38]. Other antimicrobial molecules produced by macrophages include defensins and proteases [36,39]. Although production of these various antimicrobial molecules is essential in host defense, it can also cause injury to surrounding tissue when excessive [12,36,40].

Bacterial killing occurs rapidly and does not require an extended duration of respiratory burst to be effective. Since ROS can be detrimental to host tissues, it is not

surprising that the duration of the respiratory burst response should be carefully regulated within the cell.

The respiratory burst is a self-limiting event. The duration of NADPH oxidase activity varies with the stimulus and depends in part on specific receptor-ligand interactions and signal transduction pathways [41]. Some stimuli cause immediate activation and a quick decline in activity. This type of response has been found after stimulation with zymosan, for example, where activity of the enzyme declined after 3 minutes [42]. Other stimuli, such as PMA, cause activation that persists for as long as 90 minutes [15]. The cellular events involved in termination of this process remain virtually unknown [13], although displacement of agonist from its receptor is one component [41], and the loss of $p47/67^{phox}$ from the membrane may also be involved [43]. This decline in respiratory burst activity is due to termination of oxidase activity and not to lack of substrate availability or to production of prostaglandins [43,44]. Likewise, the products of the respiratory burst do not appear to be responsible for its termination, since the addition of ROS scavengers does not prevent termination [22,45]. Some studies have suggested the presence of a cytosolic NADPH oxidase deactivator, which may function by displacing the G protein from the active oxidase complex [44].

Once terminated, respiratory burst can usually be re-stimulated, suggesting that the deactivation is reversible. Alternatively, re-stimulation could be due to the activation of reserve enzyme [44] or to regeneration of new oxidase [22]. However, in some cases, macrophages become refractory to secondary stimulus, although the desensitization may not extend to all stimuli. Berton and Gordon (1983) showed that zymosan and PMA caused quite different refractory states in macrophages [22].

Desensitization due to primary stimulation with zymosan was relatively short-lived (less than 24 hours) and was specific for homologous (zymosan) stimuli. On the other hand, PMA triggered a refractory state that lasted at least three days and was less specific, since the PMA-stimulated cells were refractory to subsequent heterologous as well as homologous stimulation. Desensitization of the macrophage respiratory burst following pre-stimulation may be due to activation of peroxisome proliferator-activated receptors (PPAR)[46]. PPAR are transcription factors with anti-inflammatory properties that bind to PPAR response elements and affect target gene expression. PPAR-λ is upregulated in activated macrophages and inhibits expression of inflammatory cytokines [47,48]. Pre-stimulation of macrophages, or activation of PPAR-λ by synthetic agonists, has been shown to attenuate respiratory burst [46]. In addition, inactivation of PPAR-λ by decoy oligonucleotides prevented the macrophage desensitization [46]. Regulation of the respiratory burst response is obviously extremely important in preventing tissue damage, yet remains poorly understood.

Despite the importance of alveolar macrophages in controlling and contributing to inflammatory processes in the lung, little is known about the effect of meconium on the various functions of alveolar macrophages. Since meconium is a particulate material, we hypothesized that alveolar macrophages exposed to aspirated meconium might generate significant amounts of ROS that could play a role in the pathology of MAS by inducing pulmonary damage. The main objective of this study was to determine whether meconium stimulates respiratory burst in alveolar macrophages. In the event that such stimulation was observed, a secondary objective was to determine whether the effect persisted beyond the immediate exposure period.

3.2 Materials and Methods

3.2.1. Cells

A continuous rat alveolar macrophage cell line, NR8383, (American Type Culture Collection, Rockville, MD) was used as a source of cells for all experiments. Cells were cultured in Ham's F12 nutrient mixture with glutamate, supplemented with 15% heat inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all components purchased from Sigma-Aldrich, St. Louis, MO). Cultures were maintained in a humidified, 5% CO_2 incubator at 37° C. This spontaneously transformed cell line grows as a mixture of adherent and non-adherent cells. On the day prior to each experiment, a cell scraper was used to dislodge adherent cells from the culture flask, and all cells were then transferred to a new flask. On the day of the experiment, non-adherent cells were collected for use in assays by pelleting.

3.2.2. Meconium

Human meconium samples were collected from soiled diapers of 5 healthy full term newborns within 8 h of delivery. The samples were cultured (Diagnostic Services, Atlantic Veterinary College, Charlottetown, PE, Canada) to determine sterility, then pooled and diluted with supplemented Ham's media to make a 20% solution. Aliquots were frozen at –70° C, and further dilutions were made as required. After desiccation, the dry matter of the 20% meconium was determined to be 47.5 mg/ml.

Equine meconium was collected aseptically from the distal colon of a newborn foal submitted for necropsy at the Atlantic Veterinary College. A portion of it was cultured and examined microscopically to confirm absence of bacterial growth. The

equine meconium was lyophilized, and stored at –70° C. When required, lyophilized meconium was solubilized in complete Ham's media at a concentration of 50 mg/ml. Further dilutions were made as indicated. For some experiments, 0.2 μm filtered meconium was obtained by centrifuging the meconium solution at 360 *g* for 10 min, and then passing the supernatant through a 0.2 μm filter.

3.2.3. Viability Assay

The viability of NR8383 cells following exposure to meconium was assessed by the trypan blue exclusion test [49]. Live cells with intact membranes exclude the dye, whereas dead cells take up the dye and stain blue. Macrophages were incubated at 2 x 10^6 cells/ml in supplemented Ham's media with various concentrations of meconium for 4 or 24 h at 37° C. Aliquots of at least 100 cells were then stained with 0.4% trypan blue, and the number of stained and unstained cells was counted separately on a hemocytometer. The percentage of live cells following meconium exposure was compared to control macrophages that were incubated for a similar length of time without meconium exposure.

3.2.4. Respiratory Burst Assay

Respiratory burst activity of meconium-exposed alveolar macrophages was assessed and compared to control macrophages using flow cytometry to measure production of a fluorescent probe. The use of this technique to assess respiratory burst in neutrophils was described by Bass *et al* [50] and has also been used for the same purpose in other phagocytes [51]. For these assays, NR8383 cells were suspended in Ham's complete media at a concentration of 2 x 10^6 cells/ml. Aliquots of 500 μl (1 million cells) were placed in 5 ml round-bottom polystyrene tubes. The cells were pre-

incubated with 5 μl of dichlorofluorescin diacetate (20 μM final) for 20 min at 37° C in the dark on a shaker platform to load the dye. Dichlorofluorescin diacetate (DCFH-DA) is a stable non-polar, non-fluorescent probe. It penetrates cell membranes by passive diffusion. Once inside, it is rapidly deacetylated by intracellular esterases to dichlorofluorescin (DCFH)[16]. This compound is polar, and therefore becomes trapped inside the cell. In the presence of ROS, it is rapidly oxidized to dichlorofluorescein (DCF), which is highly fluorescent. The ROS mainly responsible for the oxidation of DCFH is thought to be H_2O_2 [52,53]. Measurement of the fluorescent product provides a quantitative assessment of the respiratory burst in individual cells [50].

After loading with DCFH-DA, various concentrations of meconium were added to experimental samples, and the cells were further incubated for 1 h. Control samples contained only macrophages with additional media to bring the final volume in the tube to that of the experimental tubes (1ml). Some control samples were stimulated by the addition of PMA (100 ng/ml) prior to the final incubation. Following incubation, ice cold phosphate buffered saline (PBS) was added to stop the reaction, and the cells were washed twice and resuspended in 400 μl cold PBS for analysis by flow cytometry, which was performed within 30 min.

To investigate the duration of the effect of meconium on alveolar macrophages, some macrophages were incubated with meconium for 2 h, then washed and incubated in supplemented Ham's media overnight. After 24 h, DCFH-DA was loaded as described above, and cells were stimulated with 100 ng/ml PMA before flow cytometric analysis.

3.2.5. Flow Cytometry

The fluorescent intensity of 10,000 cells from each sample was measured using a Becton Dickinson FACSCalibur cytometer, equipped with an argon ion laser operating at 350 mW, 488 nm. Data was acquired in list mode using CellQuest software (Becton Dickinson, San Jose, CA), and analyzed for green fluorescence using FCS Express software (De Novo Software). Electronic gating based on forward and side scatter distribution profiles was used to eliminate debris and meconium particles from analysis. As an additional control, some of the meconium was passed through a 5.0 μm filter to remove any aggregates of meconium that could be confused with cells during flow cytometry. Fluorescence data was collected on log scale. Green fluorescence from DCF was measured at 530 ± 30 nm.

Measurement of respiratory burst included assessment of two parameters—the percentage of positive cells [54], and the net median fluorescent intensity of the sample population. The percentage of positive cells was determined by comparison of the fluorescent profile of cells loaded with DCFH-DA to the background autofluorescence of cells not loaded with DCFH-DA, using Overton histogram subtraction [55]. The cells with a fluorescent intensity greater than background were considered positive, meaning that respiratory burst had occurred. The net median fluorescent intensity (MFI) was calculated by subtracting the background MFI from the MFI of the sample population after loading with DCFH-DA, as follows:

Net MFI = (MFI of cells incubated with meconium and DCFH-DA) – (MFI of cells incubated with meconium alone). Since the fluorescent intensity of each cell is

directly proportional to the amount of DCF produced, the net MFI is directly related to the magnitude of the respiratory burst.

3.2.6. Data Analysis

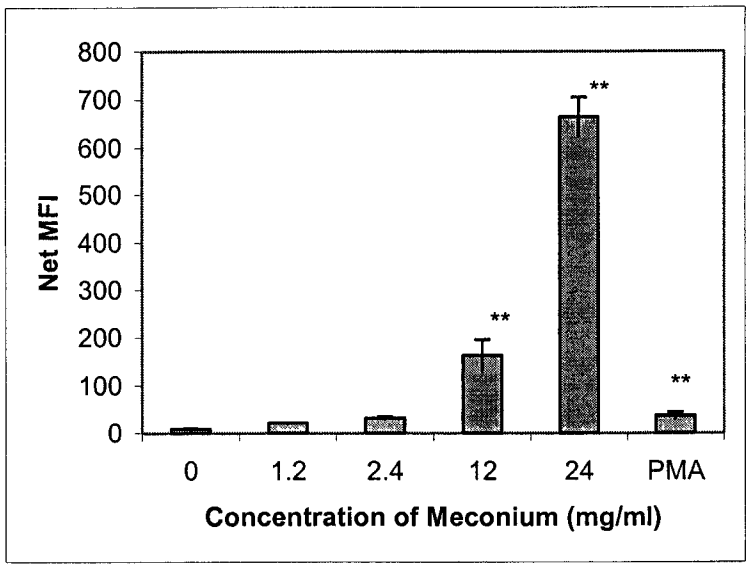
Statistical analysis was performed using InStat2 software (Graphpad Software Inc, CA). Results are reported as means ± SEM. Unless otherwise stated, analysis of variance followed by Dunnett's multiple comparison test was used to compare control values to various concentrations of meconium. Significance was accepted as $p<0.05$.

3.3. RESULTS

3.3.1. Effect of Human and Equine Meconium on Respiratory Burst in Alveolar Macrophages

Figure 6 shows that alveolar macrophages that have been incubated with human meconium demonstrate a significant, dose-dependant increase in respiratory burst activity, as measured by DCFH conversion to DCF. At a concentration of 24 mg/ml (10%), human meconium produced a net MFI that was more than 70 times greater than that of control cells (665 for meconium; 9 for control). By contrast, PMA, a commonly used stimulant of respiratory burst, produced a net MFI only 4 fold greater than control. Even at the relatively low concentration of 2.4 mg/ml, meconium stimulated a respiratory burst response similar in magnitude to that produced by PMA (net MFI of 32 for 2.4 mg/ml meconium; 38 for PMA). Not only was the magnitude of the respiratory burst significantly increased, but the percentage of cells producing an oxidative response was significantly increased as well (Fig. 6b). While 62% of control cells produced a respiratory burst, 86% of cells incubated with 24 mg/ml meconium

(a)



(b)

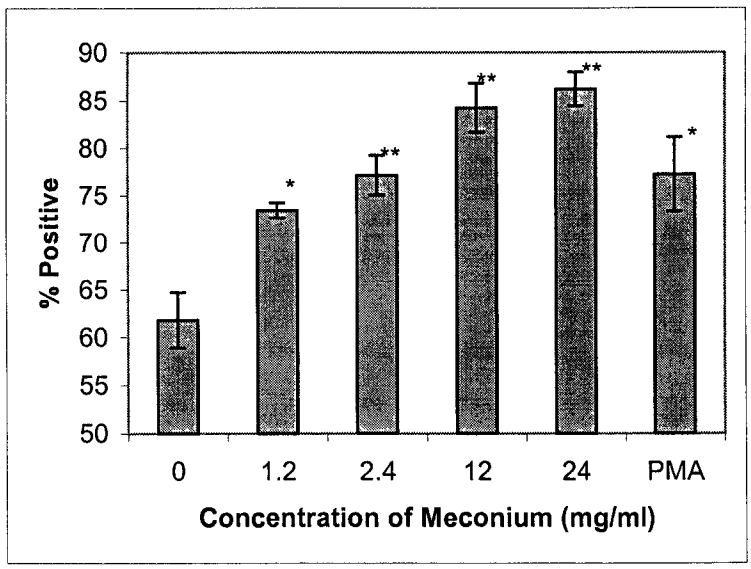


Figure 6. Human Meconium Stimulates Respiratory Burst Response in Alveolar Macrophages. Alveolar macrophages were incubated with various concentrations of meconium or with 100 ng/ml PMA, and the respiratory burst response was compared to that of control macrophages. The net MFI (a) and the percentage of positive cells (b) were determined by measuring fluorescence due to intracellular oxidation of DCFH to DCF. Results are shown as means ± SEM (n=5). Statistical comparison between meconium stimulated and control macrophages was made using ANOVA. PMA stimulated cells were compared to control macrophages using an unpaired, two-tailed Student's *t* test (*, $p<0.05$ **, $p<0.01$).

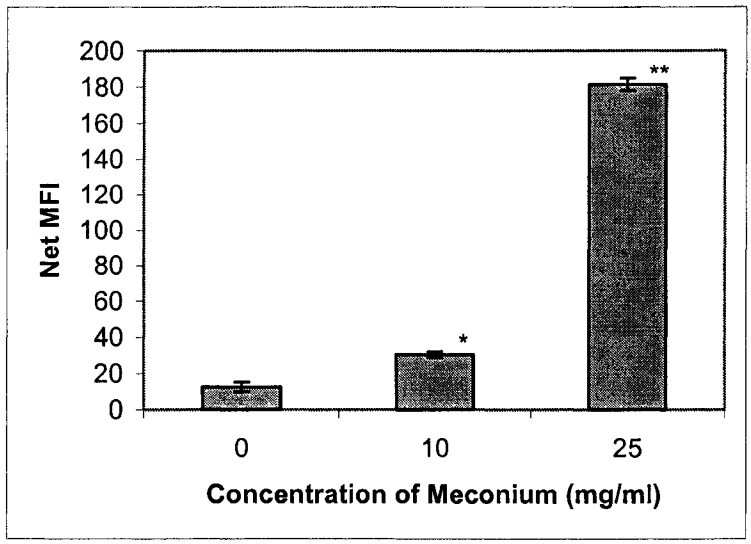
exhibited this response. The percentage of positive cells following PMA stimulation was 77%, which is the same as that induced by 2.4 mg/ml meconium.

In order to confirm that these results were not unique to human meconium, the experiment was repeated using equine meconium, and the results are shown in Fig. 7. Again, meconium stimulated a significant, dose-dependant respiratory burst in alveolar macrophages. The net MFI for cells exposed to 25 mg/ml of equine meconium was 181, while that of control cells was 13, a 14 fold increase. The percentage of responding cells increased significantly from 70% in control cells to 86% in cells stimulated with 25 mg/ml equine meconium.

3.3.2. Effect of Filtered Meconium on Respiratory Burst in Alveolar Macrophages

To determine if the observed stimulatory effect on respiratory burst was associated with phagocytosis of the particulate component of the meconium, the experiments were repeated using meconium that had been passed through a 0.2 μm filter. The results for human meconium are shown in Fig. 8. Even after filtration, human meconium stimulated a significant increase in respiratory burst. In these experiments, the net MFI increased from a mean of 13 in the control cells to 242 in the cells stimulated with 24 mg/ml meconium, an 18 fold increase. Although the net MFI of cells stimulated with 12 mg/ml meconium was increased more than 5 fold, that difference was not significant. The percent of positive cells was significantly increased at both the 12 and 24 mg/ml meconium concentration levels compared to control cells.

(a)



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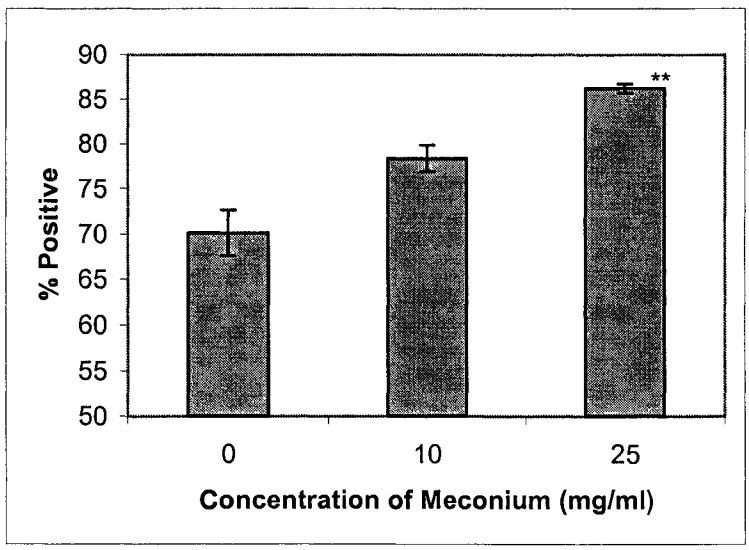
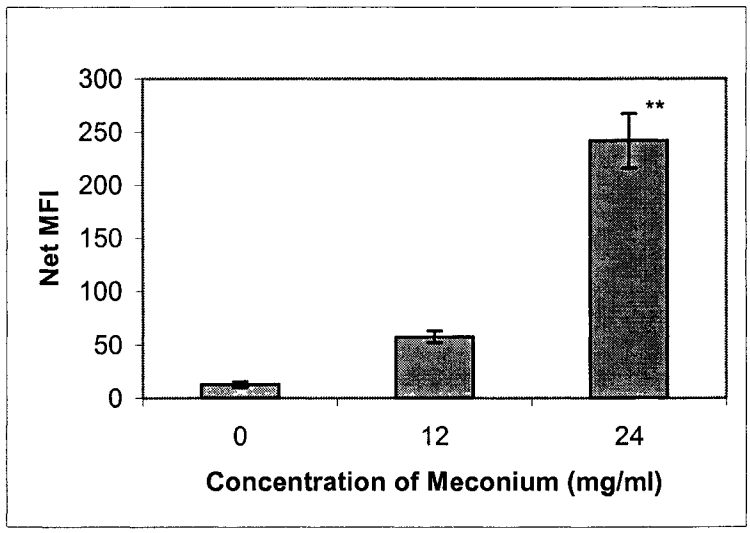


Figure 7. Equine Meconium Stimulates Respiratory Burst in Alveolar Macrophages. Alveolar macrophages were incubated with various concentrations of meconium, and the respiratory burst response was compared to that of control macrophages. The net MFI (a) and the percentage of positive cells (b) were determined by measuring fluorescence due to intracellular oxidation of DCFH to DCF. Results are shown as means ± SEM (n=3; *, $p<0.05$ **, $p<0.01$).

(a)



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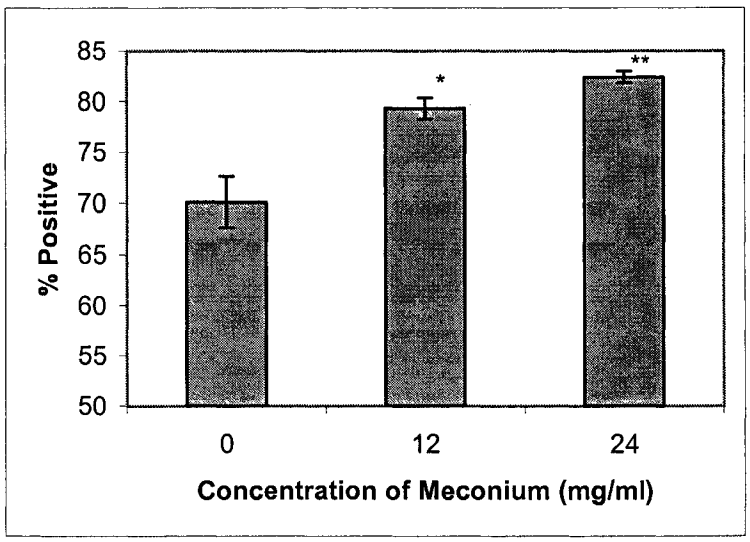


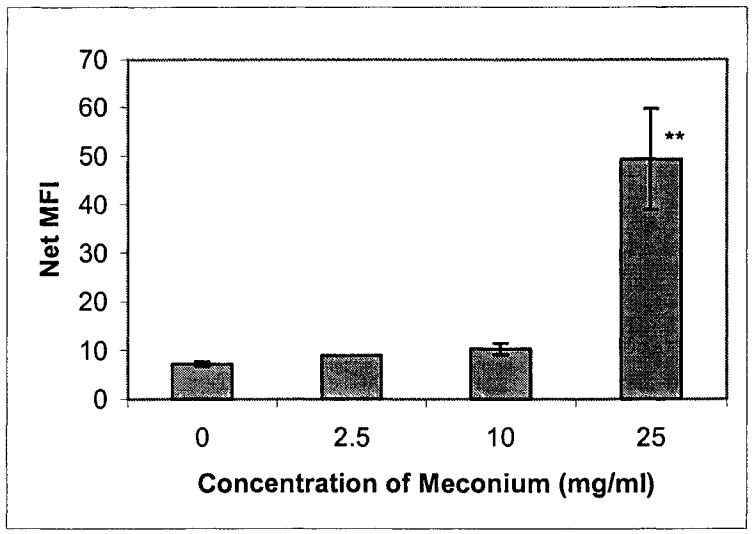
Figure 8. Filtered Human Meconium Stimulates Respiratory Burst in Alveolar Macrophages. Alveolar macrophages were incubated with 0.2 μm filtered meconium, and the respiratory burst response was compared to that of control macrophages. The net MFI (a) and the percentage of positive cells (b) were determined by measuring fluorescence due to intracellular oxidation of DCFH to DCF. Results are shown as means ± SEM (n=3; *, $p<0.05$ **, $p<0.01$).

The experiments were also performed using 0.2 μm filtered equine meconium, as shown in Fig. 9. Again, the highest concentration of meconium (25 mg/ml) produced a significant increase in net MFI (7 times greater than control), as well as in the percentage of positive cells. Interestingly, the lower concentrations of filtered equine meconium produced no detectable effect.

3.3.3. Effect of Meconium on Respiratory Burst Response to Secondary Challenge with PMA

In order to investigate whether the respiratory burst response would persist following meconium stimulation, macrophages were exposed to equine meconium for 2 h, then washed and incubated overnight. The following day (24 h later) the macrophages were loaded with DCFH-DA and stimulated with PMA. Results are displayed in Figure 10. Following overnight incubation, the meconium-exposed macrophages exhibited a dose-related decrease in respiratory burst in response to PMA stimulation. The net MFI declined from 178 in the control macrophages, to 30 and 14 in the cells exposed to 10 or 25 mg/ml of meconium, respectively. The percentage of positive cells was similarly decreased, from 84% in the control cells to 25 and 19% in the cells exposed to 10 or 25 mg/ml of meconium, respectively.

(a)



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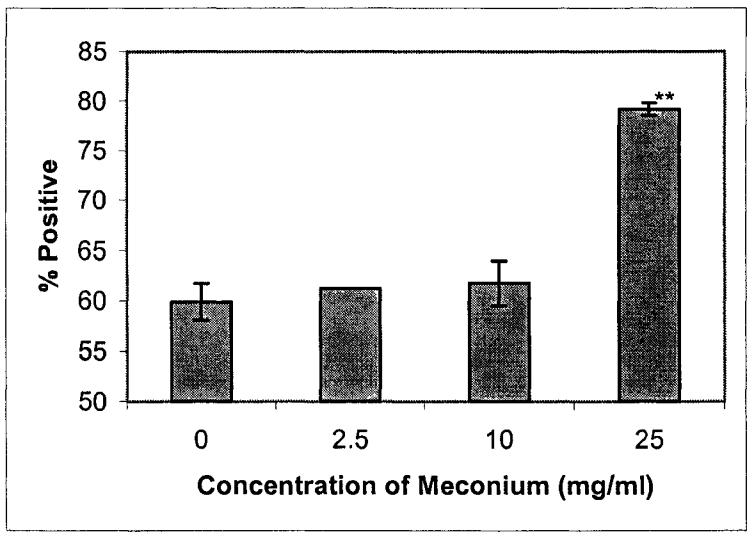
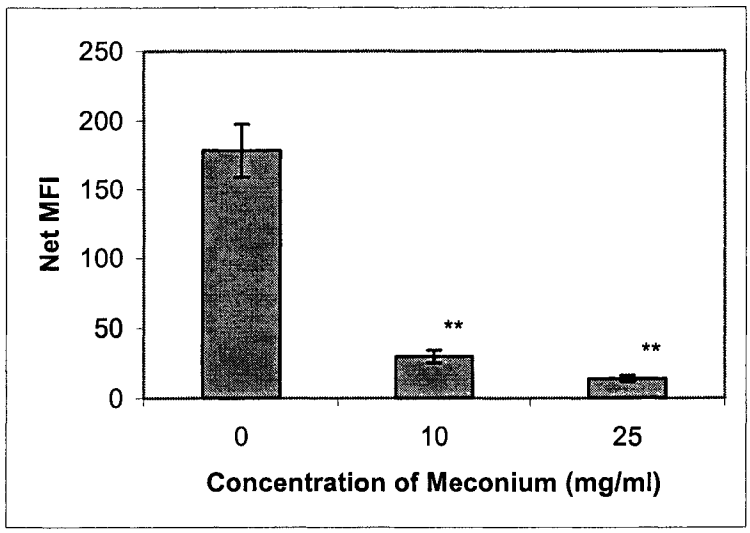


Figure 9. Filtered Equine Meconium Stimulates Respiratory Burst in Alveolar Macrophages. Alveolar macrophages were incubated with 0.2 μm filtered meconium, and the respiratory burst response was compared to that of control macrophages. The net MFI (a) and the percentage of positive cells (b) were determined by measuring fluorescence due to intracellular oxidation of DCFH to DCF. Results are shown as means ± SEM (n=3 for all concentrations except 2.5mg/ml, where n=1; **, $p<0.01$).

(a)



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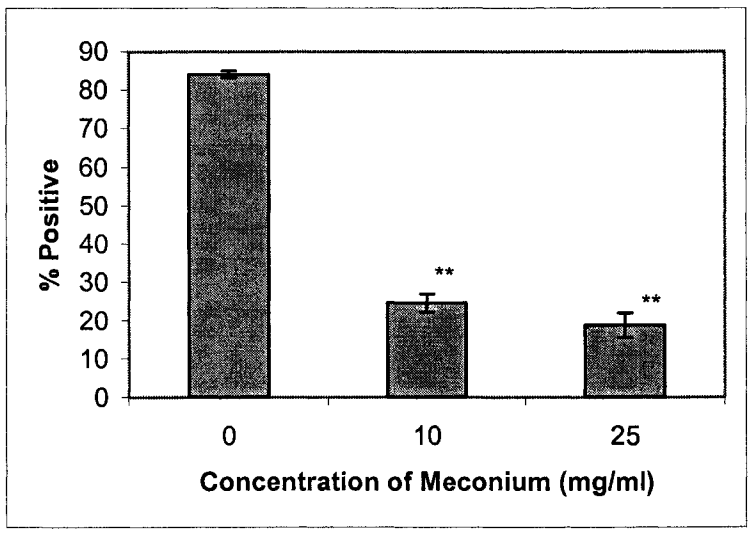


Figure 10. Meconium Attenuates the Respiratory Burst Response of Alveolar Macrophages to Subsequent PMA Stimulation. Alveolar macrophages were exposed to equine meconium for 2 h, then washed and incubated for 24 h. Net MFI (a) and percentage of positive cells (b) were determined by measuring DCF fluorescence following stimulation with PMA. Results are shown as means ± SEM (n=4 for concentrations of 0 and 25mg/ml; n=3 for 10mg/ml; **, $p<0.01$).

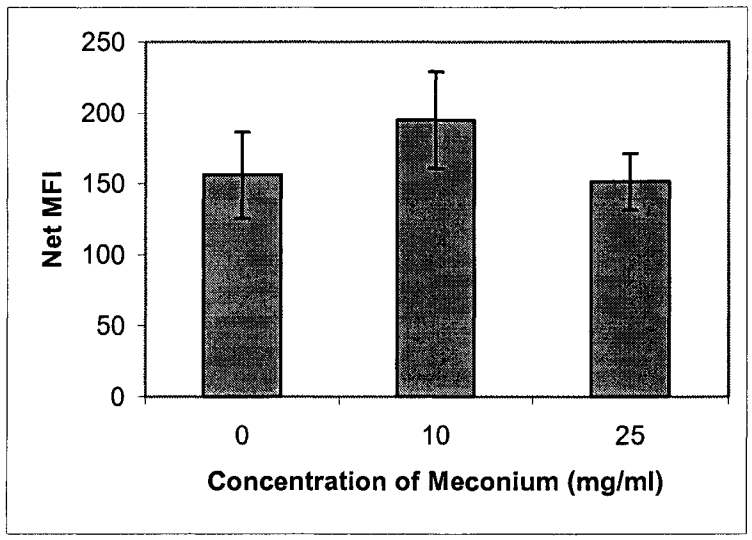
3.3.4. Effect of Filtered Meconium on Respiratory Burst Response to Secondary Challenge with PMA

To further characterize this inhibitory effect, the experiments were repeated using meconium that had been passed through a 0.2 μm filter. In contrast to the previous experiment, filtered meconium did not inhibit the respiratory burst response when macrophages were stimulated with PMA 24 h later. As shown in Fig. 11, there was no significant difference between the control and meconium-exposed macrophages for either MFI or percentage of positive cells.

3.3.5. Effect of Meconium on Cell Viability

To determine if meconium caused cell death at the concentrations used in these assays, NR8383 cell viability was assessed following incubation with meconium for 4 h. Table III shows that the viability of alveolar macrophages was not decreased after 4 h incubation with human meconium. The same result is seen in Table IV with equine meconium. To determine whether the conditions used for the secondary challenge assay might have affected cell viability, macrophages were incubated with equine meconium for 2 h, then washed and incubated for 24 h. Table V shows that even after overnight incubation, the viability of the meconium-exposed cells was not decreased compared to similarly incubated control cells.

(a)



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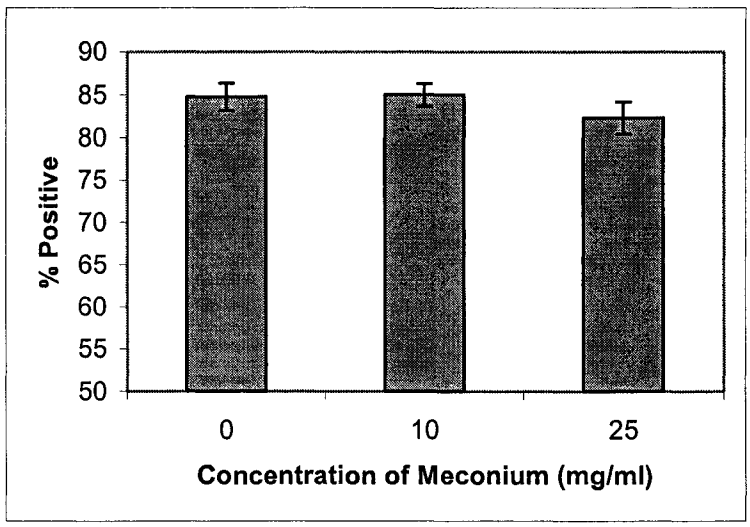

Figure 11. Filtered Meconium Does Not Affect the Respiratory Burst Response of Alveolar Macrophages to Subsequent PMA Stimulation. Macrophages were exposed to 0.2 μm filtered equine meconium for 2 h, then washed and incubated for 24 h. Net MFI (a) and percentage of positive cells (b) were determined by measuring DCF fluorescence following stimulation with PMA. Results are shown as means ± SEM (n=6).

Table III

Alveolar Macrophage Viability is Not Reduced Following Four Hours of Incubation with Human Meconium

Treatment	**% Live Cells[a]**
Control (media)	89.5 ± 0.9
Human Meconium (24mg/ml)	93.0 ± 0.9
Human Meconium (12 mg/ml)	93.0 ± 0.3

[a] Cell viability after 4 h incubation with human meconium was measured using trypan blue exclusion. Results are shown as means ± SEM (n =3).

Table IV

Alveolar Macrophage Viability is Not Reduced Following Four Hours of Incubation with Equine Meconium

Treatment	**% Live Cells[a]**
Control (media)	87.3 ± 1.2
Equine Meconium (25mg/ml)	88.7 ± 0.9
Equine meconium (10mg/ml)	90.3 ± 1.8

[a] Cell viability after 4 h incubation with equine meconium was measured using trypan blue exclusion. Results are shown as means ± SEM (n =3).

Table V

Alveolar Macrophage Viability is Not Reduced 24 h After Incubation With Equine Meconium

Treatment	**% Live Cells**[a]
Control (media)	96.3 ± 0.4
Equine Meconium (25mg/ml)	96.5 ± 0.5
Equine meconium (10mg/ml)	98.3 ± 0.5

[a] Alveolar macrophages were incubated with equine meconium for 2 h, then washed and incubated without meconium for 24 h. Cell viability was measured using trypan blue exclusion. Results are shown as means ± SEM (n =4).

3.4. DISCUSSION

3.4.1. Effect of Meconium on Respiratory Burst in Alveolar Macrophages

This study demonstrates that meconium stimulates a vigorous respiratory burst response in alveolar macrophages. The effect was dose-dependant, and probably related to the number of particles present in the meconium samples. Stimulation of respiratory burst by meconium was not entirely unexpected given that meconium is a particulate. More surprising was the magnitude of the response, which was considerably greater than that triggered by PMA. However, this result could be unique to the cell line used in these experiments. All the experiments were performed using the rat alveolar macrophage cell line, NR8383. This cell line has been found to produce a respiratory burst response when stimulated with various triggers including *Pneumocystis carinii* [56], zymosan and PMA [57]. However, in contrast to alveolar macrophages obtained by bronchoalveolar lavage (BAL), the NR8383 cell line has been reported to be less responsive to PMA than to zymosan, a particulate stimulus [57]. Since meconium is also a particulate stimulus, this characteristic of the cell line may explain why the respiratory burst response to meconium was greater than that to PMA.

Although meconium might be expected to trigger respiratory burst because of its particulate character, Kobzik reported that activation of respiratory burst does not accompany ligation of scavenger receptors or phagocytosis of unopsonized particles [28,31]. One interpretation of the results of the present study is that scavenger receptors are not involved in phagocytosis of meconium. However, the inhibitory effect observed by Kobzik was relatively minor, and only evident when cells phagocytosed small numbers of particles. Even if meconium did bind to scavenger receptors, the number of

particles present was probably great enough to mask any inhibitory effect. Furthermore, meconium is a complex and non-homogenous substance that likely binds to more than one specific receptor. The net effect of meconium on respiratory burst would depend on both the type and relative number of specific receptor-ligand interactions. The objective of this study was not to determine which receptors are engaged by meconium, although that avenue of investigation could prove interesting and informative.

3.4.2. Effect of Filtered Meconium on Respiratory Burst

Removal of particles greater than 0.2 μm from the meconium did not prevent the stimulation of respiratory burst. This could be because there are soluble factors in meconium that activate oxidative metabolism. Alternatively, it could be due to ligation of receptors by particles less than 0.2 μm in diameter. Recently there has been considerable interest in the effect of ultrafine particles (less than 0.1 μm) on alveolar macrophages [58-62]. Ultrafine particles are contaminants of polluted urban air, and may cause substantial pulmonary toxicity due to their large cumulative surface area. Pulmonary injury from inhalation of ultrafine particles is thought to be related to oxidative stress [60], and some studies have shown that they can induce inflammation and production of ROS in macrophages [63]. However, other investigators have found no increase in respiratory burst activity in macrophages following exposure to ultrafine particles [64]. In order to determine whether the stimulatory effect of filtered meconium is due to soluble factors or ultrafine particles, it would be necessary to separate, identify, and analyze the various components of meconium.

3.4.3. Effect of Meconium on Respiratory Burst in Response to Secondary Challenge

In addition to examining the role of meconium in directly stimulating respiratory burst, the effect of meconium on respiratory burst triggered by subsequent challenge with PMA was also investigated. Following receptor-mediated stimulation, macrophages may become desensitized to further stimulation, or they may become primed to exhibit enhanced respiratory burst in response to subsequent stimuli. Classic macrophage priming occurs *in vivo* in response to cytokines elaborated by T cells [36], although phagocytosis of particles may also cause *in vivo* priming in some instances [65]. Priming can also occur in the absence of lymphocyte-derived cytokines, since some stimuli, such as lipopolysaccharide (LPS)[66], fetal bovine serum (FBS)[23,67], amoebic proteins [68], and adherence [22,69] have been shown to have a priming effect on phagocytes *in vitro*. The priming phenomenon has been implicated in the production of ROS-induced pulmonary injury caused, for example, by exposure to tobacco smoke [19,70]. It was therefore important to investigate the possibility that meconium might prime macrophages to demonstrate an enhanced response to secondary challenge. Our results demonstrate that macrophages incubated with meconium were not primed, but were rendered less responsive to subsequent PMA stimulation than control macrophages.

One possible interpretation for these results is that meconium decreased the viability of the macrophages. Although macrophage viability, as determined by trypan blue exclusion, was not decreased, the possibility that meconium caused apoptotic cell death cannot be ruled out at this time. This possibility is worthy of further investigation, because it has been reported that alveolar macrophages exposed to air

pollution particles showed increased apoptosis, even though there was little evidence of toxicity by trypan blue exclusion [71].

Meconium appears to have desensitized macrophages to subsequent PMA stimulation. Unlike another particulate stimulus, zymosan, which reportedly caused desensitization only to homologous stimulation [22], meconium desensitized macrophages to PMA, a heterologous stimulus. The mechanism of this non-selective desensitization is unclear. Since PMA directly activates PKC and does not require receptor-mediated events, the desensitization triggered by meconium does not involve down-regulation of receptors. An increase in intracellular cAMP is a possible mechanism of desensitization, since agents that increase cAMP can inhibit respiratory burst [72]. However, evidence provided in Chapter 2 shows that meconium does not elevate intracellular cAMP in NR8383 cells. Desensitization of alveolar macrophage respiratory burst response by meconium might involve PPAR-γ, as has been reported for macrophages pre-stimulated with LPS and IFN-γ [46].

The duration of this inhibitory effect is also unknown at present, but it clearly persists for at least 24 hours. Further experiments should be performed to determine whether the desensitization induced by meconium is long lasting or permanent.

Filtered meconium did not cause desensitization of alveolar macrophages to secondary challenge (Fig. 6). This is probably a dose-related effect. Filtration, by removing particles larger than 0.2 μm in size, reduced the total number of particles in the meconium samples. Only the highest concentration of filtered equine meconium induced a significant respiratory burst response. Although this concentration may have been enough to trigger respiratory burst activity, it was evidently not enough to trigger

subsequent desensitization. Phagocytosis of a minimal number of particles appears to be necessary to trigger an inhibitory effect. On the other hand, it is possible that the size of the particles could be a salient characteristic in producing the desensitization. Air pollution particles 2.5-10 μm in size are reported to cause inhibition of alveolar macrophage respiratory burst following a 24 hour incubation period, while the same concentration of smaller particles (0.1-2.5 μm) does not [71].

3.4.4.' Comparison With Previously Published Studies

The findings of these experiments are not in agreement with those reported previously. Two other studies have examined the effect of meconium on phagocyte oxidative burst. Kojima *et al* (1994) reported that meconium failed to stimulate a spontaneous respiratory burst in rabbit BAL macrophages [73]. However, when these authors stimulated meconium-exposed macrophages with PMA, an enhanced response was measured, suggesting that meconium had produced a priming effect. This effect was seen at meconium concentrations from 5 to 30% but was greatest at 10%. The meconium used in their study was obtained from human newborns and 0.2 μm filtered. The lack of meconium-stimulated respiratory burst reported in their study is in direct contrast to current findings, where even filtered meconium significantly increased the respiratory burst response. One possible explanation for this discrepancy is the cell source. Kojima used freshly isolated rabbit BAL cells, whereas a rat alveolar macrophage cell line was used in the current study. Macrophages from different species are known to respond differently to various stimuli [57]. The incubation conditions were also quite different, as the macrophages in the Kojima study were suspended in Hanks solution, whereas those in the current study were suspended in culture media

with FBS. Both culture and the presence of FBS have been shown to have a strong priming effect on freshly obtained BAL macrophages [23,67]. It seems likely that the cells used in the present study were already primed by their culture conditions, and meconium provided the necessary stimulus to trigger respiratory burst.

The incubation times and the type of assay used in the Kojima study were also different from those used in the present study. Incubation time, along with other factors such as cell and particle concentration, can vary from one laboratory to the next and cause different results to be obtained [18]. Kojima incubated macrophages with meconium for 20 min, then washed and resuspended the cells, and added the chemiluminescence probe. After another 20 min incubation, PMA was added and luminescence was measured. In the present study DCFH-DA was loaded into the cells before meconium was added. Once produced, the level of DCF remains stable in the cell for at least an hour [74], allowing measurement of the cumulative product of all H_2O_2 produced over the course of the assay. In contrast, Kojima was measuring chemiluminescence at a fixed time point. Respiratory burst stimulated by the addition of meconium at least 40 minutes earlier may have therefore been overlooked.

Clark and Duff (1995) examined the effect of meconium in human neutrophils and reported a decrease in respiratory burst [75]. They used a DCFH-DA assay technique similar to that used in the present study and incubated cells with meconium for 15 min. However, they stimulated cells with PMA before measuring fluorescence, and reported respiratory burst as two parameters. Oxidative index was defined as the fluorescent intensity (FI) of PMA stimulated cells, divided by the FI of unstimulated cells; and net fluorescent intensity was defined as the difference between the FI of PMA

stimulated and unstimulated cells. Both oxidative index and net fluorescent intensity were decreased following meconium exposure. The problem with this approach relates to their definition of "stimulated" cells as those that had been exposed to PMA. The present study demonstrates that meconium is also a potent stimulus for respiratory burst. Clark and Duff's method of calculation will therefore grossly underestimate the effect of meconium, since it only identifies the additional respiratory burst due to the combined effect of meconium and PMA. The meconium-exposed cells may have already produced a near maximal respiratory burst response, and the addition of PMA therefore made little difference. The control cells however, would have a significant response to PMA alone. The concentration of meconium used in the Clark study (0.5 and 1 mg/ml) was also considerably less than that used in the present study and could explain why they did not observe a stimulation of respiratory burst in response to meconium. Finally, the Clark study was performed using neutrophils, which might be less responsive to meconium than alveolar macrophages.

3.4.5. Technical Issues

One difficulty with this assay was accounting for background autofluorescence. Many cells exhibit autofluorescence, which is thought to be due to the presence of endogenous flavoproteins [76,77]. Alveolar macrophages have a background autofluorescence that is greater than that of some other cell types and that may make flow cytometric techniques difficult to interpret due to overlap with fluorochrome emission spectra [78]. However, in this assay, although the NR8383 cells did exhibit autofluorescence, the fluorescence due to oxidation of DCFH-DA was many times greater in magnitude and therefore easily distinguished.

More problematic was the fact that meconium itself exhibited fluorescent emission. This meant that cells that had been incubated with, and subsequently phagocytosed meconium, had an increased median fluorescent intensity even when no DCFH-DA was added. Other investigators have calculated fluorescence due to respiratory burst by subtracting the fluorescence of unstimulated control cells from the fluorescence of stimulated cells [21,51]. This approach was not appropriate in this work because the stimulus for respiratory burst in this assay was meconium. Since meconium itself is fluorescent, subtracting the fluorescence of unstimulated cells would introduce a bias that would be directly proportional to the concentration of meconium. This problem was overcome by including, for each concentration of meconium used, a control sample of cells incubated with meconium, but not loaded with DCFH-DA. These control samples provided a value for the background fluorescence due to the combined effects of cellular autofluorescence and meconium, and this could then be subtracted from the DCFH-DA induced fluorescence to provide a net fluorescent intensity.

3.4.6. Biological Significance

The respiratory burst response stimulated by meconium is likely to be biologically significant. Excessive production of ROS due to respiratory burst has been implicated in pulmonary disease due to inhalation of various environmental particles. The response to meconium, at least under these experimental conditions, was very robust. If the same level of response occurs *in vivo*, then it is likely that ROS produced by alveolar macrophages are a contributing factor in the initial inflammation and pulmonary injury seen in MAS. Perhaps even more significant is the decrease in

respiratory burst response seen 24 h after meconium exposure. If this inhibition proves to be long lasting or permanent, then an important innate defense mechanism is likely to be compromised in newborn babies with MAS. Such a defect in alveolar macrophage function would likely increase the susceptibility of infants to inhaled microorganisms and would be a contributing factor in the pathophysiology of MAS. A better understanding of the effect of meconium on all aspects of alveolar macrophage function is necessary to aid in the development of appropriate treatment and preventative strategies for babies with MAS.

3.5. CONCLUSIONS

This study demonstrates that meconium stimulates a vigorous, dose-dependent respiratory burst response in alveolar macrophages. At least part of the response is triggered by soluble factors or ultrafine particles, since 0.2 μm filtration did not eliminate the respiratory burst response to meconium stimulation. However, after meconium exposure, macrophages were desensitized to secondary challenge with PMA and produced an attenuated oxidative response in comparison to control macrophages. Since respiratory burst is important in host defense, further studies are needed to determine the duration of this inhibitory effect.

3.6 Reference List

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4. AMNIOTIC FLUID STIMULATES RESPIRATORY BURST AND ENHANCES PHAGOCYTOSIS IN ALVEOLAR MACROPHAGES

4.1. Introduction

The fetus *in utero* is enclosed in a membrane-lined cavity filled with amniotic fluid. This fluid is produced by the cells of the amnion, and serves as a buffer to protect the fetus from trauma and ascending infection, while also allowing fetal movement, swallowing, urination and respiratory movements [1]. Amniotic fluid (AF) is comprised of about 98% water, with 1-2% organic and inorganic solids [2]. Early in pregnancy, the composition of AF is very similar to that of extracellular fluid, but as gestation progresses the composition changes, partly as a result of products released from the fetal kidneys and respiratory tract. The fetal lung constantly secretes water and solutes, and consequently the alveoli are filled with fluid until the time of birth [3]. Since this fluid is expelled into the surrounding AF, the composition of AF is also reflective of pulmonary development and can be used as an indicator of pulmonary maturity. Type II pneumocytes in the fetal lung begin to develop lamellar inclusion bodies by 20-24 wk of gestation, indicating that surfactant production has commenced. Various methods have been developed to assess the pulmonary maturity of the fetus by examination of surfactant phospholipids in AF collected by amniocentesis. Measurement of the relative concentrations of lecithin (phosphatidylcholine) and sphingomyelin (a nonsurfactant phospholipid) is the original and still widely used test of fetal lung maturity, but there are numerous variations on this theme. Counting of lamellar bodies is one simple method of assessment [4]. An even more straightforward

test is the visual assessment of AF turbidity, which increases concomitantly with lung maturity [5]. This increase in turbidity may be partly due to the increasing concentration of surfactant phospholipids produced in the fetal lung and transported to the AF. In addition, as gestation proceeds there is an increasing amount of exfoliated particulate matter from the fetus, including hair and epithelial cells from the membranes, skin, genitourinary and digestive tracts. Vernix caseosa is the protective layer that covers the skin of the fetus, and is composed of sebum and desquamated epithelial cells. The increase in particulate debris parallels the increase in surfactant concentration in the AF, and may in fact be a direct result, since pulmonary surfactant has been shown to induce detachment of vernix caseosa, at least in an *in vitro* system [6].

Although the fetus exhibits episodic respiratory movements from mid gestation or even earlier, these movements are relatively weak and do not normally result in aspiration of amniotic fluid [7]. Fluid pressure within the fetal lung is important for pulmonary development and is controlled by the valving mechanism of the larynx [8]. As fluid from the lung moves up the trachea, it is released through the larynx to the mouth, where it is either swallowed or expelled into the AF. The net flow of fluid is therefore out of the lung [3]. As parturition approaches, the production of this lung fluid decreases, allowing the transition from fluid-filled to air-filled alveoli. The time immediately around parturition is when infants are most at risk for AF aspiration, although they are protected by a constellation of reflexes that help defend the laryngeal airway, including apnea, rapid swallowing and coughing [8]. Nonetheless, aspiration of AF can occur either *in utero* or immediately after birth when the infant takes its first

breaths. Studies in fetal sheep have shown that *in utero* hypoxia can result in a decrease in secretion of lung fluid, as well as an increase in respiratory movements that consequently result in an influx of AF into the lung [9]. Fetal hypoxia in human infants can also initiate release of meconium from the digestive tract into the AF. In the majority of normal infants, meconium is expelled in the first 24 hours after parturition [10], but about 12% of infants expel meconium while still *in utero* [11,12]. In addition to fetal hypoxia, post-maturity is a factor in the etiology of early meconium defecation, since up to 50% of fetuses older than 42 weeks expel meconium *in utero* [13]. Meconium aspiration syndrome (MAS) occurs when a fetus inhales meconium-contaminated AF either *in utero* or during the birthing process. About 5% of infants born through meconium-stained amniotic fluid develop MAS, and this syndrome remains an important cause of respiratory distress in newborns [14].

The pathophysiology of MAS is complex. Viscous meconium plugs can lodge in bronchioles causing complete or partial airway obstruction. This results in patchy areas of atelectasis and hyperinflation, with subsequent ventilation-perfusion mismatching, hypoxia, hypercapnia, and respiratory acidosis [15]. Inflammation is also an important part of the pathophysiology. A profound pulmonary inflammatory response is apparent within several hours of meconium aspiration, with a peak influx of neutrophils, macrophages and serum proteins at 16-24 hours [16]. Bile salts and other substances in meconium are thought to irritate and injure blood vessels and other cells in the lung, particularly the type II pneumocytes, with resulting changes in surfactant production and function [16,17].

Aspiration of meconium may also put the infant at increased risk for pneumonia. Meconium has been shown to reduce host resistance to bacterial infection by an unknown mechanism. Bryan found that when sterile meconium was injected intraperitoneally to mice, or instilled intratracheally in rats, the LD_{50} for *E. coli* bacteria given concurrently was significantly reduced [18]. Furthermore, clinical findings in humans demonstrate that meconium-stained amniotic fluid is associated with intra-amniotic infection [19-21].

Meconium aspiration syndrome is well characterized clinically, and numerous studies have investigated the effect of meconium on pulmonary physiology and cellular response [22-31]. In contrast, little is known about either the incidence or the consequences of aspiration of AF without meconium contamination, although several reports have described the presence of fetal epithelial squames in the alveoli of stillborn infants and in babies who died with MAS-like symptoms [32,33].

Although aspiration of AF by human neonates is not considered a normal physiological event, the same may not be true in all species. Lopez and Bildfell [34] reported that 44% of newborn (less than 2 wk old) calves submitted for necropsy had evidence of AF aspiration, with or without meconium. Importantly, these calves were 1.5 times more likely to have pulmonary inflammation than those without evidence of aspiration. In a follow-up study, neonatal calves considered healthy at the time of birth were euthanized and examined for evidence of AF and meconium aspiration [35]. Evidence of amniotic fluid aspiration was found in 71% of these calves, leading to the conclusion that aspiration of amniotic fluid is a common event in calves at the time of parturition. In the neonatal bovine lung, amniotic fluid typically invoked only a mild

inflammatory reaction. The same was true in neonatal rats where meconium instilled intratracheally induced an acute and robust inflammatory reaction characterized by a significant influx of neutrophils, while amniotic fluid produced only mild inflammation [36]. In neonatal and adult rabbits, administration of human AF caused a slight depression in PO_2 compared to saline controls [37]. Unlike meconium, however, AF instillation did not significantly affect mortality, histology, lung weight, lung volume or radiographic appearance [37].

This lack of significant inflammation is remarkable given that AF contains keratin and squamous epithelial cells which would be expected to trigger a vigorous inflammatory response in the lung. In the past, the effect of AF on lymphocytes and natural killer cells was investigated for evidence of immunosuppressive activity that might contribute to the survival of the fetus as an allograft. Amniotic fluid has been reported to suppress lymphocyte proliferation and production of cytokines [38], to inhibit graft-versus-host reactions and prolong graft survival [39,40], as well as to inhibit the cytotoxic activity of natural killer cells [41]. The failure of aspirated or instilled AF to induce significant pulmonary inflammation could be due to the presence of anti-inflammatory substances, a number of which have been identified, including interleukin-10 [42-44], interleukin-1 receptor antagonist [45], secretory leukocyte protease inhibitor [46], and Clara cell protein [47].

Choriodecidual tissues produce both pro- and anti-inflammatory cytokines [48]. Interleukin-10 (IL-10) is found in AF and is an example of the latter. It produces its anti-inflammatory effect chiefly by inhibiting synthesis of inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α). There is some speculation

that IL-10, due to its immunosuppressant characteristics, might play a role in preventing rejection of the fetus during gestation [43], and some studies have shown that IL-10 production by the placenta is decreased at the time of parturition [48]. IL-10 suppresses release of both reactive oxygen intermediates [49] and proinflammatory cytokines by alveolar macrophages [50].

Interleukin-1 receptor antagonist (IL-1ra) is a potent anti-inflammatory molecule that exists as both secreted and intracellular isoforms [51]. The secreted isoform functions as an acute phase protein that competitively binds with IL-1 receptors on cell surfaces and thereby opposes the proinflammatory actions of IL-1. IL-1ra is found in high concentrations in amniotic fluid [45,52].

Secretory leukocyte protease inhibitor (SLPI) is a molecule with an important role in preventing excessive tissue injury during pulmonary inflammation, by inhibiting serine protease production by activated phagocytes. In addition to its anti-protease effects, SLPI has numerous anti-inflammatory activities, including suppression of nuclear factor-κB (NF-κB) activation [53] and reduction of neutrophil recruitment, possibly by inhibiting production of the complement anaphylatoxin C5a [54] and intercellular adhesion molecule (ICAM) [53]. Secretory leukocyte protease inhibitor is produced by cells of the placenta and is found in amniotic fluid [46,55].

Clara cell protein is the predominant product of Clara cells, which are the non-ciliated, non-mucous secretory cells that line the bronchiolar epithelium. During gestation, Clara cell protein is produced in the fetal lung and distributed to the amniotic fluid, where it accumulates at levels much higher than those found in fetal serum [56]. The physiologic role of this protein remains uncertain, but it has been shown to have

anti-inflammatory properties, mediated mainly by inhibition of phospholipase A2 [57]. It has also been shown to have an immunomodulatory effect by inhibiting production of various cytokines, including interferon-γ, TNF-α and IL-1, as well as inhibition of leukocyte chemotaxis [57]. It may play a role in protecting the lung from injury due to inhaled toxic particulates [58].

The presence of one or more of these anti-inflammatory molecules in AF might explain why it fails to induce the expected degree of inflammation when aspirated or instilled into neonatal lungs. If this is true, then any putative inflammatory effect of meconium might be attenuated *in vivo* by dilution with AF. Since alveolar macrophages are the cells that provide initial defense against aspirated material, it would be important to determine the effect of AF, as well as meconium, on their various functions. The objective of this study was to determine the effect of AF on alveolar macrophage phagocytosis and respiratory burst activity.

4.2. Materials and Methods

4.2.1. Cells

A continuous rat alveolar macrophage cell line, NR8383, (American Type Culture Collection, Rockville, MD) was used as a source of cells for all experiments. Cells were cultured in Ham's F12 nutrient mixture with glutamate, supplemented with 15% heat inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all components purchased from Sigma-Aldrich, St. Louis, MO). Cultures were maintained in a humidified, 5% CO_2 incubator at 37° C. This spontaneously transformed cell line grows as a mixture of adherent and non-adherent cells. On the day

prior to each experiment, a cell scraper was used to dislodge adherent cells from the culture flask, and all cells were then transferred to a new flask. On the day of the experiment, non-adherent cells were collected for use in assays by pelleting.

4.2.2. Amniotic Fluid

Amniotic fluid was collected from pregnant Sprague Dawley rats (Charles River, St-Constant, QC, Canada) at 17-19 days of gestation. Each rat was anesthetized by inhalation of halothane, and the gravid uterus was removed following a midline abdominal incision. Rats were euthanized by anesthetic overdose. The uterine extremities were clamped with hemostats, and it was suspended from a support stand. A sterile 25g needle was used to puncture individual amniotic sacs, and amniotic fluid was collected into a sterile tube as it dripped from dependant sites. A portion of the fluid collected from each rat was cultured and examined microscopically to confirm absence of bacterial growth. Aliquots of amniotic fluid were kept frozen at –70° C until needed. For all of the respiratory burst assays, and some of the phagocytosis experiments, amniotic fluid was passed through a 0.2 μm filter before use.

4.2.3. Phagocytic Assay

Phagocytic activity of alveolar macrophages incubated with AF was assessed and compared to control macrophages by quantifying the uptake of florescent latex beads using flow cytometry [59-61]. For these assays, NR8383 cells were suspended in Ham's media at a concentration of 2 x 10^6 cells/ml. Aliquots of 500 μl (1 million cells) were placed in 5 ml round-bottom polystyrene tubes. Various amounts of AF and media were added to experimental samples, so that the final volume in each tube was the same (565 μl). Control samples contained only macrophages with media.

Carboxylated fluorescent latex beads (Sigma-Aldrich) with an average diameter of 2 μm were added at a ratio of 50 beads per cell to half of the tubes. The beads were thoroughly vortexed before use, and flow cytometer analysis showed that less than 7% existed as doublets or larger aggregates. An equal volume (10 μl) of sterile phosphate buffered saline (PBS) was added to the remaining tubes. The tubes were then capped, vortexed briefly, placed in an opaque container to protect them from light, and placed in a shaking incubator at 37° C for 90 min. After incubation, 2 ml of ice-cold PBS was added to stop phagocytosis. The cells were centrifuged at 360 *g* for 5 minutes, washed with 1 ml PBS, and centrifuged again. The pellet was resuspended in 400 μl of PBS, and the cells were kept on ice until analysis by flow cytometry (less than 1 h).

4.2.4. Respiratory Burst Assay

Respiratory burst activity of amniotic fluid-exposed alveolar macrophages was assessed and compared to control macrophages using flow cytometry to measure production of a fluorescent probe. The use of this technique to assess respiratory burst in neutrophils was described by Bass *et al* [62], and has also been used for the same purpose in other phagocytic cells [63]. For these assays, NR8383 cells were suspended in Ham's complete media at a concentration of 2 x 10^6 cells/ml. Aliquots of 500 μl (1 million cells) were placed in 5 ml round-bottom polystyrene tubes. The cells were pre-incubated with 5μl of dichlorofluorescin diacetate (20 μM final) for 20 minutes at 37°C in the dark on a shaker platform to load the dye. Dichlorofluorescin diacetate (DCFH-DA) is a stable non-polar, non-fluorescent probe that penetrates cell membranes by passive diffusion. Once inside, it is rapidly deacetylated by intracellular esterases to dichlorofluorescin (DCFH)[64]. This compound is polar, and therefore becomes

trapped inside the cell. In the presence of reactive oxygen species (ROS) produced as a result of respiratory burst, it is rapidly oxidized to dichlorofluorescein (DCF), which is highly fluorescent. The ROS mainly responsible for the oxidation of DCFH is thought to be H_2O_2 [65,66]. Measurement of the fluorescent product provides a quantitative assessment of the respiratory burst in individual cells [62].

After loading with DCFH-DA, various amounts of amniotic fluid and media were added to experimental samples, and the cells were further incubated for 1 h. Control samples contained only macrophages with additional media to bring the final volume in the tube to the same as that of experimental tubes (560 μl). Some control samples were incubated with phorbol myristate acetate (PMA; 100 ng/ml), a known activator of respiratory burst, prior to the final incubation. Following incubation, ice cold PBS was added to stop the reaction, the cells were washed twice, and resuspended in 400 μl cold PBS for analysis by flow cytometry, which was performed within 30 min.

4.2.5. Flow Cytometry

The fluorescent intensity of 10,000 cells from each sample was measured using a Becton Dickinson FACSCalibur cytometer, equipped with an argon ion laser operating at 350 mW, 488 nm. Data was acquired in list mode using CellQuest software (Becton Dickinson, San Jose, CA), and analyzed for red and green fluorescence using FCS Express software (De Novo Software). Forward scatter threshold was set to eliminate cellular debris and free latex beads from analysis. Propidium iodide (3 μl, Sigma-Aldrich) was added to each tube just prior to analysis. This dye is excluded from viable cells, but penetrates cell membranes of dead cells and

intercalates into double-stranded nucleic acids, where it is excited by laser light and fluoresces red. Electronic gating was used to eliminate from analysis those cells that had taken up propidium iodide and consequently had elevated FL2 (red) fluorescence. Fluorescence data was collected on log scale. Green fluorescence from the FITC labeled beads was measured at 530 ± 30nm, and red fluorescence from the propidium iodide was measured at 585 ± 42nm. Electronic compensation was used to minimize spectral overlap between the two fluorochromes.

Measurement of phagocytosis included assessment of two parameters—the percentage of cells that were actively phagocytic, and the net median fluorescent intensity of the sample population. The percentage of phagocytic cells was determined by comparison of the fluorescent profile of cells exposed to latex beads to the background autofluorescence of cells not exposed to beads, using Overton histogram subtraction [67]. The cells with a fluorescent intensity greater than background were considered to be actively phagocytic, meaning that they were associated with one or more fluorescent beads. The net median fluorescent intensity (MFI) was calculated by subtracting the background MFI from the MFI of the sample population after exposure to latex beads. Since the fluorescent intensity of each cell is directly proportional to the number of associated fluorescent beads [60], the net MFI is directly related to the average number of beads phagocytosed per cell.

Measurement of respiratory burst included assessment of the same two parameters—the percentage of positive cells [68], and the median fluorescent intensity of the sample population. The percentage of positive cells was determined by comparison of the fluorescent profile of the macrophages loaded with DCFH-DA to the

background autofluorescence of cells not loaded with DCFH-DA, using Overton histogram subtraction [67]. The cells with a fluorescent intensity greater than background were considered positive, meaning that respiratory burst had occurred. The median fluorescent intensity (MFI) was determined from the fluorescent profile of each sample population by the flow cytometry software. Since the fluorescent intensity of each cell is directly proportional to the amount of DCF produced, the MFI is directly related to the magnitude of the respiratory burst in the sample population.

4.2.6. Data Analysis

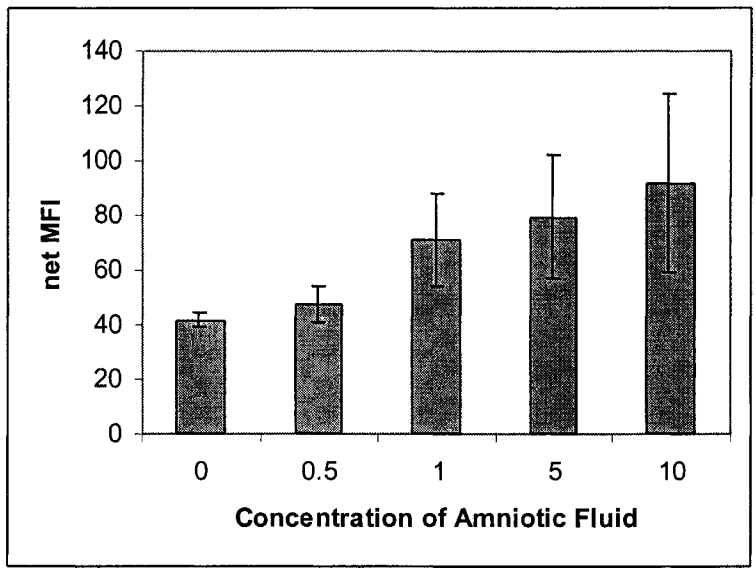
Statistical analysis was performed using InStat2 software (Graphpad Software Inc, CA). Results are reported as means ± SEM. Unless otherwise stated, analysis of variance followed by Dunnett's multiple comparison test was used to compare control values to various concentrations of amniotic fluid. Significance was accepted as $p<0.05$.

4.3. Results

4.3.1. Effect of Unfiltered Amniotic Fluid on Phagocytosis

Figure 12 shows the effect of rat amniotic fluid on the phagocytosis of latex beads by alveolar macrophages. The average net MFI for the control cells was 42, while that of the macrophages incubated with 10% AF was 92, an increase of about 120%.

(a)



(b)

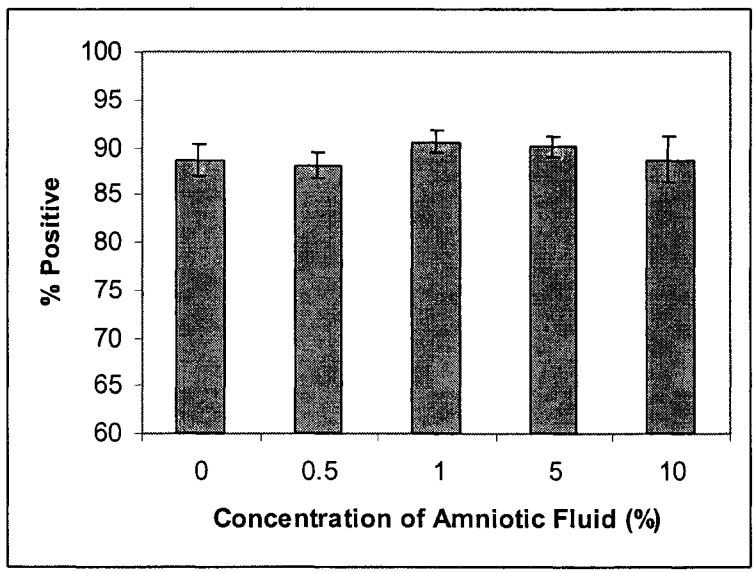


Figure 12. Unfiltered Amniotic Fluid Does Not Significantly Affect Phagocytosis by Alveolar Macrophages. Alveolar macrophages were incubated with various concentrations of unfiltered AF, and the net MFI (a) and percentage of phagocytosing cells (b) was determined after exposure to fluorescent latex beads. Results are shown as means ± SEM (n=6).

Although there appears to be a trend toward increasing net MFI with increasing concentration of AF, these differences were not significant. The day-to-day variability in net MFI was quite large, especially for the macrophages incubated with AF. By contrast, the percentage of phagocytosing cells was relatively constant (Fig. 12b). Between 88 and 91% of macrophages were associated with one or more beads, regardless of the presence of AF.

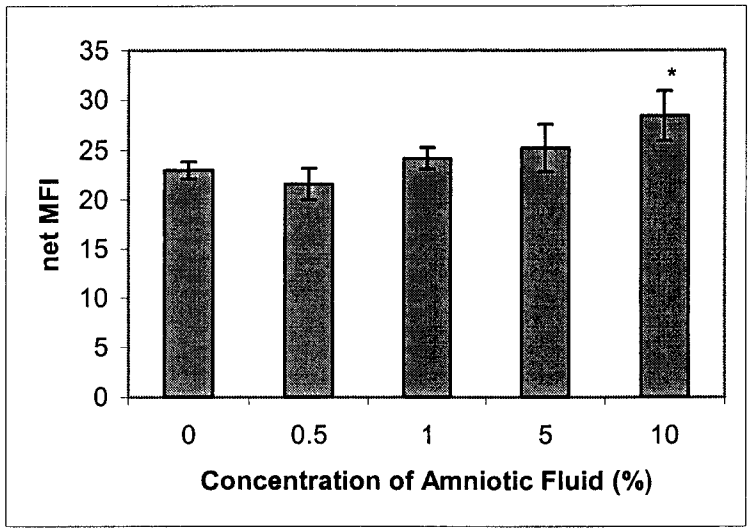
4.3.2. Effect of 0.2 μm Filtered Amniotic Fluid on Phagocytosis

In an effort to reduce the variability in net MFI, the assays were repeated using AF that had been passed through a 0.2 μm filter to remove particulate debris. Figure 13 shows the effect of filtered AF on alveolar macrophage phagocytosis. Although a dose related effect could not be demonstrated by analysis of variance, the net MFI of the macrophages incubated with 10% AF was significantly different from that of control cells (Fig. 13a). The net MFI of the 10% AF sample was 28, while that of the control cells was 23, which represents an increase of 22%. Figure 13b shows that, as with the unfiltered AF depicted in Fig. 12b, there was no effect on the percentage of phagocytosing cells.

4.3.3. Effect of Amniotic Fluid on Respiratory Burst in Alveolar Macrophages

The effect of AF on respiratory burst was also examined, and the results are depicted in Fig. 14. Only filtered AF was used in this study, because the respiratory burst response that was not due to phagocytosis of particulates was of primary interest. At both the 5 and 10% AF concentrations there was a significant increase in the average MFI compared to control cells, indicating that the magnitude of the response was

(a)



(b)

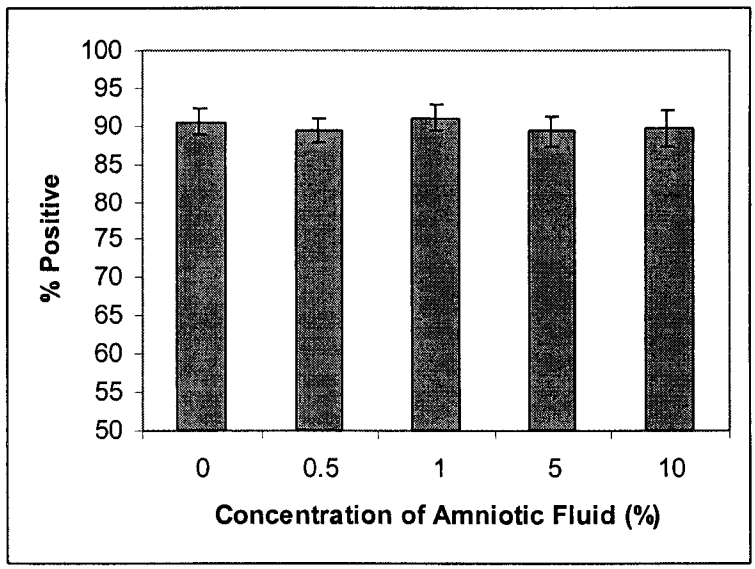
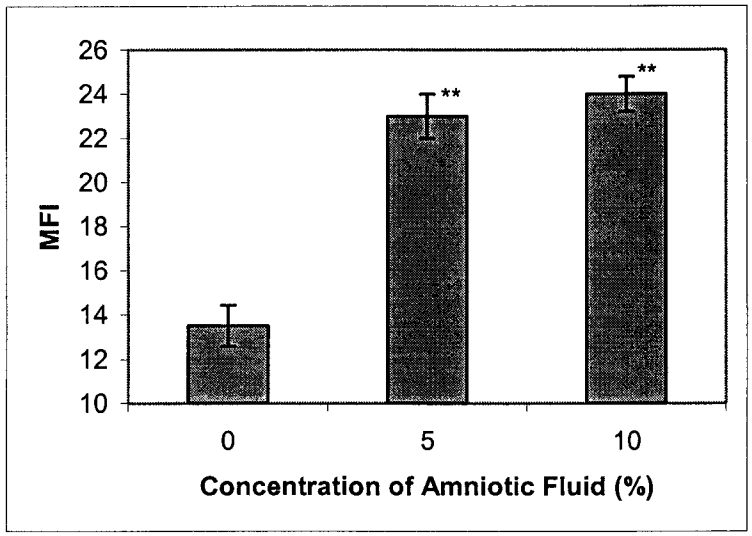


Figure 13. Filtered (0.2 μm) Amniotic Fluid Significantly Affects Phagocytosis by Alveolar Macrophages at a Concentration of 10%. Alveolar macrophages were incubated with various concentrations of filtered AF, and the net MFI (a) and percentage of phagocytosing cells (b) was determined after exposure to fluorescent latex beads. Results are shown as means ± SEM (n=5). Control cells were compared only to 10% AF exposed cells using an unpaired two tailed Student's t test (*, $p<0.05$).

(a)



(b)

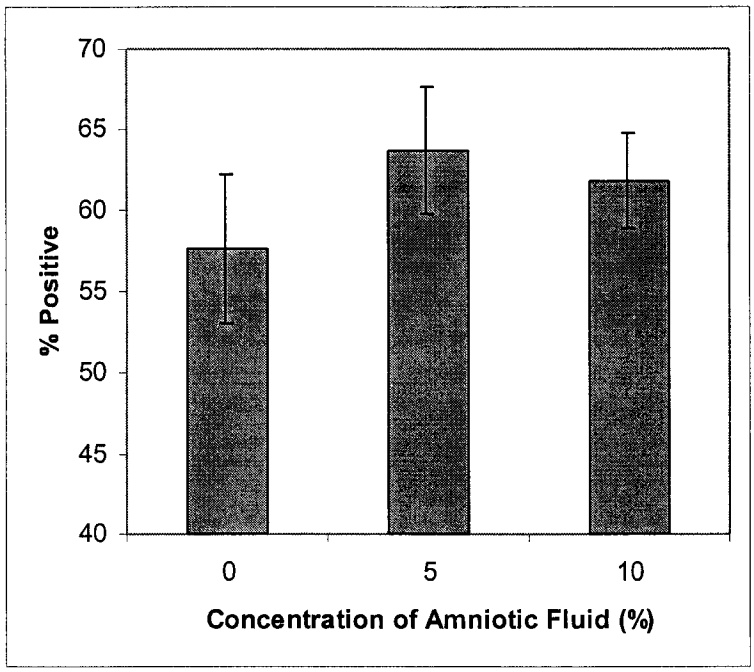
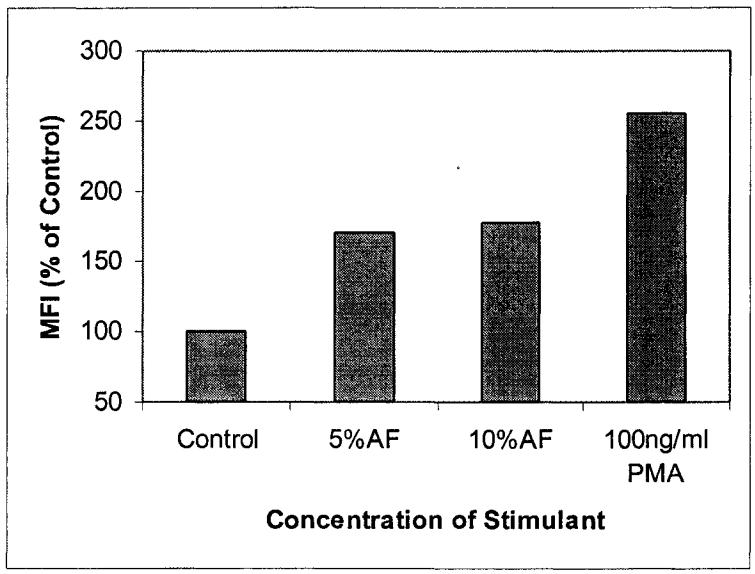


Figure 14. Amniotic Fluid Stimulates Respiratory Burst in Alveolar Macrophages. Alveolar macrophages were incubated with 0.2 μm filtered rat amniotic fluid, and the respiratory burst response was compared to that of control macrophages. The MFI (a) and the percentage of positive cells (b) were determined by measuring fluorescence due to intracellular oxidation of DCFH to DCF. Results are shown as means ± SEM (n=6; **, $p<0.01$).

increased. However, there was no significant increase in the percentage of cells producing a respiratory burst response, as depicted in Fig. 14b.

Figure 15 shows the data from Fig. 14 expressed as a percentage of control cell values, in order to allow comparison to the response produced by PMA, a known stimulant of respiratory burst activity. While the MFI observed in response to PMA stimulation was 2.5 times greater than that of the control cells, 5 and 10% AF produced a 70 and 78% increase respectively (Fig. 15a). The percentage of positive cells was increased to 119% of the control value by PMA, while AF increased the percentage of responding cells by 111 and 107% (Fig. 15b).

(a)



(b)

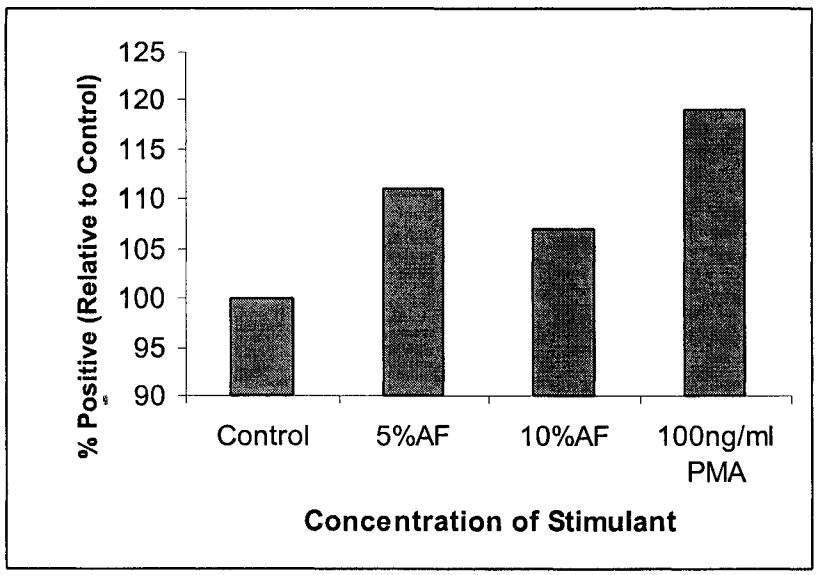


Figure 15. Amniotic Fluid Stimulates Respiratory Burst in Alveolar Macrophages. The respiratory burst response of alveolar macrophages to amniotic fluid was compared to that of control cells, and to cells incubated with 100 ng/ml of PMA. The MFI (a) and the percentage of positive cells (b) were determined by measuring fluorescence due to intracellular oxidation of DCFH to DCF, and were expressed as a percentage of the value obtained for control cells.

4.4. DISCUSSION

4.4.1. Effect of Amniotic Fluid on the Phagocytic Activity of Alveolar Macrophages

Although the effect was not significant, unfiltered amniotic fluid appears to increase the number of particles phagocytosed by alveolar macrophages, as demonstrated by the dose related increase in MFI. However, the percentage of phagocytosing cells was not noticeably affected. The alveolar macrophages used in this study avidly ingested latex beads, with close to 90% demonstrating association with one or more latex particles. Since the percentage of phagocytosing control cells was so high, the addition of AF did not produce any further increase.

The day-to-day variability seen in the net MFI of the NR8383 cells used in this study was problematic. The use of an alveolar macrophage cell line rather than macrophages obtained by bronchoalveolar lavage was chosen to reduce the variability in cellular response. However, the phagocytic activity of macrophages depends in part on their activation state, and macrophage activation state is influenced *in vitro* by a host of environmental factors such as temperature, oxygen tension [69], and adherence to plastic [70] or glassware. Furthermore, serum contains various proteins, lipids and electrolytes that might affect uptake of particles [71], and more than one batch of heat-inactivated serum was used in the media for these experiments. In addition, the AF used in the study came from different individual rats and may have therefore had slightly different compositions, and hence different effects on phagocytosis. Variability in the complement-activating potency of human AF samples collected from different individuals has been reported previously [72]. In retrospect, it would have been

preferable to have collected all the necessary amniotic fluid at the outset, and then pooled the samples. The large amount of variability in MFI prevented the attainment of significant results with the unfiltered amniotic fluid.

Filtering the AF to remove particulate matter reduced the variability in the response, but it also somewhat decreased the stimulatory effect on phagocytosis, since the net MFI of the 10% AF samples was increased 120% over that of control cells in the unfiltered, but only 22% in the filtered AF experiments. Consequently, an effect due to filtered AF could be demonstrated only at the 10% concentration, although, if higher concentrations had been used, it might have been possible to observe a dose-dependant response.

The significant response seen with 10% filtered AF suggests that at least some part of the stimulatory effect was contained in the non-particulate fraction. Hammerschmidt *et al* (1984) studied the effect of human AF on neutrophils, and although they reported no increase in activation as measured by superoxide production, aggregation, and chemotaxis, they did describe a modest activation of the complement system when AF was added to human plasma [72]. In their study, the complement-activating activity seemed to reside in the particulate fraction of the AF, since 0.2 μm filtration removed the effect.

The flow cytometric technique used to evaluate phagocytosis in this study did not permit distinction between attachment and ingestion of beads. However, some investigators have reported that this distinction may not be necessary, since the majority of cell-associated targets are eventually internalized by alveolar macrophages [73-75].

4.4.2. Effect of Amniotic Fluid on Respiratory Burst Activity of Alveolar Macrophages

Incubation of alveolar macrophages with 5 or 10% AF stimulated a significant increase in the magnitude of the respiratory burst response. However, the increase in MFI produced by incubation with AF was less than that observed in response to incubation with PMA. The percentage of AF-treated cells undergoing respiratory burst was also less than that observed in response to PMA stimulation and was not significantly different from that of control cells. This suggests that AF alone is not capable of activating alveolar macrophages sufficiently to stimulate a respiratory burst, but can increase the magnitude of the response in cells that are already activated and responding. Additional experiments in which AF-treated alveolar macrophages are subsequently stimulated with PMA are needed to determine if AF produces a priming effect in alveolar macrophages.

The stimulation of respiratory burst response seen in this study is in contrast to the results described by Hammerschmidt *et al*, who reported no increase in respiratory burst in human neutrophils incubated with human amniotic fluid [72]. One possible explanation is the difference in cells used—macrophages may be more responsive to the effect of AF than neutrophils. The AF used in the present study was of rat origin rather than human, so another possible reason for the discrepant results between their study and this one is simply that rat AF is different in composition from human. Even in the baboon, a primate with a similar fetoplacental-uterine anatomy and physiology to the human, differences in AF composition and pH as compared to human AF have been reported [76].

Besides the individual differences in AF composition that might be expected between individual rats, the stage of gestation is likely to make a considerable difference. The AF used by Hammerschmidt *et al* for their evaluation of effect on neutrophil respiratory burst was collected from women in the first trimester of pregnancy [72]. The composition of AF changes throughout gestation and concentration of some immunologically active constituents, such as proinflammatory cytokines for example, increases as parturition approaches, and may even play a role in regulating the onset of parturition [77]. The AF used in the current study was collected from pregnant rats several days before term in order to maximize the yield, although in retrospect, AF collected on the final day of gestation might be most relevant to clinical AF aspiration in the fetus or newborn.

4.4.3. Possible Stimulatory Constituents in Amniotic Fluid

Despite reported observations that AF does not cause marked pulmonary inflammation following aspiration or instillation [34-37], and in spite of its putative immunosuppressive properties [38,39], in this study AF was shown to stimulate respiratory burst and possibly to enhance phagocytosis in alveolar macrophages. This suggests that AF might contain one or more factors that activate alveolar macrophages.

One possible component of AF that could affect macrophage activity is the surfactant from the fetal lungs. Over 90% of surfactant is comprised of lipids, most of which are phospholipids, with surfactant proteins making up the remainder [78]. While the phospholipids found in surfactant have been reported to decrease phagocytosis [78] and respiratory burst [79] in alveolar macrophages, the same may not be true of surfactant proteins.

Surfactant proteins A and D (SP-A and SP-D) belong to a family of molecules called calcium-dependant lectins, or collectins [80]. The complement component C1q, and mannan-binding protein are also collectins, and the latter is found in AF, where it may play a role in enhancing innate immunity in the amniotic cavity by acting as an opsonin [81]. SP-A and SP-D can also opsonize by binding to carbohydrate ligands on microorganisms, and they are believed to play an important role in innate immunity [80]. These proteins, especially SP-A, have been shown to enhance phagocytosis in alveolar macrophages, not only by acting as an opsonin [82], but in some cases by directly activating the cell [83]. One mechanism for this activation-enhanced phagocytosis is likely up-regulation of phagocytic receptors, since SP-A increases expression of functional mannose receptors on the macrophage surface [84]. This increase in receptor expression occurs very rapidly (within 1 h) [84]. Although such receptor up-regulation is within the time frame used in this study, it is unlikely that the mannose receptor is involved in the phagocytosis of latex beads. However, there is evidence that other receptors might be up-regulated as well, since SP-A enhances phagocytosis of particles via Fcγ and complement receptors [85], and also increases levels of expression of ICAM-1, CD14, and CD11b [86]. Unopsonized latex beads are likely phagocytosed via scavenger receptors [87], but to our knowledge, the effect of SP-A on scavenger receptor expression has not been examined. In addition to their role in enhancing phagocytosis, SP-A and SP-D have been variously shown to stimulate [83,88] or to inhibit [89] production of reactive oxygen intermediates through the respiratory burst, and to increase production of inflammatory cytokines and nitric oxide in macrophages [90]. Different results in different studies could be due to use of

different cell lines, or to different densities of ligands on phagocytosable particles [84]. SP-A interacts with receptors on the surface of alveolar macrophages, and since different cell sources may have differing receptor expression, they may therefore be more or less responsive to SP-A. The activation state of the cells likely also accounts for some of the discrepancies between different published reports [80].

Amniotic fluid also contains numerous hormones, most of which are known to have some immunomodulatory effects. For example, prolactin is found in high concentrations in AF [91], and has been shown to enhance phagocytosis and respiratory burst in murine macrophages [92]. Another AF hormone that could affect macrophage function is endothelin. Endothelin is a potent vasoconstrictor that is found in relatively high concentrations in amniotic fluid, and it is believed to play a role in the etiology of amniotic fluid embolus [93]. Endothelin acts as a chemoattractant for macrophages, and also stimulates activation as measured by NF-kappaB activation [94], up-regulation in expression of CD68 and MHC molecules [95], or by release of arachidonic acid [96]. In neutrophils, endothelin-1 does not appear to stimulate respiratory burst directly, but may prime for increased respiratory burst in response to subsequent stimulation [97,98]. In alveolar macrophages, there is evidence that endothelin directly increases superoxide anion production to twice that of control cells [99].

The cytokines found in AF are another potential source of stimulation, since both pro- and anti-inflammatory cytokines have been identified [48]. The levels of some inflammatory cytokines, including TNF-α, IL-1 and IL-6, increase in amniotic fluid as gestation proceeds and may play a role in regulating parturition [77]. Some of

these inflammatory cytokines, such as TNF-α for example, have been shown to induce a respiratory burst response in alveolar macrophages [100].

Still another possible source of macrophage activation in the present study is endotoxin. Although the AF was collected aseptically and did not demonstrate bacterial growth in culture, contamination with minute amounts of endotoxin cannot be ruled out. Endotoxin is a well-known macrophage activator, and its presence could confound interpretation of experiments such as this, which are designed to measure macrophage response. Endotoxin contamination is apparently relatively common in surfactant isolated from rat lungs [101] and might also be found as a contaminant in AF.

4.4.4. Biological Significance

The increase in respiratory burst activity and the slight enhancement of phagocytosis demonstrated in this study with 10% AF are unlikely to have any real biological significance. No attempt was made to establish which components of AF might be responsible for the effect, other than to determine if it could be removed by 0.2 μm filtration. Since the effect was preserved after filtration, it cannot be due entirely to the presence of desquamated cells or other particulate debris from the fetus. If enhancement of respiratory burst is due to surfactant proteins, then the physiological relevance of this study is questionable. Alveolar macrophages in their normal *in vivo* environment are continuously exposed to surfactant from mid gestation onward. It seems unlikely that the amount of surfactant present in an aspirated bolus of amniotic fluid would significantly contribute to the activation state of alveolar macrophages *in vivo*, especially since the effect observed *in vitro* was relatively modest. However, if the effect demonstrated in this study was due to some other component of AF that is

normally not present in the alveoli at the same or greater concentrations, then AF aspirated *in utero* or at parturition could also stimulate respiratory burst and enhance phagocytosis by alveolar macrophages in neonates. Whether or not this up-regulation in alveolar macrophage function would benefit neonates by improving innate immune responses to other inhaled pathogens or particles remains to be determined.

Respiratory burst and the generation of ROS can induce pulmonary injury when excessive [102,103], but it is doubtful whether the increase in spontaneous respiratory burst demonstrated in this study would be sufficient to produce a deleterious effect *in vivo*. Previous studies have reported only minimal pulmonary inflammation in response to aspirated or instilled AF [34-37], which lends support to this conclusion. Nonetheless, further studies to examine the duration of the spontaneous respiratory burst, as well as the response to subsequent stimuli, might shed additional light on this issue. Other macrophage responses to AF, particularly the synthesis of pro and anti-inflammatory cytokines should also be examined, to determine if alterations in these functions could account for the failure of AF to induce marked pulmonary inflammation in neonates.

4.5. CONCLUSIONS

Incubation of alveolar macrophages with 5 or 10% amniotic fluid was found to increase the magnitude of spontaneous respiratory burst activity, while the percentage of cells producing this response was not significantly changed. The magnitude of the response to AF was somewhat less than that induced by PMA, a known stimulant of respiratory burst in macrophages. Incubation of alveolar macrophages with filtered

10% AF also induced a small but significant enhancement of phagocytosis that was due to an increase in the average number of associated particles per cell. The stimulation of respiratory burst induced by AF was somewhat unexpected, given that AF aspirated or instilled into the lungs causes only minimal inflammation. It would be of interest to investigate the effect of AF on other macrophage functions, such as cytokine synthesis and nitric oxide production, to determine if any anti-inflammatory effect exists.

4.6. Reference List

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5. NEITHER MECONIUM NOR AMNIOTIC FLUID INCREASES mRNA EXPRESSION OF TNF-α OR IL-1β IN ALVEOLAR MACROPHAGES

5.1. Introduction

Meconium aspiration syndrome (MAS) is an important cause of respiratory distress and mortality in newborn infants [1,2]. This syndrome occurs when the fetus expels meconium, and then inhales meconium-contaminated amniotic fluid either *in utero* or at parturition. The pathophysiology of MAS involves plugging of airways, with resultant hypoxia, hypercapnia and acidosis [3]. Surfactant dysfunction is also a component [3,4]. In addition, the aspiration of meconium causes a severe inflammatory response in the lungs, with a marked influx of neutrophils [5].

Alveolar macrophages are the cells responsible for providing first-line defense against inhaled particles and pathogens in the lungs. These cells serve a critical role in clearing particulates from the lungs by phagocytosis, and they also produce a host of different mediators that can direct and orchestrate immune responses [6]. Since these are the cells that first encounter inhaled meconium, their response to this substance likely plays a major role in the pathology of MAS.

Alveolar macrophages can be stimulated to produce a number of mediators that initiate inflammatory responses in the lung. These mediators include various chemotactic substances, such as eicosinoids, platelet activating factor, and chemokines, that recruit neutrophils and other inflammatory cells to the site of inflammation [7]. Alveolar macrophages also secrete a variety of cytokines, some of which have potent

proinflammatory effects. Tumor necrosis factor (TNF-α) and interleukin-1 (IL-1) are early response cytokines that initiate and promote inflammation. These cytokines play a central role in innate immune responses against various pathogens and are also necessary for the transition from innate to adaptive immunity [8]. IL-1 exists as two biologically equivalent isoforms: the predominantly membrane-associated IL-1α, and the secreted IL-1β [8]. Almost all cells have receptors for IL-1 and TNF-α, allowing them to respond and participate in the inflammatory response. Ligand-induced signaling through TNF-α or IL-1 receptors activates nuclear factor-κB (NF-κB), which is a transcription factor central to many inflammatory signaling pathways, and which is involved in expression of many different mediators of inflammation [8].

These proinflammatory cytokines have wide-ranging and overlapping effects on many different cell types, including both immune and non-immune cells. They are rapidly produced at sites of local inflammation and serve to amplify the inflammatory response by inciting cytokine production in other cells, thus initiating an inflammatory cascade. For example, IL-1 and TNF-α can stimulate a variety of cell types to produce chemokines that recruit inflammatory cells to the lungs [8], and they promote neutrophil extravasation at the site of inflammation by inducing expression of adhesion molecules on endothelial cells [9]. IL-1 and TNF-α also promote inflammation by affecting the function of recruited cells. For example, these cytokines prime neutrophils for enhanced phagocytosis and respiratory burst [10]. The effects of these two proinflammatory cytokines are concentration-dependant, and when large amounts are produced, systemic consequences occur. Systemic biological effects include stimulation of hematopoiesis, the induction of fever, hypotension, anorexia, acute phase

protein production, and activation of the hypothalamic-pituitary-adrenal axis. Circulating levels of TNF-α and IL-1 correlate with mortality in septic shock and acute respiratory distress syndrome [10].

However, alveolar macrophages also secrete regulatory or anti-inflammatory molecules such as transforming growth factor-β (TGF-β), interleukin-10 (IL-10), and prostaglandin E_2 (PGE_2) which function to decrease inflammatory cell activity [11,12], thus limiting the injurious effects of a sustained inflammatory reaction [13]. IL-10, for example, is a potent anti-inflammatory cytokine that inhibits the effects of proinflammatory cytokines at many different levels. IL-10 decreases macrophage synthesis of IL-1 and TNF-α, promotes the degradation of mRNA for these cytokines, attenuates surface expression of inflammatory cytokine receptors, and inhibits NF-κB translocation [13]. IL-10 also inhibits generation of reactive oxygen and nitrogen intermediates [8].

Homeostasis in the lung is a finely tuned balance that is maintained by secretion of both pro- and anti-inflammatory molecules [14]. Although other cells play a role in this process, the alveolar macrophage is the most important regulator of the immune response in the lung. The net effect of interactions between pro- and anti-inflammatory molecules depends on many factors including the activation state of macrophages [15], timing of release, receptor density, tissue responsiveness and the cytokine milieu [13].

The lung is constantly being bombarded with inhaled foreign particles. Some of these particles are infectious, and may require a systemic immune response for effective elimination. However, many of the inhaled particles are relatively inert, and a full-blown inflammatory response is undesirable, since inflammatory mediators can damage

lung tissue when produced in excess. Alveolar macrophages possess many different cell surface receptors that can recognize different types of particles. Some receptors, such as Fc and complement receptors, recognize serum opsonins. However, many inhaled pathogens are not opsonized, so alveolar macrophages also have receptors that can recognize conserved patterns found on the surface of pathogens. The nature of the receptors engaged is an important determinant of the subsequent response, because receptor binding can trigger various signaling pathways that lead to different gene expression, thus channeling the macrophage response [15]. Proinflammatory signals are generated upon particle binding to some phagocytic receptors such as Fc and mannose receptors [15]. Other receptors, such as complement [16] and scavenger receptors, do not stimulate a marked inflammatory response [17]. Some phagocytic receptors can trigger inflammatory responses directly, whereas others work in concert with co-receptors [16]. Foremost among these are the Toll-like receptors (TLR), at least 10 of which have been identified in humans in the past few years [18]. The TLRs are pattern recognition receptors that recognize conserved molecular patterns found in microorganisms [19], such as bacterial cell wall components and foreign DNA, although they also appear to respond to nonbacterial products [18]. These receptors are specifically recruited to phagosomes when particles are internalized. They sample the contents of the phagosome, and when certain patterns are recognized, TLRs are activated and trigger inflammatory responses [16]. Since different TLRs recognize distinct microbial features [20], the information obtained from activation of these receptors may enable immune cells to distinguish different types of particles and produce a response that is tailored to the threat [19].

Activation of phagocytic and Toll-like receptors initiates signaling pathways that activate various transcription factors, including NF-κB, Jun, activator protein-1 (AP-1) and Fos [21]. These transcription factors allow specific immune response genes to be expressed, resulting in the production of cytokines and other molecules that are necessary to produce an inflammatory response. Nuclear factor-κB is a principal mediator of inflammation, and its activation is required for maximal transcription of most proinflammatory molecules, including acute-phase proteins, adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), cytokines including IL-1 and TNF-α, chemokines including interleukin-8 (IL-8), and enzymes such as cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [8,22]. The most potent stimuli for NF-κB activation are lipopolysaccharide (LPS), IL-1, and TNF-α [22]. TLRs have an intracellular domain that is homologous to that of the IL-1 receptor, and many of the steps in their signaling pathways are shared [18].

The signaling pathway leading to activation of NF-κB has been relatively well characterized. Signaling through the IL-1 receptor and most of the TLRs involves the adaptor protein MyD88 that recruits IL-1 receptor activated kinase [19]. This kinase autophosphorylates and binds to another adapter molecule, TRAF6 [18]. TRAF6 complexes with NF-κB–inducing kinase (NIK), which subsequently activates inhibitory κB (IκB) kinase. Activation by TNF-α occurs by a pathway that is somewhat different early in the cascade, but also results in IκB kinase activation by NIK [8]. IκB kinase phosphorylates IκB, which causes it to degrade and release NF-κB [8]. NF-κB is then free to translocate to the cell nucleus where it initiates transcription of inflammatory genes.

A number of different stimuli can induce macrophages to produce TNF-α and IL-1 through activation of the NF-κB signaling pathway. These include endotoxins and exotoxins from bacteria, phorbol myristate acetate (PMA), calcium ionophores, complement components, and various cytokines, including IL-1 and TNF-α themselves. In addition, various particulates have been demonstrated to stimulate transcription of mRNA for these two cytokines, although the response is variable depending in part on the specific receptors engaged. Kobzik (1993) demonstrated that phagocytosis of opsonized beads caused alveolar macrophages to release TNF-α and neutrophil chemoattractant molecules, while phagocytosis of unopsonzed beads did not [23]. Other particulates that have been shown to stimulate alveolar macrophages to produce TNF-α or IL-1 include asbestos, ambient air particles, oil fly ash, cigarette smoke, silica and titanium oxide [24-28]. On the other hand, some stimuli, particularly anti-inflammatory cytokines such as IL-10 and IL-4, can inhibit expression of TNF-α and IL-1 genes.

Aspirated meconium is phagocytosed by alveolar macrophages and can be readily observed in these cells *in vivo*, although the specific receptors responsible for binding and ingestion of meconium and amniotic fluid are not known. Most complex particles, such as bacteria, can activate multiple receptors simultaneously. These receptors in turn may activate different signaling pathways leading to distinct secretory responses [15]. Since these different signaling pathways may be synergistic or antagonistic, the net effect, in terms of production of inflammatory mediators, is difficult to predict. Meconium is a complex and non-homogeneous substance, and likely binds to more than one type of receptor.

Meconium has been shown to activate NF-κB in alveolar macrophages and to induce expression of iNOS [29]. In peritoneal macrophages, meconium was found to increase expression of mRNA for TNF-α and to stimulate TNF-α secretion [30]. In addition, elevated levels of pro-inflammatory cytokines have been found in lung lavage fluid following experimental meconium instillation in newborn rabbits [31]. It seems likely, therefore, that meconium would enhance expression of mRNA coding for proinflammatory cytokines, including TNF-α and IL-1β, in alveolar macrophages. However, the meconium inhaled by newborns with MAS is diluted with amniotic fluid. Amniotic fluid may have immunosuppressive properties and contains a number of anti-inflammatory substances, such as IL-10 [32-34], IL-1 receptor antagonist [35], secretory leukocyte protease inhibitor [36], and Clara cell protein [37], that could antagonize synthesis of proinflammatory cytokines. The objective of this study was to determine whether either meconium or amniotic fluid would induce expression of mRNA for TNF-α and IL-1β in alveolar macrophages.

5.2 Materials and Methods

5.2.1. Cells

A continuous rat alveolar macrophage cell line, NR8383, (American Type Culture Collection, Rockville, MD) was used as a source of cells. Cells were cultured in Ham's F12 nutrient mixture with glutamate, supplemented with 15% heat inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all components purchased from Sigma-Aldrich, St. Louis, MO). Cultures were maintained in a humidified, 5% CO_2 incubator at 37° C. This spontaneously transformed cell line grows

as a mixture of adherent and non-adherent cells [38]. On the day prior to each experiment, a cell scraper was used to dislodge adherent cells from the culture flask, and all cells were then transferred to a new flask. On the day of the experiment, non-adherent cells were collected for use in assays by pelleting.

5.2.2. Meconium

Equine meconium was collected aseptically from the distal colon of a newborn foal submitted for necropsy at the Atlantic Veterinary College. A portion of it was cultured and examined microscopically to confirm absence of bacterial growth. The equine meconium was lyophilized, and stored at –70° C. When required, lyophilized meconium was solubilized in complete Ham's media at a concentration of 50 mg/ml.

5.2.3. Amniotic Fluid

Amniotic fluid was collected from pregnant Sprague Dawley rats (Charles River, St-Constant, QC, Canada) at 17-19 days of gestation. Each rat was anesthetized by inhalation of halothane, and the gravid uterus was removed following a midline abdominal incision. Rats were euthanized by anesthetic overdose. The uterine extremities were clamped with hemostats, and it was suspended from a support stand. A sterile 25g needle was used to puncture individual amniotic sacs, and amniotic fluid was collected into a sterile tube as it dripped from dependant sites. A portion of the fluid collected from each rat was cultured and examined microscopically to confirm absence of bacterial growth. Aliquots of amniotic fluid were kept frozen at –70° C until needed.

5.2.4. Experimental Incubation Conditions

Alveolar macrophages (3-4 x 10^6) suspended in culture media were incubated with meconium (25 mg/ml) or amniotic fluid (10% by volume) for 0.5, 1 or 2.5 h. Control cells were not exposed to meconium or amniotic fluid, but were incubated with an equal volume of additional media for the same time periods. The cells were then washed with phosphate buffered saline and centrifuged at 360 *g*. To demonstrate that increased expression of mRNA for TNF-α and IL-1β could be visualized under the experimental conditions employed, some macrophages were incubated with 10 μg/ml LPS.

5.2.5. RNA Isolation

Total cellular RNA was isolated from NR8383 cells using TRIzol reagent (Invitrogen Life Technologies, Burlington, ON). Cells were pelleted by centrifugation (360 *g*), and 1 ml of TRIzol was added and mixed by gentle pipetting. The TRIzol mixture was transferred to a 1 ml eppendorf tube (Sarstedt, Newton, NC) and was frozen at –70° C until RNA isolation was performed. On the day of RNA isolation, 200 μl of chloroform was added to each thawed sample, and the contents were mixed by vigorously inverting the tubes 20 times. After a 3 min incubation period at room temperature, the samples were centrifuged 15 min at 4° C and 15,800 *g*, in a Micromax RF centrifuge (International Equipment Company, Needham Heights, MA). Following transfer of 450 μl of the RNA supernatant to a new eppendorf tube, isopropyl alcohol (500 μl) was added to precipitate the RNA, and the contents were mixed by repeated inversion of the tubes. After incubation for 10 min at room temperature, the RNA was pelleted by centrifugation (10 min, 4° C, 15,800 *g*). The supernatant was decanted, and

the pellet was washed with 1 ml 75% ethanol, before pelleting again by centrifugation (5 min, 4° C, 15,800 *g*). After decanting the ethanol supernatant, the pellet was allowed to air dry, then resuspended in 25 µl of pyrogen free water. The quality of the isolated RNA was examined by gel electrophoresis before performing reverse transcription.

5.2.6. Reverse Transcription

Reverse transcription of the isolated mRNA was carried out in a 20 µl volume using Moloney Murine Leukemia Reverse Transcriptase (M-MLV RT; Invitrogen). Master mix for all reaction mixtures was prepared containing 200 units of M-MLV RT, 0.5 mM deoxynucleotidetriphosphates (dNTPs), 0.01 M dithiothreitol (DTT), 3 µg Random Primer hexanucleotides, and first strand buffer (all components from Invitrogen). Each reaction mixture contained 5 µl of mRNA, 6 µl of pyrogen-free water, and 9 µl of master mix. The reaction mixture was incubated at 37° C for 1h, and then at 94° C for 10 min. The cDNA product was diluted with 180 µl of pyrogen free water.

5.2.7. Polymerase Chain Reaction

Following reverse transcription, cDNA products were amplified by the polymerase chain reaction (PCR), using cytokine-specific intron spanning primer pairs, as previously described [39]. Master mix was prepared for each reaction using 0.2 mM dNTPs, 2.5 units of *Taq* DNA polymerase (Invitrogen), 50 µM primers, and reaction buffer (Invitrogen). Rat β-actin primers were synthesized by GibcoBRL Life Technologies (Rockville, MD). Primers for rat TNF-α and IL-1β were a kind gift from Dr. Andrew Stadnyk (Dept. of Pediatrics, IWK Health Centre, Halifax, NS). Primer sequences were as follows:

Rat β-actin product size 330 base pairs

Sense: (5' to 3') CTG GAG AAG AGC TAT GAG C

Antisense: (5' to 3') AGG ATA GAG CCA CCA ATC C

Rat IL-1β product size 331 base pairs

Sense: (5' to 3') CAA CAA AAA TGC CTC GTG C

Antisense: (5' to 3') TGC TGA TGT ACC AGT TGG G

Rat TNF-α product size 292 base pairs

Sense: (5' to 3') TAC TGA ACT TCG GGG TGA TCG

Antisense: (5' to 3') CCT TGT CCC TTG AAG AGA ACC

The PCR reaction was carried out in a 50 μl volume, using 11.5 μl of master mix, along with cDNA and pyrogen-free water, and the reaction mixture was overlaid with 100 μl of mineral oil to prevent evaporation. The amplification protocol used for all primers was denaturation at 92° C for 30 s, annealing at 57° C for 30 s, and primer extension at 72° C for 60 s. PCR amplification was conducted in a PTC-100 Programmable Thermal Controller (MJ Research Inc., Watertown MA) and the number of cycles in all cases was 35. PCR products were stained with ethidium bromide, and run on a 1.5 % agarose gel for visualization.

To control for variables in sample preparation, mRNA levels in different samples were normalized by comparison to the housekeeping gene, β-actin.

5.3. Results

5.3.1. Effect of Amniotic Fluid on mRNA Expression of TNF-α and IL-1β in Alveolar Macrophages

Figure 16 shows that alveolar macrophages incubated with LPS, a known inducer of inflammatory cytokine expression, demonstrated visibly increased levels of mRNA for TNF-α and IL-1β under the experimental conditions employed. IL-1β was constitutively expressed at a low level, while TNF-α was not. In contrast, Figure 17 shows that macrophages incubated with amniotic fluid for 0.5, 1 or 2.5 h did not have any change in mRNA expression for TNF-α and IL-1β, when compared to control cells incubated without amniotic fluid for the same time periods. In Figure 17, a very low level of TNF-α expression can be observed in control cells, as well as in cells incubated with amniotic fluid.

5.3.2. Effect of Meconium on mRNA Expression of TNF-α and IL-1β in Alveolar Macrophages

Figure 18 illustrates that incubation of alveolar macrophages with meconium at 25 mg/ml did not stimulate an increase in mRNA expression for TNF-α and IL-1β. Despite repeated attempts, the low basal level of gene expression for TNF-α did not allow visualization of this PCR product.

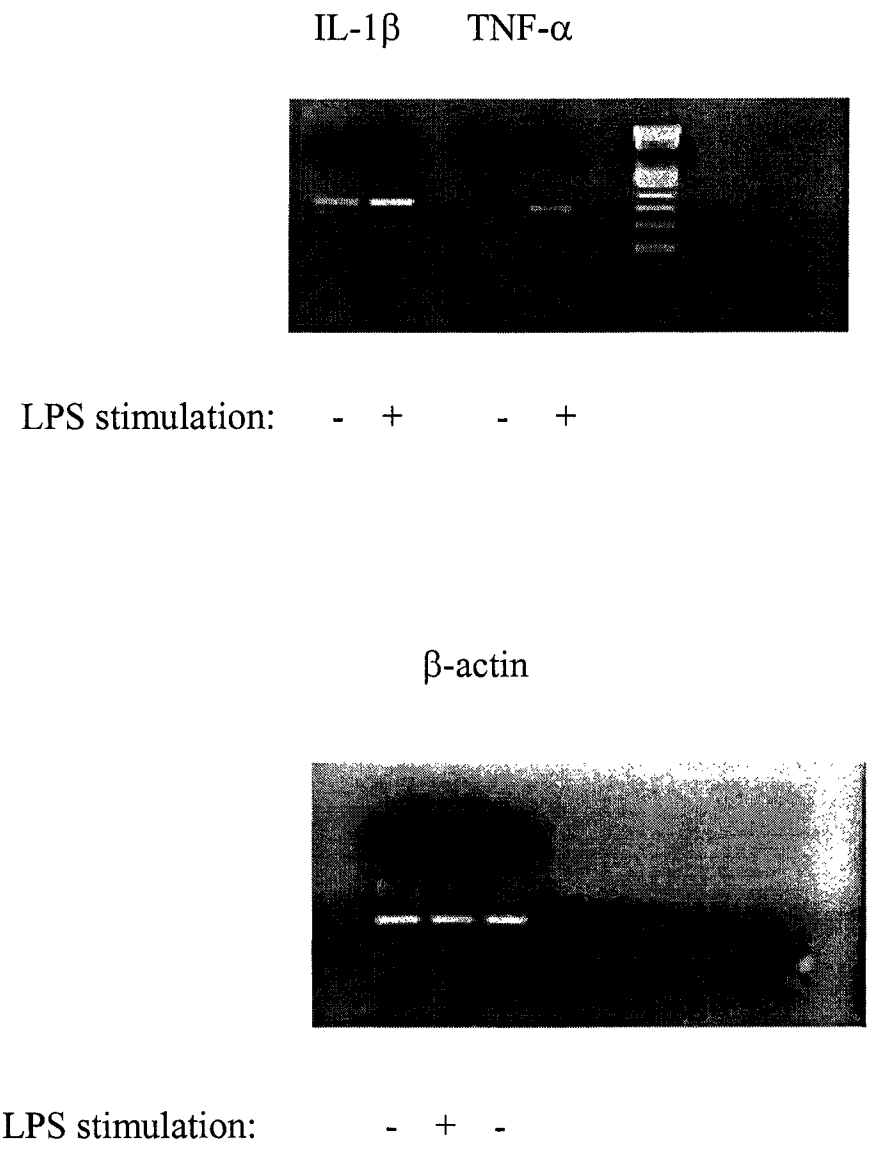

Figure 16. Effect of LPS on Transcription of IL-1β and TNF-α in Alveolar Macrophages. NR8383 cells were incubated in the presence or absence of LPS (10 μg/ml). Total RNA was isolated and reverse transcribed. PCR was performed using cytokine specific primer pairs, and products were visualized with ethidium bromide staining on 1.5% agarose gels.

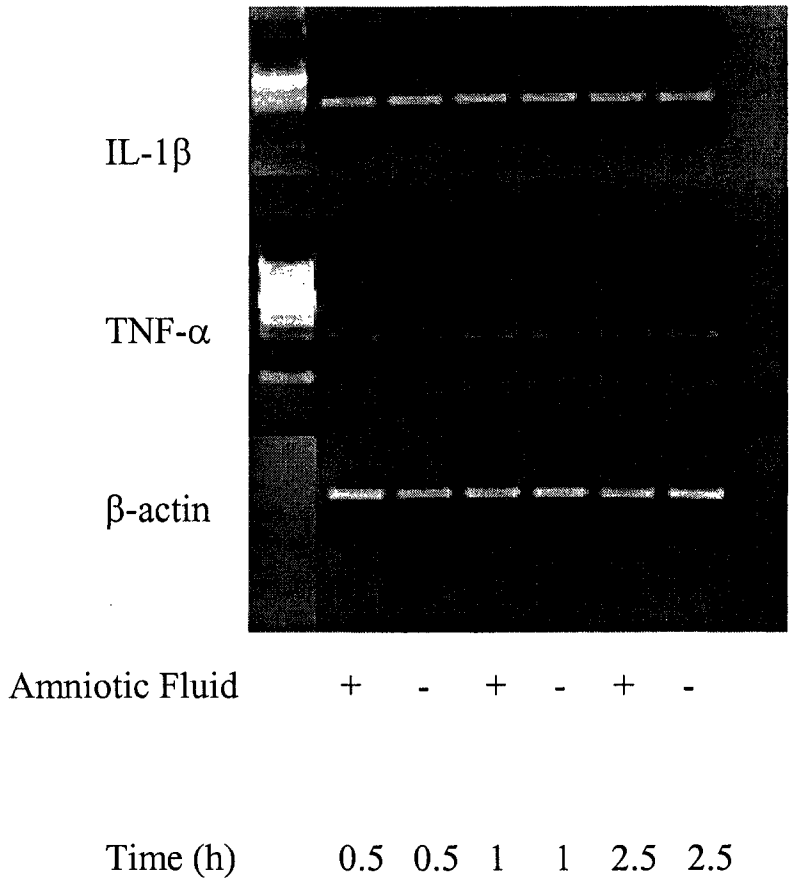

Figure 17. Incubation of Alveolar Macrophages with Amniotic Fluid Does Not Induce Transcription of mRNA for IL-1β and TNF-α. NR8383 cells were incubated in the presence or absence of 10% amniotic fluid. Total RNA was reverse transcribed, and PCR was performed using cytokine specific primer pairs. PCR products were visualized with ethidium bromide staining on 1.5% agarose gels.

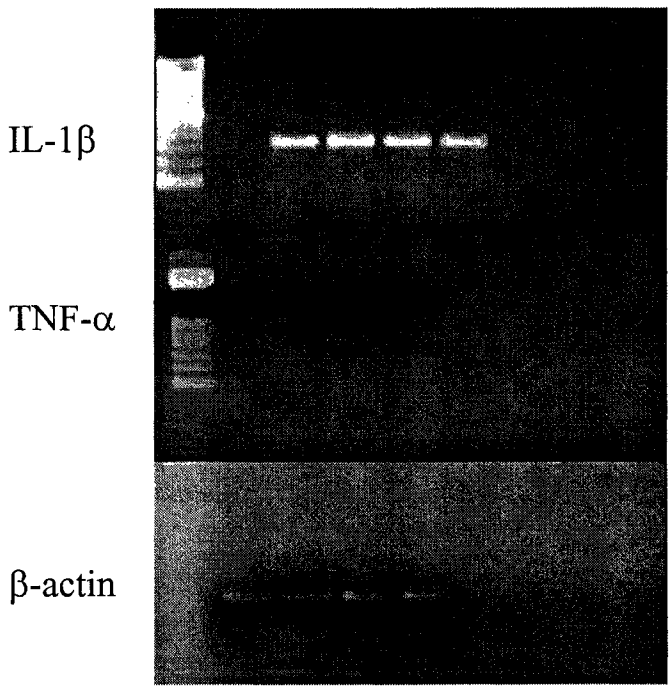

Figure 18. Incubation of Alveolar Macrophages with Meconium Does Not Increase mRNA Transcription for IL-1β or TNF-α. NR8383 cells were incubated in the presence or absence of 25 mg/ml equine meconium. Total RNA was reverse transcribed, and PCR was performed using cytokine specific primer pairs. PCR products were visualized with ethidium bromide staining on 1.5% agarose gels.

5.4. Discussion

In this preliminary study, incubation of alveolar macrophages with meconium or amniotic fluid did not affect the mRNA expression of two important proinflammatory cytokines.

Although a very low level of expression of message for TNF-α was observed in Fig. 17, no TNF-α production could be observed following RT-PCR in Fig. 16 and Fig. 18. It appears that TNF-α is not constitutively expressed in unstimulated alveolar macrophages, or is expressed at a level so low as to be undetectable most of the time. The number of alveolar macrophages used for RNA isolation in the experiment depicted in Fig. 17 was slightly higher than that used in the other experiments, which may explain this discrepancy. Another possibility is that the alveolar macrophages used in the Fig. 17 experiment may have differed in their activation state from those used in the other experiments. Macrophages *in vitro* can be activated by a variety of stimuli, including temperature changes, changes in oxygen tension [40], and adherence to plastic [41] or glassware. Although conditions were standardized in the present series of experiments, it is possible that slight variations in unmeasured environmental factors may have influenced the activation state of the cells.

The failure of amniotic fluid to generate a proinflammatory response was not surprising, and is supported by clinical observations that instilled or aspirated amniotic fluid does not result in marked pulmonary inflammation, despite the presence of potentially inflammatory components such as keratin and squamous epithelial cells [42-45]. Furthermore, a number of substances have been identified in amniotic fluid that might serve to dampen proinflammatory activity in alveolar macrophages. These

include IL-10 [32-34], IL-1 receptor antagonist [35], secretory leukocyte protease inhibitor [36], and Clara cell protein [37]. IL-10, for example, inhibits the synthesis of proinflammatory cytokines, and also promotes degradation of mRNA coding for IL-1β and TNF-α [13]. Future studies should determine whether amniotic fluid induces expression of various other cytokines, particularly anti-inflammatory cytokines, in alveolar macrophages.

The lack of increased proinflammatory cytokine mRNA expression following incubation with meconium was unexpected, since a number of studies have demonstrated that meconium stimulates proinflammatory responses in macrophages and other cells. Meconium has been shown to be a potent activator of NF-κB in NR8383 cells as detected by electrophoretic mobility shift assay and immunostaining [29]. NF-κB is a transcription factor with a principal role in inflammation, and its activation is associated with inducible expression of many inflammatory genes, including those for both IL-1β and TNF-α. In the same study, meconium also induced expression of mRNA for iNOS and increased production of nitric oxide [29]. In another study, Lally *et al* (1999) found that mouse peritoneal macrophages incubated with human meconium had increased TNF-α mRNA expression as well as TNF-α secretion [30]. Meconium also induced peritoneal macrophages to express tissue factor, or procoagulant activity, through a protein kinase C regulated pathway [30]. Furthermore, meconium has been found to induce expression of ICAM in endothelial cells [46]. Additionally, we have previously shown that meconium stimulates a robust respiratory burst response in alveolar macrophages (Chapter 3).

In addition to meconium, numerous other particulates, including latex beads, silica, asbestos and various types of air pollution particles, have been shown to stimulate production of proinflammatory cytokines in alveolar macrophages [23-25,27,28,47-49], although in some cases this stimulatory effect is believed to be due to the presence of endotoxin or transition metals [27,28,48,49].

The failure of meconium to stimulate upregulation of proinflammatory cytokine expression in this study is difficult to explain given the results of these previously published studies. One possible explanation could be that the time points chosen were inappropriate. However, in Lally's (1999) study, upregulation of TNF mRNA was seen after meconium was incubated with peritoneal macrophages for 2 h, which is similar to the time points used in the present study [30]. Furthermore, these time points are similar to those commonly used in investigating expression of early response cytokines. For example, in a murine model of endotoxemia, TNF mRNA levels peaked 30 min after stimulation, and TNF production peaked in 1h, declining to undetectable levels in 8 h [50,51].

Another potential reason for the difference between this study and previous ones is the cell source. Although Li *et al* (2001), used the same NR8383 cell line to demonstrate that meconium induces activation of NF-κB [29], this activation does not inevitably result in expression of mRNA for the two proinflammatory cytokines examined in this study. Lally (1999) used peritoneal macrophages rather than alveolar macrophages to examine the effect of meconium on mRNA for TNF-α [30]. Macrophages are known to be functionally heterogeneous [6], and although NR8383 cells are capable of producing both IL-1 and TNF-α in response to stimulation [38,52],

their responsiveness may not be equal to that of peritoneal macrophages. Indeed, it is tempting to speculate that alveolar macrophages in general might be less likely than peritoneal macrophages to respond to particulate stimuli with the production of proinflammatory mediators. This is because alveolar macrophages, unlike peritoneal macrophages, are continuously exposed to inhaled particles, and production of an inflammatory response with each encounter would likely be detrimental due to ensuing damage to host tissue.

Another possibility is that the equine meconium used in this study was qualitatively different from the human meconium used in other studies. Although previous experiments demonstrated that equine and human meconium were similar in terms of their effects on alveolar macrophage phagocytosis and respiratory burst activity (Chapters 2 and 3), their effects on cytokine synthesis could be dissimilar. This is conceivable because the effect of phagocytosis and respiratory burst might be due to the particulate nature of meconium, whereas the effect on cytokine secretion might depend more on the presence of specific chemical mediators. It would be important to directly compare the effect of meconium from these two different species on cytokine production, since we previously speculated that equine meconium could be a useful substitute for human meconium in investigating various aspects of MAS pathophysiology (Chapter 2).

The meconium concentrations used by Lally *et al* varied from 0.0001% to 1% [30], which were substantially less than that used in the present study (25 mg/ml of human meconium is approximately equivalent to 10%, as determined by weight of dry matter following dessication). Furthermore, the increase in TNF-α mRNA observed by

Lally *et al* was not dose-dependant, since the greatest effect was seen at 0.1%. The meconium concentration used by Li *et al* in assessing NF-κB activation and iNOS expression was 5 mg/ml [29], which is also less than the concentration used in the present study. Possibly the effect of meconium at high concentrations is to stimulate alternative pathways that do not result in secretion of these proinflammatory cytokines. Indeed, nitric oxide itself may provide negative feedback, since it has been shown to downregulate production of TNF-α and IL-1β by alveolar macrophages [53]. Further experiments should investigate the possibility that meconium, at concentrations less than 25 mg/ml, may induce an increase in mRNA synthesis.

Another possible explanation for the lack of inflammatory response is that meconium is toxic to alveolar macrophages at the concentration used in this study. Previous experiments showed that alveolar macrophages incubated with meconium for up to 6 h did not demonstrate decreased cell viability, as measured by trypan blue exclusion (see Chapter 2). However, it is possible that the concentrations of meconium used in this study produced subtle toxic effects that affected alveolar macrophage metabolic activity without inducing changes in cell membrane integrity. Exposure of alveolar macrophages to some types of particles has been shown to result in increased apoptosis, which is not necessarily accompanied by necrosis and decreased cell membrane integrity [47,54]. Further study is necessary to determine if meconium induces apoptosis in alveolar macrophages, and if so, the concentration at which this occurs.

Although stimulation with meconium or amniotic fluid did not directly increase cytokine-specific mRNA expression, it is possible that either of these substances could

prime macrophages for enhanced or prolonged expression following subsequent stimulation. A priming effect on cytokine production has been seen in macrophages exposed to silica particles or cotton smoke, for example [55,56]. Despite the fact that these materials did not enhance basal production of TNF-α, when cells were subsequently stimulated with LPS, their TNF-α secretion was increased. Incubating macrophages with meconium, and then stimulating them with LPS before RNA isolation could be used to explore this possibility.

5.5. Conclusions

Incubation of alveolar macrophages with either meconium or amniotic fluid was not found to increase expression of mRNA for two important proinflammatory cytokines, TNF-α and IL-1β, in this preliminary study. Further study is needed to determine if these substances induce expression of other cytokines, particularly anti-inflammatory cytokines in the case of amniotic fluid. Additional assays are also necessary to determine whether either meconium or amniotic fluid produces a priming effect on cytokine synthesis, and whether the lack of inflammatory response following meconium exposure is due to the induction of apoptosis.

5.6 Reference List

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6. GENERAL DISCUSSION AND CONCLUSIONS

Flow cytometry proved to be a useful and simple technique to evaluate both respiratory burst and phagocytic activity in alveolar macrophages, allowing large numbers of macrophages to be individually analyzed. The autofluorescence exhibited by meconium made interpretation of the results more difficult, but the problem was overcome by the use of appropriate controls.

Both human and equine meconium were found to produce similar effects on the phagocytic and respiratory burst activity of alveolar macrophages, although their effect on cytokine secretion was not compared. This finding has some practical significance, in that animal meconium may be obtained more easily than human in some circumstances, without the requirement for approval by a human ethical review board. Our results indicate that equine meconium can be substituted for human under the conditions employed in these experiments.

In these experiments, we have shown that meconium decreases phagocytic activity in alveolar macrophages. Both the percentage of actively phagocytosing cells and the average number of particles phagocytosed per cell are decreased following incubation of alveolar macrophages with equine or human meconium. When meconium was passed through a 0.2 μm filter to remove particulates, the inhibitory effect on phagocytic activity was retained, although it was diminished compared to that produced by unfiltered meconium. Since the inhibitory effect was not entirely eliminated despite the removal of particles larger than 0.2 μm, then either soluble factors or ultrafine particles remaining in the meconium samples must contribute to the effect. On the

other hand, the attenuated inhibitory effect on phagocytic activity that was produced by filtered meconium implies that at least some part of the inhibition is due to the particles present in meconium.

The particulate component of meconium could cause a decrease in the phagocytosis of latex beads by physical mechanisms, such as competition for receptor binding sites [1], or by saturation of macrophages when a maximum phagocytosable volume is achieved [2]. However, since macrophages that were incubated with both meconium and phorbol myristate acetate (PMA) had increased phagocytic uptake of latex particles compared to those incubated with meconium alone, it would seem that the meconium-induced decrease in phagocytic activity was not entirely due to saturation or competition. Activation of an inhibitory pathway is another mechanism that could explain the decrease in phagocytic activity following meconium exposure. Elevation of cellular cAMP levels and subsequent activation of protein kinase A is a well known inhibitory pathway for various macrophage functions, including phagocytosis and respiratory burst [3,4]. However, we did not find a significant increase in intracellular cAMP levels in macrophages following incubation with filtered or unfiltered meconium. This finding does not preclude the possibility that other inhibitory mediators may be activated by meconium, or that some component of the signaling pathways necessary for phagocytosis might be deactivated by exposure to meconium.

Still another possible explanation for the observed inhibition of phagocytosis, is that meconium is toxic to alveolar macrophages at the concentrations used in these studies. The viability of alveolar macrophages following incubation with meconium was investigated using trypan blue exclusion, and no decrease in viability was observed.

However, the possibility that meconium could induce apoptosis in these cells was not investigated. In this regard, there have been published accounts of macrophages being rendered apoptotic by exposure to particles, even though their cell membranes initially remained intact [5-8]. Further studies are necessary to determine whether meconium exposure induces apoptosis in alveolar macrophages.

In contrast to the observed decrease in phagocytosis, meconium stimulated a substantial increase in spontaneous respiratory burst activity, as measured by oxidation of dichlorofluorescin. Excessive production of reactive oxygen species (ROS) generated through respiratory burst activity plays a role in inducing pulmonary injury in diseases such as acute respiratory distress syndrome [9] and may contribute to the pathophysiology of meconium aspiration syndrome (MAS). Both filtered and unfiltered meconium stimulated respiratory burst, although filtration of the meconium attenuated the response. While these alterations in the two macrophage functions might at first seem contradictory, the observed increase in spontaneous respiratory burst is most likely linked to the phagocytosis of meconium particles, including ultrafine particles in the case of filtered meconium. The probable explanation for these observations is that meconium binds to one or more types of phagocytic receptors and is rapidly phagocytosed by alveolar macrophages. The binding of numerous meconium particles to phagocytic receptors stimulates respiratory burst activity in the macrophages, with resulting production of reactive oxygen species. However, as the cells take up the meconium particles, their ability to phagocytose latex beads is reduced, possibly due to a combination of the mechanisms discussed above, including macrophage saturation,

competition for receptor binding sites, activation of inhibitory pathways, and cellular toxicity.

Alveolar macrophages that had been incubated with meconium had a significantly reduced respiratory burst response when they were stimulated with PMA 24 hours later. This suggests either that meconium caused a relatively long-lasting suppressive effect or that it induced cellular toxicity. It is plausible that the increase in oxidative metabolism generated by meconium exposure is the source of a toxic effect. The observed increase in dichlorofluorescein fluorescence following the initial exposure to meconium was in the order of 70 fold, which is similar in magnitude to the 100 fold increase reported by Hiura *et al* (1999) following exposure of macrophages to diesel exhaust particles [7]. In that study, the increased generation of reactive oxygen species was associated with macrophage apoptosis.

The generation of both reactive oxygen and nitrogen species can initiate apoptosis in macrophages [8,10]. Activation of caspase-9 and caspase-3 has been shown to occur in response to elevations in ROS levels following exposure to some particulates [8], and treating macrophages with anti-oxidants can protect them from particulate-induced apoptosis [7,8]. We have shown that incubation of alveolar macrophages with meconium can generate significant levels of ROS, and others have shown that macrophages produce nitric oxide following meconium exposure [11]. Furthermore, respiratory burst activity is not the only source of reactive oxygen species in macrophages. Mitochondrial respiration, peroxisomal metabolism, and the chemical transformation of xenobiotics by enzymes such as cytochrome P450 can all generate ROS and contribute to cellular oxidative stress [12-14]. Some particulates, such as

diesel exhaust, are believed to generate ROS in alveolar macrophages primarily through the later mechanism, since methanol extraction, by removing salient chemical constituents, significantly reduces the apoptotic effect [7]. However, other particulates, including silica and asbestos, are believed to produce oxidative stress in alveolar macrophages primarily through phagocytic and inflammatory processes that lead to production of ROS [15]. Macrophages are normally protected from the damaging effects of the products of their own respiratory burst activity [7], but excessive production of ROS could overwhelm anti-oxidant defense mechanisms, and could also act in a paracrine manner to damage neighboring cells [12].

We hypothesized that meconium exposure would upregulate expression of mRNA for proinflammatory cytokines in macrophages because instilled or aspirated meconium invokes an intense inflammatory reaction in the lungs of neonates [16,17], and several studies have shown that meconium produces inflammatory responses in cultured peritoneal and alveolar macrophages, as well as airway epithelial cells [11,18,19]. Furthermore, the vigorous respiratory burst response that we observed following meconium exposure would seem to corroborate the proinflammatory character of this substance. Although the magnitude of oxidative burst does not always correlate well with production of inflammatory cytokines [20], ROS can induce activation of NF-κB, and activation of this proinflammatory transcription factor has been reported in association with meconium exposure [11]. Nonetheless, in our preliminary investigations, meconium failed to induce an increase in expression of mRNA for TNF-α or IL-1β. A possible explanation is that oxidative stress induced by

excessive production of ROS negatively affected cellular metabolism, thus preventing cytokine mRNA synthesis by alveolar macrophages in response to stimulation.

In contrast to meconium, incubation of alveolar macrophages with amniotic fluid (AF) resulted in a marginal increase in phagocytosis, as measured by an increase in the average number of latex beads taken up per cell. The magnitude of the respiratory burst response was also increased following incubation with AF although the percentage of cells producing a response did not change. This suggests that, while AF does not stimulate respiratory burst in resting macrophages, it can increase the magnitude of the response in cells that are already activated.

The meconium aspirated by neonates with MAS is necessarily mixed with AF, so the biological significance of meconium on alveolar macrophage function will depend on the influence of both of these substances. One of the questions we attempted to address is whether AF would augment or oppose the effects of meconium on alveolar macrophages. Since alveolar macrophages are crucial in defending the alveolar space from inhaled pathogens, any compromise in the functioning of alveolar macrophages could significantly impair the ability of the lung to respond to challenge by infectious microorganisms [21]. Many years ago, Bryan (1967) demonstrated that meconium administration to mice and rats reduced the LD_{50} of concurrently administered *E. coli* bacteria, and suggested that meconium reduces host resistance to bacterial infection [22]. Other studies have shown that various inhaled particulates can reduce host resistance to respiratory infection in animals [23-26], and epidemiological studies have linked air pollution particles to respiratory infection in humans [27]. The mechanism for this increased susceptibility to infection is not clear, but could be due to induction of

apoptosis in alveolar macrophages, or due simply to inhibition of phagocytic activity. In our experiments, meconium significantly decreased phagocytosis, while AF slightly stimulated phagocytic activity. Depending on the relative concentrations, AF could have a protective effect in regard to this particular macrophage function, and could allow meconium-exposed macrophages to continue to phagocytose inhaled particles and pathogens at near normal levels. On the other hand, since meconium was found to stimulate a robust spontaneous respiratory burst in alveolar macrophages, while AF increased the magnitude of the respiratory burst in cells already responding, the combined effect on ROS generation would appear to be additive, and could be of sufficient intensity to make a major contribution to the pulmonary injury and inflammation associated with MAS. Since the amount of meconium contamination in the AF aspirated by neonates is highly variable, it is difficult to predict the combined effect of these two substances on the alveolar macrophages in the lungs of babies with MAS.

An *in vitro* study such as this one can provide only a foundation for understanding the pathophysiology of a complex syndrome like MAS. There are a number of reasons why the effects observed and reported here might not reflect the responses of alveolar macrophages *in vivo*. The relative concentration of meconium and AF in the aspirated material is one factor that has already been discussed. Furthermore, even *in vitro*, considerable differences are seen in the activity of alveolar macrophages depending on the cell source and experimental conditions [28-30]. The NR8383 line has been shown to be a good model for alveolar macrophage phagocytosis and respiratory burst, and to produce levels of activity similar to that of freshly derived

rat alveolar macrophages [28,31]. Nonetheless, the level of responsiveness of NR8383 cells is probably not identical to that of human neonatal alveolar macrophages. Moreover, the *in vitro* environment is very different from that of the alveoli, where numerous other cells and chemical mediators exist that also influence macrophage activity. *In vivo* studies in neonatal animals should be performed to confirm the relevance of these results to the clinical problem of MAS.

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