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Ultrastructural changes in the lungs of neonatal rats intratracheally inoculated with meconium

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Summary. Meconium aspiration syndrome has been for many years an important cause of neonatal respiratory distress in newborn babies and sporadically reported in animals. This investigation was designed to study the ultrastructural and morphometric changes in the lungs of neonatal rats following the intratracheal inoculation of meconium. Seven-day-old Fischer-344 rats (n = 24) were randomly allocated in two groups. One group was intratracheally inoculated with saline solution and the second group received homologous meconium. Neonates were euthanatized at 1, 3 and 7 postinoculation days (PID) and lungs were examined by light and electron microscopy. Saline solution did not induce any ultrastructural changes in the lung. In contrast, meconium induced deciliation, recruitment of neutrophils and pulmonary alveolar macrophages to the bronchoalveolar space, intravascular sequestration of neutrophils and aggregation of platelets at PID 1 and 3. Other ultrastructural changes at PID 1 and 3 included interstitial edema and escape of red cells and fibrin into the alveolar space and interstitium. Interstitial edema and sequestration of neutrophils were responsible for the significant increase in thickness of alveolar septa. At PID 7 there was hyperplasia and enlargement of type II pneumocytes as well as interstitial proliferation of mesenchymal cells with intra-alveolar fibrosis. It was concluded that intratracheal inoculation of meconium in neonatal rats induces acute ultrastructural changes followed by a reparative response.

Key words: Lung, Neonatal rats, Ultrastructure, Meconium, Aspiration

Introduction

Meconium aspiration syndrome (MAS) has been recognized for many years as an important cause of neonatal respiratory distress in newborn babies (Srinivasan and Vidyasagar, 1999; Wiswell, 2001). Whereas the prevention, clinical implications and medical management of MAS are well documented and understood (Wiswell, 2001; Wiswell et al., 2002), the pathogenesis of pulmonary lesions in MAS remains enigmatic. Meconium is a complex material normally present in the fetal intestine that contains large quantities of squamous epithelial cells and keratin. These materials reach the intestine when exfoliated dermal and oropharyngeal epithelia suspended in the amniotic fluid, is subsequently swallowed into the gastrointestinal tract (Rapoport and Buchanan, 1950; Srinivasan and Vidyasagar, 1999). During fetal stress and hypoxia, increased intestinal peristalsis and relaxation of the anal sphincter cause abnormal meconium defecation into the amniotic sac. When amniotic fluid contaminated with meconium is aspirated, the airways and alveolar mucosa are irritated by the keratin, epidermal cells and intestinal secretory products like bile, resulting in a local inflammatory response in the lung (Wiswell and Bent, 1993).

The lungs of babies succumbing to MAS have a multifocal atelectasis that is often referred to as "patchy atelectasis" (Brown and Gleicher, 1981; Brady and Goldman, 1986). Microscopically, bronchi, bronchioles and alveoli contain variable amounts of meconium which appears as a bile-pigmented yellow material containing vernix caseosa, squamous cells, keratin and cellular debris (Katz and Bowes, 1992). In addition to atelectasis, air trapping and hyperinflation, aspiration of meconium induces a local pulmonary inflammation commonly referred to as chemical pneumonitis primarily centered around the terminal bronchiolar and alveolar regions where aspirated meconium typically induces edema and exudation of inflammatory cells (Tyler et al., 1978; Sun et al., 1993). Intraalveolar hyaline membranes, one of the morphologic hallmarks of

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another pediatric conditions known as "hyaline membrane disease" (neonatal respiratory distress syndrome), have also been occasionally reported in the lungs of some babies dying of severe MAS (Seo et al., 1990).

In animals, meconium-induced bronchiolitis and alveolitis have been sporadically reported in calves, foals and puppies dying in the first two weeks of life (Lopez and Bildfell, 1992; Furr, 1996). The bronchi and bronchioles of these animals showed microscopic evidence of meconium and keratin associated to mild alveolar exudation of neutrophils, macrophages and occasional giant cells (Lopez and Bildfell, 1992).

Experimentally, pulmonary lesions similar to those reported in spontaneous MAS have been reproduced under laboratory conditions with intratracheal inoculations of meconium (Tyler et al., 1978; Wiswell et al., 1992; Davey et al., 1993). Experimental studies in laboratory animals have shown that the inflammatory response concentrates in the terminal bronchioles and alveoli where neutrophils and macrophages are typically found in spontaneous cases of MAS (Tyler et al., 1978). One study has suggested that the chemical effects of meconium induce alveolar changes characterized by thickening of the alveolar wall (Tyler et al., 1978). Similarly, work in our laboratory demonstrated microscopic thickening of the alveolar wall in neonatal rats intratracheally inoculated with meconium (Martinez-Burnes et al., 2002). However, the severity of alveolar thickening induced by meconium has not been properly evaluated by ultrastructural or morphometric studies.

Although it is well known that MAS induces bronchiolitis and alveolitis, it is still unknown whether injury to bronchiolar and alveolar cells, particularly type I pneumocytes, may result from direct exposure to or the inflammatory response induced by meconium. This question could be best answered by studying with transmission electron microscopy the bronchiolar and alveolar cell morphology in neonatal animals inoculated with meconium. The objectives of this study were to evaluate the ultrastructural and morphometric changes in lungs of neonatal Fisher 344 rats inoculated with homologous meconium.

Materials and methods

Animals

Fischer-344 female and male rats obtained from commercial sources (Charles River Inc., St. Constant, Quebec, Canada) were kept at the laboratory animal facility of the Atlantic Veterinary College (AVC). Adult rats were housed individually and provided with commercial rat food and water *ad libitum*. All female rats were bred and put in a timed pregnancy program based on vaginal cytology (Baker, 1979). At the time of birth, neonates were separated by sex and male pups were used in inoculation studies while female pups were utilized as the source of meconium (Martinez-Burnes et al., 2001a). Male pups were kept with the dam and maintained on a 12/12-hour light/dark cycle at 22 °C and 50% relative humidity. Experimental protocols were approved by the local Animal Care Committee at the Atlantic Veterinary College and experiments were conducted following the guidelines of the Canadian Council on Animal Care, 1993.

Collection of meconium and preparation of inocula

Meconium was aseptically collected from the intestine of female rat pups killed with an anaesthetic overdose at the time of birth and before intake of colostrum. Meconium was maintained frozen at -80 °C until preparation of the inoculum. Prior to inoculation, samples of meconium were submitted for bacteriological analysis to assure sterility. The final concentration of unfiltered meconium was adjusted to 200 mg of meconium (wet weight) in 1 ml of saline, considered the maximum tolerated dose based on a dose range-finding study previously described (Martinez-Burnes et al., 2001a, 2002).

Intratracheal inoculation

Rat neonates were intratracheally inoculated using a methodology previously reported (Martinez-Burnes et al., 2001b). Briefly, neonates were inoculated under halothane anaesthesia and using a modified otoscope and speculum (Welch Allyn, Skaneateles Falls, NY). A sterile, 25-gauge, 8.89 cm length spinal needle (Becton Dickinson, Franklin Lakes, NJ) with the edge rounded was passed into the trachea and the inoculum was gently injected into the lungs. Pups were maintained under direct observation on a heating pad (37 °C) until recovery from the anaesthesia.

Experimental design

Seven-day-old male neonates (n=24) with an average weight of 15.6±1.1g were randomly assigned to two experimental groups (control and meconium). Neonates from the control group (n=12) received 0.05 ml of sterile saline solution and neonates from the meconium group (n=12) were inoculated with 0.05 ml of a suspension of 20% meconium (Martinez et al., 2001a). The volume of inoculum was chosen based on a dose range-finding study which showed that 0.05 ml of a 20% suspension (200 mg of meconium in 1ml of saline) was the maximum tolerated dose that inoculated to neonatal rats induce lung changes but without causing significant mortality. Four neonates per group were killed with an overdose of halothane and exsanguinated (Martinez-Burnes et al., 2001a) at 1, 3, and 7 days postinoculation (PID).

Lung fixation and processing

Following euthanasia, the lungs of neonates were fixed *in situ* by intratracheal instillation with fixative

following modified methods described in detail elsewhere (Martinez-Burnes et al., 2002). In short, the thorax was opened, the trachea was exposed and a plastic catheter (20-gauge and 32 mm length; Cathlon IVJ Critikon Canada Inc, Ontario) was inserted through a transverse slit made in the wall of the trachea. Fixative was intratracheally instilled with the catheter at a constant pressure of 20 cm of fixative using modified "Marriott bottles system" (Tyler et al., 1985). The fixative was 2% glutaraldehyde buffered to pH 7.38 at room temperature using 0.1 M sodium cacodylate and having a total osmolarity of 350 mosmol. After a minimum of 30 minutes of fixation in situ, a tight ligature was placed around the trachea to maintain the intrapulmonary pressure of the fixative once the catheter was removed. The lungs were dissected free from the thorax and stored in the same fixative for at least two hours before further manipulation. The lung volume was determined by the gravimetric method based on volume of fluid displaced by the immersed lung (Pinkerton and Crapo, 1985).

Two representative longitudinal slices (approximately 2 mm in thickness) of each animal's entire left lung and two transverse sections across the long axis of the right cranial lobes were sampled. The first slice was used for morphometry in light microscopy (LM) and the parallel second facing slice was used to obtain the blocks for transmission electron microscopy (TEM).

For LM, the glutaraldehvde fixed lung slices were processed, embedded in paraffin, sectioned at a thickness of 3 μ m and stained with hematoxylin and eosin following routine procedures. For TEM studies, two samples per lung were minced into small pieces and immersed in fresh 2% sodium cacodylate buffered glutaraldehyde for 90 minutes at room temperature. The tissue was washed in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer, dehydrated and embedded in Epon/Araldite (Marivac Ltd, Halifax, NS). Semi-thin sections (0.5 μ m thick) were stained with 1% toluidine blue in a 1% sodium tetraborate solution and observed with a light microscope. Samples containing mainly alveolar regions and terminal bronchioles in the semi-thin sections were selected and thin sections were cut at a thickness of 70-80 nm. Thin sections were stained with uranyl acetate and lead. The sections were examined and photographed using a Hitachi H7000 scanning transmission electron microscope operated at 75kV.

Morphometry

Based on preliminary studies of the rat neonatal lung, the apical lobe was selected for the morphometric assays. Five different fields were selected from each apical lobe using a systematic sampling method with a random starting point (Howard and Reed, 1998). Images for every field were captured using a digital camera (Pixera Visual Communication, Suite Model PVC100C, Pixera Corporation, Los Gatos, CA) attached to a Zeiss light photo microscope (Carl Zeiss, Ontario). The images were digitized and exported to Scion Image (Beta 3 Release, Scion Corporation, NIH-Image software), analysed at a final magnification of 6,200 X, and projected in the computer screen containing a multipurpose test grid with 84 lines and 168 test points (Weibel, 1979).

Volume fractions of the structures of interest (alveolar walls; alveolar air spaces; blood vessels larger than 25 μ m in diameter; bronchi and bronchioles; and bronchiolar air spaces) were analysed using point counting performed with the computer program of public domain <http://rsb.info.nih.gov/nih-image/> developed by the National Institute of Health (NIH) and modified for Windows by Scion Corporation (Maryland). The absolute volumes of the different lung compartments were obtained by multiplying each individual tissue compartment fraction by the total fixed lung volume. To determine the thickness of the alveolar septum, ten different fields from the apical lobe were selected using the same systematic sampling with a random starting point (Cruz-Orive and Weibel, 1981). Using a Zeiss microscope at high magnification (100x), images were captured, digitized, exported and projected in a PC high-resolution monitor at a final magnification of 10,000 X. A multipurpose test lattice overlay with 84 lines was placed over the monitor screen. The arithmetic mean thickness of the entire alveolar septa was calculated using morphometric procedures (Weibel, 1979). When the morphometry was done, the identity of the lung sample (treated vs control) was unknown to the observer.

Treatment and interactions effects were statistically evaluated by Analysis of Variance (GLM-ANOVA). Differences between groups of treatment, days postinoculation, and lobes were examined with One-Way Analysis of Variance (ANOVA) followed by the nonparametric Kruskal-Wallis test (Fisher and van Belle, 1993). A p-value <0.05 was considered statistically significant.

Results

Intratracheal inoculation of saline solution did not induce clinical signs in the neonate, and did not result in any gross, microscopic or ultrastructural changes in the lung. In contrast, inoculation of meconium caused a transient dyspnea in neonatal rats without mortality. Also, the lungs from rats inoculated with meconium exhibited a multifocal green discoloration that became more evident after perfusion with fixative. No differences were noted grossly in the distension pattern between meconium treated lungs and controls.

Meconium

In semi-thin sections of lung stained with toluidine blue, meconium was visible as an amorphous pale material in the bronchiolar and alveolar spaces. Alveolar macrophages and neutrophils were commonly observed around the meconium. Ultrastructurally, meconium appeared as a mixture of an electron-lucent matrix of a loose fine fibrillar network interspersed with elongated aggregates of electron-dense material different regions admixed with neutrophils and alveolar macrophages, and also by electron-dense aggregates of cellular debris. Enlarged alveolar macrophages exhibiting abundant debris-laden phagolysosomes were also present on the surface of the meconium.

Bronchiolar response to meconium

At PID 1 and 3, ciliated epithelial cells in bronchioles showed evidence of deciliation, particularly in areas of the lung that contained or were close to meconium or to degenerated macrophages and



neutrophils (Fig.1A). Deciliation was characterized by the presence of basal bodies at the apex of some ciliated cells and the absence of the normal length of cilia at the surface (Fig.1B). Detached cilia were commonly observed in the meconium plug (Fig. 1A). Deciliation was not observed at PID 7 nor was it detected in the control rats. No other remarkable ultrastructural changes were observed in ciliated and nonciliated epithelial cells in bronchioles of the meconium group (Fig. 1C).

Alveolar response to meconium

At the alveolar region, at PID 1 and 3 inoculation of meconium induced an apparent increase in type II pneumocytes, many of which exhibited numerous secretory granules with the characteristic lamellar arrangements of surfactant. Surfactant in the form of unfolded lamellae and myelin structures were present free in alveolar spaces close to the surface of type II pneumocytes, however, these cells did not show notable ultrastructural alterations. At PID 1 and 3, a remarkable inflammatory response in the alveolar regions was clearly associated with the presence of meconium and characterized by enlarged and activated macrophages demonstrating numerous and remarkable long cytoplasmic extensions (pseudopods) protruding into the alveolar air spaces and in close contact with meconium (Fig. 2). The interstitium beneath this region had distended interstitial spaces (edema) with increased cellularity. A layer of elongated macrophages, which contained phagolysosomes and some with lamellar structures, covered the meconium matrix (Fig. 2). Cellular debris mixed with fibrin (Inset) and characteristic latticework arrangements of surfactant were also present in the alveolar spaces (Fig. 2).

Platelet aggregations in blood vessels and in alveolar capillaries (Fig. 3A) were observed concomitantly with intracapillary sequestration of neutrophils and edematous distension of the interstitial spaces (Fig. 3B) in the lungs of some neonates inoculated with meconium mainly at PID 1 and less frequently at PID 3 and 7.

In semi-thin sections, a severe and multifocal



Fig. 2. Alveoli: meconium group, PID 1. Macrophages with multiple large pseudopods (lage arrows) in contact with meconium (M). A layer of elongated macrophages (PAM) covers the meconium. Fibrin (small arrow) and surfactant (arrowhead) are present in the alveolar space (Alv). Bar: 2 μm. **Inset:** Higher magnification of fibrin (arrow) and lattice arrangements of surfactant (arrowhead) in the alveolar space.



Fig. 3. Alveolar septum; meconium group, PID 1. **A.** A blood vessel (BV) shows aggregation of platelets (arrows). Note the edema with distension of interstitial space (*). Myofibroblast (M) with lipid droplets (L). Alveolar space (Alv). Bar: 2 μ m. **B.** Sequestration of neutrophils (PMN) in a capillary (C) associated with interstitial edema (*). Distended interstitium contains portions of myofibroblasts (M). Type II pneumocyte (EP II), Alveolar space (Alv). Bar: 2 μ m.



Fig. 4. A. Light micrograph of semi-thin resin section; alveolar space, meconium group, PID 7. Conspicuous recruitment of neutrophils (arrows) and alveolar macrophages (arrowhead) after the meconium inoculation. Bar: 20 μ m. **B.** Alveolar space; meconium group, PID 7. Enlarged neutrophils (PMN) with multiple internalized lamellar bodies (arrowheads). Alveolar space (Alv). Bar: 2 μ m. **C.** Alveoli; meconium group, PID 7. Close proximity of type II pneumocytes profiles (EP II) indicating hyperplasia. Lamellar bodies (arrows), Alveolar airspace (Alv). Bar: 2 μ m.

neutrophilic and histiocytic (proliferative) response was observed in the lungs of PID 7 neonates inoculated with meconium (Fig. 4A). At the TEM level, neutrophils and macrophages were often enlarged with numerous internalized lamellar bodies (Fig. 4B).

Distension of alveolar interstitium with less edema but with conspicuously increased cellularity was associated with areas of proliferative inflammation in the PID 7 meconium group in semi-thin sections. Distension of alveolar surface was primarily due to hypertrophy and hyperplasia of type II pneumocytes mainly in the proximal alveolar region and alveolar areas with proliferative inflammation. At TEM level, type II pneumocyte profiles were found in close proximity, possibly reflecting the increased number (hyperplasia) of these cells (Fig. 4C). An apparent increase in size and morphology of lamellar bodies in type II pneumocytes was observed in the lungs of the meconium group at PID 7. Type II pneumocytes exhibited abundant and irregular arrangement of lamellar bodies, fusion of two or more lamellar bodies, as well as release of lamellar structures to the alveolar space.

An increased thickness of alveolar septum in areas of proliferative response (Fig. 5A) was correlated with densely packed interstitial cells, including fibroblasts, myofibroblasts as well as extravascular neutrophils, and red blood cells (Fig. 5B). Isolated necrotic cells, some containing lamellar bodies and others cells with unrecognizable characteristics other than pyknotic nuclear material were observed in the interstitial space. Although perivascular spaces were markedly distended and alveolar epithelium and interstitium of rats inoculated with meconium were hyperplastic at PID 7, endothelial cells did not exhibit changes.

Focal, proliferative responses in proximal alveolar regions were observed in lungs from neonates inoculated

PMN

Fig. 5. Alveolar septum, meconium group, PID 7. A. Light micrograph of semi-thin resin section showing increased thickness of interstitium with high cellularity (arrows). Alveolar space (Alv). Bar: 20 μ m. **B.** Proliferation of fibroblastic-cells (F), myofibroblasts (M), presence of neutrophils (PMN), and red blood cell (*) in interstitial matrix. Bar: $2 \mu m$.





Fig. 6. Meconium group, PID 7. A. Light micrograph of semi-thin resin section showing a large protrusive mass (P) at the region of transition between terminal bronchiole (arrow) and alveoli (arrowhead) and protruding from the interstitium (I) to the alveolar space (Alv). Boxed area enlarged in Fig. 6C. Bar: 20 μ m. **B.** Transition of bronchiolar nonciliated cell (NC) to alveolus. Proliferation of myofibroblasts (M) and macrophages (PAM) migrating from the interstitium through discontinuities in alveolar surface to the alveolar air space (Alv). Bar: 2 μ m. **C.** Light micrograph of boxed area in Fig. 6A. The proliferative mass consists primarily of myofibroblasts (M) rich in lipid droplets (arrows), and fibroblastic cells (arrowheads) migrating from alveolar interstitium (I) to the alveolar airspace (Alv). Bar: 10 µm. **D.** Proliferative mass showing myofibroblasts (M) rich in lipid droplets (arrows). Alveolar airspace

GROUP	DAY PI	Ν	BODY WEIGHT	FIXED VOLUME	ABSOLUTE VOLUME (cm ³)				
			(g)	(g)	alv	alair*	bv	br	airbr
Control	1	4	17.6±1.47	1.222±0.048	0.382±0.023	0.606±0.066	0.126±0.049	0.033±0.029	0.073±0.071
(Saline)	3	4	20.6±1.55	1.395±0.073	0.474±0.044	0.689±0.065	0.131±0.073	0.040±0.029	0.056±0.030
	7	4	27.2±2.49	1.568±0.078	0.443±0.088	0.836±0.192**	0.125±0.028	0.069±0.043	0.090±0.069
Meconium	1	4	16.5±1.92	1.299±0.079	0.399±0.022	0.661±0.133	0.139±0.023	0.032±0.034	0.069±0.075
	3	4	18.4±2.46	1.440±0.201	0.510±0.131	0.719±0.119	0.100±0.025	0.038±0.029	0.078±0.056
	7	4	25.1±3.04	1.665±0.062	0.440±0.052	0.980±0.067**	0.128±0.065	0.049±0.022	0.055±0.021

 Table 1. Number of rats, body weight, fixed lung volume and absolute volume of the compartments of the apical lobe at different postinoculation times.

 Values are means ± SD.

alv; alveolar septa; alair: air from alveoli; bv: blood vessels > 25µm; br: bronchi and bronchioles; airbr: air from bronchi and bronchioles. *: overall significant treatment effect (p<0.05); **: significant difference (p=0.05).

Table 2. Arithmetic mean	thickness	of the	alveolar	septa	in the	apical	
lobe at different postinoculation times (Mean \pm standard deviation).							

GROUP	DAY PI	AMT (µm)
Control (Saline)	1 3 7	5.21±1.04 4.89±0.43 3.83±0.34
Meconium	1 3 7	7.22±1.77* 7.65±1.56* 7.25±0.92*

AMT: Arithmetic Mean Thickness. *: significant difference (p<0.05)

with meconium and killed at PID 7. Also, there was a notable increase in cellularity at the transition region between the terminal bronchiole and alveoli extending from the interstitium through to the alveolar space (Fig. 6A). The interstitium in these proliferative areas contained numerous myofibroblasts, fibroblasts, along with macrophages, neutrophils, red blood cells and fibrin both within the interstitium and in the alveolar space.

Fibroblasts, myofibroblasts and macrophages also protruded through alveolar gaps or defects suggesting a cell migration into the alveolar air spaces (Fig. 6B, C). Recognizable bronchiolar or alveolar epithelial cells did not cover the external surface of the proliferative process, which was in contact with the airspace, and the cells forming the intraalveolar nodules were fibroblasticlike cells and lipid-laden myofibroblasts (Fig. 6D). All type I pneumocytes, whether or not they were associated with areas of proliferative inflammation, remained without significant changes as where those in controls at PID 7.

Morphometry

Morphometric analyses did not reveal any significant changes in the fixed lung volume and volume fractions of the pulmonary compartments between the saline and meconium groups. Fixed lung volumes increased slightly after the inoculation of meconium but the magnitude of these changes was not large or severe enough to achieve statistical significance. Intratracheal inoculation of meconium did not induce significant (p>0.05) changes in the absolute volumes of the compartments with the exception of air from alveoli. Rats of the meconium group had higher volumes of alveolar air (p<0.05) (Table 1). The thickness of the alveolar septum increased significantly (p<0.01) as result of meconium inoculation. These differences between rats inoculated with saline and those inoculated with meconium remained significant (p=0.05) from PID 1 to PID 7. In rats inoculated with saline, the thickness of the alveolar septum decreased (p=0.002) from PID 1 to PID 7 (Table 2).

Discussion

The morphologic appearance of the meconium in the rat lung consisted of a mixture of a loose fibrillar matrix and an electron-dense matrix of cellular debris. Based on the known composition of meconium, the loose fine fibrillar network could be formed by the abundant mucopolysaccharides contained in meconium (Rapoport and Buchanan, 1950; Hounsell et al., 1989). The different cells contained in meconium originate from amniotic fluid, gastrointestinal tract, and fetal skin, and, along with the degenerated macrophages and neutrophils account for the electron-dense matrix of cellular debris (Rapoport and Buchanan, 1950; Kääpä et al., 1997).

The fact that meconium was surrounded by macrophages and neutrophils was not surprising since this material is known to act as a foreign body in the lung (Bacsik, 1977).

Ultrastructural changes characterized by deciliation of the bronchiolar epithelium at PID 1 and 3 were primarily associated with the presence of meconium in the airways. Damage to bronchiolar epithelium with deciliation and necrosis has been described in rabbits after the inoculation with human meconium (Tyler et al., 1978). According to some reports, meconium causes epithelial damage as a result of the chemical irritation caused by bile and various enzymes present in this material (Tyler et al., 1978; al Mateen et al., 1994). Since there was some ultrastructural evidence of the release of cytoplasmic granules from degenerated leukocytes in rats of the meconium group, it is also possible that lysosomal enzymes could have played a role in deciliation. This probable mechanism of epithelial damage has been suggested by others (Kojima et al., 1994; Shabarek et al., 1997). Also, a recent study with piglets inoculated with meconium suggests that epithelial damage in the lung may result from an increased oxidative burst of neutrophils (Soukka et al., 2002).

Inoculation of meconium induced a rapid influx of neutrophils and macrophages into alveolar spaces that persisted from PID 1 to 7. The rapid recruitment of inflammatory cells has been also documented after the inoculation of meconium in rabbits and pigs (Tyler et al., 1978; Wiswell et al., 1992; Davey et al., 1993; Soukka et al., 1997). It has been suggested that several mediators of inflammation are responsible for the attraction of leukocytes after the inoculation of meconium. Some of these mediators include tumor necrosis factor- α (TNF- α), interleukin 1, (IL-1,), and IL-8, which have been identified as important pro-inflammatory cytokines in meconium aspiration syndrome (Kojima et al., 1994; Shabarek et al., 1997; de Beaufort et al., 1998).

Intracapillary sequestration of neutrophils and platelet aggregation in neonates inoculated with meconium was closely associated with interstitial edema. Similar changes have also been described in piglets after six hours of instilled meconium and authors suggested that high concentrations of human-pancreatictype phospholipase A2 introduced with meconium may have an important role in the pathogenesis of structural changes (Soukka et al., 1997). Interestingly, local aggregation of platelets with increased production of thromboxane A2 has been proposed as the possible cause of pulmonary hypertension in infants with MAS and may play a role in the hypertensive response characteristic in this syndrome (Levin et al., 1983).

Edema, vascular sequestration of leukocytes and aggregation of platelets in the lungs of neonatal rats inoculated with meconium in the present study were suggestive of adult respiratory distress syndrome (ARDS). Since the pathogenesis of MAS shares many similarities with ARDS, it is possible that degranulation of polymorphonuclear leukocytes in alveolar capillaries takes place following aspiration of meconium (Soukka et al., 1997). The chronology of ultrastructural changes seen in the lungs of neonatal rats inoculated with meconium parallels that of ARDS (Bachofen and Weibel, 1982).

The lack of morphologic changes in type I pneumocytes may have been related to the dose of meconium. It is possible that the dose of meconium in our study was insufficient to induce obvious ultrastructural changes (Holopainen et al., 1999). It is also possible that changes in alveolar permeability were primarily centered in the endothelial side of the air-blood

barrier (Bachofen and Weibel, 1982; Fulkerson et al., 1996). Nonetheless, some studies have shown that edema, intraalveolar hemorrhage and formation of exudates can occur in ARDS without significant ultrastructural changes in the endothelium (Bachofen and Weibel, 1982). Based on this information, it is likely that the movement of plasma fluid and blood cells in the rat inoculated with meconium was due to an enlargement of the endothelial cell junctions rather than necrosis of the endothelium.

Some researchers have reported the formation of intraalveolar hyaline membranes in pig neonates inoculated with meconium (Soukka et al., 1997). Although there was an interstitial edema and intraalveolar fibrin and the leakage of protein to alveolar spaces after the inoculation of meconium has been described previously (Martinez-Burnes et al., 2001a), formation of hyaline membranes was not a feature in the neonatal rats inoculated with meconium. This discrepancy between rats and pigs could be due to species difference, artifacts of fixation or dose of meconium.

There is firm evidence in previous reports that, following acute lung injury, necrosis of type I pneumocytes is rapidly followed by hyperplasia of type II pneumocyte (Bachofen and Weibel, 1982; Sugahara et al., 1996). It is then generally assumed that proliferation of type II cells is indicative of a previous rapid loss of type I pneumocytes. However, experimental studies in rat lungs dosed with bacterial lipopolysaccharide (LPS) have demonstrated that hyperplasia of type II cells can be observed even when there is minimal ultrastructural change of type I cells (Sugahara et al., 1996). These observations may explain why, in this study, rat neonates inoculated with meconium developed hyperplasia of type II pneumocytes at PID 7 and yet type I cells did not show ultrastructural evidence of necrosis. Other possible explanation could be that subtle ultrastructural changes leading to type I cell necrosis at the early stages of lung injury were overlooked, while type II hyperplasia was easily detected in the lungs of the neonatal rats. Interestingly, necrosis of alveolar epithelium has been described after only 24 hours of experimental meconium inoculation in rabbits by light microscopy (Tyler et al., 1978). A recent study demonstrated early cellular apoptosis but not necrosis of alveolar epithelium after 6 hours of instilled meconium in piglets (Holopainen et al., 1999). The presence of edema without necrosis suggests that the dose of meconium in this study may have been insufficient to cause extensive damage to respiratory mucosa. These mild changes in neonatal rats most likely mimic the lesions found in self-limiting MAS but not in those cases with severe meconium aspiration. Therefore, the role of pulmonary apoptosis and necrosis in neonatal lungs after aspiration of meconium remain to be elucidated.

The thickening of the alveolar septum in rats inoculated with meconium that was quantified by morphometry in LM was also detected by TEM.

Distension of the interstitial space in alveolar walls at PID 1 and 3 was primarily due to edema, while at PID 7, there were also severe proliferative changes contributing to the alveolar thickening. Thickening of the alveolar wall should be added to the list of pulmonary lesions associated with acute MAS such as atelectasis, hyperinflation and inflammation. Although morphometric values for alveolar absolute volume increased in both treatment groups, rats inoculated with meconium exhibited higher absolute values in alveolar air. This finding indicates that in addition to the normal lung growth, meconium induces lung hyperinflation. This study also supports the view that changes in some volume fractions in the neonatal rat are primarily due to the normal growth and maturation of the lung and need to be carefully considered in experimental lung research.

An interesting ultrastructural finding in the rat neonates inoculated with meconium was the presence of numerous lamellar bodies in neutrophils and macrophages. Similar changes have been described in rats inoculated with other pneumotoxicants (Lopez et al., 1987) where phagocytosed lamellar bodies (myelinosomes) originated from necrotic and desquamated type II pneumocytes and released secretory granules, composed mainly of phospholipids (Ghadially, 1988). It has been recently suggested that meconium has a direct effect on the type II pneumocytes of adult rats causing an increase in surfactant secretion (Higgins et al., 1996). The increase in surfactant secretion could explain why neutrophils and macrophages of the rat neonates inoculated with meconium were filled with lamellar bodies. However, a future morphometric study focusing on intraalveolar surfactant quantity and function would be necessary to corroborate this issue.

The interstitial proliferative response that occurred in the lungs of neonatal rats at PID 7 following the inoculation of meconium was considered consistent with repair. Many studies have shown that proliferation of mesenchymal cells in the pulmonary interstitium is part of the repair process of the lung after acute injury regardless of the cause of injury (Bachofen and Weibel, 1982; Fukuda et al., 1987). Some studies have shown that intraalveolar fibrosis is a pattern of pulmonary structural remodeling in patients with diffuse alveolar damage in humans. In the early proliferative phase, activated myofibroblasts proliferated and migrated into alveolar spaces through gaps in the epithelial basement membrane and produced intraalveolar fibrosis (Fukuda et al., 1987). The same type of epithelial regeneration with hyperplasia of pneumocytes type II and cuboidal cells, and proliferation and migration of fibroblasts and myofibroblasts in alveoli was observed in human babies with respiratory distress syndrome (Takemura and Akamatsu, 1987).

The tendency for ultrastructural changes to be located at the proximal alveolar region and neighboring alveoli in the neonatal rats suggests that these areas are likely the main sites of injury and inflammatory response after the inoculation of meconium. This was not an unexpected finding since the bronchoalveolar junctions are the most common site targeted by many types of pneumotoxicants (Barry and Crapo, 1985; Pinkerton and Crapo 1985). Interestingly, this site has the highest population of type II pneumocytes and alveolar macrophages (Crapo et al., 1984).

In conclusion, results of this study show that the intratracheal inoculation of homologous meconium in seven-day-old rats induces an acute inflammation formed by recruitment of neutrophils and macrophages to the alveolar space, and sequestration of neutrophils and aggregation of platelets in capillaries. These changes parallel the leakage in the air-blood barrier with interstitial edema and escape of red cells and fibrin strands into the alveolar interstitium and alveolar space. Interstitial edema and sequestration of neutrophils are largely responsible for the early significant increase in thickness of the interstitial space at PID 1 and 3, followed by proliferation of mesenchymal cells and type II pneumocytes at PID 7. Meconium also induced deciliation of bronchiolar cells at PID 1 and 3. As part of the meconium effect and repair mechanism, there is hypertrophy and hyperplasia of type II pneumocytes, and proliferation of mesenchymal cells in the interstitium. This fibroblastic response also spills through gaps from interstitium into the alveoli causing focal intraalveolar fibrosis.

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