Meconium aspiration produces airway hyperresponsiveness and eosinophilic inflammation in a murine model

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Meconium aspiration syndrome (MAS) is an important cause of morbidity and mortality in the perinatal period and affects 2–6% of neonates when the pregnancy is complicated by meconium-stained fluid (14, 32). Prominent among the clinical features of MAS are airway obstruction, pneumonitis, pulmonary hypertension, and hypoxemia, leading to invasive therapies including extracorporeal membrane oxygenation (14, 23, 31, 32). Although the clinical outcome of MAS is variable, the mortality remains significant (4–40%) despite aggressive medical therapy (14, 23, 31, 32).

Chemical pneumonitis is a known consequence of MAS, and several histological changes have been described in clinical studies and in animal models of meconium aspiration (10, 26, 31, 32). However, limited information is available on the cellular mechanisms by which meconium produces the cascade of inflammatory reactions described in MAS. Recent work has suggested that meconium is capable of interacting with alveolar macrophages, epithelial cells, and neutrophils, thus leading to the release of important mediators of inflammation and lung injury (10, 15, 16, 18). In addition, meconium aspiration has been shown to increase the pulmonary expression of cyclooxygenase-2 in rat lungs, suggesting that metabolic products of arachidonic acid could be involved in the pathogenesis of MAS (19). More recent work has shown that meconium induces the release of inflammatory cytokines and produces significant cell death in rabbit lungs (33).

An association between meconium aspiration and the development of airway dysfunction has been suggested by clinical studies in recent years (14, 21, 25, 32). In this respect, MAS has been shown to produce long-term pulmonary sequelae including airway obstruction and hyperresponsiveness, hyperinflation, and exercise-induced bronchospasm (25). These observations were supported by a higher prevalence of asthma and airway hyperresponsiveness among survivors of MAS compared with control children (21). Although these studies suggest a potential link between neonatal MAS and altered airway function, the mechanisms by which meconium aspiration induces airway dysfunction have not been evaluated.

To this end, we have developed a murine model of MAS in an attempt to characterize the cellular mechanisms involved in the pathogenesis of MAS. In this study, we performed measurements of respiratory system mechanics in vivo to further investigate the mechanisms by which aspiration may lead to airway dysfunction. Furthermore, we evaluated the presence of inflammatory cells in the bronchoalveolar lavage fluid...
Airway hyperresponsiveness (AHR). Our findings that meconium induces AHR, eosinophilic inflammation, goblet cell hyperplasia, and cytokine imbalance thus provide new information on the pathogenesis of MAS.

METHODS

Experimental animals. Female BALB/c mice (8 wk of age), obtained from Harlan Sprague Dawley (Indianapolis, IN), were used in all studies. All the procedures employed in this study were approved by the Animal Care and Use Committee of the University of Texas Health Science Center and conformed to National Institutes of Health guidelines.

Meconium preparation and aspiration. First-pass meconium was collected from healthy term newborns under sterile conditions and was cultured to exclude bacterial contamination as previously described (17). This meconium slurry was stored at −80°C until used. Twenty percent meconium was used as the aspiration medium. After sedation with ketamine and xylazine, 50 μl of meconium were placed into the nose of a quietly breathing mouse while its mouth was closed. This technique, a modification of methods previously described by our laboratory (7–9), allows for aspiration into the lungs. Control animals received normal saline in a similar manner.

After preliminary time- and dose-response studies, in vivo experiments of lung mechanics, BALF cell analysis, and histopathological studies were performed on days 2 and 7 after the aspiration event as described below. The first time point was monitored to assess the acute functional and inflammatory responses after aspiration of meconium. The latter time point was studied to further characterize the cellular events after the initial insult to the airways.

Measurement of airway responsiveness. Airway responsiveness (AR) was measured by barometric plethysmography in unrestrained animals using whole body plethysmography (Buxco, Troy, NY) (4, 13). AR was expressed as an enhanced pause (Penh), a calculated value that reflects the pulmonary resistance measured by a conventional, two-chamber plethysmograph in ventilated animals. This value was derived from changes in the box pressure induced by bronchoconstriction during peak expiratory pressure (PEP) and peak inspiratory pressure (PIP). Penh was calculated according to the formula Penh = Pase × PEP/PIP, where Pase reflects the timing of expiration (13). The methacholine (MCh) dose-response curves were performed using a Buxco aerosol delivery system (Buxco) that includes a "push" bias flow box/aerosol controller, an ultrasonic nebulizer, and an aerosol distribution chamber. The settings for dilution flows and aerosol time period were selected according to the manufacturer’s recommendations. All the signals were filtered by a MAX II unit preamplifier, and the data obtained were stored using a Biosystem XA data acquisition and analysis software.

Mice were exposed to nebulized physiological saline for 3 min and then to increasing concentrations of nebulized MCh (3–50 mg/ml) with an ultrasonic nebulizer. Recordings were taken for 3 min after each nebulization. The Penh values measured during this period were averaged and expressed as a percentage of baseline Penh values after exposure to saline.

Results

Studies of respiratory mechanics. In control animals (n = 12), the baseline Penh was 0.79 ± 0.07, and the maximal Penh response to 50 mg/ml MCh was increased at 3.04 ± 0.6 (Fig. 1). On day 2, the baseline Penh was markedly enhanced after aspiration of meconium at 3.05 ± 0.7 and increased to 3.64 ± 0.5 when exposed to 50 mg/ml MCh (n = 8). The latter value was not different compared with control animals. On day 7, baseline Penh returned to control values: 1.05 ± 0.2 (n = 8). However, the airway responses to MCh were significantly increased by meconium aspiration as demonstrated by a leftward shift of the dose-response curve (Fig. 2) and increased maximal %Penh value: 758.2 ± 160.0%.

Analysis of BALF. The total number of cells recovered by BAL was markedly increased at 2 days after aspiration of meconium compared with control mice:
276.4 ± 10.6 × 10⁴/ml and 17.0 ± 3.6 × 10⁴/ml, respectively (P = 0.001) (Fig. 3). On day 7, the total number of BAL cells was decreased but remained greater than control values: 74.2 ± 3.7 × 10⁴/ml.

Cytokine measurements in the BAL. The levels of interferon (IFN)-γ, interleukin (IL)-4, IL-5, and IL-13 were measured in the BALF. On day 2, the concentrations of the cytokine measured were not different between meconium and control animals: 102.3 ± 21.9, 86.6 ± 17.4, 151.1 ± 29.9, and 144.3 ± 43.2% for IFN-γ, IL-4, IL-5, and IL-13, respectively (Fig. 5). Similarly, meconium did not affect the production of IFN-γ and IL-4 on day 7: 88.3 ± 11.5 and 93.6 ± 8.9%, respectively. By contrast, the concentrations of IL-5 and IL-13 were increased by aspiration of meconium at day 7: 198.9 ± 31.1 and 817.1 ± 259.3%, respectively.

Aspiration of meconium produced a significant increase in BALF lymphocytes on day 2 that persisted through day 7: 5.5 ± 0.9 × 10⁴/ml and 11.0 ± 0.9 × 10⁴/ml, respectively. BAL eosinophils were markedly increased on days 2 and 7 compared with control animals: 22.8 ± 2.3 × 10⁴/ml, 22.9 ± 1.2 × 10⁴/ml and 0.2 ± 0.2 × 10⁴/ml, respectively.

Fig. 2. Meconium aspiration increases airway responsiveness to MCh. Mice received a single airway exposure to meconium or normal saline. Airway responses to increasing concentrations of MCh were measured on day 7 and are expressed as %Penh increase from baseline (mean ± SE). After aspiration of meconium, Penh values were markedly increased compared with control animals as demonstrated by a leftward shift of the dose-response curve to MCh. *P < 0.05.

Fig. 3. Effect of meconium aspiration on bronchoalveolar lavage (BAL) fluid (BALF) cellularity. Animals received 50 μl of meconium or normal saline. BAL studies were performed at 2 (2d) and 7 days (7d) after aspiration. Meconium produced an increase in total BAL cells at 2 days that persisted through 7 days. *P < 0.05.

Fig. 4. Effect of meconium aspiration on BALF cells in mice. After aspiration of meconium or normal saline, BAL studies were performed on days 2 and 7. Aspiration of meconium produced an increase in BALF macrophages, lymphocytes, and eosinophils on day 2 that persisted through day 7. The number of neutrophils increased on day 2 and returned to control values on day 7. Open bar, control; solid bars, meconium–2 days; hatched bars, meconium–7 days. *P < 0.05.

Fig. 5. Effect of meconium aspiration on cytokine concentrations in the BAL. Cytokine levels were measured in duplicate by ELISA on days 2 and 7 after aspiration of meconium. Results were obtained in terms of pg/ml and expressed as %control (mean ± SE). Aspiration of meconium did not affect cytokine concentrations at day 2. On day 7, the levels of IL-5 (n = 12) and IL-13 (n = 7) were increased after aspiration of meconium compared with control values. Closed bars, meconium–2 days; hatched bars, meconium–7 days. *P < 0.05.
BAL cytokines are not altered by MCh challenge test (not shown).

**Histological studies of lung tissue.** Aspiration of meconium produced significant inflammatory changes within lung tissue as demonstrated by a diffuse infiltration of mononuclear cells in the alveolar and interstitial spaces on **day 2** (Fig. 6). On **day 7**, persistence of the inflammatory changes was associated with a thickened airway epithelium and a distinct positive PAS staining of goblet cells demonstrating goblet cell hyperplasia (Fig. 7).

**DISCUSSION**

The clinical features of MAS are characterized by profound functional alterations within the lung associated with an intense inflammatory reaction (31, 32). As a result, marked airway obstruction, hypoxemia, and pulmonary hypertension ensue, leading to variable degrees of pulmonary insufficiency (31, 32). Although the acute responses to meconium aspiration within the lung are widely recognized, limited information is available on the more chronic sequelae of MAS as related to morphological and functional changes of airway function. Furthermore, there is a paucity of information on the cellular mechanisms involved in the pathogenesis of MAS.

Clinical observations have suggested an association between meconium aspiration and altered airway function (14, 21, 25, 32). In children, MAS is capable of producing clinical symptoms of airway obstruction associated with other important features of chronic obstructive lung diseases: airway hyperresponsiveness (AHR) and hyperinflation (14, 32). Early work by Macfarlane and Heaf (21) described a higher prevalence of recurrent cough and wheezing among survivors of MAS, suggesting aspiration of meconium in early life may lead to airway obstruction and heightened responsiveness. Further studies on the long-term consequences of MAS revealed a higher incidence of hyperinflation and airway obstruction and hyperreactivity, as well as exercise-induced bronchospasm that persists several years after neonatal exposure to meconium (25). In light of these observations, it is possible that aspiration of meconium may induce structural and functional alterations within the lung, thus leading to long-lasting effects on airway function.

In this study, we describe alterations of AR in a murine model of meconium aspiration in vivo. After a single exposure to meconium, we found increased baseline Penh followed by enhanced airway responses to MCh, suggesting that changes in the regulation of airway smooth muscle tone and responsiveness may occur. In addition, we found aspiration of meconium produces a significant recruitment of eosinophils within BALF and lung tissue. The latter finding is of particular importance due to the frequent association...
between eosinophilia and AHR in the mouse model (1, 2, 20, 22, 24). Together, our findings suggest aspiration of meconium produces functional and cellular changes that mimic asthma-like airway dysfunction in a murine model.

Although complex interactions between inflammatory and immune cells are involved in the asthma-like phenotype seen in the allergen-driven model, it is widely accepted that the development of altered airway function is dependent on CD4+ T lymphocytes and that eosinophils are the effectors of this response (1, 2, 20, 22, 24). In this respect, recent studies have further defined the cell signaling involved in the recruitment and activation of eosinophils within the lung and their association with AHR (1, 22). Several cytokines appear to play a critical role in the pathological and functional changes seen in mammalian models of asthma (1, 20, 22). IL-5, IL-3, IL-13, and granulocyte-macrophage colony-stimulating factor are involved in the recruitment and activation of eosinophils (1, 2, 20, 24, 27). IL-4 is involved in the isotype switching in B cells from IgG2a and -2b to produce IgG1 (in mice) and IgE (hallmark of atopy in human asthma) (11, 20). In addition, IL-4 is involved in activation of mast cells, thus leading to the release of several proinflammatory cytokines and chemotactic factors that could contribute to the altered airway function (11, 20).

In our murine model, meconium aspiration produces an environment favorable for the development of eosinophilic inflammation and AHR that is associated with increased levels of IL-5 and IL-13. The latter findings are especially compelling in light of the known properties of these molecules in the pathogenesis of airway dysfunction and inflammation. In this respect, recent evidence has increasingly recognized the role played by IL-5 and IL-13 in the development of the functional and morphological features of human and experimental asthma (12, 28–30). Furthermore, overexpression of Th2 cytokines such as IL-5 and IL-13 is critically involved in mucin gene activation and the development of goblet cell hyperplasia (5, 6). As a result, the thickened airway epithelium and goblet cell hyperplasia with enhanced mucus production may contribute to the increased AR seen in our murine model. Collectively, these findings provide new insights into the cell signaling involved in MAS-induced airway dysfunction and offer the opportunity to investigate potential therapeutic intervention in our murine model.

In summary, we have developed a murine model in an attempt to determine the effects of meconium aspiration on airway mechanics in vivo. In addition, we sought to characterize the inflammatory response produced by aspiration of meconium within the lung. Our results demonstrate that meconium aspiration produces AHR, eosinophilic inflammation, goblet cell hyperplasia, and a Th2-prone environment, thus providing the first evidence of meconium-induced experimental asthma in a mouse model. Of particular interest is the fact that AHR and inflammation are produced by a stimulus (meconium) that is believed to produce long-term sequelae on airway function in chil-

Fig. 7. Periodic acid-Schiff (PAS) staining of lung sections obtained from control animals (A) and mice exposed to meconium on days 2 (B) and 7 (C). A: representative section of a control animal. B: no evidence of PAS staining on day 2. C: the airway epithelium is thickened as a result of marked goblet cell hyperplasia in the airways with distinct positive staining on day 7.
dren (14, 21, 25, 32). Because meconium is a complex mixture of protein, fat, carbohydrates, cellular debris, and biliary products (3), the actual component(s) that induces altered airway function and inflammation is not apparent from our study. Additional work with fractionated components of meconium will be necessary to further define this aspect of MAS pathogenesis. Finally, airway exposure to meconium in younger animals will be needed to provide further support to our observations obtained in adult mice to model a neonatal disorder.

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