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A Rat Model for Arrest of Alveolarization Induced by Antenatal Endotoxin Administration

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ABSTRACT: A possible association between intrauterine inflammation and impairments of lung development has been suggested. The purpose of this study is to determine the influence of a potent proinflammatory agent, intra-amniotic lipopolysaccharide (LPS), on lung development. At 21 d gestation, an intra-amniotic injection of 1 μg LPS was administered to two subgroups of WKAH rats. One subgroup received only LPS and the other received LPS plus a fetal intraperitoneal dose of 0.25 μg granulocyte-colony stimulating factor (hrG-CSF) to produce peripheral blood neutrophilia. A third subgroup received hrG-CSF only, and a control group received maternal intraamniotic and fetal intraperitoneal normal saline. All pups were delivered by cesarean section at 22 d (term, 22.5 d) and maintained under identical conditions. Left upper lungs were obtained for morphometric analysis at 1, 3, 7, 14, 21, 45, and 60 d of age. Morphometric analysis indicated that changes in alveolar surface density (*Sv*), average alveolar radius (*r*), and numerical density of alveoli (*nv*) all showed that there were fewer and larger alveoli in rat lungs that had been exposed to LPS, but not to hrG-CSF alone or saline. LPS-exposed alveoli showed fewer secondary septa, suggesting an arrest of alveolarization. No destructive changes were observed in any alveoli. We concluded that these changes could be caused purely by intra-amniotic LPS. These abnormalities closely mimic those of new bronchopulmonary dysplasia. The LPS damage model may be applicable to further studies of the pathophysiology of new BPD. **(*Pediatr Res* 59: 396–400, 2006)**

As a result of numerous studies conducted of lung development, some lung diseases are being reassessed as abnormalities of morphogenesis (1,2). Intrauterine infection/inflammation is widely recognized as one of the most important factors related to an arrest of alveolarization, and recent studies strongly suggest that the inflammatory response to intrauterine infection is an important pathogenetic factor in postnatal lung aberration (3,4). LPS is one of the potent proinflammatory stimulating agents associated with intrauterine inflammatory processes. Intra-amniotic LPS administration inhibited postnatal lung development, resulting in fewer and larger alveoli in premature lambs and baboons (5), and, at the same time, it accelerated the maturation of surfactant systems (6–8). Intratracheal LPS instillation in newborn rats was also reported to result in fewer and larger alveoli (9,10).

In humans, five stages of lung morphogenesis, 1) embryonic phase, 2) pseudoglandular phase, 3) canalicular phase, 4) saccular phase, and 5) alveolar phase, have been identified (11–13). Secondary alveolar septa are formed during the alveolar phase, which occurs between 36 wk of gestation and 24 mo postnatal (12,13). These five stages of lung structural development are observed in most mammals, however, different degrees of lung maturity at the same gestational stage are observed in various species (14,15). The lung structures of a newborn lamb are in the alveolar phase, which corresponds to those of human lung at 36–38 wk of gestation, when alveolarization is well underway (14,15).

The lungs of the newborn rat exhibit structural immaturity compared with those of human term newborns. Full-term rats are born with their lungs in the saccular phase, which corresponds to human lung at 26–28 wk of gestation. Rat lung alveolarization takes place predominantly in the first 2 wk after birth, which corresponds to approximately 35 wk of gestation in human lung. However, the rat surfactant system is more advanced at birth than that of the human. In rat, gas exchange can be performed despite the relative structural immaturity of the lungs. This uncoupling of alveolarization and surfactant generation makes rat a good model in which to study alveolarization.

Our aim in this study is to determine the effects of LPS exposure on perinatal lung development. We hypothesized that an intraamniotic exposure to LPS would produce fewer and larger alveoli in newborn rat lungs. To test this hypothesis, we analyzed rat lung morphology from birth to adulthood. Because much of the morphologic lung development in rat takes place after birth, rat was chosen as the model system in which to investigate the longitudinal effects from birth to adult. In addition, based on our previous findings that fetal neutrophilia was strongly associated with lung abnormality in human premature infants (3), we used antenatal administration of hrG-CSF in this study.

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Abbreviations: BPD, bronchopulmonary dysplasia; **hrG-CSF,** human recombinant granulocyte-colony stimulating factor; **LPS,** lipopolysaccharide

METHODS

This animal experiment was performed at Hokkaido University, and the protocol was approved by the Animal Care and Use Committee of Hokkaido University Graduate School of Medicine.

Animals. Timed-pregnancy WKAH/htm (term, 22.5 d) rats, weighing 380–500 g, were used. Rats were maintained in the School of Medicine facility, and were allowed food and water *ad libitum*. Lighting was provided from 0600 to 1800 h.

Drug administration. Pregnant rats were anesthetized with intraperitoneal injection of Nembutal (pentobarbital 50 mg/mL; Abbott Laboratories, Abbott Park, IL) at the dose of 0.1 mL/100 g body weight. Rats were then into four groups. In group 1, a midline abdominal section was performed and 1 μg LPS (*Escherichia coli* 055:B55, solubilized in saline; Sigma Chemical Co., St. Louis, MO) was injected into each amniotic sac, together with an injection of 0.25 μg hrG-CSF (Glan, filgrastin recombinant; Kirin Brewery Co., Ltd., Japan) directly to each fetal abdominal cavity through peritoneum. In a preliminary study, the LD_{50} of LPS was determined to be 0.2–0.4 mg/kg fetuses. The dose of hrG-CSF used in this study was calculated to increase fetal peripheral blood neutrophils from 7,000–10,000 to 30,000–50,000/mm^3. In group 2, rats were treated by an intraamniotic injection of 1 μg LPS alone, and in group 3, rats were treated by a fetal intraperitoneal injection of 0.25 μg hrG-CSF alone. In group 4 (control), rats were treated by intraperitoneal and intraamniotic injection of normal saline. All injections were performed at 21 d gestation, and surgery was performed 24 h later at 22 d. After removing uteri containing 1–15 fetuses from the mother rat, newborn rats were delivered from the uterus. Placentas were separated by cutting the umbilical cord. Because the newborn rats were not vaginally delivered, simple resuscitation procedures, such as wiping amniotic fluid away and body stimulation to induce crying, were used. The pups received no supplemental oxygen or artificial ventilation. After being kept in an incubator for 1 h to recover their body temperature, pups were weighed and given to the foster rats. Foster rats, weighing 450–600 g, which had been raised in the same facility under aseptic condition, were randomly divided into four groups. Each foster mother was given only pups from a single group; pups from different groups were not mixed. Each foster mother was given five to eight pups. The number of pups given to a foster mother was controlled to equalize litter size, eliminate survival rate bias, and account for differences in feeding behavior.

Tissue fixation and processing. The method of tissue processing and lung fixation has been reported previously (16,17). At least five pups from each group were examined at 1, 3, 7, 14, 21, 45, and 60 d of age. After blood samples were collected for leukocyte counts, animals were killed by an intraperitoneal injection of Nembutal. Animals then received an intratracheal instillation of 10% buffered formalin at pressure of 20 cmH_2O for 20 min, and the lungs were removed. Lungs were further fixed in buffered formalin an additional 48 h. The left lung was processed for light-microscopic morphometry. The lung was dissected into a few slices along vertical axes that were arranged in the cassette casein in a parallel fashion, then cut into 4-μm-thick sections and stained with hematoxylin and eosin. Elastica van Gieson stain was applied to the selected sections for assessment of elastic tissue content. All placentas were collected, fixed in buffered formalin for 48 h, and processed for microscopic examination. Placenta was cut into 4-μm-thick sections, stained with hematoxylin and eosin, and examined for the presence of chorioamnionitis according to the criteria of Navarro and Blanc (18,19).

Light-microscopic morphometry. Morphometry is known to be a suitable method for analyzing lung structures (20). Lung specimens were analyzed at a magnification of $\times$400 using MICD (Microcomputer Imaging Device Research Inc., St. Catharine's, ON, Canada). Ten images from one lung were transferred to a PICT format and analyzed using Stereology Toolbox (Morphometrix, Davis, CA). The images were selected by a masked observer, and examined randomly. The PICT images were superimposed on the grid according to their vertical axis, and the numbers of points in alveolar space (*Vav*) and intersections between alveolar walls and cycloids (*Sv*) were counted. The total number of points and intersections in each left lung were $>$400.

Calculated variables. Using the two determined variables defined above (*Vav and Sv*), the following two variables were calculated: *nv*, numerical density of alveoli (number of alveoli in a unit of lung volume), and *r*, average alveolar radius. The formulas for calculations have been reported previously (16).

Statistical analysis. Body weight, number of leukocytes, *Sv, r,* and *n* of the four groups were compared by one-way ANOVA, and those on each sampling point by Scheffé test. The ratio of live birth to total number of pups and the survival rate of pups in the four groups were compared by χ^2 test and long-rank test, respectively. A $p < 0.05$ was considered to be statistically significant.

RESULTS

Survival and weight gain. The number of pregnant rats examined was 9, 7, 10, and 10 in groups 1, 2, 3, and 4 respectively. The number of pups including stillborns for each mother rat was 8.5 $\pm$ 3.3, 9.3 $\pm$ 3.0, 8.3 $\pm$ 2.7, and 6.9 $\pm$ 2.8 for groups 1, 2, 3, and 4, respectively. The total number of pups treated antenatally was 72, 57, 43, and 47 in groups 1, 2, 3, and 4, respectively. The ratio of live birth to the total number of pups was 0.86 $\pm$ 0.13 (7.2 $\pm$ 2.9/8.5 $\pm$ 3.3) for group 1, 0.89 $\pm$ 0.13 (8.3 $\pm$ 3.2/9.3 $\pm$ 3.0) for group 2, 0.96 $\pm$ 0.10 (8.0 $\pm$ 2.7/8.3 $\pm$ 2.7) for group 3, and 0.97 $\pm$ 0.05 (6.7 $\pm$ 2.7/6.9 $\pm$ 2.8) for group 4. Thus, the live birth rate was significantly lower in groups 1and 2 than in groups 3 and 4 ($p < 0.001$). In addition, the pups in groups 1 and 2 were more likely to die within 2 wk of delivery than in groups 3 and 4 (Fig. 1). We were unable to determine the cause of death in these newborns because of the foster mothers' cannibalistic behavior. Postnatal weight gain was retarded in the rats in groups 1 and 2 compared with those in groups 3 and 4 (Fig. 2). There was essentially no weight gain from 45 to 60 d of age in group 1.

Peripheral blood samples. The leukocyte count was 22,844 $\pm$ 947.5/mm^3, 10,700 $\pm$ 424.3/mm^3, 27,995 $\pm$ 15,384.2/mm^3, and 5,500 $\pm$ 1,060/mm^3, in groups 1($n = 2$), 2 ($n = 2$), 3 ($n = 5$), and 4 ($n = 5$), respectively. The groups treated with GCSF showed significant increases of leukocytes compared with the control group ($p = 0.012$). The groups treated with LPS showed significant increases in leukocytes compared with the control group ($p = 0.001$), but there were no significant differences between groups 1 and 2 ($p = 0.193$). Although the small number of data in group 1 limits comparison, groups 1 and 3 do not appear different ($p = 0.673$).

Placenta and umbilical cord. Chorioamnionitis (Blanc: 3) was present in all placentas (35/35) in group 1 and 89.5% (31/35) in group 2. Only one placenta (1/35) exhibited chorioamnionitis (Blanc: 2) in group 3. No placenta (0/35) exhibited chorioamnionitis in group 4.

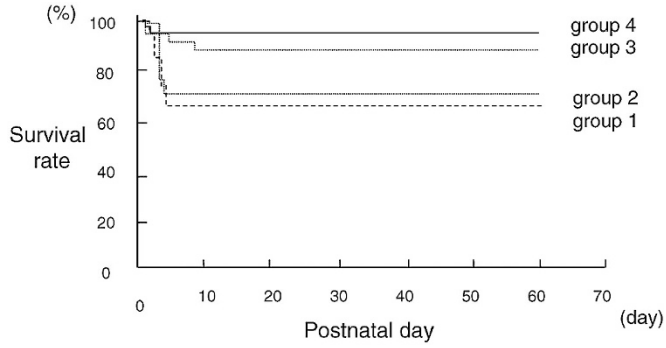

Figure 1. Survival rate. Pups in group 1 were treated by both an intraamniotic LPS injection and a fetal intraperitoneal hrG-CSF injection. Group 2 was treated by LPS alone, and group 3 was treated by hrG-CSF alone. Pups in group 4 were treated by normal saline and served as controls. The total number of pups treated antenatally was 72, 57, 43, and 47 in groups 1, 2, 3, and 4, respectively. Kaplan-Meier plot shows that the survival rate of pups exposed to LPS (groups 1 and 2) was significantly lower than that of pups without antenatal exposure to LPS (groups 3 and 4) ($p < 0.001$). The antenatal administration of hrG-CSF alone had no significant effect on the survival rate ($p = 0.325$).

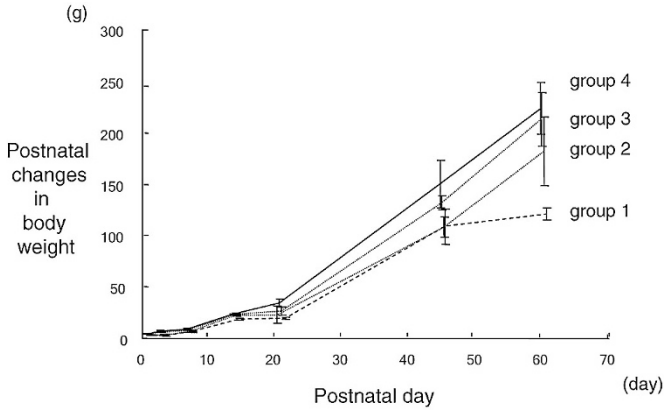

Figure 2. Postnatal changes in body weight. Vertical bars indicate the mean ± SD. Each group is represented by five rats. Body weights in groups 1 and 2 were significantly lower than in groups 3 and 4 at any postnatal sampling point. A plateau of weight gain was observed in group 1 between 45 and 60 d. A significant difference between groups 1 and 2 was seen only at 60 d ($p = 0.012$), and a difference between groups 3 and 4 was seen only at 3 d ($p < 0.001$).

Light-microscopy. Fewer and larger alveoli were detected the 1 d of age in groups 1 and 2 (Fig. 3). The alveoli were more abnormal at 2 wk, with fewer secondary septa. This appearance was observed at all ages up to 60 d of age. There was no evidence of alveolar destruction, cellular infiltration, intraalveolar edema, or fibrosis. Abnormal distribution of collagen and elastic fibers stained by Elastica van Gieson was not found in either of these groups.

Alveolar surface density. Five lung specimens from each group were subjected for morphometric analysis at each postnatal sampling point (Fig. 4). Ten images for each lung specimen were analyzed and their average value was considered as one data for one pup. As a result, 140 lung specimens with 1400 images were analyzed. The same samples were used for the analysis of average alveolar radius and numerical density of alveoli. Postnatal changes in the *Sv* values did not differ between group 1 and group 2 except at two sampling points, and those in group 3 and group 4 were essentially the same. However, the *Sv* values were significantly lower in group 1 and group 2 than in group 3 and group 4 at every sampling point.

Average alveolar radius. The *r* values did not differ between groups 1 and 2 or between groups 3 and 4 (Fig. 5). However, the *r* values in groups 1 and 2 (no statistical differences between them) were significantly larger than in groups 3 and 4 (no statistical differences between them) at every sampling point.

Numerical density of alveoli. The *nv* values in groups 1 and 2 remained small at every sampling point, whereas they increased with age in groups 3 and 4 (Fig. 6). Differences for *nv* values between group 1 and group 2, and those between group 3 and group 4, respectively, were not statistically significant.

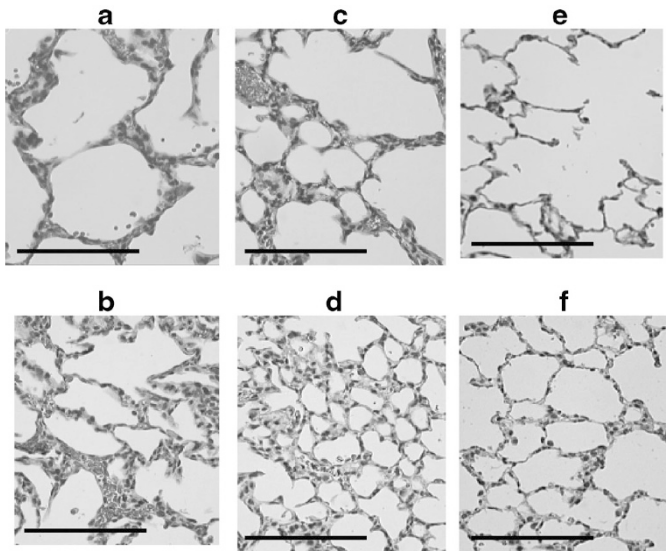

Figure 3. Fewer and larger alveoli induced by antenatal LPS. Scale bars indicate 0.1 mm. Representative photomicrographs of lung sections (hematoxylin and eosin stains, ×400 magnification) on 3 d (saccular stage) of group 1 (*a*) and group 4 (*b*), on 14 d (alveolar stage) of group 1 (*c*) and group 4 (*d*), on 60 d (adult lung) of group 1 (*e*) and group 4 (*f*). Histologic findings in group 4 are representative of normal lung developmental stages. Lung specimens from group 1 and group 2 displayed larger alveoli and fewer secondary septa than the lungs from group 3 and group 4. There was no evidence of alveolar destruction, cell infiltration of alveoli, intraalveolar edema, or fibrosis.

DISCUSSION

We examined the effects of antenatal intraamniotic endotoxin on rat lungs. LPS is a potent proinflammatory stimulus that has been used in animal models of intrauterine inflammation to mimic chorioamnionitis. Recently, several studies have reported on the relationship between antenatal LPS administration and postnatal lung structural changes in animal models (5–10). This finding of fewer and larger alveoli associated with LPS exposure is important. In our study, fewer and larger alveoli were detected on d 1, becoming more apparent at 2 wk and persisting up to the end of our observation at 60 d. We could not detect any sign of alveolar destruction. Instead, the alveoli exposed to LPS remained morphologically immature, with fewer secondary septa, which suggests LPS caused early structural developmental arrest. Based on these findings, we speculated that LPS exposure inhibited lung development,

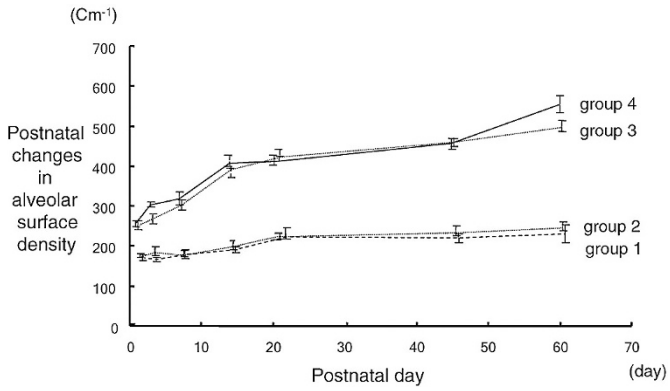

Figure 4. Postnatal changes in alveolar surface density. Vertical bars indicate the mean ± SD. Each group is represented by five rats. Lower *Sv* values indicate a reduced surface area for gas exchange. The *Sv* values in groups 1 and 2 were significantly lower than in groups 3 and 4 at any postnatal sampling point ($p < 0.001$). Changes in the *Sv* values in group 1 were similar to those in group 2. A significant difference between groups 1 and 2 was seen only at 1 d ($p = 0.003$) and 3 d ($p = 0.017$). *Sv* values in group 3 were similar to those in group 4 except at 3 d ($p = 0.001$) and 60 d ($p = 0.002$).

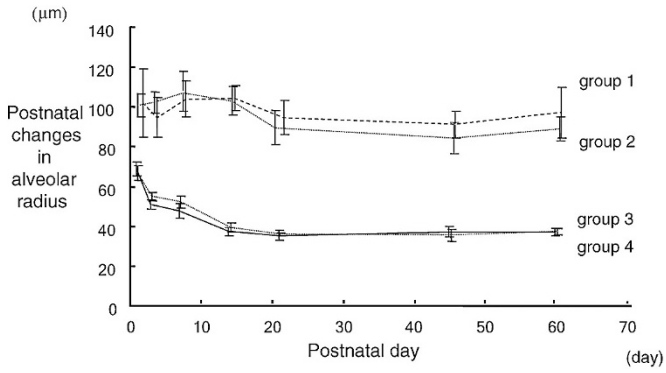

Figure 5. Postnatal changes in the alveolar radius. Vertical bars indicate the mean ± SD. Each group is represented by five rats. The *r* values were significantly larger in groups 1 and 2 than in groups 3 and 4 at any postnatal sampling point ($p < 0.05$). There were no significant differences in the *r* values between groups 1 and 2 ($p > 0.05$), or between groups 3 and 4 ($p > 0.05$) at any postnatal sampling point.

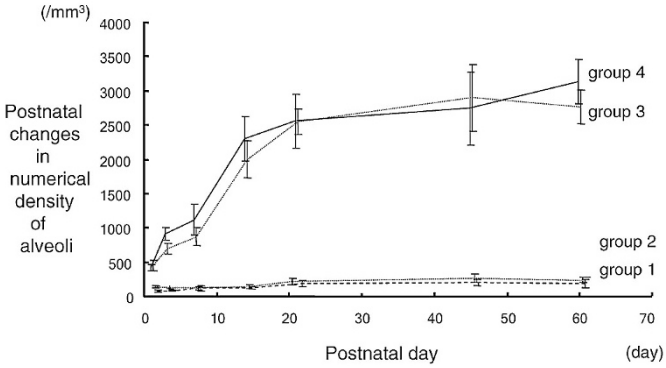

Figure 6. Postnatal changes in the numerical density of alveoli. Vertical bars indicate the mean ± SD. Each group is represented by five rats. Groups 1 and 2 displayed a striking difference with groups 3 and 4 for the *nv* values ($p < 0.05$); *nv* increased rapidly in groups 3 and 4, while remaining at lower levels in groups 1 and 2, especially during the first 14 d. There were no significant differences for the *nv* values between groups 1 and 2 ($p > 0.05$), or between groups 3 and 4 ($p > 0.05$) at any postnatal sampling point.

which was manifested by a decreased formation of new alveoli, an arrest of alveolarization. Recent studies suggest that inflammatory factors in amniotic fluid may play an important role in lung growth. For example, transforming growth factor-β1 was shown to inhibit branching morphogenesis in embryonic lung development (21). Prenatal inhibition of FGF signaling caused emphysema in the postnatal period (22). Increased expression of GATA-6 inhibited postnatal alveolarization (23). An imbalance of TIMP-MMP (tissue inhibitor of matrix metalloproteinases-matrix metalloproteinase) system using knockout technology in mouse produced dilation of alveoli (24,25). Thus, studies to identify the effect of LPS on rat lung development are warranted.

Although several studies have reported the relationship between LPS and altered lung structure, only a few studies have assessed lung growth over time (16,17). Our longitudinal study enables us to assess the effect of LPS exposure on each lung developmental phase. In addition, the characteristics of rat lung enabled us to eliminate postnatal treatment so that we could isolate the effects induced by antenatal LPS administration on lung development. Moss *et al.* (26) reported that structural changes similar to those in our study were present in lamb lungs at birth after intraamniotic LPS administration but were not detectable at 60 d of age. In their study, LPS was administered at 125 d gestation, when alveolarization is already underway in lamb lungs (14,15). We concluded that lungs in a prealveolarization stage of development are more vulnerable to damage (27) and will result in more postnatal abnormalities. Willet *et al.* (28) reported that intraamniotic LPS administration produced fewer and larger alveoli in lamb lungs, however, subsequent mechanical ventilation clearly affected lung morphology through a variety of mechanisms. Thus, it is difficult to conclude that the observed pathologic changes were caused solely by intervention of antenatal factors. Although these models may be useful animal models for arrested alveolarization, another long-term experimental model is needed in which the influence of LPS can be assessed without the confounding variables introduced by postnatal treatment and in which more developmental stages were assessable after birth.

There are several potential limitations to the present study. Franco *et al.* (9,10) reported that intratracheal instillation of LPS at 2 mg/kg to neonatal rats caused lung structural changes, whereas we used 0.2–0.4 mg/kg of LPS for intraamniotic administration. Our results have clearly shown that survival rates and body weights of newborn rats were affected in the LPS-treated groups. Furthermore, the induced lung structural changes did not improve with lung maturation. In a previous study, we reported that antenatal administration of dexamethasone in rat impaired postnatal lung growth, exhibited by fewer and larger alveoli, however, in contrast with the present findings, the impairment of lung growth improved in adulthood (16). It is not clear whether these differences are due to difference between the effects of applied agents or to difference in experimental animals (29). In addition, a plateau in weight gain in group 1 between 45 and 60 d occurred, without similar finding in group 2, which was otherwise similar. This may be due to some synergistic action of LPS and hrG-CSF in group 1. Other organs such as brain, heart, and kidney, were not examined in this study. Further experiments are required to assess for possible changes in these organs.

Our results also showed unexpectedly that intraperitoneal hrG-CSF administration was not related to the observed structural changes. Although a sufficient number of peripheral blood samples could not be obtained, the available data indicated that the most striking increase in leukocytes was found in group 3, which was treated by G-CFS only. In group 3, an increase in leukocytes was found in all blood samples we examined. According to these findings, we presumed that the leukocyte count in peripheral blood is not directly related to the lung structural changes.

A last issue to be discussed is the similarity between this experimental model and of the so-called "new" BPD. The mechanism of BPD is likely to be multifactorial. It has been traditionally considered that BPD is mediated by barotraumas to premature lungs (30,31). However, one particular type of BPD, called new BPD, worsens in the early postnatal period despite absence of mechanical ventilation, and it is frequently associated with chorioamnionitis (31,32). Its morphologic features were characterized by fewer and larger alveoli indicating

aberration of normal lung development, more specifically, an arrest of alveolarization (1,2). In spite of the recent progress in neonatal care, new BPD is remains a persistent clinical problem and may have some characteristic differences from the other forms of BPD in its pathogenesis. The effects of antenatal LPS on alveolar development may help understanding mechanisms leading to new BPD.

In conclusion, the major finding of this study is that proinflammatory stimulus induced by LPS plays an important role in abnormal postnatal lung development. Intra-amniotic LPS administration induced an arrest of alveolarization, resulting in fewer and larger alveoli in rat lungs, which mimics lung morphology of new BPD in human premature infants.

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