Comparison of postnatal lung growth and development between C3H/HeJ and C57BL/6J mice

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Soutiere, Shawn E., and Wayne Mitzner. Comparison of postnatal lung growth and development between C3H/HeJ and C57BL/6J mice. J Appl Physiol 100: 1577–1583, 2006. Previous work by our group has demonstrated substantial differences in lung volume and morphometric parameters between inbred mice. Specifically, adult C3H/HeJ (C3) have a 50% larger lung volume and 30% greater mean linear intercept than C57BL/6J (B6) mice. Although much of lung development occurs postnatally in rodents, it is uncertain at what age the differences between these strains become manifest. In this study, we performed quasi-static pressure-volume curves and morphometric analysis on neonatal mice. Lungs from anesthetized mice were degassed in vivo using absorption of 100% O2. Pressure-volume curves were then recorded in situ. The lungs were then fixed by instillation of Zenker’s solution at a constant transpulmonary pressure. The left lung from each animal was used for morphometric determination of mean air space chord length (Lma). We found that the lung volume of C3 mice was substantially greater than that of B6 mice at all ages. In contrast, there was no difference in Lma (62.7 μm in C3 and 58.5 μm in B6) of 3-day-old mice. With increasing age (8 days), there was a progressive decrease in the Lma of both strains, with the magnitude of the decrease in B6 Lma mice exceeding that of C3. C3 lung volume remained 50% larger. The combination of parenchymal architectural similarity with lung air volume differences and different rates of alveolar septation support the hypothesis that lung volume and alveolar dimensions are independently regulated.

MATERIALS AND METHODS

Animals. Breeders from two standard inbred strains (C3 and B6) were purchased from Jackson Laboratories (Bar Harbor, ME). Neonatal male and female progeny that were 1 (day of birth), 2, 3, and 8 days of age were used in this study. Progenitors were housed in an animal facility and provided with food and water ad libitum. The protocol was approved by the institutional animal care and use committee of the Johns Hopkins Medical Institutions.

Quasi-static P-V curves. Each animal was weighed (Table 1) and anesthetized with pentobarbital sodium intraperitoneally at a dose of 40 mg/kg BW. After anesthesia, a cannula was inserted into the trachea with the aid of a binocular microscope and secured with either 4–0 suture or, for the smallest animals (all 1 day and some 2 day), cyanoacrylate adhesive (Advanced Formula Instant Krazy Glue, Elmer’s Product’s, Columbus, OH). The cannula was then connected to a ventilator. The animal was ventilated at a tidal volume of 10 ml/kg with 100% O2 for 5 min before the cannula was closed with a stopcock for 15 min to degas the lungs. Quasi-static P-V curves were immediately performed in situ with a system detailed in previous studies (22, 26), with a few modifications to accommodate the very small volume. Briefly, the system consists of a water-filled syringe mounted on a dual-infusion withdrawal syringe pump (model 900–610, Harvard Apparatus, Dover, MA). A 1-ml air-filled vertical column was connected to the syringe and the tracheal cannula.

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Mass-specific lung volume was calculated by dividing \( V_{30} \) by the animal’s BW. Two animals died (one from each strain at 8 days of age) from the anesthesia before complete degassing of the lung. The P-V data from these animals were excluded from the analysis. However, the lungs from these animals were processed for morphometry.

Fixation. Following the quasi-static P-V curve in 1-, 3-, and 8-day-old animals, lung fixation was performed as previously described (27). Briefly, the tracheal cannula was connected to a 7-cm H\(_2\)O column of Zenker’s fixative (EM Science, Gibbstown, NJ) for 4 h. Zenker’s fixative, a mercuric chloride-acetic acid fixative, at 7 cm H\(_2\)O was selected because we have found that it provides good tissue preservation, lung inflation, and alveolar recruitment at relatively low distending pressures.

Furthermore, fixing at a higher pressure of 14 cm H\(_2\)O produced no significant change in either \( L_{\text{aw}} \) or lung volume determined by water displacement (27). The first attempts at fixing 1-day animals were unsuccessful (\( n = 2 \) C3 animals), presumably because the surface tension of the fixative at the end of the small tracheal cannulas was sufficient to prevent flow. Pinching and releasing the tubing that connected the column of fixative to the tracheal cannula broke this small meniscus, allowing fixative to flow into the lung, resulting in satisfactory fixation in all subsequent 1-day mice. At the end of the fixation period, the trachea was ligated, and lungs and heart were removed en bloc and placed in water for at least 48 h. The heart and right lung were then dissected away and discarded. The top 1 mm of

### Table 1. Body weight and lung volume parameters in neonatal mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age, days</th>
<th>( n )</th>
<th>( BW, ) g</th>
<th>( V_{30}, ) ml</th>
<th>( \frac{V_{30}}{BW}, ) ( \mu )l/g</th>
<th>( C_{\text{compliance}} ), ml/cm H(_2)O ( \cdot ) ( \text{ml}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>1, 3</td>
<td>2</td>
<td>1.423 ± 0.093</td>
<td>0.057 ± 0.007</td>
<td>39.81 ± 2.39</td>
<td>0.179 ± 0.033</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>1, 3</td>
<td>2</td>
<td>1.870 ± 0.084</td>
<td>0.068 ± 0.008</td>
<td>37.34 ± 5.84</td>
<td>0.130 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>2, 3</td>
<td>2</td>
<td>2.432 ± 0.053</td>
<td>0.075 ± 0.011</td>
<td>30.66 ± 3.87</td>
<td>0.238 ± 0.033</td>
</tr>
<tr>
<td></td>
<td>4, 6</td>
<td>2</td>
<td>4.690 ± 0.154</td>
<td>0.187 ± 0.020</td>
<td>40.65 ± 2.51</td>
<td>0.145 ± 0.022</td>
</tr>
<tr>
<td></td>
<td>8, 6</td>
<td>2</td>
<td>6.070 ± 0.122</td>
<td>0.259 ± 0.014</td>
<td>50.48 ± 2.42</td>
<td>0.150 ± 0.033</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>1, 3</td>
<td>2</td>
<td>1.293 ± 0.119</td>
<td>0.098 ± 0.011*</td>
<td>73.27 ± 5.17†</td>
<td>0.234 ± 0.051</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>1, 3</td>
<td>2</td>
<td>2.163 ± 0.103</td>
<td>0.144 ± 0.015†</td>
<td>64.99 ± 3.98†</td>
<td>0.124 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>4, 6</td>
<td>2</td>
<td>2.197 ± 0.136</td>
<td>0.157 ± 0.009‡</td>
<td>72.71 ± 1.52†</td>
<td>0.188 ± 0.045</td>
</tr>
<tr>
<td></td>
<td>8, 6</td>
<td>2</td>
<td>5.070 ± 0.112</td>
<td>0.259 ± 0.014‡</td>
<td>50.48 ± 2.42*</td>
<td>0.150 ± 0.033</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n \), no. of animals. Body weight (BW) and respiratory system mechanics are shown in two standard inbred mouse strains at 1, 2, 3, and 8 days postparturition. \( V_{30} \), lung volume at 30 cm H\(_2\)O. Lung volume and mass-specific lung volume were significantly greater in C3H/HeJ (C3) than in C57BL/6J (B6) mice at all ages studied: *\( P < 0.05 \), †\( P < 0.001 \). There were no differences between strains in either BW or respiratory system-specific compliance at each age.
the left lung was removed and discarded, and then three serial 2-mm sections were removed by cutting perpendicular to the main axis of the lung. Left lungs <7 mm in length were sectioned into a cranial, middle, and caudal region. Blocks were washed in Weigert’s iodine followed by sodium thiosulfate, infiltrated with glycol methacrylate, and then embedded in catalyzed glycol methacrylate (Polysciences, Warrington, PA). Two-micrometer-thick sections were cut with a microtome and stained with methylene blue.

Tissue sampling. The process of selecting lung sections was standardized, as described elsewhere (27). From each section, three to four nonoverlapping 1,130 × 904–926 µm fields were sampled, deliberately avoiding the large airways and blood vessels. Each region was photographed for digital analysis, and conventional morphometric methods were then used (16, 17, 27, 34). The mean chord length was determined by quantifying the length of air spaces between alveolar walls; this is analogous to the more conventional mean linear intercept. Using a Macintosh computer running NIH Image software, photographs were converted into binary images. A grid with parallel lines spaced at 35 µm was then overlaid onto the image, and the length of each chord, defined by the intercept with alveolar walls, was measured. Images and specific analytic and computational details are described elsewhere (13). Using at least twelve 1,130 × 904-µm fields from each left lung, ~9.2 mm² were subjected to analysis, yielding 2,500–3,700 chords per lung. To reduce optical and histological processing noise, and for comparison with previous studies (citations), chords <8 and >250 µm were excluded. Although these exclusions might alter the absolute magnitude of the Lma, relative comparisons between strains should be unaffected. Using mean values of Lma and V30 (from the peak of the second inflation), we estimated alveolar surface area with the Tomkeieff-Hennig method (SÅ1) (6, 35).

Statistical analysis. Statistical differences in Lma and V30 between strains were analyzed by t-tests, and differences within a strain were analyzed by one-way ANOVA with the Bonferroni correction for multiple pairwise comparisons using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). The regressions of V30 as a function of BW (fit through the origin) and V30 as a function of postnatal age (1–3 days) were obtained by sum of least squares regression. For all statistics, a 95% confidence level was considered statistically significant.
RESULTS

Lung volume. Table 1 shows the animal weights, $V_{30}$, and $V_{30}$ normalized to BW at the different ages studied for both strains. No distinction for the sex of the animals was made because it has been shown that, in one murine species, there are no differences in lung development between the sexes during the early postnatal period (5). The data show the expected increase in BW and increase in $V_{30}$ in each strain with age. In the B6 strain, lung volume increases at the same rate as BW, as evidenced by the fairly constant mass-specific lung volume of $\approx 36 \mu l/g (P > 0.05$ for all pairwise comparisons). In contrast, mass-specific lung volumes start out much higher in the C3 animals ($P < 0.001$ for C3 vs. B6, 1-, 2-, and 3-day pairwise comparisons), then decrease significantly by day 8 (C3 at day 3 vs. C3 at day 8, $P < 0.01$), although still remaining higher than for the B6 animals ($P = 0.022$). Figure 1 shows the $V_{30}$ from each strain at all ages examined, plotted as a function of BW. Figure 2 illustrates $V_{30}$ from each strain at all ages, plotted as a function of postnatal age. Figure 2 includes the lung volume data on 14 adult males from Tankersley et al. (32). The lung volume in the C3 mice at each age is statistically greater than that in the B6 mice. The data also show that the significantly greater $V_{30}$ values in C3 animals are evident as early as our first measurements on postnatal day 1 ($P = 0.0425$). Because there is a considerable variation in animal BW at each age, we plotted the $V_{30}$ as a function of weight. In the C3 strain, there is a clear ($R^2 = 0.83$) linear increase in $V_{30}$ and BW (0.070 ml/g BW). Although weak, this trend is also present in the B6 strain ($R^2 = 0.37$), with the slope of the regression over the first 3 days of postnatal life in the B6 strain (0.033 ml/g BW) being approximately one-half that in the C3 strain.

Lung morphometry. Representative low-power photomicrographs of lung sections from 3- and 8-day B6 and C3 mice are shown in Figs. 3 and 4, respectively. At 3 days, there are no obvious gross morphological differences in lung architecture between strains. However, at 8 days, there appears to be gross qualitative differences in alveolar size between strains. Figure 5 compares the $L_{ma}$ data from the strains at 3 and 8 days. At 3 days, the $L_{ma}$ from the C3 animals was 62.7 $\mu$m and not statistically distinguishable from the B6 $L_{ma}$ of 58.5 $\mu$m ($P = 0.1228$). However, because lung volume was so much larger, the $S_{at}$ in C3 mice (91.3 cm²) was approximately twice that of B6 mice (46.5 cm²). Table 2 contains the summary data for $L_{ma}$ and $S_{at}$. At 8 days, the 56.6-$\mu$m $L_{ma}$ from the C3 animals was statistically different from the 46.1-$\mu$m $L_{ma}$ in the B6 animals ($P = 0.0014$). In addition, the $L_{ma}$ from 3 to 8 days showed a substantial 21.2% reduction in the B6 strain ($P < 0.0001$), a decrease twice that seen in C3 mice over the same period. In fact, the 9.8% reduction in $L_{ma}$ from 3 to 8 days in C3 mice did not meet our established statistical confidence level ($P = 0.0717$). At 8 days, $S_{at}$ was similar in both strains (160.6 cm² in C3 and 146.4 cm² in B6) (Table 2).

Figure 6 plots the time course for changes in $L_{ma}$, incorporating the adult data from another previously published morphological study that used these same strains (27). Figure 6 shows that, at 8 days, the $L_{ma}$ for B6 animals decreased 53% of the total decrement in alveolar size observed from 3 to 49 days; in the C3 strain, however, it had decreased only 35% of the total change over the same time period.

DISCUSSION

There have been many studies on postnatal morphometry of the murine lung (1, 5, 18) and lung mechanics (23, 29–31). However, few of these have been in the mouse, and none has attempted to correlate structure with function in the mouse. The primary objective of this study was to identify at what point the differential lung mechanics and morphometric phenotypes found in adult C3 and B6 mice first appear. The results of our study show that the differences in lung volume are already present at postnatal day 1. We suspect that this difference exists prenatally, perhaps reflecting an earlier initiation of lung growth or a more rapid growth from the same starting time (or both). The differences that appear in lung morphology, however, were not present until postnatal day 3. These differences continued to postnatal day 8, which is considered to be the rapid alveolar proliferation period of lung development in mice (1).

Table 2. Morphometric parameters in neonates from two common inbred strains of mice at 3 and 8 days of age

<table>
<thead>
<tr>
<th>Age, days</th>
<th>C57BL/6J</th>
<th>C3H/HeJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Means ± SE</td>
<td>n</td>
</tr>
<tr>
<td>Mean air space chord length, $\mu$m</td>
<td>3</td>
<td>62.4 ± 2.01</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>58.5 ± 1.22</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>46.1 ± 1.33</td>
</tr>
<tr>
<td>Total alveolar surface area, cm²</td>
<td>1</td>
<td>38.3 ± 10.04</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>46.5 ± 6.16</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>146.4 ± 10.25</td>
</tr>
</tbody>
</table>

*n. No. of animals. Mean air space chord length was significantly larger in C3 strain (*$P = 0.0014$) than in the B6 strain at day 8 only. As B6 mice matured, they exhibited a significant decrease in mean air space chord length from 3 to 8 days (*$P < 0.0001$). C3 mean air space chord length did not decrease ($P = 0.072$). Tomkeieff-Hennig estimates of alveolar surface area were considerably greater in C3 compared with B6 at day 3 but not at day 8 postnatal age.
It is worth noting that this seminal paper by Amy et al. (1), characterizing postnatal mouse lung development, was done in Swiss-Webster albino mice, an outbred strain. This work was done at a time many years before the explosion of availability of genetically varied mouse strains. Given the fact that there are so many functional and structural differences in lungs of mouse strains, we suspect that the time course of this alveolarization phase may also turn out to be quite strain dependent.

Selection of experimental time points. Experimental time points were selected based on the postnatal growth of the mouse lung (1) and the rat lung (4, 5). From postnatal days 1–3, the mouse lung contains no true alveoli, consisting primarily of sacculae. These are characterized as large smooth-walled structures, frequently with a double capillary system. At postnatal day 4, the lung appears very heterogeneous, and shallow alveoli are recognizable. Most of the lung still consists of primary sacculae, which are being subdivided by secondary crests. Postnatal days 4–14 are characterized by rapid alveolarization, generated by the subdivision of primary sacculae into alveoli, reducing the distance between alveolar walls. In rats, ~70% of the decrement in mean alveolar diameter had occurred by day 7 (5). With increasing age (to ~6 wk), there are increasing numbers of alveoli reported to be formed by growth processes different from that of septation (14, 18, 19).

Thus our morphometric analysis at day 3 postnatal would be representative of the lung just before the anticipated rapid alveolarization, and day 8 should represent immediately after the peak of rapid septation. Beyond day 8, alveolar septation clearly continues, as demonstrated in Fig. 6, but other putative alveogenic mechanisms may become dominant.

Lung morphometry. We used $L_{ma}$ a conventional morphometric method (7, 16, 17, 34), to determine air space chord lengths. It is also the same technique used previously to examine variation in lung morphometry in these strains (26, 27). At 3 days, during the saccular stage of lung development (1), we found no difference in lung morphometry between the C3 and B6 strains. This suggests that the alveolar septation process and the molecular events underlying them may be similar up to this age. From day 3 to day 8, however, there was a significant 21.2% $L_{ma}$ decrease in the B6 strain. The much smaller 9.8% $L_{ma}$ reduction in the C3 strain did not reach standard confidence levels ($P = 0.072$). The result is that, at 8 days, there are substantial differences in $L_{ma}$ between strains.

Although air space size was similar in these strains, $S_{A}$ in C3 was twice that in B6 at 3 days, simply resulting from the additional lung volume. At 8 days, $S_{A}$ was similar between strains, because of the increased rate of septation in the B6 strain. The growth and alveolarization in the period between 3 and 8 days clearly accounts for some of the structural differences observed later in adulthood. This finding agrees with observations in other species. For example, by administering dexamethasone to rats during the septal subdivision of saccule stage of lung development (4) (postnatal days 4–13), Massaro et al. (18) provided evidence for a critical period during which the gas-exchange sacculae present at birth must be subdivided. If this fails to occur, the lung maintains an emphysematous appearance. In addition, they observed that $S_{A}$ could increase without a change in alveolar size, similar to what we observed in the C3 strain. They speculated that other unknown mechanisms of forming alveoli must be present, and that these unidentified means must be differentially regulated. Other investigators have emphasized that the complexity and rapidity make this rapid developmental stage inherently susceptible to disturbance (20).

Lung volume. The effects of lung maturation on lung mechanics have also been studied extensively in other species (23, 25, 28–31), and, although at least one study examined respiratory system mechanics in 1- to 2-day-old mice (10), we know of no studies that followed mouse mechanics throughout the perinatal period, nor any that have shown significant differences in respiratory system mechanics among strains within a species at early postnatal ages. We found, in this study, that differences in lung volume between C3 and B6 strains were present as early as at 1 day, an observation suggesting its presence prenatally. However, we know of no literature on prenatal lung mechanics in mice.

Although the lung volume differences at 1 day continued through subsequent days, the margin of difference between the strains was reduced by 8 days. This likely reflects a combination of a rapid burst in lung growth in the B6 and a slowing of the growth rate in C3 animals. This interpretation is supported by Table 1, which shows lung volume data normalized to BW. There is a direct proportionality between lung volume and BW, as reflected by the constant value of $36 \mu l/g$ in B6 mice. In the C3 strain, this specific lung volume is substantially higher ($73 \mu l/g$ at day 1), but it falls...
off dramatically (50 μL/g) by 8 days. That the lung-to-BW ratio is higher in neonates than adults has been described by Fisher and Mortola (8, 9), who examined the ratio of lung dry weight to BW in a number of newborn mammals, ranging from the rat to piglet. Burri et al. (5), in their extensive study on the postnatal rat lung, also found a similar drop in specific lung volume after day 10 in the rat, equivalent to the period of alveolar proliferation in mice. Our present results show that the pattern is much more complex in mice. Although the pattern in rats seems similar to that observed in the C3 strain, the B6 mice do not show this same trend. Reasons for this genetic variability are not clear.

Our results are not the first to show that lung maturation and growth may progress separately. In fact, it has been shown that maturation can be accelerated at the expense of lung growth. Experimental glucocorticoid treatment that produced increases in both surface activity and alveolar stability was accompanied by a reduction in the number of cells and lung weight, as well as an inhibition of the outgrowth of new interalveolar septa (2, 3, 18, 33). It is also accepted that pulmonary epithelial differentiation and functional maturation are mostly under endocrine control, whereas pulmonary tissue growth is highly influenced by physical factors, which sustain expansion and determine luminal volume of both fetal and juvenile lungs (11, 12, 15, 36).

In summary, our results demonstrate that differences at birth are already present in the lung P-V phenotype between C3 and B6 strains, despite a similar morphometric structural appearance. The structural differences become manifest at postnatal age day 8, subsequent to the rapid alveolarization phase of mouse lung development. At present, we can only speculate about the potential pathophysiological consequences of both this altered rate of alveolar development and the resultant differences in lung and alveolar size. Nevertheless, it is not difficult to imagine not only that susceptibility to environmental insults on the development of emphysema in mouse models might be quite dependent on the different baseline lung structure in different strains but also that these differences might provide clues to the mechanisms involved. Such speculation, however, awaits further experimental investigation.

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REFERENCES


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