Alveoli increase in number but not size from birth to adulthood in rhesus monkeys

Dallas M. Hyde, Shelley A. Blozis, Mark V. Avdalovic, Lei F. Putney, Rachel Dettorre, Nathanial J. Quesenberry, Paramjit Singh, and Nancy K. Tyler

1California National Primate Research Center, 2Department of Psychology, and 3Department of Internal Medicine, Division of Pulmonary and Critical Care, University of California, Davis, Davis, California

Submitted 1 December 2006; accepted in final form 8 June 2007

Hyde DM, Blozis SA, Avdalovic MV, Putney LF, Dettorre R, Quesenberry NJ, Singh P, Tyler NK. Alveoli increase in number but not size from birth to adulthood in rhesus monkeys. Am J Physiol Lung Cell Mol Physiol 293: L570–L579, 2007. First published June 22, 2007; doi:10.1152/ajplung.00467.2006.—Postnatal developmental stages of lung parenchyma in rhesus monkeys is about one-third that of humans. Alveoli in humans are reported to be formed up to 8 yr of age. We used design-based stereological methods to estimate the number of alveoli \( N_{\text{alv}} \) in male and female rhesus monkeys over the first 7 yr of life. Twenty-six rhesus monkeys (13 males ranging in age from 4 to 1,920 days and lung volumes from 41.7 to 602 cm\(^3\); 13 females ranging in age from 22 to 2,675 days and lung volumes from 43.5 to 380 cm\(^3\)) were necropsied and lungs fixed, isotropically oriented, fractionated, sampled, embedded, and sectioned for alveolar counting. Parenchymal, alveolar, alveolar duct core air, and interalveolar septal tissue volumes increased rapidly during the first 2 yr with slowed growth from 2 to 7 yr. The rate of change was greater in males than females. \( N_{\text{alv}} \) also showed consistent growth throughout the study, with increases in \( N_{\text{alv}} \), best predicted by increases in lung volume. However, mean alveolar volume showed little relationship with age, lung volume, or body weight but was larger in females and showed a greater size distribution than in males. Alveoli increase in number but not volume throughout postnatal development in rhesus monkeys.

stereology; parenchyma; alveolar ducts; postnatal development

ALVEOLAR NUMBER AT BIRTH in human infants has been conservatively estimated to be at least half of the number of human adults (40, 49). In contrast, the number of conducting airways is completely developed by birth in humans, but airway size increases with lung growth (3, 32). Comparison of lung morphology in macaques and humans shows that there are similarities in segmental arrangement, structure and branching pattern of airways, arterial structure, and arterial changes after birth (32, 41). Although there are differences in the number of lobes, the number of generations of different types of airways, and the number and size of alveoli, the overall structure in the monkey is more similar to that in man than is the structure of the lung of other laboratory animals (41). The general developmental stages in the rhesus monkey are the following: embryo, 21–45 days gestation; fetus, 45–165 days gestation; newborn, 24 h postnatal; neonate, 0–1 mo; infant, 1–12 mo; juvenile, 12–24 mo; adolescent, 2–4 yr; and young adult, 4–8 yr (18). Stages of human lung development show alveolarization by formation of secondary interalveolar septa from ~36 wk of gestation to ~1–2 yr of age and microvascular maturaton by remodeling of interalveolar septa and restructuring of the capillary bed from birth to 2–3 yr of age (5, 40). The majority of alveoli are produced postnatally in humans to reach the adult number of ~450 million alveoli (35). One morphometric study of lungs of children from 26 days to ~5 yr of age identified two phases of postnatal lung development and growth: 1) from birth to ~18 mo of age characterized by alterations in volumetric proportions of parenchymal compartments, and 2) 18 mo to adulthood with proportional growth of all lung compartments (49). In the first phase, there is a disproportionate increase in components that are concerned with gas exchange (air space and capillary volumes) with a proportional decrease in interstitial tissue mass.

Since many human infants are exposed to a variety of inhaled infectious diseases and irritants such as air pollutants and environmental tobacco smoke that damage the lung, it is important that we understand the nature of these insults on the developmental process of the lung. This need necessitates the use of laboratory animals that represent good models of human lung development in which we can study normal lung development and its potential perturbation. Hence, we used precise, design-based stereological methods to sample whole rhesus monkey lungs to establish the normal parameters of alveolar growth. The time of postnatal developmental stages of lung parenchyma in the rhesus monkey is about a third that of the human. Thus we hypothesize that the most rapid phase of alveolar development in rhesus monkeys will be within the first year of life; however, we included animals from 4 to 2,675 days (7 yr) because somatic growth is complete in rhesus macaques by 6 yr of age (7, 42).

MATERIALS AND METHODS

Animals, Necropsy, and Tissue Collection

All monkeys selected for these studies were California National Primate Research Center colony-born rhesus macaques (Macaca mulatta). All monkeys were given a comprehensive physical examination, including a chest radiograph and complete blood count, and were determined to be healthy monkeys. Care and housing of animals complied with the provisions of the Institute of Laboratory Animal Resources and conformed to practices established by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animal studies conformed to applicable provisions of the Animal Welfare Act and other federal statutes and regulations relating to animals (Guide for the Care and Use of Laboratory Animals, 5th ed.). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Morphometric Estimates of Lung Structure

Lung volume. Lung volume (V_l) without the trachea and extrapulmonary bronchi was estimated by its buoyant weight in PBS (36). The right cranial lung lobes from three additional monkeys (7 mo, 2 yr 9 mo, and 5 yr 4 mo) were estimated by their buoyant weight in PBS and by the Cavalieri method, a volume estimate of the sectioned (5-mm slabs) fixed lobe using point counting to estimate slab areas that were multiplied by the slab thickness to estimate volume (34).

Estimation of Alveolar Number

The method for counting alveoli is based on the mathematical concept of the Euler characteristics of structures (26, 35). This provides an estimate of the number of holes in a two-dimensional (2-D) net (alveolar opening rings). To estimate the number of alveolar opening rings, the number of bridges (B) and islands (I) that appear on one section of a "disector" pair but not the other are counted (see Fig. 5; Ref. 26). Bridges (the more frequent event) are connections that appear between two separate profiles in one section but not the other. Islands (the rare event) are new isolated profile. The Euler characteristic \( \Delta \chi = (1-B)/2 \). The total number of alveoli (N_{alv}) in a lung \( (alv,lung) \) was calculated using the fractionator principle

\[
N_{alv,lung} = -\chi \frac{\sum \Delta \chi}{SF}
\]

where SF is the total sampling fraction, comprised of the sampling fractions of bars, blocks, heights, and areas (Table 2). The height sampling fraction was estimated as the ratio of the disector height (i.e., sections 1 and 3 for a disector height of 10 \( \mu m \)) to the block width on a microscope section (where the block width = block depth) (see Fig. 7; Ref. 26). The area sampling fraction was estimated as the ratio of the sampling field area to the field displacement to the next sampled field in \( x \times y \) at a magnification of \( \times 420 \) using the Computer-Assisted Stereological Toolbox software system (CAST; Visiopharm, Hørsholm, Denmark).

Estimation of Processing Shrinkage

An unbiased estimate of the global volume change is the following factor for shrunken global volume \( (F_{sgv}) \), \( F_{sgv} = (S_{A_{after}}/S_{A_{before}})^{2/3} \), where the block area before processing, \( S_{A_{before}} \), is estimated from the photographs, and section area after shrinkage, \( S_{A_{after}} \), is estimated by point counting; the summation is over all blocks for an animal (Table 2). All estimated absolute surfaces and volumes on histological sections were corrected for global shrinkage by dividing with the above factor under the explicit assumption that shrinkage is uniform across all tissue components (12).

Calculation of Number-Weighted Mean Alveolar Volume

Number-weighted mean alveolar volume \( (\bar{V}_{n,alv}) \) was calculated from previously described values as \( V_{n,alv} = V_{l} \times (V_{alv,lung}/N_{alv,lung}) \), where the units are converted to \( \mu m^3 \) (see Fig. 1; Ref. 20). In the number-weighted mean alveolar volume, each alveolus has an equal statistical "weight" regardless of its volume.

Calculation of Volume-Weighted Mean Alveolar Volume

Volume-weighted mean alveolar volume (\( \bar{V}_{v,alv} \)) was calculated using the point-sampled intercept method that estimates the volume of structures provided that the tissue is sampled under IUR conditions (see Fig. 1; Ref. 20). A CAST-Grid system was used to make the calculation as \( V_{v,alv} = (\pi/3) \times T_{IAS} \times I_{IAS} \), where \( I_{IAS} \) is the mean of the cubed point-sampled intercepts of alveoli, \( F_{sv} \) is the factor for shrunken global volume, and the units are in \( \mu m^3 \). In the volume-weighted mean alveolar volume, each alveolus is "weighted" by its

### Table 1. Morphological and morphometric parameters in rhesus monkeys

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age Range, days</th>
<th>BW Range, kg</th>
<th>V_l, cm³</th>
<th>V_v, cm³</th>
<th>V_par, cm³</th>
<th>V_np, cm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>22–2,675</td>
<td>0.47–3.9</td>
<td>43.5–380</td>
<td>183.6±123.24*</td>
<td>176.9±118.16*</td>
<td>6.7±5.61*</td>
</tr>
<tr>
<td>Male</td>
<td>4–1,920</td>
<td>0.41–12.66</td>
<td>41.7–602</td>
<td>197.7±176.62*</td>
<td>190.8±172.04*</td>
<td>7.0±5.59*</td>
</tr>
</tbody>
</table>

*Values are expressed as means ± 1 SD. BW, body weight; V_l, lung volume; V_par, parenchymal volume; V_np, nonparenchymal volume.
Fig. 1. An illustration of an isotropic uniform random (IUR)- and smooth fractionator-sampled monkey lung. 1) An agar-embedded lung is placed on a uniform clock and cut along a uniformly random direction in the agar, not in the tissue. Faces are labeled A–D to follow the orientation of the cut agar faces. 2) The agar block is then made to rest on the face just cut; the 90°-edge is now in the 0-0 direction of the nonuniformly divided (cosine-weighted) clock. 3) Using a new random number, the cut is again made in the agar. 4) The resulting block is re-embedded in the slicing machine with the last cut face (isotropic face) parallel to the cutting direction of the slicing machine. 5) and 6) Slabs are cut at a constant thickness of 5 mm and laid out on a table. 7) Each slab is then cut into bars of a width identical to the slab thickness at 5 mm. 8) The bars are sorted according to the area of the upper surface from largest to smallest. 9) Every second bar is pushed down out of the row, providing the smooth fractionator sampling sequence as illustrated by the arrows. Using a random start from 1 to 3 in the bottom row (2 in this case), every 3rd bar is sampled for a sampling fraction of 1/3. 10) The bars are cut into 15-mm-long bricks. 11) The bricks are sorted according to the area of the upper surface from largest to smallest. 12) Every second bar is pushed down out of the row, providing the smooth fractionator sampling sequence as illustrated by the arrows. Using a random start from 1 to 3 in the bottom row (2 in this case), every 3rd bar is sampled for a sampling fraction of 1/3. 13) Each of the sampled bricks is put into embedding molds for embedding, sectioning, and staining.
volume. Consequently, when there is any distribution of alveolar sizes in a lung, the volume-weighted mean alveolar volume will always be greater than the number-weighted mean alveolar volume. It becomes evident that the volume-weighted mean alveolar volume has size information embedded in it that can be estimated.

Calculation of the Coefficient of Variation of the Distribution of Number-Weighted Alveolar Volumes

The coefficient of variation of the distribution of number-weighted alveolar volumes (\(CV_{n\,alv}\)) was calculated from previously described values (20) as

\[
CV_{n\,alv} = \sqrt{\frac{\nu_{alv}}{\nu_{alv}} - 1.}
\]

Variance and Efficiency of Stereological Estimators

The observed biological variation among individuals is large for features of interest in biological tissues, and it is useful to know whether it is worth increasing the precision of the stereological sampling or including more animals in the study (22). It is possible to divide the observed variance (OCV) into its two components, the true biological variation (CV) and the average sampling variation of the counting noise (CE) (22). This is calculated by

\[
OCV^2(N) = CV^2(N) + CE^2(N).
\]

For sensible fractionator sampling designs (19), the dominating component of sampling variation is the counting noise. Because of the very sparse sampling, the counting noise was calculated by the formula \(CE^2\) noise \((N) = 1/(\Sigma \beta + \Sigma \lambda)\), where the summation is over one animal (Table 3). Note that both bridges (B) and islands (I) contribute to the counting noise of alveolar number estimation. Contributions to stereological variation for ratio estimators like volume, number, surface, and length densities have been derived (9). However, we used some simple guidelines that usually suffice for stereological sample size within an animal (primary sampling unit): 100–200 probe interactions (e.g., point hits, intersections, or feature counts), 50 fields, and 10 blocks (25).

Statistical Analysis

Statistical analyses were based on linear and nonlinear regression (SAS Institute, Cary, NC). A series of models were fitted to each outcome, considering age, \(V_L\), and body weight as predictors. Five functions were considered for each outcome: linear, quadratic, two-piece linear spline, a two-parameter exponential function, and a three-parameter exponential function. For example, when considering an outcome as a function of age, the models fitted to each outcome were

Linear: \(y = \beta_0 + \beta_1 \text{Age} + e\)

Quadratic: \(y = \beta_0 + \beta_1 \text{Age} + \beta_2 \text{Age}^2 + e\)

Two-piece linear spline: \(y = \beta_0 + \beta_1 \text{Age} + e\) if \(\text{Age} \leq \tau\), and \(y = \beta_0 + \beta_1 \tau + \beta_2 (\text{Age} - \tau) + e\) if \(\text{Age} > \tau\)

Two-parameter exponential: \(y = \exp(-\beta_1 \text{Age}) + e\)

Three-parameter exponential: \(y = \exp(-\beta_0 - \beta_1 \text{Age}) + e\)

Assessment of model fit was based on the coefficient of determination \(R^2\). The test for \(R^2\) is whether it is statistically different from 0. For the linear and two-parameter exponential functions, the minimum \(R^2\) value is 0.23. For the quadratic and three-parameter exponential functions, the minimum \(R^2\) value is 0.21. The best fitting function for each outcome is reported. Age, \(V_L\), and body weight were centered to the sample means of 743 days, 190.7 cm$^3$, and 2.92 kg, respectively. Sex was treated as a moderator of model parameters to allow for sex differences in all regression coefficients. Statistical significance was defined as \(P \leq 0.05\).

RESULTS

Ages in males ranged from 4 to 1,920 days, whereas females ranged from 22 to 2,675 days (Table 1). \(V_L\) in males ranged from 41.7 to 602 cm$^3$ compared with females that ranged from 43.5 to 380 cm$^3$ (Table 1). Body weight in males ranged from 0.41 to 12.66 kg compared with females that ranged from 0.47 to 3.9 kg (Table 1). \(V_{\text{par}}\) comprised 96% of the entire lung in both sexes without the trachea and extrapulmonary bronchi. \(V_{\text{par}}\) showed a three-parameter exponential function with age [adjusted (Adj) \(R^2 = 0.93\) and \(V_L\) (Adj \(R^2 = 0.99\)] with males showing a significantly increased nonlinear change rate compared with females (Table 4; Fig. 2). \(V_{\text{par}}\) showed a quadratic function with body weight (Adj \(R^2 = 0.92\) with males showing a significant increased volume at the mean value of 2.92 kg compared with females (Table 4). \(V_{\text{alv}}\) and \(V_{\text{ad}}\) showed quadratic functions with age (\(\text{alv},\) Adj \(R^2 = 0.85\); \(\text{ad},\) Adj \(R^2 = 0.91\)) and \(V_L\) (\(\text{alv},\) Adj \(R^2 = 0.97\); \(\text{ad},\) Adj \(R^2 = 0.89\)) with males showing significantly increased linear or nonlinear change rates compared with females (Table 4; Figs. 3 and 4). \(V_{\text{alv}}\) showed a linear function with body weight (Adj \(R^2 = 0.84\)) with males showing significantly increased linear change rate and an increased volume at the mean value of 2.92 kg compared with females (Table 4). \(V_{\text{alv}}\) showed a quadratic function with body weight (Adj \(R^2 = 0.93\)) with males showing a significantly increased linear change rate and an increased volume at the mean value of 2.92 kg compared with females (Table 4; Fig. 5). \(N_{\text{alv, lung}}\) showed a quadratic function with age (Adj \(R^2 = 0.75\)) and \(V_L\) (Adj \(R^2 = 0.82\)) but a quadratic function with body weight (Adj \(R^2 = 0.79\)) (Table 4; Fig. 5). \(N_{\text{alv, lung}}\) showed a quadratic function with age (Adj \(R^2 = 0.74\); Table 4; Fig. 6) and a two-parameter exponential function with \(V_L\) (Adj \(R^2 = 0.81\)) with males showing a significantly increased nonlinear change rate compared with females (Table 4). \(N_{\text{alv, lung}}\) also showed a two-parameter exponential function with body weight (Adj \(R^2 = 0.73\)) with

### Table 2. Fractionator values for developing rhesus monkey lungs

<table>
<thead>
<tr>
<th>Sex</th>
<th>SF, bar</th>
<th>SF, block</th>
<th>h (dis), mm</th>
<th>B Width, mm</th>
<th>SF, height</th>
<th>dx, dy</th>
<th>a (fra)</th>
<th>SF, area</th>
<th>SF</th>
<th>F$_{avg}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>0.10*</td>
<td>0.27*</td>
<td>0.01*</td>
<td>8.5*</td>
<td>1.11×10^{-3}*</td>
<td>1.67</td>
<td>0.04</td>
<td>0.01</td>
<td>3.99×10^{-7}*</td>
<td>0.51*</td>
</tr>
<tr>
<td>Male</td>
<td>0.06*</td>
<td>0.31*</td>
<td>0.01*</td>
<td>9.0*</td>
<td>1.12×10^{-3}*</td>
<td>1.53</td>
<td>0.04</td>
<td>0.02</td>
<td>3.44×10^{-7}*</td>
<td>0.51*</td>
</tr>
</tbody>
</table>

*Mean of all animals. SF, sampling fraction; F$_{avg}$, factor for shrunken global volume; h(dis), disector height; B Width, block width; dx, dy, field displacement; a(fra), sampling field area.

### Table 3. Estimates of the variation and mean counts for the number of alveoli in the lung

<table>
<thead>
<tr>
<th>Sex</th>
<th>$\Sigma\Delta N$</th>
<th>CE Noise</th>
<th>CV Biological</th>
<th>CV Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>164</td>
<td>0.05</td>
<td>0.68</td>
<td>0.73</td>
</tr>
<tr>
<td>Male</td>
<td>172</td>
<td>0.05</td>
<td>0.72</td>
<td>0.77</td>
</tr>
</tbody>
</table>

$\Sigma\Delta N$, Euler characteristic; CE noise, average sampling variation of the stereological measurement; CV biological, biological variability; CV total, the sum of CE noise and CV biological.
females showing a significantly increased nonlinear change rate and an increased number at the mean value of 2.92 kg compared with males (Table 4). The mean Euler characteristic for females (−164) and for males (−172) was sufficient to give an average sampling variation of the stereological measurement of 0.05 for both sexes, whereas the dominant variance in the study was biological (variability between animals; Table 3). $S_{\text{IAS}}$ showed quadratic functions with age (Adj $R^2 = 0.90$) and $V_L$ (Adj $R^2 = 0.98$) with no difference between sexes (Table 4; Fig. 7). However, $S_{\text{IAS}}$ showed a linear function with body weight (Adj $R^2 = 0.93$) with females showing a significantly increased linear change rate and an increased surface at the mean value of 2.92 kg compared with males (Table 4; Fig. 8). $V_n$ was best fit by quadratic functions with age (Adj $R^2 = 0.90$).
\( R^2 = 0.13 \) and body weight (Adj \( R^2 = 0.39 \)), whereas with \( V_L \) (Adj \( R^2 = 0.32 \)), a three-parameter exponential function provided the best fit, indicating little consistency in change with age or \( V_L \) (Table 4; Fig. 9). However, in females, \( V_n \) and body weight showed a significantly increased nonlinear change rate and an increased alveolar volume at the mean value of 2.92 kg compared with males (Table 4). It should be noted that the mean values for \( V_n \), the number-weighted mean alveolar volume, were 12% and for \( V_v \), the volume-weighted alveolar volume, were 29% greater in females than males (Table 5). Furthermore, \( CV_n \) had two-parameter exponential functions with age (Adj \( R^2 = 0.48 \), \( V_L \) (Adj \( R^2 = 0.48 \), and body weight (Adj \( R^2 = 0.42 \) (Table 4; Fig. 10), indicating a greater distribution of alveolar volumes with age, \( V_L \), and body weight. \( CV_n \) was also 14% greater in females than males, an indication of a greater distribution of alveolar volumes in females than males (Table 5). Furthermore, for females, \( CV_n \) and body weight showed a significantly increased linear change rate and an increased alveolar volume distribution at the mean value of 2.92 kg compared with males (Table 4). As expected, the global shrinkage because of dehydration and paraffin embedding was very pronounced: the lung tissue shrunk to 51% of the fixed volume of the monkey (Table 2).

**DISCUSSION**

\( V_{par}, V_{alv}, V_{ad}, \) and \( V_{ias} \) all increased rapidly during the first 2 yr of life in rhesus monkeys and then slowly grew from 2 to 7 yr. The rate of change was greater in males than females especially from 2 to 7 yr in proportion to somatic growth. \( N_{alv} \) also showed consistent growth throughout the 7 yr, but increases in \( N_{alv} \) were best predicted by increases in \( V_L \). However, \( V_n \) showed little relationship with age, volume, or body weight, and in females, \( V_n \) was larger, and alveoli showed a greater size distribution of volume than in males. Alveoli increase in number but not volume throughout all of the postnatal developmental/growth stages (infant, 1–12 mo; juvenile, 12–24 mo; adolescent, 2–4 yr; and young adult, 4–8 yr) in rhesus monkeys.

**Oxygen Diffusion and Lung Parenchyma**

Oxygen exchange between air and blood occurs by diffusion across the air-blood barrier in the lung parenchyma. Oxygen flow by diffusion from air to blood is governed by Fick’s law that has a permeability coefficient, surface, and thickness of the barrier between air and blood (16). A morphometric model of
diffusion capacity that was proposed by Weibel (43) and subsequently refined by Weibel and colleagues (44) showed a direct correlation of diffusion capacity to alveolar surface and an indirect correlation to thickness of the blood air barrier. The morphometric estimate of diffusion capacity of oxygen in monkey lungs was very similar to dogs and scaled linearly with body weight (17). Additional studies using the morphometric estimate of diffusion capacity of oxygen in monkey lungs concur with these initial findings (1, 27). When Gehr and colleagues (17) compared the scaling of morphometric parameters (like alveolar surface area) with maximal oxygen consumption, they concluded that bigger animals required a larger pulmonary diffusion capacity to admit the flow of oxygen required by the organism. We would expect the increase in surface area in monkeys during the first 7 yr of life to show a proportional increase in pulmonary diffusion capacity even though there is no data on the maximal oxygen uptake in rhesus monkey lungs during postnatal development. It is noteworthy that membrane diffusion capacity and capillary blood volume showed an age-related increase consistent with alveolarization from birth to 8 wk of age in lambs (10).

Lung Growth Differences Between Sexes

In humans, female lungs tend to be smaller than male lungs throughout childhood and even in adolescence when girls attain greater height earlier than boys. As girls attain their maximum adult height in late puberty, their lung growth ceases, whereas that of boys continues longer, in some cases into early adulthood (2). In boys and girls, the growth of the lung parenchyma and its airways occurs independently, but this dysanapsis is more pronounced in boys (2). The configuration of the adult female lung is the result of proportional growth of its airways in relation to its parenchyma, but that of the adult male lung is the result of dysanaptic growth where growth of the airways has lagged behind that of the lung parenchyma. Our measurements of parenchymal volume in rhesus monkey lungs show very similar trends over the first 7 yr of life that correspond to growth into early adulthood in humans. In rodents, females have smaller alveoli and more alveoli and alveolar surface area per body weight than males (28, 29). Our observations in rhesus monkey lungs show similar results to those seen in rodents with more alveoli and alveolar surface area per body weight in females compared with males. However, rhesus monkeys have larger alveoli per body weight in females compared with males, a result opposite that observed.

Fig. 6. The log of the number of alveoli in the lung ($N_{alv, lung}$) vs. age (days) for females (solid line and filled circles) and males (dashed line and open circles) is plotted according to a quadratic function (Table 4).

Fig. 7. The surface of interalveolar septa ($S_{IAS}$) in the lung (m$^2$) vs. age (days) for females (solid line and filled circles) and males (dashed line and open circles) is plotted according to a quadratic function (Table 4).

Fig. 8. The surface of interalveolar septa in the lung (m$^2$) vs. body weight (kg) for females (solid line and filled circles) and males (dashed line and open circles) is plotted according to a linear function (Table 4).

Fig. 9. The log of the mean alveolar number-weighted volume ($V_{alv, n}$) vs. age (days) for females (solid line and filled circles) and males (dashed line and open circles) is plotted according to a quadratic function (Table 4).
Studies of human lungs show a rapid alveolar growth phase investigations of human postnatal lung development (40, 49). M. mulatta than our study of Thus these results from the study of there was little alveolar multiplication after birth (24). Therefore, the exact time at which alveolar multiplication ceases in humans is still obscure. Differences between studies may lie in the stereological approach used to estimate alveolar number. Traditionally, the estimation of alveolar number has been done by assumption of a specific geometric shape, a rotatory ellipsoid (45). Human acinar reconstructions identified six different geometric shapes for alveoli and thereby illustrate the difficulty in selecting one geometric shape as a mean of the representing all six shapes.

Recently, we (26) introduced an approach to alveolar counting that used the smooth fractionator (19), a rigorous design-based sampling approach to the lung and the Euler characteristic to estimate the number of alveoli in lung without bias. Estimation of the total number of any feature in an organ or any containing space with the fractionator is direct, and there is no need to know the reference volume. This method is unaffected by global and differential shrinkage, swelling, and distortion of the containing space during embedding and sectioning. Euler number estimation uses physical dissectors that are true volume probes (39). Euler number estimation of alveoli makes no assumption regarding the size, shape, or orientation of the structures to be counted in contrast to 2-D analyses. In essence, the Euler number count is directed toward counting the rings of alveolar mouth openings. Another approach that has been applied to rodent lungs estimates the number of alveoli in the lung indirectly by selecting individual alveoli using serial sections and a dissector and then making volume estimates by Cavalieri (33) or point-sample intercept (30) methods and dividing the mean alveolar volume into the volume of alveoli in the lung. This indirect approach is extraordinarily time consuming and is complicated by the need to define alveolar mouth openings in three dimensions as a (curved) wall of the alveolar air space in serial sections. The alveolar counting procedure used in this paper is one of counting discontinuities of a well-defined structure, the alveolar wall. Except for the necessity of a well-trained observer who can concentrate on the ends of the interalveolar septal walls, our experience is that the counting is straightforward and unproblematic (26).

**Sampling**

We previously optimized sampling for alveolar estimation in adult rhesus monkeys (26). One refinement in this study was that we used a thinner slab thickness of 5 mm to increase our sampling in infant lungs but maintained a 5-µm section thickness. This allowed us to achieve the appropriate sample total of 100–200 Euler counts per lung (Table 3) (21). Although the sampling and processing steps were monitored and corrected for shrinkage necessary for estimates of mean alveolar volumes, the fractionator design for the total number of alveoli is independent of shrinkage (26). Since only the counting noise is easily known in fractionator designs, it must be kept low by a sensible design (19). The estimated CE noise of 0.05 (Table 3) for both males and females easily meets this requirement. The total observed CV of 0.77 and 0.73 in males and females, respectively, indicate that the remaining biological variability of alveolar number during postnatal development is very high. Of course, this is not surprising since we are dealing with a developmental change of ~11-fold in alveolar number in both males and females over the first 7 yr of life in rhesus monkeys. We can have confidence in our individual animal estimates because of a value of 0.05 for CE noise.

**Number of Alveoli**

The general description of rhesus monkey body weights and lung volumes in this study are similar to previously published

---

**Table 5. Estimates of the means and variation of volumes of alveoli in the lung**

<table>
<thead>
<tr>
<th>Sex</th>
<th>$V_{n\text{alv}} \times 10^{-9}$</th>
<th>$\tilde{V}_{alv} \times 10^{-9}$</th>
<th>CV$_{n\text{alv}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>5.36±1.844</td>
<td>16.40±8.776</td>
<td>1.37±0.629</td>
</tr>
<tr>
<td>M</td>
<td>4.79±1.605</td>
<td>12.67±7.433</td>
<td>1.20±0.474</td>
</tr>
</tbody>
</table>

Values are expressed as means ± 1 SD. $\tilde{V}_{alv}$, volume-weighted mean alveolar volume.

---

**Fig. 10.** The coefficient of variation of the distribution of number-weighted alveolar volumes ($CV_{n\text{alv}}$) vs. age (days) for females (solid line and filled circles) and males (dashed line and open circles) is plotted according to a 2-parameter exponential function (Table 4).
values for these species (7). A precise morphometric study of regional differences in rat lungs fixed by intratracheal instillation at 20 cmH₂O pressure showed significant decreases in the volume and surface densities of interalveolar septa in subpleural compared with central lung regions (48). These investigators recommended that for quantitative light microscopic analysis of lung tissue, the most appropriate sampling unit is at minimum the entire lobe (48). When monkey lung lobes are fixed by intratracheal instillation at 30 cmH₂O pressure, more variation in alveolar number was observed between cranial and caudal lobes than between the entire left lung of adult monkeys (26). In this study, we analyzed all six lobes of the rhesus monkey lung that were fixed by intratracheal instillation at 30 cmH₂O pressure, a fixation pressure that mimics total lung capacity. Our use of smooth fractionator sampling followed by stratified random sampling of disectors on sections guaranteed an alveolar number that was independent of inflation fixation variation and variations in alveolar size. Our finding of the greater increase in lung volumes and alveolar surface area in males compared with females over all ages is very similar to that reported for postnatal human lung growth (40). The greater numbers of alveoli in males compared with females over all ages is also very similar to that reported for postnatal human lung growth (40). At what age alveolar multiplication ends in humans is still open to question, primarily because of the stereological methods used and the variability between individuals. Our data in rhesus monkeys clearly documents alveolar addition in the first 7 yr that has not been previously reported in nonhuman primates. However, the best predictor of alveolar number in a rhesus monkey lung is V₁.

Alveolar Volume

Mean alveolar volume showed a poor relationship with age and V₁, implying that with increases in V₁, alveoli are added and do not enlarge to any significant degree during postnatal growth in rhesus monkeys. It is noteworthy that females had mean alveolar volumes that were 8% greater than males. CVₙₐᵥ, a measure of the size distribution of number-weighted alveolar volume, showed a steady increase with age for both males and females. Furthermore, CVₙₐᵥ was 40% greater in females than males. It is possible from the greater size distribution that the greater mean volume in females is the result of a subpopulation of larger alveoli in females that are not found in males. Because little is known about the development of lung parenchyma, most review articles on sex differences in lung development focus on the airways (4). Since this study is the first to measure CVₙₐᵥ, more studies are needed to estimate the effect of increasing age in both sexes into adulthood (7–18 yr) and with old age (≥18 yr in rhesus monkeys). Does this greater CVₙₐᵥ during postnatal development in females persist into adulthood and even advance with old age? If so, then perhaps there is an anatomical basis to the compelling evidence that women are more susceptible than men to the development of chronic airflow limitation (8). Furthermore, women who have chronic obstructive pulmonary disease (COPD) seem to have a greater risk of hospitalization than men. The greater severity of disease among women is consistent with evidence of a greater predisposition to develop COPD and the appearance of COPD at an earlier age than men (8).

The use of the fixed V₁ as estimated by the immersed buoyant weight in PBS may have overestimated our tissue and air space volumes. A comparison of right cranial lung lobe volumes from three monkeys spanning the range of this study showed an overestimation of 4.9–16% by the buoyant weight method compared with the Cavalieri method. There was no apparent relationship with age as the percentage of difference was least in the youngest (6.5%) and oldest (4.9%) and greatest in the middle age range monkey (16%). A comparison of lung volumes by these two methods in dog lungs showed that the volume of the intact fixed lung under positive pressure is systematically higher by 13–25% than that measured after sectioning and release of airway pressure (47). Only lung volumes estimated by the Cavalieri method were used in the morphometric calculations of dog lungs (47). The lower shrinkage from immersion to the slab volume estimate in monkey compared with dog lungs could be the result of a smaller size or fixative difference. Further studies are needed to investigate the shrinkage differences of these two fixatives (2.5% buffered glutaraldehyde and 1% glutaraldehyde-1% paraformaldehyde in cacodylate buffer), but we strongly recommend that investigators report both volume estimators for future morphometric studies.

We have shown that alveoli are added in rhesus monkey lungs proportional to age, V₁, and body weight into young adulthood in both males and females. Furthermore, we recommend that previous reports of postnatal development in nonhuman primates and humans be reconsidered because of the potential bias of sample, tissue shrinkage, and geometric assumption of alveolar shape.

ACKNOWLEDGMENTS

The support of Primate Services at the California National Primate Research Center for animal handling, care, and necropsy support and especially the efforts of Sona Santos were critical to this study and are gratefully acknowledged. We thank Kathy West for drawing Fig. 1. We thank Dr. Suzette Smiley-Jewell for editing the manuscript.

GRANTS

This work was supported by National Institute of Environmental Health Sciences Grants P01-ES00628 and P01-ES11617 and National Center for Research Resources Grant RR-00169.

REFERENCES


