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A STEREOSCOPIC SCANNING ELECTRON MICROSCOPE STUDY OF PULMONARY HYPOPLASIA IN CHONDRODYSTROPHIC MICE

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Abstract

Pulmonary hypoplasia is a life threatening condition in newborns resulting from a generalized underdevelopment of the lungs. The lung disorder is usually secondary to conditions outside the lung such as thoracic volume reduction. The precise mechanism by which thoracic volume reduction prevents normal lung development and growth is unknown. As a model for human pulmonary hypoplasia associated with lethal skeletal dysplasia, a stereoscopic SEM study of chondrodystrophic (*cho*) fetal mouse lungs fixed by intratracheal instillation with 3% glutaraldehyde was conducted. In comparison with lungs of phenotypically normal littermates, the mutant's lungs appeared unaffected with respect to structure of major bronchiolar airways and in the morphology and amount of surfactant precursors (multilamellar bodies). The primary saccules within the mutant's lungs were significantly smaller and more numerous relative to those of normal littermates. These observations provide evidence that the lungs for this type of pulmonary hypoplasia are ultrastructurally normal with respect to upper airways, but that the primary saccules, or units of function in neonatal breathing in the rodent, are significantly smaller. This effect, however, does not appear to inhibit differentiation of type II pneumocytes or production of surfactant.

KEY WORDS: pulmonary hypoplasia, surfactant, lung, respiratory distress, thoracic volume, mouse, scanning electron microscopy, dwarfism, chondrodystrophy

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Introduction

Pulmonary hypoplasia refers to a generalized underdevelopment of lung tissue due to inhibited cellular proliferation and decreased expansion of the future alveolar space. Clinically this disorder is considered present if the neonate's lung:body-weight ratio is less than 67% of normal and if the volume of potential air space for oxygen exchange, estimated histologically, is less than normal[5,31,52,102,108]. This condition has been reported associated with other congenital malformations such as diaphragmatic hernia[3,11,15,28,30,85,89], fetal akinesia[9,27,68,69], oligohydramnios[35,45,78,83,105], prune-belly syndrome[48], pleural effusions[10,17,97], anencephalus[76,84,85] certain chondrodystrophies[24,25,36,87,107], or any combination of the aforementioned anomalies[62,80]. The severity of pulmonary hypoplasia is usually proportional to the severity of the associated anomaly[18,37,60,82].

There are more than eighty genetically distinct forms of skeletal dysplasia observed in humans[86,98], 13 of which are lethal[24,25,87]. Some of the more common lethal short-limb dwarfisms are thanatophoric dysplasia[54,63], short-rib polydactyly syndromes, and achondrogenesis. These chondrodystrophies decrease the thoracic cavity volume which is thought to cause secondary pulmonary hypoplasia that frequently leads to respiratory failure and ultimately death[25,34,63,64,80]. Only a few cases of pulmonary hypoplasia associated with chondrodystrophy have been reported in humans and these have only been studied at the clinical level[5,24,34].

At the experimental level studies have been carried out in which surgically induced diaphragmatic hernia, oligohydramnios, fetal akinesia, pleural effusions and diminished fetal breathing have been shown to cause secondary pulmonary hypoplasia[10,29,32,33,42,43,65,69-71,77,78,103,109]. These studies support the hypothesis that both fetal breathing and sufficient thoracic volume are necessary for normal lung development. It is possible that the confined thoracic space inhibits the development of the bronchial tree and alveoli which would contribute to

the respiratory distress experienced by newborns with pulmonary hypoplasia[51].

Another possible consequence of thoracic volume reduction is an effect on the production and secretion of surfactant, a phospholipid that coats the alveolar surface[13,49,57,58]. Surfactant lowers the surface tension within the alveoli, allowing them to remain open[20-22,40,53,72,73,79,96]. Atelectasis, or defective expansion of the alveoli, is often associated with newborns who for a number of reasons do not produce enough surfactant[12,44,75]. Surfactant is also thought to keep the blood-air barrier of the lung relatively free of surface water, thereby increasing the clearance rate of foreign particles and allowing better gas exchange[44,74,75]. Surfactant is synthesized and assembled as lamellar bodies within type II pneumocytes [6,19,38,53,55,56,59,66,90]. These cells become differentiated during the pseudoglandular period of development at approximately day 14 of gestation in the mouse and during week 11 of gestation in human[104]. The ability of these cells to synthesize surfactant is apparently determined by the source of their epithelium, not by epithelial-mesenchymal interactions[47,104]. The lamellar bodies are secreted into the alveolar lumen as concentric layers of membranous structures and have been observed to have irregular strands connecting them to their secretory cell of origin[16,90,101]. These spheres then disperse to form tubular myelin which eventually coats the alveolar lumen[8,91,106,110,111]. Because of relatively new technologies for diagnosing and treating infants with immature surfactant systems, the occurrence of respiratory distress syndrome is less frequent than in the past[4,23,39,41,67,75,81,92,100]. Nevertheless, any child born lacking a mature surfactant system is at risk for respiratory failure. If the thoracic volume reduction impairs the differentiation of type II pneumocytes and thereby inhibits the production and secretion of lamellar bodies, this could be a factor in the respiratory distress of the lethal chondrodystrophies.

To avoid surgically induced artifacts and to gain a better understanding of the mechanism(s) by which thoracic volume reduction induces pulmonary hypoplasia, we are currently studying three genetically distinct mouse models of pulmonary hypoplasia, viz. *cho*, *cmd*, and *Dmm*[14,88,93,95]. Each mutant has thoracic dystrophy and dies of respiratory failure. We have reported that two of the mutants, *cho* and *cmd*, have the characteristics of pulmonary hypoplasia[50,93] and in *cho* we have reported that thoracic volume reduction during the fetal period is associated with the onset of pulmonary hypoplasia[46]. The *cho* mutant has also been shown to have a narrowed tracheal lumen which may exacerbate the problem of pulmonary hypoplasia caused by the decreased thoracic volume[94]. The aforementioned parameters of bronchiolar development, alveolarization (primary saccule maturation in the mouse[2]), and the pulmonary surfactant system have been thought to be vital factors for normal lung function. In order to confirm the previously reported decrease in size of the *cho's* primary saccules and to ascertain further abnormalities in the mutant bronchiolar lining and surfactant system, we performed a stereoscopic SEM study on these parameters in *cho* mouse fetal lungs.

Materials and Methods

Tissue Sampling

Normal and mutant littermate fetuses were obtained from timed pregnancies resulting from overnight matings of heterozygous (cho\+) C57BL mice. The dams were fed Wayne Breeder Blox and water ad libitum and kept under a 12 hour light-dark cycle. The day a vaginal plug was observed was considered day 0 of gestation.

One day before parturition (gestation day 18) the pregnant females were killed by exposure to ether for 15 minutes, their uteri were removed and placed in Hank's solution, and the fetuses were removed from the uterus and weighed. The lungs, trachea, and larynx were removed intact from each of the normal and mutant fetuses.

Intratracheal fixation and processing of lungs for SEM

A 16 mm, 30 gauge blunt tipped needle was inserted into the trachea and the trachea was ligated to the needle[61]. The needle was then attached to a 12 mm I.D. glass column filled to a height of one meter with 3% glutaraldehyde in cacodylate buffer, pH 7.3. The fixative was introduced into the lungs at a flow rate of 7.0 µl/sec, or 30% less than that used for fixing adult mouse lungs[7]. The lungs were immersed in Hank's solution while being fixed via intratracheal instillation, and after two hours the saline bath was changed to 3% glutaraldehyde and the lungs were fixed for an additional 14 hours[26]. The left lung of each fetus was processed through a graded series of alcohol then critical-point dried and adhered to 12 mm aluminum stubs. The interior of the lung was then exposed by carefully teasing the tissue apart prior to being sputter-coated with gold. Lungs from a pair of normal-mutant littermates from three litters were examined with a JEOL SEM. We assumed the fracturing of the lungs to be random and the primary saccules to be spherical. This allowed the measurement of 10 primary saccule diameters from each of several intermediate magnification (180 X) scanning electronmicrographs per fetus (n=3,3). Differences between normal and mutant mean saccule diameters were tested for significance by the t-test. A probability of 0.05 or less was considered significant.

Processing of lungs for Transmission Electron Microscopy

For transmission electron microscopy, whole lungs were fixed without intratracheal instillation in 2% glutaraldehyde and 3% acrolein in cacodylate buffer, pH 7.3. The tissues were processed through a graded alcohol series, cleared in acetone, and embedded in Spurr's epoxy[99]. Thin sections were stained with lead citrate and uranyl acetate. Lungs from a pair of normal-mutant littermates from four litters were examined with a Philips 400 EM. The diameter of 10 individual lamellar bodies was estimated from several electron micrographs at a final magnification of 16,200 for each fetus (n=4,4). The t-test was used to detect differences between normal and mutant with a 0.05 probability being considered significant.

Results

Gross internal lung morphology

The primary bronchus of the normal lung fractured frontally and examined with SEM was large and well distended, and further patent airway branching was evident in the secondary bronchi. Associated with these airways were large blood vessels. Most of the lung interior was comprised of well distended primary saccules (Fig. 1A).

The mutant's lung interior appeared markedly affected (Fig. 1B). The primary saccules appeared more numerous and much smaller than normal. Blood vessels and the bronchial airways appeared normal in the mutant, however, no attempt was made to assess this morphometrically.

Airways and primary saccules

Bronchiolar passages could be distinguished from primary saccules by the difference in their lumenal surface at higher magnification (200 X; Fig. 2A vs. 5A). The lumenal surface of a normal primary bronchus was convoluted (Fig. 2A) and showed numerous tufts of cilia and distinct cellular boundaries (Figs. 3A, 4A). Whereas, primary saccules had no convolutions or cilia but did contain multilamellar bodies (5A, 6A). The average primary saccule diameter from three normal fetuses was 59.9 $\pm$ 2.8 µm (n=3).

The mutant's bronchial lumenal surface appeared normal with respect to convolution, tufts of cilia, and cellular boundaries (Fig. 2B, 3B, 4B). The average diameter per primary saccule from three mutant fetuses was 29.9 $\pm$ 4.0 µm, or 50% of normal. The difference in size of primary saccules between normal and mutant at 200 magnification was apparent (Figs. 5A, 5B).

Evidence for lung maturation: the surfactant system

Surfactant precursors, or multilamellar bodies, were predominantly observed within the primary saccules of normal fetal mouse lungs, in association with the lumenal wall (Figs. 6A, 7A) . However, it was not uncommon to observe them within the bronchiolar airways. These multilamellar bodies were comprised of a cluster of numerous single lamellar bodies with diameters ranging from .22-1.00 µm (n=3). Strands of tubular myelin, like those in Figure 8B, that presumably unwound from the tightly compacted multilamellar bodies, were frequently observed throughout the normal lungs. In addition, more loosely organized material was also observed within the normal's primary saccules (Fig. 8A, 9A).

In the mutant, multilamellar bodies of comparable size, shape, and number, relative to the normal, were also observed within the primary saccules (n=3; Figs. 6B, 7B). Strands of tubular myelin in contact with multilamellar bodies and loosely organized material were also frequently observed throughout the mutant lungs (Figs. 7B, 8B, 9B).

With transmission electron microscopy, the primary saccules of the normal contained multilamellar bodies consisting of varying numbers of single lamellar bodies that ranged in diameter from .25-1.23 µm (n=4). Most lamellar bodies were coiled, but some showed tubular myelin in a cross-hatched configuration (Fig. 10). There was no apparent difference between normal and mutant with respect to size or ultrastructure of multilamellar bodies (n=4,4).

Discussion

In previous studies we compared the gross morphological, histological, and biochemical parameters of hypoplastic lungs in chondrodystrophic mouse fetuses with that of unaffected littermates[46,50,93]. These studies confirmed the presence of pulmonary hypoplasia in two forms of chondrodystrophy, cho and cmd. The present SEM study confirms our previous observations of relatively smaller, more numerous primary saccules within the lungs of mouse fetuses with pulmonary hypoplasia[46,50,93,94].

The mutant's bronchiolar airways, viewed under SEM, did not appear atypical. The lumenal surface of the bronchial tree appeared normal in the mutant, despite its airways developing in a relatively confined space. The patent state of the mutant's airways, however, may not be representative of the in situ condition. The intratracheal fixation technique used in the present study may have caused some of the mutant's possibly collapsed airways to appear normal[94]. However, since no attempt was made to measure the diameter of bronchiolar airways, we do not know how they compare between normal and mutant. We are presently performing 3-D computer reconstructions of serial sections and microcastings of the bronchiolar airways to determine if there are any volumetric or branching differences between normal and mutant.

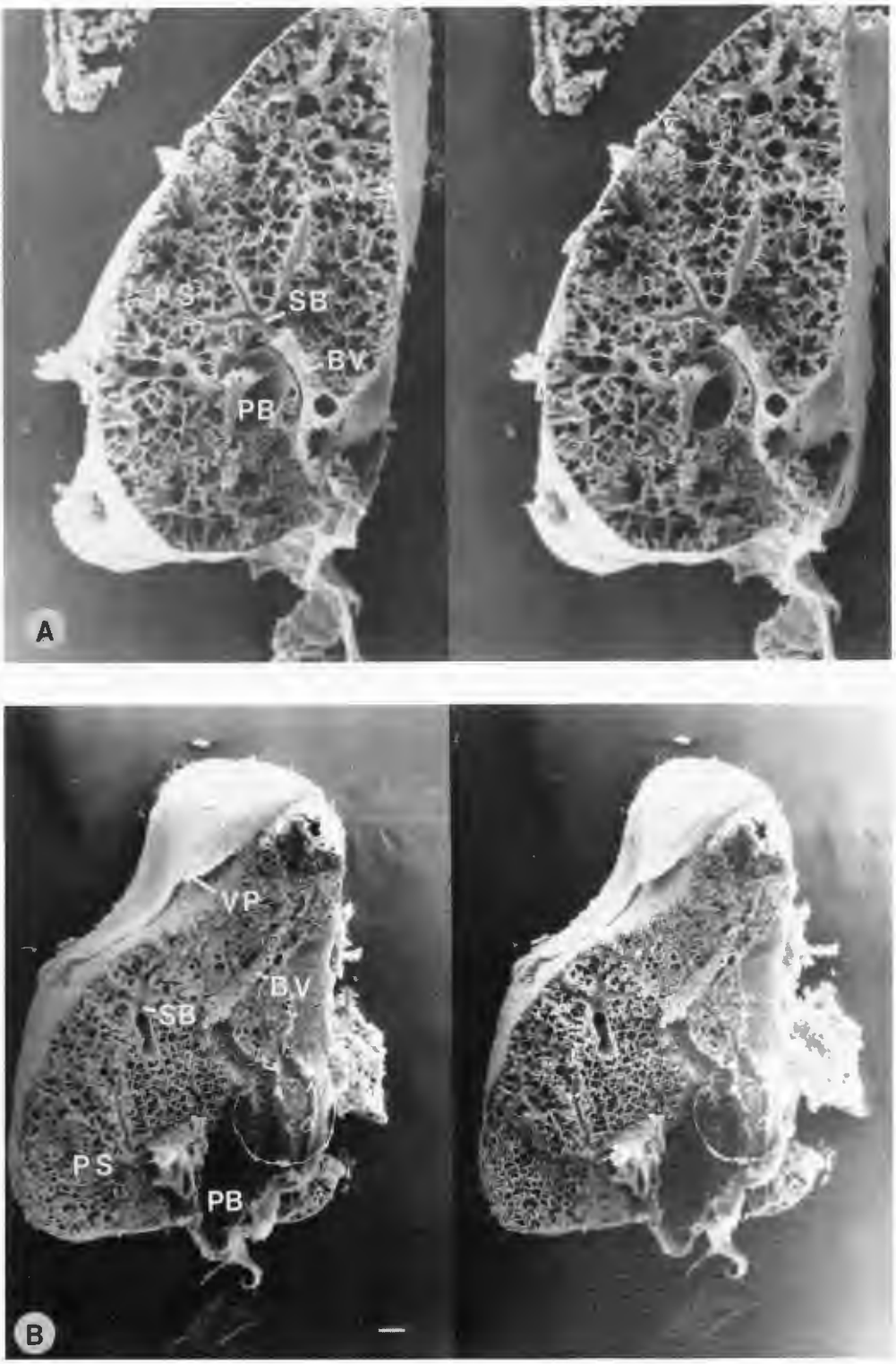

Figure 1. SEM stereopairs of the superior left lobe of day-18 fetal mouse lungs. The normal lung (A), fractured frontally, shows the primary bronchus (PB), blood vessel (BV), secondary bronchi (SB), and numerous primary saccules (PS). The hypoplastic lung (B), fractured frontally, shows typical primary bronchi (PB), secondary bronchi (SB), blood vessels (BV), visceral pleura (VP), and atypically reduced primary saccules (PS). (Bar = 100 μm).

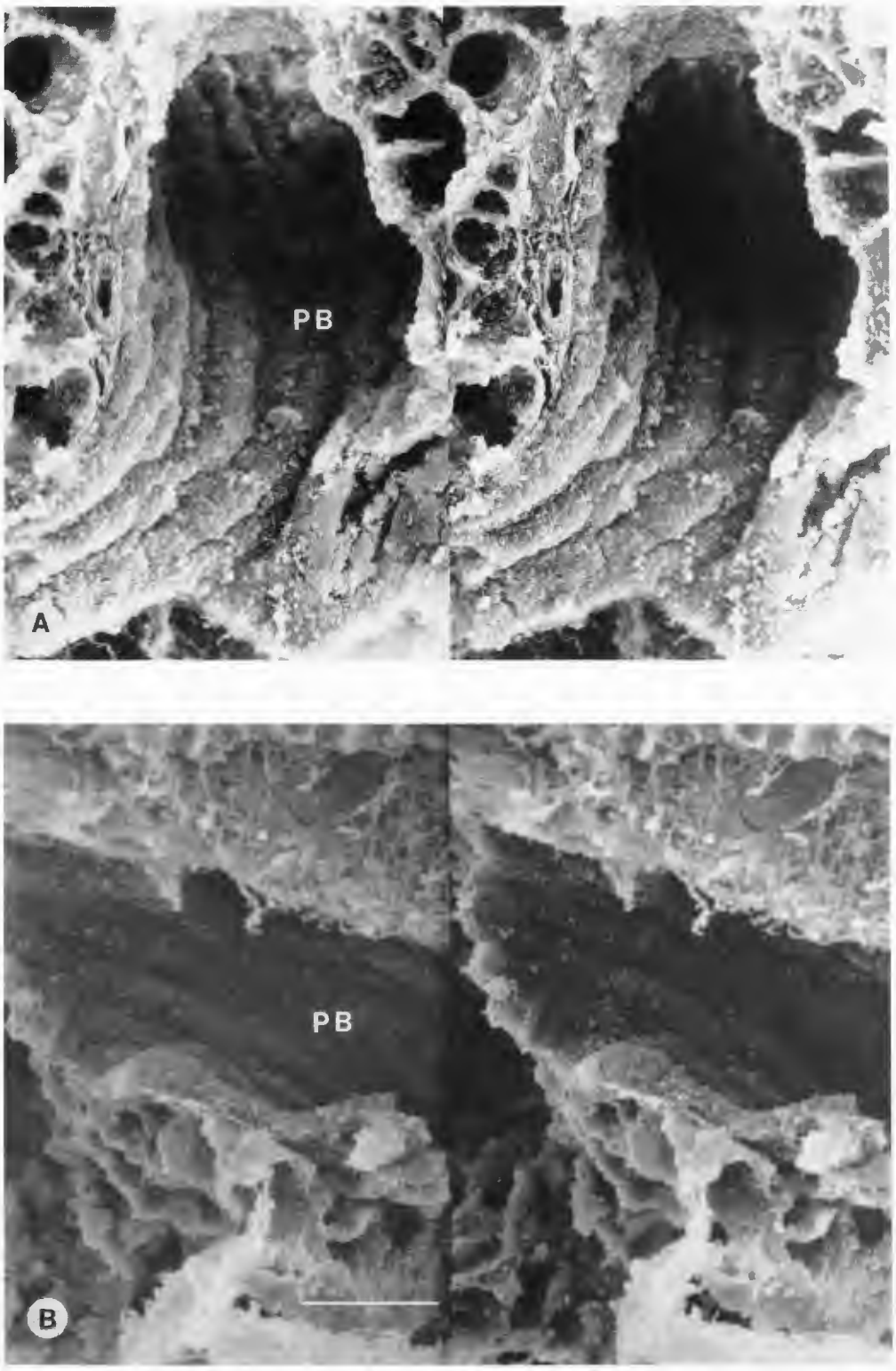

Figure 2. Primary bronchi (PB) of normal (A) and hypoplastic (B) fetal mouse lungs. The internal linings of the bronchi from normal and mutant fetuses appear convoluted. (Bar = 100 μm).

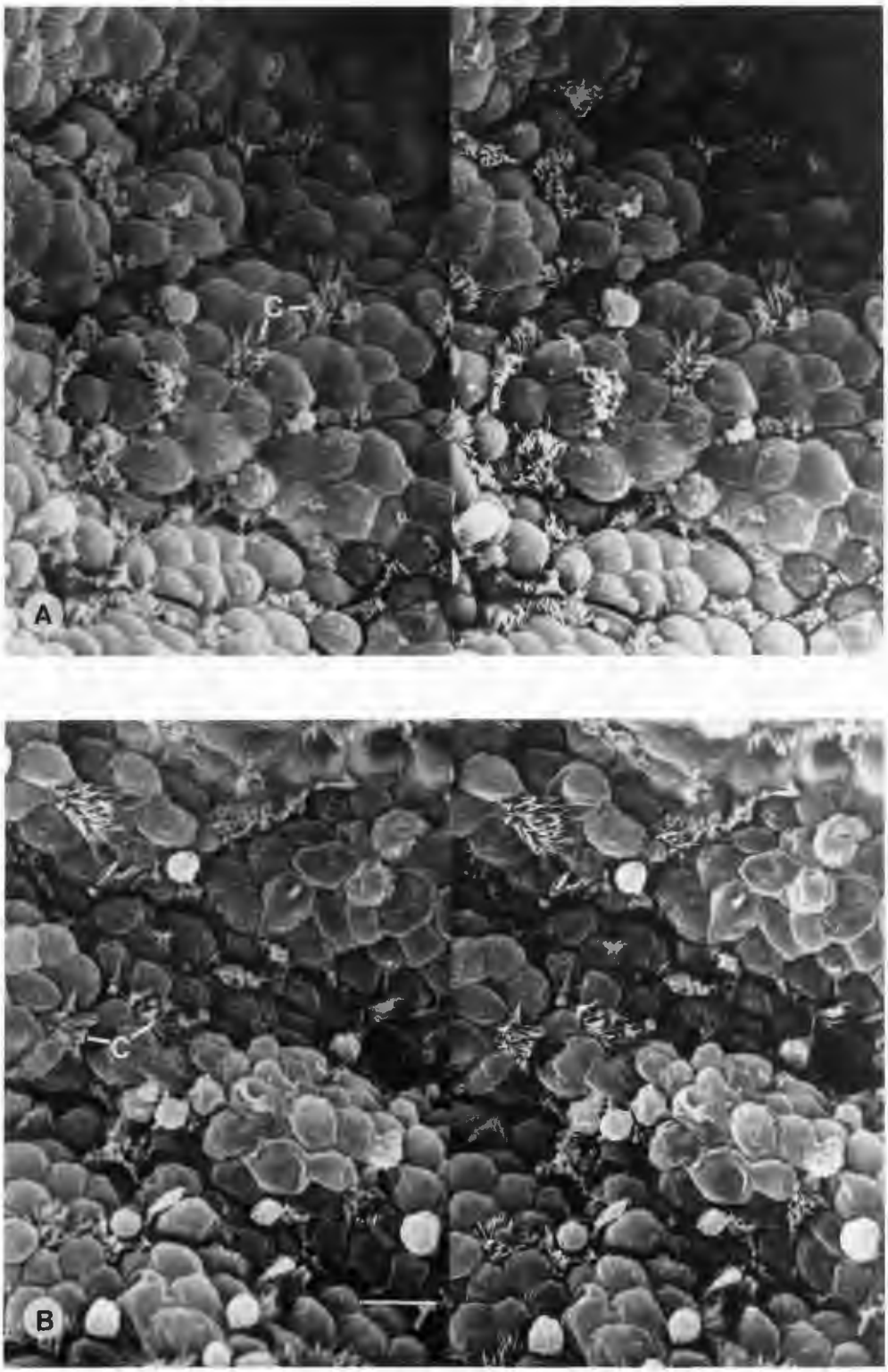

Figure 3. Lumenal surface of normal (A) and hypoplastic (B) fetal bronchi. No differences in overall structure or spatial relationship of cilia (C) were observed between normal and mutant. (Bar = 10 μm).

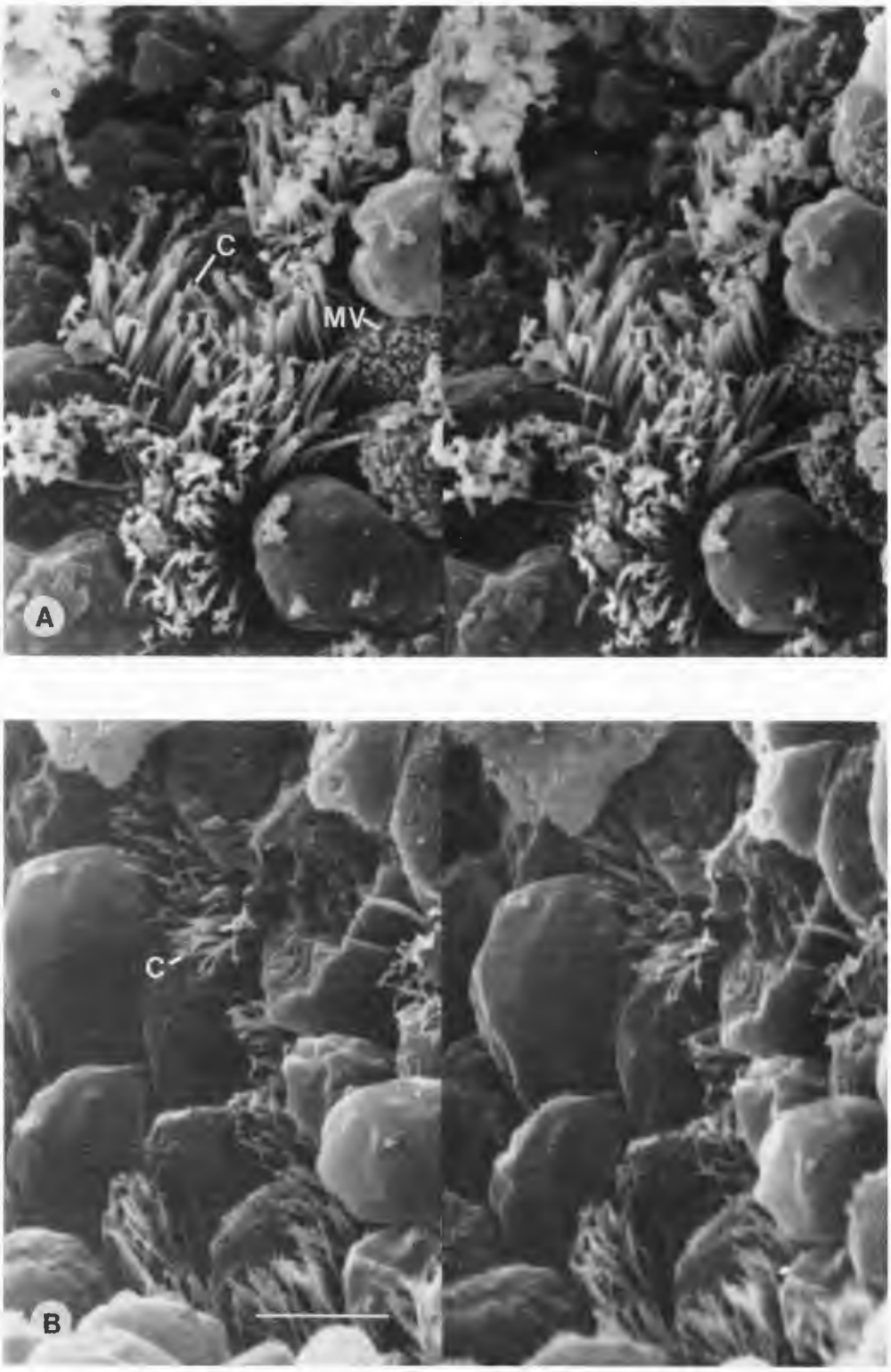

Figure 4. Cells lining the bronchiolar airways of normal (A) and hypoplastic (B) fetal mouse lungs. No difference was observed between the normal and mutant with respect to bronchiolar cilia (C) and microvilli (MV). (Bar = 10 μm).

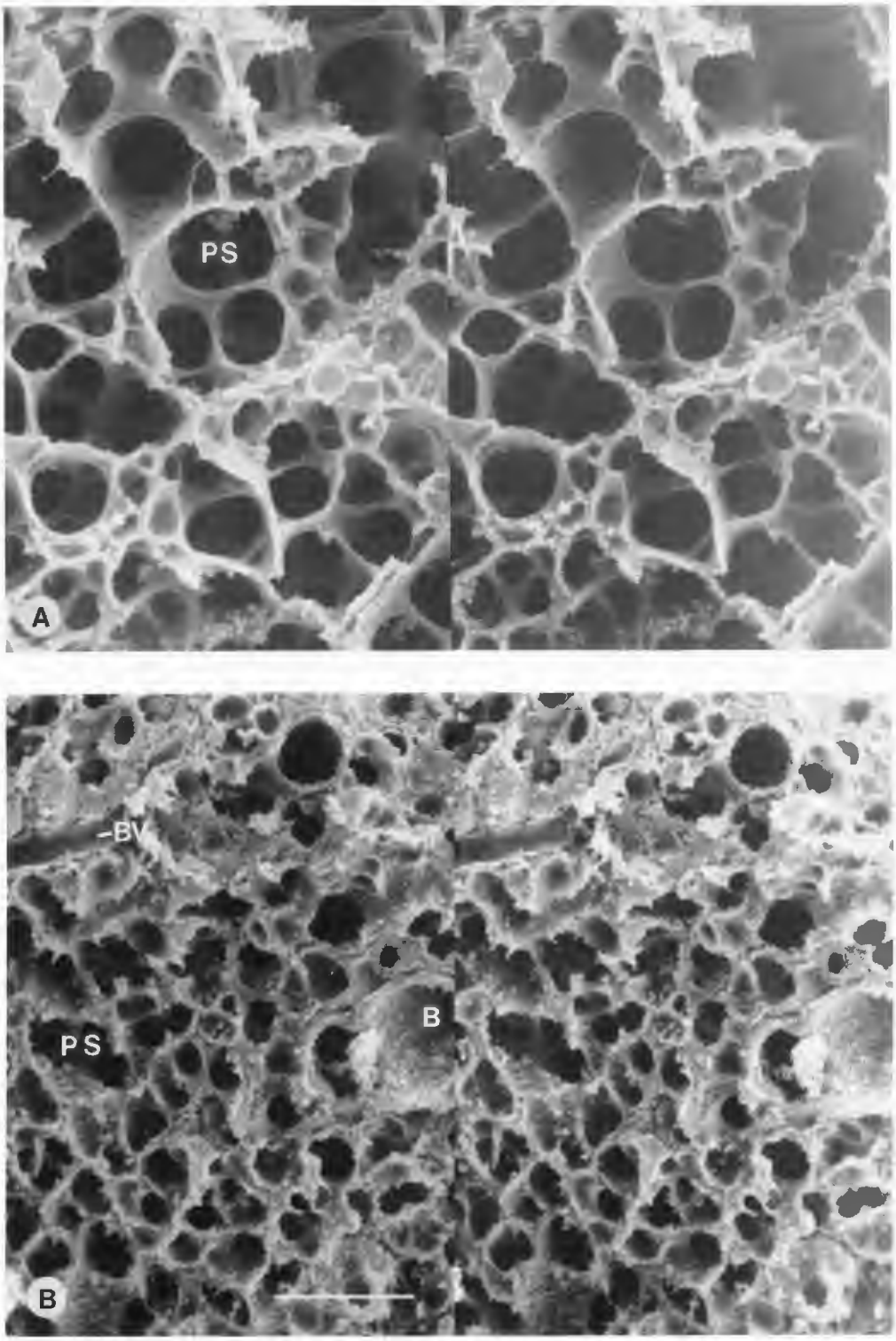

Figure 5. Stereopairs of primary saccules of day-18 fetal mouse lungs. The well-distended primary saccules (PS) shown are typical for a normal lung (A) in contrast with the relatively undistended primary saccules (PS) of the hypoplastic lung (B). Normal appearing bronchiole (B) and blood vessel (BV) are depicted in the mutant. (Bar = 100 μm).

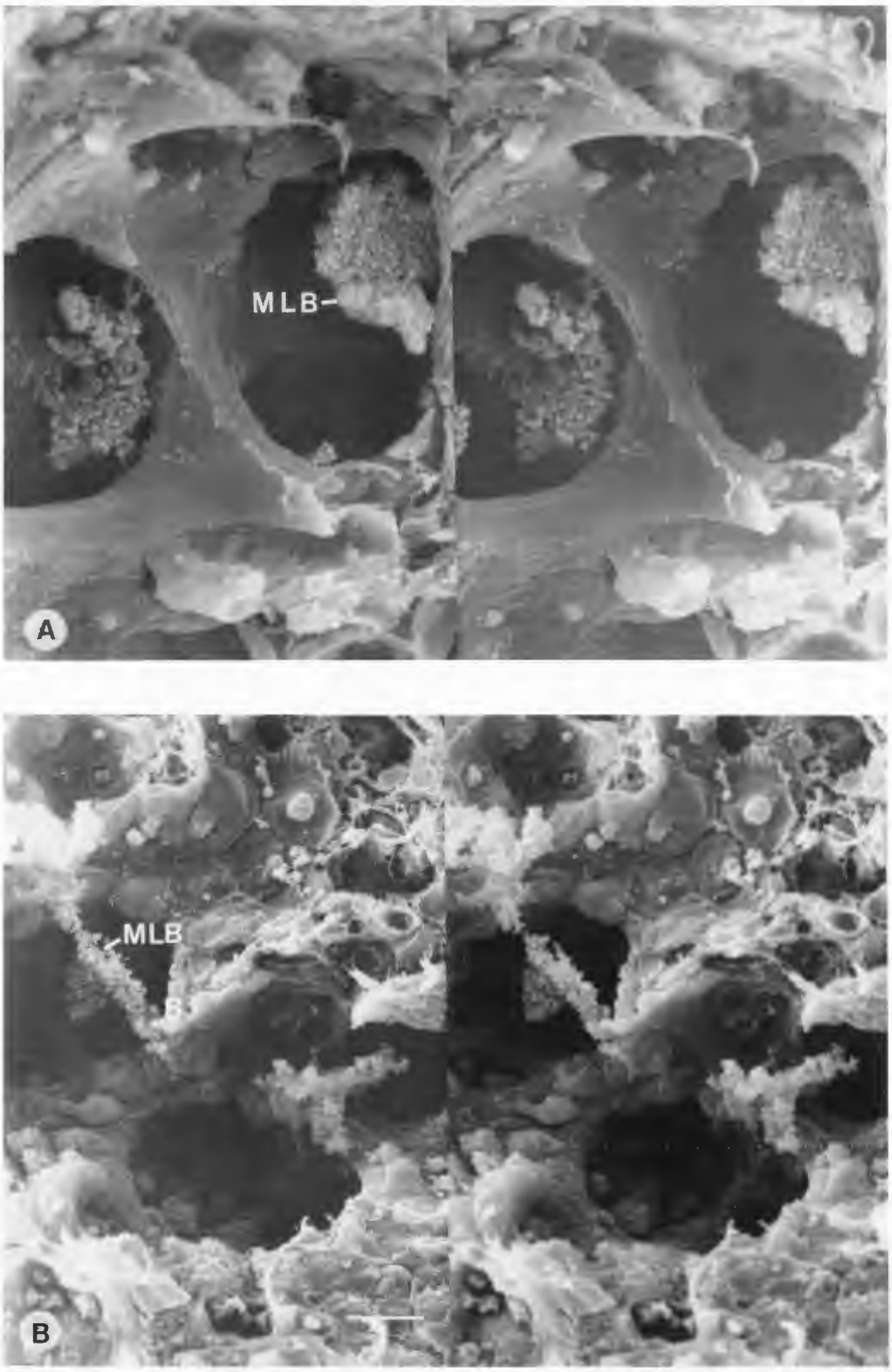

Figure 6. Compact multilamellar bodies (MLB) were routinely observed within primary saccules of both normal (A) and mutant (B) fetal lungs. (Bar = 10 μm).

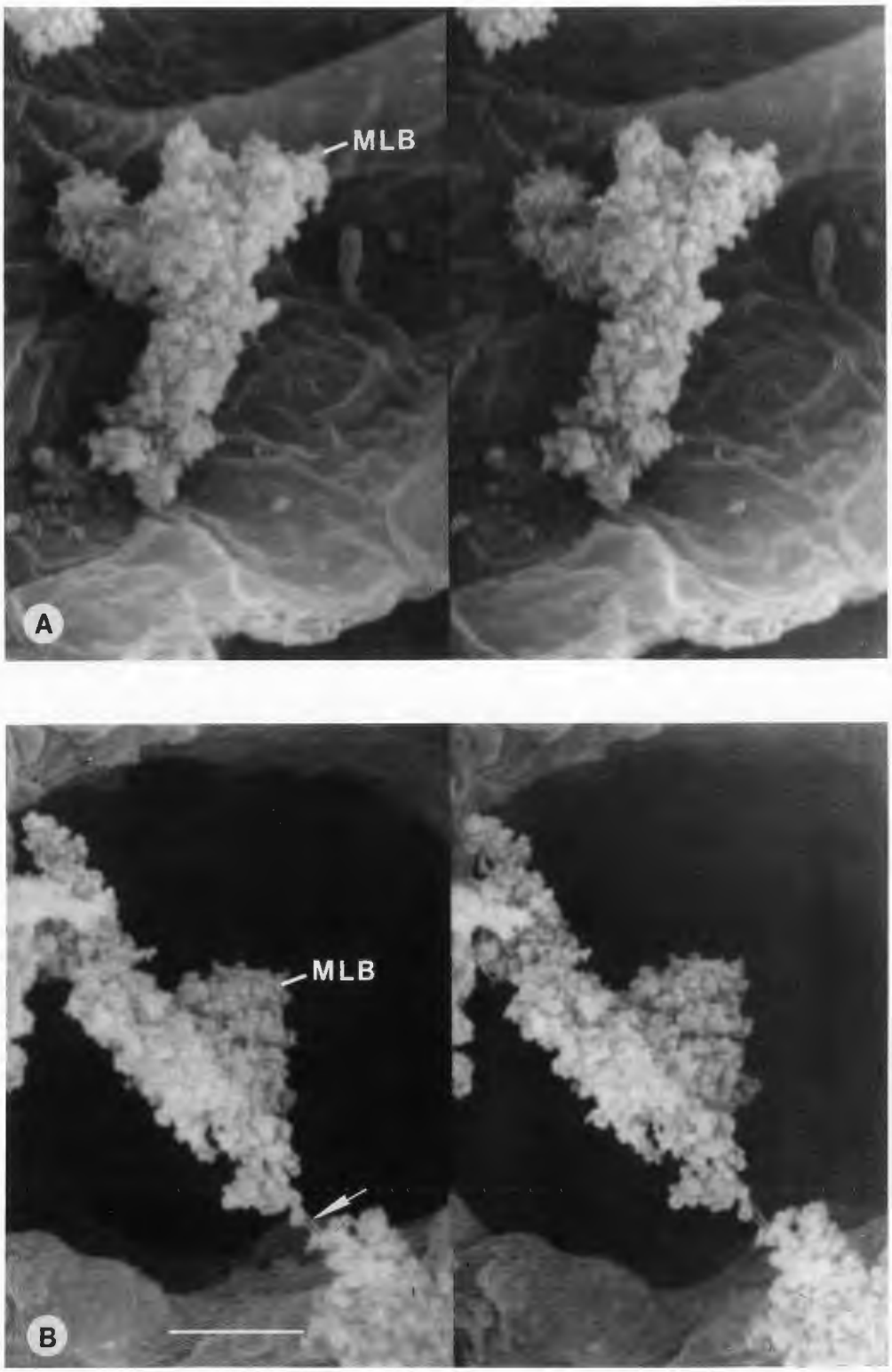

Figure 7. Multilamellar body (MLB) shown within a primary saccule of the normal lung (A). In the mutant (B) MLB's appeared normal. This particular MLB spanned the primary saccule. Note the strand of tubular myelin (arrow) connecting two clusters of lamellar bodies. (Bar = 5 μm).

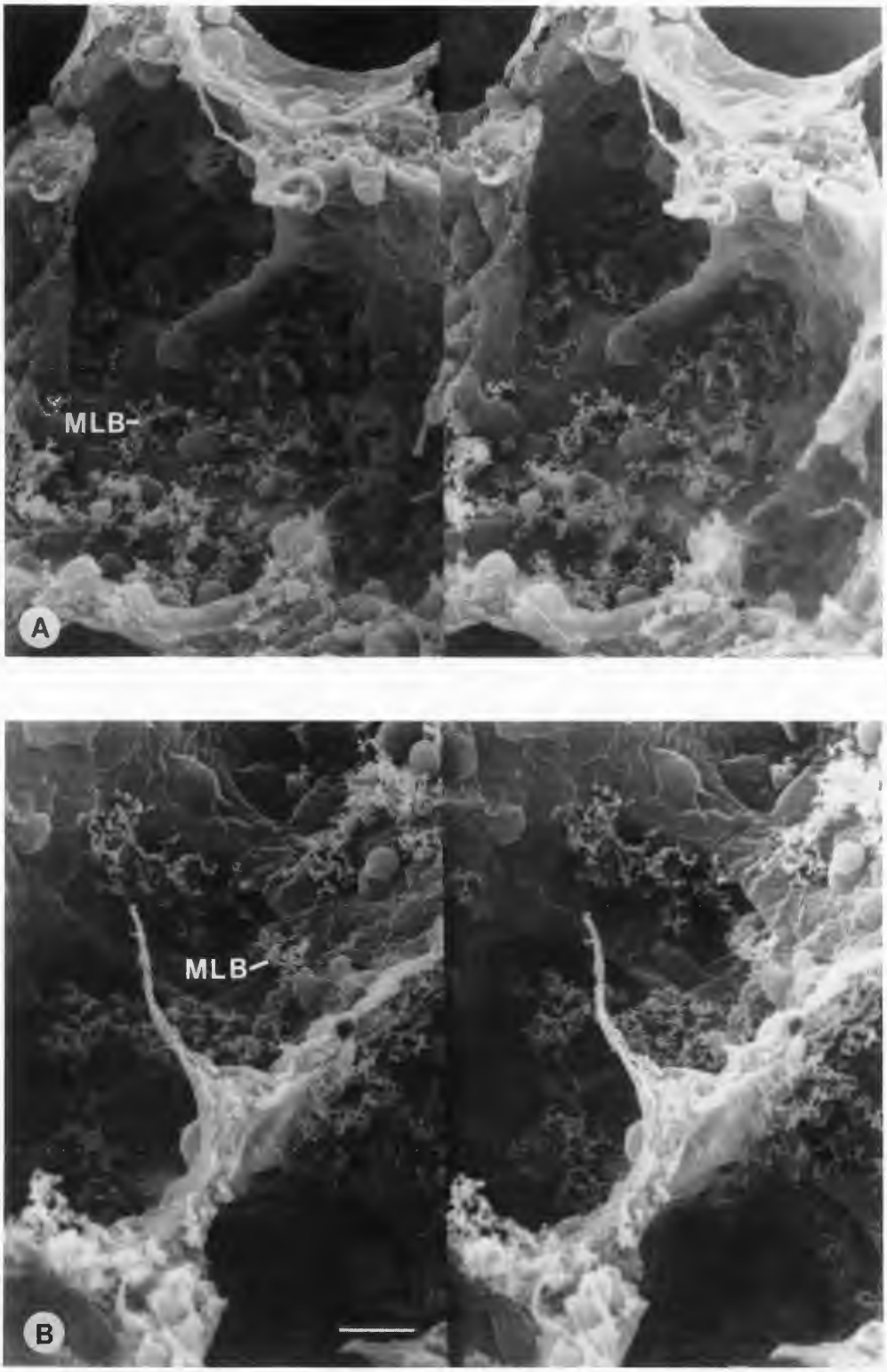

Figure 8. Multilamellar bodies (MLB) less compact than those shown in Figs. 6 and 7 were found throughout the primary saccules of both normal (A) and hypoplastic lungs (B). (Bar = 10 μm).

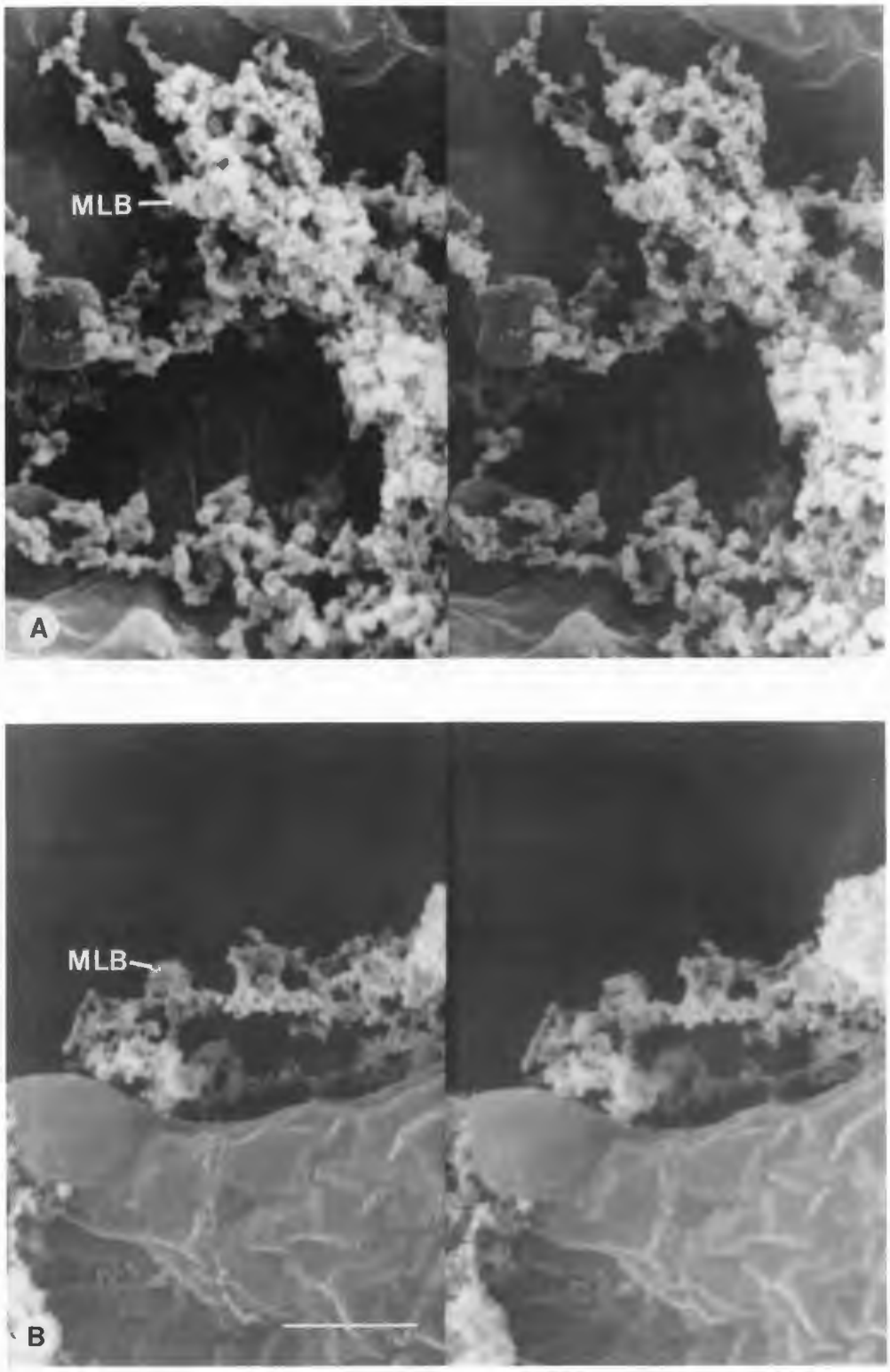

Figure 9. Higher magnification of less compact multilamellar bodies shown in Figure 8. In both normal (A) and mutant (B) the MLB also appears in a more diffuse configuration. (Bar = 10 μm).

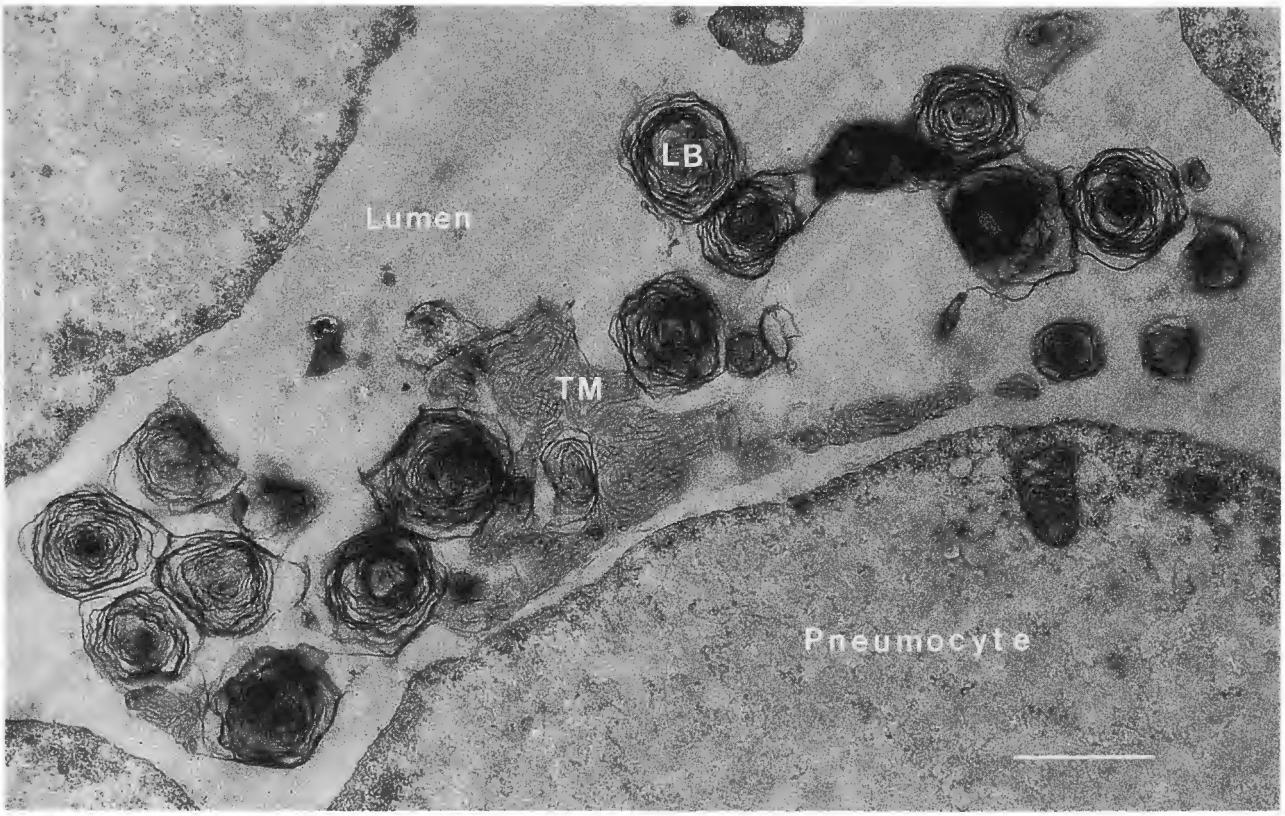

Figure 10. Transmission electronmicrograph of a multilamellar body (MLB; cf. Figs. 6-7) within the lumen of a primary saccule. Note the type II pneumocytes surrounding the lumen and their secretory product, the multilamellar body. As shown with SEM, the MLB consists of many single lamellar bodies (LB) which appear to be in the process of unwinding to form tubular myelin (TM). Tubular myelin in thin section appears cross-hatched and is presumed to be the surfactant precursor.
(Bar = 1 μm).

One of the parameters thought to be of greatest consequence to the demise of human newborns with pulmonary hypoplasia is the size of the alveoli. From histological sections, the area of the mutant's primary saccules (precursors of alveoli) was estimated to be 24% of normal[93], which equates to a diameter 49% of normal. From scanning electronmicrographs the mutant's lungs showed a comparable decrease in average saccule diameter (50% of normal). Stereoscopic visual evidence also showed the mutant's primary saccules to have less volume.

Remnants of multilamellar bodies were found within the bronchial tree. Their presence at a site other than the primary saccule is most likely due to the flow of fluid in and out of the fetal lungs. The net flow of fluid in the fetal lungs has been reported to be outward[1]. The finding of multilamellar bodies in the airways indicates that the material was not drastically mobilized by the intratracheal fixation procedure.

The mutant's multilamellar bodies observed under SEM were morphometrically similar to those observed in the normal. They were present in both mutant and normal lungs in similar quantities and configuration, thus ruling out atelectasis, caused by a lack of surfactant, as a possible factor in the lethality of the *cho's* disorder.

The present stereoscopic SEM study has provided a new perspective of pulmonary hypoplasia in chondrodystrophic mice. The observations made in the present study support our previous findings of reduced primary saccule size in pulmonary hypoplasia. The mechanism by which the reduction in thoracic volume inhibits growth but not differentiation (i.e. surfactant system) of the type II pneumocytes remains to be demonstrated.

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Discussion with Reviewers

M.R. Harrison: Are there other demonstrable changes in the pulmonary vascular bed of the mutant mice compared to normals? One might expect to see a decreased number of vessels per unit lung.

Authors: The present study did not lend itself to such a morphometric assessment. We are, however, presently carrying out research which addresses this very question. We will use a computer-aided 3-D image analyzing system to reconstruct serial sections of lungs. We are also attempting to create microvascular casts of the lungs. Both of these methods should allow the assessment of branching and measurement of the volume of the vasculature.

D.E. Schraufnagel: How are the mutants detected?

Authors: At day 18 the mutants are detected by the presence of cleft palates, micromelia, and a shortened snout, all of which are 100% penetrent. These parameters can be used to distinguish the mutants as early as day 15, however, detection of days 13 and 14 mutants must be accomplished through a histological screen of the cartilage. See Seegmiller et al., 1971, 95.

D.E. Schraufnagel: Could the fetal lungs have been inflated with fixative before they were excised?
Authors: This is a possibility. It should be performed to provide an estimate of the mutant's lung capacity to expand *in vivo*.

Reviewer III: Are there data to demonstrate that the structures observed by SEM are, as stated, actually lamellar bodies?
Authors: Based on TEM we conclude that the structures viewed with SEM are lamellar bodies of varying size (.22-1.23 μm). We are presently carrying out a phospholipid assay of the lungs to determine their surfactant content. We are also attempting to reconstruct a 3-D computer generated image of serial sectioned multilamellar bodies viewed with TEM.

Reviewer IV: Why was such a high perfusion/fixation pressure (100 cm) required?
Authors: The "high pressure" was required to overcome the resistance of the 30 gauge needle, which was greatly reduced in size compared to that used in previous experiments. The flow rate achieved by this procedure was comparable to previously reported flow rates.

Reviewer V: How may a reader obtain information cited in reference number 26?
Authors: The information can be found in a book (in press) entitled Models of Lung Disease: Microscopy and Structural Methods, In: Lung Biology in Health and Disease Series, Joan Gill, ed., Marcel Dekker, New York.