Absence of Tubular Myelin in Lungs of Infants Dying With Hyaline Membrane Disease

DAPHNE E. deMELLO, MD, E. Y. CHI, PhD, E. DOO, MD, and DAVID LAGUNOFF, MD

From the Departments of Pathology, St. Louis University School of Medicine and Cardinal Glennon Children's Hospital, St. Louis, Missouri, and University of Washington, Seattle, Washington

Immaturity of the pulmonary surface active material synthesizing system with deficiency of surface active material in the premature lung is an accepted cause of hyaline membrane disease. Lamellar bodies, the intracellular form of surface active material, are produced and secreted from Type II pneumocytes and converted to tubular myelin in the alveolar lumen. Tubular myelin, in turn, gives rise to the surface monolayer, which has the greatest surface active property. Thus, lung sections were studied by light and electron microscopy from 35 infants who died of histologically confirmed hyaline membrane disease and 19 infants who died of other causes. Tubular myelin was not identified ultrastructurally in lungs of infants who died of hyaline membrane disease, despite the presence of abundant lamellar bodies. In contrast, 16 of 19 infants dying of other causes had easily identifiable tubular myelin in addition to lamellar bodies. The absence of tubular myelin in the hyaline membrane disease patients suggests an abnormality in the conversion of lamellar bodies to tubular myelin. The authors speculate that this abnormal lamellar body turnover may be important in the pathogenesis of hyaline membrane disease. (Am J Pathol 1987, 127:131–139)

HYALINE membrane disease (HMD), or idiopathic respiratory distress syndrome (IRDS), is a major cause of perinatal mortality.[1] Immaturity of the surface active material (SAM) synthesizing system, with resultant deficiency, is now widely accepted as the cause of the disease. Current therapy is successful in many, but not all, cases. The reasons for therapeutic failures are not always clear.

Pattle[2] and Clements[3] defined the importance of SAM for normal respiratory function. Subsequently Avery and Mead[4] highlighted the connection between HMD and a deficiency of pulmonary SAM. SAM occurs in the lung in three ultrastructurally distinct forms: intracellular and extracellular lamellar bodies (LB), extracellular tubular myelin (TM), and a surface monolayer.[5–8] Lamellar bodies are extruded from Type II pneumocytes where they are produced and are transformed into tubular myelin. Both can spread at air/water interfaces and exhibit surface activity.[9,10] TM appears to give rise to the phospholipid monolayer at the air/fluid interface in the lung that constitutes the actual functional surfactant.[7,10]

In infants dying with HMD, studies have shown a clear correlation between a low number of lamellar bodies, particularly extracellular lamellar bodies, and decreased surface activity of lung extracts. The decreased number of lamellar bodies in immature lungs is temporary; their number increases as Type II cells increase.[11] An excess of Type II cells rich in lamellar bodies may be found in some infants, perhaps as a consequence of oxygen therapy.[12,13] For adequate surface activity, the lipid must spread at the air/fluid interface. Tubular myelin appears to be an intermediate form between lamellar bodies and the surface layer of lipid, but there is little evidence on the sequence of events either in normal lungs or in lungs of patients with IRDS. It is possible that in addition to, or as an alternative to, inadequate formation of LBS, a block in secretion of LB, a failure to form tubular myelin in the alveolar space, or a defect in the production of a surface active monolayer could contribute to respiratory distress in the newborn.

Supported in part by NHLBI Pediatric Pulmonary SCOR HL 19187 (W. A. Hudson, PI).

Accepted for publication November 12, 1986.

Address reprint requests to Daphne E. deMello, MD, Department of Pathology and Laboratory Medicine, Cardinal Glennon Children's Hospital, 1465 South Grand Boulevard, St. Louis, MO 63104.

Table 1—Subject Data

Number of patients	Gestational age (weeks)	Birth weight (g)	Postnatal survival	Males	Females
35 (HMD group)	23–38	540–2820	4 1/2 hours to 7 days	22	13
19 (control group)	40	2000–4248	2 days to 5 months	10	9

In an effort to study this process, we examined by light and electron microscopy the lungs of 35 infants dying with HMD. The lungs of 19 infants dying without HMD were studied as controls.

Materials and Methods

Samples of lung tissue were obtained at autopsy from 35 infants that died of hyaline membrane disease, at the Neonatal Intensive Care Units at the University of Washington and at Cardinal Glennon Children's Hospital. The gestational age of the infants ranged from 23 to 38 weeks, and the birth weights ranged from 540 to 2820 g. The postnatal age of the infants was 5 hours to 7 days. There were 22 boys and 13 girls (Table 1).

Autopsy lung samples were also obtained from 19 infants dying of causes other than HMD. One sample was from a surgical specimen. These were all full-term infants with birth weights of 2000–4248 g, and postnatal ages ranged from 2 days to 5 months. There were 10 boys and 9 girls (Table 1). The clinical diagnoses of this group of infants are listed in Table 2.

Tissue was taken from the right upper lobe apex in the form of 2-mm slices for light microscopy and 1-mm cubes for electron microscopy. The tissue was fixed in chilled 2% glutaraldehyde with 2% paraformaldehyde in a 0.1 M sodium cacodylate buffer, pH 7.2, for 4 hours. For light microscopy the tissue was washed two times in 0.1 M cacodylate buffer for 15 minutes per wash and dehydrated through graded alcohol concentrations. Plastic embedding, using the JB-4 protocol,[14] was followed by sectioning at 1 or 2 μ with a Sorvall-JB4 microtome. Sections were stained with hematoxylin and eosin (H&E), toluidine blue, periodic acid–Schiff, and methenamine silver.

For electron microscopy, aldehyde-fixed tissue cubes were rinsed in three 15-minute changes of 0.1 M sodium cacodylate and then postfixed in 1% 0s04 in 0.1 M sodium cacodylate for 1 hour. After distilled water rinses, the tissue cubes were stained in 0.5% uranyl acetate in distilled water for 30 minutes. After dehydration in increasing concentrations of alcohol and infiltration with propylene oxide–Epon mixtures, tissue cubes were embedded in Epon 812.[15] Sections 0.5 μ thick were cut on a Sorvall MT-1 microtome and stained with toluidine blue for light microscopy. Thin sections for electron microscopy were cut on a Sorvall MT-2 microtome, mounted on carbon-coated copper grids, and stained with uranyl acetate and lead citrate.

Tissue for freeze-fracture was cut into 1–2-mm cubes and fixed as described above. Cacodylate buffer rinses (0.1 M) were followed by incubation in 30% glycerol in 0.1 M sodium cacodylate for 12–24 hours. The tissue was placed on a Balzer copper freezing disk and frozen in Freon-22 cooled by liquid nitrogen.[16] Fracturing was done on a Balzer 301 machine, and replicas were coated with carbon and platinum.

Specimens were examined by two observers (either EC or DED). The identity of the cases was not known at the time of examination.

Results

Infants With HMD

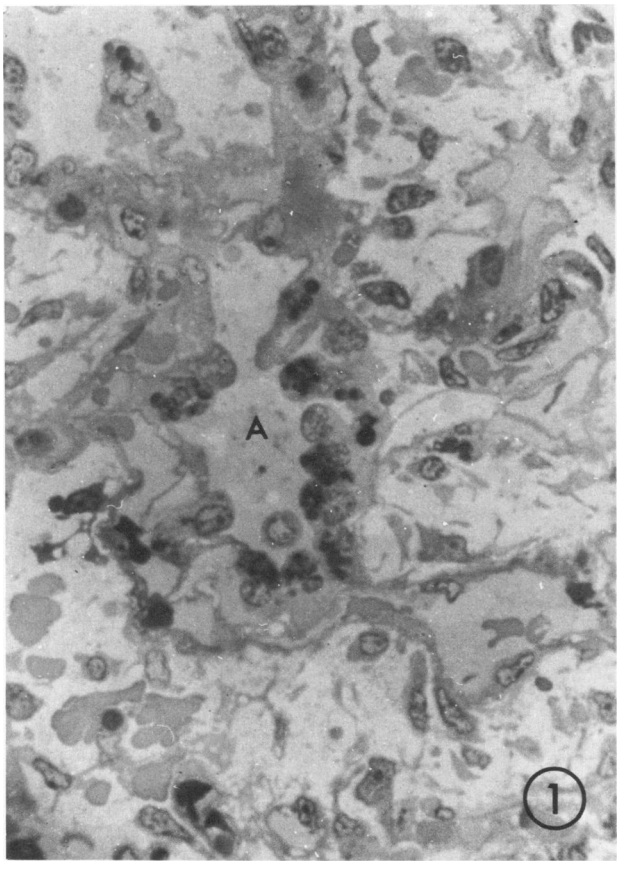
Light Microscopy

On H&E staining of lung the following features of HMD were consistently noted: the presence of hyaline membranes, irregular aeration of air spaces with widespread atelectasis, pulmonary congestion and/or hemorrhage, bronchial epithelial sloughing, and pulmonary edema. Dilated lymphatics, septal wall thickening, and partial reepithelialization of the alveoli were less consistently noted. Epon-embedded tissue sectioned for light microscopy revealed a paucity of alveolar Type I cells and concomitant hyperplasia of Type II cells containing lamellar bodies (Figures 1 and 2).

Table 2—Diagnoses in Control Group

1. Kasabach–Merritt syndrome
2. Galactosemia
3. Anoxic encephalopathy (cervical cord injury)
4. Volvulus, peritonitis, sepsis
5. Congenital heart disease (8) (2)*
6. Intussusception, sepsis
7. Meconium aspiration, bronchopneumonia, ECMO*
8. Short-limbed dwarfism
9. Congenital lobar emphysema
10. Persistent fetal circulation (2)
11. Bronchopulmonary dysplasia

The number in parentheses is the number of cases.
*Patients without TM. ECMO, extracorporeal membrane oxygenator.



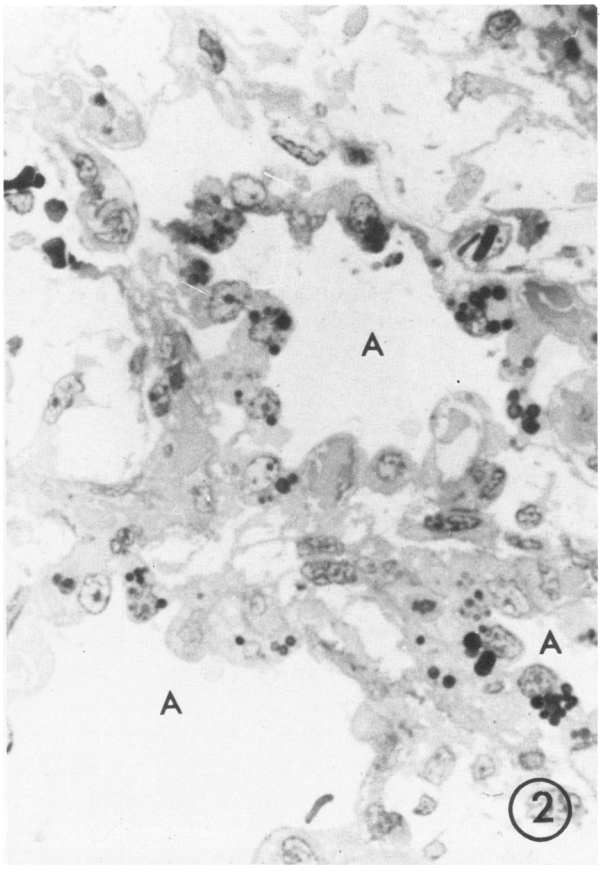


Figure 1—The lung tissue from a premature (27 weeks) infant who died 82 hours after birth. In the alveoli (*A*), the pneumocytes of the epithelial lining are mostly Type II cells containing many lamellar bodies. Glycol methoerylate embedded tissue, sectioned at 1 μ. (Toluidine blue, ×900) **Figure 2**—The lung tissue of a premature (30 weeks) infant who died 6 days after birth. The alveoli (*A*) are expanded; the pneumocytes are dominated by Type II cells. Glycol methoerylate embedded tissue, sectioned at 1 μ. (Toluidine blue, ×900)

Electron Microscopy

Postmortem autolysis[17,18] was apparent in all cases. Lamellar bodies, however, were well preserved. Typical lamellar bodies were observed intracellularly, in alveolar edema fluid and embedded in hyaline membranes (Figure 3a). No significant structural differences were noted between intracellular and extracellular lamellar bodies.

Most lamellar bodies, whether intracellular or extracellular, contained dense areas, sometimes positioned at the core of the lamellar body but usually extending to the outermost lamella, from a point just peripheral to the center. Structures within these dense areas included granular and amorphous masses, clusters of pinwheel formations, or woven or cross-hatched formations (Figure 4a). Lamellas typically inserted into these dense regions. Lamellar bodies with more than one dense area had lamellae extending between the dense areas (Figure 5a). Intensive search failed to uncover tubular myelin within the alveoli of any of the 35 infants with HMD studied by electron microscopy. Freeze-fracture findings generally corroborated TEM observations. Lamellar bodies were found intra- and extracellularly (Figure 3b). Fracture faces of the lamellas were smooth (Figure 4b); lamellar body dense areas exhibited a general graininess (Figure 5b).

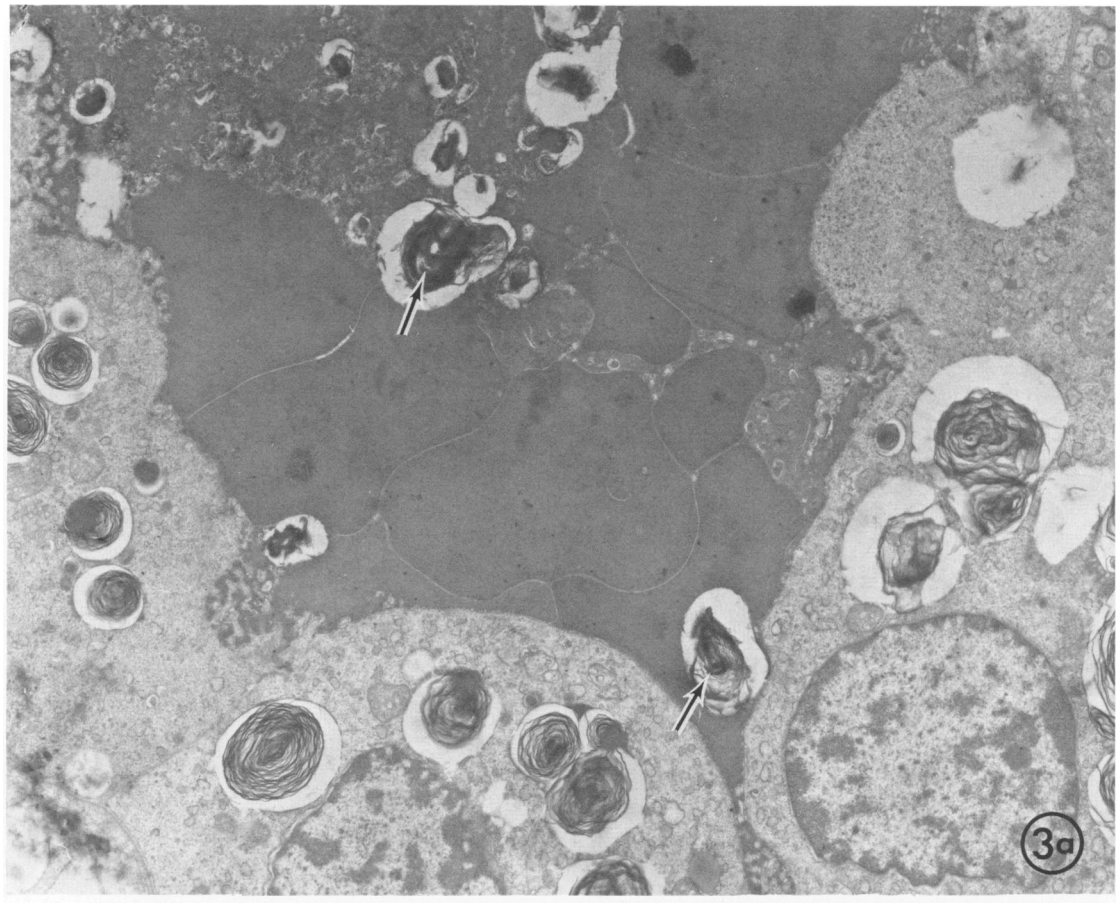
Infants Without HMD

Light Microscopy

H&E staining of lung tissues from the 19 infants without HMD revealed no eosinophilic hyaline membranes. In general, the lungs appeared structurally normal; two infants had acute bronchopneumonia. Compared with the lungs of infants with HMD, a greater area of the alveolar lining epithelium was covered by Type I cells in the lungs of the control cases.

Electron Microscopy

Extracellular lamellar bodies found within alveolar spaces were ultrastructurally similar to those in the lungs of infants with HMD.


3a


LB
3b

In the extracellular alveolar compartment, clumps of tubular myelin, identified by the characteristic lattice pattern, were readily observed in 16 of the 19 control cases. No obvious relationship between the number of extracellular lamellar bodies and the amount of tubular myelin was noted.

The tubular myelin lattice in cross-section consisted predominantly of 30 × 35-nm rectangles. Incorporated into the lattice structure were dense areas, reminiscent of the dense regions within lamellar bodies (Figure 6). These dense areas often appeared to be masses of tightly compressed, intersecting bilayer membrane and were frequently encircled by a loose bilayer membrane. Tubular myelin appeared to arise from lamellar bodies as described for other mammalian species (Figure 7). Often, several lamellar bodies were seen to participate in the formation of a single continuous mass of tubular myelin.

Discussion

In this study of the lungs of 35 infants with HMD, in no instance was tubular myelin observed, although Type II cells and lamellar bodies were present and frequently abundant. In contrast, lamellar bodies and tubular myelin forms of SAM were readily observed in the lungs of infants dying with primary diseases other than HMD.

Lamellar bodies are thought to transform into tubular myelin and then into the alveolar lining monolayer.[13,19,20] Failure of this transition may result from 1) intrinsic lamellar body defects; 2) defects in, or deficiencies of, nonlamellar body material required to complete the transformation; or 3) improper environmental conditions for the transformation.

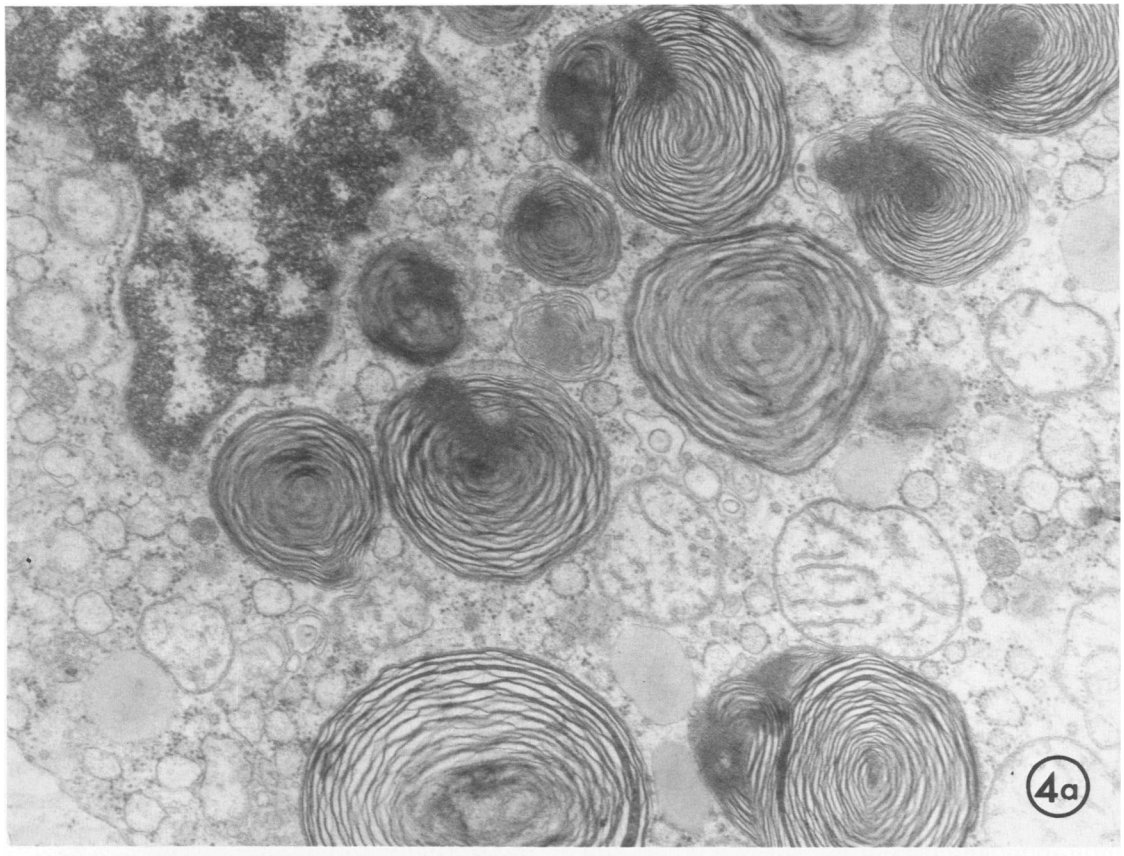
Infants with HMD have a lower percentage of palmitic acid and higher percentages of 18-carbon and 20-carbon fatty acids in surfactant isolated from pharyngeal and tracheal aspirates, compared with gestational age-matched controls.[21] Hallman et al[22] earlier found that phosphatidylglycerol, a minor surfactant phospholipid, was completely absent from lung washes of infants with HMD, but appeared after recovery from the disease. Since the composition of surfactant probably determines its ability to transform to tubular myelin as well as to serve as an effective surface active material, it is possible that intrinsic lamellar body defects in HMD could account for their failure to form tubular myelin.

It has been postulated that formation of tubular myelin from lamellar bodies requires nonlamellar body components.[23,24] Absence of essential extrinsic components could account for the absence of tubular myelin in infants with HMD. Chi and Lagunoff[25] have shown that intramembranous particles are not present in lamellar bodies, but become evident first in membranes transitional between lamellar bodies and tubular myelin. It was proposed that the particles, possibly hydrophobic proteins, may play a significant role in the organization of tubular myelin. Groniowski[26,27] and Walski[28] suggested that a glycoprotein on the cell membrane of the Type II pneumocyte may participate in the transformation of lamellar bodies into tubular myelin, and proposed a scheme for the incorporation of such glycoproteins into the lamellar body structure during the process of extrusion from the Type II pneumocyte. It is tempting to speculate that a defect in the incorporation of non-LB components might explain the absence of intraalveolar TM, despite the presence of abundant intracellular LB in the HMD patients. Sanders et al[19] have shown that lamellar bodies isolated in Ca^{2+}-free sucrose form tubular myelin lattices when Ca^{2+} is added, which indicates the importance of the ionic environment in the transformation to tubular myelin.

Alternatively, the absence of tubular myelin in the lungs of patients dying with HMD may be related to the oxygen therapy and positive pressure ventilation they received. However, high concentrations of oxygen have been associated with increases in both lamellar bodies and tubular myelin in alveoli of rats,[13] which suggests that oxygen therapy does not suppress surfactant maturation, but may enhance lamellar body and tubular myelin formation. Furthermore, many of the control infants in whom TM was found had also received oxygen therapy and positive pressure ventilation. Another formal possibility we cannot refute is that TM forms in patients with HMD but is unstable either in life or in the postmortem interval before autopsy.

Because neonatal intensive care units are referral centers, the patient population studied is skewed toward severely affected infants. It could be argued that a minimum number of days is required for the transformation of extracellular lamellar bodies to tubular myelin, and that patients who can be maintained for this period would be expected to live, whereas those dying in the interim might have little or no tubular myelin. In the experiments of Sanders et al[19] the conversion of LB to TM occurred promptly

Figure 3a—Electron micrograph of lung tissue (6 hours after death) of a premature (33 weeks) infant who died 5 days after birth. Note that the alveolar epithelial cells are Type II; materials released from lamellar bodies can be seen in the alveolar space (*arrows*). (×9000) **b**—Freeze-fractured alveolus, same case. The Type II cell is fully developed and contains many lamellar bodies (*LB*). (×10,000)



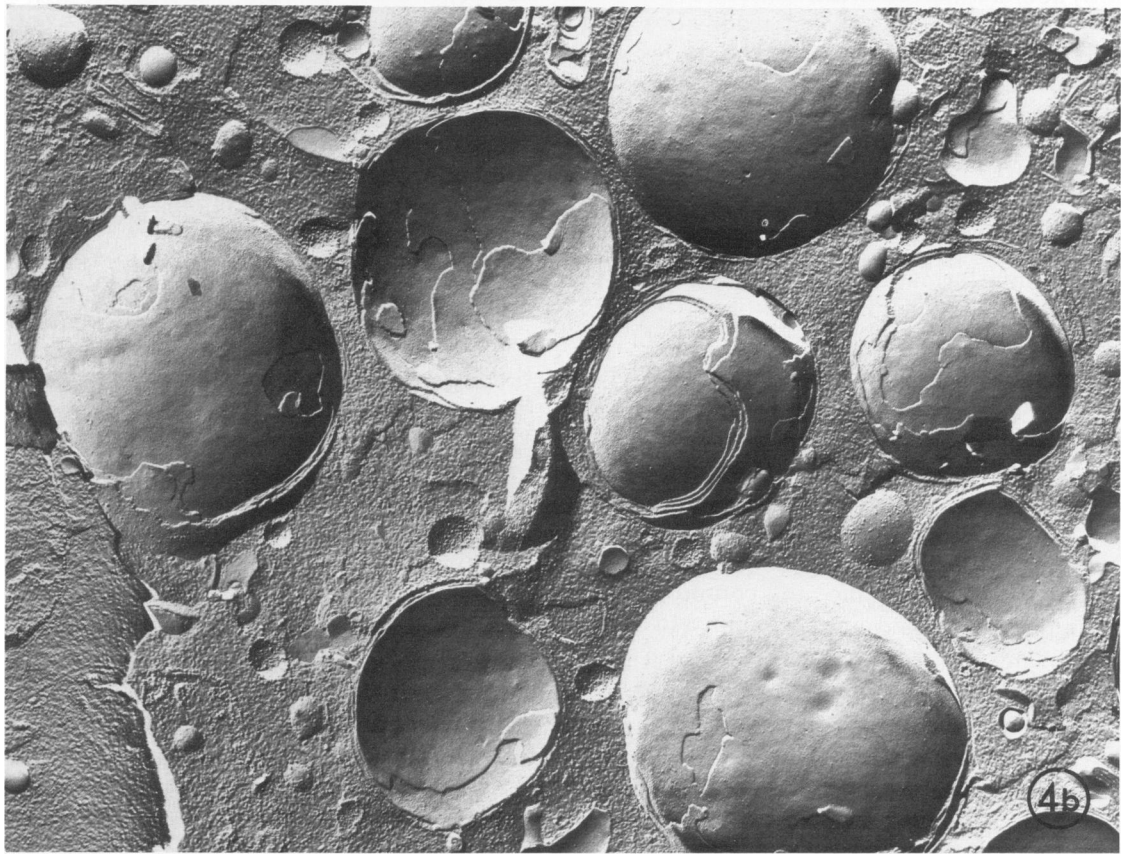


Figure 4a—Transmission electron micrograph of a Type II pneumocyte in a premature infant; the lamellar bodies are well developed. (×27,000) **b**—Freeze-fractured Type II pneumocyte, same case. The lamellar bodies contain only smooth multilayered membranes. No intramembranous particles can be seen. (×34,000)

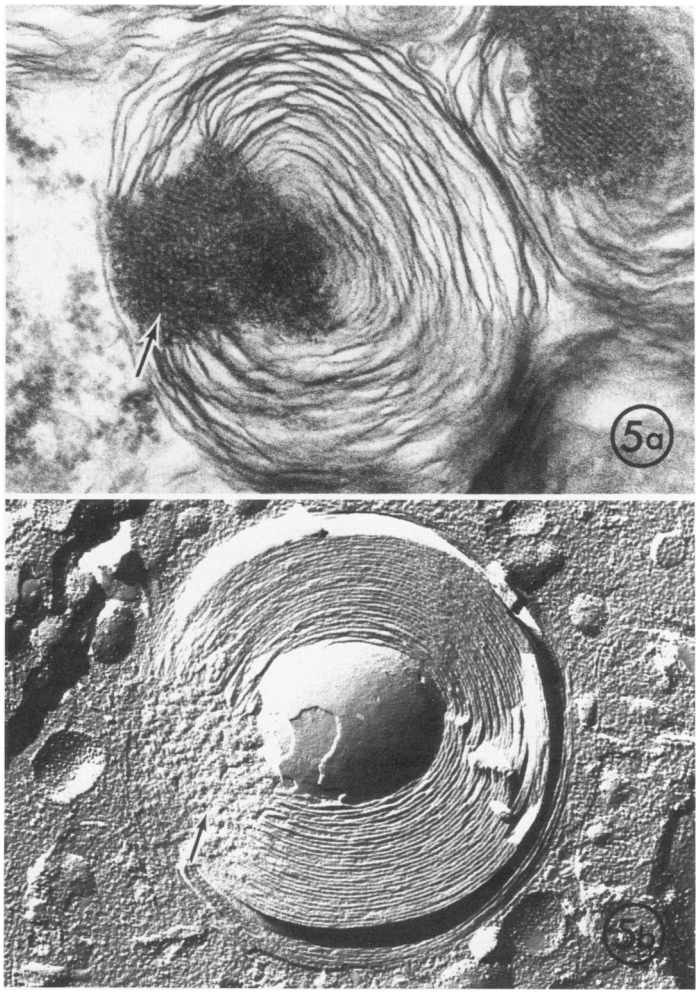


Figure 5a—A lamellar body in a Type II cell. Higher magnification. Note the tubular-like structures in the dense area; the membrane layers seem to radiate from it (*arrows*). (×50,000) **b**—Freeze-fractured lamellar body shows the dense area contains no defined structure (*arrow*). (×42,000)

upon the addition of Ca^{2+}, suggesting that there is no necessity for a time lapse in order that this transformation might occur. Furthermore, TM was absent in all HMD patients, even those who survived 3 or more days, making this an unlikely explanation for its absence. Our ability to find tubular myelin in the infants without HMD as long as 60 hours after death, makes us confident that the consistent absence of tubular myelin in the HMD infants is not attributable to a generally manifest postmortem instability of tubular myelin.

We were unable to identify TM in three control infants. Two of these infants had acute bronchopneumonia, which may have played a role in obscuring the TM in these infants. The third infant had unexplained atelectasis of the right upper lobe, which was the portion of lung sampled for the study. What role the absence of TM played in the development of atelectasis in this case is uncertain.

The absence of TM in all 35 of the HMD patients, and its presence in 16 of 19 control patients, is statistically highly significant (chi-square analysis, $P < 0.005$).

Obviously, the ideal control population would be the group of infants who recover from HMD, or a group of comparably premature infants without HMD. However, because lung samples from such infants are not likely to become available, the study of bronchoalveolar lavages obtained during routine pulmonary toilet on intubated infants might be useful. Ten lavages from premature infants and 6 from full-term infants were examined in a pilot study. The fluid was centrifuged at 27,000g for 20 minutes at 0 C according to the method outlined by Finley et al.[29] The sediment was diced and examined by electron microscopy. Many lamellar bodies, some intact and some partially disrupted, were seen, in addition to large amounts of amorphous particulate matter. TM was

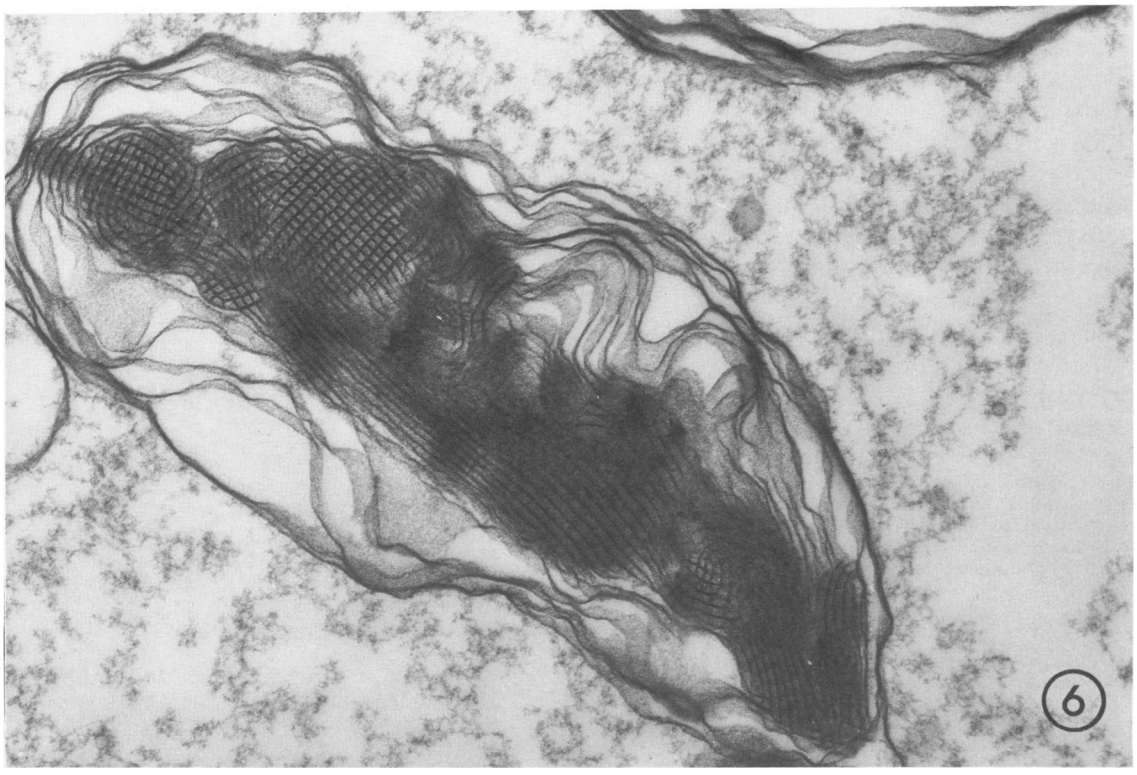


Figure 6—Alveolar tubular myelin of a full-term infant who died 1 day after birth. The lattice structure is clearly present in the secreted lamellar body. (×40,000)

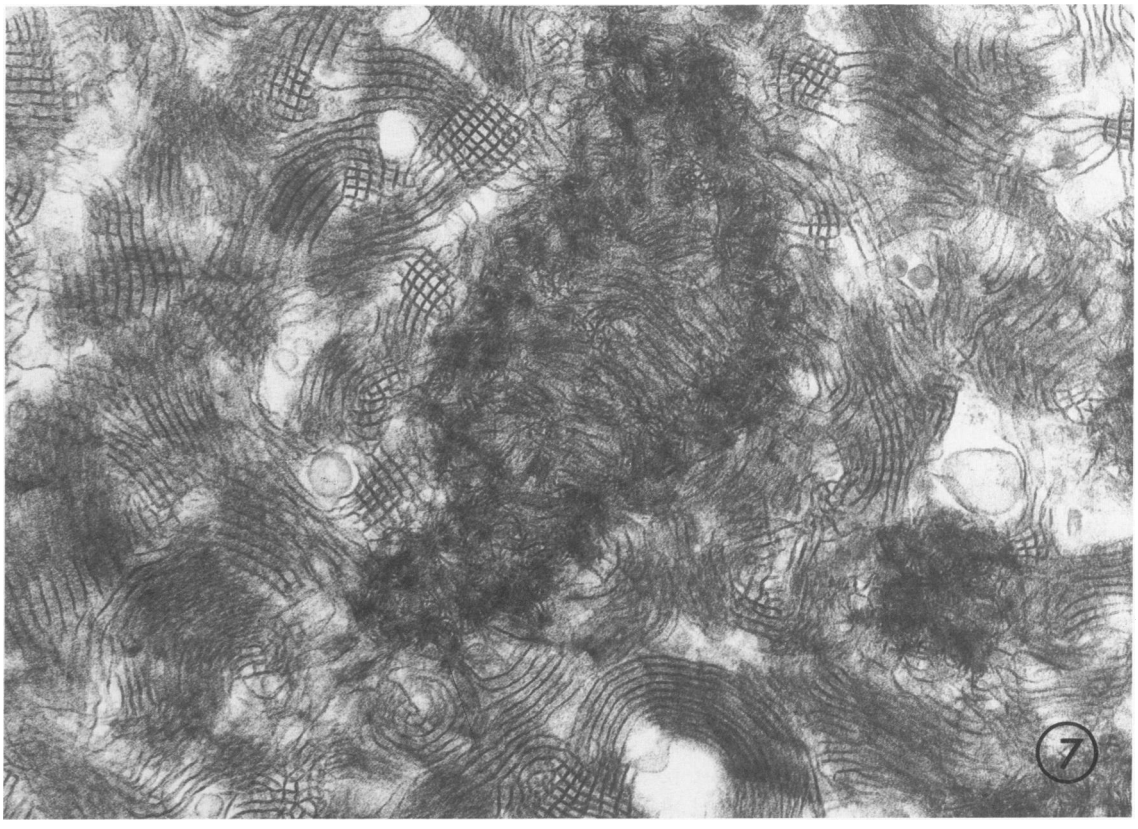


Figure 7—Tubular myelin from the lung of a term infant who died 2 days after birth. (×40,000)

not identified in either group, although Finley et al[29] were able to demonstrate TM in lavages from dog lungs using this technique. Hook et al[30] have studied bronchoalveolar lavage effluents of patients with pulmonary alveolar proteinosis. Although in such patients large amounts of TM fill the alveolar spaces,[31] Hook et al[30] found that TM constituted only 1.6% of the total particle volume. The remainder, as in our experience, was made up of multilamellated structures and miscellaneous material.

In spite of our inability to provide completely satisfactory controls, we believe that our observations on the lungs of patients dying with HMD and the available control lungs are consistent enough to be worthy of consideration in an effort to explain the failure of therapy in a substantial group of patients.

References

1. Farrell PM, Avery ME: Hyaline membrane disease. Am Rev Respir Dis 1975, 111:657–688
2. Pattle RE: Properties, function and origin of the alveolar lining layer. Nature 1955, 175:1125–1126
3. Clements JA: Surface tension of lung extracts. Proc Soc Exp Biol Med 1957, 95:170–172
4. Avery ME, Mead J: Surface properties in relation to atelectasis and hyaline membrane disease. Am J Dis Child 1959, 97:517–523
5. Kikkawa Y: Morphology of alveolar lining layer. Anat Rec 1970, 167:389–400
6. Weibel ER, Gil J: Electron microscopic demonstration of an extracellular duplex lining layer of alveoli. Respir Physiol 1968, 4:45–57
7. Weibel ER, Kistler JS, Tondury G: A sterologic electron microscope study of "tubular myelin figures" in alveolar fluids of rat lungs. Z Zellforsch Microsk Anat 1966, 69:418–427
8. Sorokin SP: A morphologic and cytochemical study on the great alveolar cell. J Histochem Cytochem 1967, 14:884–897
9. Gil J, Reiss OK: Isolation and characterization of lamellar bodies and tubular myelin from rat lung homogenates. J Cell Biol 1973, 58:152–171
10. Paul GW, Hassett RJ, Reiss OK: Formation of lung surfactant films from intact lamellar bodies. Proc Natl Acad Sci USA 1977, 74:3617–3620
11. Balis JU, Delivonia M, Conen PE: Maturation of postnatal human lung and the Idiopathic Respiratory Distress Syndrome. Lab Invest 1966, 15:530–546
12. Anderson WR, Strickland MB, Tsai SH, Haglin JJ: Light microscopic and ultrastructural study of the adverse effects of oxygen therapy on the neonate lung. Am J Pathol 1973, 73:327–348
13. Kistler GS, Caldwell PRB, Weibel ER: Development of fine structural damage to alveolar and capillary lining cells in oxygen-poisoned rat lungs. J Cell Biol 1967, 33:605–628
14. Chi EY, Smuckler EA: A rapid method for processing liver biopsy specimens for 2 m sectioning. Arch Pathol Lab Med 1976, 100:457–462
15. Luft JH, Improvement in epoxy resin embedding methods. J Biophys Biochem Cytol 1961, 9:409–414
16. Chi EY, Lagunoff D, Koehler JK: Abnormally large lamellar bodies in type II pneumocytes in Chedick-Higashi syndrome in beige mice. Lab Invest 1976, 34(2):166–173
17. Bachofen M, Weibel ER, Roos B: Postmortem examination of human lungs for electron microscopy. Am Rev Respir Dis 1975, 111:247–256
18. Lauweryns JM: Hyaline membrane disease in newborn infants. Hum Pathol 1970, 1:175–204
19. Sanders RL, Hassett JR, Vatter AE: Isolation of lung lamellar bodies and their conversion to tubular myelin figures in vitro. Anat Rec 1980, 198:485–501
20. Sanderson RJ, Vatter AE: A mode of formation of tubular myelin from lamellar bodies in the lung. J Cell Biol 1977, 74:1027–1031
21. Shelley SA, Kovacevic M, Paciga JE, Balis JU: Sequential changes of surfactant phosphatidylcholine in hyaline membrane disease of the newborn. N Engl J Med 1979, 300:112–116
22. Hallman M, Feldman BH, Kirkpatrick E, Gluck L: Absence of phosphatidylglycerol (PG) in respiratory distress syndrome in the newborn. Pediatr Res 1977, 11:714–720
23. Williams MC: Freeze-fracture studies of tubular myelin and lamellar bodies in fetal and adult rat lungs. J Ultrastruct Res 1978, 64:352–361
24. Williams MC, Mason R: Development of the type II cell in the fetal rat lung. Am Rev Respir Dis 1977, 115:37–47
25. Chi EY, Lagunoff D: Linear arrays of intramembranous particles in pulmonary tubular myelin. Proc Natl Acad Sci 1978, 75:6225–6229
26. Groniowski J: Presumable role of surface glycoprotein of alveolar pneumonocytes in transformation of lamellar bodies into tubular myelin. Acta Med Pol 1979, 20:393–396
27. Groniowski JA: Fine structural basis of pulmonary surfactant. Int Rev Exp Pathol 1983, 25:183–238
28. Groniowski JA, Walski M: Further studies on the tubular myelin structure of extracellular alveolar lining layer. Acta Med Pol 1979, 20:15–16
29. Finley TN, Pratt SA, Ladman AJ, Brewer L, McKay MB: Morphological and lipid analysis of the alveolar lining material in dog lung. J Lipid Res 1968, 9:357–365
30. Hook GER, Gilmore LB, Talley FA: Multilamellated structures from the lungs of patients with pulmonary alveolar proteinosis. Lab Invest 1984, 50:711–725
31. Gil J: Histological preservation and ultrastructure of alveolar surfactant. Ann Rev Physiol 1985, 47:753–763