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Immunogold Localization of SP-A in Lungs of Infants Dying from Respiratory Distress Syndrome

Daphne E. deMello,* Sarah Heyman,* David S. Phelps,† and Joanna Floros‡

From the Departments of Pathology, Cardinal Glennon Children's Hospital and St. Louis University School of Medicine, St. Louis, Missouri; and Pediatrics,† and Cellular and Molecular Physiology,‡ The Pennsylvania State University, College of Medicine, Hershey, Pennsylvania*

Prematurely born infants can develop the neonatal respiratory distress syndrome (RDS) because of a deficiency of pulmonary surfactant. This lipoprotein complex synthesized by type II pneumocytes has different ultrastructural forms—intra- and extracellular lamellar bodies, which within the alveoli are transformed into tubular myelin, and this in turn gives rise to the surface monolayer, the functionally active form of surfactant. We have previously shown that at autopsy RDS lungs lack tubular myelin and have decreased immunoreactivity for antisera to surfactant protein A (SP-A), an important component of tubular myelin. Therefore, we proposed a role for SP-A in the conversion of lamellar bodies to tubular myelin and in the pathogenesis of RDS. To explore this possibility further, we compared in 14 RDS and 14 control lungs the distribution of SP-A in ultrathin sections, using affinity-purified rabbit anti-human-SP-A IgG and goat anti-rabbit IgG-conjugated with 10 nm colloidal gold particles. In controls, gold label was present in lamellar bodies, endoplasmic reticulum, on the cytoplasmic membrane of type II cells, and on lamellar bodies and tubular myelin either within alveoli or macrophages. In RDS lungs, reduced label was present in the same intracellular compartments and organelles, except in tubular myelin, which is absent. It is postulated that if SP-A is indeed necessary for the conversion of lamellar bodies to tubular myelin, in RDS either there is a deficiency of adequate amounts of functional SP-A or some other important component of surfactant is missing. (Am J Pathol 1993, 142:1631–1640)

The neonatal respiratory distress syndrome (RDS) results from a deficiency of pulmonary surfactant.[1] Surfactant, a lipoprotein complex secreted by type II pneumocytes, contributes to alveolar stability during respiration.[2] It consists primarily of lipids (90%) and proteins (10%).[3,4] Inside the cell, the lipoprotein complex has the ultrastructural form of lamellar bodies that, when secreted from the type II pneumocytes, are transformed into tubular myelin within the alveolar space. Tubular myelin gives rise to the phospholipid monolayer which constitutes functional surfactant at the air fluid interface of the alveolus.[5,6] Four surfactant proteins have been identified to date, SP-A, SP-B, SP-C, and SP-D. Several studies have shown that SP-A, SP-B, and SP-C play an important role in enhancing the surface-active functions of surfactant lipids. SP-A, a 35-kd hydrophilic protein, has been localized to intracellular and extracellular lamellar bodies, to tubular myelin, and to the surface monolayer at the air liquid interface.[7–12,24]

In previous studies,[13,14] we have shown that tubular myelin is absent in the lungs of infants dying from RDS. These RDS lungs also showed reduced immunoreactivity on immunostaining for SP-A and the hydrophobic proteins, suggesting that surfactant proteins are necessary for the conversion of lamellar bodies to tubular myelin. To evaluate abnormalities in production or transport of surfactant proteins, using immunogold localization, we compared the subcellular distribution of SP-A in the lungs of 14 infants dying from RDS and of 14 control infants dying from other causes.

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

Materials and Methods

Tissues

The lungs of 14 infants dying from RDS were randomly selected from the autopsy files of Cardinal Glennon Children's Hospital. The diagnosis of RDS was based on clinical, radiographic, and histopathological findings. The gestational ages of these infants ranged from 24 to 37.5 weeks, their birth weights ranged from 590 g to 2350 g and postnatal ages from 3 hours to 20 days. There were 9 boys and 5 girls (Table 1).

Autopsy lung tissue from 14 control term or near-term infants dying from causes other than RDS were obtained. These infants' birth weights ranged from 1970 g to 4000 g and their postnatal ages from 1 day to 60 days. There were 8 boys and 6 girls. The clinical diagnoses are indicated in Table 2. In all infants the postmortem interval, before tissue sampling, was less than 24 hours.

Immunogold Staining

Tissue from the midlung region of the right or left lower lobe was diced to 1-mm cubes and immersion-fixed in 2% glutaraldehyde in phosphate-buffered saline with 0.1 mmol/L $CaCl_2$ for 2 to 4 hours. The tissue was then postfixed in 1% osmium tetroxide for one hour followed by dehydration in increasing concentrations of acetone. Tissue blocks were embedded in London Resin White (Polysciences, Inc. Warrington, PA, #17411). Ultrathin sections were obtained on formvar-coated nickel grids and used for immunostaining. Immunostaining for SP-A was performed as follows: the grids were washed in drops of distilled water for 5 minutes and incubated for 15 minutes at room temperature on drops of 0.15 mol/L glycine in 0.1% bovine serum albumin (BSA) in Tris, pH 7.5. After a 10-minute block in 10% normal goat serum, the primary antibody incubation was done. The antibody specificity for SP-A was established by Western blotting and immunoprecipitation and any cross-reactivity with serum components was eliminated by immunoaffinity chromatography. The detailed characterization of this affinity-purified rabbit anti-human SP-A IgG fraction, has been previously described.[15,16] The grids were placed on 15 µl drops of antibody that had been diluted with 1% BSA (final concentration of IgG 8 µg/ml), at room temperature for two hours. The grids were then washed three times for five minutes each in 0.1% BSA. This was followed by a second blocking step with incubation of the grids in drops of 10% normal goat serum for 10 minutes. Grids were then incubated with the secondary antibody, a goat anti-rabbit IgG conjugated with 10-nm colloidal gold particles (Amersham Life Sciences, Arlington Heights, IL, #RPN 421), in a 15-µl drop for half hour. After washes in distilled water, 0.1% BSA in Tris, and finally distilled water, the grids were counterstained with 2% aqueous uranyl acetate and Reynolds lead citrate. Grids were dried and examined in the Jeol 100 Electron Microscope. For two control and three RDS lung samples, the fixation and processing procedure was modified as follows:[17] 1-mm cubes of lung tissue were fixed in a 1% glutaraldehyde/2% paraformaldehyde solution in 0.1 mol/L $CaCl_2$ for at least 2 hours. After several washes in a 3.5% sucrose solution in phosphate-buffered saline, the tissue was washed in 0.1 mmol/L maleate (maleic acid) 3.5% sucrose buffer, pH 6.5, and then stabilized in 2% uranyl acetate in maleate sucrose buffer for 2 to 4 hours at 4 C in the dark. After several washes in sucrose maleate buffer, the tissue was dehydrated in acetone and embedded in LR White. Subsequent immunogold staining of thin sections was as described before, but instead of the final counterstaining step, the grids were exposed to osmium fumes for one hour. Staining controls were treated identically, except for the primary antibody step in which the anti-SP-A antibody was replaced either by a rabbit antibody to human lactoferrin (Biogenex, San Ramon, CA), diluted with 1% BSA to a final concentration of 7.5 µg/ml, or with 1% BSA/Tris, pH 7.4.

Grids were examined without knowledge of the patient's clinical course or gestational age. For each case, at least six grids, each containing five to 10 sections, were examined. To identify specific label

Table 1. *Respiratory Distress Syndrome*

Gestational age	Sex	Race	Birth weight (g)	Postnatal age
1) 24 weeks	F	W	590	3 days
2) 24–25 wks	M	B	800	2 days
3) 24–25 wks	M	B	700	1 day
4) 25 weeks	F	W	680	14 days
5) 27 weeks	M	B	727	1 day
6) 28 weeks	M	W	950	3 hours
7) 28 weeks	F	W	760	20 days
8) 29 weeks	M	W	1180	4 days
9) 31 weeks	M	W	1410	1 day
10) 32 weeks	M	W	2100	5 days
11) 32 weeks	F	W	2000	5 days
12) 32 weeks	M	B	1690	8 days
13) 36 weeks	F	B	1877	5 days
14) 37.5 weeks	M	W	2350	10 hours

Table 2. *Controls*

Gestational age	Sex	Race	Birth weight (g)	Postnatal age	Clinical diagnosis
1) 36 weeks	F	W	2525	15 days	PFC
2) 37 weeks	F	W	2340	2 days	AHP
3) 37 weeks	M	W	3940	1 day	CHD
4) 39 weeks	M	B	2740	26 days	CHD
5) 40 weeks	F	W	3090	2 days	CHD
6) 40 weeks	M	W	3775	3 days	CHD
7) 40 weeks	M	W	3600	4 days	PA
8) 40 weeks	M	B	3475	5 days	PA
9) 40 weeks	F	W	3172	9 days	CHD
10) 40 weeks	F	W	3700	12 days	CDH
11) 40 weeks	M	W	4000	21 days	PFC
12) 40 weeks	M	W	2310	60 days	CHD
13) 42 weeks	F	B	1970	2 days	IUGR
14) 42 weeks	M	W	3200	10 days	HP

CHD: congenital heart disease; AHP: alobar holoprosencephaly; PA: perinatal asphyxia; CDH: congenital diaphragmatic hernia; HP: hypophosphatasia; PFC: persistent fetal circulation; IUGR: intrauterine growth retardation.

on a structure of interest, the ratio of the number of gold particles it contained to the number of gold particles on other structures within the same section was determined. A threefold increase was required as evidence of specific labeling. Background label was defined as the number of gold particles per square area of tissue section that received primary incubation with lactoferrin or Tris. To be valid, specific label had to be at least three times greater than background. In controls, the specific label was up to 10 times greater than background.

The density of gold label on lamellar bodies was determined using the Bioscan Optimas 3.01 computerized image analysis system. The number of gold particles per measured cross-sectional area of intracellular or extracellular lamellar body was calculated to yield the density of gold particles per lamellar body for each case. The resulting data was analyzed for statistical significance using the unpaired student's *t*-test.

Results

Control Infants

Type II Cells, Intracellular Labeling

Gold label was present in the Golgi region, smooth and rough endoplasmic reticulum, and lamellar bodies. The smaller lamellar bodies nearer the basal aspect of the cell had less label per unit area than larger lamellar bodies toward the apical aspect of the cell (Figure 1). Gold particles seemed to be evenly distributed throughout the cut surface of the lamellar bodies, including the central amorphous region. Gold label was present on both sides of the cell membrane abutting the alveolar lumen. Generally, the uranyl acetate–stabilized tissue revealed poorer tissue preservation than the osmium-postfixed tissue. However, uranyl acetate stabilization increased specific gold label throughout, but most significantly on the cell membrane (Figure 2). Negative staining control grids had a background of rare gold particles on mitochondria and type II cell nuclei.

Extracellular Labeling

More gold label was present in alveolar lamellar bodies than in intracellular lamellar bodies, and the pattern of distribution was diffuse throughout the lamellar body, including the central amorphous core. In tubular myelin also, gold label was present throughout the cross-hatch grid. The intensity of label was consistently greater in tubular myelin than in alveolar lamellar bodies, and this was also evident in lamellar bodies in which the peripheral lamellae were in the process of forming tubular myelin (Figure 3A). Although rare gold particles were present at the corners of the tubular myelin grid, there was a predilection for labeling to occur at a fixed interval from the corners of the cross-hatch (Figure 3A). Phagocytosed lamellar bodies and tubular myelin within alveolar macrophages also contained gold label, although the intensity in the phagocytosed organelles was considerably less than in the extracellular organelles. Occasionally, tubular myelin appeared altered with thick coarse lamellae or as an amorphous electron dense mass within alveoli. This appearance, which generally correlated with overall poor cellular preservation and prolonged postmortem interval, was interpreted as degeneration, and in such areas, the intensity of SP-A label was significantly increased (Figure 4). Label on red blood cells in the field did not achieve

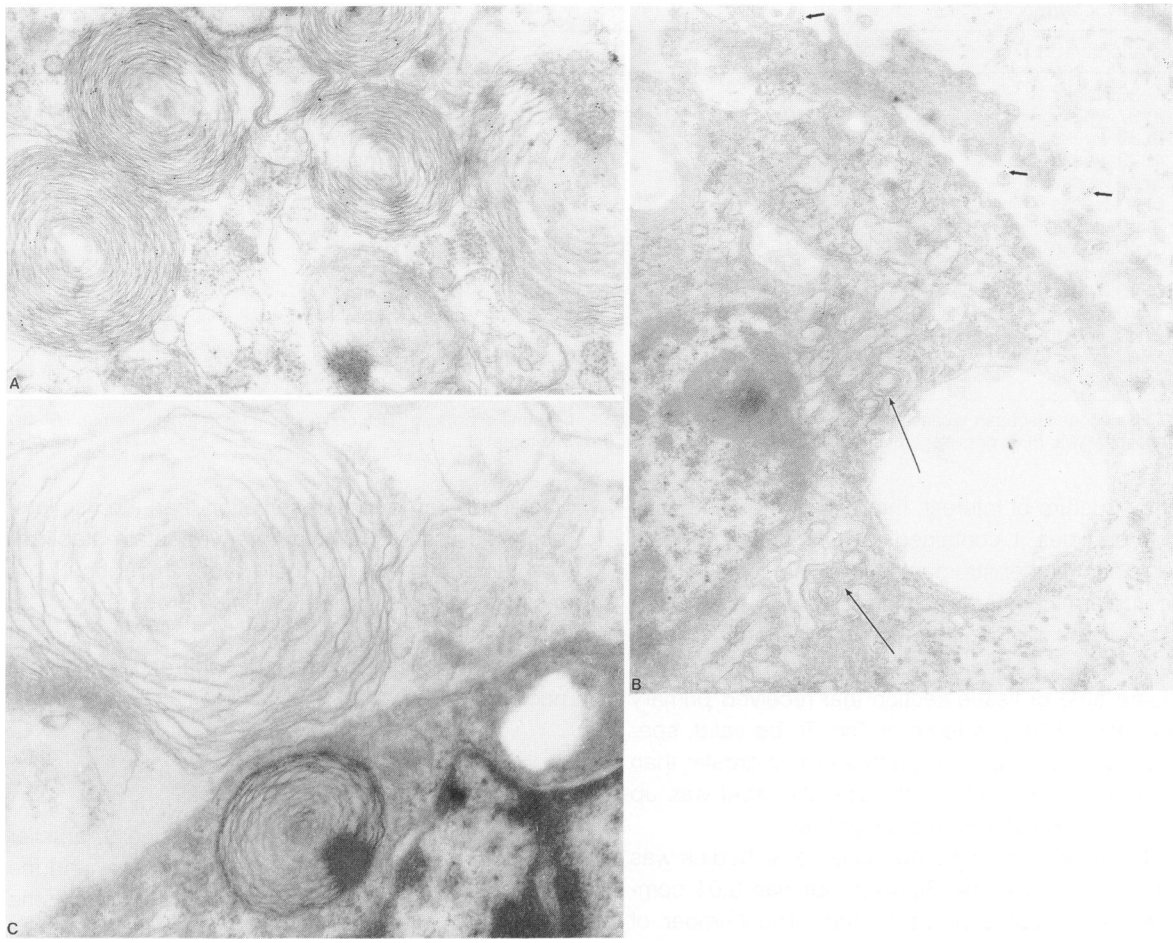


Figure 1. **A:** *A type II pneumocyte in a control infant showing gold label for SP-A scattered throughout the cut surface of large lamellar bodies near the cell apex. (Osmium fixation, mag. ×45000).* **B:** *A type II pneumocyte in an infant dying from RDS contains clusters of gold label for SP-A in the smooth endoplasmic reticulum and on the cytoplasmic membrane in the airspace (small arrows). The large empty space containing a few gold particles represents a leached lamellar body, whereas immature lamellar bodies near the nucleus (long arrows), are unlabeled. (Osmium fixation mag. ×30000).* **C:** *Negative staining control: a type II pneumocyte in a control infant's lung, in which an antibody to lactoferrin instead of SP-A was used during the primary incubation step. Note the absence of gold particles on intracellular and extracellular lamellar bodies. (Osmium fixation mag. ×45000).*

the intensity of that on tubular myelin or on type II cell membranes.

RDS Infants

Type II Cells, Intracellular Labeling

The more immature lungs and those with the shortest postnatal age had fewer lamellar bodies within type II cells compared with the more mature RDS lungs or those with a longer postnatal age. Gold label was present in the same sites as those in control infants. In the smooth endoplasmic reticulum, however, labeled foci showed an increase in gold particles compared with controls. Lamellar bodies had fewer particles than those in controls (see quantitative analysis). Label was rarely seen on the luminal aspect of the cell membrane. (Figure 1B) Uranyl acetate stabilization resulted in only a slightly increased specific gold label throughout the cell (Figure 2B). Even on the luminal membrane of type II cells, label was considerably less than that seen in uranyl acetate–stabilized control lungs.

Extracellular Labeling

Alveolar lamellar bodies had an increased gold label compared with intracellular lamellar bodies (see quantitative analysis). Gold particles had no predilection for any portion of the lamellar bodies and were present throughout the surface. The label on alveolar lamellar bodies was lower than that in controls (see quantitative analysis). Tubular myelin was not identified in RDS lungs. In one patient, however, several unraveling alveolar lamellar bodies were seen forming a haphazardly arranged mesh of

lamellae (Figure 3B). The mesh contained a few gold particles. Amorphous dense alveolar masses containing SP-A label like those in controls (Figure 4) were not seen.

Quantitative Analysis

In one RDS lung (24 to 25 weeks gestation), intracellular lamellar bodies were not identified. In the remaining RDS patients, the mean gold density per lamellar body was significantly lower than in the 14 control lungs ($P < 0.004$), see Table 3. Extracellular lamellar bodies were identified in nine of the RDS lungs and in 12 controls. Here also, the mean gold density per lamellar body was significantly lower in the RDS lungs compared to the controls ($P < 0.0004$), see Table 3.

The lamellar body gold density was also compared between older RDS infants (>30 weeks gestation, $n = 6$) and the youngest control infants (<3 days postnatal age, $n = 4$): intracellular lamellar bodies were identified in all, and the gold density was significantly lower in the RDS patients compared to the controls ($P < 0.01$), see Table 3. Of these patients, extracellular lamellar bodies were identified in four RDS infants and in all four controls. Compared with controls, in the RDS infants there was a decreased gold density on extracellular lamellar bodies ($P < 0.001$, Table 3). In the RDS and control patients where both extracellular and intracellular lamellar bodies were identified, the density of gold label in each group (RDS or control) was significantly greater in extracellular than in intracellular lamellar bodies: RDS $P < 0.01$; controls $P < 0.001$.

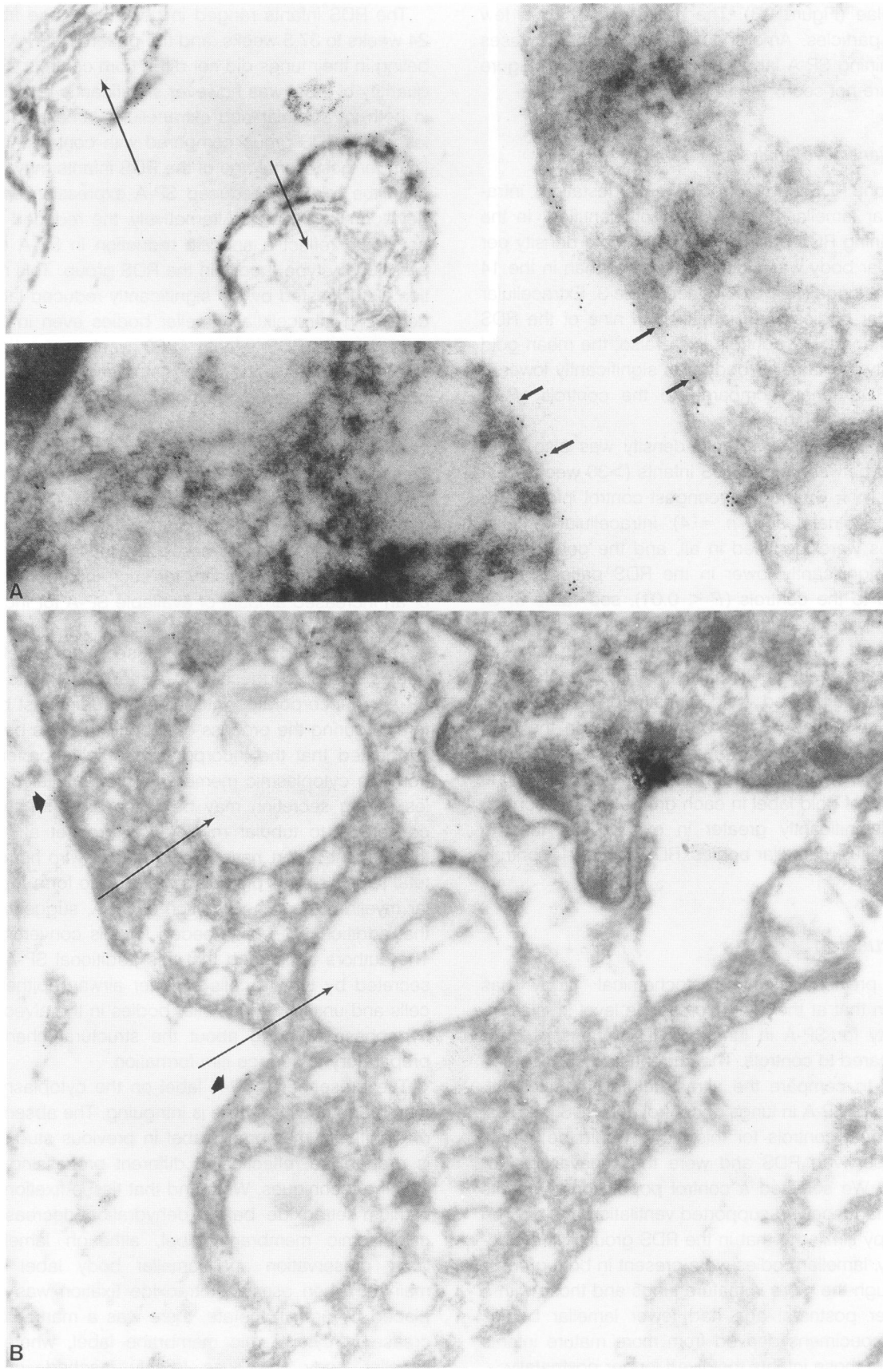
Discussion

Our previous immunohistochemical study has shown that at the light microscope level immunoreactivity for SP-A in lungs of RDS infants is lower compared to controls. The current study was undertaken to compare the ultrastructural immunolocalization of SP-A in lungs of control and RDS patients. The ideal controls for this study would be infants who survived RDS and were thus unavailable for study. We selected a control population of infants who had received supported ventilation and oxygen therapy similar to that in the RDS group. Ultrastructurally, lamellar bodies were present in both groups, although the more immature lungs and those with a shorter postnatal age had fewer lamellar bodies than specimens derived from more mature infants or from those infants that lived longer postnatally.

The RDS infants ranged in gestational age from 24 weeks to 37.5 weeks, and the pattern of SP-A labeling in their lungs did not differ from controls. The quantity of label was however significantly reduced in both intracellular and extracellular lamellar bodies in the RDS group compared with controls. The younger gestational age of the RDS infants may explain the relatively reduced SP-A expression on a developmental basis. Alternatively, the reduced label could reflect a specific reduction in SP-A expression by type II cells in the RDS group. This notion is suggested by the significantly reduced label density in intracellular lamellar bodies even in the more mature RDS infants (>30 weeks gestation) when compared to the youngest controls (<3 days postnatal). Furthermore, although both groups show increased SP-A label in extracellular versus intracellular lamellar bodies, the magnitude of the increase is greater in the controls than in the RDS infants. Whether this finding reflects a more efficient mechanism for SP-A incorporation into extracellular lamellar bodies, the increased availability of other key components necessary for such incorporation, or an increased amount of available SP-A for incorporation is currently unknown.

The increased SP-A label in secreted lamellar bodies further suggests that sizeable amounts of SP-A are incorporated into lamellar bodies just before or during the process of secretion. It has been suggested that the incorporation of glycoproteins from the cytoplasmic membrane into lamellar bodies during secretion may be responsible for their conversion to tubular myelin.[18–20] Froh et al[21] in their recent report have shown that *in vitro* human fetal lamellar body preparations failed to form tubular myelin without the addition of SP-A, suggesting that additional SP-A is needed for this conversion. The authors concluded that this additional SP-A is secreted by type II cells or other airway epithelial cells and united with lamellar bodies in the alveolar hypophase to bring about the structural change preparatory to surface film formation.

The presence of SP-A label on the cytoplasmic membrane of type II cells is intriguing. The absence of significant membrane label in previous studies[7] is probably a reflection of different processing or staining techniques. We found that tissue fixation in osmium tetraoxide before dehydration decreased cytoplasmic membrane label, although lamellar body preservation and lamellar body label remained. When osmium tetraoxide fixation was replaced by uranyl acetate, there was a marked increase in cytoplasmic membrane label, whereas lamellar body lipid was largely leached, even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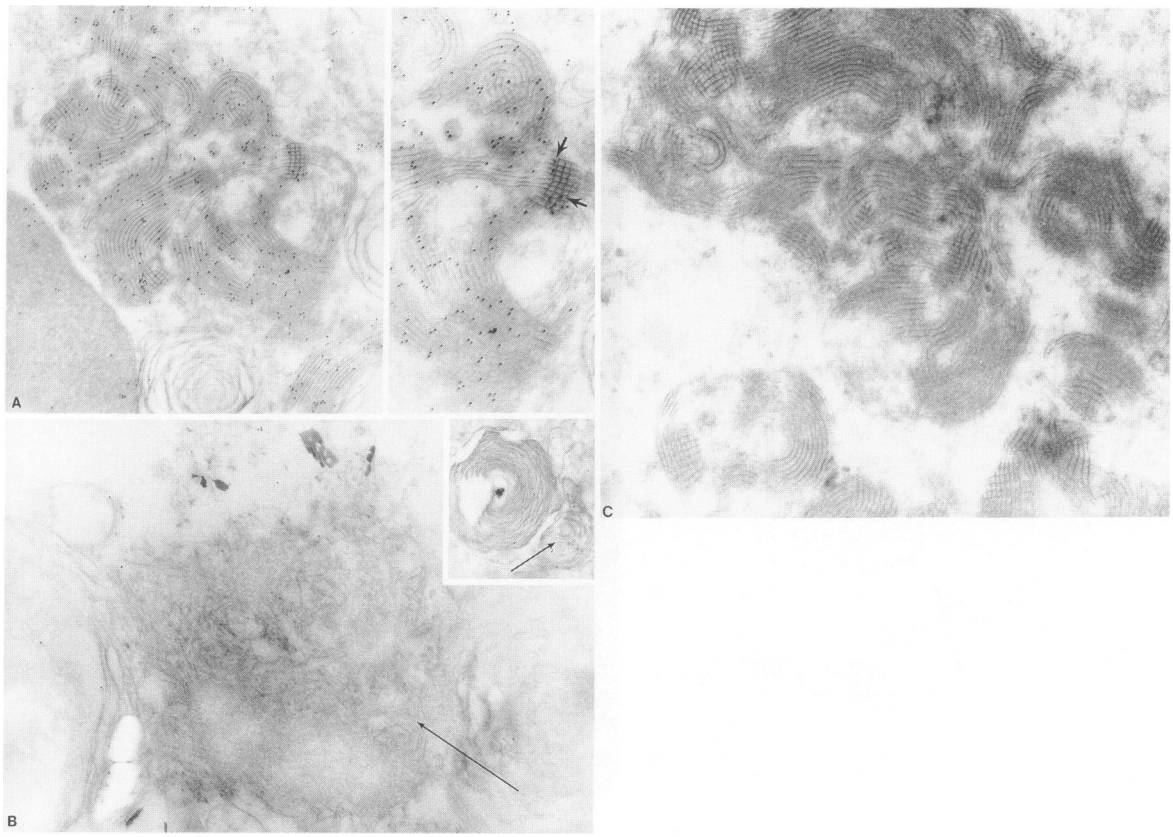

Figure 3. A: *Abundant gold label for SP-A in intra-alveolar tubular myelin in the lung of a control infant. Compare the intensity of label with that on a red blood cell in the lower left corner. High-power view of SP-A labeled tubular myelin (inset), shows that in regions of cross-hatch, gold label is a set distance from the corners (arrows). (Osmium fixation, mag. ×45000).* B: *In an RDS infant's lung, lamellar bodies attempt to form tubular myelin (long arrows) that lacks the regular grid structure. Note the sparse label for SP-A on both lamellar bodies and the haphazard membrane sheets. (Osmium fixation, mag. ×75000).* C: *Negative staining control: a control infant's lung in which the primary antibody incubation step was performed with an antibody to lactoferrin. Note the complete absence of label on tubular myelin. (Osmium fixation, mag. ×45000).*

though immunoreactivity for SP-A persisted. One could argue that the SP-A cytoplasmic membrane label represents sites of attachment for secreted SP-A which would facilitate incorporation into lamellar bodies during secretion, thus aiding their conversion to tubular myelin. Alternatively, these sites could represent the route of reentry of SP-A into the cell for recycling after its release from degenerating alveolar lamellar bodies or tubular myelin. The recent identification of an SP-A-specific membrane receptor[22,23] correlates with this hypothesis. A third possibility is that the presence of SP-A in this location could provide for its ready exit from, or entry into, the surface monolayer at the air/liquid interface during the inspiratory and expiratory phases of respiration.[24]

The pattern of intracellular SP-A label in control lungs could be indicative of the pathway taken by SP-A from the site of modification in the Golgi to the site of secretion at the alveolar luminal surface. The presence of label in both smooth and rough endoplasmic reticulum suggests the existence of two secretory pathways or of a secretory pathway and a pathway for recycling of SP-A. Interestingly, in rats the instillation of liposomes ([^{14}C]lipids and [^{125}I]-SP-A) by Young and coworkers[25] resulted in radiolabel not only in lamellar bodies but also in Golgi-rich and microsomal fractions. In our RDS samples, the subcellular distribution of SP-A was similar to that seen in controls, but the intensity of label generally was considerably less, suggesting an overall decreased synthesis. The focal collections of SP-A in the endoplasmic reticulum of RDS lungs may be indicative of a burst of SP-A synthetic activity or a delay/defect in transport to the cell surface. It is unlikely that the observed changes in the RDS infants

Figure 2. A: *In a control infant, intracellular lamellar bodies within type II pneumocytes have been leached of lipid during tissue processing (long arrows, inset). Cell membranes separated by an alveolar space show abundant gold label for SP-A on the inner and outer aspects (small arrows). (Uranyl acetate fixation, mag. ×45000).* B: *An RDS infant's lung shows gold label for SP-A on a type II cell membrane (small arrows). Intracellular lamellar bodies (long arrows) have been leached of lipid during tissue processing. (Uranyl acetate fixation, mag. ×30000).*

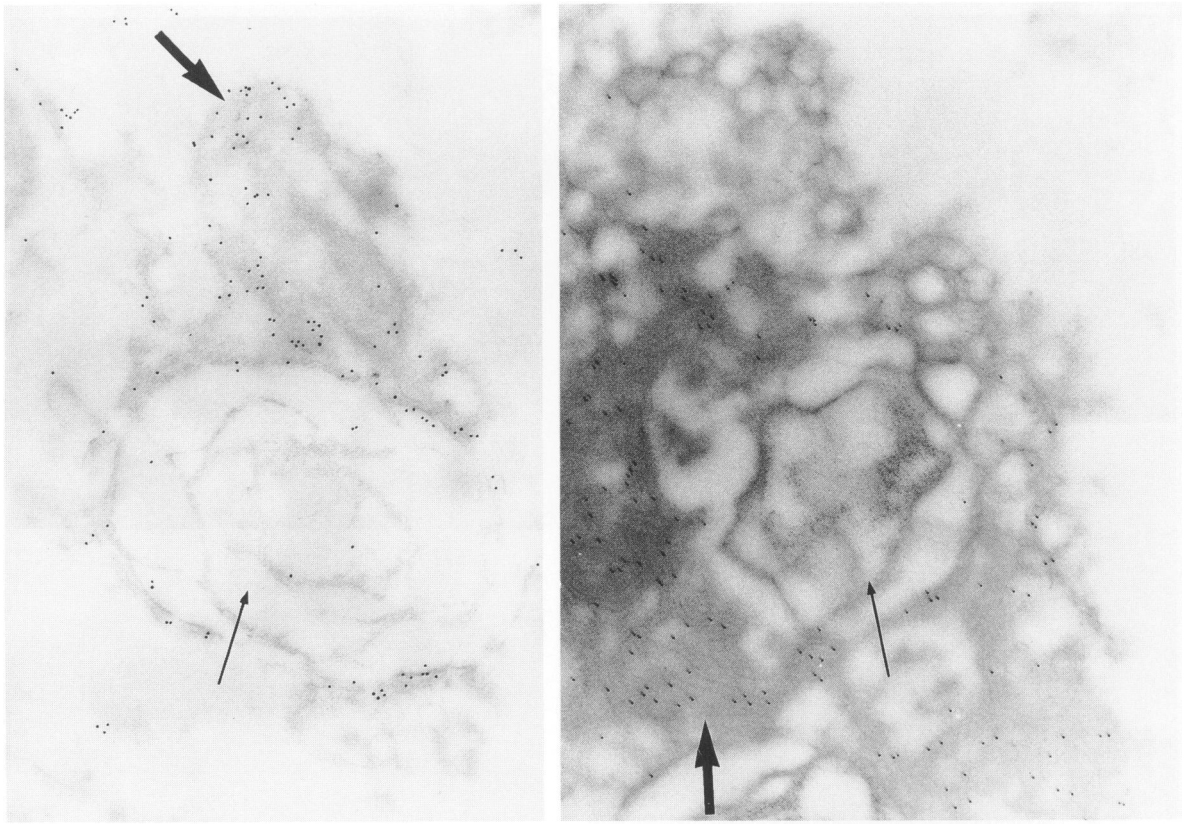

Figure 4. *Abundant gold label on degenerating tubular myelin in the alveolar space of a control infant's lung. Note the difference in intensity of gold label between tubular myelin (thick arrows) and the lamellar bodies (thin arrows). (Osmium fixation, mag. ×45000).*

Table 3. *Quantitative Analysis—Mean Gold Density/Lamellar Body*

	RDS		Control	
	All ages	>30 weeks	All ages	<3 days postnatal
Intracellular (mean ± SD)	1.57×10^{-6} $\pm 1.30 \times 10^{-6}$* (n = 13)	1.84×10^{-6} $\pm 1.26 \times 10^{-6}$‡ (n = 6)	2.31×10^{-6} $\pm 2.85 \times 10^{-6}$* (n = 14)	5.26×10^{-6} $\pm 4.26 \times 10^{-6}$‡ (n = 4)
Range	2.36×10^{-7} to 3.84×10^{-6}		2.08×10^{-7} to 1.09×10^{-5}	
Extracellular (mean ± SD)	3.01×10^{-6} $\pm 2.57 \times 10^{-6}$† (n = 9)	1.69×10^{-6} $\pm 1.77 \times 10^{-6}$§ (n = 4)	8.6×10^{-6} $\pm 9.9 \times 10^{-6}$† (n = 12)	1.06×10^{-5} $\pm 1.72 \times 10^{-5}$§ (n = 4)
Range	5.9×10^{-7} to $7.46. \times 10^{-6}$		3.27×10^{-7} to 3.62×10^{-5}	

* $P<0.004$; † $P<0.0004$; ‡ $P<0.01$; § $P<0.001$.

were due to ventilatory therapy or high levels of inspired oxygen as the control infants were similarly treated.

In control infants, the immunogold label within tubular myelin was consistently greater than that present on both intra- and extracellular lamellar bodies. Generally, there was a relative increase in gold label on the peripheral lamellae involved in forming tubular myelin compared with the label on the central portions of the lamellar body. The increased label in tubular myelin may reflect increased SP-A incorporation during the process of transformation from lamellar body to tubular myelin. This SP-A may reach the extracellular space via other means besides the lamellar body. Or the process of transformation from lamellar body to tubular myelin may allow SP-A antigenic determinants to become more accessible to antibody binding resulting in the increased label in tubular myelin. The observation that degraded tubular myelin shows an even higher amount of SP-A label may in part support this view, suggesting that steric hindrance or

accessibility for antibody binding contributes to differential SP-A label.

In vitro studies suggest that SP-A is a necessary component of tubular myelin and that critical concentrations of each tubular myelin component is required for the formation of this complex structure.[26,27] It is likely that our inability to detect tubular myelin in RDS tissues[13] relates to insufficient amounts of at least SP-A. Exactly how the *in vivo* incorporation of SP-A into the lipid bilayer of lamellar bodies promotes its conversion to tubular myelin is unclear.

The predominant site of gold label in the tubular myelin grid is a slight distance from the corner. A similar distribution of gold particles labeling SP-A within the tubular myelin lattice 20 nm from a corner has been previously reported.[28] This distance probably reflects the size of the primary and secondary antibodies and the nature of their attachment to the epitopic site of SP-A, or it could reflect steric hindrance of the various molecules at their attachment sites.

In conclusion, we have shown that the pattern of SP-A label is similar in the lungs of RDS and control infants but the intensity of labeling differs significantly between the two groups. Moreover, in controls, a gradient of SP-A labeling in intracellular lamellar bodies, extracellular lamellar bodies, and tubular myelin is seen. In RDS infants, although a gradient in intracellular and extracellular lamellar bodies is present, the intensity is much less than in controls. The reduced SP-A expression in RDS may be an important factor in the pathogenesis of this disease.

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