Immunocytochemical Localization of Lysozyme and Surfactant Protein A in Rat Type II Cells and Extracellular Surfactant Forms

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_J Histochem Cytochem_ 1992 40: 1491

DOI: 10.1177/40.10.1527372

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What is This?
Immunocytochemical Localization of Lysozyme and Surfactant Protein A in Rat Type II Cells and Extracellular Surfactant Forms

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Received for publication January 28, 1992 and in revised form June 4, 1992; accepted June 13, 1992 (2A2575).

Using immunogold labeling of fixed, cryosubstituted tissue sections, we compared the distribution of lysozyme, an oxidant-sensitive lamellar body protein, with that of surfactant protein A (SPA) in rat Type II cells, extracellular surfactant forms, and alveolar macrophages. Morphometric analysis of gold particle distribution revealed that lysozyme and SPA were present throughout the secretory and endosomal pathways of Type II cells, with prominent localization of lysozyme in the peripheral compartment of lamellar bodies. All extracellular surfactant forms were labeled for both proteins with preferential labeling of tubular myelin and unilamellar vesicles. Labeling of tubular myelin for SPA was striking when compared with that of lamellar bodies and other extracellular surfactant forms. Lamellar body-like forms and multilamellar structures were uniformly labeled for lysozyme, suggesting that this protein is rapidly redistributed within these forms after secretion of lysozyme-laden lamellar bodies. By contrast, increased labeling for SPA was observed over peripheral membranes of lamellar body-like forms and multilamellar structures, apparently reflecting progressive SPA enrichment of these membranes during tubular myelin formation. The results indicate that lysozyme is an integral component of the lamellar body peripheral compartment and secreted surfactant membranes, and support the concept that lysozyme may participate in the structural organization of lung surfactant.

KEY WORDS: Rat lung surfactant; Type II cell; Lamellar body; Tubular myelin; Lysozyme; Surfactant protein A; Freeze-substitution; Immunogold labeling.

Introduction

Alveolar Type II pneumocytes are specialized cells responsible for the synthesis, storage, secretion, and recycling of surfactant phospholipids, surfactant-associated proteins, and other functional components of lung surfactant (5,15,17). Surfactant phospholipids, composed primarily of dipalmitoylphosphatidylcholine (DPPC), are stored in lamellar bodies, which are functionally multicompartimented organelles involved in both the secretory pathway and the endosomal–lysosomal system of these cells. In addition to phospholipids, lamellar bodies contain surfactant-associated proteins (SAP), including SPA (4,6,11,26,29,37-39) and lysosomal enzymes (1,12,18,20,21). We have also previously reported that lysozyme is a prominent lamellar body protein which is rapidly depleted after acute ozone-induced oxidant injury (30,32). On secretion into the alveolar hypophase, surfactant membranes are organized into various forms including tubular myelin, believed to be the primary source of phospholipid molecules that produce the monomolecular surface film at the alveolar air–liquid interface. There is also in vitro evidence that the surfactant-associated proteins SPA and SP-B play a role in the organization of tubular myelin (10,35,41).

There is little information about the structural interrelationships of surfactant phospholipids and their associated proteins within lamellar bodies and secreted surfactant membranes. This is in part due to the difficulty of preserving surfactant phospholipid membranes and tissue antigenicity for ultrastructural immunocytochemical studies of lamellar bodies and extracellular surfactant membranes. In attempts to preserve surfactant membrane morphology, researchers have employed aldehydes, osmium tetroxide, tannic acid, and/or uranyl acetate, in combination with dehydration and epoxy embedding, to produce tissue with excellent morphology but poor post-embedding antigenicity (6). Other workers used cryosubstitution (7) or cryoultramicrotomy (37-39) techniques to preserve tissue antigenicity but did not adequately preserve intracellular and extracellular surfactant membrane morphology.

A recently published technique involving cryosubstitution of
aldehyde-fixed, uranyl acetate-post-fixed lung tissue was reported to provide an adequate compromise in the morphology and antigenericity of the alveolar surfactant system (37). With this technique, the present study compares the distribution of lysozyme and SPA in Type II cells, extracellular surfactant, and alveolar macrophages. The results indicate that lysozyme, like SPA, is a surfactant-associated protein with preferential localization within the peripheral compartment of lamellar bodies and in tubular myelin. A portion of this study has appeared in abstract form (16).

Materials and Methods

Antibodies. Anti-human lysozyme, which crossreacts with rat lysozyme, was obtained commercially (Accurate Chemical and Scientific; Westbury, NY). Antibodies to SPA were produced from the 72 Kd dimer of SPA, isolated from rat surfactant by preparative gel electrophoresis, and injected into rabbits (31). On immunoblotting of non-disulfide-reduced SAP, the anti-SPA serum labeled the 72 Kd dimer of SPA and showed weaker reactions for higher molecular aggregates of this protein, as well as for the 28-36 Kd monomers of SPA. Reduction of the SAP with dithiothreitol, followed by immunoblotting with the anti-72 Kd serum, yielded reaction product primarily with the 28-36 Kd monomer. Non-immune rabbit serum was used as a control primary serum. The secondary antibody was goat anti-rabbit IgG conjugated to 10-nm gold particles, purchased from Janssen Biochemicals (Accurate Chemical and Scientific).

Tissue Preparation. Lung tissue was obtained from three barrier-reared adult Fischer 344 rats. These animals came from separate litters, and were shipped from Charles River Breeding (Kingston, NY) in filtered crates, housed in cages with filter tops, and acclimated for at least 1 week before use. The rats were housed according to National Research Council guidelines, with free access to food and water, and were sacrificed by intraperitoneal injection of pentobarbital. The lungs of these rats were inflation-fixed via intratracheal intubation with 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, at 4°C and 20 cm pressure. After an initial 15-min fixation, the lungs were maintained in an inflated state during removal from the chest cavity and were immersed in additional fixative at 4°C. After 1 hr of fixation the left lung was removed, cut into 2-mm thick slices, and placed in cold 0.1 M phosphate buffer, pH 7.2, for 10 min to remove the glutaraldehyde component of the fixative. Sliced lung tissue was then diced into 0.5-mm cubes and placed in cold buffered 2% paraformaldehyde for an additional 4-hr fixation. Tissue blocks were infiltrated overnight with 2:3 m sucrose at 4°C on a tissue rotator, frozen in liquid nitrogen the following day, and introduced to the pre-cooled chamber of a Phillips 301 TEM at 60 kV with a 20-μm objective aperture. Nine separate sets of gold-labeled grids were photographed for this study. Each set consisted of a minimum of six grids, with at least two grids for each of the three primary antisera. Select grids were post-stained with uranyl acetate and lead citrate alone to enhance identification of cell organelles.

Morphometry. Serial sections from one block were used for the morphometric analysis of label density for lysozyme or SPA in Type II cells and extracellular surfactant forms found in an alveolar pouch. Pouches are observed in the corners of some alveoli; freely communicating with the alveolar space through a small opening formed by attenuated Type 1 epithelium, and contain one or more Type II cells (23). Evaluation of lamellar bodies and extracellular surfactant forms in the same sections permitted correlation of label density and distribution patterns for lysozyme or SPA. For these studies, a total of 24-30 cells and over 200 μm² of surfactant forms per immunolabeling group were photographed.

For the morphometric analysis of lamellar bodies, the total area of these organelles was divided into central and peripheral compartments. The peripheral compartment of lamellar bodies was defined as the amorphous, phospholipid membrane-free region and the first three phospholipid membranes of cross-sectioned lamellar bodies that were adjacent to a clearly defined limiting membrane. Tangential sections through this peripheral compartment did not reveal a limiting membrane around the lamellar body. The extracellular surfactant membranes were classified into subtypes as described previously (3). These subtypes include the following: (a) lamellar body-like forms, defined as circular, densely coated surfactant membranes similar in size and appearance to lamellar bodies but lacking a limiting membrane; (b) multinlamellar structures—multiple, loosely coiled parallel surfactant membranes arranged in various configurations; (c) unilamellar vesicles, surfactant membranes arranged in the form of vesicles, sometimes in the form of vesicles within a vesicle; and (d) tubular myelin—surfactant membranes arranged in the characteristic lattice form of adiacent tubules. The outer three membrane profiles of lamellar body-like forms cut in cross-section were considered to be the outer membranes for the morphometric studies.

Immunocytochemistry. Sections on grids from cryosubstituted lung tissue were immunolabeled as described previously (38), with minor modifications. All sections to be labeled were first conditioned for 20 min on 5% newborn calf serum in 20 mM Tris buffer with 150 mM NaCl and 2.5 mM KCl (TBS), pH 8.2, at room temperature. The sections were rinsed two times each for a total of 30 sec at room temperature on separate drops of TBS, pH 8.2, containing 0.1% bovine serum albumin, 0.2% coldwater fish gelatin (Sigma; St Louis, MO) and 0.05% Tween 20 (TBS-AGT), to prevent nonspecific labeling. The sections were incubated with either a primary rabbit antibody or non-immune rabbit serum (control) at 1:50 or 1:100 dilutions in TBS-AGT for 1-2 hr at room temperature in a moisture chamber.

After primary antibody labeling, all sections were rinsed six times for 5 min each on drops of TBS-AGT, and gold labeling of the sections was accomplished with goat anti-rabbit IgG-gold antibody at a 1:50 dilution in TBS-AGT, pH 8.2, for 1 hr at room temperature. All sections were rinsed successively six times for 5 min each on drops of TBS-AGT, then on TBS, and finally on distilled water. The sections were dried with #30 Whatman filter paper and, without further staining, viewed and photographed in a Philips 301 TEM at 60 kV with a 20-μm objective aperture. Nine separate sets of gold-labeled grids were photographed for this study. Each set consisted of a minimum of six grids, with at least two grids for each of the three primary antisera. Select grids were post-stained with uranyl acetate and lead citrate alone to enhance identification of cell organelles.
LYSOZYME AND SPA IMMUNOLABELING IN RAT ALVEOLI

Results

Processing of rat lung tissue by the freeze-substitution technique enabled excellent preservation of lamellar bodies and extracellular surfactant forms for immunocytochemistry (Figure 1). In alveolar Type II cells, specific labeling for lysozyme and SPA was observed over the organelles of the secretory-endocytic pathway, including endoplasmic reticulum, trans-Golgi vesicles, multivesicular bodies, and lamellar bodies. The labeling for lysozyme and SPA over these organelles was consistently higher than labeling over the nucleus and mitochondria of the Type II cells \((p<0.01)\), as shown in Table 1. Both proteins were characteristically found within the peripheral compartment of lamellar bodies (Figures 2a–2c; Table 1), whereas the central areas of these organelles showed minimal nonspecific labeling that was not significantly different than over the nucleus of the Type II cells. In sections labeled for lysozyme, the particle density over the peripheral compartment of lamellar bodies was higher than that observed over any other Type II cell organelles. Minimal nonspecific labeling was observed over Type II cells of control sections incubated with non-immune rabbit serum in place of the primary antibody (Figure 2d; Table 1).

In all forms of extracellular surfactant membranes, label density for lysozyme was significantly higher than labeling found over control sections (Table 2). Prominent labeling was observed over tubular myelin and unilamellar vesicles (Figure 3a), and in these locations the particle density was similar to that observed in the peripheral compartment of lamellar bodies. In cross-sectional profiles of tubular myelin labeled for lysozyme, 96% of the 169 gold particles counted were located within 20 nm from a membrane profile, but only 57% of these particles were within 20 nm from a corner of the lattice. The multilamellar structures and lamellar body-like forms showed a similar labeling density, which was significantly lower than the labeling density over tubular myelin and unilamellar vesicles. No significant difference was observed in the particle density between central and outer membranes of lamellar body-like forms labeled for lysozyme.

All extracellular surfactant forms were also specifically labeled for SPA, with the exception of the central area of lamellar body-like forms, which had a low label density similar to that of the central membranes of lamellar bodies. Tubular myelin showed a striking labeling for SPA when compared with all other surfactant forms (Figures 3b and 3c; Table 2). For SPA a total of 415 particles associated with cross-sectional profiles of tubular myelin were counted. Of these particles, 98% were located within 20 nm from a membrane profile and 64% within 20 nm from a lattice corner. Unilamellar vesicles showed a higher label density for SPA than multilamellar structures or lamellar body-like forms. In addition, gold labeling...
Table 1. Mean gold particle density over Type II cell organelles (counts/μm² ± SEM).a,b

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Anti-lysozyme (μm²)</th>
<th>Anti-SP-A (μm²)</th>
<th>Non-immune rabbit serum (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus</td>
<td>1.6 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(72 μm²)</td>
<td>(80 μm²)</td>
<td>(92 μm²)</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>2.4 ± 0.3</td>
<td>0.05 ± 0.03</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(38 μm²)</td>
<td>(46 μm²)</td>
<td>(34 μm²)</td>
</tr>
<tr>
<td>Golgi complex</td>
<td>13.6 ± 1.8c,d</td>
<td>10.9 ± 1.3c,d</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(17 μm²)</td>
<td>(34 μm²)</td>
<td>(26 μm²)</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>17.1 ± 1.8c,d</td>
<td>11.1 ± 1.2c,d</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(48 μm²)</td>
<td>(64 μm²)</td>
<td>(57 μm²)</td>
</tr>
<tr>
<td>Multivesicular bodies</td>
<td>17.1 ± 3.8c,d</td>
<td>5.7 ± 2.7c,d</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>(4 μm²)</td>
<td>(3 μm²)</td>
<td>(5 μm²)</td>
</tr>
<tr>
<td>Total lamellar body compartment</td>
<td>14.8 ± 1.8c,d</td>
<td>1.2 ± 0.2c,d</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(162 μm²)</td>
<td>(148 μm²)</td>
<td>(141 μm²)</td>
</tr>
<tr>
<td>Lamellar body peripheral area</td>
<td>27.1 ± 2.8c,d</td>
<td>2.5 ± 0.3c,d</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(65 μm²)</td>
<td>(53 μm²)</td>
<td>(54 μm²)</td>
</tr>
<tr>
<td>Lamellar body interior</td>
<td>2.8 ± 0.6</td>
<td>0.6 ± 0.2c,d</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(97 μm²)</td>
<td>(95 μm²)</td>
<td>(87 μm²)</td>
</tr>
</tbody>
</table>

a All data were obtained from randomly divided grids of serially sectioned tissue.

b Numbers in parenthesis indicate total area of each organelle studied.

c,d Gold particle density significantly different than background level over nucleus and mitochondria (p<0.01).

Table 1 shows the mean gold particle density over Type II cell organelles, comparing the labeling for lysozyme and surfactant-associated protein-A (SP-A). The data are presented in counts per square micrometer ± standard error (SEM). The values indicate that SP-A labeling was significantly higher over multilamellar structures than over lamellar body-like forms, primarily owing to the absence of labeling in the central regions of the latter. It is of interest that cryosubstitution medium resulted in reduced contrast of nuclear heterochromatin and mitochondrial cristae. However, the use of uranyl acetate in the substitution fluid instead of osmium tetroxide promoted stabilization of phospholipid membranes during low-temperature dehydration. This approach eliminated the need to etch sections with sodium metaperiodate or hydrogen peroxide, which is a common step in immunolabeling sections of osmiolated tissue and results in the loss of the phospholipid membranes of Type II cell lamellar bodies (22).

Subcellular Localization of SPA and Lysozyme in Type II Cells

Previous immunocytochemical studies have indicated that SPA, a major surfactant-associated protein, is localized within the synthetic and secretory pathway of alveolar Type II cells and Clara cells (4,29,39). However, there is considerable controversy concerning the localization of SPA within lamellar bodies. Coalson et al. (6) reported gold labeling for SPA over lamellar bodies but not over any other organelle of Type II cells in rat lung embedded in Epon-Araldite. In their study, phospholipid membranes of the lamellar bodies were not retained during tissue osmication and ethanol dehydration. With cryosections of rat lung tissue, Walker et al. (39) reported a low level of labeling for SPA over the phospholipid membranes, but not over the peripheral area of lamellar bodies. In Lowicryl HM20 freeze-substituted human lung, Voorhout et al. (37) reported that labeling for SPA was not found over lamellar bodies, although heavy labeling for SP-B was localized predominately over the well-preserved phospholipid membranes of these organelles. However, these researchers also found labeling for both SP-B and SPA over membranes of small lamellar bodies and remnants of large lamellar bodies in gold-labeled cryosections. The apparent distribution of label for SPA over the membranes of lamellar bodies in frozen thin sections may be the result of an antigen solubilization artifact, a phenomenon that has been observed even after glutaraldehyde fixation during immunolabeling of cryosections from lipid-rich rat brain tissue (36).

In isolated rat lamellar bodies, SPA has been reported to constitute only 1% of the total protein present, in contrast to SP-B, which has been estimated to constitute 28% (26). This may account for the apparent lack of lamellar body labeling for SPA in Lowicryl-embedded Type II cells reported by Voorhout et al. (37) and the low labeling reported herein. However, the low level of SPA detected in isolated lamellar bodies may be in part due to its peripheral localization which could result in loss of SPA during the lamellar body isolation procedure. There is evidence that the SPA content of isolated lamellar bodies is insufficient for their in vitro transformation to tubular myelin and rapid formation of surf-
peripheral compartment of lamellar bodies, thus confirming ear-
face film consisted of unstained cores and reactive rims. More recently, it was reported that pulmonary lysozyme is a secretory protein of Type I1 according to earlier observations from this laboratory (34). The results of the present study clearly demonstrate that lysozyme is determined. 

Lysozyme was first identified in alveolar Type I1 pneumocytes and macrophages in immunohistochemical studies by Spicer et al. (34) of paraffin sections of rat lung. In Type I1 cells, lysozyme was described as occurring in "a morula-like cluster of bodies which consisted of unstained cores and reactive rims." More recently, it was reported that pulmonary lysozyme is a secretory protein of Type I1 cells (33) and is expressed at high levels in these cells (25). The results of the present study clearly demonstrate that lysozyme is found in both the secretory pathway (endoplasmic reticulum, Golgi apparatus, multivesicular bodies, and lamellar bodies) and the endocytic pathway (multivesicular bodies and lamellar bodies) of the Type I1 cell, with a striking preferential localization within the peripheral compartment of lamellar bodies. It should be emphasized that the precise distribution of lysozyme and SPA in the endosomal-lysosomal compartment of these cells has not been determined.

Recent immunohistochemical studies have reported that SPA and lysozyme are found in both lamellar bodies and lysosomes isolated from Type I1 cells (11,28). The localization of lysozyme and SPA in multivesicular bodies and in the periphery of lamellar bodies may be due in part to re-uptake of these proteins, along with extracellular surfactant material, as suggested by Kalina and Socher in their studies of gold-labeled SPA uptake by cultured rat Type I1 cells (23). It is of interest that, in addition to SPA and lysozyme, the lysozyme enzymes acid phosphatase (1,21) and cathepsin H (22), have been shown to have a similar peripheral localization pattern in lamellar bodies. Eckenhoff and Somlyo (9) found sulfur, an element common to proteins, to be concentrated in the periphery of lamellar bodies in elemental analysis studies of cryofixed adult rat lung sections, but a similar pattern was not found in fetal rat lung Type I1 cells (8), possibly due to an inability of these cells to recycle surfactant components. The above findings are consistent with the concept that lamellar bodies are functionally multicompart- mented organelles involved in the storage, secretion, recycling, and degradation of many proteins whose regulation and functions within the lung surfactant system have not been as yet defined.

### Table 2. Mean gold particle density over extracellular surfactant forms (counts/μm² ± SEM)^a,b^ 

<table>
<thead>
<tr>
<th>Surfactant form</th>
<th>Anti-lysozyme</th>
<th>Anti-SPA</th>
<th>Non-immune rabbit serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubular myelin</td>
<td>31.2 ± 4.6</td>
<td>161.0 ± 21^d^</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>(12 μm²)</td>
<td>(26 μm²)</td>
<td>(31 μm²)</td>
</tr>
<tr>
<td>Unilamellar vesicles</td>
<td>23.0 ± 5.3</td>
<td>22.4 ± 3.4^d^</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(25 μm²)</td>
<td>(27 μm²)</td>
<td>(23 μm²)</td>
</tr>
<tr>
<td>Multilamellar structures</td>
<td>7.8 ± 0.6^e^</td>
<td>11.9 ± 1.4^d^</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(196 μm²)</td>
<td>(106 μm²)</td>
<td>(139 μm²)</td>
</tr>
<tr>
<td>Total lamellar body-like forms</td>
<td>10.1 ± 2.1^f^</td>
<td>4.5 ± 0.8^e^</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(49 μm²)</td>
<td>(49 μm²)</td>
<td>(43 μm²)</td>
</tr>
<tr>
<td>Outer membranes of lamellar body-like forms</td>
<td>10.0 ± 2.0</td>
<td>8.3 ± 1.3</td>
<td>0.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>(18 μm²)</td>
<td>(21 μm²)</td>
<td>(12 μm²)</td>
</tr>
<tr>
<td>Central membranes of lamellar body-like forms</td>
<td>13.5 ± 5.6</td>
<td>0.6 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(31 μm²)</td>
<td>(28 μm²)</td>
<td>(31 μm²)</td>
</tr>
</tbody>
</table>

^a^ All data obtained were from serial sections of one alveolar pouch. ^b^ Grids were divided at random among incubation groups. ^c^ Gold particle density significantly lower than tubular myelin and unilamellar vesicles. ^d^ Significantly different from all other surfactant forms.

### Distribution of SPA and Lysozyme in Extracellular Surfactants

A novel approach of the present study is the morphometric evaluation of serially sectioned alveolar pouches, which facilitated the comparative evaluation of the immunolabeling pattern among extracellular surfactant forms. In these pouches, both SPA and lysozyme show prominent localization within tubular myelin when compared with other extracellular surfactant forms.

In our gold labeling studies of tubular myelin, both SPA and lysozyme were localized at the membranes with a particle density higher than 95%, but not at any specific region of the lattice structure. The tubular myelin lattice in our preparations measured 60 nm from one corner of the lattice to its nearest neighboring corner. Using a distance of 20 nm from any corner as a defining parameter in particle counting, each 60 nm side of a lattice would have 2/3 or 40 nm assigned to corners. Our gold particle counts reflect approximately 2/3 (64%) of the particles labeling SPA occurring within 20 nm of a corner, and thus revealing no preferential association of particles with corners of the lattice. Voorhout et al. (38) reported that in isolated surfactant fractions 79% of the gold label for SPA was localized within 20 nm from the corners of the tubular myelin lattice. This level of localization is only 12% higher than the predicted level (67%) of random distribution, which contrasts with the striking gold label density (98%) for the lattice membranes reported herein. Voorhout et al. (38) also found no SPA labeling of other surfactant forms present in tubular myelin-enriched fractions isolated from lavage fluid. This is at variance with our results that were obtained by in situ fixation of secreted surfactant membranes. Procedures for isolating tubular myelin from lung lavage may have promoted artifactual loss or translocation of SPA before fixation. In addition, the use of osmium tetroxide in the cryosubstitution medium by Voorhout and co-workers may have reduced the number of antigenic sites present in their preparations owing to protein denaturing.

On the basis of our morphometric data, there is a significant difference between the distribution of SPA and lysozyme in densely coiled lamellar body-like forms and in multilamellar structures. The peripheral membranes of lamellar body-like forms reveal an in-

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**Figure 3.** Immunogold labeling of extracellular surfactant forms in an alveolar pouch for (a) lysozyme and (b) SPA, showing heavy labeling of tubular myelin (TM). (c) High-magnification photograph of tubular myelin labeled for SPA, showing striking association of gold particles with membranes of the lattice. MP, membrane-associated particles. (d) Control section incubated with non-immune rabbit serum. MS, multilamellar structure; LBF, lamellar body-like form; V, unilamellar vesicle. Original magnifications: a,b,d × 40,000; c × 100,000. Bars = 0.25 μm.
Figure 4. Extracellular surfactant in an alveolar pouch showing labeling pattern of various surfactant forms for SP-A. In addition to tubular myelin (TM), prominent labeling (arrowheads) is noted in association with some unilamellar vesicles (V) and multilamellar structure outer membranes (arrows). LBF, lamellar body-like form. Original magnification × 40,000. Bar = 0.25 μm.

Increased label density for SP-A when compared with the peripheral compartment of lamellar bodies, suggesting that, after secretion peripheral membranes of unwinding lamellar bodies become enriched with extracellular SP-A. A further increase in labeling for SP-A was found in multilamellar structures, which apparently represent intermediate forms before the formation of tubular myelin. In contrast, label for lysozyme over the central membranes of lamellar body-like forms increases fivefold over label in the center of lamellar bodies, with a two- to threefold decrease in label over peripheral membranes, suggesting a uniform spreading of lysozyme throughout these structures after secretion. The above contrasting changes may reflect the existence of different modulatory actions by these proteins during secretion and unwinding of surfactant membranes.

Unilamellar vesicles also show increased labeling for SP-A when compared with all other surfactant forms except tubular myelin. Wright et al. (42) reported that SPA enhances the in vitro uptake of DPPC vesicles by Type II cells. There is evidence that in vitro conversion of tubular myelin to unilamellar vesicles requires serine protease activity (13) and may represent a mechanism of surfactant degradation. Endocytosis of unilamellar vesicles by Type II cells could provide a mechanism for recycling of surfactant, resulting in the accumulation of SPA and lysozyme in the peripheral compartment of lamellar bodies.

Biochemical studies have indicated that lysozyme is an oxidant-sensitive lamellar body protein which is rapidly depleted during acute ozone-mediated alveolar injury and subsequent repair (30). This lysozyme deficiency is associated with time-dependent structural defects in the organization of stored and secreted surfactant membranes (3). In addition, other investigators have recently reported that in vitro exposure of SPA to ozone results in structural and functional changes of this protein, which may contribute to ozone-induced defects in the lung surfactant system (27). On the basis of these observations and the results of the present study, we are presently exploring the hypothesis that lysozyme, in concert with SPA and other surfactant-associated proteins, participates in the regulation of secretion, organization, and recycling of surfactant membranes.
LYSOZYME AND SPA IMMUNOLABELING IN RAT ALVEOLI

Figure 5. Immunogold labeling of phagocytized surfactant membranes (SM) for (a) lysozyme and (b) SPA in alveolar macrophages. N, nucleus; M, mitochondria; L, primary lysosome containing endogenous lysozyme; SM, surfactant membranes; TM, tubular myelin; arrowheads, gold particles. Original magnification × 40,000. Bars = 0.25 μm.

Literature Cited


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