In chyloptysis, SP-A affects the clearance of serum lipoproteins entering the airways

ANTONELLA ALBERTI, FRANCO RAVENNA, DANIELA QUAGLINO, MAURIZIO LUISETTI, MAURIZIO MURACA, LORENZO PREVIATO, GORETTA BALDO ENZI, ROBERTA BRUNI, AND ALDO BARITUSSIO

Institute of Internal Medicine and Centro del Consiglio Nazionale delle Ricerche per lo Studio dell’Invecchiamento, University of Padua, 35128 Padua; Institute of Respiratory Medicine, University of Ferrara, 44100 Ferrara; Institute of General Pathology, University of Modena, 41100 Modena; and Institute of Respiratory Diseases, Istituto di Ricovero e Cura a Carattere Scientifico Polidinico San Matteo, University of Pavia, 27100 Pavia, Italy; and Department of Pediatrics, King/Drew Medical Center, Los Angeles, California 90059

Alberti, Antonella, Franco Ravenna, Daniela Quaglino, Maurizio Luissetti, Maurizio Muraca, Lorenzo Previato, Goretta Baldo Enzi, Roberta Bruni, and Aldo Baritussio. In chyloptysis, SP-A affects the clearance of serum lipoproteins entering the airways. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L737–749, 1998.—Serum lipoproteins may enter the airways and appear in sputum (chyloptysis) when the lymphatic circulation is impaired by inflammation, neoplasia, or an abnormal proliferation of smooth muscle cells. While analyzing the bronchoalveolar lavage fluid of a patient with chyloptysis, we noticed that surfactant could not be separated from contaminating serum lipoproteins. On macroscopical density gradient centrifugation, we noticed that surfactant had a lower than normal density and could not be separated from contaminating serum lipoproteins. On incubating it with 125I-labeled very low density lipoproteins (VLDLs), low-density lipoproteins, and high-density lipoproteins, we found that surfactant was not separated from contaminating lipoproteins. On incubating it with 125I-labeled very low density lipoproteins, we speculated that lipoproteins might interact with surfactant components. To clarified this point we immobilized surfactant protein (SP) A on microtiter wells and incubated it with 125I-labeled very low density lipoproteins (VLDLs), low-density lipoproteins, and high-density lipoproteins. We found that SP-A binds lipoproteins. Studying in greater detail the interaction of SP-A with VLDLs, we found that the binding is time and concentration dependent; is inhibited by unlabeled lipoproteins, phospholipids, and antibodies to SP-A; is increased by Ca2+; and is unaffected by methyl-α-D-mannopyranoside. Whole surfactant is a potent inhibitor of binding. Furthermore, we found that SP-A increases the degradation of VLDLs by alveolar macrophages and favors the association of VLDLs with alveolar surfactant. We conclude that SP-A influences the disposal of serum lipoproteins entering the airways and speculate that binding to alveolar surfactant might represent an important step in the interaction between exogenous substances and the lung.

Lung surfactant; surfactant protein A; alveolar macrophages; lymphangiolemioomatosis

LUNG ARCHITECTURE PREVENTS circulating fluid, protein, and cells from flooding the air spaces, the primary barrier being the alveolar epithelium. Tight junctions are important for the barrier function of the alveolar epithelium. They surround the cells circumferentially near the apical surface and allow them to maintain polarized apical and basolateral domains (reviewed in Ref. 24). If alveolar flooding occurs, excess fluid must be removed to restore gas exchange. Recent experiments indicate that Na+ absorption by type II cells is the driving force for clearing liquid from the alveolar spaces, that water follows Na+ movement through specific water-transporting proteins, and that most proteins are cleared by restricted diffusion through paracellular routes (reviewed in Ref. 8). It is also considered that intraluminal degradation of proteins, removal through receptor-mediated endocytosis, and translocation by pinocytosis are all clearance pathways of minor importance (8). In addition to the removal of particulate matter, alveolar macrophages seem to play an important, albeit ill-defined, role in the removal of proteins from the airways (8).

Serum lipoproteins migrate from the vascular space into the interstitial where they deliver lipids to cells, contribute to the reverse transport of cholesterol, and are possibly involved in processes such as the removal of lipopolysaccharide endotoxins and the defense against infectious agents (18). In the lung, lipoproteins come in contact with the basolateral portion of alveolar cells and usually cannot be detected in the alveolar spaces. Serum lipoproteins, together with other components of the interstitial fluid, may enter the airways when the blood-gas barrier is grossly disrupted or when the lymphatic circulation is impaired by inflammation, neoplasm, or an abnormal proliferation of smooth muscle cells. The last condition is the hallmark of lymphangiolemioomatosis (36). This rare disease is characterized by the abnormal proliferation of smooth muscle cells in thoracic and abdominal lymphatics and in lung bronchovascular axes, with obstruction of bronchioles, arterioles, venules, and lymphatics and formation of cysts throughout the lung. Lymphangiolemioomatosis occurs almost invariably in women of childbearing age and presents clinically with dyspnea, spontaneous pneumothorax, chylothorax, hemoptysis, and chyloptysis (presence of serum lipoproteins in sputum). Although pneumothorax and chylothorax are frequent clinical findings, chyloptysis is rare (35). We are not aware of studies addressing the destiny of lipoproteins entering the alveoli.

Among the patients with lymphangiolemioomatosis we are currently following, one has frank chyloptysis. Her bronchoalveolar lavage fluid (BALF) has an abnormal concentration of triglycerides, cholesterol, and free fatty acids and contains proteins associated with serum lipoproteins, such as apolipoproteins (apo) A1, A2, B, C2, C3, and E. While analyzing the BALF by continuous density gradient centrifugation, we noticed that surfactant had a lower than normal density and could not be separated from contaminating lipoproteins. On
the basis of these observations, we speculated that surfactant may interact with lipoproteins entering the airways and sought to determine which surfactant components are involved in the interaction.

We started by studying the interaction between surfactant protein (SP) A and serum lipoproteins because it has been shown that SP-A is able to bind synthetic lipid vesicles (19), lipopoly saccharides (16, 38), viruses (39), bacteria (reviewed in Ref. 41), and components of the plasma membrane of type II pneumocytes and alveolar macrophages (22, 28). We then studied the effect of adding SP-A to BALF containing lipoproteins. Finally, we analyzed the effect of SP-A on the degradation of lipoproteins by alveolar macrophages. We found that SP-A binds lipoproteins entering the airways, associates them with alveolar surfactant and alveolar macrophages, and increases their degradation by alveolar macrophages.

MATERIALS AND METHODS

Media and chemicals. Dipalmitoyl phosphatidylcholine (DPPC), egg phosphatidylcholine (PC), phosphatidylglycerol from egg lecithin (PGL), cholesterol, and thrombin were obtained from Sigma (St. Louis, MO). Before use, phospholipid purity was checked by thin-layer chromatography (29). 125I was from Amersham. DMEM and trypsin-EDTA were from Gibco BRL (Paisley, UK). Lipoprotein-poor fetal bovine serum was prepared by bringing fetal bovine serum (Sigma) to a density of 1.25 g/ml with NaBr, centrifuging it twice at 120,000 g for 48 h, and discarding the floating components at each step. At the end, lipoprotein-poor fetal bovine serum was dialyzed against PBS, filtered (0.2 µm; Minisart, Sartorius), and stored frozen.

Patients. The present investigation originated from the study of a female patient who, at 48 yr of age, 2 yr before her menopause, sought the advice of a physician for shortness of breath, persistent sputum, chronic hypoxia, radiological signs of interstitial lung disease, chyloptysis, and chylothorax. Hematologic laboratory values and blood chemical findings were normal. She was diagnosed as having lymphangioleio myomatosis after a chest computed tomography and transbronchial lung biopsy (36). Over 5 yr, we obtained 20 samples of BALF from this patient. BALF characteristics were compared with those of BALF obtained from 11 patients with idiopathic alveolar proteinosis and from 27 normal subjects under observation for hemoptysis. To obtain BALF, a fiberoptic bronchoscope was wedged into a segmental bronchus of the right lower lobe, and five 20-ml aliquots of sterile isotonic saline were instilled at room temperature and recovered by gentle suction. Usual recoveries varied between 60 and 70% of the volume introduced. BALF was filtered through sterile gauze and either examined immediately or stored frozen.

Analysis of lung lavage fluid. Proteins were measured in the presence of 1% SDS (29). SP-A was measured by antigen inhibition ELISA using rabbit IgG directed against human SP-A isolated from BALF obtained from patients with alveolar proteinosis. After reduction, SP-A migrated as a main band of 32–34 kDa and a minor band of 65 kDa during polyacrylamide gel electrophoresis (1, 14). Cholesterol and triglycerides were measured by enzymatic methods with commercial kits from Boehringer Mannheim (Mannheim, Germany) and Merck (Darmstadt, Germany), respectively. apoA1,-A2, -B, -C2, -C3, and -E were measured by immunodiffusion. We used a kit from Daiichi (Tokyo, Japan) to measure apoAI and a kit from Behring to measure apoB, -A2, -C2, -C3, and -E. In some experiments, apoAI and apoB were also analyzed by immunoblotting with sheep IgG anti-apoAI and anti-apoB obtained from Serotec.

To analyze lipids further, aliquots of lavage fluid were extracted according to Bligh and Dyer (4), and the organic phase was used for quantification of lipid phosphorus (2), for thin-layer chromatography (30, 35), and for the assay of free fatty acids. Free fatty acids were isolated from the other lipids by sorbent chromatography (17), esterified, and analyzed on a gas chromatograph (5890 series II, Hewlett-Packard, Palo Alto, CA) with a 30-m DB-23 fused-silica capillary column (J&W Scientific, Rancho Cordova, CA).

Fractionation of lung lavage fluid by ultracentrifugation. In some experiments, we examined components of lavage fluid isolated by flotation. To this end, filtered lavage fluid was centrifuged at 150 g for 10 min, and the resulting supernatant was brought to a density of 1.3 g/ml with solid NaBr. After adjustment of the pH with solid Tris, the suspension was overlaid with a solution of NaBr in saline with a density of 1.2 g/ml and centrifuged at 100,000 g for 48 h in an SW 28 rotor. At the end, materials floating on top of the gradient were collected, dialyzed against distilled water, delipidized two times with chloroform-methanol (2:1), solubilized, reduced, and fractionated by polyacrylamide gradient gel (3–10%) electrophoresis. After electrophoresis, proteins were either stained with Coomassie R-250 or transferred onto nitrocellulose, incubated overnight with sheep IgG anti-human apoB (1:1,000), and then reacted with a secondary antibody conjugated with alkaline phosphatase (Immuno AG, Vienna, Austria).

In other experiments, 4 ml of filtered lavage fluid were deposited on top of a continuous gradient of sucrose made by mixing equal amounts of 0.1 and 0.8 M sucrose in 145 mM NaCl, 10 mM HEPES, 1.25 mM CaCl2, and 1.0 mM MgCl2, pH 7.4. The tube was centrifuged at 100,000 g for 48 h in an SW 28 rotor, and its content was fractionated starting from the bottom. On the fractions, we checked the density by refractometry, measured lipid phosphorus (2) and SP-A (1), analyzed the lipid composition by thin-layer chromatography (35), and studied the distribution of apoB by immunoblotting after polyacrylamide gradient gel electrophoresis.

Isolation of surfactant from lung lavage fluid. Fresh lavage fluid was filtered through gauze and centrifuged at 150 g for

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Table 1. Lavage composition in normal subjects, patients with alveolar proteinosis, and a patient with chyloptysis

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Protein</th>
<th>Phospholipid</th>
<th>Cholesterol</th>
<th>Triglycerides</th>
<th>Fatty Acids</th>
<th>SP-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>28</td>
<td>170 ± 14</td>
<td>46 ± 5</td>
<td>2 ± 1</td>
<td>24 ± 3</td>
<td>1 ± 1</td>
<td></td>
</tr>
<tr>
<td>Alveolar proteinosis</td>
<td>12</td>
<td>5,060 ± 821†</td>
<td>885 ± 196†</td>
<td>11 ± 2†</td>
<td>76 ± 22*</td>
<td>24 ± 7</td>
<td>769 ± 309†</td>
</tr>
<tr>
<td>Chyloptysis</td>
<td>1</td>
<td>33,470</td>
<td>598</td>
<td>262</td>
<td>587</td>
<td>152</td>
<td>2</td>
</tr>
</tbody>
</table>

Values are means ± SE in ng/ml except for values for patient with chyloptysis that are averages of 6–20 determinations (n, in parentheses, range) and value for fatty acids in normal subjects that is average of 2 determinations of pooled lavages; n, no. of subjects; SP-A, surfactant protein A. Significant difference compared with normal (control) subjects: *P < 0.05; †P < 0.01.
10 min to sediment cells. The supernatant was then centrifuged at 85,000 g for 90 min. The resulting pellet was suspended in 1 M sucrose, 145 mM NaCl, 10 mM Tris·HCl, 1.25 mM CaCl₂, and 1.0 mM MgCl₂, pH 7.4, and centrifuged at 100,000 g for 36 h in an SW 28 rotor. Floating materials were collected, diluted with water, and pellet at 100,000 g for 60 min.

Electron microscopy. Freshly obtained BALF was filtered through gauze and centrifuged at 100,000 g for 90 min. The supernatant was examined by negative staining (1% uranyl acetate). The pellet was fixed in osmium tetroxide (3% in 0.1% cacodylate), stained with uranyl acetate (2%), dehydrated, embedded in Spurr resin, sectioned from top to bottom, and examined by transmission electron microscopy, taking samples at several levels.

Cells from BALF were stained with crystal violet and counted in a Neubauer hemocytometer. Differential counts of sedimented cells were done after May-Grunwald-Giemsa staining.

Isolation and labeling of serum lipoproteins. Very low density lipoproteins (VLDLs; density 0.95–1.006 g/ml), low-density lipoproteins (LDLs; density 1.019–1.063 g/ml), and high-density lipoproteins (HDLs; density 1.063–1.210 g/ml) were isolated from the serum of normolipemic subjects (apoE phenotype E 3/3) after an overnight fast (13) and were labeled with 125I (10). Labeled lipoproteins were metabolically active as judged from binding to circulating lymphocytes, had a specific radioactivity of 150–300 cpm/µg protein, and were >95% precipitable with 10% (vol/vol) TCA. They were stored at 4°C. Labeled VLDL was used within 1 mo, whereas labeled LDL and HDL were used within 1 wk.

Binding of SP-A to lipoproteins present in lung lavage fluid. Four separate 4-ml aliquots of filtered BALF, freshly obtained from the patient with chyloptysis, were diluted to 10 ml and adjusted to the following composition: 145 mM NaCl, 10 mM HEPES, 1.25 mM CaCl₂, 1.0 mM MgCl₂, and 1,250 U/ml of aprotinin (Antagosan, Behring), pH 7.4. SP-A was added to three aliquots to final concentrations of 5, 10, and 20 µg/ml, while the remaining aliquot served as the control. The samples were then incubated for 1 h at 37°C with gentle shaking. At the end, they were cooled, deposited on top of a continuous gradient of sucrose (0.1–0.8 M), and centrifuged at 100,000 g for 48 h in an SW 28 rotor. Gradients were then fractionated starting from the bottom. On the fractions, we measured lipid phosphorus and studied the distribution of

Fig. 1. Apolipoprotein (apo) B in lung lavage fluid. A: protein composition in very low density lipoproteins (VLDLs; 10 µg of protein) and floating components isolated from lavage fluid of patient with chyloptysis (SURF; 100 µg of protein). VLDLs were isolated from normal serum. To isolate floating components, lavage fluid was brought to a density of 1.3 g/ml with solid NaBr, overlaid with a solution with density of 1.2 g/ml, and centrifuged at 100,000 g for 48 h. Proteins associated with materials floating on top of tube and VLDL proteins were then fractionated on 3–10% gradient PAGE and stained with Coomassie. or, Origin; fr, front. Single arrowhead, apoB 100; double arrowhead, apoB 48. B: detection of apoB in plasma (30 µg of protein), VLDL (10 µg of protein), and SURF (50 µg of protein). After PAGE, proteins were transferred onto nitrocellulose and reacted with anti-apoB antibody. Plasma and VLDLs contained apoB-100. Lavage fluid contained apoB and apoB degradation products. C: apoB in lavage fluid obtained from normal subjects (a–d), a patient with alveolar proteinosis (e), and patient with chyloptysis (f). Floating components were isolated from 1 ml of lavage fluid as in A and analyzed as in B. apoB was present only in patient with chyloptysis. Nos. on right, molecular mass in kDa.
apoB by immunoblotting after polyacrylamide gradient gel electrophoresis.

To study the direct effect of SP-A on the centrifugal behavior of VLDLs, two aliquots of 125I-VLDL (4 ml each, 350 µg triglycerides/ml) were incubated for 1 h at 37°C in the absence and presence of 20 µg SP-A/ml and were then centrifuged at 100,000 x g for 48 h on a continuous gradient of sucrose (0.1–0.8 M). Finally, the gradients were fractionated, and the distribution of radioactivity was analyzed.

Binding of VLDLs, LDLs, and HDLs to immobilized SP-A. SP-A obtained from patients with alveolar proteinosis (100 µg/ml in 5 mM Tris) was added to 96-well microtiter plates (50 µl/well; Nunc), and incubated for 18 h at 4°C. The wells were then washed two times with PBS, pH 7.4, blocked for 1 h with 300 µl of 3% BSA in PBS at room temperature, and washed again two times with PBS. Various concentrations of labeled lipoproteins (4–280 ng protein in 40 µl of 0.5% BSA in PBS) were then added to the wells and incubated at 37°C for 0.5–8 h. Finally, unbound lipoproteins were removed by aspiration, and the wells were washed five times with PBS. Bound materials were dissolved overnight with 100 µl of 0.5% SDS in 0.1 N NaOH and counted. The binding of lipoproteins to wells coated with SP-A was considered total binding. The binding in the presence of a 25-fold excess of nonlabeled lipoproteins was considered nonspecific binding. Specific binding was calculated by subtracting nonspecific binding from total binding. Under the present experimental conditions, nonspecific binding was comparable to binding in wells not coated with SP-A. Each assay was done in triplicate and replicated two to five times. For all lipoproteins studied, binding to SP-A was saturable and time dependent, equilibrium being reached at 37°C in 4–6 h.

To characterize further the binding of lipoproteins to SP-A, we incubated immobilized SP-A with 125I-VLDL (1 µg protein/ml) for 6 h and examined the effect on binding of excess nonlabeled VLDL, 0–5 mM CaCl2, 1 mM EDTA, 1 M methyl α-D-mannopyranoside, and 1 mg/ml of rabbit IgG anti-human SP-A or IgG obtained from preimmune rabbits (6). We also examined the effect on binding of 0.01–1 mg phospholipid/ml. Phospholipids were added either as a dispersion containing unilamellar liposomes prepared by sonication (50% DPPC, 25% egg PC, 15% PGL, and 10% cholesterol), as a sonicated Bligh and Dyer (4) extract of surfactant, or as whole surfactant isolated from BALF obtained from normal subjects. Dispersion of phospholipids and Bligh and Dyer surfactant extracts was obtained by letting the dried lipids hydrate in PBS for 1 h at room temperature and then sonicating them at 30 W for 20 s at 42°C. The sonication was repeated five times, with 15-s intervals. Surfactant extracts contained 1% cholesterol. To see whether cholesterol influences the binding of VLDLs to SP-A, in a separate set of three experiments, we measured the binding of 125I-VLDL to SP-A in the presence of liposomes containing cholesterol (50% DPPC, 25% egg PC, 15% PGL, and 10% cholesterol) or not containing cholesterol (56% DPPC, 28% egg PC, and 16% PGL). In these experiments, the liposomes were added at a concentration of 0.1 mg phospholipids/ml.

Isolation of alveolar macrophages. Alveolar macrophages were isolated from lung lavage fluid obtained from normal subjects who were under observation for hemoptysis (28). The lavage fluid was filtered through a 100-µm nylon cell strainer (Falcon) and centrifuged at 150 g for 10 min. The resulting pellet was washed two times with DMEM containing (per liter) 2 mM glutamine, 3 g of glucose, and 3.7 g of Na2HCO3 and brought to a concentration of 3–6 x 106 cells/ml. Macrophages comprised >90% of the cells obtained. This suspension was then transferred to six-well Falcon plates (2 ml/well), and the cells were left to adhere to plastics for 3 h at 37°C. In each plate, three wells contained cells while the remaining wells served as controls (blank wells).

Effect of SP-A on the catabolism of VLDLs by alveolar macrophages. To wells containing adhering macrophages and to blank wells, we added 2 ml of DMEM containing 125I-VLDL (2 µg protein/ml), 10% lipoprotein-poor fetal calf serum, 5,000 U/ml of penicillin, 5,000 mg/ml of streptomycin, and 0–12 µg/ml of SP-A. The wells were then incubated at 37°C for 15 h.

Fig. 2. Distribution of phospholipids and surfactant protein (SP) A after density gradient centrifugation of lung lavage fluid. Four milliliters of lavage fluid obtained from a normal subject, a patient with alveolar proteinosis, and a patient with chyloptysis were centrifuged over continuous gradients of sucrose (0.1–0.8 M). Gradients were then fractionated, and alternate fractions were analyzed. Nos. in parentheses, content of bottom fraction. Data are representative of 2 experiments.
To measure the degradation of VLDLs by alveolar macrophages, 1 ml of medium, collected after gentle panning of the plate, was mixed with 0.1 ml of 100% ice-cold TCA, left on ice for 30 min, and centrifuged at 16,000 g for 10 min. Aliquots of the resulting supernatant were counted. Counts recovered in blank wells, representing spontaneous degradation, were 8.4 ± 1.7% (SE) of applied radioactivity. Degradation of VLDLs by macrophages, estimated after subtraction of spontaneous degradation and expressed as nanograms of VLDL protein degraded per 10⁶ cells per hour, was usually <2% of the added VLDL.

To measure the radioactivity associated with the cells, the remaining medium was aspirated and discarded, and the wells were washed two times with 5 ml of 1 mM EDTA in PBS and then incubated at 37°C for 15 min with trypsin-EDTA. The resulting suspension was used for cell counting and to measure radioactivity. Blank wells usually contained <2% of applied radioactivity. Radioactivity associated with cells was estimated after subtraction of the counts associated with blank wells.

The association of 125I-VLDL with alveolar macrophages was also studied in the following way. Wells containing macrophages and blank wells were treated for 1 h at 4°C with 3% albumin (fatty acid free; Sigma) in DMEM (3 ml/well). Wells were then incubated for 1 h at 4°C with 125I-VLDL (0–8 µg/ml) in 1.8 ml of DMEM containing 1% albumin. Fluid was then removed, and the wells were washed five times with 4 ml of PBS. Finally, well content was solubilized for 16 h with 3 ml of 0.5% SDS in 0.1% sodium hydroxide and counted. The radioactivity associated with macrophages was calculated by subtracting the radioactivity present in blank wells from that present in wells containing macrophages. In similar experiments, done at 4°C, we studied the effect of increasing concentrations of SP-A (0–12 µg/ml) or of a 25-fold excess of nonlabeled VLDL on the binding of 125I-VLDL to alveolar macrophages. Under the conditions chosen, macrophages bound <1% of the added VLDL.

Statistical analysis. Differences among several groups were analyzed by one-way ANOVA. Differences between two groups were analyzed with the paired-sample t-test. The level of significance accepted was 5%. Values are reported as means ± SE.

RESULTS

BALF composition. In our patient with lymphangioleiomyomatosis and chyloptysis, the concentration of protein, phospholipids, cholesterol, triglycerides, and free fatty acids was extraordinarily increased with respect to the normal subjects, whereas the concentration of SP-A was close to the normal concentration (Table 1). The phospholipid composition was similar to that of the normal subjects with the exception of phosphatidylglycerol, which was present in low amounts (1.2 vs. 5.5 ± 1.2% in normal subjects). Patients with alveolar proteinosis had very high concentrations of proteins, phospholipids, and SP-A. Cholesterol, triglycerides, and free fatty acids were also increased (Table 1). In the patient with chyloptysis, BALF contained 400,000–900,000 cells/ml (normal range 120,000–190,000 cells/ml). Cells were 85% lymphocytes (normal range 4–10%), 6% macrophages (80–98%), 7% neutrophils (0–5%), and 2% eosinophils (0–3%). Because at biopsy there was no infiltration of the interstitium with inflammatory cells, we assumed that the excess lymphocytes represented contamination of the air spaces with...
Most macrophages had foamy cytoplasm. When analyzed by immunodiffusion, BALF from the patient with chyloptysis contained measurable amounts of apoAI, apoAII, apoCII, apoCIII, and apoE. These proteins were undetectable in BALF obtained from the normal subjects.

When analyzed by immunoblotting, apoB was present in BALF obtained from the patient with chyloptysis but was absent in BALF obtained from the normal subjects or from patients with alveolar proteinosis. In fact, in our patient with chyloptysis, phospholipids and SP-A distributed around the middle of the gradient, whereas in normal subjects and especially in patients with proteinosis, a large fraction of these components sedimented closer to the bottom of the tube (Fig. 2). Furthermore, in the patient with chyloptysis, large amounts of cholesterol, triglycerides, and fatty acids were associated with chylomicrons isolated from a patient with chylomicronemia (data not shown). The antiserum anti-ApoB recognized apoB-100, apoB-48, and all apoB degradation products obtained by incubating LDLs with purified thrombin (23). We assumed that proteins recognized by the antiserum, with a molecular mass lower than that of apoB-100 or apoB-48, were degradation products of apoB. In plasma collected from a normal subject in the presence of 2 mg/ml of aprotinin, 2 mM EDTA, 1 mg/ml of peptatin A, and 1 mM phenylmethylsulfonyl fluoride and analyzed immediately, the antiserum recognized only apoB-100 (Fig. 1).

The abundance of different forms of apoB present in the lavage fluid of the patient with chyloptysis changed greatly from time to time.

After centrifugation of BALF over a continuous gradient of sucrose, we noticed several differences between the patient with chyloptysis, the normal subjects, and patients with alveolar proteinosis. In fact, in our patient with chyloptysis, phospholipids and SP-A distributed around the middle of the gradient, whereas in normal subjects and especially in patients with proteinosis, a large fraction of these components sedimented closer to the bottom of the tube (Fig. 2). Furthermore, in the patient with chyloptysis, large amounts of cholesterol, triglycerides, and fatty acids were associated with chylomicrons isolated from a patient with chylomicronemia (data not shown). The antiserum anti-ApoB recognized apoB-100, apoB-48, and all apoB degradation products obtained by incubating LDLs with purified thrombin (23). We assumed that proteins recognized by the antiserum, with a molecular mass lower than that of apoB-100 or apoB-48, were degradation products of apoB. In plasma collected from a normal subject in the presence of 2 mg/ml of aprotinin, 2 mM EDTA, 1 mg/ml of peptatin A, and 1 mM phenylmethylsulfonyl fluoride and analyzed immediately, the antiserum recognized only apoB-100 (Fig. 1).

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with the phospholipid band, whereas these components were not detectable in the normal subjects (Fig. 3). Finally, only BALF from the patient with chyloptysis formed a white layer of floating material enriched in triglycerides and apoB (Figs. 3A, fraction 48, and 4, top, fractions 48 and 50).

After density gradient centrifugation of BALF obtained from the patient with chyloptysis, apoB migrated both with the phospholipid band (density 1.012–1.029 g/ml) and with components floating at the top of the gradient (density < 1.006 g/ml; Fig. 4, top, fractions 48 and 50). A closer scrutiny of these floating components reveals that they were especially enriched in apoB-48 besides containing apoB-100 and apoB degradation products. Floating materials thus contained chylomicrons and VLDLs.

After density gradient centrifugation of BALF obtained from our patient with chyloptysis, apoAI, a component of chylomicrons and HDLs, was mostly associated with the phospholipid band but was also detectable at the top of the gradient and toward the bottom of the tube (data not shown).

Binding of SP-A to serum lipoproteins present in BALF. Adding increasing amounts of SP-A (0–20 µg/ml) to the lavage fluid of the patient with chyloptysis had dramatic effects on the centrifugal behavior of the lavage components. In fact, the white layer of material floating at the top of the gradient became progressively smaller and, at a concentration of 20 µg SP-A/ml, physically disappeared. At this concentration of SP-A, lavage phospholipids moved toward sections of the gradient of lower density (Fig. 5), floating apoB disappeared, and all apoB was detected within the phospholipid band in fractions in which the density was 1.018–1.044 g/ml (Fig. 4, bottom).

Because SP-A might bind chylomicrons and VLDLs and change their density, we incubated labeled 125I-VLDL (350 µg triglycerides/ml) with SP-A (20 µg/ml) and then fractionated it by continuous density gradient centrifugation. We found that after incubation with SP-A, the apparent density of a large proportion of VLDLs remained unchanged (<1.006 g/ml), whereas a minor fraction became denser, migrating in sections of the gradient with a density of 1.007–1.024 g/ml (Fig. 6). We speculate that, on incubation with SP-A, the density of chylomicrons should change less than that of the VLDLs and conclude that the observed changes in the distribution of apoB cannot be due solely to an increase in the density of chylomicrons and VLDLs. Our results are better explained by assuming that SP-A added to the lavage fluid of our patient with chyloptysis bound VLDLs and chylomicrons and associated them with alveolar surfactant that then became less dense than normal.

The present results also indicate that in our patient with chyloptysis, the ability of SP-A to bind apoB-containing lipoproteins had been exceeded by the large amount of lipoproteins present in the airways.

Electron microscopy. In normal subjects, the pellet obtained by centrifuging BALF at 100,000 g for 90 min contained lamellar bodylike structures, few myelin figures, and vesicles with a diameter of 100–600 nm, whereas the supernatant contained small protein aggregates (Figs. 7 and 8). In patients with alveolar proteinosis, the pellet contained many lamellar bodylike structures, empty vesicles with diameters of 100 to >1,000 nm, cell debris, and abundant amorphous material, whereas the supernatant contained vesicles with 60- to 450-nm diameters (Figs. 7 and 8). In the patient with chyloptysis, the pellet contained numerous cells, cell debris, fibrin, lipid droplets, and a few vesicles immersed in a granular electron-dense material. Lamellar bodylike structures were scarce and poorly organized. There were few myelin figures (Fig. 7). The supernatant was characterized by an irregular network consistent with the presence of abundant lipid moieties and was covered by a layer of floating material containing a large number of lipid globules (Fig. 8).

Binding of VLDLs to SP-A. To characterize further the binding of SP-A to lipoproteins, we incubated labeled VLDL, LDL, and HDL with SP-A immobilized on a solid support. We found that SP-A can bind VLDL, LDL, and HDL and that the binding is concentration dependent and saturable (Figs. 9 and 10). Because the nonspecific binding of LDL and HDL is higher than that of VLDL, we used labeled VLDL to better characterize the binding of lipoproteins to SP-A. We found...
that binding is inhibited by the addition of cold VLDLs or liposomes made with DPPC, egg PC, PGL, and cholesterol, is increased by Ca\(^{2+}\), is unaffected by methyl α-D-mannopyranoside, and is attenuated by a polyclonal antiserum to SP-A (Fig. 9). IgG anti-apoB (1 mg/ml) did not influence binding (data not shown).

The binding of VLDLs to SP-A is inhibited to a different extent by different surfactant components (Fig. 11). In fact, whole surfactant inhibited the binding more efficiently than surfactant lipids or Bligh and Dyer (4) surfactant extracts, suggesting that SP-A associated with surfactant can bind VLDLs. Furthermore, surfactant extracts that contain surfactant lipids, SP-B, and SP-C inhibited binding less efficiently than liposomes made of pure lipids (Fig. 11), suggesting that SP-B and SP-C are not involved in the binding of VLDLs to alveolar surfactant and may possibly inhibit binding. Finally, liposomes containing cholesterol inhibited binding with the same efficiency as liposomes free of cholesterol (data not shown), suggesting that phospholipids are involved in the binding of VLDLs to SP-A.

These experiments were done with surfactant obtained from normal subjects; however, surfactant obtained from patients with alveolar proteinosis can also bind VLDLs (data not shown).

Effect of SP-A on the degradation of VLDLs by alveolar macrophages. In three experiments, we incubated alveolar macrophages with a fixed concentration of \(^{125}\)I-VLDL (2 µg protein/ml) in the presence of increasing concentrations of SP-A (0–12 µg/ml). We found that alveolar macrophages can degrade VLDLs and that the degradation rate is influenced by SP-A. However, the shape of the dose-response curve varied among experiments, the greatest effect being observed at concentrations of SP-A ranging from 3 to 12 µg/ml (Fig. 12, inset). For this reason, in a larger series of

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**Fig. 7.** Transmission electron microscopy of pellet obtained by centrifuging lavage fluid at 100,000 g for 90 min. A–C: lower part of pellet. D–F: upper part of pellet. In normal subjects (A and D), pellet contained lamellar bodylike structures, few myelin figures (arrowhead), and vesicles 160–600 nm in diameter. In patients with alveolar proteinosis (B and E), pellet contained many lamellar bodylike structures, empty vesicles 100 to >1,000 nm in diameter, and amorphous material (*). In patient with chyloptysis (C and F), pellet contained few lamellar bodylike and myelin figures, lipid globules (arrows), and abundant amorphous material. Bars, 1 µm.

**Fig. 8.** Negative staining of supernatant obtained by centrifuging lung lavage fluid at 100,000 g for 90 min. In normal subjects (A), supernatant contained protein aggregates (arrowheads). In patients with alveolar proteinosis (B), supernatant contained vesicles 60–450 nm in diameter. In patient with chyloptysis, supernatant was characterized by an irregular network consistent with presence of abundant lipid moieties (C) and was covered by a layer of floating material containing many lipid globules (D, arrows). Bars, 1 µm.
In the past, SP-A has received much attention because of its involvement in the regulation of the surfactant life cycle. Recent in vitro experiments, however, indicate that this protein may also be important in the defense against invading organisms and in the modulation of the immune response in the lung. Thus it has been shown that SP-A binds different types of pathogens and inactivates some of their toxic products (16, 38, 39, 41), stimulates phagocytosis and chemotaxis (37, 42), increases the production of reactive oxygen species by alveolar macrophages (40), and influences the production of cytokines such as granulocyte-macrophage colony-stimulating factor and tumor necro-

Fig. 9. Binding of $^{125}$I-VLDL to SP-A. Labeled VLDL was incubated with SP-A immobilized on plastic microwell plates, and binding was estimated as a function of VLDL concentration (top left), incubation time (top right), and various additions (bottom). Binding in presence of a 25x excess of nonlabeled VLDL was considered nonspecific binding. Specific binding ($\Delta$) was calculated by subtracting nonspecific binding ($\bigcirc$) from total binding ($\bigcirc$). Effect of incubation time and additions was studied in presence of 1 $\mu$g $^{125}$I-VLDL protein/ml, and data shown represent specific binding. Effect of Ca$^{2+}$ was studied in presence of 10 mM HEPES; all other incubations were done in PBS. Effect of IgG anti-SP-A was compared with that of preimmune rabbit IgG. Values are means ± SE; n, no. of experiments. DPPC, dipalmitoyl phosphatidylcholine; PC, phosphatidylcholine; PGL, phosphatidylglycerol from egg lecithin; Chol, cholesterol; ns, not significant. $p$, $P$ values compared with no addition by paired $t$-test.

Fig. 10. Binding of $^{125}$I-low-density lipoprotein (LDL; top) and $^{125}$I-high-density lipoprotein (HDL; bottom) to SP-A. Labeled lipoproteins were incubated in microwells coated with SP-A (total binding). Binding to wells coated with SP-A in presence of excess nonlabeled lipoproteins represented nonspecific binding. Specific binding was estimated by subtracting nonspecific binding from total binding.

DISCUSSION

In the past, SP-A has received much attention because of its involvement in the regulation of the surfactant life cycle. Recent in vitro experiments, however, indicate that this protein may also be important in the defense against invading organisms and in the modulation of the immune response in the lung. Thus it has been shown that SP-A binds different types of pathogens and inactivates some of their toxic products (16, 38, 39, 41), stimulates phagocytosis and chemotaxis (37, 42), increases the production of reactive oxygen species by alveolar macrophages (40), and influences the production of cytokines such as granulocyte-macrophage colony-stimulating factor and tumor necro-

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SP-A also affects the association of VLDLs with alveolar macrophages. In fact, when $^{125}$I-VLDL was incubated with alveolar macrophages at 37°C for 15 h, the radioactivity associated with the cells was 5.0 ± 1.0% of the total radioactivity in the absence of SP-A and 12.7 ± 2.6% in the presence of 6 $\mu$g SP-A/ml ($p < 0.01$ by paired $t$-test). The effect of SP-A on the association of VLDLs with alveolar macrophages was confirmed in companion experiments in which alveolar macrophages were exposed to $^{125}$I-VLDL at 4°C for 1 h (Fig. 13). Those experiments showed that VLDLs bind to alveolar macrophages and that binding is saturable because the presence of a 25x excess of nonlabeled VLDL decreased binding by >90% (not shown). As shown in Fig. 13, the association of VLDLs with macrophages at 4°C increased significantly (over 20 times) in the presence of SP-A.

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Lipids in the Airways

The function of circulating lipoproteins is to transport lipids. They provide body tissues with triglycerides (via chylomicrons and VLDLs) and cholesterol (via LDLs and HDLs) and retrieve excess lipids from the periphery via HDLs. The process is carefully directed by apoproteins that interact with specific receptors (18). Lipoproteins entering the airways cannot be expected to behave according to these lines because they may not find the appropriate receptors; may become entangled in mucus; or may be modified by the oxidative environment, a condition known to influence the fate of circulating lipoproteins (21). Furthermore, due to the presence of the mucociliary escalator, lipoproteins may escape from the lung through the larger airways.

The patient we studied had lymphangiomyomatosis, a disease that impairs lymph formation and flow in several organs and may stop lymph flow through the thoracic duct. She had chylothorax and presented, in sputum, lipoproteins originating from both the liver (e.g., VLDLs, which contain apoB-100) and the intestine (e.g., chylomicrons, which contain apoB-48) (18). The mechanism by which the lipoproteins entered the airways remains unclear. In fact, lipoproteins could have moved from the extravascular spaces into the airways together with other components of lung interstitial fluid, or they could have arrived through direct connections between the airways and abnormal lymph vessels carrying intestinal lymph. In both cases, it is likely, although we did not prove it, that chylomicrons entered the airways mostly after meals.

Surfactant appears to interact with lipoproteins entering the airways. In fact, in our patient with chylothorax, surfactant partitioned with serum lipoproteins during density gradient centrifugation of BALF. Furthermore, surfactant isolated from the normal subjects was able to bind VLDLs.

SP-A (but not SP-B or SP-C) appears to play a central role in the binding of lipoproteins to surfactant. This is indicated by the binding of VLDLs, LDLs, and HDLs to immobilized SP-A and by the observation that SP-A...
added to the lavage fluid of our patient with chyloptysis was able to associate with alveolar surfactant all floating lipoproteins containing apoB (chylomicrons and VLDLs).

In vitro, the binding of VLDLs to SP-A can be prevented by the addition of phospholipids, implying either that phospholipids bind SP-A to a site close to the binding site of VLDLs or that the binding of VLDLs to SP-A involves the phospholipid coat of the lipoprotein. This view is in agreement with current knowledge. In fact, several groups (11, 14, 19) have demonstrated that SP-A can bind liposomes of different phospholipid composition and aggregate them through the formation of protein-protein cross bridges. These authors have also shown that the presence of Ca$^{2+}$ is necessary for the formation of protein-protein bonds but not for the binding of SP-A to phospholipids (19).

We noticed that Ca$^{2+}$ increases the binding of VLDLs to immobilized SP-A. Because the binding of SP-A to the phospholipid coat of the lipoprotein is not expected to depend on Ca$^{2+}$ (19), the observed effect may be due to lipoprotein aggregation. The present results, however, do not exclude the possibility of Ca$^{2+}$-induced changes in the binding affinity of immobilized SP-A.

Besides binding lipoproteins, SP-A affects both the binding and degradation of VLDLs by alveolar macrophages. The mechanism by which SP-A increases the degradation of VLDLs by alveolar macrophages is unknown. It may be due to increased binding of lipoproteins to macrophages, to lipoprotein aggregation, or to a direct effect of SP-A on these cells by a process similar to that observed in the uptake of certain bacterial strains (9).

In vitro experiments and studies with intact animals indicate that alveolar macrophages might take up and degrade a large fraction of alveolar surfactant (7, 26, 43). Recently, Wright and Youmans (43) showed that the uptake of surfactant lipids by alveolar macrophages is increased by SP-A, almost doubling at a concentration of 30–40 µg SP-A/ml. They have calculated that, in the presence of 80 µg DPPC/ml and 15 µg SP-A/ml, alveolar macrophages can take up 0.7 µg DPPC·10$^6$ cells$^{-1}$·h$^{-1}$. In contrast, after the uptake of fluorescent liposomes by the mouse lung epithelial cell line MLE-12, Horowitz et al. (15) found that SP-A increases the adhesion of liposomes to the cell membrane but does not influence uptake. It is possible that this discrepancy is due to differences between cell types, to differences in the concentrations of SP-A used, or to the fact that SP-A might not affect to the same extent aggregation and uptake as our data suggest (Figs. 12 and 13).

On the basis of the present results, the rate of degradation of VLDLs by alveolar macrophages appears to be much smaller than that of surfactant lipids, even considering that proteins make up 8–10% of VLDL mass (18) and that the concentration of VLDLs to which the macrophages were exposed in vitro was far from saturation. However, it has been shown that rat peritoneal macrophages take up unmodified lipoproteins much more slowly than lipoproteins subjected to modifications such as acetylation, oxidation, aggregation, association with immune or matrix protein complexes, or glycosylation (5, 12, 21, 32). Because lipoproteins entering the airways may undergo some of these modifications, phagocytosis by alveolar macrophages might be greater than the present data suggest, and the intervention of SP-A could be significant.

The uptake of modified lipoproteins by macrophages is mediated by several receptors such as macrophage scavenger receptors type I and II, LDL receptor-related protein, FcgRII-B2, CD 36 monocyte surface antigen, and macrosialin (21, 32, 33). These mechanisms of uptake are not suppressed by increased levels of cell cholesterol, as normally happens with lipids entering the cell via the LDL receptor, and are thought to be responsible for the formation of foamy macrophages that are so characteristic of the early steps of atherogenesis (32). The presence in the BALF of our patient of foamy macrophages suggests that an unrestrained mechanism of lipid uptake might have been at work.

The degradation of serum lipoproteins by alveolar macrophages has not been studied to an extent comparable to that of other macrophage populations; however, the interest in this subject is likely to increase because it has been found that alveolar macrophages express scavenger receptors (27); that a secreted form of macrophage scavenger receptor binds to substances like crocydolite asbestos (34), lipopolysaccharide (31), and lipoteichoic acid (12); and that scavenger receptors type I and II have a short collagenous domain that makes them members of the family of defense collagens. Defense collagens include C1q complement component, mannan binding protein, conglutinin, and SP-A and -D (21).

To summarize, in patients with chyloptysis, SP-A interacts with lipoproteins entering the airways and influences their fate, favoring binding to alveolar surfactant and degradation by alveolar macrophages. Binding to alveolar surfactant, which covers a much greater area than that covered by the mucociliary escalator, might represent an important step in the interaction between exogenous substances and the lung.

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Address for reprint requests: A. Baritussio, Istituto di Medicina Interna, Policlinico, Via Giustiniani 2, 35128 Padua, Italy.

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