Time course of lung parenchyma remodeling in pulmonary and extrapulmonary acute lung injury

Flavia B. Santos,¹ Lilian K. S. Nagato,¹ Nicolau M. Boechen,² Elnara M. Negri,³ Alberto Guimarães,¹ Vera L. Capelozzi,⁴ Debra S. Faffe,¹ Walter A. Zin,¹ and Patricia R. M. Rocco²

Laboratories of ¹Respiration Physiology, and ²Pulmonary Investigation, Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro, Rio de Janeiro; ³Department of Thoracic Medicine, Hospital Antonio Candido Camargo, São Paulo; and ⁴Department of Pathology, Faculty of Medicine, University of São Paulo, São Paulo, Brazil

Submitted 8 April 2005; accepted in final form 11 August 2005

Santos, Flavia B., Lilian K. S. Nagato, Nicolau M. Boechen, Elnara M. Negri, Alberto Guimarães, Vera L. Capelozzi, Debra S. Faffe, Walter A. Zin, and Patricia R. M. Rocco. Time course of lung parenchyma remodeling in pulmonary and extrapulmonary acute lung injury. J Appl Physiol 100: 98–106, 2006. First published August 18, 2005; doi:10.1152/japplphysiol.00395.2005—The aim of this study is to test the hypothesis that the early changes in lung mechanics and the amount of type III collagen fiber do not predict the evolution of lung parenchyma remodeling in pulmonary and extrapulmonary acute lung injury (ALI). For this purpose, we analyzed the time course of lung parenchyma remodeling in murine models of pulmonary and extrapulmonary ALI with similar degrees of mechanical compromise at the early phase of ALI. Lung histology (light and electron microscopy), the amount of elastic and collagen fibers in the alveolar septa, the expression of matrix metalloproteinase-9, and mechanical parameters (lung-resistive and viscoelastic pressures, and static elastance) were analyzed 24 h, 1, 3, and 8 wk after the induction of lung injury. In control (C) pulmonary (p) and extrapulmonary (exp) groups, saline was intratracheally (it; 0.05 ml) instilled and intraperitoneally (ip; 0.5 ml) injected, respectively. In ALIp and ALIexp groups, mice received Escherichia coli lipopolysaccharide (10 μg it and 125 μg ip, respectively). At 24 h, all mechanical and morphometrical parameters, as well as type III collagen fiber content, increased similarly in ALIp and ALIexp groups. In ALIexp, all mechanical and histological data returned to control values at 1 wk. However, in ALIp, static elastance returned to control values at 3 wk, whereas resistive and viscoelastic pressures, as well as type III collagen fibers and elastin, remained elevated until week 8. ALIp showed higher expression of matrix metalloproteinase-9 than ALIexp. In conclusion, insult in pulmonary epithelium yielded fibroelasticogenesis, whereas mice with ALI induced by endothelial lesion developed only fibrosis that was repaired early in the course of lung injury. Furthermore, early functional and morphological changes did not predict lung parenchyma remodeling.

collagen; elastin; extracellular matrix; lung mechanics

THE EVOLUTION OF PATIENTS with acute respiratory distress syndrome (ARDS) and/or acute lung injury (ALI) is quite variable and has been the subject of great controversy (3, 29, 45). ARDS is typically described as a stereotyped response to lung injury with transition from acute alveolar capillary damage to a proliferative phase, independent of the initial etiology (20, 41). Although various causes of ARDS result in a uniform pathology in the late stage, evidence indicates that the pathophysiology of early ARDS may differ according to the type of the primary insult (17, 48).

Two different forms of ARDS are described: pulmonary ARDS, with direct effects on lung epithelial cells, and extrapulmonary ARDS, reflecting lung involvement secondary to a systemic inflammatory response, being the main target damage to the pulmonary endothelial cell (3, 14, 30, 33). It has been recognized that pulmonary and extrapulmonary ARDS are not identical, and differences could be detected radiographically, functionally, and therapeutically (ventilatory strategies, positive end-expiratory pressure, drugs) (3, 11, 14, 17). However, there are contradictions among the different studies addressing these issues because 1) the distinction between pulmonary and extrapulmonary ARDS is not always clear and simple, 2) it is possible that direct and indirect insults coexist in the same subject, making it difficult to evaluate these two entities separately, and 3) these patients present different degrees of lung injury (14, 30, 33). To rule out these limitations and to better understand the mechanisms regulating the inflammatory and fibroproliferative responses, we recently developed BALB/c mice models of pulmonary and extrapulmonary ALI induced by Escherichia coli lipopolysaccharide (LPS) with similar functional changes early in the course of lung injury (21). We observed that, given the same pulmonary mechanical dysfunction, independently of the etiology of lung injury, insult to pulmonary epithelium yielded more pronounced inflammatory responses. Although an exaggerated inflammatory response underlies the pathogenesis of pulmonary ALI at the early phase, the amount of collagen fiber was similar in pulmonary and extrapulmonary ALI (21), suggesting that inflammation and fibrosis could be dissociated. On the other hand, it has been described that inflammation and fibrosis are probably intimately linked, although the exact degree to which inflammation drives fibrosis remains unclear (8). Because Menezes and colleagues (21) analyzed the morphofunctional changes only 24 h after the induction of lung injury, we could not assert whether a progressive fibrosis occurred without an increase in lung inflammation.

In addition, some studies have attempted to show that the severity and outcome of ALI depend on the balance between alveolar epithelial and/or vascular endothelial injuries and their repair mechanisms (15, 45). Efficient alveolar epithelial repair may reduce the development of fibrosis, since the presence of an intact alveolar epithelial layer suppresses fibroblast proliferation and matrix deposition (1, 15, 45). On the other hand,
pulmonary endothelium is also critical for the repair and remodeling of the alveolar capillary membrane (28).

Thus the aim of this study is to test the hypothesis that the early changes in lung mechanics and the amount of collagen fiber in alveolar septa do not predict the time course of lung parenchyma remodeling in ALI. To that end, lung histology (light and electron microscopy), the amount of elastic and collagen fibers in the alveolar septa, the expression of matrix metalloproteinase (MMP)-9, and mechanical parameters [static elastance (Est), resistive and viscoelastic/inhomogeneous pressures] were analyzed at 24 h, 1, 3, and 8 wk after the induction of lung injury.

**MATERIALS AND METHODS**

**Animal Preparation**

Sixty BALB/c mice (20–25 g) were randomly divided into four main groups. In control groups, the animals received sterile saline solution (0.9% NaCl) intratracheally (it; 0.05 ml; Cp group; n = 6) or intraperitoneally (0.5 ml, Cexp group; n = 6). In ALI groups, the animals received E. coli LPS (555–55, 10 μg it (ALIp group, n = 24) or 125 μg ip (ALIexp group, n = 24) suspended in saline solution with total volumes equal to 0.05 and 0.5 ml, respectively). These doses of E. coli LPS were able to yield a 1.5-fold increase in lung Est compared with control groups (21). Animals from ALIp and ALIexp groups were analyzed at 24 h, 1, 3, and 8 wk (n = 6 at each time point). For intratracheal instillation, mice were anesthetized with sevoflurane, a 1-cm-long midline cervical incision was made to expose the trachea, and LPS or saline was instilled with a bent 27-gauge tuberculin needle. The cervical incision was closed with 5.0 silk suture and the mice returned to their cage. The animals recovered rapidly after surgery.

**Pulmonary Mechanics**

Twenty-four hours, 1, 3, and 8 wk after E. coli LPS administration, the animals were sedated with diazepam (1 mg ip) and anesthetized with pentobarbital sodium (20 mg/kg body wt ip). The trachea was cannulated, and the cannula (0.8-mm ID) was tied firmly in place with a suture placed around the trachea. Then the animals were paralyzed with gallamine triethyliodide (2 mg/kg), and a constant-flow ventilator provided artificial ventilation (Samay VR15, Universidad de la Republica, Montevideo, Uruguay) with a frequency of 100 breaths/min, a tidal volume of 0.2 ml, flow of 1 ml/s, and positive end-expiratory pressure of 2.0 cmH2O. The anterior chest wall was surgically removed.

A pneumotachograph (1.5-mm ID, length = 4.2 cm, distance between side ports = 2.1 cm) was connected to the tracheal cannula for the measurements of airflow and changes in lung volume (23). The pressure gradient across the pneumotachograph was determined by means of a Validyne MP45-2 differential pressure transducer (Engineering Corp, Northridge, CA). The flow resistance of the equipment, tracheal cannula included, was constant up to flow rates of 26 ml/s and amounted to 0.12 cmH2O·ml−1·s−1. Equipment resistive pressure (flow resistance of the equipment × airflow) was subtracted from pulmonary resistive pressure so that the present results represent intrinsic values. Tracheal pressure was measured with a Validyne MP-45 differential pressure transducer (Engineering Corp). All signals were conditioned and amplified in a Beckman type R Dynograph (Schiller Park, IL). Flow and pressure signals were also passed through eight-pole Bessel filters (902LPF, Frequency Devices, Haverhill, MA) with the corner frequency set at 100 Hz, sampled at 200 Hz with a 12-bit analog-to-digital converter (DT2801A, Data Translation, Marlboro, MA), and stored on a microcomputer. All data were collected using LABDAT software (RHT-InfoData, Montreal, Quebec, Canada).

Lung resistive (ΔP1) and viscoelastic/inhomogeneous (ΔP2) pressures, total pressure drop (ΔPtot = ΔP1 + ΔP2) and Est were computed by the end-inflation occlusion method (5, 6). Briefly, after end-inspiratory occlusion, there is an initial fast drop in tracheal pressure (ΔP1) from the preocclusion value down to an inflection point followed by a slow pressure decay (ΔP2), until a plateau is reached. This plateau corresponds to the elastic recoil pressure of the lung. ΔP1 selectively reflects airway resistance in normal animals and humans, and ΔP2 reflects stress relaxation, or viscoelastic properties of the lung, together with a small contribution of time-constant inhomogeneities (pendelluft) (5, 39). Lung Est was calculated by dividing the elastic recoil pressure of the lung by the tidal volume.

**Histological Study**

Light microscopy. Heparine (1,000 IU) was intravenously injected immediately after the determination of respiratory mechanics. The trachea was clamped at end expiration, and the abdominal aorta and vena cava were sectioned, yielding a massive hemorrhage that quickly killed the animals. Then the lungs were removed en bloc at end expiration. Two investigators, who were unaware of the origin of the material, examined the samples microscopically.

The left lung was quick-frozen by immersion in liquid nitrogen, fixed with Carnoy’s solution (24), and embedded in paraffin. Four-micrometer-thick slices were obtained by means of a microtome and stained with hematoxylin and eosin. Morphometric analysis was performed with an integrating eyepiece with a coherent system made of a 100-point and 50 lines (known length) grid coupled to a conven-

Table 1. Morphometrical parameters and cellularity in lung parenchyma

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normal Area, %</th>
<th>Alveolar Collapse, %</th>
<th>Lm, μm</th>
<th>PMN, %</th>
<th>MN, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp</td>
<td>95.80±0.15</td>
<td>4.20±0.15</td>
<td>40.99±1.20</td>
<td>15.55±0.73</td>
<td>21.47±0.93</td>
</tr>
<tr>
<td>ALIp24</td>
<td>49.50±1.99*</td>
<td>50.50±1.90*</td>
<td>30.02±0.57*</td>
<td>30.71±2.80*</td>
<td>4.19±1.34*</td>
</tr>
<tr>
<td>ALIp1</td>
<td>77.92±2.06††</td>
<td>22.09±2.06†</td>
<td>33.55±0.34††</td>
<td>27.88±1.40*</td>
<td>2.92±0.58*</td>
</tr>
<tr>
<td>ALIp3</td>
<td>85.32±0.46‡‡‡</td>
<td>14.80±0.46‡‡‡</td>
<td>35.47±0.90‡‡‡</td>
<td>30.80±1.62*</td>
<td>1.76±0.83*</td>
</tr>
<tr>
<td>ALIp8</td>
<td>90.42±0.30‡‡‡‡</td>
<td>9.60±0.29‡‡‡‡</td>
<td>36.08±0.91‡‡‡‡</td>
<td>22.83±1.48</td>
<td>2.06±0.50*</td>
</tr>
<tr>
<td>Cexp</td>
<td>95.64±0.63</td>
<td>4.34±0.63</td>
<td>39.23±1.96</td>
<td>10.95±1.50</td>
<td>21.25±1.10</td>
</tr>
<tr>
<td>ALIexp24</td>
<td>55.00±0.63*</td>
<td>45.00±0.56*</td>
<td>29.74±2.13*</td>
<td>33.10±3.55*</td>
<td>4.82±1.33*</td>
</tr>
<tr>
<td>ALIexp1</td>
<td>95.00±2.47</td>
<td>5.00±2.46</td>
<td>38.60±0.48</td>
<td>15.81±1.29</td>
<td>1.85±0.41</td>
</tr>
<tr>
<td>ALIexp3</td>
<td>91.99±2.48</td>
<td>8.01±2.48</td>
<td>38.26±0.24</td>
<td>17.80±1.10</td>
<td>1.08±0.08</td>
</tr>
<tr>
<td>ALIexp8</td>
<td>90.24±2.54</td>
<td>6.76±2.52</td>
<td>39.48±0.23</td>
<td>10.93±1.16</td>
<td>4.65±1.19</td>
</tr>
</tbody>
</table>

Values are means ± SE of 6 animals in each group. Data were gathered from 10 random, noncoincident fields per mouse. In control (C) pulmonary (p) and extrapulmonary (exp) groups, saline was intratracheally instilled (it; 0.05 ml) and intraperitoneally injected (0.5 ml), respectively. In acute lung injury (ALI) groups at 24 h, 1, 3, and 8 wk, ALIp and ALIexp groups mice received E. coli LPS (10 and 125 μg ip, respectively). Lm, mean linear intercept between alveolar walls; PMN, polymorphonuclear cell fractional area; MN, mononuclear cell fractional area. *Significantly different from C group (P < 0.05). †Significantly different from ALIp24 (P < 0.05). §Significantly different from ALIexp1 (P < 0.05). ||Significantly different from ALIexp3 (P < 0.05).
tional light microscope (Axioplan, Zeiss, Oberkochen, Germany). The fraction areas of collapsed and normal alveoli were determined by the point-counting technique at a magnification of ×200 across 10 random noncoincident microscopic fields (46). Lung tissue distortion was assessed by measuring the mean linear intercept between alveolar walls (Lm) at a magnification of ×200. Lm was determined by counting the number of intercepts between the eyepiece lines and the alveolar septa of each microscopic field. Lm was expressed as the relation between the line length (1.250 μm) and the total number of intercepts (Lm = \Σ line length/number of intercepts). Polymorphonuclear and mononuclear cells and pulmonary tissue were evaluated at ×1,000 magnification. Points falling on polymorphonuclear and mononuclear cells were counted and divided by the total number of points falling on tissue area in each microscopic field (46). Slices also underwent specific staining methods to quantify collagen (Picosirisius-polarization method) (22) and elastic fibers (Weigert’s resorcin fuchsin method with oxidation) (47) in the alveolar septa. The alveolar septa quantification was carried out with the aid of a digital analysis system and specific software (Image-Pro Plus 4.1 for Windows; Media Cybernetics, Silver Spring, MD) under ×200 magnification. The images were generated by a microscope (Axioplan, Zeiss, Oberkochen, Germany) connected to a camera (Sony Trinitron charge-coupled device, Sony, Tokyo, Japan) and fed into a computer through a frame grabber (Oculus TCX, Coreco, St. Laurent, PQ, Canada) for offline processing. The thresholds for collagen and elastic fibers, kept constant throughout the measurements, were established after enhancing the contrast up to the easy identification of the fibers as either black (elastic) or birefringent (collagen) bands. The area occupied by fibers was quantified by digital densitometry, adjusting the threshold level of measurement to the gray density of fibers of the collagenous and elastic systems. To avoid any bias resulting from septal edema or alveolar collapse, the areas occupied by the elastic and collagen fibers were measured in each alveolar septum and were divided by the length of each studied septum. The results were expressed as the amount of elastic and collagen fibers per unit of septum length (μm²/μm).

**Transmission electron microscopy.** To obtain a stratified random sample, three slices of 2 × 2 × 10 mm from three different segments of the right lung (cranial, middle, and caudal lobes) and then fixed with 2.5% glutaraldehyde and 0.1 M phosphate buffer (pH = 7.4) for 60 min at −4°C. The slices were then rinsed in phosphate buffer, postfixed with 1% osmic tetroxide in phosphate buffer for 30 min, and rewashed three times in phosphate buffer. Finally, the slices were dehydrated in an acetone series and then placed in a mixture of 1:1 acetone:Epon overnight before embedding in Epon for 6 h. After fixation, the material was kept for 48 h at 60°C before undergoing ultramicrotomy for transmission electron microscopy.

**Immunohistochemistry**

Strips (2 × 2 × 10 mm) from the right lung were fixed with 4% paraformaldehyde and embedded in paraffin for immunohistochemical study. MMP-9 was identified by immunohistochemical staining with the peroxidase-labeled-streptavidin biotin method+ technique (Dako, Carpinteria, CA). Antigen retrieval was carried out by pressure-cooking the slides for 3 min in 10 mM citric acid buffer at pH 6, and endogenous peroxidase activity was blocked by placing the slides in 2% hydrogen peroxide for 30 min. The slides were then rinsed in deionized water followed by Tris-buffered saline containing 0.1% bovine serum albumin and incubated in 20% normal rabbit serum for 10 min. Then slides were incubated with primary antibody (mouse monoclonal 56-2A4 at 1:100 dilution; Dako, Carpinteria, CA) overnight at 4°C. Slides were then washed in Tris-buffered saline and incubated sequentially with biotinylated rabbit antimouse IgG (Dako) at a dilution of 1:1,000. This was followed by streptavidin combined in vitro with biotinylated horseradish peroxidase at a dilution of 1:1,000 (Dako). The reaction product was developed using diaminobenzidine tetrahydrochloride. Finally, the slides were counterstained with hematoxylin, dehydrated, and mounted in resinous mountant. Known positive controls were included in each run, as well as negative controls without the primary antibody (31). Any brown cytoplasmic staining of cells (macrophages, neutrophils, and alveolar cells) characterized positive expression for MMP-9, and we used morphometry and an eyepiece grid with 100 points and 50 lines to quantify the staining as follows. First, at ×100 magnification, we selected the region of highest expression. Then the grid was used at ×400 to count the fraction of points overlaying positively stained structures. The average of 10 microscopic fields provided the final result as a percentage of stained structures (31).
Statistical Analysis

SigmaStat 2.0 statistical software package (Jandel, San Raphael, CA) was used. The normality of the data (Kolmogorov-Smirnov test with Lilliefors’ correction) and the homogeneity of variances (Levene median test) were tested. If both conditions were satisfied, one-way ANOVA test followed by Bonferroni’s post hoc test were used to assess differences among each pulmonary or extrapulmonary groups. In the negative case, Kruskal-Wallis ANOVA was used followed by Dunn’s test. Two-way ANOVA was used to compare pulmonary and extrapulmonary effects along time using the etiology of lung injury and the time course of ALI as the two factors for analysis. Spearman correlation test was run to identify associations between functional and morphological data. In all instances, the significance level was set at 5% (α = 5%).

RESULTS

The survival rate was 100% in both ALI groups. During the experiment, the animals did not lose weight (initial weight: 20–25 g; final weight at 8 wk: 35–40 g).

The fraction area of alveolar collapse and the number of polymorphonuclear cells were higher and Lm was lower in ALI than in control at 24 h (Table 1 and Fig. 1). In ALIexp, lung parenchyma was repaired at the first week with all morphometrical parameters similar to those in the control group (Table 1 and Fig. 1). However, in ALIp, the fraction area of alveolar collapse remained high until the week 8, although it decreased progressively along time. Lm was higher in ALI at 1, 3, and 8 wk than at 24 h but remained lower than the control group. The number of polymorphonuclear cells persisted elevated until week 3 and returned to control values at 8 wk. Mononuclear cell content was smaller in both ALI groups compared with the control (Table 1).

Collagen fiber content in the alveolar septa was significantly higher in both ALIp and ALIexp groups compared with control groups 24 h after the induction of lung injury (Figs. 2 and 3). The amount of collagen fiber remained elevated until week 8 in ALIp, whereas it returned to control values at 1 wk in ALIexp. Elastic fiber content increased in ALIp group at 1 wk and remained increased until 8 wk, whereas there was no elastogenesis in the ALIexp group (Fig. 3).

Figures 4 and 5 show the time course of ultramicroscopy of lung parenchyma in ALIp and ALIexp groups, respectively. In control groups, alveolar spaces and epithelial cells were preserved (Figs. 4A and 5A). At 24 h, ALIp group showed cytoplasmatic degeneration of type II pneumocyte. In ALIexp, there was no lesion in type II pneumocyte, although endothelial damage was present. In both groups, the alveolar interstitium was thickened because of increased amounts of extracellular matrix elements, such as collagen fibers at 24 h (Figs. 4B and 5B). In ALIp, the elastic fiber content was increased at 1 wk (Fig. 4C), and the ratio between types I and III collagen fibers increased progressively along time (Figs. 4A and 5A). Figure 6 shows the immunohistochemical localization of type III collagen in the alveolar septa at 24 h, 1, 3, and 8 wk after intratracheal instillation of saline (C) or E. coli LPS (ALI; 10 μg it (p) or 125 μg ip (exp), respectively). Boxes show interquartile (25–75%) range, whiskers encompass range, and horizontal lines represent median values. *Significantly different from C group (P < 0.05).
increased progressively during the time course of lung injury (Fig. 4, D and E). In ALIexp, there was a small amount of type III collagen fiber and no elastogenesis at 3 and 8 wk (Fig. 5, D and E). In addition, fibroblasts lost their myofibroblastic features (lower amount of cytoplasmic organelles) at 8 wk (Fig. 5 E).

At 24 h, all mechanical parameters (ΔP1, ΔP2, ΔPot, and Est) increased to the same extent in ALIp and ALIexp groups compared with the control groups (Figs. 6 and 7). In ALIp, Est returned to control values at 3 wk, whereas resistive and viscoelastic pressures remained elevated until 8 wk. In ALIexp, all mechanical data returned to control values at 1 wk (Figs. 6 and 7).

Considering Cc and ALIp groups together, Est and ΔP2 were well correlated with the fraction area of alveolar collapse and with polymorphonuclear cell content. ΔP2 was also correlated with the amount of collagen fiber in the alveolar septa (Table 2). Taking into consideration Cexp and ALIexp groups together, Est was significantly associated with the fraction area of alveolar collapse and polymorphonuclear cell content (Table 2).

ALIp showed more cells expressing MMP-9 than ALIexp at 24 h. In this moment, neutrophils expressed more MMP-9 than macrophages, but at 1 wk macrophages expressed a higher amount of MMP-9 in the ALIp group. In the ALIexp group, both neutrophils and macrophages expressed MMP-9, but at 1 wk the expression of MMP-9 by macrophages was higher than by the other cells (Fig. 8).

**DISCUSSION**

In the present study, with the use of models of pulmonary and extrapulmonary ALI with similar mechanical compromise in the early phase of lung injury, pulmonary ALI induced fibroelastogenesis followed by an increase in the mechanical parameters. Extrapulmonary ALI developed only fibrosis that was repaired early in the course of lung injury.

ALI was induced by intratracheal instillation or intraperitoneal injection of *E. coli* LPS. Gram-negative sepsis is the most common precipitating condition for ARDS development. The LPS components of gram-negative bacteria (endotoxin) play a major role in initiating the inflammatory processes that result in ALI/ARDS. LPS produces a well-characterized model of ALI that can mimic morphological and functional changes observed in clinical situations secondary to the presence of circulating LPS (13, 34, 38, 42). We used controlled models of pulmonary and extrapulmonary ALI induced by two different doses of *E. coli* LPS with similar functional changes (21). The use of these models is advantageous because we can precisely control the moment of the analysis, and the animals breathe spontane-
ously, avoiding the side effects resulting from mechanical ventilation. Furthermore, we can provide additional insights into the pathogenesis of the endotoxin response after endothelial or epithelial lesion.

Respiratory mechanical parameters were measured by the end-inflation occlusion method. This method allows the identification of elastic, resistive, and viscoelastic and/or inhomogeneous lung mechanical components (5, 6). It is well known that ALI increases lung Est and resistive and viscoelastic/inhomogeneous pressures early in the course of lung injury (34, 35). Our results showed that resistive and viscoelastic pressures and Est increased to the same extent in the ALIp and ALIexp groups at 24 h (Figs. 6 and 7), as previously reported (21). The early increase in lung resistive pressure in both ALI groups could be attributed to the reduction in bronchial caliber caused by thickened airway walls (39). However, resistive pressure remained elevated until week 8 in ALIp, whereas it returned to control values at 1 wk in the ALIexp group (Fig. 6). These changes probably reflect the different release of endogenous mediators following endotoxemia-yielding active airway constriction (9, 12, 21). The early increase in viscoelastic pressure until week 8 in ALIp could be attributed to the presence of alveolar collapse, distortion of patent alveoli, edema, inflammation with neutrophils (Table 1), as well as to changes in extracellular matrix components (Figs.

Fig. 5. Electron microscopy of time course of extrapulmonary ALI. A: control group. Note the preserved epithelium and the presence of capillary (C) and neutrophil (N) in the alveolar septa. B: lung parenchyma 24 h after ALI induction showed no epithelial lesion, but endothelial damage was observed. Collagen fibers (*) were located in the thick part of the alveolar wall with normal elastin, thus characterizing a process of fibrosis. C–E: 1, 3, and 8 wk, respectively, after ALI induction, there is a small amount of type III collagen fibers (*) and no elastogenesis. At 8 wk (E), the fibroblast (F) lost myofibroblastic features (lower amount of cytoplasmic organelles). Inset: detail of fibroblast cytoplasm (Fcy) with small number of mitochondria.

Fig. 6. Values are means + SE of 6 animals in each group (10–15 determinations per animal). Stacked bar chart plot data in which shaded bars represent the resistive pressure (ΔP1) and the open bars are the viscoelastic/inhomogeneous (ΔP2) pressure dissipations. The whole column represents the total pressure (ΔPtot) variation in each group. In Cp and Cexp, saline was intratracheally instilled (0.05 ml) and intraperitoneally injected (0.5 ml), respectively. In ALIp groups, E. coli LPS was intratracheally instilled (10 μg), and in ALIexp group, E. coli LPS was intraperitoneally injected (125 μg). The mechanical measurements were performed 24 h, 1, 3, and 8 wk after the induction of ALI. *Significantly different from C group (P < 0.05).
and 3). In ALIexp, viscoelastic pressure returned to control values at 1 wk, reflecting endothelial lesion repair and control of the lung inflammatory process (Fig. 6 and Table 1). In this context, Rojas and coworkers (38) also observed a transient lung injury when endotoxin was intraperitoneally injected. The early changes in Est in ALIp could result from surfactant dysfunction because pneumocyte type II degeneration was observed (Fig. 4) (16). Type II cells produce surfactant, are important to active alveolar liquid clearance, and represent the progenitor cells that regenerate the alveolar epithelium after injury (18, 44). Although electron microscopy evidenced a preserved type II pneumocyte in ALIexp (Fig. 5), the alveolar capillary membrane lost its integrity and proteinaceous fluid leaked into alveolar space and degraded surfactant, thus changing the tensoalveolar properties, probably leading to alveolar collapse and increased Est (Fig. 6) (45). The absence of epithelial lesion (Figs. 4 and 5) in the ALIp group could probably explain the early recovery of the mechanical and morphometrical parameters in the ALIexp group (Figs. 6, 1, 3, and 7, and Table 1) (15). Efficient alveolar epithelial repair may reduce the development of fibrosis in animal models of ALI because the presence of an intact alveolar epithelial layer suppresses fibroblast proliferation and matrix deposition (1, 15).

Remodeling and repair are also consequences of inflammation, reflecting the architectural response of the lung to injury, and depend on the primary site of lung injury (18, 21, 25, 39, 43). Different cell types are actively involved in the inflammatory response to diverse pathogenetic mechanisms causing ARDS. These cell types include neutrophils, macrophages, monocytes, endothelial and epithelial cells, as well as pulmonary interstitial cells such as fibroblasts (7, 18). Menezes and colleagues (21) showed that intratracheal instillation of E. coli LPS yields more pronounced inflammatory responses than does the indirect insult induced by intraperitoneal E. coli LPS administration. In the indirect insult, the inflammatory mediators released from extrapulmonary cells into the systemic circulation increase the endothelial barrier permeability and activate and recruit several inflammatory mediators (15, 28). This leads to disseminated intravascular coagulation and an acute inflammatory response, which initiates interstitial myo-

Table 2. Correlation matrix between physiological and morphometric parameters

<table>
<thead>
<tr>
<th>ALIp</th>
<th>ALIexp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collapse, %</td>
<td>PMN, %</td>
</tr>
<tr>
<td>Est, cmH₂O/ml</td>
<td>0.87 (0.001)</td>
</tr>
<tr>
<td>ΔP₂, cmH₂O</td>
<td>0.57 (0.02)</td>
</tr>
</tbody>
</table>

Est, pulmonary static elastance; ΔP₂, viscoelastic/inhomogeneous pressure; Collapse, fraction area of alveolar collapse; PMN, polymorphonuclear cells; NS, nonsignificant. P values are shown in parentheses. Correlation was performed on data from Cp, ALIp, Cexp, and ALIexp groups.
fibroblast migration into the alveolar clot. These cells move to and proliferate in the airspace. On the other hand, the loss of epithelial integrity in ALI has a number of consequences: it contributes to alveolar flooding, disrupts epithelial fluid transport, and leads to surfactant abnormalities and fibrosis. In this context, type II cells migrate and begin to proliferate along the alveolar septa in an attempt to cover the denuded basement membrane and reestablish the continuity of alveolar epithelium in the direct insult (Fig. 4). Within the alveolar wall, fibroblasts proliferate and migrate through the basement membrane into the fibrinous intra-alveolar exudate (Fig. 4). If the fibrinous exudates can be resolved, restoration of normal lung architecture may be achieved (Fig. 5). However, if alveolar type II cells migrate over the surface of the organizing granulation tissue and thereby transform the intra-alveolar exudates into interstitial tissue, interstitial fibrosis of the lung may develop (Fig. 4) (43). In the present study, type III collagen fiber, which is more flexible and susceptible to breakdown, appeared early in the course of both ALI groups, in accordance with previous studies (2, 10, 19, 21, 35–37), indicating that the biochemical processes implicated in collagen synthesis are indeed able to react very quickly to the aggression independently of the etiology of lung injury. Several studies describe that an elevated amount of procollagen type III in the bronchoalveolar lavage fluid reflects collagen synthesis at the site of disease and may be used as a marker of the reparative process. Furthermore, higher levels of procollagen type III are strongly associated with increased risk for fatal outcome (2, 10, 19). In the ALIp group, there was a progressive increase in the ratio of types I and III collagen fibers. On the other hand, type III collagen fiber content returned to control values at 1 wk in ALIexp. It results that type III collagen is probably not a good marker to predict the evolution of ALI.

The mechanisms triggering fibrogenesis seem to differ from those of elastogenesis (35). Elastic fiber content did not increase early in the course of lung injury in both ALI groups, but ALIp mice showed an increase in the amount of elastic fiber at 1 wk that remained until 8 wk. These findings are in accordance with those of Negri and colleagues (25), who suggested that elastic fiber deposition depends on both the phase of lung injury and on the primary site of disease. Furthermore, the presence of elastosis in ALIp could be attributed to the intensity of lung injury, considering that there is a threshold degree of lung injury responsible for triggering elastogenesis (26, 35–37). The absence of collagen and elastic fiber deposition late in the course of ALIexp could be explained by the absence of direct epithelial alveolar injury and consequently different fibroblast activation (Fig. 5) (1).

In the pathogenesis of ALI, degradation of the extracellular matrix is considered one of the crucial events (18). Various proteolytic enzymes are involved in this condition, such as neutrophil elastase and MMPs (18). Among these MMPs, MMP-9 has been widely studied because of its pathogenetic role in many lung diseases (4, 27, 40). MMPs present many functions: participation in the regulation of lung matrix turnover, promoting angiogenesis; contribution to lung immunoprotection through the migration of inflammatory cells into the infected or damaged lung; degradation of type IV collagen fiber, basement membrane, and elastin, and probably involvement in epithelial movement across basement membranes (49). In the present study, the level of MMP-9 increased at 24 h in both ALI groups as well as the amount of collagen fibers (Figs. 4, 5, and 8). We observed that neutrophils expressed higher levels of MMP-9 at 24 h, whereas MMP-9 was expressed mainly by macrophages in the late phase of lung injury. Interestingly, although MMPs appeared to be related to the destruction of extracellular matrix, they also have been involved in the initiation and progression of fibrosis and have profound effects on the release of growth factors and cytokines (49). In this context, in the present study, the level of MMP-9 was higher in the ALIp group, probably contributing to the on-going fibrosis, with no significant effect in initiating the fibrotic process (Figs. 2 and 8). Furthermore, we cannot rule out a potential role of MMP-9 in promoting and/or perpetuating elastogenesis (Figs. 3 and 8). MMP-9 expression decreased during the time course of the lesion in the ALIp and ALIexp groups, suggesting that there is an imbalance between matrix-degrading activity and metalloproteinases contributing to lung tissue changes (32). Several studies (27, 32, 49) investigated the role of these proteinases in ALI, but there are controversies about their contribution to the development of ALI, as well as about the relationship between MMPs and disease severity or pathological changes. MMPs alone certainly do not determine the clinical course of ARDS (32), but the understanding of the role of MMP in different clinical stages may probably lead to new therapeutic strategies.

In conclusion, early functional changes did not predict the behavior of lung parenchyma remodeling, and the amount of collagen fiber at the early phase of lung injury is probably not a marker of the reparative process. Given the same pulmonary mechanical dysfunction at the early phase of ALI, insult to pulmonary epithelium yielded fibroelastogenesis, whereas mice with ALI induced by endothelial lesion developed only fibrosis that was repaired early in the course of lung injury. Furthermore, the higher expression of MMP-9 in ALIp is possibly associated with the on-going fibrosis. These experimental models allow the testing of novel therapeutic approaches focusing on the inhibition of the fibroproliferative process.

ACKNOWLEDGMENTS

We express our gratitude to Antonio Carlos de Souza Quaresma and Veronica Cristina dos Santos for skillful technical assistance.

GRANTS

This work was supported by The Centers of Excellence Program [Programa de Apoio a Núcleos de Excelência do Ministério de Ciência e Tecnologia (PRONEX)-MCT and PRONEX-FAPERJ], The Brazilian Council for Scientific and Technological Development (CNPq), São Paulo State Research Supporting Foundation (FAPESP), and Carlos Chagas Filho Rio de Janeiro State Research Supporting Foundation (FAPERJ).

REFERENCES
