Pulmonary lymphatics and edema accumulation after brief lung injury

Dean E. Schraufnagel, Narasimhan P. Agaram, Aamir Faruqui, Sajal Jain, Leena Jain, Karen M. Ridge, and Jacob Iasha Sznajder

Departments of Medicine and Pathology, Section of Respiratory and Critical Care Medicine, University of Illinois at Chicago, Michael Reese Hospital, and Northwestern University, Chicago 60612; and Pulmonary and Critical Care Medicine, Northwestern University, Chicago, Illinois 60611

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In previous studies of hyperoxic lung injury, an increased amount of initial, saccular, and conduit lymphatics was observed after 7 days of hyperoxia (8, 22). In those studies, the increased lymphatic casting could have resulted from filling existing lymphatics and potential tissue spaces or from lymphatic proliferation. This study was undertaken to determine whether similar lymphatic filling could take place in a model of injury over a brief period. Mechanical ventilation with overinflation was chosen as a model to cause lung damage and edema (6, 26). In addition, we sought to determine the sites of this early edema formation and the structure of these compartments.

With light and scanning electron microscopy of lymphatic casts, we studied where fluid accumulates and the relations of the lymphatics to the blood vessels and airways. The casting resin that is injected into the pulmonary vasculature is viscous and hardens in ~1 min to show where fluid goes transvascularly within that time. Vascular casting is a sensitive method of detecting small amounts of lymphatic filling (1, 8, 19, 22). Spontaneously breathing animals with negative intrathoracic pressures were used in addition to animals ventilated at low tidal volume and with positive pressure, because we believed that the positive pressure may decrease focal fluid collections that we could detect with this casting technique.

METHODS

Animal preparation. The experiment was approved by the Animal Use and Care Committee at Michael Reese Hospital. The animals were handled according to National Research Council guidelines (14). Male pathogen-free Sprague-Dawley rats, weighing 289–304 g, were anesthetized intraperitoneally with pentobarbital sodium (Nembutal, Abbott Laboratories, North Chicago, IL), with an initial dose of 50 mg/kg and termination when the animal did not respond to a tail pinch. After anesthesia, a tracheostomy was performed with a 14-gauge plastic tube placed and secured ~5 mm into the trachea. The tubing was attached to a small animal ventilator (model 683, Harvard Apparatus, South Natick, MA). The rat was ventilated at a frequency of 70 breaths/min. The low-

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tidal-volume animals were ventilated at 3–5 ml and peak airway pressure of 8–10 cmH2O. The high-tidal-volume group was ventilated at 12–16 ml and peak airway pressure of 35 cmH2O. Inspiratory pressure was monitored by a Honeywell transducer attached to a strip chart recorder (model 2400, Gould, Cleveland, OH). A third group of rats were anesthetized and tracheostomized and allowed to breathe spontaneously for 25 min. All animals breathed only room air.

Thirty-five animals (divided into three groups) were used for measurement of wet-to-dry lung weight ratio. After ventilation, the rat’s right upper lobe was tied off, removed, and placed in a preweighed microcentrifuge tube. The centrifuge tube was then placed in a lyophilizer and dried overnight. The tube was reweighed, and the tube weight was subtracted from the final weight. The tube was then returned to the vacuum for several more hours. The tube was again removed and reweighed. This process was repeated until a constant weight, defined as <0.001-g difference between the two measurements, was obtained.

**Light microscopy.** Distribution of edema was assessed by light microscopy in 15 animals (five in each group). After ventilation, or the 25 min without ventilation, the lungs were removed en bloc and fixed. The trachea was filled with a 10% formalin solution at 25 cmH2O pressure. The lungs remained inflated with formalin for ≥24 h and then processed for routine light microscopy with dehydration and paraffin embedding. One block was made for each animal and positioned to give a coronal section through the apex of the lung. The light-microscopic slides were stained with hematoxylin and eosin and by the elastin-van Gieson method.

To measure the diameters of noncapillary vessels, the elastin-van Gieson-stained slides were moved to select fields at random. Vessels were identified as arteries or veins on the basis of their elastic laminae and, to a lesser extent, their position near or distal to an airway (10). The vessel diameter was the average of three diameters that allowed it to encompass the vessel edge. We recorded their diameters and the cuff diameter. For each animal, measurements for 10 arteries and 10 veins were tabulated.

To assess damage to bronchial epithelia, we studied epithelial cell sloughing severity by viewing the elastin-van Gieson-stained slides with the ×10 magnification objective. No detectable epithelial abnormality was scored 0. If the epithelial uplifting from the basement membrane was <100 μm long and no sloughed cells were found, the score was 1. Epithelial uplifting >100 μm or >20 clumps of epithelial cells was scored 2. More extensive damage of the epithelium, with areas of denudation or sheets of bronchial cells in the airway or alveoli, was scored 3.

Alveolar edema was graded by selecting random alveolar fields using the ×40 magnification objective to view the hematoxylin-and-eosin-stained slides. No evidence of edema was scored 0; a few strands of fibrin or debris could be present in the whole field, and still the edema would be scored 0. If fibrin strands or clumps were found in several, but less than half, of the alveoli and at least one erythrocyte was present per alveolus, then the score was 1. If pink, flocculent material or clumps were present in more than half of the alveoli and erythrocytes were present in most alveoli, the score was 2. If pink material or blood was found in almost all (>80%) alveoli, the score was 3. If dense proteinaceous material and erythrocytes flooded all alveoli, the score was 4.

Interlobular septal widening from edema was identified by scanning the entire slide using a ×4 magnification objective. The lengths of all widened septa were measured with the reticle and the ×10 magnification objective and recorded as a total length in millimeters of widened interlobular septa per slide per animal. Because no animal had five widened septa, five entries were recorded for each animal, with up to five zeros being assigned to those animals with no widened interlobular septa.

**Lymphatic casting and scanning electron microscopy.** For casting and scanning electron microscopy, we used 15 additional animals. After ventilation or tracheostomy-nonventilation, the abdomen was opened and the caudal vena cava was cannulated. The vasculature was rinsed with 50 ml of heparinized (5,000 U/l) normal saline warmed to 40°C. Fifteen milliliters of partially polymerized methyl methacrylate (Mercox, Ladd Research Industries, Burlington, VT) mixed with ~2 g of accelerator (50% benzoyl peroxide) were injected at a constant flow rate into the caudal vena cava over 1 min with an infusion pump (model 351, Sage Instruments, Cambridge, MA). The methacrylate was allowed to harden for 1 h, and the lungs were separated and placed in a sodium hydroxide solution until the tissue was digested.

The casts were rinsed in detergent, water, and alcohol and cut into ~1 × 8 × 8-mm pieces. The specimens were fastened to aluminum studs using double-sided tape. They were sputter coated with palladium-gold and viewed with a scanning electron microscope (model JSM-35C, JEOL). The accelerating voltage was 10 kV, and the working distance was 15 mm (18, 18a).

From each animal, four studs of the cut surface and four studs of the pleural surface were made. On the pleural surface, we recorded the presence and type of lymphatics found on 10 randomly selected fields at ×200 magnification. Random field selections were carried out by placing a numbered grid over the microscope’s cathode ray tube with the specimen image at the lowest magnification that allowed it to fill the screen. A number was selected from a random-number table, and the area under the number was moved to the center of the screen. We then increased the magnification. On the cut surface, 10 arteries and 10 veins per stud were found at ×200 magnification. We recorded their diameters and the presence of adjacent lymphatics. If we could not determine whether a vessel was an artery or a vein, it was classified as indeterminate and the same measurements were carried out.

Fig. 1. Wet-to-dry lung weight ratio. Ratio was greater in animals ventilated at high tidal volume (Vt) than in the other two groups (P < 0.01). There was no significant difference between animals ventilated at low tidal volume and spontaneously breathing (Spont) animals.

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(up to 10 indeterminate vessels per stud or until we had 10 arteries and 10 veins). If no lymphatic was present around a vessel, a score of 0 was assigned. If the vessel was partially encircled by lymphatics, a score of 1 was assigned, and if the perivascular lymphatics completely surrounded the vessel, a score of 2 was assigned.

On the basis of morphology, different types of lymphatics and prelymphatic interstitial spaces can be cast by injecting a resin through permeable pulmonary vasculature (19, 22) and air space (8). The lymphatic casts around the large blood vessels have been termed saccular and conduit lymphatics, and those on the pleural surface have been termed initial and conduit lymphatics (19).

Analysis. The specimens were coded and examined in a blinded fashion. We used analysis of variance to compare the three groups of animals for continuous data. We used Scheffe's procedure ($P < 0.05$) to locate differences detected by the analysis of variance. For discontinuous or rank data, we performed the nonparametric Kruskal-Wallis test. For proportional data, such as the presence or absence of lymphatics around vessels in the different tidal volume groups, we used contingency table analysis to generate a $\chi^2$ statistic. In addition to the rank order (0–2) cast lymphatic data, we used the presence or absence (0–1) of lymphatics. Values are means ± SE. Statistical analysis was performed with SPSS statistical software (version 7.5, SPSS, Chicago, IL).

RESULTS

There were no external differences in the animals, except frothy tracheal discharge during ventilation in two animals ventilated at high tidal volume. The lungs of the group ventilated at high tidal volume appeared more erythematous and edematous. The wet-to-dry lung weight ratio was 5.97 ± 0.27 for animals ventilated at high tidal volume, 4.98 ± 0.06 for animals ventilated at low tidal volume, and 4.95 ± 0.04 for nonventilated animals. The difference between the values of the animals ventilated at high tidal volume and the others was significant ($P < 0.0001$; Fig. 1).
Light microscopy. Light-microscopic viewing of the slides as unknowns did not distinguish the groups, but the measurements showed that the arterial cuffing ($P < 0.0001$; Figs. 2 and 3), venous cuffing ($P < 0.01$; Figs. 4 and 5), alveolar edema ($P < 0.01$; Fig. 6), and septal thickening ($P < 0.001$; Figs. 7 and 8) were greater in the animals ventilated at high tidal volume than in the other groups. Septal widening was not observed in the group ventilated at low tidal volume, but this was not different from the nonventilated group, in which average septal widening was only 150 μm per animal. The epithelial sloughing and damage were minimal in all animals and not different among the groups. The scores were $<0.5$ in each group. The different measures of edema were usually found together in most animals.

Scanning microscopy of casts. The group ventilated at high tidal volume had more arterial (Figs. 9 and 10) and venous cuffing (Figs. 11 and 12) than the others. The cuffing involved the prelymphatic space and saccular and conduit lymphatics (19). Lymphatics in the interlobular septa were cast in the group ventilated at high tidal volume. Lymphatics of the pleural surface were scant in all animals.

There were lymphatic casts around $\sim 18\%$ of the arteries of the unventilated animals, $5\%$ of the arteries of the animals ventilated at low tidal volume, and $29\%$ of the arteries of the animals ventilated at high tidal volume ($P < 0.01$; Fig. 9). For this parameter, the groups differed from each other ($P < 0.05$; Table 1). The animals ventilated at high tidal volume and the unventilated animals had more saccular and conduit lymphatics than the animals ventilated at low tidal volume ($P < 0.01$) but were not different from each other. In the group ventilated at high tidal volume, more lymphatics completely surrounded the arteries. A similar pattern of lymphatics was observed around
veins (Fig. 11; *P* < 0.01) and indeterminate vessels (*P* < 0.001) in all the groups.

The larger vessels generally had more cast lymphatics than smaller vessels. Cast lymphatics around arteries were not different from those around veins when all groups were taken together. Nonventilated animals had more periarterial than perivenous casts (18 vs. 8%, *P* < 0.01).

There was no difference in cast lymphatics on the pleural surface between the groups. Of the 10 fields selected randomly and viewed at ×200 magnification, <10% had cast lymphatics in each of the groups (9, 8, and 6% in the nonventilated, low-tidal-volume, and high-tidal-volume groups, respectively). Cast conduit lymphatics were present on the pleural surface in ≤3% of the ×200 magnification fields (2, 3, and 1% in the nonventilated, low-tidal-volume, and high-tidal-volume groups, respectively).

### DISCUSSION

Mechanical ventilation with high tidal volumes, even for a short period of time, causes increased edema formation (6, 27) and impairs clearance of fluid from alveoli (25). Using this ventilated rodent model, we studied the structure and filling of different lymphatic forms in rat lungs as they underwent acute changes (25 min) caused by high tidal ventilation. The short duration of the ventilation was chosen to show the changes in early edema formation, which contrasts with our previous studies of hyperoxic lung injury, where an increase in initial, saccular, and conduit lymphatics was observed after 7 days of hyperoxia (8, 22). Undiluted Mercox is a viscous material with a viscosity of 27 cP before addition of the benzoyl peroxide accelerator (2). After the accelerator is added, the viscosity increases logarithmically with time. The increased viscosity with our method makes extrusion of the resin through the 20-gauge needle difficult after a little more than 1 min. In <5 min the resin hardens to become rigid (20). This method, therefore, identifies lymphat-

**Table 1. Cast lymphatics**

<table>
<thead>
<tr>
<th>Type</th>
<th>Spontaneous</th>
<th>Low VT</th>
<th>High VT</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arterial lymphatics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saccular</td>
<td>18 ± 3</td>
<td>5 ± 2</td>
<td>29 ± 3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Conduit</td>
<td>19 ± 3</td>
<td>2 ± 1</td>
<td>17 ± 3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Partial</td>
<td>13 ± 3</td>
<td>5 ± 2</td>
<td>21 ± 3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Complete</td>
<td>5 ± 2</td>
<td>0 ± 0</td>
<td>8 ± 2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Venous lymphatics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saccular</td>
<td>8 ± 2</td>
<td>9 ± 2</td>
<td>25 ± 3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Conduit</td>
<td>16 ± 3</td>
<td>2 ± 1</td>
<td>14 ± 2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Partial</td>
<td>5 ± 2</td>
<td>9 ± 2</td>
<td>18 ± 3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Complete</td>
<td>2 ± 1</td>
<td>7 ± 7</td>
<td>7 ± 2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Indeterminate vessel lymphatics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saccular</td>
<td>18 ± 7</td>
<td>5 ± 4</td>
<td>35 ± 6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Conduit</td>
<td>18 ± 7</td>
<td>0 ± 0</td>
<td>13 ± 4</td>
<td>&lt;0.038</td>
</tr>
<tr>
<td>Partial</td>
<td>15 ± 6</td>
<td>3 ± 3</td>
<td>11 ± 4</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Complete</td>
<td>3 ± 3</td>
<td>3 ± 3</td>
<td>24 ± 5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as percentage. VT, tidal volume.
Lymphatics that fill within this short time interval. The ventilation was stopped just before the lungs were cast, so that mechanical factors from ventilation could not affect intralymphatic and interstitial pressure, which in turn could affect the filling.

The structure of these lymphatic casts as viewed by electron microscopy was identical to that in models of chronic hyperoxic lung injury (22) and other models of acute pulmonary edema (19). The edema fluid moves from capillaries into the interstitium and from there into characteristic tissue spaces (prelymphatics) that are intimately connected to the initial lymphatics and conduit lymphatics.

With ventilator-induced lung injury, perivascular lymphatics become more engorged than other types of lymphatics (Figs. 2, 4, 5, and 10–12). The perivascular lymphatic filling is not associated with significant pleural lymphatic filling, in contradistinction to our chronic hyperoxic model (8, 22). In the group ventilated at high tidal volume, edema formation was most apparent in the periarterial, perivenous, and interlobular septa and alveoli. There was more lymphatic filling in the larger vessels than in the smaller vessels, which could mean that the stress and edema-forming forces were greater in the proximal vessels or that the large vessels had more lymphatic capacity and greater ability to be recruited. It could also be that smaller lymphatics feed into the larger lymphatics and the brief time was sufficient for them to fill more completely.

In a model of chronic lung injury caused by exposure of rats to 85% oxygen for 7 days, all lymphatic beds were greatly increased (8, 22). It is well known that this hyperoxic stimulus causes significant proliferation of alveolar epithelial cells and lung fibroblasts (3). These studies raised the question whether the increase in lymphatic capacity was the result of the formation of new lymphatics caused by cell proliferation in response to chronic hyperoxia or the recruitment of preexisting lymphatics. This short-term study provides strong evidence to support the notion that prelymphatics exist, ready to be rapidly recruited in situations where edema develops.

The ready casting of lymphatics by injection of a viscous, partially polymerized methacrylate into the pulmonary vasculature shows that the model of ventilator-induced lung injury produces a permeability edema for this high-molecular-weight resin. Webb and Tierney (29) observed alveolar and perivascular edema when they ventilated rats for a short duration with high peak inspiratory pressure. The eosinophilic character of the edema suggested that it was protein rich. This was confirmed by several investigators (6, 7, 12, 16). However, the mean light-microscopic alveolar edema score for our animals was only 0.24, and no slide had a score of 3 or 4. A score of 3 or 4 might be expected with severe proteinaceous and hemorrhagic pulmonary edema, which we have seen with neurogenic pulmonary edema (21, 23) and prolonged (≥60-min) mechanical ventilation (12). Light microscopy was unable to detect major airway damage in this model.

There were more perivascular cast lymphatics in the spontaneously breathing, anesthetized tracheostomized rats than in rats ventilated at low tidal volume. This may have resulted from the positive-pressure ventilation, which is known to shift intrapulmonary fluid (15). It is also possible that breathing efforts of the unventilated animals and the greater negative intrathoracic pressure could have increased vascular transudation. Negative-pressure pulmonary edema has been reported in patients with upper airway obstruction (4), but lymphatic filling was only mildly increased in our spontaneously breathing animals, as detected by scanning electron microscopy of the casts. This method may be more sensitive than the wet-to-dry weight ratio of the lung. Although the nonventilated animals were anesthetized, breathing movements were observed as the resin was injected.

The study could not distinguish a significant difference in lymphatic compartments around the arteries and veins. Heavy resin accumulation around these blood vessels may make it difficult or impossible to distinguish the blood vessel type, which is dependent on the characteristic surface features seen under the electron microscope (13). The presence of indeterminate vessels may have masked an arteriovenous difference. When the vessel wall is visible, small veins can be distinguished from small arteries much better by electron microscopy of vascular casts than by light microscopy (18).

This study provides evidence that brief ventilation with high tidal volumes produces edema and that the perivascular and septal lymphatics are readily available to accept the increased amount of fluid. The structure of the lymphatics in brief lung injury is identical to that in chronic edema. Edema can be scored by light-microscopic measurements and visualized and scored by casting the lymphatics through the vascular space with an appropriate resin. The casting technique provides a “snapshot” of the lymphatic filling that occurs within ~60 s.

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