Upregulation of alveolar epithelial fluid transport after subacute lung injury in rats from bleomycin

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Folkesson, Hans G., Gerard Nitenberg, Bonnie L. Oliver, Christian Jayr, Kurt H. Albertine, and Michael A. Matthay. Upregulation of alveolar epithelial fluid transport after subacute lung injury in rats from bleomycin. *Am. J. Physiol.* 275 (*Lung Cell. Mol. Physiol.* 19): L478–L490, 1998.—Alveolar epithelial fluid transport was studied 10 days after subacute lung injury had been induced with intratracheal bleomycin (0.75 U). An isosmolar Ringer lactate solution with 5% bovine serum albumin and ^{125}I-labeled albumin as the alveolar protein tracer was instilled into the right lung; the rats were then studied for either 1 or 4 h. Alveolar fluid clearance was increased in bleomycin-injured rats by 110% over 1 h and by 75% over 4 h compared with control rats ($P < 0.05$). The increase in alveolar fluid clearance was partially inhibited by amiloride (10^{-3} M). Alveolar fluid clearance decreased toward normal levels in rats that were studied 60 days after bleomycin instillation. Remarkably, the measured increase in net alveolar fluid clearance occurred in the presence of a significant increase in alveolar epithelial permeability to protein. Moreover, the increase in alveolar epithelial fluid clearance occurred even though the mRNA for the α-subunit of the epithelial sodium channel was decreased in alveolar epithelial type II cells isolated from these rats. In addition, ^{22}Na uptake by isolated alveolar epithelial type II cells from rats treated with bleomycin demonstrated a 52% decrease in uptake compared with type II cells from control rats. Morphological results demonstrated a significant hyperplasia of alveolar type II epithelial cells 10 days after bleomycin injury. Thus, these results provide evidence that proliferation of alveolar epithelial type II cells after acute lung injury may upregulate the transport capacity of the alveolar epithelium, even though the expression of epithelial sodium channels is reduced and the uptake of ^{22}Na per cell is also reduced. These results may have clinical relevance for the resolution of alveolar edema in the subacute phase of lung injury.

alveolar liquid clearance; alveolar type II cells; acute lung injury; pulmonary edema

THE FUNCTION of the alveolar epithelial barrier has been studied after removal of ventilation (36) or pulmonary blood flow (20) as well as after acute lung injury from oleic acid (43), acid aspiration (13), and exposure to 100% oxygen (18, 46, 47). Alveolar epithelial transport also has been investigated after intravenous endotoxin (42) and alveolar endotoxin (17) as well as after gram-negative bacteremia (34) and injury with *Pseudomonas aeruginosa* in models of bacterial pneumonia (44). In several of these models, there was an acute upregulation of alveolar fluid transport secondary to increased vectorial transport of sodium by alveolar epithelial cells (17, 18, 34, 35, 47). However, only a few studies have addressed the transport function of the alveolar epithelial barrier at later time points after acute lung injury. Most of these studies were done in hyperoxic lung injury over 2–3 days. The function of alveolar epithelial transport in the subacute phase after lung injury is an important clinical issue because patients often have persistent respiratory failure as the acute phase of acute lung injury transforms to a subacute phase (2, 6, 24).

Intratracheal bleomycin is a good experimental model of lung injury because it produces a lung injury that transforms from an acute to a subacute lung injury that is associated with an influx of inflammatory cells and acute loss of alveolar type I epithelial cells followed by regeneration of large numbers of alveolar type II epithelial cells; it is also associated with a variable degree of pulmonary fibrosis depending on the dose of bleomycin (5, 23, 38, 45). In this study, we tested the hypothesis that during subacute lung injury net alveolar fluid clearance would increase in association with hyperplasia of alveolar type II epithelial cells.

In preliminary studies, we found that 10 days after bleomycin-induced lung injury, the alveolar epithelial barrier was capable of net alveolar fluid transport, even though there was a significant increase in bidirectional protein flux across both the endothelial and epithelial barriers of the lung. Therefore, our first objective was to quantify alveolar fluid clearance in the subacute phase (10 days) after intratracheal instillation of bleomycin. The studies of alveolar fluid clearance were carried out simultaneously with measurements of bidirectional protein flux across the endothelial and epithelial barriers of the lung. Because net alveolar liquid clearance was markedly increased, the second objective was to examine several possible mechanisms that may explain the substantial increase in alveolar fluid clearance. The third objective of these studies was to carry out experiments 60 days after instillation of bleomycin to examine the functional capacity of the alveolar epithelial barrier at a time when resolution of most of the subacute lung injury would have occurred. Northern blot analysis was done to study whether expression of the α-subunit from the epithelial sodium channel (α-ENaC) in alveolar epithelial type II cells was altered by bleomycin exposure. In addition, sodium uptake was measured in vitro in cells isolated from bleomycin-treated rats to compare with in vitro sodium uptake in cells from control rats. Morphological studies were done to complement the physiological and molecular data.

METHODS

Animals

Male Sprague-Dawley rats (n = 84) weighing 300–350 g (Simonsen, Gilroy, CA) were used. The rats were kept in a 12:12-h day-night rhythm at 20°C, with food and water ad libitum. The protocol for these studies was approved by the University of California, San Francisco Animal Research Committee.

Bleomycin Instillation

Bleomycin (0.75 U; Blenoxane, Nippon Kayaku, Tokyo, Japan) was intratracheally instilled 10 or 60 days before the experiments as previously described (15). Control rats received an intratracheal instillation of sterile 0.9% NaCl 10 days before the experiments. During ether anesthesia, the rats were placed on a slanted board (20° from vertical) hanging from their upper incisors. The bleomycin (0.75 U) was delivered via the mouth into the trachea with a modified syringe needle in a volume of 1 ml/kg body weight. A distribution control experiment in which sterile saline with Evans blue dye was instilled into three rats demonstrated that the instillation procedure resulted in a patchy distribution of blue in all lobes of both lungs. After the instillation, the rats were allowed to recover in their cages, where they remained until the fluid clearance measurements were done 10 or 60 days later. Immediately after the bleomycin instillation and every second day, all rats received 5 mg of enrofloxacin (Baytril, Miles Agricultural Division, Animal Health Products, Shawnee Mission, KS) subcutaneously to minimize the risk of infection.

Based on the results of preliminary studies, we determined that 0.75 U of bleomycin in 0.3 ml of sterile saline was the optimal dose to cause moderate lung injury. The time period of 10 days after bleomycin instillation was selected as optimal for the subacute injury to develop, as other investigators have described (23, 45). The longer time period of 60 days was chosen as a time point when much of the subacute injury had resolved, as described earlier (23).

Surgical Preparation for Fluid Clearance Measurements and Ventilation

Ten and sixty days after intratracheal instillation of bleomycin, the rats were anesthetized with pentobarbital sodium (50 mg/kg body weight intraperitoneally; Nembutal, Abbott Laboratories, Chicago, IL). Anesthesia was maintained with one-half of the initial dose of pentobarbital sodium hourly. A 0.2-mm-ID (PE-240, Clay Adams, Becton Dickinson, Parsippany, NJ) endotracheal tube was inserted through a tracheostomy. A PE-50 catheter (Clay Adams, Becton Dickinson) was inserted in the right carotid artery to monitor systemic blood pressure and to obtain blood samples.

Pancuronium bromide (0.3 mg/kg body weight; Pavulon, Organon, West Orange, NJ) was given hourly for neuromuscular blockade. The rats were kept in the right decubitus position and ventilated with a constant-volume piston pump (Harvard Apparatus, Dover, MA) with an inspired oxygen fraction of 1.0 and peak airway pressures of 12–20 cmH_2O and supplemented with a positive end-expiratory pressure of 3 cmH_2O. The respiratory rate was adjusted to maintain arterial P_{CO_2} between 35 and 45 mmHg during the baseline period.

Preparation of Alveolar Instillate

Five percent bovine serum albumin (Sigma, St. Louis, MO) was prepared in Ringer lactate. Anhydrous Evan's blue dye (1 mg; Aldrich Chemical, Milwaukee, WI) and ^{125}I-labeled human serum albumin (0.5 μCi; Merck-Frosst, Montreal, Canada) were added to the 5% albumin solution. In some studies [see *Group 5: Effect of amiloride (apical sodium-channel inhibitor) on alveolar fluid clearance*], 10^{-3} M amiloride (Sigma) was added to the instillate.

A sample of the instillate was saved for total protein measurements, radioactivity counts, and water-to-dry weight ratio measurements so that we could subtract the dry weight of the added protein in the lung water calculation.

General Experimental Protocol

In all studies in which fluid clearance was measured, the following general protocol was used. After surgery, a 1-h baseline of stable heart rate and blood pressure was required before alveolar fluid instillation. As a vascular protein tracer, 3 μCi of ^{131}I-labeled human serum albumin (Merck-Frosst) were injected intra-arterially 15 min into the baseline period. This vascular tracer was used to calculate the flux of plasma protein into the air spaces (see *Lung endothelial and epithelial barrier protein permeabilities*). Blood samples were taken every 20 min during the remaining baseline period for arterial blood gases and radioactivity measurements.

An instillation tube (PE-50, Clay Adams, Becton Dickinson) was passed through the tracheal tube and positioned in the right lung. Then, with 1-ml syringes, 1 ml of fluid was instilled over 20 min for the 1-h experiments and 1.4 ml of fluid were instilled over 30 min for the 4-h experiments. The volume instilled was adjusted because of the different duration of the experiments. After instillation, the tubing was withdrawn. Prior studies by our group (14, 19, 25) demonstrated that the rate of alveolar fluid clearance was independent of the instilled volume.

Arterial blood samples were obtained for measurement of ^{131}I-albumin and ^{125}I-albumin activity and arterial blood gas determinations hourly after the instillation.

At the end of each experiment, the abdomen was opened, and the animal was exsanguinated by transecting the abdominal aorta. Urine was sampled by puncturing the bladder, and the radioactivity count was measured to check the stability of the isotope label attached to the tracer proteins. The lungs were then removed through a midline sternotomy. An alveolar fluid sample (0.1–0.2 ml) was obtained by gently passing the sampling catheter (PE-50) into a wedged position in the instilled area of the right lung. After centrifugation, total protein concentration and radioactivity of the alveolar fluid sample were measured. In a prior study, Berthiaume et al. (9) reported that the concentration of the protein tracers in the fluid sampled by a small catheter wedged into the distal airways was the same as in an alveolar micropuncture sample. Right and left lungs were homogenized separately for extravascular lung water measurements and radioactivity counts.

In some experiments, alveolar fluid was used for measurement of catecholamines and/or transforming growth factor (TGF)-α. Ultrastructural morphological studies were done in four bleomycin-instilled rats (two at 10 days and two at 60 days) and two control rats.

Specific Protocols

Group 1: Effect of bleomycin on extravascular lung water. To evaluate the effect of alveolar bleomycin on the extravascular lung water content of both lungs, rats (n = 6) were intratracheally instilled with 0.75 U of bleomycin. Control rats (n = 5) were instilled intratracheally with 0.9% NaCl. Ten days after instillation, the rats were anesthetized and surgically pre-

pared as described in *General Experimental Protocol*. Then, after baseline and 1 h of ventilation but without additional fluid instillation, the rats were exsanguinated, and the lungs were removed and processed for gravimetric determination of extravascular water. The extravascular lung water and dry weights of these noninstilled lungs from bleomycin-treated rats were used for lung liquid clearance calculations (see *Lung liquid clearance*).

Group 2: Effect of bleomycin on alveolar cell counts. Recruitment of inflammatory cells associated with intratracheal bleomycin instillation into the distal air spaces was quantified 10 days after bleomycin instillation ($n = 4$ rats). Air space leukocyte counts were obtained by bronchoalveolar lavage (15) after exsanguination. Four control rats were intratracheally instilled with 1 ml/kg body weight of 0.9% NaCl 10 days before the bronchoalveolar lavage. For bronchoalveolar lavage, a tracheostomy was made, and the lungs were lavaged with 0.9% NaCl. The saline entered the lungs over 3 min at 10 cmH_2O pressure; then the lavage was recovered. This procedure was repeated three times, the recovered fluid was pooled, and the volume was measured. Typically, a lavage volume of 20 ml was recovered. After centrifugation of the lavage fluid, the pelleted cells were resuspended in 1 ml of 0.9% NaCl. Total cell number was determined with a hemocytometer. The percentage of different cell types present was determined by observing cell morphology on cytocentrifuge preparations (Shandon Cytospin 3, Shandon Scientific, Cheshire, UK) with a modified Wright-Giemsa stain (Diff-Quik, American Scientific Products, McGaw Park, IL).

Group 3: Control rats. Alveolar fluid clearance was measured 10 days after intratracheal instillation of 0.9% NaCl in control rats. After the baseline period, ~3 ml/kg body weight of the 5% albumin solution with ^{125}I-labeled human serum albumin were instilled into one lung. The rats ($n = 7$) were initially studied for 4 h. Control rats ($n = 6$) were also done over 1 h. Then, the rats were killed and processed as described in *General Experimental Protocol*.

Group 4: Effect of bleomycin on bilateral protein permeability and alveolar fluid clearance. These studies were done 10 days after intratracheal bleomycin instillation into the air spaces. After the baseline period, 3 ml/kg of the 5% albumin solution with ^{125}I-labeled human serum albumin were instilled into one lung for the 4-h studies ($n = 8$). Initially, 4-h experiments for measuring the alveolar liquid clearance were done, but the alveolar epithelial permeability to protein was significantly increased (Table 1). It was possible to account for the loss of alveolar epithelial protein because we had included the alveolar protein tracer ^{125}I-albumin in the instilled fluid and measured its escape into the blood. Thus the alveolar liquid clearance calculation could be corrected for the loss of protein during the 4 h of experiments (see *Alveolar fluid clearance* for details). As an additional control, 1-h studies ($n = 6$ rats) were done because less of a correction would be necessary because the loss of the alveolar protein tracer would be less (see RESULTS). After these time intervals, the rats were exsanguinated and processed as described in *General Experimental Protocol*.

Group 5: Effect of amiloride (apical sodium-channel inhibitor) on alveolar fluid clearance. These studies were done 10 days after intratracheal bleomycin instillation. To determine the effect of amiloride on the bleomycin-stimulated alveolar fluid clearance over 1 h ($n = 9$ rats), we used amiloride (10^{-3} M), which inhibits apical uptake of sodium by alveolar epithelial type II cells. Control rats ($n = 4$) were instilled with the 5% albumin solution with 10^{-3} M amiloride and studied for 1 h. An amiloride concentration of 10^{-3} M was used because ~50% of amiloride is protein bound and another significant fraction escapes from the air spaces, resulting in functional concentrations closer to 10^{-4} M (29, 46). The rats were processed as described in *General Experimental Protocol*.

Group 6: Recovery studies 60 days after bleomycin instillation. To study alveolar fluid clearance several weeks after lung injury, studies were done 60 days after instillation when lung morphology had returned toward a near-normal pattern (see *Effect of Alveolar Bleomycin on Lung Morphology*). The rats were studied for 1 h after fluid instillation and then processed as described in *General Experimental Protocol*.

Group 7: Morphological studies of the lung after bleomycin instillation. To assess the qualitative effects of bleomycin on alveolar and lung morphology, electron-microscopic studies were carried out. Four rats were instilled with bleomycin and killed 10 ($n = 2$) or 60 ($n = 2$) days later. Control rats ($n = 2$) were intratracheally instilled with 0.9% NaCl 10 days before the morphological studies were done. The rats were anesthetized, and the lungs were fixed by vascular perfusion. Ringer lactate solution was used to clear blood from the pulmonary vasculature before fixation. When the lungs were blood free, the perfusate solution was changed to 2.5% glutaraldehyde-1% paraformaldehyde in phosphate-buffered saline. The lungs were perfused for 1–2 min with the fixative and then left in the chest for 15 min with fixative poured on top. Then, the lungs were excised and immersed in fixative until they were processed. We embedded and sectioned 10 blocks (2/lung lobe) in epoxy resin (Embed 812, EM Sciences, Ft. Washington, PA). A JEOL 100S transmission electron microscope was used to observe and photograph the tissue sections (1 thin section/tissue block).

Table 1. *Effect of 10 days of intratracheal bleomycin on movement of protein from air spaces across lung alveolar epithelial barrier in rats*

Group	n	Time, h	Alveolar Protein Tracer, % of instilled	
			Lung	Blood
Control	6	1	98 ± 2	0.5 ± 0.4
Bleomycin (0.75 U)	6	1	92 ± 6*	6.3 ± 5.3*
Control	7	4	95 ± 1	1.0 ± 1.2
Bleomycin (0.75 U)	8	4	70 ± 15*	21.8 ± 10.4*

Values are means ± SD; n, no. of rats. Alveolar protein tracer was ^{125}I-albumin. *$P < 0.05$ (by Student's t-test) compared with control group at each time point.

Measurements

Airway pressure, hemodynamics, and pulmonary gas exchange. Airway pressure, heart rate, and systemic arterial pressure were measured with calibrated pressure transducers (PD23 ID, Gould, Oxnard, CA) and recorded continuously on a Grass polygraph (model 7, Grass Instruments, Quincy, MA). Arterial blood gases and pH were measured at 30-min intervals. The mean systemic arterial pressure was calculated.

Lung endothelial and epithelial barrier protein permeabilities. Two methods were used to measure the protein permeability of the alveolar epithelium to albumin (10). The first method required measurement of residual ^{125}I-albumin (the alveolar protein tracer) in the lung. The second method required measurement of the accumulation of the alveolar protein tracer ^{125}I-albumin in the blood.

For measuring the clearance of the alveolar tracer protein ^{125}I-albumin from the lung, several measurements were necessary. First, the total radioactivity instilled in the lungs

[^{125}I-albumin in counts·min^{-1} (cpm)·g^{-1}] was calculated by multiplying the radioactivity in aliquots of the instillate by the volume instilled. To calculate the quantity of ^{125}I-albumin in the lungs at the end of the experiment, the average of duplicate radioactivity counts from the lung homogenate from the instilled lung was multiplied by the volume of the lung homogenate. To measure the accumulation of ^{125}I-albumin from the alveolar spaces into the blood, the radioactivity count in the plasma samples was multiplied by the estimated plasma volume (in ml). The plasma volume was calculated by the following relationship

$$\text{plasma volume} = \text{body weight (in g)} \times 0.07 \times (1 - \text{hematocrit}) \quad (1)$$

To estimate the clearance of the vascular tracer protein ^{131}I-albumin (^{131}I-albumin$_{vascular,lung}$) into the extravascular compartments of the lungs (interstitium and air spaces; ^{131}I-albumin$_{extravascular,lung}$), the total extravascular ^{131}I-albumin accumulation in the alveolar liquid recovered from the air spaces and in the lung homogenate (^{131}I-albumin$_{total,lung}$) was measured and is expressed as extravascular plasma equivalents. Then, the amount of extravascular ^{131}I-albumin was calculated by the following equation

$$^{131}\text{I-albumin}_{extravascular,lung} = {}^{131}\text{I-albumin}_{total,lung} - {}^{131}\text{I-albumin}_{vascular,lung} \quad (2)$$

To calculate ^{131}I-albumin$_{vascular,lung}$, the ^{131}I-albumin counts in the last arterial blood sample were multiplied by the blood volume in the lungs corrected for the hematocrit. If the amount of ^{131}I-albumin in 1 ml of plasma was known, the extravascular ^{131}I-albumin represents the volume of plasma that leaked out from the blood vessels during the experiment.

TCA precipitation was carried out on the instillates, urine samples, and selected samples from each experiment; it was established that both of the tracers, ^{125}I and ^{131}I, were always >98% bound to the protein in all samples.

Alveolar fluid clearance. The progressive concentration of both the instilled unlabeled albumin and the instilled ^{125}I-labeled albumin over 1 or 4 h was used to quantify fluid clearance from the distal air spaces, as our group has done before (9, 10, 14, 19, 20, 25, 36). Alveolar fluid clearance is expressed as final-to-instilled protein or ^{125}I-albumin concentration ratio and as a percentage of instilled volume. The term alveolar does, however, not imply that all fluid reabsorption occurs at the alveolar level. Some reabsorption may occur across distal bronchial epithelium (7).

Because rats instilled with saline 10 days before the alveolar liquid clearance studies had the same progressive increase in alveolar protein concentration as normal noninstilled rats (14, 34), an earlier liquid instillation per se does not change the ability to measure fluid clearance from the air spaces of the lung. In addition, there was a good correlation between the concentration of the instilled albumin and the ^{125}I-labeled albumin in the control rats. Therefore, we used the concentration of the instilled unlabeled albumin as an index of alveolar epithelial fluid clearance in these experiments.

Alveolar epithelial barrier injury is frequently associated with an increased permeability to protein (13). Therefore, measurement of alveolar epithelial fluid clearance under pathological conditions, such as in this study after bleomycin-induced lung injury, can be done only after the loss of the alveolar protein tracer is accounted for (12, 42). Because we included a radiolabeled tracer protein (^{125}I-albumin) in the alveolar instillate, it was possible to estimate the loss of protein from the air spaces by measuring the percentage remaining in the lung; it was then possible to correct the alveolar fluid clearance calculation. Because we accounted for the loss of the alveolar protein tracer, the modified values of the instilled ^{125}I-labeled albumin and calculations based on these values are described as "corrected." The correction is explained as follows. If 100,000 cpm/g of ^{125}I-albumin were instilled into the lungs and 4 h later there was an increase in the alveolar ^{125}I-albumin concentration to 110,000 cpm/g, then the increase would be 10%. However, if 10% of the ^{125}I-albumin had entered the blood circulation, then the actual quantity of the alveolar protein tracer in the air spaces would have been ~90,000 cpm/g. Thus the actual increase to 110,000 cpm/g would represent an increase of 20,000 cpm/g instead of 10,000 cpm/g or a 22% increase instead of a 10% increase. To provide further validation, we also did shorter experiments over 1 h, during which time lower quantities of the alveolar protein tracer left the air spaces. These studies demonstrated that there was a significantly higher alveolar epithelial fluid clearance in the bleomycin-exposed rats compared with the control rats (see RESULTS), strengthening the accuracy of the corrections for the loss of alveolar protein tracer in the 4-h experiments. Therefore, the corrected progressive increase in ^{125}I-albumin concentration was estimated by *Eq. 3*

$$\text{corrected F/I } {}^{125}\text{I-albumin} = {}^{125}\text{I-albumin}_{asp}/({}^{125}\text{I-albumin}_{inst} - {}^{125}\text{I-albumin}_{blood}) \quad (3)$$

where F/I is the final-to-instilled ^{125}I-albumin ratio, ^{125}I-albumin$_{asp}$ is the ^{125}I-albumin count in the air space liquid sample, ^{125}I-albumin$_{inst}$ is the ^{125}I-albumin count in the instillate, and ^{125}I-albumin$_{blood}$ is the total ^{125}I-albumin count in the blood circulation.

One assumption is that the quantity of ^{125}I-albumin that has escaped from the air spaces into the lung interstitium is <5%. This is reasonable because a lung lymph study by Berthiaume et al. (8) showed that alveolar protein was rapidly cleared from the interstitium by the lymphatics and the circulation.

Alveolar fluid clearance was also calculated from the concentrations of unlabeled albumin in the instillate and aspirate. Protein was measured by the biuret method. Then, alveolar liquid clearance (ALC; in percentage of instilled volume) was calculated from *Eq. 4*

$$\text{ALC} = [(V_I - V_A)/V_I] \times 100 \quad (4)$$

where V_I is the volume of instilled fluid, V_A is the alveolar fluid volume at *time t* (*t* equals either 1 or 4 h) and is calculated from the increase in alveolar protein concentration over the time of experiment. *Equation 4* was also used to calculate alveolar fluid clearance from the corrected ^{125}I-albumin data.

Lung liquid clearance. Extravascular lung water was determined after 1 or 4 h as in prior studies by our group (10, 12, 20, 25, 36). Before exsanguination, a blood sample was obtained to measure hemoglobin concentration and the water-to-dry weight ratio of blood to calculate the blood volume in the lungs. Each lung was homogenized separately, and extravascular lung water was determined by calculating the water-to-dry weight ratio. The volume of excess extravascular lung water (in ml) in the instilled experimental lung was calculated as the difference between the water-to-dry weight ratios of the experimental and contralateral lungs multiplied by the dry weight of the experimental lung. The dry weight of the instilled experimental lung was corrected for by the dry weight of the instilled protein remaining in the lung at the

end of the experiment. To determine the mass of protein remaining in the lung, the dry weight of the instillate was multiplied by the fraction of ^{125}I-albumin remaining in the lung. This value was then subtracted from the total dry weight of the experimental lung as shown in *Eq. 5*

$$\text{excess extravascular lung water} = |[W_e/(D_e - P)] - (W_c/D_c)| \times (D_e - P) \quad (5)$$

where W and D are the extravascular lung water and blood-free dry weights, respectively, of the experimental (e) and control (c) lungs and P is the blood-free dry weight of the instilled solution multiplied by the fraction of ^{125}I-albumin remaining in the lung after 1 or 4 h. *Equation 5* does not account for the possibility that some of the circulating plasma may enter the instilled experimental lung. To estimate the quantity of plasma that entered the instilled lung, we measured the transfer of the vascular protein tracer ^{131}I-albumin into the extravascular spaces of the instilled lung (see *Lung endothelial and epithelial barrier protein permeabilities*).

Cell isolation and culture. Alveolar type II cells were isolated from bleomycin-treated ($n = 5$) and control ($n = 4$) Sprague-Dawley rats by standard methods (22). Isolated alveolar epithelial type II cells were plated ($3–4 \times 10^5$ cells/cm^2) on 12-well culture plates (Corning, New York, NY) and maintained in Dulbecco's modified Eagle's medium (at the University of California, San Francisco Cell Culture Facility) containing 10% heat-inactivated fetal bovine serum, 50 U/ml of penicillin-streptomycin, and 4 mM glutamine at 37°C in a humidified atmosphere of 95% air-5% CO_2. The culture medium was changed every other day. The cells were examined daily and studied at confluence after 2–4 days. The average time for study of the cells in the control and bleomycin groups was the same.

^{22}Na influx studies. After removal of the culture medium, cells from either bleomycin-treated or control rats on 12-well dishes were rinsed two times and preincubated for 20 min at 37°C in a buffered Na-free solution composed of (in mM) 137 *N*-methylglucamine, 5.4 KCl, 1.2 $MgSO_4$, 2.8 $CaCl_2$, and 15 HEPES (pH 7.4). At the end of the preincubation, the Na-free solution was replaced by the uptake solution composed of (in mM) 14 NaCl, 35 KCl, 96 *N*-methylglucamine, and 20 HEPES (pH 7.4) containing 1 mM ouabain and 0.5 μCi/ml of ^{22}NaCl (37 MBq/mg Na; Amersham, Oakville, Ontario) in the absence or presence of 100 μM amiloride. After a 5-min incubation, uptake was stopped by washing the cells three times with 1 ml/well of an ice-cold solution containing (in mM) 120 *N*-methylglucamine and 20 HEPES, pH 7.4. The cells were then solubilized in 0.5% Triton X-100. Tracer activities were determined with a gamma counter, and the remaining volume of each sample was used for assessing the protein content per well. ^{22}Na influx was determined in the absence or presence of amiloride. Results are expressed in nanomoles per milligram of protein per 5 min.

Determination of plasma concentrations of epinephrine. In four rats, 1 ml of blood was collected in a heparinized tube just before instillation of a 5% rat albumin solution, as done before by Pittet et al. (34). Blood samples were immediately centrifuged at 3,000 *g* for 5 min at 4°C; 0.5 ml of plasma was transferred to an Eppendorf tube and quickly frozen to −70°C in acetone and dry ice. Samples were stored at −70°C until analyzed. Plasma samples were spiked with an internal standard and absorbed on activated alumina at an alkaline pH. Epinephrine was eluted with 0.1 M perchloric acid, analyzed by reverse-phase HPLC with a C_8 column, and detected by the amperometric method with an electrochemical detector. Correlation coefficient and detection limit of this method were 0.96 and 10 pg/ml, respectively.

Northern blot analysis. Primary alveolar epithelial type II cells were isolated from the lungs of control (saline-instilled; $n = 2$) and injured (bleomycin-instilled; $n = 2$) Sprague-Dawley rats at 10 days postinstillation by standard methods (22). Total cellular RNA was extracted from aliquots of 3×10^6 cells immediately after isolation with TriZOL Reagent (GIBCO BRL, Gaithersburg, MD) as previously described (30). For Northern blot analysis, total RNA from equal numbers of cells was denatured in sample loading buffer (Sigma), size fractionated on 1% agarose containing 2.2 M formaldehyde, and transferred to nylon membranes. Membranes were hybridized to the cDNA probe for the α-subunit of rat ENaC (rENaC) in 6× saline-sodium citrate (1× is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 2× Denhardt's solution, 0.1% SDS, 10% dextran sulfate, and 100 μg/ml of salmon sperm DNA at 65°C for 18 h. The α-rENaC probe was kindly provided by Dr. Yves Berthiaume (Université de Montreal, Montreal, Canada) and consists of a 446-bp fragment from nucleotide 985 to nucleotide 1431 of the α-rENaC sequence. After hybridization, membranes were exposed to an autoradiographic film at −80°C for 5 days. Equivalent loading of RNA was verified by photography of ethidium bromide-stained gels and hybridization to a β-actin probe (Oncor, Gaithersburg, MD).

Statistics

All data are means ± SD. Time-dependent effects of bleomycin were analyzed by repeated-measures ANOVA followed by Student-Newman-Keuls test post hoc. The effects of bleomycin were compared with the control group for alveolar protein concentration and alveolar fluid clearance with a one-way ANOVA followed by Student-Newman-Keuls test post hoc. Statistical significance was set as $P < 0.05$.

RESULTS

Effect of Alveolar Bleomycin on Extravascular Lung Water

There was no difference in extravascular lung water between the right and left lungs of bleomycin-instilled rats. Therefore, we pooled the data from the left and right lungs for each rat. The results demonstrated a moderate but significant increase in the water-to-dry weight ratio for the bleomycin-instilled rats (4.75 ± 0.49 g water/g dry lung; $n = 6$) compared with control saline-instilled rats (3.80 ± 0.20 g water/g dry lung; $n = 5$; $P < 0.05$ by Student's *t*-test). This result is consistent with the presence of mild interstitial edema, a finding that was confirmed by the histological studies (see *Effect of Alveolar Bleomycin on Lung Morphology*). This lung water-to-dry weight ratio in rats exposed to bleomycin was used as the water-to-dry weight ratio of the control lungs for the lung liquid clearance calculations. The extravascular lung water in rats ($n = 6$) 60 days after bleomycin instillation was 4.16 ± 0.48 g water/g dry lung.

Effect of Alveolar Bleomycin on Bronchoalveolar Lavage Cell Count

The total number of neutrophils and macrophages recovered by bronchoalveolar lavage on *day 10* was greater in rats exposed to bleomycin than in rats previously instilled with saline (Table 2). The bronchoalveolar lavage inflammatory cells were predominantly

Table 2. *Effect of intratracheally instilled bleomycin on BAL cell counts*

Group	*n*	BAL WBC, $\times 10^6$	BAL PMN, %
Control	4	8 ± 3	2 ± 2
Bleomycin (0.75 U)	3	10 ± 5	24 ± 4*

Values are means ± SD; *n*, no. of rats. BAL, bronchoalveolar lavage; WBC, white blood cells; PMN, polymorphonuclear leukocytes (neutrophils). *$P < 0.05$ (by Student's *t*-test) compared with control group.

neutrophils. In the peripheral circulation, there were no changes in white blood cell count (data not shown).

Effect of Alveolar Bleomycin on Protein Movement Across the Epithelial and Endothelial Barriers of the Lung

To study the effect of bleomycin on alveolar epithelial protein flux, measurements of the appearance of the alveolar protein tracer ^{125}I-albumin in the blood as well as residual ^{125}I-albumin in the lung at the end of the experiments were done. The quantities of ^{125}I-albumin are expressed as a percentage of the instilled amounts (in cpm). There was a significantly higher loss of ^{125}I-albumin across the alveolar epithelium into the blood in rats instilled with bleomycin than in control rats, especially in the 4-h studies (Table 1).

The lung endothelial protein flux was measured by the use of the vascular protein tracer ^{131}I-albumin. The endothelial protein flux is expressed as extravascular plasma equivalents in the lung, representing milliliters of plasma that had moved from blood vessels into the extravascular compartment of the lung. The extravascular plasma equivalents were increased after bleomycin instillation compared with those of control rats in the 1- and 4-h studies (significant only at 4 h; Table 3). The amount of plasma that leaked out of the vascular space was relatively low but increased after bleomycin exposure similarly in both the 1- and 4-h studies.

Alveolar and Lung Liquid Clearances

Alveolar fluid clearance was measured from the progressive increase in the instilled unlabeled protein and tracer (^{125}I-albumin) concentrations in bleomycin-instilled and saline-instilled control rats. The final-to-initial ^{125}I-albumin concentration ratios at 1 and 4 h (Fig. 1) were similar to the instilled protein data (Table 4) when the loss of alveolar protein tracer into the bloodstream (as described in METHODS) had been accounted for in the calculations. There was a significant increase in alveolar fluid clearance in the rats instilled 10 days earlier with bleomycin (Figs. 1 and 2, Table 4) compared with the control rats in all studies. The alveolar fluid clearance calculated from the concentration of either instilled protein or ^{125}I-albumin (corrected values) was similar to the gravimetrically measured lung liquid clearance (Fig. 3).

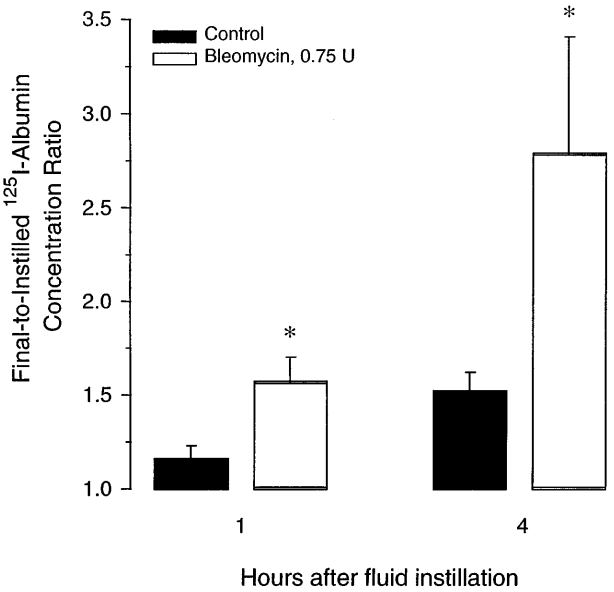

Fig. 1. Alveolar fluid clearance (expressed as final-to-instilled ^{125}I-albumin concentration ratio) in rats treated with intratracheal bleomycin 10 days before experiments. Rats were studied for 1 and 4 h after instillation of 5% albumin solution. Instilled ^{125}I-albumin alveolar concentrations were corrected for loss of tracer into blood. Data are means ± SD. Final-to-instilled ^{125}I-albumin concentration ratio increased significantly more in bleomycin-instilled compared with control (saline-instilled) rats in all studies (1 and 4 h). *$P < 0.05$ vs. control rats at different times of experiments.

Table 3. *Effect of 10 and 60 days of intratracheal bleomycin on endothelial permeability to protein in rats*

Group	*n*	Extravascular Plasma Equivalents, ml	
		1 h	4 h
Control	6	0.02 ± 0.02	0.05 ± 0.04
Bleomycin (0.75 U)			
10 days	6	0.05 ± 0.05	0.17 ± 0.12*
60 days	6	0.02 ± 0.02	ND

Values are means ± SD of endothelial permeability measured as extravascular plasma equivalents of ^{131}I-albumin; *n*, no. of rats. ND, not done. *$P < 0.05$ (by Student's *t*-test) compared with control group.

Effect of the Sodium-Channel Inhibitor Amiloride on Bleomycin-Stimulated Fluid Clearance

To determine whether the increase in fluid clearance from the distal air spaces in these rats was mediated by

Table 4. *Effect of 10 and 60 days of intratracheal bleomycin on alveolar liquid clearance in rats*

Group	*n*	Time, h	Alveolar Protein Concentration, g/dl		Final-to-Instilled Protein Concentration Ratio
			Instilled	Final	
Control	6	1	4.71 ± 0.56	5.51 ± 0.74	1.16 ± 0.13
Bleomycin (0.75 U) 10 days later	6	1	4.51 ± 0.46	7.41 ± 1.35	1.64 ± 0.18*
Control	7	4	4.66 ± 0.50	8.20 ± 0.74	1.76 ± 0.16
Bleomycin (0.75 U) 10 days later	8	4	4.93 ± 0.93	13.23 ± 2.97	2.69 ± 0.70*
Recovery for 60 days	6	1	5.00 ± 0.28	6.80 ± 0.78	1.36 ± 0.11*†

Values are means ± SD; *n*, no. of rats. $P < 0.05$ (by 1-way ANOVA) compared with: *control group; †bleomycin group (both matched for time of study).

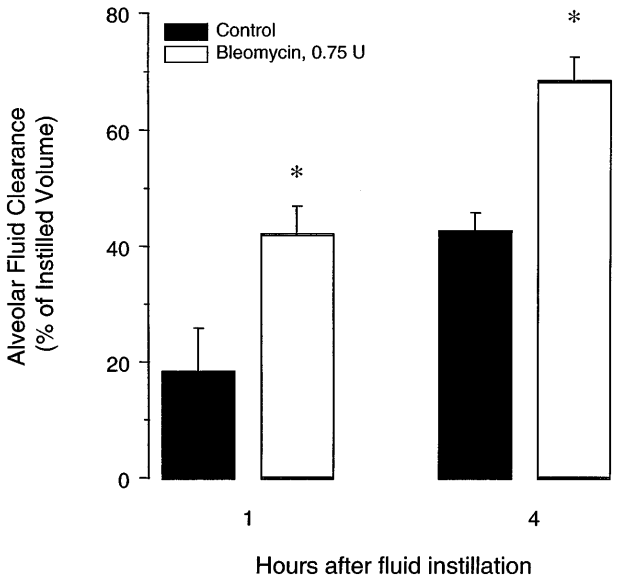

Fig. 2. Alveolar fluid clearance (expressed as percentage of instilled volume) in rats treated with intratracheal bleomycin 10 days before experiments. Rats were studied for 1 and 4 h after instillation of 5% albumin solution. Data are means ± SD. Intratracheal instillation of bleomycin to distal air spaces of lung caused a significant increase in alveolar fluid clearance compared with control (saline-instilled) lungs at all time points. *$P < 0.05$ vs. control rats at different times of experiments.

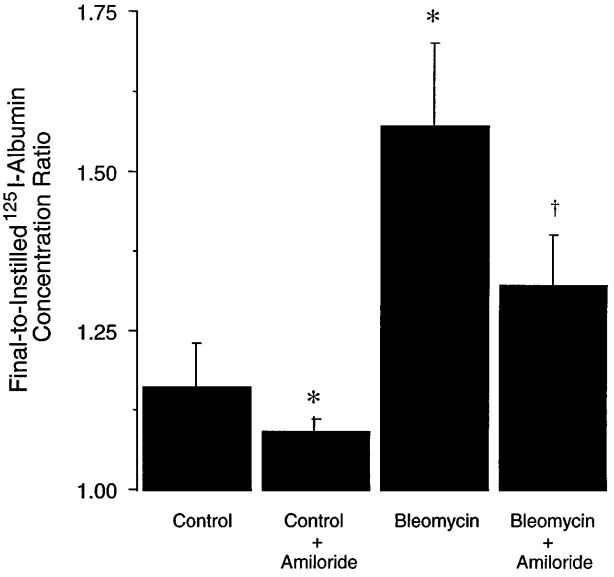

Fig. 4. Effect of amiloride on bleomycin (0.75 U)-stimulated alveolar fluid clearance (expressed as final-to-instilled ^{125}I-albumin concentration ratio) compared with control rats. Rats were studied for 1 h after instillation of 5% albumin solution. Data are means ± SD. Amiloride (10^{-3} M) instilled into distal air spaces of control rats inhibited alveolar fluid clearance by ~50% as shown before (14, 17, 19). *$P < 0.05$ vs. control groups at different times of experiments. †$P < 0.05$ vs. bleomycin-instilled rats at different times of experiments.

an increase in the uptake and transport of sodium across the lung epithelial barrier, amiloride (10^{-3} M), a sodium-transport inhibitor, was added to the instilled solution for the 1-h studies. The addition of amiloride partially reduced (by 43%) the increase in the final-to-instilled alveolar protein tracer (^{125}I-albumin) ratio caused by bleomycin (Fig. 4). Amiloride was also instilled in control rats and inhibited alveolar fluid clearance by 54% in these rats over 1 h (Fig. 4).

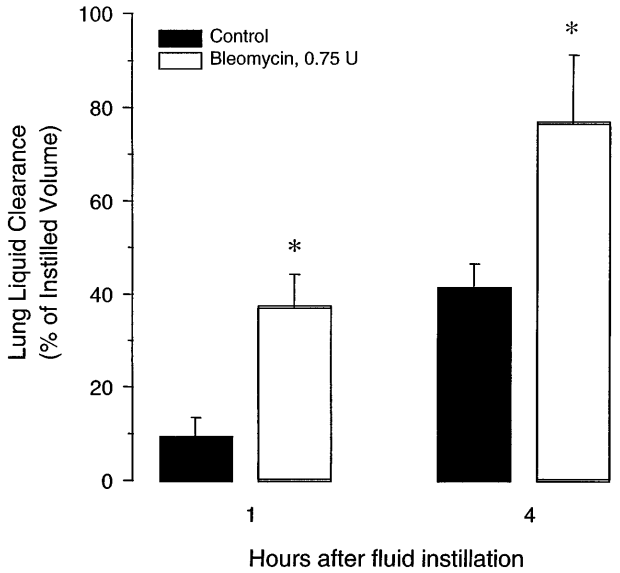

Fig. 3. Lung liquid clearance in rats treated with intratracheal bleomycin 10 days before experiments. Rats were studied for 1 and 4 h after instillation of 5% albumin solution. Data are means ± SD. Intratracheal instillation of bleomycin caused a significant increase in lung liquid clearance at all time points compared with control rats. *$P < 0.05$ vs. control rats at different times of experiments.

Effect of Recovery From the Injury

In rats studied for 1 h 60 days after intratracheal instillation of 0.75 U of bleomycin ($n = 6$), there was a near-complete recovery in all the parameters studied compared with control and bleomycin-instilled rats studied 10 days after instillation of bleomycin (Table 3). The increase in the bidirectional permeability to protein was 50% less than at 10 days (data not shown). The final-to-instilled ^{125}I-albumin concentration ratio was still elevated (1.36 ± 0.11 vs. 1.16 ± 0.13 in control rats) but was significantly reduced compared with 1.64 ± 0.18 in rats treated with bleomycin 10 days before fluid instillation (Fig. 5). Similar results were obtained for alveolar fluid clearance based either on protein concentration ratios or on final-to-instilled ^{125}I-albumin concentration ratios (Table 4).

^{22}Na Influx Studies

Uptake studies of ^{22}Na in isolated alveolar epithelial type II cells demonstrated that total individual epithelial cell sodium uptake was significantly decreased 10 days after bleomycin treatment (Fig. 6). Furthermore, the sensitivity to amiloride inhibition of the epithelial cell sodium uptake was significantly decreased in bleomycin-treated cells (Fig. 6). In control cells, amiloride inhibited 48% of the sodium uptake, whereas in cells isolated from rats 10 days after bleomycin treatment, amiloride inhibited only 20% of the sodium uptake.

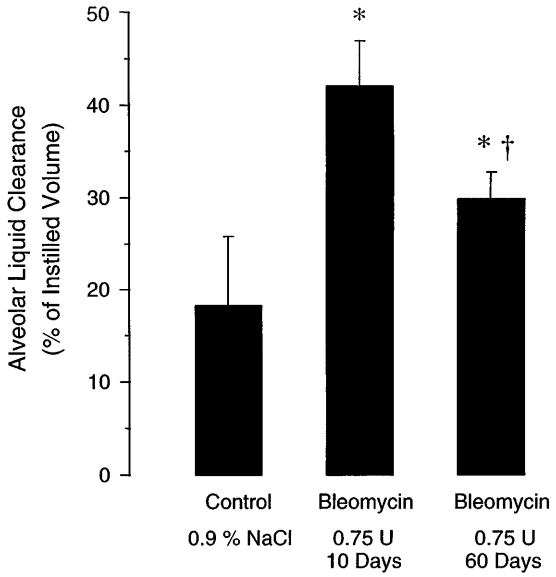

Fig. 5. Effect of exposure to bleomycin 60 days earlier on alveolar fluid clearance in control (saline-instilled) rats and in rats instilled either 10 or 60 days before experiment with 0.75 U of bleomycin. Rats were studied for 1 h after instillation of 5% albumin solution. Data are means ± SD. Instilled ^{125}I-albumin alveolar concentrations were corrected for loss of tracer into blood. Increase in alveolar fluid clearance observed 10 days after bleomycin exposure was significantly reduced at 60 days but did not return to control levels. *$P <$ 0.05 vs. control group. †$P < 0.05$ vs. 10-day bleomycin exposure.

Epinephrine and TGF-α Measurements

In the three plasma samples obtained at the beginning of the experiments, epinephrine levels were normal (<14 pg/ml; $n = 2$ rats) or minimally elevated in rats instilled 10 days before with 0.75 U of bleomycin (200 pg/ml; $n = 1$ rat). TGF-α was not detected in any of the alveolar fluid samples from four bleomycin-instilled rats studied on *day 10*.

Northern Blot Analysis of α-ENaC Expression

Northern blot analysis was done to determine whether the observed increase in alveolar liquid clearance at 10 days post-bleomycin instillation was associated with a change in the level of expression of α-ENaC mRNA. The results show that the level of mRNA expression of the α-subunit of rENaC decreased significantly in alveolar epithelial type II cells isolated from rats instilled with bleomycin compared with control rats (Fig. 7). When the same blots were probed for β-actin mRNA as a control, there was an equal level of expression in all lanes.

Effect of Alveolar Bleomycin on Lung Morphology

Bleomycin instillation caused cellular injury from which the lungs recovered. The most conspicuous evidence of injury 10 days after bleomycin instillation was hyperplasia of alveolar epithelial cells. Groups of cuboidal, hyperplastic epithelial cells were observed (Fig. 8). Transmission electron microscopy enabled identification of the hyperplastic cells as alveolar type II epithelium on the basis of numerous, large lamellar bodies in the cytoplasm and short microvilli along the apical membrane (Fig. 8*B*). These morphological characteristics of the hyperplastic cells were in contrast to the morphological appearance of alveolar type II epithelial cells in the control lungs (Fig. 8*A*). In the latter lungs, the alveolar type II epithelial cells were solitary and short, and their cytoplasm had small lamellar bodies.

Recovery from injury was seen primarily in the lungs of rats that had bleomycin instilled 60 days before morphological observation (Fig. 8*C*). Their lungs had fewer alveolar type II epithelial cells that were flatter and more widely spaced apart than in the lungs of rats treated with bleomycin 10 days before the structural observation (Fig. 8*C*). Another indication of recovery

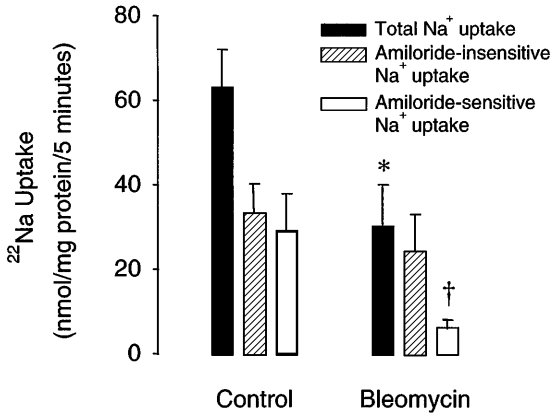

Fig. 6. ^{22}Na uptake in alveolar epithelial type II cells isolated from control and bleomycin-instilled rats. Overall Na uptake was significantly reduced 10 days after bleomycin instillation. Fraction of Na uptake that was sensitive to amiloride inhibition was also significantly reduced after bleomycin instillation (29 ± 9 and 6 ± 2 nmol·mg protein^{-1}·5 min^{-1} for control and bleomycin-treated rats, respectively). *$P < 0.05$ (by unpaired Student's *t*-test) compared with control rats. †$P < 0.05$ (by unpaired Student's *t*-test) compared with baseline.

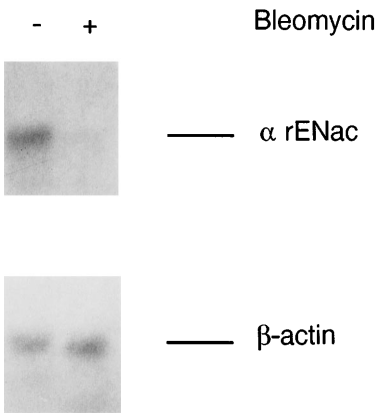

Fig. 7. Northern blot analysis of α-subunit of rat epithelial Na channel (α-rENaC) expression in isolated alveolar epithelial type II cells from rats exposed to bleomycin (+) 10 days before isolation and in control rats instilled with 0.9% NaCl (−) at the same time. Level of mRNA expression of α-rENaC decreased dramatically in alveolar epithelial type II cells isolated from rats instilled with bleomycin compared with control rats. When the same blots were probed for β-actin mRNA as a control, there was an equal level of expression in all lanes.

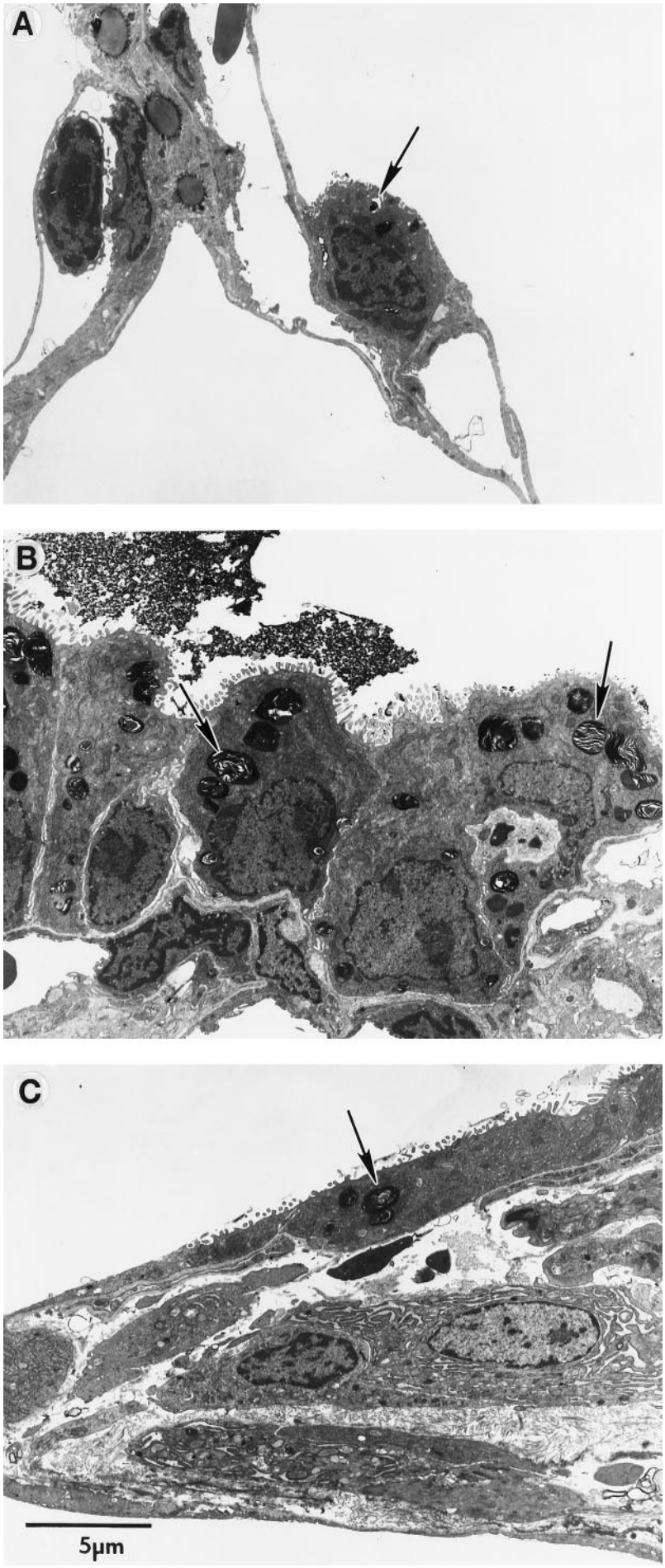

Fig. 8. Ultrastructural appearance of lungs of rats after intratracheal instillation of bleomycin. *A*: control lung (saline instilled). *B*: 10 days after instillation of bleomycin. *C*: 60 days after instillation of bleomycin. Compared with appearance of alveolar type II epithelial cell (*A*, arrow) in control lungs, alveolar type II epithelial cells (*B*, arrows) have microvilli and large lamellar bodies and were hyperplastic and hypertrophic in lungs of rats treated with bleomycin 10 days earlier. Alveolar type II epithelial cell (*C*, arrow) with lamellar bodies was flatter, and more alveolar type I epithelial cells were apparent in lungs of rats treated with bleomycin 60 days earlier. *A–C*, same magnification.

was that much of the air space surface was lined by alveolar type I epithelial cells (Fig. 8*C*).

DISCUSSION

There were two remarkable findings in these studies. First, the alveolar epithelium was able to remove excess alveolar fluid even though epithelial protein flux across the epithelial barrier was increased. Second, net fluid transport across the alveolar epithelium was markedly upregulated in the subacute phase after bleomycin-induced lung injury. The mechanism for this increased transport capacity may depend on an increased number of alveolar type II epithelial cells or an increase in sodium uptake by individual epithelial cells. However, because the message for the α-subunit of the ENaC was downregulated as well as the ^{22}Na uptake in individual epithelial cells isolated after bleomycin exposure, it is more likely that an increase in the number of alveolar epithelial type II cells is responsible for the increased clearance of excess fluid from the distal air spaces in bleomycin-exposed lungs.

It is remarkable that the alveolar epithelium was able to remove excess alveolar fluid even though the epithelial permeability to protein was increased 10 days after intratracheal bleomycin instillation. Ten days after bleomycin instillation, there was still an increased bidirectional flux of protein across the alveolar barrier. There is clinical precedent for this observation: some patients with acute lung injury can remove some excess alveolar fluid even though flooding of the air spaces with protein-rich edema fluid occurred within the preceding hours (26). Also, one prior experimental study (43) from our laboratory found that net alveolar fluid clearance occurred within 8 h after the development of oleic acid-induced lung injury in sheep. Interestingly, physiological (43) and morphological (27) studies showed evidence of increased endothelial and epithelial permeability to protein at these early time points. Also, in one earlier study (21) of intratracheally administered bleomycin in rats, increased solute movement across the alveolar-capillary barrier was observed. In the bleomycin-treated rats with subacute lung injury in this study, upregulated alveolar sodium and fluid transport capacities may explain, in part, why the extravascular lung water of these rats was increased only to 4.75 ± 0.49 g water/g dry lung, a finding consistent with interstitial edema alone. Clinically, many patients with acute respiratory distress syndrome after 7–14 days have radiographic (1) and morphological (6) evidence of less lung edema, although they may still have respiratory failure from fibrosing alveolitis (24). A possible mechanism in the recovering patients as well as in the rats in our study could be that the proliferation of alveolar type II epithelial cells enables the alveolar epithelium to clear excess alveolar fluid even in the presence of increased protein flux across the alveolar epithelium.

Because inflammation in the lung is frequently associated with an increased epithelial-endothelial permeability to protein, it was important to correct the fluid clearance calculations for the loss of the alveolar protein tracer ^{125}I-albumin into the blood. Therefore, we calculated alveolar epithelial fluid clearance using the increase in ^{125}I-albumin concentration over 1 and 4 h, taking into account the disappearance of the labeled albumin into the blood circulation. The alveolar fluid clearance calculations were, when this correction was used, almost identical with the unlabeled and labeled albumin concentrations over 1 and 4 h. Because we measured a net increase in fluid movement across the alveolar epithelium, the epithelium must have been sufficiently tight to remove excess alveolar fluid. This conclusion was strengthened by the observation that the alveolar protein concentration rose significantly over plasma protein concentration. Further support for this comes from the results of our shorter 1-h studies. In these studies, the same observation of an increased alveolar fluid clearance was observed even though the alveolar protein tracer left the air spaces to a lesser extent.

Because the alveolar epithelium retained its ability to remove excess alveolar fluid, the second objective of these studies was to identify a mechanism to account for the increase in alveolar fluid clearance. Because the increase in fluid clearance was inhibitable by amiloride, we concluded that active sodium uptake and transport were responsible. A significant fraction of the bleomycin-stimulated alveolar fluid clearance was inhibited by amiloride. However, inhibition by amiloride in control rats was greater (54%) than the inhibition by amiloride in bleomycin-exposed rats (43%). Similar results were obtained when we measured individual cell sodium uptake in the ^{22}Na uptake studies, in which amiloride inhibited 48% under control conditions while inhibiting only 20% after bleomycin treatment. There are at least four possible mechanisms that could explain the stimulatory effect on alveolar fluid clearance in the bleomycin experiments: *1*) an increased number of amiloride-sensitive apical sodium channels per alveolar type II epithelial cell as suggested in other studies (47), *2*) an increased number of alveolar type II epithelial cells, *3*) upregulation of amiloride-insensitive sodium channels after bleomycin exposure, and/or *4*) the new epithelial cells that appeared had a greater fraction of amiloride-insensitive sodium channels than the native type II cells. The modest decrease in amiloride sensitivity after bleomycin exposure suggests the latter two possibilities. Our morphological data provide circumstantial evidence for an effect from the increase in the number of alveolar epithelial type II cells because there was marked alveolar type II epithelial cell hyperplasia, suggesting that an increased number of alveolar type II epithelial cells could be responsible for the stimulated alveolar fluid clearance after bleomycin exposure. Also, other earlier investigations (3, 5) of bleomycin-induced lung injury have demonstrated marked alveolar epithelial type II cell hyperplasia. The molecular studies and the functional measurements of ^{22}Na uptake further support the morphological data. In the Northern blot studies, α-ENaC mRNA expression was lower in the bleomycin-exposed rats. Therefore, it

is less likely that upregulation of α-ENaC was responsible for the increase in alveolar fluid clearance. Also, the functional sodium uptake and transport, measured as ^{22}Na uptake, was decreased in the alveolar epithelial type II cells isolated from bleomycin-exposed rats. Whether the decrease in sodium transport across alveolar epithelial type II cells was primarily in the new type II cells or in all cells could not be deduced from our data. Taken together, these data suggest that the increased alveolar fluid transport after bleomycin exposure was related primarily to the increase in alveolar epithelial type II cell number. However, the stimulatory response may also be a result of another amiloride-insensitive sodium channel that was upregulated by bleomycin exposure. However, the absolute uptake of ^{22}Na was reduced in vitro so this seems less likely. It is also possible that an upregulated activity of basolaterally located Na^+-K^+-ATPase could increase the alveolar fluid clearance after bleomycin exposure. This has been shown under other conditions (28, 31). But this explanation seems less likely because sodium uptake was clearly reduced after bleomycin exposure. If sodium pump number and/or activity were increased, then we would have expected overall sodium uptake to have increased.

The hyperplasia observed after bleomycin exposure is not inhibited by antibodies to TGF-α (45), suggesting that this growth factor was not primarily involved in mediating the hyperplastic effect from bleomycin. Also, in our studies, there was no TGF-α detected in the alveolar fluid. This is an important finding because Folkesson et al. (14) reported that TGF-α can acutely upregulate alveolar epithelial vectorial sodium and fluid transport in rats.

Hyperoxia is another experimental model that has been used to induce alveolar type II epithelial cell hyperplasia (4, 11, 46, 47). That model, however, has provided different results regarding the effects on alveolar epithelial fluid clearance. Both the time and dose of oxygen administered significantly affect the pathological findings after hyperoxia. High doses are often associated with significant edema formation and increased lung lymph flow (37). In several studies (28, 31), alveolar type II cell Na^+-K^+-ATPase is upregulated after subacute hyperoxia, suggesting that alveolar fluid clearance may be increased in this condition. With the patch-clamping technique, Na^+-conductive pathways have been demonstrated to be upregulated after subacute hyperoxia in rats (18, 47). Yue et al. (47) and other investigators (4, 11) have found that apical sodium channels in the individual alveolar type II epithelial cells were upregulated after sublethal hyperoxic lung injury. This may have led to an upregulation of alveolar fluid clearance even in the presence of a leaky alveolus, similar to our findings after bleomycin exposure. A study of sublethal exposure to hyperoxia of isolated perfused rat lungs demonstrated that both amiloride-sensitive Na^+ channels and Na^+-K^+-ATPase were upregulated as a mechanism for the observed increase in alveolar epithelial fluid clearance after hyperoxia (28, 31). In contrast, other in vivo studies demonstrated no net effect on alveolar epithelial fluid clearance (16) or even a decreased fluid clearance after exposure to hyperoxic conditions (39).

Alveolar fluid clearance may be affected by endogenous mediators such as epinephrine (34). For example, endogenous catecholamines released from septic shock (34) or hemorrhagic shock (33) are each associated with stimulated alveolar fluid clearance. In the present study, plasma epinephrine was not elevated, suggesting that the observed increase in alveolar fluid clearance after bleomycin was not primarily mediated by catecholamines. Therefore, the marked degree of alveolar type II epithelial cell hyperplasia provides a good alternative explanation, particularly because the upregulated alveolar fluid clearance returned to almost normal around 60 days after intratracheal bleomycin when alveolar epithelial type II cell hyperplasia was not evident.

Another indicator of the acute phase from bleomycin-induced acute lung injury is an increased permeability of the alveolar epithelial-endothelial barrier to protein. It has been demonstrated that, after intratracheal instillation of bleomycin, there was an increase in lung microvascular permeability to ^{125}I-labeled albumin (40). However, this increase in protein permeability of the microvascular endothelium develops over time because a study of acute bleomycin administration in sheep did not cause an increased vascular protein permeability immediately after bleomycin administration (41). Also, exposure to subacute hyperoxia results in increased epithelial-endothelial FITC-labeled Dextran 20000 flux as well as in sucrose flux (32). In this study, we found an increased endothelial permeability to albumin and an increased alveolar epithelial permeability to albumin. However, Wangensteen et al. (40) found that, 5 days after bleomycin instillation, the capillary endothelial permeability to protein appeared normal even though the total extravascular albumin space was increased threefold above the control value. In our study, the endothelial and epithelial permeabilities to protein remained elevated for up to 10 days after bleomycin instillation. Remarkably, even though the increase in alveolar epithelial permeability to protein was significant, the alveolar epithelium was sufficiently intact to actively remove excess alveolar fluid at an even higher rate than under normal conditions.

There are some limitations in a complex in vivo model such as this one. First, our estimations of the alveolar fluid clearance assume that once the alveolar protein tracer ^{125}I-albumin left the air spaces, it will not reenter. This could happen if there were large leaks of protein-rich fluid into the air spaces. We measured both extravascular lung water and plasma equivalents and did not find significant alveolar edema, but there was interstitial edema. Second, the patchy distribution of bleomycin may have resulted in areas that were more or less affected by bleomycin, and thus the capacity to transport sodium and fluid may not be homogeneous. However, over the 10 days of this study, it is likely that these differences were not critical.

A second possible limitation is that Northern blot data do not provide evidence of a change in protein expression at the epithelial cell membrane. It only demonstrates what happens at the mRNA level and not necessarily at the level of the functional protein, apical ENaC. However, the consistent decrease in mRNA expression of α-ENaC and the decrease in sodium transport, measured as ^{22}Na uptake, suggest a real decrease in the sodium-transporting capacity of the individual alveolar type II epithelial cell. Thus it is more likely that the increase in type II cell number is the principal mechanism explaining the elevated alveolar epithelial fluid clearance capacity after intratracheal bleomycin.

In summary, after subacute lung injury, the capacity of the alveolar epithelium to remove excess alveolar fluid is markedly upregulated even though there is some injury to the barrier properties of the epithelium. The increase in fluid transport may be explained, in part, by an increase in the number of alveolar type II epithelial cells, resulting in a greater transport capacity of the alveolar epithelium. These studies do not, however, rule out other mechanisms that may upregulate the transport capacity of the alveolar epithelium. These observations may be clinically relevant because sustained upregulation of alveolar fluid clearance by catecholamine-independent mechanisms may be an important recovery mechanism from pulmonary edema during the subacute phase after lung injury.

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