

# Local Abnormalities of Coagulation and Fibrinolytic Pathways that Promote Alveolar Fibrin Deposition in the Lungs of Baboons with Diffuse Alveolar Damage

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## Abstract

Because alveolar fibrin is a prominent histologic feature of diffuse lung injury in baboons, we hypothesized that local abnormalities of pathways of fibrin turnover would favor fibrin deposition in the alveolar space. To test this hypothesis, procoagulant and fibrinolytic activities were characterized in serial bronchoalveolar lavage (BAL) of baboons with evolving diffuse alveolar damage (DAD) induced by exposure to 100% O<sub>2</sub>. BAL procoagulant activity, characterized mainly as the tissue factor-Factor VII complex, was markedly increased after induction of DAD. Extrinsic pathway inhibitor was likewise increased in BAL during evolving DAD but was insufficient to control coagulation. Urokinase-like fibrinolytic activity was usually detectable in baseline BAL but was undetectable after 7 d of O<sub>2</sub>. DAD BAL contained significantly increased plasminogen levels, plasmin inhibitor activity sufficient to neutralize all plasmin produced by BAL plasminogen activator found in control BAL and detectable plasminogen activator inhibitor-1. Antiplasmin activity was due, in part, to increased  $\alpha_2$ -antiplasmin. These changes correlated with quantitatively increased alveolar fibrin deposition demonstrated by histologic and morphometric analyses. Multiple abnormalities of pathways of fibrin turnover occur concurrently in the alveolar compartment of the lungs of baboons with DAD, which collectively predispose to diffuse alveolar fibrin deposition.

## Introduction

Alveolar fibrin deposition is a histologic hallmark of acute alveolitis and is particularly prominent in the Adult Respiratory Distress Syndrome (ARDS)<sup>1</sup> as well as acute lung injuries in experimental animals including diffuse alveolar damage (DAD) in baboons (1-6). We hypothesized that abnormalities of pathways of fibrin turnover occur in alveolar fluids in asso-

ciation with the high grade alveolitis that characterizes evolving DAD and that these abnormalities favor alveolar fibrin deposition. To test this hypothesis, we analyzed the procoagulant and fibrinolytic activities in serial, unconcentrated bronchoalveolar lavage (BAL) fluids of baboons with evolving DAD induced by protracted exposure to 100% O<sub>2</sub>. The histology of this lung injury is characterized by extensive alveolar fibrin, hyaline membranes, varying degrees of alveolitis, and early fibrotic repair. The baboon model of DAD, induced by 100% O<sub>2</sub>, was chosen for this study as it bears strong morphologic as well as pathophysiologic resemblance to ARDS, which is likewise characterized by DAD (5, 6).

As a result of microvascular and interstitial injury and/or local elaboration, substrates and inhibitors of coagulation and fibrinolysis may enter the alveoli, a compartment from which they are normally sequestered in the absence of inflammation. Access of such mediators is suggested by the recent demonstration that the composition of alveolar fluids in ARDS is similar to that of plasma (7). We postulated that abnormal entry or local elaboration of these reactants establishes conditions in which local fibrin deposition is favored. Supportive evidence includes recent observations that procoagulant activity is significantly increased in BAL of bleomycin-treated marmosets (3) and patients with ARDS (8), which, in both cases, is temporarily associated with extensive alveolar fibrin deposition.

The role of extravascular fibrin deposition in the pathophysiology of acute lung injury and DAD, in particular, is currently unclear. However, fibrin or fibrin-derived peptides are known to influence endothelial cell function (9, 10) vascular permeability (11) and inflammatory cell traffic (12) and thus may influence the inflammatory response (13). Fibrin also serves as a provisional matrix upon which fibroblasts may aggregate and secrete collagen (14), suggesting that fibrin could influence in vivo tissue repair and scarification. Moreover, fibrinolytic and coagulation reactants may activate the complement and kinin-forming systems (15). For these reasons, and because extensive extravascular, primarily alveolar fibrin deposition occurs in the severely injured lung, knowledge of the local abnormalities that promote alveolar fibrin deposition is essential to understanding the pathogenesis of ARDS and related forms of lung injury. In this study, we demonstrate that multiple abnormalities of pathways of fibrin turnover occur in the lower respiratory tract fluids of baboons with evolving DAD and that these abnormalities functionally predispose to the local deposition of alveolar fibrin.

## Methods

**Animals.** The 17 young adult baboons (*Papio cynocephalous*) studied were born in captivity, had no history of respiratory disease and were free of parasites. Baseline studies, including chest x rays, pulmonary function tests and arterial blood studies were normal. The protocols used to obtain these physiologic measurements were as previously described (5). Animals were anesthetized with ketamine and main-

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1. *Abbreviations used in this paper:* ARDS, adult respiratory distress syndromes; BAL, bronchoalveolar lavage; CHF, congestive heart failure; DAD, diffuse alveolar damage; EPI, extrinsic pathway inhibitor; FRC, functional residual capacity; NPP, normal pooled plasma; PA, plasminogen activator; PAI, plasminogen activator inhibitor; RLL, right lower lobe; TLC, total lung capacity; t-PA, tissue PA; u-PA, urokinase PA.

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tained under continuous deep sedation with diazepam and muscular paralysis using pancuronium (5). Throughout the course of these experiments, the animals were kept on continuous mechanical ventilation in an intensive care unit-like setting. Details of the protocols for mechanical ventilation and long term management of these baboons have previously been described (5, 6). Briefly, animals were ventilated via cuffed endotracheal tubes using a volume cycled ventilator (MA-1; Puritan-Bennett Corp., Kansas City, KS). Additional instrumentation of these animals included placement of a balloon-tipped pulmonary artery catheter, an arterial catheter, and urinary bladder catheter. Trended measurements included electrocardiographic monitoring, determination of systemic and pulmonary vascular pressures, cardiac output determinations and arterial blood gas studies. In addition, daily chest x rays were followed and serial pulmonary function testing including determination of functional residual capacity (FRC), diffusing capacity (DLCO) and total lung capacity (TLC) were performed.

After collection of baseline data on the first day of each experiment, DAD was induced by exposure to 100% oxygen ( $n = 10$  animals). Alternatively, control baboons were mechanically ventilated while receiving 21% oxygen ( $n = 7$  animals). To prevent bronchopulmonary infection, animals were given ampicillin and polymyxin. Animals received a prophylactic antacid (Maalox; William H. Rorer, Inc., Fort Washington, PA) every 4 h. Fluids were administered intravenously on the basis of blood chemistries, urinary output, and hemodynamic measurements. A fiberoptic bronchoscope (pediatric model; Olympus Corp., Irving, TX) was used to perform BAL, which consisted of the instillation of 90 ml of saline into a single lobe. Baboons exposed to 100% O<sub>2</sub> or controls ventilated with 21% O<sub>2</sub> were instilled with 90 ml of saline. The recovered fluid was centrifuged at 2,500  $g$  at 4°C for 10 min. The pellet was used to determine total and differential white cell counts, while the supernatant was centrifuged at 12,000  $g$  for 15 min and the supernatant frozen at -70°C until used. In preliminary experiments, we found that <sup>125</sup>I radiolabeled Factor Xa or plasminogen were not altered by the lavage processing procedure. The physical form of these proteins, when added to BAL alone or to BAL supplemented with  $1 \times 10^6$  human neutrophils/ml and then processed as described above, was virtually identical. Plasminogen remained in the 90,000  $M_r$  form in saline, as well as baseline or DAD BAL with or without BAL processing. Factor Xa migrated at 53,000 in saline and in baseline BAL but was, in part, complexed at 120,000  $M_r$  in DAD BAL, with or without BAL processing. DAD was induced by exposing baboons to 100% inspired oxygen for 5–9 d. The infusion of dopamine was used to treat severe systemic hypotension which was associated with severe, edematous lung injury necessitating the use of positive end expiratory pressure (PEEP) in three of these animals. BAL was obtained before the initiation of 100% oxygen and at either 5, 7, and in some cases, 9 d after exposure.

Seven control animals were mechanically ventilated with 21% oxygen for 5–11 d. These animals were maintained under the same conditions as those described above. BAL was obtained at day 0, 7, and 11 of control mechanical ventilation as described above.

**Histologic and morphometric studies.** Autopsy was performed immediately after the death or elective sacrifice of each animal. Lungs were perfused for 15 min with phosphate-buffered 4% formaldehyde-1% glutaraldehyde fixative (4CF-1G) at 28 cm H<sub>2</sub>O pressure using a rolling pump (16).

Specimens were prepared for light microscopy and transmission electron microscopy as previously described (5). Lung blocks were obtained from the right upper and right middle lobes (one each), studied by routine light microscopy and used for correlation with microbiologic results. The right lower lobe (RLL) was dedicated entirely for pathologic and morphometric studies. After weighing and intrabronchial fixation with phosphate-buffered 4% paraformaldehyde at 20 cm H<sub>2</sub>O constant airway pressure for 48 h (17), the RLL lung volume was determined by volume displacement and transverse 1.0-cm sections were taken at three levels through each lobe (18). The entire surface of each transverse slice of the RLL was totally sampled and blocked for light microscopic studies with an average of 14 blocks being examined.

Determination of both the extent and severity of DAD (either exudative, reparative, or mixed patterns) and pneumonias (graded histologically as organizing, mild, moderate, or severe) was accomplished. Electron microscopy specimens were obtained from 5 or more representative areas of the usual 14 light microscopic sample sites. 50 blocks were fixed in Zetterqvist's osmium fixative of which 20 were then embedded and thick sectioned. Six blocks were then thin sectioned and stained with Reynolds' lead citrate and uranyl acetate. The presence of fibrin within the alveolar space was confirmed by its characteristic periodicity on electron microscopy (19). Volume density determinations of the presence of fibrin within the alveolar space were accomplished on specimens of the 100% oxygen treated animals and were compared with a group of nonventilated healthy baboon controls using established techniques (20). Three animals were randomly selected from each group for analysis.

**Coagulation assays.** Single-stage coagulation assays in which BAL was exposed to either normal pooled human plasma or human plasmas deficient in Factors VII, VIII, or X were performed as previously described (3, 8). All assays were performed in duplicate using the same lot of each deficient plasma to assay all BAL samples. Extrinsic pathway inhibitor (EPI) activity was measured in a three-stage coagulation assay, as previously described (21). A standard curve was constructed from pooled normal human plasma and showed a direct linear relationship between the logarithm of the clotting time and the logarithm of the plasma containing EPI between 0.2–8%, assuming 100 ng EPI/ml plasma (21).

**Amidolytic assay of Factor Xa.** Conversion of Factor X to Xa by baboon BAL was determined by an amidolytic assay performed as previously described, with slight modifications (8). In this assay, 45  $\mu$ l of BAL was incubated with 30  $\mu$ l of 0.15 M NaCl, 0.02 M Tris, pH 7.4 (TBS), and 15  $\mu$ l human Factor X (10 U/ml). This mixture was incubated at 37°C for up to 30 min. 75- $\mu$ l aliquots were added to a spectrophotometer cuvette containing 25  $\mu$ l of 4 mM benzoylisoleucyl-glutamyl-glycyl-arginine-*p*-nitroanilide (S2222; Kabi Group, Inc., Greenwich, CT) and 200  $\mu$ l of 50 mM Tris, 175 mM NaCl, and 7.5 mM EDTA, pH 8.4. The initial rate of increase in absorbance at 405 nm was determined on a response spectrophotometer (Gilford Instruments, Inc., Oberlin, OH). The picomoles of Factor X hydrolyzed per minute per milliliter of BAL was calculated based on the initial rate of increase of absorbance generated by human Factor Xa standards as previously described (3).

**Proteolytic cleavage <sup>125</sup>I-labeled human Factor X.** Factor X was labeled with Na <sup>125</sup>I as previously described (8). Baboon BAL was incubated at 37°C with a mixture of radiolabeled and unlabeled human Factor X (5  $\mu$ g/ml) in polypropylene tubes for 30 min. Aliquots were removed and subjected to nonreduced SDS polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiographic development of the dried gel as we have previously described (3, 8).

**Neutralization of BAL Factor X activating activity by antibodies to Factor VII and tissue factor.** Equal volumes of baboon BAL and rabbit antibodies (IgG fraction) to Factor VII (50  $\mu$ g/ml), tissue factor (100  $\mu$ g/ml), or nonimmune IgG were incubated for 1 h at 37°C after which BAL Factor X activating activity was determined in the Factor Xa amidolytic assay. Characterization of the neutralizing antibody to human Factor VII has been previously described (22). Antibody to bovine tissue factor, which cross-reacts with human tissue factor (23, 24), was generously provided by Dr. Ronald Bach (Yale University, New Haven, CT).

**Fibrin plate assay of BAL fibrinolytic activity.** Plasminogen activator (PA) activity and plasminogen independent fibrinolytic activity of baboon BAL was assayed in <sup>125</sup>I-fibrin plates as previously described (25, 26). The sensitivity of the assay performed as described was 0.0001 IU/ml based on urokinase standards (American Diagnostics, Inc., Greenwich, CT). PA activity of BAL was determined by calculation of the time at which 50% of trypsin releasable radioactivity was achieved by the sample.

**Neutralization of fibrinolytic activity in baboon BAL.** Baboon BAL was preincubated with a final concentration of 160  $\mu$ g/ml of the IgG

fraction of antibodies to urokinase (u-PA), tissue plasminogen activator (t-PA), control goat IgG or normal saline solution for 30 min at 37°C, and analyzed in the <sup>125</sup>I-fibrin plate assay. The goat antibodies were purchased from American Diagnostica (Greenwich, CT), and their specificities confirmed. The t-PA was generously provided by Dr. Eugene Levin (Research Institute of Scripps Clinic, La Jolla, CA).

**Characterization of PA and PA inhibitory activity in baboon BAL.** PA of baboon BAL was also characterized by fibrin gel autography, as described by Levin and colleagues (27). Briefly, BAL samples or standards were subjected to SDS-PAGE using 10% acrylamide resolving gels (3). The gel was then soaked in 0.1 M sodium phosphate pH 7.2 with 2.5% Triton X-100, blotted dry and placed on top of a freshly prepared fibrin indicator gel. The indicator gel was allowed to develop at room temperature in a moist chamber, and the resulting clear lysis zones were photographed. Reverse fibrin gel autography was performed as previously described (28). Samples were subjected to SDS-PAGE, the gel soaked in Triton X-100 and then placed over a fibrin-agar indicator plate supplemented with 0.075 IU/ml of urokinase (final concentration). Regions of the gel containing inhibitor were revealed by the formation of opaque, lysis-resistant zones in the clear, lysed indicator gel.

**Antiplasmin activity.** Antiplasmin activity of BAL samples was measured by incubating with equal volumes of 5 µg/ml plasmin (American Diagnostica) diluted in normal saline and BAL samples at 37°C for 30 min. Aliquots were analyzed in the <sup>125</sup>I fibrin plate assay to determine the residual fibrinolytic activity of the sample-plasmin mixtures.

Alternatively, antiplasmin activity was measured by a decrease in the initial rate of plasmin-mediated hydrolysis of the chromogenic substrate H-D-Val-Leu-Lys-p-nitroanilide (S-2251; KABI Group, Inc.) (29). Briefly, 50 µl of plasmin (10 µg/ml in TBS) was mixed with 50 µl of normal saline or BAL samples, incubated at 37°C for 10 min and then added to prewarmed spectrophotometer cuvettes containing 25 µl of 3.5 mM S-2251 and 175 µl of TBS. Initial rates of cleavage of the substrate were assessed by the change in absorbance at 405 nm using a spectrophotometer (DU 30; Beckman Instruments, Inc., Fullerton, CA) and were linear for at least 5 min. The rates were converted to micrograms per milliliter of plasmin based upon the amidolytic activity generated by plasmin standards. Residual plasmin activity of the plasmin-BAL samples was expressed as percent inhibition relative to the plasmin standard mixed with saline. Expression of amidolytic activity by plasmin was unaffected by addition of up to 2 mg/ml asolectin (American Lecithin Co., Woodside, NY), which represented 100 times the phospholipid content we previously reported in BAL of patients with ARDS (8). Addition of up to 10 mg/ml of bovine serum albumin likewise did not interfere with plasmin-mediated amidolytic activity.

Goat antibody to human  $\alpha_2$ -antiplasmin ( $\alpha_2$ AP) and control goat IgG were purchased from American Diagnostica and bound to Affigel-10 beads (Bio-Rad Laboratories, Richmond, CA), according to the manufacturer's instructions at a ratio of 5 mg IgG/ml of resin.  $\alpha_2$ AP (American Diagnostica) was shown to inhibit plasmin, when it was diluted to 10 µg/ml in TBS and then mixed for 10 min at 37°C with 10 µg/ml plasmin. This inhibitory capacity was removed if  $\alpha_2$ AP was incubated for 1 h at 37°C with antibody to  $\alpha_2$ AP bound to Affigel-10 but was unchanged after adsorption with control beads. BAL samples were incubated with either anti- $\alpha_2$ AP or control beads 1:1 (vol/vol) for 1 h at room temperature, and the beads were then sedimented by a flash spin using a Beckman microfuge. Remaining antiplasmin activity was measured in two assays. To 60 µl of supernatant was added 60 µl of plasmin standard (6 µg/ml) for 10 min at 37°C and the plasmin-mediated amidolytic activity was determined. Alternatively, the supernatants were mixed 1:1 with a plasmin standard (1.25 µg/ml in normal saline) for 30 min at 37°C and plasmin-mediated release of radiolabeled fibrin was measured in the <sup>125</sup>I fibrin plate assay.

**Radioimmunoassays.** Double antibody competitive equilibrium radioimmunoassays were modified from those previously reported for human plasminogen and  $\alpha_2$ AP (29). Proteins were radioiodinated by the dilute chloramine T method (30) to specific activities of 1.7–6.6

µCi/µg. To 250 µl of labeled protein (0.5 nM) was added 250 µl of competing human protein (for standard curve) or bronchoalveolar fluid (diluted 1:4) and 250 µl of antibody diluted to give ~ 30% binding of the iodinated ligand. After incubation at 4°C for 16–20 h, 500 µl of goat anti-rabbit (Pel-Freez Corp., Rogers, AR) IgG was added and incubated at 4°C for 6 h. The immunoprecipitates were collected by centrifugation at 2,000 g for 15 min, and 500 µl of supernatant was counted in a gamma counter. In control studies, semilogarithmic graphs of the inhibition of <sup>125</sup>I-protein binding to its specific antibody seen by normal baboon plasma was parallel to the competition curves observed for human plasma permitting an estimate of these proteins present in biological fluids. The buffer used for the assay contained 50 mM Tris/HCl (pH 8.0), 25 mM NaCl, 10 mM benzamidine, 1 mM PMSF, 5 U/ml aprotinin, 0.75% normal rabbit serum, and 0.02% NaN<sub>3</sub>. Plasminogen was purified from human plasma by the method of Deutsch and Mertz (31) and human  $\alpha_2$ AP was bought from American Diagnostica. Rabbit antibodies to human plasminogen and  $\alpha_2$ AP were generously provided by Dr. Edward F. Plow and Dr. Lindsey A. Miles (Research Institute of Scripps Clinic, La Jolla, CA).

**Characterization of the form of plasminogen-related antigen in baboon BAL.** BAL plasminogen related antigen was adsorbed by addition of 20 µl of lysine-Sepharose 4B (coupled by CNBr; 32) to 1 ml of BAL sample and mixed by rotation at room temperature for 1 h. The beads were collected by centrifugation for 30 s using a Beckman Microfuge and washed once in normal saline. Plasminogen-related antigen was eluted by boiling the antibody-Sepharose beads for 4 min in the 2% SDS, 1% glycerol, 62.5 mM Tris-HCl, pH 6.8. Supernatants were applied to 4% acrylamide stacking gels and run on 10% acrylamide resolving gels by the method of Laemmli (33). Electrophoretic transfer to nitrocellulose was performed according to Towbin et al. (34) using a Bio-Rad transfer cell with a recirculating water bath (4°C) at 60 V for 90 min. The nitrocellulose paper was "blocked" by incubation with 0.5 M NaCl, 0.01 M Tris, pH 7.5, 3% gelatin for 30 min at room temperature, incubated with a 1:200 dilution of rabbit anti-human plasminogen overnight at 4°C, and reacted with 0.1 µg/ml <sup>125</sup>I goat anti-rabbit IgG, radiolabeled according to the method of Fair et al. (30), for 3 min at ambient temperature. Plasminogen derivatives were localized by autoradiography, and the molecular weights of plasminogen species were determined by the relative migration of transferred prestained standards (Bio-Rad Laboratories).

**Human pleural fluids.** Pleural fluids were collected by standard thoracentesis technique from hospitalized patients in order to examine the specificity of abnormalities of fibrin turnover observed in BAL. Pleural fluids were harvested from patients with congestive heart failure (CHF, *n* = 6), patients with pneumonia and parapneumonic effusions (*n* = 4), empyema (*n* = 3), and patients with carcinoma of the lung and associated pleural effusions (*n* = 16), and were immediately collected in polypropylene tubes containing 3.8% sodium citrate in a ratio of 9:1, pleural fluid/citrate. The fluids were immediately centrifuged at 400 g for 10 min, after which the pleural fluid total white cell and differential counts and protein content were determined (3, 8). The cell-free pleural fluids were then immediately frozen at -70°C until further processing was completed. Pleural fluids were harvested for clinical indications, and the collection of these fluids for this study was approved by the Human Subjects Institutional Committee of The University of Texas Health Center at Tyler.

**Statistical analysis.** Nonparametric analysis of BAL variables was used as these variables are not known to be normally distributed. Intergroup variation for BAL variables was first determined by Kruskal Wallis 1-way analysis of variance. Specific comparison of BAL variables between groups was done using the Mann Whitney U-Wilcoxon Rank Sum W test. The data are presented as box graphs that show the medians, ranges of 50% of the data points (boxes) and the entire range of values.

## Results

In the 100% O<sub>2</sub> group, DLCO was usually reduced at day 4 of exposure, suggesting pulmonary oxygen toxicity and chest x

rays showed diffuse bilateral infiltrates suggesting the development of pulmonary edema by day 5. Hypotension requiring infusion of dopamine was observed in three animals usually on the fifth to sixth day after oxygen exposure and was believed to be due to the effects of positive pressure mechanical ventilation and worsening lung injury. One animal in the oxygen-treated group succumbed to an episode of hypotension. The others were electively sacrificed at 6 to 9 days after exposure to 100% O<sub>2</sub>. BAL obtained at baseline revealed about 1 × 10<sup>6</sup> total white cells with greater than 90% macrophages on differential white cell count. At all time points following injury in oxygen-challenged baboons, BAL contained 10<sup>6</sup> to 10<sup>7</sup> total white cells and a neutrophilic pleocytosis (neutrophils ≥ 30%) was observed. About 50% of the instilled saline was recovered during the lavage procedure. Total protein content in BAL from control and treated baboons was followed for up to 11 d (Fig. 1). In the oxygen-treated group, an increase in total protein was seen which continued up to day 9 after hyperoxia to 5–6.25 mg/ml. In baboons ventilated with 21% O<sub>2</sub>, the BAL total protein rose from a baseline of 0.02–0.05 mg/ml to a median of 0.2 mg/ml, which was significant (*P* = 0.02), but was 10-fold less than that level observed in injured animals with DAD.

**Histologic and morphometric observations.** Histologic changes in the 100% oxygen-treated group included exudative DAD characterized by protein-rich pulmonary edema and hyaline membrane formation (Fig. 2 A). Fibrin was abundant in the intraalveolar exudate of these animals (Fig. 2 B). Superimposed upon this process was a pneumonitis characterized by increased numbers of neutrophils (PMNs) in respiratory bronchioles and surrounding alveoli (not shown).

The presence or absence of pneumonia, and DAD was determined histologically in each animal (Table I). Analysis for exudative DAD indicated a diffuse presentation in 7 of 10

baboons treated with 100% O<sub>2</sub> and focal in 2. None of the control mechanically ventilated baboons developed DAD. Pneumonia was observed in nearly every animal, which varied from mild to moderate in both control 21% and 100% O<sub>2</sub>-treated baboons. Volume density determinations of the presence of fibrin within the alveolar space revealed a significant difference in the animals with DAD compared to a group of nonventilated baboon controls (*n* = 3 in each group). By this morphometric analysis, grossly more fibrin was present in the lungs of animals with DAD as a result of 100% O<sub>2</sub> exposure (Table II). Minimal to no alveolar fibrin was present in the three control animals.

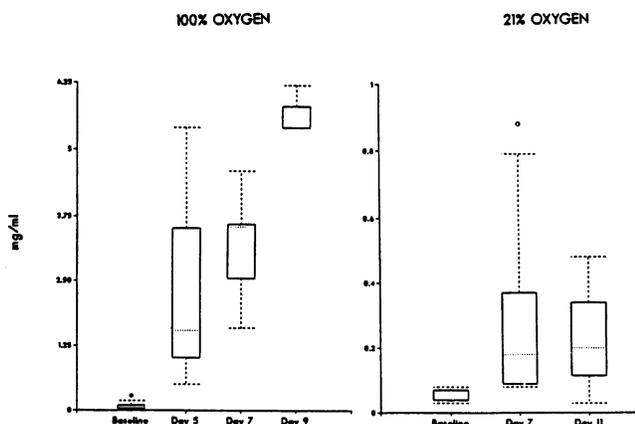
**Characterization and quantitation of procoagulant activity present in baboon BAL.** Normal baboon BAL was capable of shortening the recalcification time of normal pooled plasma as well as both Factor VII and VIII deficient plasma but not that of plasma deficient in Factor X (Table III). The recalcification times of BAL in normal pooled and each deficient plasma were decreased after lung injury. The dependence on Factor X in each coagulation profile suggested the presence in baboon BAL of an activator of Factor X. Support for a Factor X activator in baboon BAL was shown by activation of Factor X to Factor Xa in amidolytic assays and cleavage of purified human Factor X to form the 55,000 *M<sub>r</sub>* Factor Xa product (data not shown).

BAL Factor X activating activity was similar to that have previously reported in BAL of patients with ARDS and marmosets with bleomycin-induced lung injury (3, 8) and suggested that tissue factor-Factor VII complex was the major procoagulant activity present in baboon BAL. Further, this activity was sedimentable by ultracentrifugation at 100,000 *g* for 2 h at 4°C, suggesting the presence of a tissue factor moiety. The Factor X activating activity was completely neutralized in the presence of the antibody to Factor VII (Table IV). The antibody to tissue factor was also effective in inhibiting the Factor X activating activity. Therefore, tissue factor associated with Factor VII accounts for the major procoagulant activity present in baboon BAL.

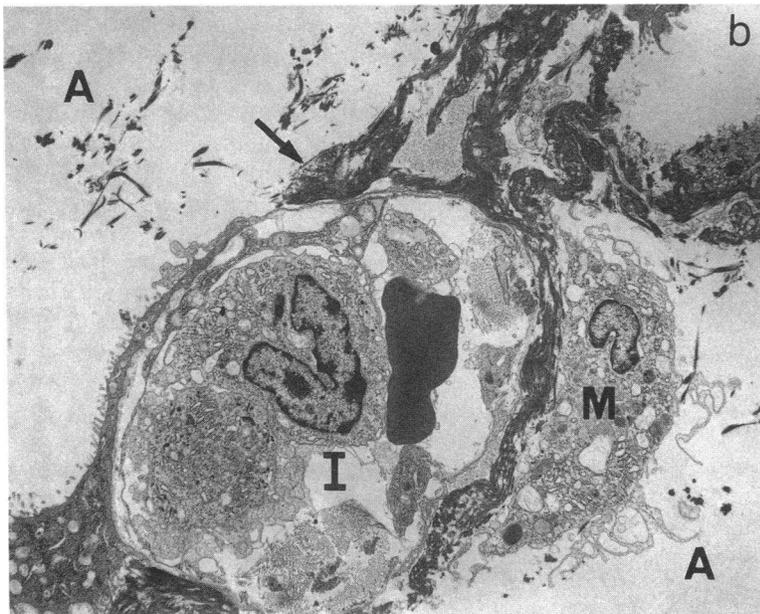
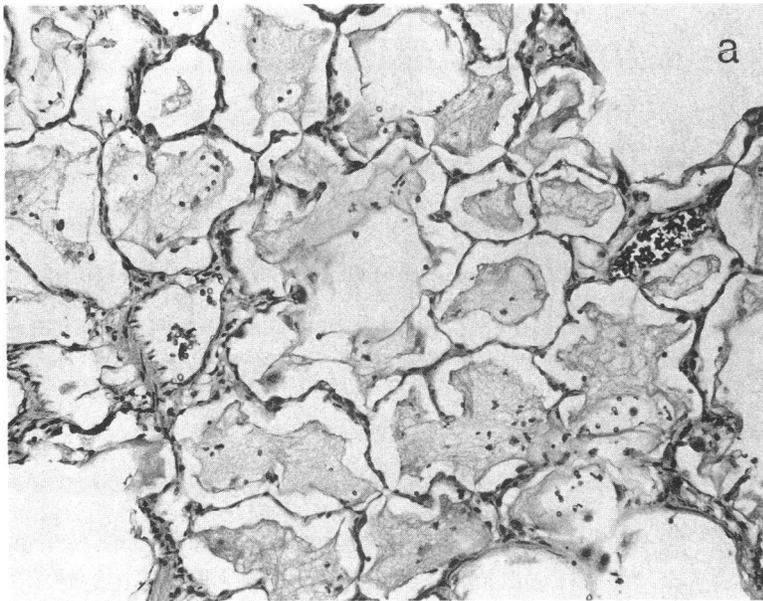
As shown in Fig. 3, the recalcification times of baseline baboon BAL in normal pooled plasma were significantly longer than those obtained any time after lung injury (*P* = 0.04). Animals given 21% O<sub>2</sub> also showed shorter recalcification times over the 11 d. Transformation of the recalcification times to procoagulant units demonstrated that for each time measurement after lung injury significantly greater amounts of BAL procoagulant activity were present (*P* < 0.01) relative to baseline. This was the case for each DAD animal studied. BAL procoagulant activity was variably increased in 21% O<sub>2</sub> control baboons and was usually of lesser magnitude. In animals ventilated with 21% O<sub>2</sub>, a focal bronchopneumonia was often observed and found to correlate with increased procoagulant activity present in BAL observed on day 7 (*P* = 0.04), but BAL procoagulant activity returned to baseline levels by day 11.

Because the increase in procoagulant activity in BAL of baboons with DAD was the result of the assembly and expression of the extrinsic pathway of coagulation, i.e., Factor VII associated with tissue factor, we investigated the possible regulation of this complex by determining the degree of Factor X activation relative to the presence of the recently characterized extrinsic pathway inhibitor (EPI;21). The rate of Factor Xa formation was observed to increase (*P* = 0.04) and remain

## BABOON BAL PROTEINS



**Figure 1.** Changes in total protein concentration in serial BAL samples of baboons treated with 100% oxygen or 21% oxygen. The data are presented as box plots: open boxes represent the fields which contain 50% of the values and contain the median value (· · ·). The range of values is indicated by the dashed lines and the circles represent outlying values. Confidence limits are described in the text.



*Figure 2.* Histologic changes in the lungs of 100% O<sub>2</sub>-treated baboons. (a) Protein rich edema and a few extravasated RBCs are in the alveolar spaces. Hematoxylin and eosin;  $\times 140$ . (b) Fibrin and an alveolar macrophage (M) envelope the alveolar wall. The type I epithelial lining is focally lost (arrow). A monocyte and RBC are in the interstitium (I). A, Alveolar space. Lead citrate and uranyl acetate;  $\times 7,800$ . The arrow indicates alveolar fibrin.

elevated following DAD, while a transient increase in Factor Xa generation was noted for 21% O<sub>2</sub> controls (Fig. 4). Injury with 100% O<sub>2</sub> indicated a clear inverse relationship between Factor X activation and the presence of EPI. No correlation was evident between these parameters during the period of control ventilation, although modest elevations were noted, presumably as a result of focal bronchopneumonia.

In preliminary experiments, we found that the addition of baboon plasma, or lysed or live human neutrophils to BAL shortened the recalcification times of BAL in normal pooled human plasma but failed to increase the Factor X activating activity of BAL. No procoagulant activity was observed in the neutrophil preparations and extremely small amounts were found in baboon plasma by recalcification, but not amidolytic assays. These data indicate that the influx of neutrophils into the lung was not responsible for the observed increase in procoagulant activity.

*Characterization and quantitation of the fibrinolytic activity present in baboon BAL.* Fibrinolytic activity was usually but not invariably detectable in unconcentrated baseline BAL (Fig. 5). In baboons ventilated with 21% O<sub>2</sub>, fibrinolytic activity was either stable or rose somewhat after several days of mechanical ventilation, but was not significantly changed from baseline levels. In baboons with 100% O<sub>2</sub> injury, the BAL fibrinolytic activity was reduced at day 5 and undetectable by days 7 and 9. In the 100% oxygen-treated group, one animal (No. 4081) developed only mild lung injury. In this animal fibrinolytic activity was detectable at all time points after the administration of 100% O<sub>2</sub>. Thus, depressed fibrinolytic activity present in baboon BAL appeared to be associated with the induction of DAD. The addition of lysed or live human neutrophils increased the total fibrinolytic activity found in baseline baboon BAL. Addition of lysed or live neutrophils to BAL of baboons with evolving DAD that lacked fibrinolytic activity

**Table I. Histologic Features of 100% and 21% O<sub>2</sub>-treated Baboons**

Group	Animal No.	DAD		Pneumonia	
		Focal	Diffuse	Mild	Moderate
100% O <sub>2</sub>	4081*	0		×	
	3825	×		0	
	3933	×		×	
	3917, 4060, 4253		×	×	
	4054, 4055 4390, 4564		×		×
21% O <sub>2</sub>	4128, 4196, 4242	0		×	
	4260, 4304				
	4171, 4238	0			×

0, none; \* No widespread pulmonary edema. Bronchopneumonia was diagnosed as follows: mild, the presence of scattered neutrophilic infiltrates localized to terminal bronchioles and surrounding alveoli; moderate, when extension of this process caused confluence of infiltrates between adjacent lobules; and severe, when the exudative process was extensively confluent both grossly and microscopically and was occasionally associated with tissue necrosis.

did not generate detectable fibrinolytic activity and the addition of baboon plasma to BAL inhibited BAL fibrinolytic activity.

Because fibrinolysis is initiated by specific plasminogen activators (PA), we characterized the components of the fibrinolytic system in BAL. The fibrinolytic activity present in baboon BAL was mainly plasminogen dependent. Plasminogen independent fibrinolytic activity was not detectable in un-concentrated BAL at any time point by the fibrin plate assay (data not shown). Further, PA activity could be neutralized by an antibody to urokinase but not by nonimmune IgG controls or an antibody specific for t-PA in the <sup>125</sup>I fibrin plate assay (Table V). When the molecular weight of the PA was investigated by fibrin gel autography, the major zone of fibrinolytic activity present in baboon BAL was found to comigrate with urokinase standards (Fig. 6 a) and be neutralized by an antibody to urokinase (Fig. 6 b). Additional higher molecular

**Table II. Morphometric Analysis of Tissue Fibrin Deposition in Baboons with DAD\***

Animal No.	Histology	Density
4564	DAD	6.96
4054	DAD	9.18
4390	DAD	17.09
971	Normal	0
967	Normal	0.06
968	Normal	0

\* Controls with normal lung histology were unventilated, healthy baboons. DAD samples selected for study were randomly chosen from animals having typical histologic DAD. Volume densities, used as an index of alveolar fibrin deposition, are expressed as a percentage: Volume density = 100 × fibrin, hyaline membranes/airspace.

**Table III. Recalcification Times of Baboon BAL in Normal-pooled and Factor-deficient Human Plasma\***

	Factor VIII	Factor VII	Factor X	NPP
<b>Baseline</b>				
(n = 4)	239.2±24.6	311.0±84.3	>500	271.6±69.0
DAD (n = 5)	74.8±15.3	69.7±16.1	281.5±74.2	65.3±13.3

\* VII, VIII, X, plasma-deficient in Factor VII, VIII, or X. NPP, normal-pooled plasma. Recalcification times are expressed in seconds. All assays were done in duplicate, using the same lots of plasma. The mean±standard deviation of the measured recalcification times are shown.

weight zones of fibrinolysis were identified at *M<sub>r</sub>* 85,000 and 110,000 and were most prominent in DAD BAL. Lytic zones were undetectable or grossly attenuated in baseline baboon BAL (n = 9 samples) when plasminogen was deleted from the indicator plates. By contrast, prominent zones of fibrinolysis at *M<sub>r</sub>* 110,000 but not 85,000 were found in BAL samples from baboons with DAD when plasminogen was deleted (data not shown). The 85,000 *M<sub>r</sub>* zone of fibrinolysis was not inhibited in the presence of either of our antibodies to u-PA or t-PA. These experiments indicate that urokinase-like fibrinolytic activity was present in baboon BAL and that plasminogen independent fibrinolytic activity could also be detected in BAL of baboons with evolving DAD using fibrin gel enzymography.

Because the BAL from baboons with DAD demonstrated lytic zones at higher molecular weight and because these BAL samples had no detectable fibrinolytic activity when studied by the fibrin plate assay, we reasoned that specific inhibitor(s) of PA or plasmin were likely to be present. Determination of BAL PA inhibitors (PAI) was monitored by reverse fibrin autography. Baseline BAL harvested before lung injury contained no detectable PAI activity, whereas these samples contained detectable u-PA activity by the fibrin plate assay and fibrin gel autography. However, after induction of lung injury leading to DAD, lysis-resistant zones were detected at 45,000 *M<sub>r</sub>* in some (three of six) DAD BAL samples, indicating the presence of PAI. In separate experiments, we found that this zone of PAI migrated near that of Hep G2 cells, which are known to produce PAI-1 (35) and this zone could be inhibited by an antibody to PAI-1 (Fig. 7).

The absence of detectable fibrinolytic activity in BAL of baboons with DAD in the fibrin plate assay was not associated with local insufficiency of plasminogen substrate in these fluids. Increased levels of plasminogen were detectable in BAL of baboons with DAD or pneumonitis compared to baseline BAL (P = 0.04) (Fig. 8). Injury with 100% O<sub>2</sub> led to a progressive increase in plasminogen to very high levels over the course of 9 d (Fig. 8). Ventilated 21% O<sub>2</sub> controls showed a modest increase with time, but the levels were well below those measured in baboons with DAD.

We next investigated whether the plasminogen had been cleaved within the BAL. Plasminogen was isolated from BAL using lysine-agarose absorption and the plasminogen-related proteins separated by SDS-PAGE, transferred to nitrocellulose, and localized by immunoblotting (Fig. 9). The major plasminogen species in control or DAD BAL was the Glu-/plasminogen with an *M<sub>r</sub>* 90,000. Activation of the native pro-

Table IV. Neutralization of Procoagulant Activity of Baboon BAL by Antibodies to Factor VII and Tissue Factor

Baboon BAL No.	+Buffer	+IgG	+VII IgG	%INH	+Buffer	+IgG	+TFIgG	%INH
3933	0.163	0.127	0	100.0	0.109	0.091	0.036	60.4
4055	0.309	0.291	0	100.0	0.055	0.073	0	100.0
4353	0.055	0.055	0	100.0	0.055	0.055	0	100.0
	$\bar{x}$ %INH			100.0				86.8

Baboon BAL was incubated with buffer (0.15 M NaCl, 0.02 M Tris HCl, pH 7.4), the IgG fraction of rabbit nonimmune immunoglobulin (IgG) or the IgG fraction of antibodies to tissue factor (TFIgG) or Factor VII (VII IgG) as described in Methods. Aliquots of the reaction mixture were studied in the Factor Xa amidolytic assay, and the data are expressed in pmol/min per ml of Factor Xa activity based upon Factor Xa standards. %INH, % inhibition of nonimmune IgG. The concentration of TFIgG and its nonimmune control was 100  $\mu$ g/ml; that of VII IgG and its nonimmune control was 50  $\mu$ g/ml. %INH,  $1 - (\text{activity in the presence of specific antibody} / \text{activity in the presence of nonimmune IgG}) \times 100$ .  $\bar{x}$ , mean value. Tissue factor antibody neutralization experiments of all samples and VII antibody neutralizations of sample 4253 were done using a different batch of Factor X in separate experiments. The BAL samples selected for these analyses were randomly chosen from a large group of samples harvested mainly during evolving DAD and were found to have readily detectable Factor X activating activity in initial screening assays done to establish trends in serial BAL.

tein to Lys-77-plasminogen ( $M_r$  81,000) and the A chain of plasmin (Lys-77-Arg 560; 56,000  $M_r$ ) were noted in DAD samples.

Because the depressed fibrinolytic activity present in DAD BAL was associated with elevated BAL total protein, we hy-

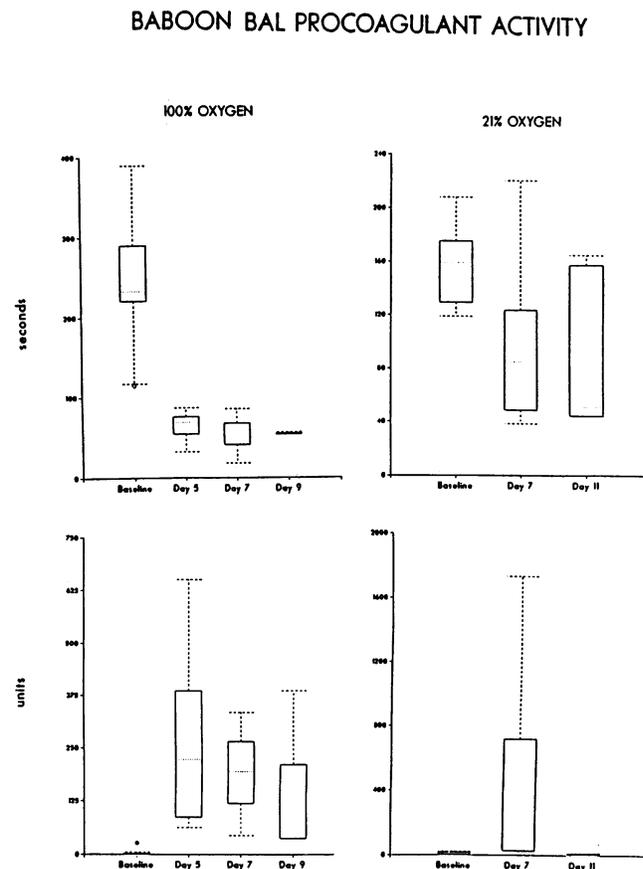
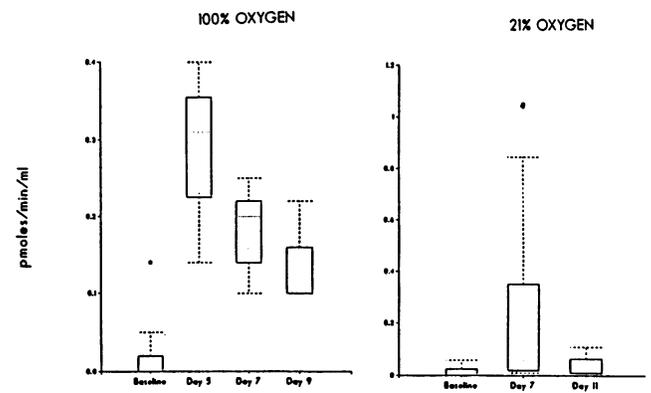


Figure 3. Procoagulant activity in serial BAL samples of baboons treated with 100% oxygen and 21% oxygen. Data are presented as recalcification times in seconds (top row) and in procoagulant units relative to a thromboplastin standard curve (bottom row) in the box plot format described in Fig. 1. Confidence limits are described in the text.

BABOON BAL FACTOR X ACTIVATING ACTIVITY



BABOON BAL EXTRINSIC PATHWAY INHIBITOR

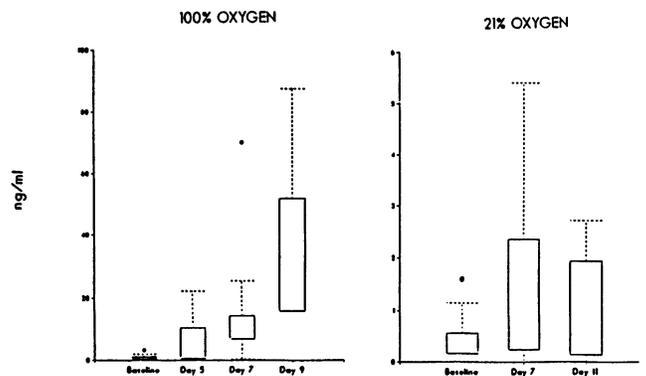
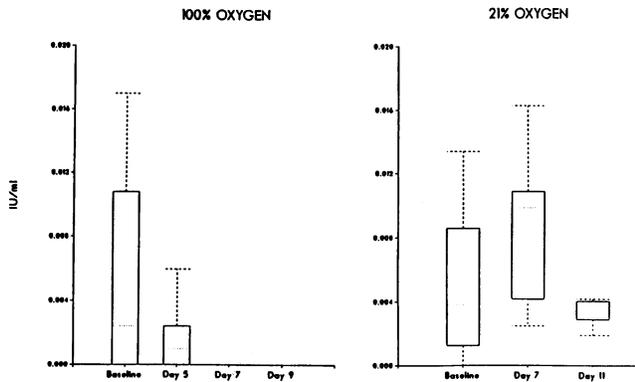


Figure 4. Factor X activating activity and presence of the extrinsic pathway inhibitor in serial BAL samples of baboons treated with 100% oxygen or 21% oxygen. Data are presented as Factor Xa amidolytic activity (pmol/min per ml) in the top row and concentration of the extrinsic pathway inhibitor (ng/ml) relative to a normal human plasma standard (bottom row) in the box plot format described in Fig. 1. Confidence limits are described in the text.

## BABOON BAL FIBRINOLYTIC ACTIVITY



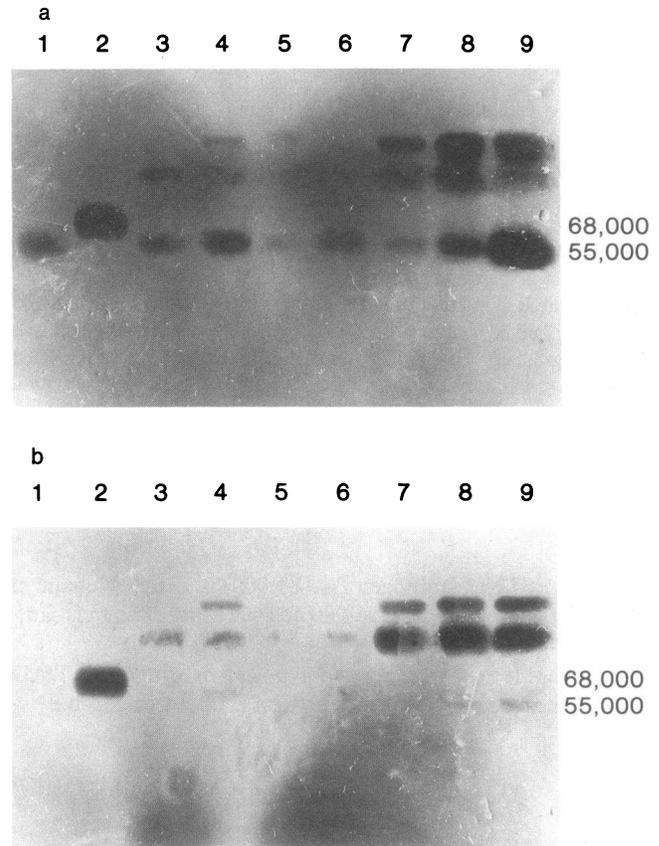
**Figure 5.** Fibrinolytic activity in serial BAL samples of baboons treated with 100% oxygen or 21% oxygen. Data are presented in IU/ml relative to urokinase standards. Median values were 0 and the data range approached 0 on days 7 and 9 of 100% oxygen treatment. The box plot format is described in Fig. 1. Confidence limits are described in the text.

pothesized that baseline fibrinolytic activity was inhibited by inhibitor(s) derived from plasma that had leaked into the alveolar space. The presence of inhibitors of fibrinolysis was initially suggested on the original  $^{125}\text{I}$ -fibrin plates. To determine whether plasmin inhibition was responsible for the depressed BAL fibrinolytic activity after lung injury, we incubated these BAL samples with purified plasmin at a concentration predetermined to equal or to exceed the highest levels generated by the PA found in control baboon BAL. As shown in Table VI, fibrinolytic activity of the plasmin standards at 2.5 or 5  $\mu\text{g}/\text{ml}$  was completely inhibited by the addition of BAL lacking fibrinolytic activity from baboons with DAD, indicating the presence of an active inhibitor of plasmin. Plasmin inhibitory activity was also found to increase, by amido-

**Table V. Neutralization of Fibrinolytic Activity in Baboon BAL**

Baboon BAL No.	+NSS	+IgG	+uPAIgG	%INH	+tPAIgG	%INH
4055	0.0941	0.0928	0	100	0.0955	0
4771	0.0083	0.0082	0	100	0.0076	7
4189	0.0958	0.1000	0	100	0.1000	0
$\bar{x}$ %INH				100		2.3

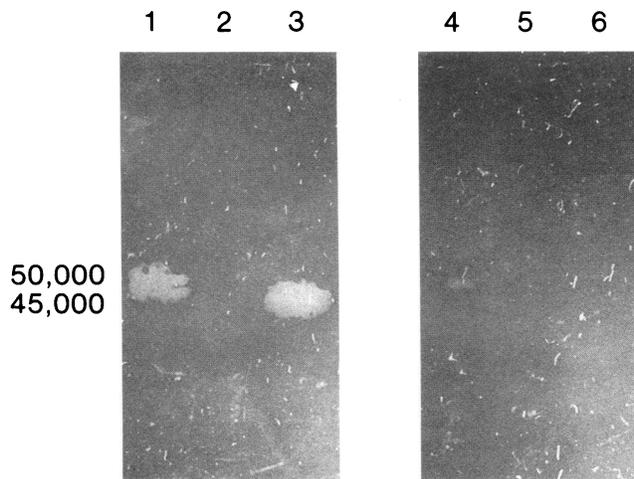
BAL samples were added 1:1 with normal saline solution (NSS), the IgG fraction of goat nonimmune IgG, or the IgG fraction of antibodies to urokinase (uPAIgG) or tissue plasminogen activator (tPAIgG) as described in Methods and the average of duplicate assays of fibrinolytic activities was determined in the  $^{125}\text{I}$  fibrin plate assay. The final concentration of all antibodies used was 160  $\mu\text{g}/\text{ml}$ . The fibrinolytic activities shown are in IU/ml based upon urokinase standards. %INH, inhibition compared to IgG control.  $\bar{x}$ , mean value. The BAL samples selected for these analyses were chosen from a large group of those harvested mainly before lung injury, which were found to contain readily detectable fibrinolytic activity.



**Figure 6.** Fibrin plate enzymography of baboon plasminogen activator activity in BAL. Samples (30  $\mu\text{l}$ ) were electrophoresed on SDS-10% polyacrylamide gels under nonreduced conditions and fibrinolytic activity localized using a plasminogen and fibrin containing agarose gel. (a) The fibrin enzymography plate was impregnated with 100  $\mu\text{g}/\text{ml}$  nonimmune goat IgG. Lane 1, 0.5 IU/ml urokinase standard. Lane 2, 0.130 U/ml tPA standard. Lanes 3-6 contain neat baseline or control BAL. Lanes 7-9, BAL from baboons with evolving DAD. (b) The same samples were run in an enzymography gel impregnated with 100  $\mu\text{g}/\text{ml}$  of the IgG fraction of goat antibody to human urokinase.

lytic assay, in serial BAL during the evolution of DAD (data not shown). Baseline BAL contained little antiplasmin activity; mean fibrinolytic activity generated by 2.5  $\mu\text{g}/\text{ml}$  plasmin mixed with baseline baboon BAL = 0.2864 IU/ml ( $n = 4$  samples) vs 0.3046 IU/ml generated by the plasmin standard mixed with normal saline in the fibrin plate assay. Whereas plasmin inhibitory activity was observed upon injury, little antiplasmin activity was found in baseline BAL or that of control 21%  $\text{O}_2$  baboons by either fibrinolytic or amidolytic assays.

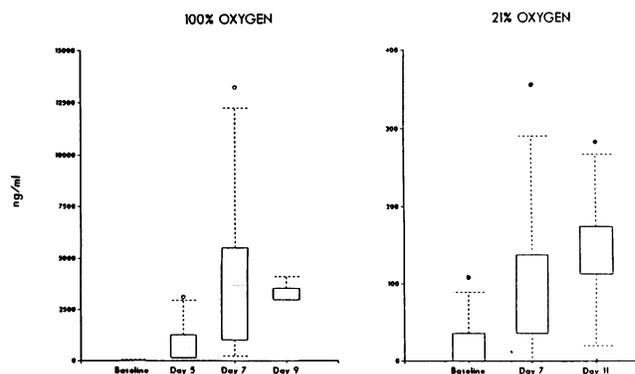
In order to determine whether  $\alpha_2\text{AP}$  contributed to the depressed fibrinolytic activity in BAL of baboons with evolving DAD, we adsorbed these BAL samples with goat antibody to  $\alpha_2\text{AP}$  or control goat IgG. The adsorbed samples were then tested for their ability to neutralize a 1.25- $\mu\text{g}$  plasmin standard in the fibrin plate assay (Table VII). The samples ( $n = 5$ ) chosen for these experiments contained no detectable fibrinolytic activity and were obtained from baboons with acute lung injury and evolving DAD. As shown in Table VII, inhibition of plasmin-mediated fibrinolytic activity could be partially re-



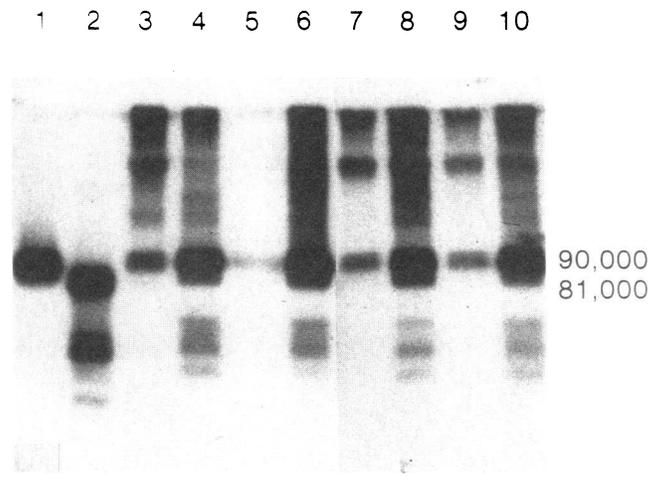
**Figure 7.** Presence of plasminogen activator inhibitor by reverse autoradiography of baboon BAL. Samples (30  $\mu$ l) were electrophoresed on an SDS-10% polyacrylamide gel under nonreducing conditions. PAI was localized using an agarose gel containing fibrin, plasminogen, and 0.075 IU/ml of urokinase. Lanes 1 and 4, PAI secreted from Hep G2 cells; lanes 2 and 5, baseline BAL; lanes 3 and 6, BAL from animal with evolving DAD. The fibrin gel overlay for the samples in lanes 1–3 was impregnated with nonimmune rabbit IgG at a final concentration of 20  $\mu$ g/ml. The overlay for lanes 4–6 contained the IgG fraction of rabbit antibody to PAI-1, 20  $\mu$ g/ml.

versed in undiluted samples by adsorption with antibody to  $\alpha_2$ AP. Complete or nearly complete neutralization of BAL-mediate inhibition of plasmin activity could be achieved with dilution of BAL in two samples. This effect corresponded to the measured concentration of  $\alpha_2$ AP antigen in BAL samples and was about one-fourth that determined in the other samples. Baseline BAL contained little or no detectable antiplasmin activity and the  $\alpha_2$ AP antigen was below the detection limits of our radioimmunoassay (30 ng/ml). Thus, BAL sam-

### BABOON BAL PLASMINOGEN



**Figure 8.** Plasminogen in serial BAL samples of baboons treated with 100% oxygen or 21% oxygen. The concentration of plasminogen ( $\mu$ g/ml) was determined by radioimmunoassay. The data are presented as box plots which are described in Fig. 1. Confidence limits are described in the text.



**Figure 9.** Immunoblot for plasminogen present in baboon BAL. Samples were absorbed with lysine-agarose beads, and the bound material separated on SDS-7% polyacrylamide gels under reducing conditions. After transfer to nitrocellulose, the plasminogen derivatives were localized using rabbit antiplasminogen,  $^{125}$ I-goat anti-rabbit IgG and autoradiography. Lane 1, 100 ng of Glu-plasminogen standard; lane 2, 100 ng of a mixture of Lys-plasminogen and plasmin; lanes 3, 5, 7, and 9, plasminogen species present in neat baseline BAL before induction of DAD; and lanes 4, 6, 8, and 10, plasminogen derivatives absorbed from neat BAL of the same baboons with evolving DAD.

ples obtained during development of DAD, which lacked fibrinolytic activity, contained significant amounts of antiplasmin activity.

To examine the specificity of the abnormalities of fibrin turnover we detected in BAL of baboons with DAD, we measured the procoagulant and fibrinolytic activities present in human pleural fluids which served as non-BAL controls. All pleural fluids from patients with CHF were transudative, whereas those from patients with pneumonia, empyema or carcinoma of the lung fulfilled criteria for exudates (36). Proportionately larger numbers of neutrophils were contained in

**Table VI.** Antiplasmin Activity of Baboon BAL

Plasmin concentration	Sample	IU/ml	% Inhibition
5.0 $\mu$ g/ml	NSS	1.829	—
	4054 7-22	0	100
	4055 7-23	0	100
	4253 4-16	0	100
	4153 4-18	.007	99
2.5 $\mu$ g/ml	NSS	.740	—
	4054 7-22	0	100
	4055 7-23	0	100
	4253 4-16	0	100
	4153 4-18	.007	99

% Inhibition = Units NSS – Units BAL/Units NSS  $\times$  100. All samples were run in duplicate in the fibrin plate assay described in Methods. NSS, normal saline. BAL samples used in these experiments were all from baboons with DAD. Samples used in these experiments were randomly selected from the pool of BAL harvested after lung injury that were found to contain depressed fibrinolytic activity.

Table VII. Absorption of Antiplasmin Activity in Baboon BAL by Antibody to  $\alpha_2$  Antiplasmin

Sample	Residual fibrinolytic activity		$\alpha_2$ AP
	+Control IgG	+ $\alpha_2$ AP IgG	
	IU/ml		
4054 Neat	0	0	670
(7-22) 1:2	0	0	
1:4	0	0	
1:8	0	0	
4253 Neat	0	0	112
(4-16) 1:2	0	0.0497	
1:4	0.0563	0.0978	
1:8	0.1405	0.1363	
4253 Neat	0	0	140
(4-18) 1:2	0.0080	0.0152	
1:4	0.0252	0.0706	
1:8	0.1043	0.1461	
4055 Neat	0	0	590
(7-23)			
4060 Neat	0.0265	0.0496	716
(8-5)			

Absorption experiments were performed as described in Methods. Control IgG, goat nonimmune IgG,  $\alpha_2$ AP IgG, goat antihuman  $\alpha_2$ AP IgG. Fibrinolytic activities of serial dilutions of BAL samples in the presence of the plasmin standard (1.25  $\mu$ g/ml) were determined by  $^{125}$ I-fibrin plate assay. All BAL samples used in these experiments were from baboons with 100% O<sub>2</sub> exposure. Concentrations of  $\alpha_2$ AP were determined by radioimmunoassay.

exudative fluids based on total white cell and differential counts (Table VIII). Exudative pleural effusions rarely (4 of 23 exudates) contained detectable plasminogen-dependent fibrinolytic activity in the  $^{125}$ I-fibrin plate assay, whereas this fibri-

nolytic activity was detectable in five of the six pleural fluid samples from patients with CHF. Shorter recalcification times, indicating increased procoagulant activity, were detectable in pleural fluids of patients with pneumonia or lung cancer compared to patients with congestive heart failure which did not differ from the recalcification times in normal pooled plasma of pleural fluids from patients with empyema. These data indicate that similar trends of both procoagulant and fibrinolytic activities occur in exudative pleural fluids and DAD BAL. Increased procoagulant activity in the setting of high-grade pleural inflammation (empyema) does not invariably occur (Table VIII), whereas it is consistently observed in BAL of all baboons during evolving DAD (Fig. 3).

## Discussion

Tissue fibrin deposition represents the net effect of procoagulant mechanisms which favor fibrin formation and fibrinolysis which favors its dissolution. The balance between these processes may therefore be inferred to control fibrin deposition in the alveolar space, such as occurs in the setting of acute lung injury. Extravascular fibrin deposition, chiefly within the alveoli characterizes the exudative phase of DAD in patients with the adult respiratory distress syndrome (ARDS) (1), and may occur in association with the high grade alveolitis associated with the interstitial lung diseases (2). We chose to study the baboon model, because extensive alveolar fibrin formation uniformly accompanies the lesion of DAD in baboons with induced ARDS (5), because of phylogenetic proximity of the model to humans and because of availability of immunologic reagents which cross-react with baboon proteins. Animals were studied under conditions approximating those currently used in the care of patients with ARDS including invasive instrumentation and mechanical ventilation. We studied a model of DAD, induced by protracted exposure to hyperoxia, which is germane to clinical circumstances associated with ARDS in humans (37). Superinfection, as observed in this

Table VIII. Procoagulant and Fibrinolytic Activities in Neutrophil-containing Pleural Fluids

Group	Total white cells	PMN	LDH	Total protein	Recalcification time in NPP	Fibrinolytic activity
	per mm <sup>3</sup>	%	IU/ml	mg/ml	s	IU/ml
Cancer (n = 16)	1,675	22.0	225	4.15	110.0 (48.5-245.0) (P = 0.005)*	0
Empyema (n = 3)	8,800	100.0	2,920	4.00	178.2 (63.5, 178.2, 431.4) (P = 0.439)*	0
Pneumonia (n = 4)	1,300	14.0	376	4.40	118.2 (81.1-206.2) (P = 0.055)*	0
CHF (n = 6)	288	11.5	65	1.85	205.3 (179.0-285.5)	0.0495 (0-0.4)

Median values are shown with the range of minimum and maximum values for procoagulant activities and fibrinolytic activity of the CHF group. Procoagulant activity, recalcification time of pleural fluid in NPP. Plasminogen-dependent fibrinolytic activity was measured in the  $^{125}$ I-fibrin plate assay. CHF, congestive heart failure; cancer, lung cancer; pneumonia, parapneumonic effusion. PMN, polymorphonuclear granulocytes; LDH, lactic dehydrogenase. Confidence limits of procoagulant activities vs. CHF are shown.

study, commonly accompanies the underlying derangements associated with ARDS in humans. Further, because trended BAL data are difficult to obtain in patients with ARDS, this model provided a unique opportunity to examine evolving abnormalities of fibrin turnover in primates with comparable lung injury.

Lung injury induced by 100% O<sub>2</sub> in baboons was associated with an increase in BAL procoagulant activity. Recalcification times in deficient plasmas, ultracentrifugation, and antibody neutralization studies indicated that the procoagulant activity was attributable to the expression of the extrinsic activation complex; that is, tissue factor associated with Factor VII. Of interest was the apparent inverse relationship between the level of EPI and Factor Xa generation after oxygen injury. With the 100% oxygen-induced injury, there was a gradual increase in EPI with a concomitant decrease in Factor Xa production. Because the lung is relatively rich in the components of the extrinsic pathway; tissue factor and Factor VII (38), the potential for the EPI to control initiation of coagulation may be diminished, which results in a net expression of procoagulant activity. The increase in lower respiratory tract procoagulant activity in baboons with DAD is similar to that which we and others have previously described in the BAL of human subjects and marmosets (3, 8, 38, 39). The observation of modestly augmented procoagulant activity in BAL of some of the baboons mechanically ventilated with 21% oxygen is not surprising, in as much as these animals often developed superinfection with histologic evidence of purulent pneumonitis.

Fibrinolytic activity present in baboon BAL was found to be immunologically cross-reactive with antibodies to human urokinase. It has previously been reported that urokinase-like fibrinolytic activity is readily detectable in BAL of normal human subjects (39). We found that a similar, if not identical, PA was usually present in baboon BAL under normal circumstances. In the absence of clinically apparent lung disease or superimposed pneumonitis, BAL contained relatively large amounts of fibrinolytic activity and at that same time had relatively small amounts of procoagulant activity. The net effect of these combined processes would be to favor fibrin dissolution. This baseline procoagulant-fibrinolytic balance in alveolar lining fluids probably explains the absence of fibrin within the alveoli under normal circumstances. Previously, it has been found that fibrinogen is not a resident protein of the alveolar lining fluids and that fibrin is not normally found at extravascular or intravascular sites within the normal lung (1, 40). Our morphometric and histologic findings support these observations as well as the notion that the local balance between procoagulant activity and fibrinolysis in alveolar fluids is a crucial determinant of alveolar fibrin deposition within the lung.

Following the induction of DAD, abnormalities of the local procoagulant-fibrinolytic balance were apparent. The pattern observed in BAL was that of augmented procoagulant activity in association with depressed fibrinolytic activity. These disturbances would be expected to additively favor tissue fibrin deposition. We found that this local disruption of the procoagulant-fibrinolytic balance was temporally associated with alveolar fibrin formation both histologically and morphometrically in baboons with DAD. These observations are unlikely to be solely attributable to the contamination of DAD BAL by plasma-derived or leukocyte proteases as: (a) the addition of baboon plasma, and to a lesser extent, lysed or live

neutrophils could shorten the recalcification times of BAL in plasma but did not generate increased Factor X-activating activity and (b) the addition of lysed or live neutrophils, in quantities comparable to those originally harvested, failed to generate increased fibrinolytic activity in DAD BAL. Similar trends of procoagulant and fibrinolytic activities occurred in association with exudative pleural disease, indicating that locally disordered fibrin turnover is not specific to the alveolar space in DAD but may also accompany exudative processes in the pleural compartment. The lack of consistently increased procoagulant activity in empyema fluids is consistent with previous reports (41) and indicates that high-grade inflammation in the pleural space is not invariably associated with increased local procoagulant activity, such as consistently occurs in BAL during evolving DAD.

In addition to PA, low levels of substrate plasminogen are present in the native form in the normal lung. It is probable that PAI keeps the system regulated under normal homeostasis. Upon injury, plasminogen is increased, but we paradoxically measured a decrease in fibrinolysis. Depressed fibrinolytic activity present in BAL obtained from animals with evolving DAD was contingent upon antiplasmin activity in these BAL samples, which was potent enough to inhibit expression of levels of fibrinolytic activity generated by levels of PA present in control BAL. Baseline baboon BAL samples or those of control baboons ventilated with 21% O<sub>2</sub> contained little or no antiplasmin activity, while BAL samples from baboons with evolving DAD contained large amounts of antiplasmin activity. These observations suggest that inhibition of plasmin by constituent(s) of BAL from baboons with evolving DAD is in large part responsible for the depressed fibrinolytic activity found in these samples. Inhibition of plasmin was, in part, due to  $\alpha_2$  antiplasmin, which likely entered the alveolar space as a result of microvascular injury, but could be locally elaborated as well. This observation does not preclude the possibility that other inhibitors, notably PAI-1 or PAI-2, could also depress fibrinolytic activity in the alveolar lining fluids in the setting of lung injury. Indeed, we found PAI activity, due to PAI-1, in BAL of baboons after, but not before induction of DAD, suggesting that a dual mechanism of inhibition (one for PA and one for plasmin) may account for the local depression of fibrinolytic activity in the injured primate lung.

The virtually universal occurrence of alveolar fibrin in the inflamed alveolar compartment suggests the possibility that tissue fibrin deposition and the procoagulant-fibrinolytic balance in alveolar fluids may be of importance in the pathogenesis of acute lung injury and repair. In a process analogous to that which occurs during normal wound healing (42), it is likely that intraalveolar fibrin provides an abnormal matrix for the subsequent deposition of fibrous tissue. It has previously been shown that fibrin deposits occur at sites of pulmonary scar formation (43, 44). In addition, fibrin and fibronectin form a suitable matrix for attraction of fibroblasts with resultant secretion of collagen *in vitro* (14). The relationship between the observed local abnormalities of pathways of fibrin turnover and fibrin deposition in the pathogenesis of DAD and accelerated pulmonary fibrosis remains to be established and is contingent upon the results of specific interventions that are designed to interrupt these pathways and arrest alveolar fibrin deposition in DAD. Our data provide the rationale for such interventions and suggest the feasibility of strategies which target abnormalities of either coagulation or fibrinolytic

pathways in DAD. We conclude that locally disordered fibrin turnover, based upon multiple, concurrent, prothrombotic abnormalities of pathways of both coagulation and fibrinolysis, accompanies increased alveolar fibrin deposition in the lungs of baboons with DAD.

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