Protein Permeability in the Adult Respiratory Distress Syndrome

Loss of Size Selectivity of the Alveolar Epithelium

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Abstract

Small amounts of plasma protein normally reach the alveolar epithelial surface by a size-selective process that restricts the passage of very large molecules. Size selectivity may be compromised in the lungs of patients with the adult respiratory distress syndrome (ARDS). To assess this question, bronchoalveolar lavage fluid (BALF) from normal volunteers ($n = 11$), cardiac edema patients ($n = 3$), and ARDS patients ($n = 8$) was compared. Mean total protein in ARDS BALF was >12 times the levels in normals or cardiac edema patients. BALF/plasma total protein ratios and measurements of epithelial lining fluid protein also separated the patient groups. The large proteins IgM and $\alpha2$-macroglobulin were found in ARDS BALF at >90 times the concentrations of normal or cardiac edema fluid. The relationship of distribution coefficient vs. log molecular weight for seven proteins (54,000–900,000 mol wt) hyperbolically increased in normals but was flat in ARDS patients. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a paucity of high molecular weight proteins in normal and cardiac edema BALF, but demonstrated the full spectrum of plasma proteins in ARDS BALF. We conclude that normal size selectivity is preserved in cardiac edema but is destroyed by the alveolar–capillary injury of ARDS.

Introduction

Alterations in water and solute flux across the endothelial membrane in the pulmonary edema states have been extensively studied in sheep and dog chronic lung lymph fistula models (1–5). Studies of lymph flow rates, protein composition, and lymph/plasma protein concentration ratios have shed considerable light on the comparative pathophysiology of permeability pulmonary edema and hydrostatic pulmonary edema, whose respective clinical analogues are the adult respiratory distress syndrome (ARDS)[1] and left heart failure. Similar studies of transendothelial

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1. *Abbreviations used in this paper:* ARDS, adult respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; CBB, Coomassie Brilliant Blue; DC, distribution coefficients; ELF, epithelial lining fluid; FIO$_2$, inspired oxygen concentration; ICU, intensive care unit; PAGE, polyacrylamide gel electrophoresis; SAB, sample application buffer.

water and protein flux in human pulmonary edema, however, are hindered by the inaccessibility of pulmonary lymph.

In contrast, water and solutes that reach the alveolar epithelial surface can be recovered in humans by aspiration of tracheobronchial edema fluid (6–11) or by bronchoalveolar lavage (12–16). In the evaluation of human pulmonary edema, Anderson et al. (10) have demonstrated increased clearance of ^{131}I-labeled human albumin from plasma to tracheobronchial fluid in patients with ARDS as compared with patients with cardiac pulmonary edema. Other studies have differentiated these groups of patients using tracheobronchial aspirate/plasma total protein ratios (6–9). However, these techniques may not be applicable to all pulmonary edema patients, since they require airway flooding with a sufficient quantity of recoverable alveolar fluid that is uncontaminated by central airway secretions. This limitation might be overcome by the use of segmental bronchoalveolar lavage, a technique that more directly samples alveolar epithelial lining fluid (ELF) (17).

Although increased protein permeability is well documented in ARDS, little information is available on the distribution of variously sized plasma proteins between blood and ELF in this disorder. ELF obtained from normal subjects contains a spectrum of soluble proteins derived from plasma (12–17). Although many of these proteins are quantitatively proportioned as in plasma, very large molecules such as IgM and $\alpha2$-macroglobulin are much more sparsely distributed to the alveolar surface (15). In contrast to the normal lung, increased protein permeability in ARDS may be associated with loss of this size restriction so that all plasma proteins are distributed equally between plasma and ELF. In this context, Fowler et al. (18) have demonstrated the presence of fibrinogen and other high molecular weight proteins in ARDS lavage fluid.

In the present study, both groups of pulmonary edema patients and normal volunteers were evaluated by bronchoalveolar lavage. The protein composition of recovered lavage fluid was analyzed by a variety of techniques to answer three questions. First, how do bronchoalveolar lavage fluid (BALF) total protein concentrations and BALF/plasma protein ratios differ in ARDS patients, cardiac pulmonary edema patients, and normal volunteers? Second, how do the pulmonary edema states differ in their distribution of variously sized proteins between plasma and ELF? Third, do BALF protein measurements differentiate human pulmonary edema states more reliably than similar measurements of tracheobronchial secretions?

Methods

Patient groups. The ARDS group ($n = 8$) consisted of Medical Intensive Care Unit (ICU) patients with noncardiogenic pulmonary edema who were hospitalized between October 1982 and March 1984. All had hypoxemic respiratory failure which required mechanical ventilation with positive end expiratory pressure. Diagnostic criteria for ARDS (19) included the following: (*a*) clinical history of a catastrophic event known to predispose to ARDS, excluding exacerbations of chronic pulmonary

disease or left ventricular failure; (b) clinical respiratory distress (tachypnea > 20 breaths/min, labored breathing); (c) chest roentgenogram demonstrating diffuse pulmonary infiltrates; (d) physiologic parameters of intrapulmonary shunting ($PO_2 < 50$ mmHg, when inspired oxygen concentration (FIO_2) > 0.6), and normal pulmonary capillary wedge pressure (<15 mmHg). In addition, no patient had bronchoscopic or clinical evidence of airway or parenchymal pyogenic infection. Patients with ARDS due to inhalation of caustic agents (e.g., gastric acid aspiration, noxious fume inhalation) were excluded from the study.

ICU patients with acute cardiogenic pulmonary edema requiring mechanical ventilation comprised one control group ($n = 3$), hereafter referred to as the cardiac edema group. Diagnostic criteria included: (a) absence of predisposing catastrophic events that might lead to noncardiogenic pulmonary edema; (b) chest roentgenogram demonstrating cardiomegaly and signs of hydrostatic pulmonary edema; (c) right heart catheterization data demonstrating elevated pulmonary capillary wedge pressure (>24 mmHg); and (d) no evidence of airways or lung parenchymal pyogenic infection. A second control group consisted of normal volunteer subjects ($n = 11$) who had no history of pulmonary disease or cigarette smoking. Written informed consent was obtained from all subjects according to a protocol approved by the Ohio State University Human Subjects Review Committee.

Study design. Patients with pulmonary edema generally underwent bronchoalveolar lavage within 24 h of intubation. Early study was deemed necessary to minimize potentially confounding factors associated with the ICU setting. These include: (a) airway inflammatory response to bacterial colonization and/or endotracheal trauma in the intubated patient, and (b) the effects of therapeutic interventions such as oxygen toxicity, mechanical ventilation, diuretic and fluid management, and inotropic agents. Bronchoalveolar lavage was repeated at 72-h intervals in those patients who continued to require mechanical ventilation.

Bronchoalveolar lavage. Bronchoalveolar lavage was performed as previously described (14). After topical lidocaine anesthesia, a 4.8-mm diam flexible fiberoptic bronchoscope (model BF-B2, Olympus Corp. of America, New Hyde Park, NY) was passed through the endotracheal tube of pulmonary edema patients (transnasally in normal volunteers) and wedged into a right middle lobe or lingular subsegment. Five successive 20-ml aliquots of 0.9% saline were instilled and immediately aspirated under low suction.

Lavage fluid processing. BALF samples were strained through monolayers of coarse-mesh surgical gauze and centrifuged at 500 g for 15 min. 1.0 M Tris-HCl (pH 7.4) was added to the decanted supernate to achieve a final concentration of 0.05 M Tris. After passage through a 0.45-μm filter, the cell-free lavage supernates were concentrated by pressure dialysis to 2–3 ml over a YM5 membrane (Amicon Corp., Lexington, MA) and stored at −80°C. The cell pellet was resuspended and washed twice in Hanks' balanced salt solution without calcium or magnesium. A small aliquot was then cytocentrifuged at 35 g for 10 min, air dried, and stained by a modified Wright-Giemsa stain. A differential count was performed on a minimum of 200 cells.

Total protein assay. Accurate determinations of total protein in serum and BALF were necessary for calculation of distribution coefficients (DC) and for gel electrophoresis sample loading. Although the G250 Coomassie Brilliant Blue (CBB) spectrophotometric dye-binding assay of Bradford (20) offers rapid, reliable quantitation of single-purified proteins or of fixed proportion protein mixtures (21), accuracy is impaired when protein mixtures of variable proportion must be compared. This occurs because equal concentrations of different serum proteins in CBB solution vary markedly in their optical densities (21, 22). These protein-specific sensitivity differences are substantially diminished when sodium dodecyl sulfate (SDS) is added to the reaction medium. The resulting absolute values strongly correlate with the biuret method, for which differential sensitivity among proteins is minimal (22). Thus, the Bio-Rad protein microassay procedure (after Bradford) was modified as follows: (a) 2 vol of dye reagent concentrate (Bio-Rad Laboratories, Richmond, CA) (a mixture of CBB G250, methanol, and phosphoric acid) were gently mixed with 1 vol of 0.024% SDS. (b) 0.3 ml of this mixture was added to 0.7 ml of sample (plasma or concentrated BALF), and the solution was

gently mixed by inversion. (c) After incubation at 22°C for 15 min, absorbance readings were made at 595 nm on a Beckman DU-8 spectrophotometer. Corresponding protein concentrations were determined from a regression line generated from seven different concentrations of a reference standard (mean correlation coefficient 0.9994±0.0001). The reference standard was prepared from deep-frozen aliquots of a 70-μg/ml mixture of 65% bovine serum albumin and 35% gamma globulin (Bio-Rad Laboratories). Each sample was assayed in triplicate at different dilutions and the resultant standard deviation was <5% of the mean for each trio.

BALF total protein concentration refers to micrograms (μg) of protein contained in 1 ml of Tris-buffered cell-free lavage fluid before its concentration over the YM5 membrane (Amicon Corp.). A representative calculation (ARDS patient 5) follows:

total volume of unconcentrated BALF = 67 ml;
total volume of YM5 concentrate = 2.5 ml;
total protein concentration of YM5 fluid = 24,040 μg/ml. Therefore,

$$\text{BALF total protein} = \frac{(24,040 \ \mu g/ml)(2.5 \ ml)}{67 \ ml} = 897 \ \mu g/ml. \text{ Direct assay}$$

of unconcentrated BALF strongly correlated with the above calculations; i.e., protein loss on the YM5 membrane was <5%.

Measurement of individual proteins. Concentrations of plasma and BALF protein components were determined by radial immunodiffusion (Behring Diagnostics, Sommerville, NJ). The proteins, their molecular weights, and their assay ranges included: α-1 antitrypsin (54,000 mol wt), 6–85 mg/dl; albumin (68,000 mol wt), 42–585 mg/dl; transferrin (90,000 mol wt), 15–891 mg/dl; haptoglobin (100,000 mol wt), 20–290 mg/dl; ceruloplasmin (150,000 mol wt), 3.5–50 mg/dl; α2-macroglobulin (820,000 mol wt), 25–350 mg/dl; and IgM (900,000 mol wt), 1.1–18 mg/dl. IgM levels were also quantitated by immunonephelometry when there was adequate sample (lower limit, 1.1 mg/dl). Assays were performed using plasma or concentrated BALF samples, and concentrations of the individual proteins were expressed as micrograms per milliliter of plasma or unconcentrated BALF.

In some instances, even YM5-concentrated BALF was too dilute to be quantitated by radial immunodiffusion. After 12-h dialysis against distilled water, these samples were further concentrated by lyophilization and then reconstituted in 20–45 μl of Tris-saline buffer, depending on the number of assays required. To control for nonspecific protein losses during this secondary processing, a 5-μl aliquot of the lyophilized preparation was always reserved for repeat triplicate total protein assay. Repeat protein assay of these samples ensured accurate concentration ratios of component protein to total protein, which were necessary for determination of DC.

ELF measurements. Measurements of ELF provided information about the dilutional effects of bronchoalveolar lavage. For the measurement of ELF volumes and proteins, additional ARDS patients ($n = 5$) and normal volunteers ($n = 10$) were evaluated by an identical bronchoalveolar lavage procedure. Measurement of ELF was accomplished by the urea dilution method described by Rennard et al. (17). This method assumes that the small molecule urea is freely diffusible between plasma and ELF. Therefore, urea measurements in simultaneous plasma and BALF samples allow for calculation of ELF volume (milliliters) and expression as a percentage of BALF (ml ELF/100 ml BALF). ELF concentrations of total protein and albumin were calculated for each patient group.

Estimation of alveolar–capillary membrane protein permeability. BALF total protein concentration was used as an index of alveolar-capillary membrane permeability (23). A concentration ratio of BALF total protein to plasma total protein (BALF/plasma) was calculated for each patient and expressed as the ratio × 100. In additional patients, protein permeability was estimated by ELF total protein concentration and ELF/plasma total protein ratios.

Permeability of proteins of various molecular weights. The presence of variously sized plasma proteins in the alveolar spaces was demonstrated by quantitative and qualitative methods. The quantitative methods included intergroup comparison of BALF concentrations of the very high molecular weight proteins α2-macroglobulin and IgM, and also the cal-

culation of DC for individual proteins as described by Bell et al. (15), according to the following formula:

$$DC = \frac{X_{plasma}/TP_{plasma}}{X_{BALF}/TP_{BALF}},$$

where X = concentration of component protein in plasma or BALF, and TP = concentration of total protein in plasma or BALF. Using this formula, DC = 1.0 indicates that a protein constituent comprises an equal percentage of plasma and alveolar lining fluid total protein. Values of DC > 1.0 indicate increasing restriction of a particular protein from the alveolar lining fluid. Values of DC <1.0 indicate enhanced access of a particular protein to the alveolar lining fluid.

This quantitative data regarding the permeability of selected proteins was complemented by the qualitative molecular weight profiling of plasma and BALF by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE). BALF from the three experimental groups was compared side-by-side in slab gels. The particulars of gel preparation follow.

SDS-PAGE. Resolving gel solutions consisted of total acrylamide concentrations of 3.8–5.0% (2.6% of total acrylamide as bis), 0.375 M Tris-HCl (pH 8.9), 0.5 M urea, and 0.2% wt/vol SDS. Stacking gel solutions consisted of total acrylamide concentrations of 3.5–4.0% (2.6% of total acrylamide as bis), 0.125 M Tris-HCl (pH 6.8), 0.5 M urea, and 0.1% SDS. Polymerization was induced by tetramethylethylenediamine (TEMED) and ammonium persulfate.

Samples were prepared by quantitative alcohol precipitation followed by resuspension in 50 μl of sample application buffer (SAB). 1 vol of sample (adjusted to 425 μg protein/ml) was mixed with 2 vol of absolute ethanol, ice bathed for at least 2 h, centrifuged at 8,800 *g* for 20 min, and gently decanted. Protein precipitate was reconstituted in SAB composed of 0.1 M Tris-HCl (pH 6.8), 1.0 M urea, 2.0% SDS, 0.002% wt/vol bromphenol blue, and 20% glycerol. No reducing agent was included in the SAB.

Reconstituted samples were denatured in boiling water for 5 min, centrifuged, and applied to the gel. Reservoir buffer (pH 8.3) consisted of 0.192 M glycine, 0.025 M Tris-HCl, and 0.1% SDS. Electrophoresis was performed at 20 mA per gel, constant current, until the dye front reached the bottom of the gel (~6 h). The gel was then fixed and stained with the Silver Stain Kit (Bio-Rad Laboratories) and photographed.

Statistical methods. Data are expressed as the mean±SEM. The significance of intergroup differences was assessed by nonparametric analysis (Wilcoxon's (24) rank sum test for independent samples) because measured parameters could not be assumed to be normally distributed. The null hypothesis was rejected at the 0.05 level. Data cited from other publications mentioned in the Discussion section were similarly analyzed (Table IV).

Results

Table I displays the relevant physiologic data of the pulmonary edema patients at the time of the initial lavage, which was within 24 h of intubation for five of eight ARDS patients and for all three cardiac edema patients. As expected, ARDS patients had significantly lower values for pulmonary capillary wedge pressure ($P < 0.05$) than cardiac edema patients. ARDS patients also had lower values for static lung compliance ($P < 0.05$). The mean alveolar–arterial oxygen pressure difference was higher in ARDS patients but not significantly different from cardiac edema patients. Seven of eight ARDS patients survived, compared with one of three cardiac edema patients.

Bronchoalveolar lavage was well tolerated by all patient groups. 20 such procedures were performed on the eight ARDS patients. 8 of these represented the initial evaluation of each ARDS patient and the other 12 represented serial studies performed on six of the patients. Examination of the airways at bronchoscopy revealed no evidence of purulent secretions or blood. There were no significant differences in percent recovery

Table I. Physiologic Data of Pulmonary Edema Patients at Initial Bronchoalveolar Lavage

	Patient	Age	Diagnosis	Days*	AaPO₂‡	Cst§	PCWP‖	%PMN¶	Outcome**
ARDS (*n* = 8)	1	28	Histiocytic lymphoma, septic shock	<1	580	19	3	95	S
	2	19	Toxic shock syndrome	<1	514	22	5	77	S
	3	37	Urinary tract infection, sepsis, acute tubular necrosis	1	548	28	15	94	D
	4	64	Atypical pneumonia syndrome (unknown pathogen)	1	517	27	14	89	S
	5	32	Atypical pneumonia syndrome (unknown pathogen)	3	453	21	12	97	S
	6	63	Non-Hodgkin's lymphoma, septic shock	<1	545	26	5	96	S
	7	71	Ruptured pancreatic pseudocyst, sepsis	7	547	33	8	26	S
	8	37	Intrauterine device endometritis, septic shock	13	187	19	7	8	S
	Mean	43.8			486	24.4‡‡	8.6‡‡	72.8‡‡	
	SEM	6.8			45	1.8	1.6	12.5	
Cardiac edema (*n* = 3)	9	66	Cardiogenic shock, arrhythmias	<1	557	32	25	12	S
	10	67	Gastrointestinal bleed, acute tubular necrosis, 40-lb fluid overload	<1	384	31	24	3	D
	11	65	Cardiogenic shock	<1	107	39	30	1	D
	Mean	66.0			349	34.0	26.3	5.3	
	SEM	0.6			131	2.5	1.9	3.4	

* Days from intubation to first lavage. ‡ Alveolar–arterial oxygen tension difference (mmHg) calculated from the alveolar air equation after 20 min on 100% oxygen, zero positive end expiratory pressure, except patients 8 and 11 on 45 and 35% FIO₂ respectively. § Static compliance, milliliters per centimeter H₂O. ‖ Pulmonary capillary wedge pressure, millimeters of mercury. ¶ Percent neutrophils present in lavage cell differential. ** S, survived; D, died. ‡‡ Significantly different from hydrostatic edema patients, $P < 0.05$.

of BALF between any of the three patient groups. Of the 100 ml of saline instilled, recovery was 45.3±3.4% from ARDS patients, 36.7±2.3% from cardiac edema patients, and 48.4±3.2% from normal volunteers. Neutrophils comprised 72.8±12.5% of recovered lavage cells in ARDS patients compared with only 5.3±3.4% of recovered cells in cardiac edema patients ($P < 0.05$). The normal volunteer group had a mean age of 23.5±1.2 yr and a mean neutrophil percentage of 0.7±0.3%.

BALF total protein as an index of protein permeability. Consistent with the concept that ARDS is a form of high permeability pulmonary edema, ARDS BALF total protein concentrations were markedly increased compared with cardiac edema patients and normal volunteers (Fig. 1). Total protein concentration of ARDS BALF was 1,000±200 μg/ml when the initial BALF values were averaged for the eight patients. The BALF protein concentration was even higher (1,271±127 μg/ml) when the "late" lavages performed on days 7 and 13 (Fig. 1, open circles) were excluded. In contrast, both cardiac edema patients and normal subjects had BALF total protein concentrations that were substantially lower (<10%) than the ARDS group (cardiac edema group 59±11.0 μg/ml; normals 81±9.4 μg/ml; $P < 0.01$, both comparisons). The mean BALF total protein concentration in cardiac edema patients did not differ significantly from those in normal subjects.

BALF/plasma total protein ratio as an index of protein permeability. Simultaneous samples of BALF and plasma taken at the time of the initial lavage procedure were available from five of the ARDS patients. The mean BALF/plasma protein ratio (×100) for these initial lavages, all performed within 4 d of onset of ARDS, was 2.84±0.37 (range 1.89 to 4.02). In contrast, the BALF/plasma protein ratio averaged 0.11±0.02 (range 0.08 to 0.15) for cardiac edema patients and 0.11±0.01 (range 0.04 to 0.17) for normal subjects. Again, the mean BALF/plasma protein ratio in ARDS patients was significantly greater than that in cardiac edema patients ($P < 0.05$) and normal subjects ($P < 0.01$). The BALF/plasma ratios of cardiac edema patients and normal subjects were not significantly different.

ELF measurements. Intergroup differences in BALF total protein and BALF/plasma total protein ratios were confirmed by measurements of ELF volumes and ELF protein concentrations (Table II). Measurements of ELF theoretically negate differences that might occur secondary to the dilutional effects of lavage. Consistent with the presence of pulmonary edema, ARDS patients had increased ELF volumes. ARDS ELF volumes averaged 8.3±2.0 ml/100 ml BALF compared with only 0.95±0.06 ml/100 ml BALF for normals ($P < 0.01$). ARDS ELF concentrations of albumin and total protein were not significantly dif-

Table II. ELF Measurements in ARDS Patients and Normals

	Patient	ELF volume	ELF albumin	ELF total protein	ELF/plasma albumin	ELF/plasma total protein
		ml/100 ml*	mg/ml	mg/ml		
ARDS	S.B.	15.0	4.5	9.1	0.22	0.21
(n = 5)	J.B.	10.5	3.7	11.0	0.17	0.26
	C.C.	9.0	2.6	7.2	0.10	0.16
	T.L.	3.6	7.6	18.1	0.17	0.27
	M.V.	3.2	5.7	16.8	0.29	0.34
	Mean	8.3‡	4.8	12.4	0.19§	0.25‡
	SEM	2.0	0.8	1.9	0.03	0.03
Normals	R.R.	0.81	7.2	16.0	0.16	0.23
(n = 10)	D.C.	0.89	3.3	11.1	0.08	0.18
	R.B.	0.85	4.1	9.3	0.09	0.15
	T.M.	1.14	2.7	4.0	0.07	0.06
	J.H.	1.07	5.4	10.7	0.12	0.15
	T.M.	0.96	5.2	9.6	0.11	0.14
	A.A.	1.33	2.4	4.9	0.06	0.07
	R.E.	0.62	6.1	10.8	0.13	0.14
	D.N.	0.73	7.1	8.2	0.14	0.12
	S.D.	1.10	2.2	5.1	0.06	0.08
	Mean	0.95	4.6	9.0	0.10	0.13
	SEM	0.06	0.6	1.1	0.01	0.02

* ml ELF/100 ml BALF.
‡ Significantly different from normal ELF ($P < 0.01$).
§ Significantly different from normal ELF ($P < 0.05$).

ferent from normal ELF concentrations. However, because the ARDS patients had very low plasma albumin and total protein, ARDS ELF/plasma ratios were significantly increased compared with normal ratios. ARDS ELF/plasma total protein ratios averaged 0.25±0.03 compared with 0.13±0.02 for normals ($P < 0.01$). ARDS ELF/plasma ratios for albumin were also increased (Table II).

Serial bronchoalveolar lavage studies. Six ARDS patients were evaluated with serial bronchoalveolar lavage. Analysis of the serial data provided information about the time course of the permeability alterations, as well as their relationship to gas exchange abnormalities (Fig. 2). During the first 5 d of ARDS, every BALF/plasma total protein ratio remained at least two times as high as the highest cardiac edema or normal volunteer BALF/plasma ratio (Fig. 2, *top*). There was a gradual downward trend in the BALF/plasma ratio with time, and the ARDS ratios approximated those of the control groups by day 7. Serial BALF/plasma ratios were also compared with changes in gas exchange as assessed by the alveolar-arterial oxygen pressure difference (Fig. 2, *bottom*). Although gas exchange gradually improved in all patients, visual comparisons of Fig. 2 (*top and bottom*), reveal that trends in BALF/plasma protein ratios could not be used to predict trends in the adequacy of gas exchange.

Serial lavage data also demonstrated a significant correlation between lung protein permeability (micrograms total protein per milliliter BALF) and the degree of lung inflammation (percent lavage neutrophils). This correlation was described by the formula: protein concentration = 13.3 (percent neutrophils) + 97 ($r = 0.63$, $P < 0.01$, data not shown).

Measurement of the high molecular weight proteins $\alpha2$-macroglobulin and immunoglobulin M. To determine if the protein permeability changes in ARDS extended to very large proteins, BALF was analyzed for the presence of $\alpha2$-macroglobulin (820,000 mol wt) and IgM (900,000 mol wt). Both proteins were readily detectable by radial immunodiffusion in ARDS BALF,
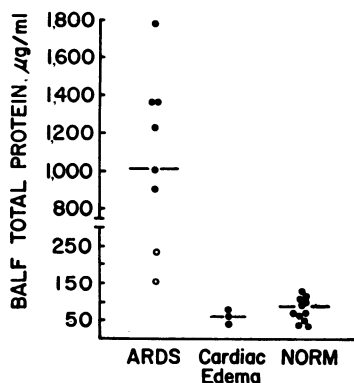

Figure 1. BALF total protein levels in ARDS, cardiac pulmonary edema, and normal volunteers (NORM). In the ARDS group, open circles represent first lavages obtained at 7 and 13 d after onset of ARDS; solid circles represent first lavages obtained before the 5th day.

Figure 3. BALF levels of the high molecular weight proteins α2-macroglobulin and IgM in ARDS patients and normal volunteers. The concentrations of both proteins were significantly increased in ARDS BALF.
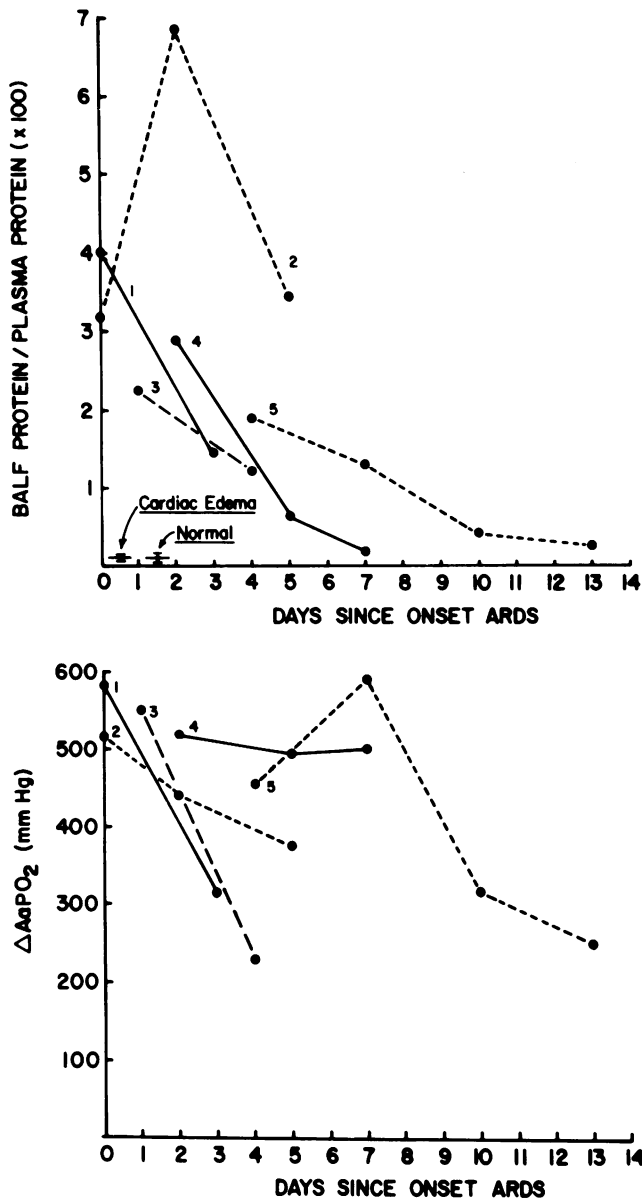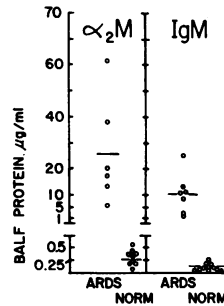
Figure 2. (Top) BALF/plasma ratios. Serial BALF/plasma protein ratios as a function of time since onset ARDS. The means and ranges of cardiac pulmonary edema patients and normal volunteers (NORM) are depicted at left for comparison. (Bottom) ΔAaPO₂. Serial changes in alveolar–arterial oxygen tension differences (mmHg) on zero positive end expiratory pressure and 100% FIO₂ as a function of time since onset ARDS. Note that trends in BALF/plasma protein ratios do not reliably predict trends in the adequacy of gas exchange for a given individual.

which contained 25.8±8.3 μg/ml of α2-macroglobulin and 10.3±2.9 μg/ml of IgM (Fig. 3). In contrast, these proteins could not be measured by radial immunodiffusion in normals unless the BALF was further concentrated by lyophilization. Using simultaneously measured total protein to extrapolate these values back to unconcentrated BALF, normal BALF contained 0.24±0.05 μg/ml of α2-macroglobulin and 0.11±0.02 μg/ml of IgM (Fig. 3). Thus, ARDS BALF contained >90-fold concentrations of both proteins compared with normal BALF ($P < 0.01$, both comparisons). Even after lyophilization, α2-macroglobulin could not be detected in cardiac edema BALF. IgM was 0.14 μg/ml in one of the cardiac edema patients. In the other two

cardiac edema patients, IgM immunodiffusion precipitin rings were visible but were below the lower limit of quantification.

DC for individual serum proteins. Since ARDS BALF contained markedly increased concentrations of protein, including high molecular weight proteins, it appeared that the permeability defect of ARDS was associated with destruction of the normal size-restrictive property of the alveolar–capillary membrane. Table III demonstrates the marked increases in component protein concentrations of ARDS BALF compared with normal BALF. These differences become more important when simultaneous plasma protein component concentrations are used to calculate DC. Fig. 4 depicts the curves of DC vs. molecular weight ($\log_{10}$ scale) for ARDS patients and normal volunteers. In normal volunteers, the DC for lower molecular weight proteins approximated unity. Thus, the respective DC values for α1-antitrypsin, albumin, and transferrin were 0.76±0.08, 0.81±0.10, and 0.46±0.06. As expected, the higher molecular weight proteins had higher DC; the respective values for haptoglobin, ceruloplasmin, α2-macroglobulin, and IgM were 6.04±1.23, 3.97±1.26, 18.2±3.2, and 14.0±2.5. In contrast to normal volunteers, the DC curve generated from ARDS patient data is flattened, which indicates that a size-indiscriminate bulk flux of plasma proteins has replaced the usual size-selective access to the alveolar spaces (Fig. 4). Alpha-1 antitrypsin, albumin, transferrin, haptoglobin, and ceruloplasmin were distributed virtually equally between plasma and BALF as evidenced by their respective DC: 1.34±0.24, 1.12±0.08, 0.48±0.11, 1.32±0.28, and 1.27±0.12. Even α2-macroglobulin (DC, 2.16±0.41) and IgM (DC, 3.51±0.74) were only minimally restricted from the alveolar spaces in ARDS patients. In the ARDS patients studied serially, DC for α2-macroglobulin and IgM were persistently low, with no significant reversion toward normal within the time period studied.

PAGE of BALF protein. The DC data of selected proteins suggested that the size-selective barrier function of the alveolar–capillary membrane is lost in ARDS. To further assess the validity of this concept, proteins were analyzed according to molecular size by PAGE. Electrophoretic profiles of equal amounts (75 μg) of BALF protein and matched plasma protein from four ARDS patients were nearly identical (Fig. 5). Virtually all plasma bands in each patient could also be identified in the matched BALF protein profile. Interestingly, some bands present in BALF were not seen in plasma, and most likely represented products of the intense local inflammatory response.

When normal and ARDS BALF protein profiles were compared by PAGE, the ARDS profile consistently contained a spectrum of higher molecular weight protein bands not visible in normal BALF, even though equal amounts of total protein were loaded (Fig. 6). These findings confirm what one would predict from the previously noted high molecular weight diver-

Table III. Bronchoalveolar Lavage Protein Concentrations*

	BALF‡	Recovery	Total protein	α1 AT	ALB	TRF	HPT	CER	α2 M	IgM
	ml									
ARDS (n = 8)	45.3	45.3%	1,000	91.8	548	91.9	58.7	9.6	25.8	10.3
	±3.4		±200	±27.6	±142	±27.9	±9.6	±2.9	±8.3	±2.9
Normals (n = 11)	48.4	48.4%	81	4.4	36.5	6.0	0.47	0.13	0.24	0.11
	±3.2		±9.4	±0.7	±3.1	±2.0	±0.12	±0.06	±0.05	±0.02

All ARDS component protein concentrations were significantly higher than normals ($P < 0.01$). * Micrograms per milliliter. ‡ Volume of instilled fluid recovered from 100 ml/lobe lavage. α1 AT, α1-antitrypsin; ALB, albumin; TRF, transferrin; HPT, haptoglobin; CER, ceruloplasmin; and α2 M, α2-macroglobulin.

gence in DC between normal and ARDS patients. Cardiac edema BALF electrophoretic protein profiles closely resembled normal BALF.

Discussion

Protein permeability of the pulmonary microcirculation has been extensively studied in animal lung lymph fistula models which trace the transendothelial movement of protein from plasma to pulmonary lymph (1–5). Such models have demonstrated increased lymph flow rates in both types of pulmonary edema. Lymph/plasma protein concentration ratios, however, drop to about half of baseline after induction of cardiac edema (3) but remain unchanged or increase slightly after induction of permeability edema (2). Since evaluation of pulmonary lymph is not clinically feasible in man, similar assessment of the pulmonary edema states in humans has depended on techniques that recover alveolar lining fluid. Since this fluid is normally separated from interstitial lymph by an epithelial cell interface, one would expect its protein composition to be further modulated by epithelial permeability characteristics specific to the edema-forming process. Indeed, the protein content of tracheobronchial aspirates of human edema fluid has been used to differentiate cardiac pulmonary edema from permeability pulmonary edema (ARDS) (6–11). However, a potential problem

with this method is contamination of pulmonary edema fluid by central airway exudate. In the present study, we hypothesized that bronchoalveolar lavage would collect a more representative alveolar sample and thus allow for better characterization of epithelial protein permeability in human pulmonary edema. We present evidence that the protein content of BALF and BALF/plasma protein ratios can be used to unequivocally separate ARDS patients from heart failure patients and normal controls. We have further demonstrated that the increased protein permeability of ARDS is associated with a loss of the normal size-selective access of plasma protein to the alveolar epithelial surface.

Protein permeability of the normal alveolar capillary membrane. The normal pulmonary parenchymal barrier that separates air from blood is composed of endothelial, interstitial-lymphatic, and epithelial compartments. Each of the three compartments is endowed with traits that protect against alveolar flooding (25). The ultimate physical defense against alveolar flooding, however, is the alveolar epithelial lining, which is rendered virtually impermeable to intercellular protein flux by tight
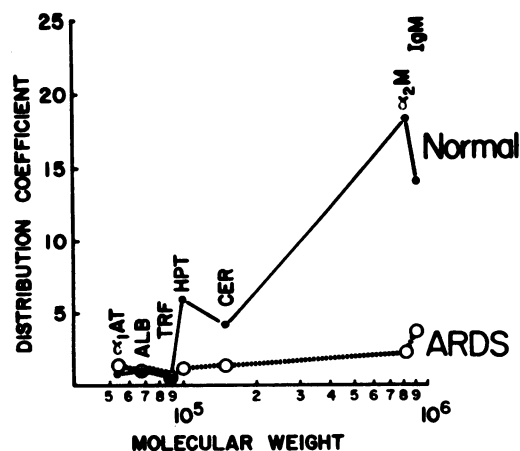

Figure 4. Molecular weight–dependent divergence of DC of normal volunteers and ARDS patients. In ARDS, plasma proteins were found in plasma and BALF in similar proportions. α1AT, alpha-1 antitrypsin; ALB, albumin; TRF, transferrin; HPT, haptoglobin; CER, ceruloplasmin; and α2M, α2-macroglobulin.
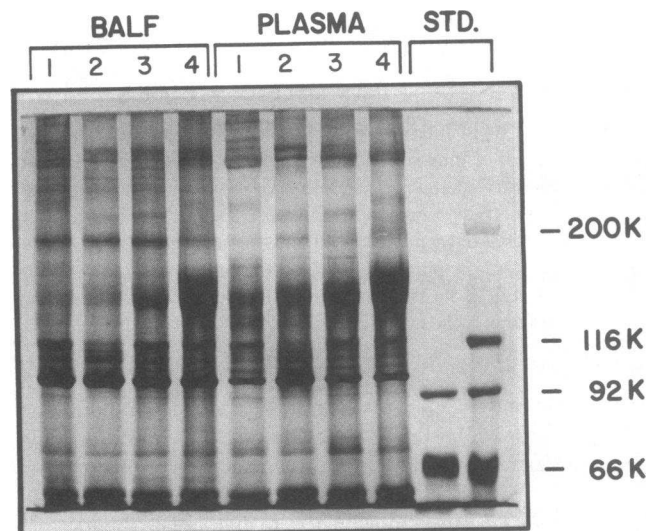

Figure 5. Polyacrylamide slab gel electrophoresis of first BALF protein (lanes 1–4) and matched plasma protein (lanes 5–8) from four ARDS patients. The electrophoretic protein profiles for ARDS BALF protein and plasma protein were virtually identical (5% gel, 75 μg protein per lane. Lane 10 standards are myosin, β-galactosidase, phosphorylase B, and bovine serum albumin, with respective molecular weights of 200,000, 116,000, 92,500, and 66,000.)
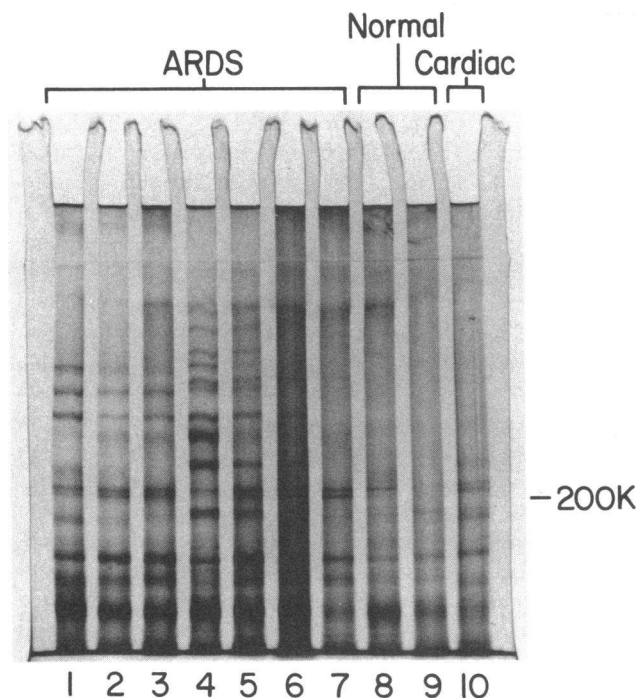
Figure 6. Polyacrylamide slab gel electrophoresis of BALF protein from three ARDS patients (lanes *1-3*), plasma and three serial BALF protein samples from a single ARDS patient (lanes *4-7*), two normal volunteer BALF samples (lanes *8-9*), and a single cardiac edema BALF sample (lane *10*). A larger proportion of high molecular weight proteins is present in ARDS BALF (3.8% gel, 20 μg protein per lane).

junctions composed of an intricate network of junctional fibrils (26). That this ultrastructural finding represents an impediment to solute flux is supported by estimated equivalent pore radii of alveolar epithelium (determined from experimentally measured reflection coefficients) of 0.6–1.0 nm (27). Analagous correlates for the "leakier" nonfenestrated endothelial barrier include ultrastructurally demonstrable gaps in some endothelial intercellular junctions measuring 4 nm in width (28) and estimated equivalent pore radii of 4.0–5.8 nm. More recent study of pulmonary endothelial permeability using endogenous protein fractions of varying hydrodynamic radii has generated osmotic reflection coefficients consistent with a two "pore" model with equivalent pore radii of 8 and 20 nm, accommodating 81 and 16% of hydraulic flow, respectively (4). Conceivably, such pores would allow free diffusion of albumin (hydrodynamic radius 3.5 nm) through the endothelium. In contrast, the equivalent pore radius of the epithelium would appear to restrict all but the very smallest solutes from the alveolar lining fluid.

Given the high resistance of alveolar epithelium to intercellular transport of protein, alternate mechanisms must be invoked to account for the wide variety of plasma proteins that we and others have harvested from the alveolar spaces of normal human volunteers by bronchoalveolar lavage (12–17). Some investigators have suggested that the protein harvested is an artifact of mechanical injury induced by the lavage procedure itself (29). However, several lines of evidence contradict this hypothesis. First, red blood cells are very rarely seen in cell pellets of normal lavages. Second, other methods of evaluating the alveolar epithelial surface have also confirmed the presence of plasma proteins (29–31). Third, Gorin and Stewart (32) have used a radio-

labeled albumin marker in sheep with chronic lung lymph fistulas to demonstrate that BALF is not significantly contaminated by plasma or interstitial fluid proteins. If the variably sized proteins harvested from the alveolar surface by bronchoalveolar lavage are not the result of sampling injury, and are too large to pass through intercellular conduits, then how do they gain access to the epithelial surface? The most tenable explanation appears to be transcellular vesicular transport. Except for the perinuclear areas, the organelle population of type I epithelial cytoplasm is almost exclusively plasmalemmal vesicles, numbering 150–250/ μm^3. The vesicles, occupying $\sim$2% of the cytoplasmic volume, average 70 nm in diameter (33), a size large enough to accommodate even large plasma proteins like IgM, whose molecular diameter is $\sim$30 nm (34).

Nonselectivity of protein flux in ARDS. Using DC, Bell et al. (15) have shown that plasma proteins <200,000 mol wt are distributed approximately equally between plasma and BALF of normal volunteers, i.e., each component protein in this size range comprises about the same percentage of BALF total protein as plasma total protein. They demonstrated that high molecular weight proteins as α2-macroglobulin and IgM are restricted from the alveolar spaces, which is evidenced by their very high DC. Thus, the concept has evolved that plasma proteins reach the alveolar epithelial surface by a size-selective process that restricts high molecular weight species. If the proteins reach the alveolar epithelial surface by vesicular transport, it is plausible that size selection occurs during the pinocytotic formation of new vesicles. This phenomenon may relate to the higher diffusibility of smaller passenger proteins and/or to the physical bottleneck that occurs as the walls of the invaginated plasmalemma close over the newly formed vesicle.

In the present study, comparisons of normals and cardiac edema patients with ARDS patients revealed significant differences in the distribution of variously sized plasma proteins into BALF. The protein electrophoretic profiles of cardiac edema BALF, compatible with preservation of epithelial integrity, did not differ significantly from normal BALF, as both groups revealed restricted access of high molecular weight proteins to the alveolar surface. In contrast, ARDS BALF electrophoretic profiles contained the full spectrum of plasma proteins. Loss of the normal restriction of larger molecules from the alveolar spaces was evidenced by the drop in their DC toward unity. These findings imply that in ARDS there is bulk flux of plasma proteins into the alveoli.

Although the data presented supports the concept of altered epithelial permeability in ARDS, it does not permit direct measurement of permeability as is possible with intravenously injected tracer proteins (32) or with various scintigraphic techniques (35–41). The present data also cannot be manipulated to estimate epithelial pore size. The traditional assessment of endothelial permeability in lung lymph-fistula animal models involves determination of osmotic reflection coefficients (σ) of variously sized proteins using subinjurious hydrostatic gradients to maximize transendothelial fluid flux. Under such conditions, σ is approximated by $1 - (C_L/C_P)$, where C_L/C_P is the lymph to plasma protein concentration ratio for a given protein (42, 43). The experimentally determined σ and the known solute radius (a) for each of the variously sized proteins are then plotted among pore radius (R) curves predicted by the Levitt equation (42): $\sigma = 16/3(a/\mathrm{R})^2 - 20/3(a/\mathrm{R})^3 + 7/3(a/\mathrm{R})^4 - 0.354(a/\mathrm{R})^5$. Pore size ranges are then extrapolated from the pore radius curves containing the data point clusters (42). In the current study of

human epithelial permeability in ARDS, we obtained the BALF/plasma ratios, and could adjust for the dilutional effects of lavage by correcting to ELF/plasma ratios. However, the latter cannot be used to predict σ because they are probably not filtration independent, equilibrium concentration ratios obtained at maximal transepithelial fluid flux. Indeed, therapeutic concerns necessitate maintenance of low capillary hydrostatic pressure in ARDS patients. Moreover, continuous transepithelial flux is abrogated by the interstitial–lymphatic sump and the hydrostatic back pressure imposed once dependent alveolar spaces are flooded. Although normal volunteer ELF probably represents an equilibrium state, it is a bidirectional vesicular equilibrium rather than a unidirectional hydraulic one. Because of these difficulties, we chose to evaluate epithelial membrane integrity by assessing changes in size selectivity as manifested by reduction in DC for large proteins. This methodology is more practical to apply to the human epithelium, since ongoing maximal transepithelial flux need not be assumed, and dilutional factors are not a concern (i.e., BALF component protein and total protein are expressed as ratios relative to plasma).

As expected, serial bronchoalveolar lavage studies of ARDS patients demonstrated successive declines in BALF total protein and BALF/plasma total protein ratios. However, serial DC for α2-macroglobulin and IgM showed no significant improvement over the period studied. Although the reason for this apparent discrepancy is unknown, histopathologic studies of ARDS suggest that the endothelium survives injury to the alveolar–capillary barrier better than the epithelium because of its more efficient reparative processes (44, 45). Restoration of the epithelium, however, must await type II cell proliferation and subsequent squamous transdifferentiation (46). Thus, in the present study, successive improvements in BALF protein and BALF/plasma protein ratios may be explained by alveolar protein clearance in the face of a restored endothelial barrier. Restoration of normal DC for α2-macroglobulin and IgM may lag behind because they require protein replenishment by the selectively restricted cargo of a critical mass of functioning epithelial cells.

Edema fluid assessment of the pulmonary edema states in humans. Several clinical studies comparing cardiac pulmonary edema and ARDS have utilized catheter aspiration of tracheobronchial fluid to assess alterations in alveolar protein permeability (6–9). The chief criterion for entry into these studies was pulmonary edema of sufficient magnitude to allow collection of several milliliters of edema fluid from the airways. The data in Table IV has been compiled from patients in the above cited studies in whom (*a*) edema fluid and plasma protein values were known, and (*b*) pulmonary capillary wedge pressure measurements and clinical diagnosis suggested the existence of either hydrostatic or permeability mechanisms, but not both. Nonparametric analysis reveals statistically significant differences in mean bronchial aspirate protein levels of cardiac edema and ARDS groups, although there is considerable overlap of ranges. Much better separation of the ranges occurs when edema/plasma protein ratios are compared, where the mean ratio in ARDS patients is roughly twice that of cardiac edema patients. In contrast, the BALF data in the present study reveals logfold differences in means and widely separated ranges whether BALF protein concentrations alone or BALF/plasma ratios are examined.

The finding that BALF protein analysis provides better separation of patient groups than tracheobronchial protein analysis suggests that the latter technique samples alveolar edema fluid mixed with variable proportions of central airway secretions. In this context, the numerous bronchial irritants that accompany mechanical ventilation (such as high inspired oxygen concentrations, bacterial colonization, secretion stasis, and suction catheter and endotracheal tube trama) probably lead to serous exudation from the endobronchial wall. This may well be enhanced by the high capillary hydrostatic pressures of cardiac edema. Mixed with alveolar edema fluid, this serous exudate would raise the protein concentration of cardiac edema bronchial aspirate but not significantly alter the already exudative protein concentrations of ARDS aspirate. The true proportion of alveolar protein could thus be widely variable. Conversely, contamination with airway exudate is likely to be insignificant with a "wedged" bronchoalveolar lavage procedure because (*a*) the dependent secretions that would be harvested by bronchial aspirate are not only bypassed by the bronchoscope, but are unlikely to contaminate the nondependent right middle lobe (or lingula) site of lavage; and (*b*) should bronchial exudate form at the subsegmental level, its contribution to total protein would be dwarfed by that of the exponentially larger alveolar surface.

The excellent separation of patient groups by BALF protein

Table IV. Comparison of Bronchial Aspirate and Bronchoalveolar Lavage in the Assessment of Pulmonary Edema States

Study	PCWP	n	Edema fluid protein			Ratio of edema protein to plasma protein		
			Mean	Range	P	Mean	Range	P
1976: bronchial	CPE	0	—	—		—	—	
aspirate (6)	ARDS <15	4	4.9	3.4–7.1 g/dl		0.78	0.57–1.11	
1979: bronchial	CPE >20	3	3.5	2.7–4.4 g/dl	<0.05	0.59	0.50–0.70	<0.05
aspirate (7)	ARDS <18	11	5.3	3.8–7.2 g/dl		0.95	0.66–1.33	
1979: bronchial	CPE >20	3	2.6	1.5–3.5 g/dl	<0.05	0.46	0.40–0.56	<0.01
aspirate (8)	ARDS <20	18	4.2	2.5–7.5 g/dl		0.93	0.58–2.68	
1981: bronchial	CPE >15	8	3.4	2.0–4.4 g/dl	NS	0.47	0.26–0.69	<0.01
aspirate (9)	ARDS <15	12	4.2	2.4–6.3 g/dl		0.77	0.51–0.98	
Present study:	CPE >24	3	59	40–78 μg/ml	<0.01	0.11*	0.08–0.15	<0.05
bronchoalveolar lavage	ARDS <15	8	1,000	147–1,778 μg/ml		2.84*	1.89–4.02	

CPE, cardiac pulmonary edema; PCWP, pulmonary capillary wedge pressure, millimeters of mercury. * Number represents BALF/plasma ratio × 100.

analysis was supported by ELF measurements. In the present study, bronchoalveolar lavage of normal volunteers yielded 0.95 ± 0.06 ml ELF/100 ml BALF with ELF albumin concentrations of 4.6 ± 0.6 mg/ml. These values are very similar to the data reported by Rennard et al. (17) (1.0 ± 0.1 ml, 3.7 ± 0.3 mg/ml), and support the conclusion that ELF albumin concentrations are $\sim10\%$ of plasma values. In ARDS patients, ELF volumes were increased approximately eightfold, and ELF total protein values were 25% of plasma values. Thus ELF/plasma total protein ratios clearly separate ARDS patients from normal subjects (0.25 compared with 0.13). The urea method is subject to error if appreciable amounts of urea are diffused into the lavage fluid from plasma during the brief time of the lavage. This potential source of error would appear to be minimal since the present study followed the same protocol as Rennard et al. (17), in which five 20-ml aliquots were instilled and immediately withdrawn. The urea method has not previously been used in ARDS patients, and it remains theoretically possible that the flux of urea from plasma to instilled saline lavage fluid occurs at a faster rate in ARDS patients compared with normals. In this case, the urea method would overestimate the volume of ARDS ELF and thus underestimate the calculated ELF protein concentrations. Therefore, in the present study, differences between ARDS and normal ELF/plasma protein ratios may represent minimum values.

Acknowledgment

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