PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **184**, 83–91 (1987)

Crossed Immunoelectrophoretic Analysis of ARDS Lavage Proteins (42449)

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Abstract. Serum proteins in normal and ARDS bronchoalveolar lavages were analyzed using crossed immunoelectrophoresis. Normal lavages demonstrated relatively few proteins (albumin, α_1-antitrypsin, transferrin, and haptoglobin) in low concentrations. In contrast, substantial amounts of all identifiable serum proteins were detected in ARDS lavages. IgA was apparently locally produced. Two of the largest proteins, β-lipoprotein (mol wt > 2 million) and IgM (mol wt ~ 900,000) were found to be complexed as evidenced by their coprecipitation in a single spike in ARDS lavage. Electrophoretic modifications of ARDS albumin and α_1-antitrypsin precipitation peaks and partial identity spurring of the α_1-lipoprotein peak with other precipitation loops indicated possible complex formation between these proteins and other possibly pathogenic lung fluid constituents. Similarly, modifications of orosomucoid and Gc-globulin peaks indicated possible molecular alterations resulting from interactions with other components. The relatively few protein modifications exhibited in ARDS lavages together with α_1-antitrypsin–protease complex formation confirm the relative absence of substantial proteolytic activity in ARDS edema fluids obtained within 12 hr of the onset of the syndrome demonstrated in previous studies. © 1987 Society for Experimental Biology and Medicine.

Adult respiratory distress syndrome (ARDS)[5] is an acute, often fatal condition involving diffuse lung damage that occurs in 150,000 to 200,000 patients a year (1). Although diverse triggering events cause ARDS to develop (2, 3), its pathology is surprisingly consistent. Injury to Type I alveolar epithelial cells and increased microvascular permeability results in interstitial and alveolar edema. This leakage of protein-rich fluid into alveoli is accompanied by little visible endothelial damage in the initial stages (2, 4). Subsequently, this exudation is accompanied by cellular infiltration and hyaline membrane deposition, followed eventually by fibrosis in some cases.

Bronchoalveolar lavage has proven to be extremely useful for characterization of lung cellular and proteinaceous constituents (5). Normally, albumin, transferrin, secretory IgA, and IgG have been found in lavage fluid, with α_1-antitrypsin and free secretory piece present less frequently, and α_2-macroglobulin rarely detected (5, 6). Various studies have demonstrated quantitative changes in the lung fluid proteins of patients with pulmonary disease (6–8). Recent investigations of bronchoalveolar lavage from ARDS patients have revealed extensive alterations in the cellular content of alveolar fluid (9–11), but, in general, information about protein constituents is lacking.

In the present study, crossed immunoelectrophoresis (X-IEP), a technique uniquely capable of simultaneous quantitative and qual-

[1] Supported by the Charles E. Culpeper Foundation, Inc., and, in part, by USPHS-NIH Grant ST32AI070-35-05.

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[5] Abbreviations used: X-IEP, crossed immunoelectrophoresis; PA, prealbumin; Ct, carbamylated transferrin; Or, orosomucoid; Alb, albumin; α_1Lp, α_1-lipoprotein; α_1At, α_1-antitrypsin; α_1B, α_1B-glycoprotein; α_1X, α_1-antichymotrypsin; IαI, inter-α-trypsin inhibitor; Gc, Gc-globulin; Cr, ceruloplasmin; α_2HS, α_2HS-glycoprotein; α_2M, α_2-macroglobulin; Hpt, haptoglobin; Tr, transferrin; Hpx, hemopexin; IgA, immunoglobulin A; βLp, β-lipoprotein; C3, C3 component; IgG, immunoglobulin G; IgM, immunoglobulin M; ARDS, adult respiratory distress syndrome.

0037-9727/87 $1.50
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itative comparisons of proteins in complex solutions (12–14), was used to characterize alterations of lung fluid during the development of ARDS. We have confirmed the exudation of most of the serum proteins. Furthermore, our analyses have revealed abnormal electrophoretic and antigenic characteristics of a few serum proteins in these lavage fluids, probably through interactions with other constituents of the lung.

Materials and Methods. *Lavage samples.* Bronchoalveolar lavages were obtained from seven healthy nonsmokers and from six patients with ARDS after informed consent. The ARDS patients were identified in the intensive care units of the University of Colorado and Denver Veterans' Administration Medical Centers. These patients had so-called "distant" or "nonthoracic" etiologies of ARDS, specifically diabetic ketoacidosis, postcardiopulmonary bypass, drug overdose (colchicine), drug reaction (protamine), and gram negative sepsis (Table I) (9). The patients satisfied the following criteria for entry into the lavage study:

(1) intubated, undergoing mechanical ventilation;

(2) total static pulmonary compliance ≤50 cc/cm H_2O;

(3) nonthoracic etiology of ARDS;

(4) pulmonary capillary wedge pressure ≤12 mm/Hg;

(5) arterial to alveolar PO_2 ratio < 0.2;

(6) less than 12 hr from onset of ARDS to lavage.

Alveolar lavage was obtained through a fiberoptic bronchoscope using ten 30-ml aliquots of sterile 0.9% saline, except in two patients (PU and GA) whose edema fluid was aspired directly by tracheal suctioning. The bronchoscope was gently wedged into the lateral segment of the right middle lobe. Retrieved lavage (approximately 167 ml ± 6 ml) was iced and centrifuged twice for 10 min, once at a low speed (450*g*) and once at a high speed (50,000*g*) to remove the cellular and lipid fractions, respectively. All samples were concentrated 10–15× on an Amicon PM-10 filter and stored at −70°C until analysis. Of these lavages, 100 μl was utilized for X-IEP analyses.

Crossed immunoelectrophoresis. X-IEP was performed as previously described (12, 15) using rabbit antiserum to normal human serum,

TABLE I. (DATA FROM REF. (9))

ARDS patient	Hours after diagnosis	Total lavage protein (mg/ml)	Etiology
OR	12	3.75	Pneumonia: *Streptococcus pneumoniae*
TH	6	6.96	Drug overdose: colchicine
OL	8	13.2	Septicemia: *Serratia marcescens*
MD	6	12.5	Septicemia: *Escherichia coli*
PU	2	17.3	Drug reaction: protamine
GA	12	18.0	Diabetic ketoacidosis
Normal lavages	Range: 0.25–1.78 Mean: 0.65		

Mean cell counts

	Total cell number (cells, mm^3)	Percentage of total cells: Neutrophils	Macrophages	Lymphocytes
ARDS lavage	11,500 ± 0.5	91 ± 0.88	7 ± 1.2	2 ± 1.3
Normal lavage	2,100 ± 0.5	1 ± 0.4	97 ± 1.3	2 ± 0.1

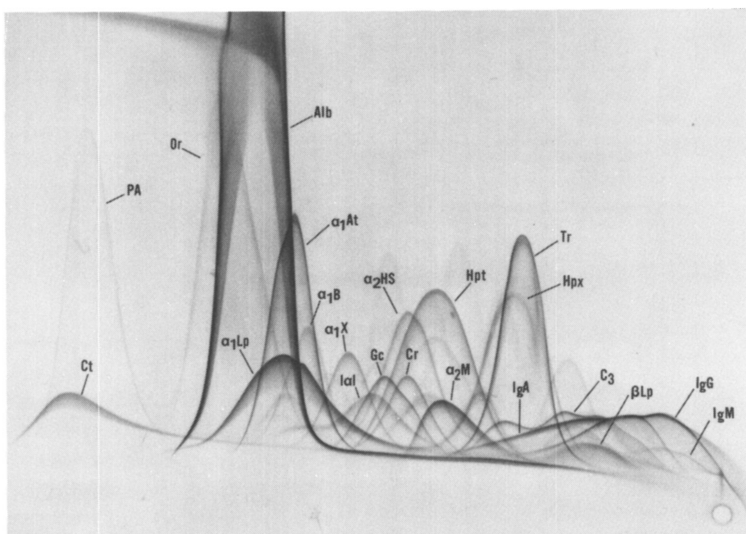

FIG. 1. X-IEP profile of pooled, normal human serum using polyspecific rabbit antiserum. Ct, carbamylated human transferrin (internal control antigen of known concentration); PA, Prealbumin; Or, orosomucoid; Alb, albumin; α_1Lp, α_1-lipoprotein; α_1At, α_1-antitrypsin; α_1B, α_1B glycoprotein; α_1X, α_1-antichymotrypsin; IαI, inter-alpha-trypsin inhibitor; Gc, Gc-globulin; α_2HS, α_2HS glycoprotein; Cr, ceruloplasmin; Hpt, haptoglobin; α_2M, α_2-macroglobulin; Hpx, hemopexin; Tr, transferrin; IgA, immunoglobulin A; βLp, β-lipoprotein; C3, C3 complement; IgG, immunoglobulin G; IgM, immunoglobulin M.

prepared in this laboratory (15). Serum proteins and qualitative deviations were identified by comparing lavage patterns with a standard, normal serum pattern (Fig. 1). The concentrations of the different proteins were determined from the areas under their respective loops of precipitation using previously established methods (12, 16). The ratios between these areas and that of an added standard antigen, carbamylated transferrin (Ct; 17), were determined. The same concentration of carbamylated transferrin was added to each sample as a constant to be used for quantitative analyses and for comparisons of different lavages. Single precipitation loops were identified using monospecific antiserum blockade as previously described (18, 19).

Results. *Quantitative alterations.* Figure 2 shows the marked differences in protein content between normal and ARDS lung fluids. Normal lung lavages concentrated 10× revealed only three or four proteins (albumin, α_1-antitrypsin, transferrin, and occasionally haptoglobin). When detected, IgG was evident in only trace amounts. The low protein concentrations and the uniformity of these patterns among normal subjects demonstrated that the lavage procedure, in itself, did not produce edematous lung tissue damage during sampling.

In contrast, the ARDS patients exhibited much more variability with moderate to extreme elevations of proteins compared with the normal subjects. X-IEP analyses revealed numerous proteins in their lavages. These included 18 of 20 serum proteins (Table II). Prealbumin, orosomucoid, albumin, α_1-B glycoprotein, hemopexin, transferrin, and the two immunoglobulins, IgA and IgG, were readily identified in all samples. Gc-globulin (transcalciferin) and α_2-HS glycoprotein were found less frequently. Only the fluid from patients PU and GA, with edema extensive enough to sample directly (Fig. 2) demonstrated substantial amounts of α_1-lipoprotein. All other samples contained only trace or undetectable amounts of this protein. In general, protein concentrations did not correlate with the time after diagnosis.

Of the antiproteases, α_1-antitrypsin and α_1-antichymotrypsin were commonly detected, with inter-α-trypsin inhibitor less so. Notably

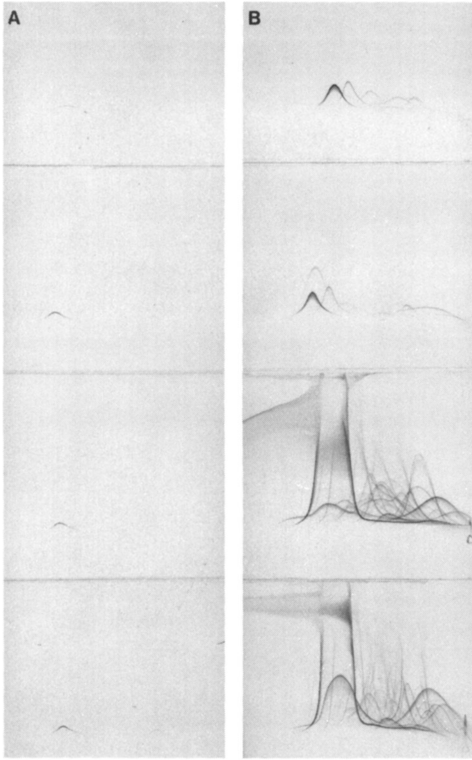

FIG. 2. Comparisons of normal vs ARDS lavages. (A) Lavages from healthy subjects. Only Alb, α_1At, Hpt, and Tr were detected. (B) Lavages were from ARDS patients. The lower two lavages collected without saline. Almost all serum proteins were readily detected (except βLp and IgM).

α_2-macroglobulin, a high molecular weight protein (mol wt = 725,000), was evident in all ARDS lavages.

Two macromolecular serum proteins, β-lipoprotein (mol wt > 2 million) and IgM (mol wt ~ 900,000), were not readily identified in any samples, including those from PU and GA. But, these proteins could be identified using a monospecific antiserum blockage during the first direction electrophoresis shown in Fig. 3. Application of monospecific antiserum against β-lipoprotein (Fig. 3B) only revealed the disappearance of a precipitation spike at the origin (arrow in Fig. 3A). Similarly, more specific antiserum to IgM used either in mixture with lavage or for blockage affected this spike. Ouchterlony analysis (not shown) with those antisera confirmed the presence of β-lipoprotein and IgM in ARDS lavage. Their coprecipitation was revealed by X-IEP.

For most proteins, ARDS X-IEP patterns showed the lavages to contain antigens in proportions similar to those in serum. But, this was not true for IgA. Thus, comparisons of IgA and IgG (Table III) showed IgA to be high relative to the normal IgA/IgG ratio of 1:1 in serum X-IEP profiles (13). IgA lavage area ratios range from approximately 2 to 10 times those of IgG.

Qualitative alterations. Several electrophoretic abnormalities were found in the ARDS lavage patterns as compared with normal serum patterns, some of which are depicted in Figs. 4 and 5. A cathodic shift of the orosomucoid precipitation loop was common to all ARDS patients, as were cathodic tails for both orosomucoid and α_1-antitrypsin. The patients with markedly increased exudation (PU and GA) exhibited more of these abnormalities including albumin loops with unusual cathodic tails and Gc-globulin precipitation loops with an *anodic* tail. One patient, GA, also demonstrated a new unidentified loop.

In addition, precipitate spurs of partial identity associated with α_1-lipoprotein indicated the presence in ARDS lavages of both free α_1-lipoprotein and α_1-lipoprotein bound to some unknown, more cathodic lung component(s). In one ARDS patient (GA), sufficient α_1-lipoprotein was bound to significantly reduce the amount of free α_1-lipoprotein in the lavage fluid (Table II).

Discussion. Previous X-IEP analyses of pathological sera documented numerous protein abnormalities associated with pulmonary diseases, and with alterations of ARDS serum being the most extensive (13). In that study, the concentrations of virtually all proteins were severely depressed in serum from ARDS patients. Only four acute-phase proteins (orosomucoid, α_1-antitrypsin, α_1-antichymotrypsin, and haptoglobin) were either elevated or at normal levels. The increased permeability of the pulmonary microvasculature and resulting edema associated with ARDS were thought to contribute significantly to this profound, general loss of circulating proteins (2, 20, 21). A correspondingly marked increase in serum proteins could be expected in ARDS lavage. The present study supports this interpretation, for there were indeed great increases in all identifiable serum proteins in such lavages. Analyses of lavages with similar total

TABLE II. ARDS LAVAGE PROTEIN CONCENTRATIONS[a]

Patient	PA	Or	Alb	α_1LP	α_1AT	α_1B	α_1X	IαI	Gc	CR	α_2HS	α_2M	Hpt	Hpx	Tr	C_3	IgA	IgG
OR (12)[b]	*	0.52	0.46		0.04	0.01	0.16					0.03	0.02	0.05	0.06		0.17	0.02
TH (6)	0.05	0.22	0.75	*	0.40	0.02	0.03			0.03		0.03	0.18	0.05	0.11	0.03	0.03	0.01
OL (8)	1.81	0.95	0.98	*	1.14	0.03	0.87	0.01	0.07	0.07	0.07	0.05		0.12	0.30	0.03	0.17	0.10
MD (6)	0.12	2.18	1.13	*	2.19	0.04	1.41	0.08	0.09	0.09		0.06	0.79	0.33	0.21	0.05	0.29	0.03
PU (2)	5.83	7.39	7.83	1.51	5.80	1.47	2.66	0.29	1.41	1.04	1.33	0.26	1.11	1.64	3.41	0.83	1.51	0.69
GA (12)	3.73	6.34	11.93	0.48	10.45	1.08	7.07	0.22	0.98	1.05	1.20	0.49	7.19	1.34	2.46	0.64	1.76	0.42

[a] Protein concentrations expressed as carbamylated transferrin units.
[b] Hours after diagnosis.
*Only a trace detected.

protein concentrations also suggest possible selectivity in the passage of some proteins (e.g., PA, Or, α_2HS, Hpt, and IgG levels in OL and MD lavages).

These increases were best seen relative to X-IEP patterns of normal lavages, when both kinds of lavage were used after 10-fold concentration. Although the number of serum antigens detected by X-IEP in these normal lavages was fewer than previous studies detected with up to 200-fold concentrations (5–7), the 10-fold concentration was necessary because of the small sample volumes available to us. This was also fortuitous for the ARDS–normal lavage comparisons, because the greater amounts of protein in ARDS lavages prevented parallel comparisons at higher lavage concentrations.

Unexpectedly, the two antigens of largest molecular weight, β-lipoprotein (mol wt $>$ 2 million) and IgM (mol wt $\sim$ 900,000) coprecipitated as a single spike above the origin of ARDS X-IEP patterns. Complexing between immunoglobulins and other serum proteins in normal and pathological sera have been reported previously (22, 23). The sera of our ARDS patients showed no evidence of β-lipoprotein–IgM complex formation (13). Consequently, the antigens must have formed the complexes observed in the patients' lavages during or after entering the lungs. The meaning of this observation is unknown.

IgA levels demonstrated a possible exception to wholesale transudation of serum proteins from plasma to the lungs, according to the following reasoning. In X-IEP, with this polyspecific antiserum, the ratio of IgA to IgG is 1:1 as a baseline for normal serum (13). This ratio disagrees with the actual milligram per milliliter IgA:IgG ratio for normal serum (24) for technical reasons associated with immunochemical measurement of the IgG loop of precipitate (25, 26). But, this is not important in the comparisons being discussed here, because the technical problem is equally present in X-IEP analyses of lavage fluid as in serum when using the same antiserum and thus is a mathematical constant. Changes from this baseline would still agree with other forms of measurement. While the IgA:IgG ratio is 1:1 in normal serum, then, it was 3:1 in ARDS sera (13), apparently because of a greater loss of IgG than of IgA to the lungs. The IgA:IgG

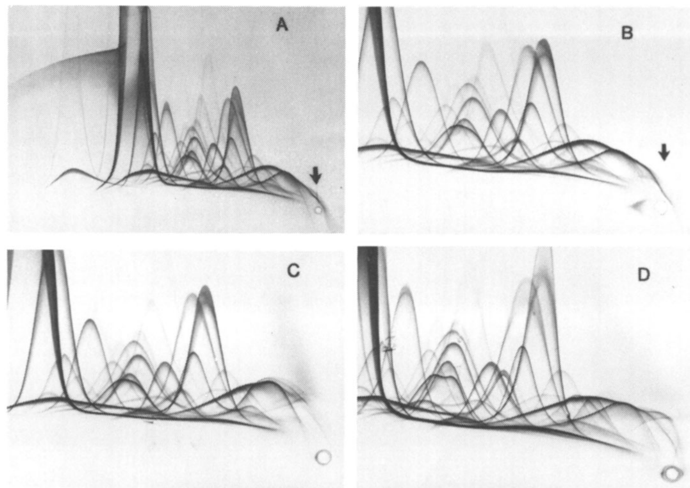

FIG. 3. βLp and IgM in ARDS lavage (diluted 1:1 with buffer). Monospecific antisera to identify βLp and IgM were used. Monospecific antiserum was placed *anodic* to the antigen well to specifically precipitate and identify βLp (B), or was mixed with antigen (C) and placed *above* the well (D) to identify IgM. The cathodic areas of the lavages in these panels have been enlarged. (B) and (C), the precipitation spike (arrow) disappeared while becoming less intense in (D), indicating the coprecipitation and possible complex between βLp and IgM. The nonspecific precipitations above IgG in (C) and (D) result from extraneous proteins in the monospecific antiserum (1, 13).

ratio in normal lavage should be about 20:1 (6) because much more IgA is being synthesized in the lungs. Since the corresponding losses of other serum proteins in ARDS seemed to be to the lungs (see above), one would predict a decrease in IgA:IgG ratio in ARDS lavage due to a larger transudative influx into the lungs of IgG than IgA. While all ARDS lavages contained a lowered ratio, as expected, some lavages had IgA:IgG ratios much higher than that corresponding to the transudated serum flooding the lungs. These data and reasoning indicate that IgG leaks into ARDS lungs like most of the other serum antigens, but the lung, itself, may contribute some portion of the IgA, either from direct mixing of transudated serum with previously existing lung fluid or by continued, local production of IgA.

We can suggest several explanations for the qualitative or electrophoretic abnormalities we saw in ARDS lavages. The overall cathodic

TABLE III. X-IEP RATIOS OF IgA TO IgG IN ARDS LAVAGE

Patient	IgA/IgG
OR	8.50
TH	3.00
OL	1.70
MD	9.67
PU	2.19
GA	4.19

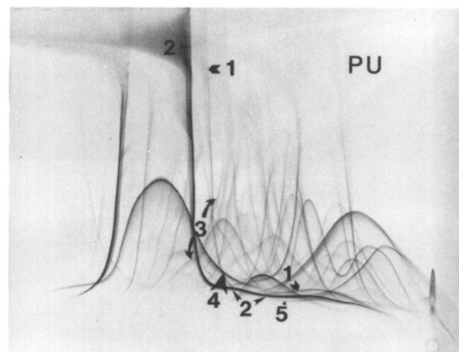

FIG. 4. Qualitative alterations in the ARDS lavage of patient PU. (1) α_1At with cathodic tail; (2) abnormal Alb tail; (3) Gc-globulin peak with *anodic* tail; (4) spur of partial identity with α_1Lp; (5) unidentified protein.

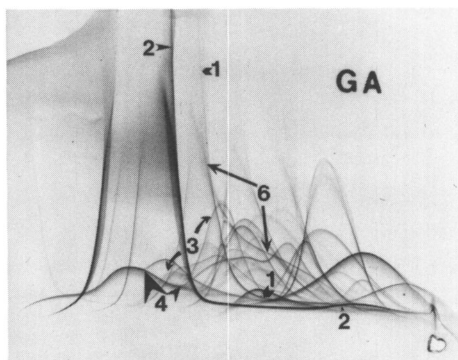

FIG. 5. Qualitative alterations in the lavage of patient GA. Same key as in Fig. 4. (1) α_1-At with cathodic tail; (4) spurs of identity with α_1-Lp; (6) Or peak shifted cathodally, with cathodic tail.

shift and the marked cathodic tail of acid carbohydrate-rich orosomucoid (27) probably resulted from changes in its sialic acid residues (28). On the other hand, cathodic tailing of the α_1-antitrypsin without its general shifting could have been due to limited complexing of this antiprotease with proteases (29, 30). Similarly, albumin, with its well-known carrier characteristics, most likely formed its abnormal cathodic tail by complex formation. The reasons for the anodic head on transcalciferin (Gc-globulin) (31) is obscure. They could include limited proteolysis, binding to anodically migrating molecule, or oxidant activity (32). Nevertheless, such an alteration in Gc-globulin could affect Vitamin D binding and transport, and thereby impact macrophage function (31, 33, 34).

We did see some direct evidence of binding and complex formation in the partial identity reaction of α_1-lipoprotein with two other loops of precipitation. These other loops were not detected in X-IEP patterns of normal lavage, nor in normal or ARDS sera (13). But, they must be normal serum antigens, since they were being detected by antiserum to normal serum. We have seen them in sera from extensively burned patients (35). They may be antigens with pathologically changed mobilities and acquired affinity for α_1-lipoprotein, or they could be pathologic derivatives of this lipoprotein itself. That the abnormalities in X-IEP patterns of ARDS lavages might be artifacts of our procedure for concentrating the lavages is a less likely explanation, judging from our experiences with other similarly concentrated body fluids.

Previous analyses of these and other ARDS lavages found an absence of neutral proteinase activity (9). McGuire *et al.* (10) and Cochrane *et al.* (36) also demonstrated that early ARDS lavages lack protease activity, while also showing that neutrophil elastase was complexed with α_1-antitrypsin using classic immunoelectrophoresis. We have confirmed the formation of α_1-antitrypsin–protease complexes using X-IEP.

We have also indirectly confirmed the relative absence of protease activity. In the only study of the multiple effects of adding a high concentration of protease (trypsin) to human serum, X-IEP analyses revealed that all but a few serum proteins exhibited markedly abnormal loops of precipitation (37). Within minutes, many peaks decreased or vanished as they underwent qualitative alterations (e.g., splitting; formation of so-called "flying" loops of precipitate). Since we did not observe similar extensive changes, there appeared to be little or no protease activity at this stage of ARDS, although multiple affects of neutrophil elastase and cathepsin G have yet to be specifically analyzed.

Other reports on the presence of proteases in lavages from ARDS (38) or emphysema (39) could differ from our finding because of sampling times. Thus, there might not be free proteases within 12 hours, but there could be activity in samples taken later in the development of an acute illness such as ARDS or during a chronic disease like emphysema, as also suggested by the findings of others (10, 36).

The X-IEP analyses reported here support the belief that plasma proteins constitute the predominating macromolecules of protein-rich ARDS pulmonary edema fluid. Also, they have detected possibly pathogenic modifications of some plasma proteins that could affect some of their primary physiological functions. Further investigations with polyspecific antiserum against lung constituents would detect any changes in these components and indicate possible interactions with plasma proteins. Thus, X-IEP analyses could lead to an in-

creased understanding of the causes and control of ARDS.

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Received October 22, 1985. P.S.E.B.M. 1987, Vol. 184.
Accepted September 30, 1986.