

# Stimulation of Human Leukocyte Elastase by Platelet Factor 4

## PHYSIOLOGIC, MORPHOLOGIC, AND BIOCHEMICAL EFFECTS ON HAMSTER LUNGS IN VITRO

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**ABSTRACT** The purpose of this study was to determine if human platelet factor 4 (PF<sub>4</sub>) stimulates human leukocyte elastase (HLE) against lung elastin. Lung elastin was purified from hamster lungs and tritiated by reduction with NaB<sup>3</sup>H<sub>4</sub>. We found that HLE activity against this substrate is increased by concentrations of PF<sub>4</sub> as low as 1.6 μg/ml, and that this stimulation increased linearly with additional PF<sub>4</sub>. Lungs removed from hamsters and inflated with solutions containing buffer alone, low dose HLE, HLE plus PF<sub>4</sub>, or PF<sub>4</sub> alone were incubated for 2 h at 37°C. Whereas low-dose HLE failed to lower lung elastin when compared to control animals, HLE stimulated by PF<sub>4</sub> lowered lung elastin by 20%. PF<sub>4</sub> alone had no effect. Furthermore, low-dose HLE failed to alter the mechanical properties of hamster lungs as measured by pressure-volume curves in saline, although there was a significant loss of lung elasticity in the mid- and high-lung volume ranges in lungs treated with HLE and PF<sub>4</sub>. Morphologic studies revealed that low dose HLE resulted in a minimal emphysemalike lesion whereas HLE plus PF<sub>4</sub> caused a significantly more severe lesion. PF<sub>4</sub> is capable of stimulating HLE against lung elastin, and this effect may have a role in the pathogenesis of emphysema.

### INTRODUCTION

The leukocytes of many species, including man, contain numerous proteases, among which is an elastase

with activity at neutral pH (1, 2). Purification of human leukocyte elastase (HLE)<sup>1</sup> and its subsequent administration to experimental animals has produced a disease-model that closely resembles human pulmonary emphysema (3, 4). Inasmuch as alveolar macrophages contain a similar neutral elastase (5), and because both leukocytes and macrophages are found in the lung during periods of inflammation, it has been attractive to postulate that proteases from these cells are responsible for much of the connective tissue injury found in emphysema (6). Support for this protease-pathogenesis theory comes from work showing that patients with severe obstructive lung disease have higher levels of HLE in their circulating leukocytes (7, 8), and from the demonstration that the severity of physiologic abnormalities in patients with emphysema correlates with levels of HLE (9). In addition, the level of elastolytic enzyme is increased in the alveolar macrophages of cigarette smokers (10), and we have recently shown that animals with pneumococcal pneumonia contain from 40 to 70% more elastase in both circulating and lung-derived leukocytes (11).

Some of the factors that modulate the activity of HLE after its release from the leukocyte are known. Alpha-1 protease inhibitor (α<sub>1</sub>Pi) irreversibly inactivates the enzyme (12, 13), and α<sub>2</sub>-macroglobulin appears to be a weak, and perhaps incomplete inhibitor of HLE (14). The low molecular weight bronchial inhibitor described by Tegner (15) is capable of complexing with free HLE

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<sup>1</sup> *Abbreviations used in this paper:* HLE, human leukocyte elastase; L<sub>m</sub>, mean linear intercept; NBA, N-t-BOC-L-alanine-p-nitrophenyl ester; PBS, phosphate-buffered saline; PF<sub>4</sub>, platelet factor 4; P<sub>L</sub>, transpulmonary pressure; PV, pressure volume; SDS, sodium dodecyl sulfate; TLC<sub>25</sub>, total lung capacity at a P<sub>L</sub> of 25 cm H<sub>2</sub>O; V<sub>1 max</sub>, total volume obtained.

present in bronchial secretions; Weinbaum et al. (16) have described a high molecular weight, bronchial mucous inhibitor that also inactivates HLE. Fewer studies have been conducted to investigate factors that might increase the activity of HLE. Jordan and co-workers (17) have shown that activity of pancreatic elastase (peak activity at pH 8.8) is markedly increased if the elastin substrate is first incubated with anionic detergents such as sodium dodecyl sulfate (SDS) or with anionic fatty acids. Because the mechanism of this stimulation rests with the enhanced attraction of cationic enzyme for anionic substrate, and because HLE is also a cationic enzyme at neutral pH (1, 18), it is not surprising that HLE shows increased activity toward SDS-elastin (19). We have recently shown that platelet factor 4 (PF<sub>4</sub>), a cationic protein (*M<sub>r</sub>* 7,500–7,800) that is liberated by platelets during the release reaction, is capable of stimulating HLE against elastin from bovine neck ligament (20). The effects of PF<sub>4</sub>-stimulated HLE against lung elastin have not been reported.

The objectives of our investigation were to study the physiologic, morphologic, and biochemical effects of PF<sub>4</sub>-stimulated HLE against lung elastin. For these studies we used male Golden Syrian hamster lungs in vitro, and elastin purified from the lungs of these animals.

## METHODS

**Animals.** Adult male Golden Syrian hamsters weighing from 110 to 130 g were obtained from a commercial source (Simonson Labs Inc., Gilroy, Calif.). Animals were housed three per cage, and fed a normal diet and water ad lib. until used.

**HLE.** Fresh buffy coats from human blood were obtained from the San Diego Veterans Administration Medical Center. The buffy coat was mixed with an equal volume of 4% dextran (250,000 mol wt) in saline and allowed to settle for 30 min at 4°C. A sample of the supernatant solution was analyzed for total erythrocyte count on a hemocytometer, and the remainder was centrifuged at 200 *g* at 4°C for 20 min. To the resulting supernatant fluid, 10 ml of water was added for every 10<sup>9</sup> cells; after 1 min isotonicity was restored by the addition of an equal volume of 0.3 M KCl. The pellet obtained after centrifugation at 200 *g* for 15 min (4°C) was washed twice with 0.02 M phosphate in 0.15 M NaCl, pH 7.4, (phosphate-buffered saline; PBS). Lysosomal granules were recovered from these leukocytes as described (18, 21), and the granules lysed by repeated freeze-thawing (five times). The supernatant fluid obtained after centrifugation at 27,000 *g* for 20 min (4°C) was stored at –20°C until purified.

Purification of this crude lysosomal extract was performed by affinity chromatography on a Sepharose-elastin column (1.6 × 20 cm) as described (21). The activity of the collected fractions (5.0 ml) was measured as esterase activity using *N*-t-BOC-L-alanine-*p*-nitrophenyl ester (NBA, Pierce Chemical Co., Rockford, Ill.) according to the method of Vissar and Blout (22). The pooled active fractions were concentrated with 70% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and then dialyzed against PBS and concentrated by ultra-filtration (UM-2 membrane, Amicon Corp., Lexington, Mass.) to contain >10,000 NBA esterase U/ml. Polyacrylamide gel electrophoresis of this purified enzyme

using the method of Reisfeld et al. (23) demonstrated the typical elastase isoenzyme pattern (18, 21). SDS-polyacrylamide gel electrophoresis of this enzyme revealed a molecular weight of 22,000–24,000.

**Human PF<sub>4</sub>.** Purification of human PF<sub>4</sub> for use in these experiments was carried out by a published method (24), and the fractions from the heparin Σ-aminocaproic acid-Sepharose affinity column, which contained PF<sub>4</sub>, were pooled. PF<sub>4</sub> activity on all samples was measured by the method of Poplawski and Niewiarowski (25) as modified by Levine and Wohl (24). The heparin-neutralizing activity of PF<sub>4</sub> ranged from 8–15 μg PF<sub>4</sub>/U heparin. Before use in these experiments, PF<sub>4</sub> in column buffer was dialyzed against PBS. The activity of PF<sub>4</sub> was unaffected by this buffer change. Some of this PF<sub>4</sub> was radioiodinated with <sup>125</sup>I, according to the method of David and Reisfeld (26).

**Protein measurement.** Protein determination on all samples was made by the method of Lowry et al. (27).

**Lung elastin purification.** Lungs were removed from hamsters that had been anesthetized (Surital, Parke-Davis, Detroit, Mich.). The lungs were trimmed free of large airways and blood vessels, minced with scissors, homogenized in cold 0.15 M NaCl (Polytron homogenizer, Brinkmann Instruments, Westbury, N. Y.), and washed repeatedly with cold 0.15 M NaCl until blood free. The homogenate was defatted with chloroform:methanol (3:1, vol:vol) and the elastin was purified according to the method of Partridge, et al. (28). The final residue obtained was washed with water and lyophilized. 200 μg samples were hydrolyzed in 5.7 N HCl in evacuated tubes at 108°C for 24, 48, and 72 h. Amino acid analysis of the hydrolysates was performed on a Durrum D-500 amino acid analyzer (Durrum Instrument Corp., Sunnyvale, Calif.) using a lithium citrate gradient. The amino acid composition of the residue agreed to within 5% with previously published data for hamster lung elastin (29).

**Radiolabeling of hamster lung elastin.** The lung elastin obtained above was tritiated according to a modification of the procedure of Lent et al. (30). A standardized mixture of NaBH<sub>4</sub> and NaB<sup>3</sup>H<sub>4</sub> (222 Ci/mole, New England Nuclear Corp., Boston, Mass.) was prepared according to the method of Blumenfeld and Gallup (31), and reduction was allowed to proceed for 1 h at a pH of 8.5–9.0. The specific activity of the <sup>3</sup>H-elastin obtained was 1.54 × 10<sup>5</sup> cpm/g.

**Effects of PF<sub>4</sub> and other proteins on elastolytic, proteolytic, and esterolytic activity of HLE.** Reduced, tritiated lung elastin was suspended in 0.01% Triton-X in 0.1 M phosphate buffer containing 0.15 M NaCl (pH 7.4) and stirred at room temperature for 1 h. This detergent treatment of the elastin was necessary to prevent clumping of the substrate and allow for reproducible pipetting. The pretreatment of elastin with Triton-X had no measurable effect on the elastolytic rate with either HLE or pancreatic elastase. The suspension was centrifuged (2,000 *g* for 10 min), the supernatant fluid decanted, and the Triton-treated <sup>3</sup>H-elastin was suspended in PBS at a concentration of 200 mg/ml and stored at 4°C. To test the effect of PF<sub>4</sub> on HLE using <sup>3</sup>H-hamster lung elastin as the substrate, 100 μl of suspended <sup>3</sup>H-elastin was added to 1.5-ml Eppendorf micro test tubes (Brinkmann Instruments) containing 900 μl of the following solutions: PBS (<sup>3</sup>H background), 5 μg purified trypsin in PBS (Worthington Biochemical Corp., Freehold, N. J.); tube added to measure nonspecific proteolysis of substrate), 5 μg HLE in PBS, and 5 μg HLE that contained PF<sub>4</sub> at various concentrations. Before the addition of <sup>3</sup>H-elastin these tubes were allowed to preincubate for periods of time ranging from 2 to 30 min at room temperature. Incubation of <sup>3</sup>H-elastin with the test solutions was carried out at 37°C in a shaking water bath for 3 h. The reaction was terminated by cooling the tubes to 4°C, and the tubes were

spun in a microcentrifuge (Brinkmann Instruments) at 4,000 g for 8 min at 4°C. 400  $\mu$ l were pipetted into 20-ml screw-top scintillation vials containing 10 ml of Biofluor (New England Nuclear Corp.), and these vials were counted in a scintillation counter (Mk II, Nuclear Chicago, Chicago, Ill.).

The effects of PF<sub>4</sub> on the esterolytic activity of HLE were carried out using NBA as the substrate (22). The effects of PF<sub>4</sub> on the nonspecific proteolytic activity of HLE was measured using casein (purified powder, Sigma Chemical Co., St. Louis, Mo.) which had been radioiodinated with <sup>125</sup>I (26) as the substrate. The <sup>125</sup>I-casein assays were carried out according to the method of Katchman, et al. (32). Experiments were also carried out to test the effect of other proteins on the elastolytic activity of HLE (using <sup>3</sup>H-elastin as substrate). These proteins included poly-1-lysine (Types II, III, and V, Sigma Chemical Co.), bovine serum albumin (Sigma Chemical Co.), and ovalbumin (Sigma Chemical Co.). The methodology used was identical to that outlined for PF<sub>4</sub>.

**Effect of PF<sub>4</sub> and HLE on lung elasticity in vitro.** Hamsters were anesthetized by intraperitoneal injection of 0.1 mg phenobarbital/g body wt, and the trachea was cannulated with an 18 gauge polyvinyl catheter with a stopcock attached. Animals were mechanically ventilated (Small Animal respirator, Harvard Instrument Co., Ayer, Mass.) with 100% O<sub>2</sub> using a tidal volume of 3.0 ml at a rate of 60 breaths/min. After 15 min the animals were asphyxiated by occlusion of the tracheal cannula at end expiration. This technique allowed for total degassing of the lungs. The thorax was opened and the lungs were perfused via the right ventricle with cold PBS until bloodless. The lungs and heart were removed en block by careful dissection. The preparation was discarded at this stage if the lungs did not appear small and liverlike. Lungs were rinsed in 0.15 M NaCl, blotted dry, and weighed.

3 ml of the following solutions were instilled into the lungs through the tracheal cannula: PBS (pH 7.4), 40  $\mu$ g HLE in PBS, 40  $\mu$ g HLE plus 44  $\mu$ g PF<sub>4</sub> (ratio PF<sub>4</sub>: HLE = 3.4:1), and 44  $\mu$ g PF<sub>4</sub> alone. If the fluid did not distend the lungs uniformly, the preparation was discarded. The preparation was incubated at 37°C in a humidified incubator for 2 h. The amount of HLE used was determined in preliminary experiments designed to find the dosage of HLE that would alter lung compliance minimally. At the end of the incubation period, as much solution as possible was removed from the lungs using gentle suction; the preparation was blotted dry and weighed.

Inflation and deflation pressure-volume curves were measured in saline using a modification of the method described by Karlinsky, et al. (33). The preparation was suspended in a large saline-filled tank, and the tracheal cannula was connected through a saline-filled tube to a 10-ml syringe mounted in a Harvard variable-speed infusion-withdrawal pump (Harvard Instruments Co.). The saline in the syringe was colored with a small amount of methylene blue so that small leaks could be detected. Transpulmonary pressure (P<sub>L</sub>) was measured using a PR-23 differential pressure transducer (Statham Instruments, Oxnard, Calif.) which was placed at the level of the lungs and connected to the infusion-withdrawal line through a saline-filled side arm. P<sub>L</sub> was measured as the difference between intrapulmonary pressure and water pressure at the level of the lungs. The pressure signal was fed into a DR-12 recorder with multitrace (Electronics for Medicine Inc., Pleasantville, N. Y.), and photographed. The volume injected was determined by prior calibration of the variable-speed infusion system. Pressure-volume (PV) curves were obtained in each animal after first predicting (34) the total lung capacity at a P<sub>L</sub> of 25 cm H<sub>2</sub>O (TLC<sub>25</sub>). As many as seven 1-ml instillations of saline were made, each during 15 s. After each increment, 5 s was permitted to elapse, and P<sub>L</sub> points were

recorded. Infusions continued until the predicted TLC<sub>25</sub> was reached. If no leaks were noted, and a plateau in P<sub>L</sub> had not been reached, an additional 0.5 ml was added. Withdrawals of saline were then made at the rate of 1 ml/15 s, and at low lung volumes a decreased rate of 0.5 or 0.25 ml/15 s was used. Two PV cycles were performed in each pair of lungs, the first with an infused volume of 4.0 ml to standardize the volume history. Infusions to predicted TLC<sub>25</sub> were made on the second cycle. Any preparation was discarded that had experienced a large leak (by sight) or a minimal leak that did not stop on the first deflation.

PV curves were constructed from PV points obtained during the second infusion-withdrawal cycle. Zero volume was set to the volume of lung prior to incubation, or tissue volume. Volume at each P<sub>L</sub> was expressed as the percentage of the total lung volume obtained (% V<sub>L,max</sub>). Inflation and deflation PV curves were fitted to the plotted points, and P<sub>L</sub> values for 40, 60, 80, and 100% of V<sub>L,max</sub> obtained by interpolation. Chord compliance was calculated as previously described (33) as the slope of the line drawn between 40 and 60% of V<sub>L,max</sub> for mid-lung volume; between 80 and 100% V<sub>L,max</sub> for high-lung volume. Data were subjected to analysis of variance followed by Scheffe's test for intergroup differences (35). P values < 0.05 were considered significant.

**Morphologic studies.** Four lungs from each of the above defined groups were fixed in 10% formalin at a pressure of 12.5 cm H<sub>2</sub>O. After overnight fixation the lungs were stored in formalin until all the lungs to be embedded were fixed. Lungs were blocked and embedded in paraffin at the same time to equalize any artifact induced, and were sectioned to a thickness of 5  $\mu$ m. Sections were stained with hematoxylin and eosin. The distance between alveolar walls was measured as the mean linear intercept (L<sub>m</sub>). L<sub>m</sub> was measured in 20 microscopic fields at  $\times$ 200 magnification. The alveolar internal surface area was calculated as described (36).

**Biochemical studies of hamster lungs incubated in vitro.** Hamster lung-heart preparations were prepared as described above for physiologic studies, and inflated with 3.0 ml of one of the following solutions: PBS (pH 7.4), 40  $\mu$ g HLE in PBS, 40  $\mu$ g HLE plus 44  $\mu$ g PF<sub>4</sub>, and 44  $\mu$ g PF<sub>4</sub> alone. The HLE and PF<sub>4</sub> were from the same batches used in the physiologic studies. The lungs were incubated, as above, for 2 h at 37°C. After incubation, as much of the solution that could be obtained by gentle suction was removed via the tracheal cannula. Five lungs were studied in each group, and the lungs were pooled into four groups. The pooled lungs were homogenized with a polytron homogenizer (Brinkmann Instruments) and washed free of blood with cold PBS. The homogenates were defatted in chloroform: methanol (3:1, vol:vol) and then extracted overnight at 4°C in 0.15 M NaCl. The homogenates were collected by centrifugation, washed with cold H<sub>2</sub>O, and lyophilized. Aliquots of these homogenates were analyzed for total hydroxyproline (37), and the amount of collagen contained was calculated by multiplying the amount of hydroxyproline by 7.7 (38). The small contribution of elastin to hydroxyproline content was ignored. 10–30 mg of the homogenates was extracted for 1 h in boiling 0.1 N NaOH, and the residue remaining was analyzed for elastin content using the micro method of Naum and Morgan (39). Six determinations were made for collagen and elastin content in each pool of saline-extracted lungs. Data were subjected to analysis of variance and intergroup differences were determined using Scheffe's test.

## RESULTS

**Effects of PF<sub>4</sub> and other proteins on HLE activity.** Purified PF<sub>4</sub> resulted in no change in the esterolytic

activity of 5  $\mu\text{g}$  of HLE against the synthetic substrate NBA (Fig. 1). The nonspecific proteolytic activity of HLE against  $^{125}\text{I}$ -casein was essentially unchanged at low  $\text{PF}_4$  concentrations, and was modestly stimulated (20% increase) at  $\text{PF}_4$  concentrations  $>13 \mu\text{g}/\text{ml}$  (molar ratio of  $\text{PF}_4$ :HLE = 8:1). Control tubes containing HLE without added  $\text{PF}_4$  released  $1.2 \times 10^3$  cpm from  $^{125}\text{I}$ -casein after the 2-h incubation, and background tubes containing casein substrate and either  $\text{PF}_4$  or PBS alone released  $<100$  cpm. The activity of HLE against  $^3\text{H}$ -elastin was increased by as little as 1.6  $\mu\text{g}/\text{ml}$   $\text{PF}_4$  (molar ratio of  $\text{PF}_4$ :HLE = 1:1), and HLE stimulation progressed linearly as the concentration of  $\text{PF}_4$  increased (Fig. 1). Control tubes (5  $\mu\text{g}$  HLE alone) released  $1.9 \times 10^4$  cpm from  $^3\text{H}$ -elastin in 3 h, and background tubes released from 500–800 cpm. As described under Methods,  $\text{PF}_4$  and HLE were allowed to preincubate for periods up to 30 min. However, the length of time of this preincubation did not alter the amount of stimulation of HLE seen. Each of the polylysine types that were tested also possessed HLE stimulatory activity against  $^3\text{H}$ -elastin with polylysine V (30,000 mol wt) resulting in the most significant stimulation. All polylysines were active at a molar ratio of polylysine:HLE of 1:1. Ovalbumin and bovine serum albumin had no effect on the elastolytic activity of HLE. All enzyme assays were linear with time and protein concentrations.

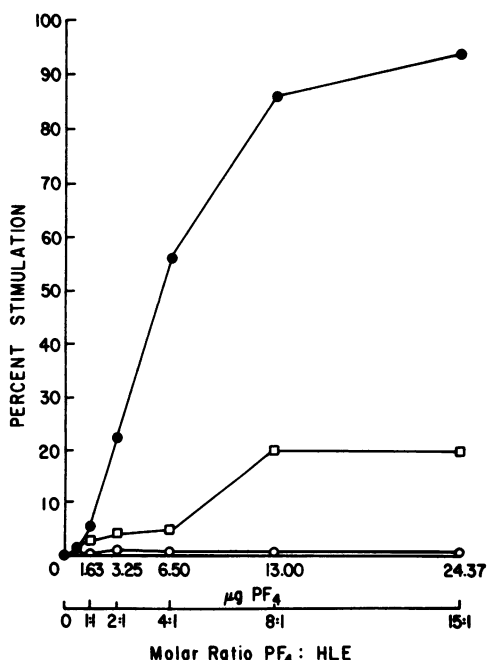


FIGURE 1 Stimulation of HLE by  $\text{PF}_4$  either reduced, tritiated hamster lung elastin (●), NBA (○), or  $^{125}\text{I}$ -casein (□) as the substrate. Each point is the average of two separate determinations. ALL incubations were carried out at  $37^\circ\text{C}$  for 3 h in a total volume of 1.0 ml.

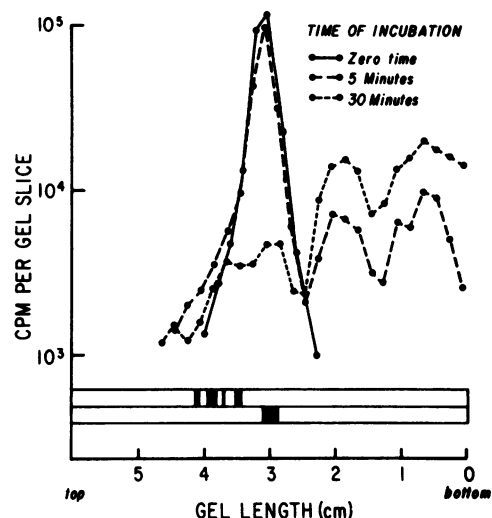


FIGURE 2 Proteolysis of  $^{125}\text{I}$ - $\text{PF}_4$  by purified HLE.  $\text{PF}_4$  was incubated with enzyme for varying times, as indicated above, and the mixture was run on SDS polyacrylamide gel electrophoresis (PAGE) (10% acrylamide). Gels were sliced, and 2-mm gel fragments were counted for radioactivity. Coumassie blue stained SDS-PAGE gels of purified HLE (upper) and  $\text{PF}_4$  are shown for reference.

Initial attempts to recover  $\text{PF}_4$  after the incubation with HLE yielded conflicting results. In an attempt to determine the fate of  $\text{PF}_4$  incubated with HLE, we studied the effect of HLE on radiolabeled  $\text{PF}_4$  at the time of mixing (zero time), 5 min, and 30 min after mixing. We found that HLE was capable of cleaving  $\text{PF}_4$  into at least two radiolabeled fragments (Fig. 2), and that this  $\text{PF}_4$  proteolysis was present within 5 min of incubation. At the end of 30 min  $>75\%$  of the native  $\text{PF}_4$  had been fragmented.

*Changes in lung elasticity induced by  $\text{PF}_4$ -HLE mixture.* A total of 52 lungs (13 in each group) were successfully degassed and removed from hamsters. Nine lungs developed visible leaks during inflation with saline, and were discarded. Data from the 43 lungs studied are summarized in Table I. The mean body weights of the four groups of animals were the same, so the predicted  $\text{TLC}_{25}$  of the groups was similar. Therefore, the  $V_{t \max}$  of the lungs in each group were similar. The amount of fluid that was retained in the lungs following incubation with the mixture of HLE and  $\text{PF}_4$  was significantly greater than in the control lungs ( $P < 0.001$ ) or lungs treated with HLE alone ( $P < 0.01$ ).

Mean  $\pm$  SE values for  $P_L$  at 40, 50, 60, 70, 80, 90, and 100%  $V_{t \max}$  are plotted for inflation in Fig. 3 and for deflation in Fig. 4. Inasmuch as the values of  $V_{t \max}$  for the four groups were similar (Table I), comparisons of  $P_L$  at percentages of  $V_{t \max}$  are valid. Because both the inflation and deflation PV curves for the groups treated with  $\text{PF}_4$  alone were identical to the control curves, they were omitted for the sake of clarity. While the in-

TABLE I  
Mean  $\pm$  SE Body Weights, Total Lung Volumes, and Trapped Fluid Volumes  
in Four Groups of Hamster Lungs Treated In Vitro

|                        | Control         | HLE             | PF <sub>4</sub> | HLE and PF <sub>4</sub> | F*    |
|------------------------|-----------------|-----------------|-----------------|-------------------------|-------|
| n                      | 13              | 11              | 8               | 11                      |       |
| Weight, g              | 120.1 $\pm$ 3.2 | 124.0 $\pm$ 3.2 | 124.7 $\pm$ 3.5 | 121.2 $\pm$ 2.9         | 1.031 |
| Total fluid volume, ml | 7.62 $\pm$ 0.08 | 7.86 $\pm$ 0.12 | 7.72 $\pm$ 0.13 | 7.85 $\pm$ 0.10         | 1.303 |
| Trapped fluid, ml      | 0.92 $\pm$ 0.07 | 1.08 $\pm$ 0.07 | 1.05 $\pm$ 0.09 | 1.89 $\pm$ 0.11         | 4.51† |

\* F ratio from analysis of variance.

†  $P < 0.01$ ; Scheffe's test shows 1.89 significantly different from 0.92 ( $P < 0.001$ ), and from 1.08 and 1.05 ( $P < 0.01$ ).

flation and deflation curves of the lungs treated with elastase alone were shifted only slightly upwards and to the left over the entire volume range, the addition of PF<sub>4</sub> to HLE in the incubation fluid resulted in a statistically significant leftward shift of the PV curve during both inflation and deflation. This decrease in elastic recoil pressure was noted at both mid and high volumes.

Comparisons of chord compliance during inflation and deflation over both mid- and high-volume ranges are given in Table II. The chord compliance of lungs treated with HLE alone or PF<sub>4</sub> alone were similar to the chord compliance of control lungs. Lungs treated with a mixture of HLE and PF<sub>4</sub> had chord compliances that were significantly greater than control lungs (or elastase treated lungs) at mid-lung volume and high-lung volume. This difference was significant during inflation and during deflation with saline.

**Lung connective tissue content.** The content of lung elastin and of lung collagen in each preparation is shown in Table III. Treatment with HLE alone resulted in a small and not significant decrease in lung

elastin expressed either as milligrams of elastin per lung or as a percentage of the freeze-dried weight. Treatment with PF<sub>4</sub> alone had no effect on lung-elastin content. Lungs incubated with a mixture of HLE and PF<sub>4</sub> experienced a significant lowering of lung-elastin content when compared with control preparations or preparations incubated with HLE alone. The collagen content, expressed either as milligrams collagen per lung or as a percentage of freeze-dried weight was not altered in any of the treatment groups (Table III).

**Morphologic studies.** Representative histologic preparations from each treatment group are shown in Fig. 5. Except for some perivascular edema, the PBS control preparations showed no abnormalities. The preparations incubated with PF<sub>4</sub> alone showed no significant morphological differences from the control preparation (Figs. 5A and 5B). Lungs that had been incubated with HLE alone demonstrated patchy areas of emphysematous changes, with increased alveolar size and dilatation of alveolar ducts (Fig. 5C). The  $L_m$  in this group of lungs was significantly greater than the  $L_m$  of control animals ( $P < 0.05$ , Table IV), and the internal surface area significantly reduced ( $P < 0.05$ ,

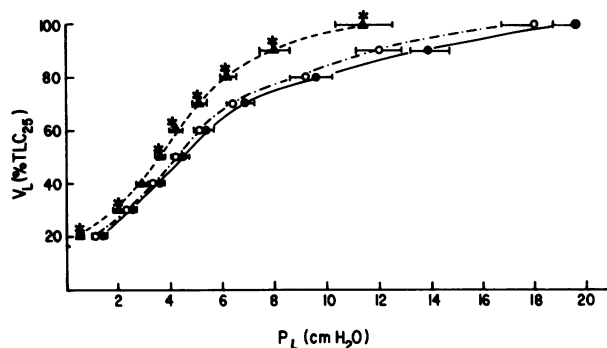


FIGURE 3 Mean inflation PV curves. Mean inflation saline-filled PV curves of in vitro control lungs, lungs treated with low-dose HLE and lungs treated with low-dose HLE plus PF<sub>4</sub>. Bars show SE of means. Stars indicate data points significantly different from control values by Scheffe's test ( $P < 0.01$ ). See text for discussion.  $V_L$ , lung volume; ●, control; ○, HLE; ▲, HLE plus PF<sub>4</sub>.

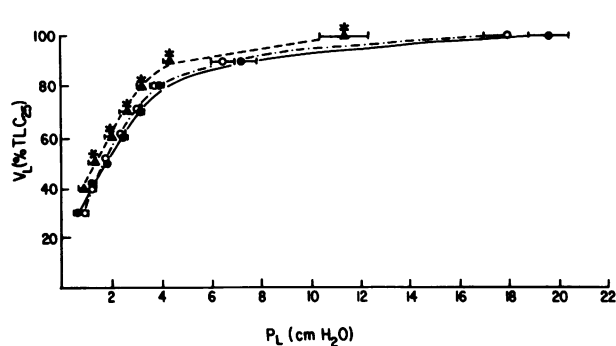


FIGURE 4 Mean deflation PV curves. Mean deflation saline-filled PV curves of in vitro control lungs, lungs treated with low-dose HLE, and lungs treated with low-dose HLE plus PF<sub>4</sub>. Bars show SE of means. Stars indicate data points significantly different from control values by Scheffe's test ( $P < 0.01$ ). See text for discussion.  $V_L$ , lung volume; ●, control; ○, HLE; ▲, HLE plus PF<sub>4</sub>.

**TABLE II**  
*Comparison of Mean ± SE Inflation and Deflation Chord Compliances over Mid- and High-Lung Volume Ranges in Four Groups of Hamster Lungs Treated In Vitro*

| Volume range | Percent total fluid volume/cm H <sub>2</sub> O P <sub>L</sub> |            |                   |                           |                     |
|--------------|---|------------|-------------------|---------------------------|---------------------|
|              | Control*  | HLE*       | PF <sub>4</sub> * | HLE and PF <sub>4</sub> * | F†                  |
| 40–60%       |   |            |                   |                           |                     |
| Inflation    | 11.78±0.38  | 13.18±0.35 | 11.27±0.42        | 15.47±0.47                | 20.12§              |
| Deflation    | 14.11±0.61  | 15.30±0.58 | 13.08±0.42        | 17.03±0.64                | 7.36 <sup>¶</sup>   |
| 80–100%      |   |            |                   |                           |                     |
| Inflation    | 2.08±0.14   | 2.25±0.20  | 2.00±0.12         | 4.90±0.70                 | 12.83 <sup>¶¶</sup> |
| Deflation    | 1.32±0.07   | 1.54±0.15  | 1.20±0.06         | 2.79±0.27                 | 19.30**             |

\* n values: control, 13; elastase, 11; PF<sub>4</sub>, 8; elastase and PF<sub>4</sub>, 11.

† F ratio from analysis of variance.

§ F, *P* < 0.001; Scheffe's test: 15.47 significantly different from 11.78 (*P* < 0.001) and from 13.18 (*P* < 0.01).

<sup>¶</sup> F, *P* < 0.01; Scheffe's test: 17.03 significantly different from 14.11 (*P* < 0.05).

<sup>¶¶</sup> F, *P* < 0.01; Scheffe's test: 4.90 significantly different from 2.08 and from 2.25 (*P* < 0.001).

\*\* F, *P* < 0.001; Scheffe's test: 2.79 significantly different from 1.32 and from 1.54 (*P* < 0.01).

Table IV). Lungs that had been incubated with a mixture of HLE and PF<sub>4</sub> showed the most severe damage on histologic sectioning, with marked subpleural air-space dilatation and alveolar duct enlargement (Fig. 5D). The L<sub>m</sub> for these preparations was significantly increased when compared with either the control or HLE alone preparations (Table IV), and the internal surface area of these lungs was significantly reduced (Table IV).

## DISCUSSION

Previous data from our laboratory have shown that PF<sub>4</sub> is capable of stimulating HLE when bovine ligamentum nuchae elastin is used as the substrate (20). The experiments presented in this paper show that PF<sub>4</sub> is capable of stimulating the elastolytic activity of HLE

when either purified hamster lung elastin or intact lungs are used as the substrate. The stimulation of the elastolytic activity of HLE is not specific to PF<sub>4</sub> since similar stimulation was seen with poly-L-lysine.

It has been postulated that for a protein to have elastolytic activity, it must first be adsorbed onto the insoluble substrate, elastin (40, 41). Because elastin contains a high percentage of nonpolar amino acids (29) and carries a small negative charge (40, 42), adsorption can take place either by hydrophobic interactions or via electrostatic forces. In keeping with this hypothesis, the reaction between porcine pancreatic elastase and elastin is heavily dependent on the charge difference between the basic enzyme and elastin. It can be nearly completely inhibited in the presence of 0.2 M NaCl (41), and enhanced when elastin has been precoated with hydrophobic anionic ligands (42, 43). Initial data

**TABLE III**  
*Mean ± SE Elastin and Collagen Content of Four Groups of Hamster Lungs Incubated In Vitro*

|                             | Control    | HLE        | PF <sub>4</sub> | HLE and PF <sub>4</sub> | F*                 |
|-----------------------------|------------|------------|-----------------|-------------------------|--------------------|
| Elastin, mg/lung†           | 2.24±0.09  | 2.17±0.06  | 2.25±0.07       | 1.81±0.07               | 8.62 <sup>¶</sup>  |
| Elastin, % freeze-dried wt  | 3.82±0.10  | 3.60±0.09  | 4.08±0.09       | 3.11±0.07               | 8.10 <sup>¶¶</sup> |
| Collagen, mg/lung§          | 5.83±0.58  | 5.92±0.19  | 6.06±0.43       | 5.84±0.16               | 1.49               |
| Collagen, % freeze-dried wt | 10.56±0.55 | 11.51±0.37 | 11.20±0.71      | 10.54±0.29              | 1.10               |

\* F value from analysis of variance.

† Elastin determinations are mean ± SE of eight separate determinations.

§ F, *P* < 0.01; Scheffe's test: 1.81 significantly different from 2.24 (*P* < 0.001), and from 2.17 (*P* < 0.01).

<sup>¶</sup> Collagen determinations are mean ± SE of six separate determinations.

<sup>¶¶</sup> F, *P* < 0.01; Scheffe's test: 3.11 significantly different from 3.82 and 3.60 (*P* < 0.01).

**TABLE IV**  
*L<sub>m</sub> and Internal Surface Area in Four Groups of Hamster Lungs Incubated In Vitro\**

|                        | Control    | HLE        | PF <sub>4</sub> | HLE and PF <sub>4</sub> | F†     |
|------------------------|------------|------------|-----------------|-------------------------|--------|
| L <sub>m</sub> , μm    | 32.14±0.11 | 39.74±0.11 | 34.40±0.09      | 45.9±0.12               | 33.34§ |
| ISA, cm <sup>2</sup> ‡ | 631.3±18.5 | 523.6±3.9  | 650.9±6.0       | 489.1±5.0               | 20.33¶ |

Data represented as mean±SE.

\* All lungs were fixed with neutral buffered formalin (10%) at an inflation pressure of 12.5 cm H<sub>2</sub>O.

† F value from analysis of variance.

§ F, *P* < 0.01: Scheffe's test shows 45.9 significantly different from 32.14 and 39.74 (*P* < 0.01). Also, 39.74 is significantly different from 32.14 (*P* < 0.05).

‡ ISA, internal surface area.

¶ F, *P* < 0.01: Scheffe's test shows 498.1 significantly different from 631.3 and 523.6 (*P* < 0.01). Also, 523.6 is significantly different from 631.3 (*P* < 0.05).

concerning HLE have shown that its adsorption onto elastin is much less charge dependent than the pancreatic enzyme (1), and unpublished observations in our laboratory have shown that HLE retains 75% of its activity against elastin at salt concentrations as high as 0.45 M. Therefore, although Gertler (41) has shown that poly-L-lysine is adsorbed onto elastin and effectively inhibits the subsequent adsorption (and elastolytic activity) of pancreatic elastase, our data show that such electrostatic forces are less important in determining the activity of HLE. Indeed, poly-L-lysine is, like PF<sub>4</sub>, a potent stimulator of HLE activity.

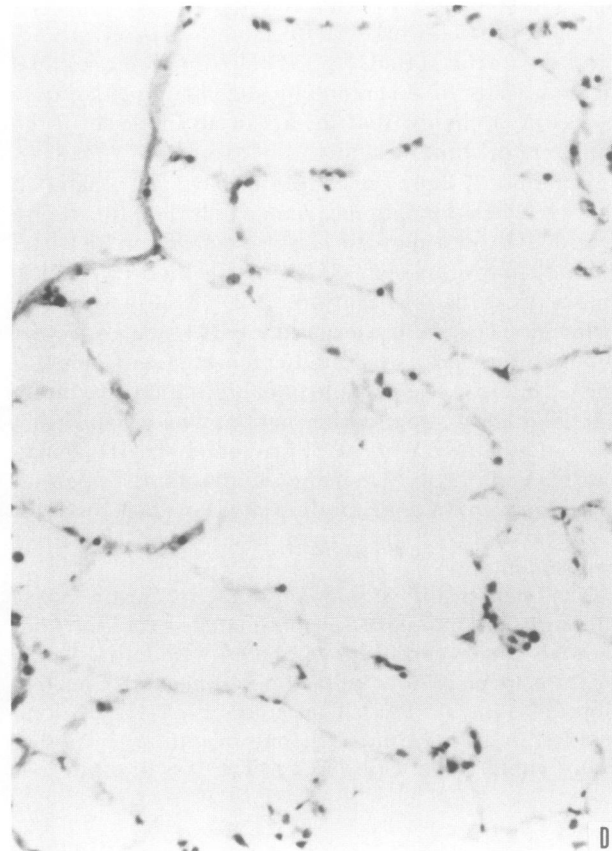
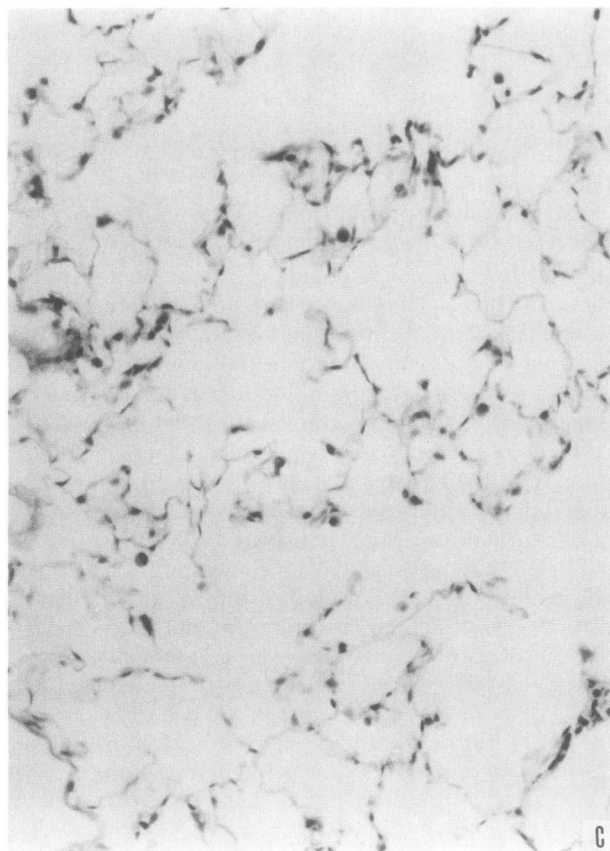
Kagan and co-workers (42), and Jordan et al. (43) have shown that the adsorption of hydrophobic, anionic ligands leads to a conformational change in soluble α-elastin, a change that results in an increase in the number of binding sites for pancreatic elastase. Furthermore, these investigators have demonstrated that such a conformational change enhances the elastolytic activity of pancreatic elastase more than can be accounted for by this increase in enzyme binding (42, 43). Inasmuch as PF<sub>4</sub> is a basic protein by virtue of its lysine-rich region near the carboxyterminus (44), it is likely that this protein, and its radioiodinated fragments, are adsorbed onto the elastin molecule. Preliminary data from our laboratory support this notion. We propose that PF<sub>4</sub>, and the fragments of PF<sub>4</sub> generated by HLE, may cause a conformational change in elastin and open up previously unavailable binding sites for attack by HLE. Confirmation of this hypothesis awaits further experiments.

The statistically significant loss of elastic recoil pressure and increase in compliance over the mid-volume range seen in lungs treated with an HLE-PF<sub>4</sub> mixture are consistent with stimulation of HLE against lung elastin by PF<sub>4</sub>. The amount of HLE alone needed to give a similar change in mid-lung volume chord compliance was >65 μg. The loss of elastic re-

coil pressure measured at lung volumes close to TLC<sub>25</sub> in the HLE-PF<sub>4</sub> lungs cannot be explained by effects on elastin alone if we view the PV diagram of the normal lung according to the hypotheses of Stenikar (45) and Mead (46). These hypotheses postulate that the PV relationship measured in the normal lung can be partitioned according to the elastic qualities of the two main connective tissue elements of the lung, elastin and collagen. Elastin, which is a highly extensible fiber and can be stretched to 130% of its resting length (47), is believed to be responsible for the shape of the PV curve at low and mid-lung volumes. Collagen, with a high elastic modulus allowing for fiber lengthening of only 2% (48), is believed to be responsible for the shape of PV curve at high-lung volumes. Experiments that have tested these hypotheses using either intact lungs (33) or isolated alveolar walls (49, 50) have generally been in agreement.

Our analyses of elastin and collagen content in lungs from each experimental group failed to show any significant lowering of lung-collagen content to account for the increased compliance seen at high-lung volumes in the HLE-PF<sub>4</sub> group. An explanation for this dichotomy can be found if, rather than viewing the elastin and collagen network of the lung as functioning independently, we agree with the postulate that the two connective tissue elements interact at specific contact points (33, 51), and that this elastin-collagen interaction allows for smooth transition from one part of the PV curve to another. HLE stimulated by PF<sub>4</sub> could be viewed as having the ability to attack lung elastin more completely than HLE alone, resulting in a disruption of these elastin-collagen contact points and a loss of elastin recoil pressure at high-lung volumes.

The light microscopic data demonstrate that although 40 μg of HLE alone did not result in a significant shift in the PV curve or a lowering of lung elastin content, there was alveolar over-distension in these lungs with a





significant increase in  $L_m$ . We postulate that at this dose of HLE elastin fibers have been damaged, but not completely solubilized. Such elastin "nicking" would result in a minimal physiologic lesion, as was found in this study. Support for this hypothesis comes from in vivo work where electron micrographs reveal such minimal damage in lung zones which are abnormal on light microscopic examination following the instillation of papain (52), and from in vitro studies that show that a significant number of peptide bonds in elastin must be split before complete solubilization takes place (40).

The present study demonstrates that  $PF_4$  is capable of stimulating HLE activity against lung elastic tissue in vitro. This stimulation enhances the biochemical, physiological, and morphological effects of HLE in the isolated hamster lung. Although this work was done in vitro, it is quite possible that such a mechanism is operable in vivo. If true, then in the presence of  $PF_4$ , an otherwise nonpathogenic dose of HLE might be stimulated so that significant elastic tissue disruption takes place.

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FIGURE 5 Histologic sections of hamster lungs after intratracheal instillation of test solutions. All lungs were incubated for 2 h at 37°C and then inflation fixed in neutral buffered 10% formalin at an inflation pressure of 12.5-cm  $H_2O$ . 5A: control lung treated with PBS, pH 7.4. 5B: Lung treated with 44  $\mu$ g of  $PF_4$  in 3.0 ml of PBS. 5C: Lung treated with 40  $\mu$ g HLE alone. 5D: Lung treated with 40  $\mu$ g HLE plus 44  $\mu$ g  $PF_4$ . All lungs are stained with hematoxylin and eosin. Original magnification  $\times 200$ .

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