Digestion of Lung Proteins by Human Leukocyte Granules *in Vitro*[1] (36493)

AARON JANOFF, ROBERT A. SANDHAUS, VERNE D. HOSPELHORN, AND RONALD ROSENBERG
(Introduced by Arthur C. Upton)

Health Sciences Center, State University of New York at Stony Brook, Stony Brook, New York 11790; Will Rogers Hospital, Saranac Lake, New York 12983; New York University School of Medicine, New York, New York 10016

The discovery of a correlation between inherited deficiency of serum alpha-1-antitrypsin and incidence of familial emphysema (1, 2) suggested a role for proteases in the pathogenesis of the latter disease. Development of anatomic changes in animal lungs resembling those seen in diffuse, panacinar emphysema of man, following endotracheal administration of exogenous proteases (3, 4) lent further support to the "protease-pathogenesis" hypothesis. In its present state, this hypothesis implicates leukocytes as one potential source of endogenous proteases participating in destruction of lung tissue in emphysema (5–9). Neutral proteases of human polymorphonuclear leukocytes (PMN) are inhibited by alpha-1-antitrypsin (6, 7, 10) and thus may play a role in emphysema associated with abnormally low levels of this inhibitor. We studied the ability of human PMN granules to digest proteins of human and mouse lungs *in vitro*. Similar studies with proteases isolated from purulent human sputum have recently been reported (7). Preliminary results of our experiments are presented below.

Materials and Methods. Lung substrates. Fractions containing crude connective tissue and connective tissue partially freed of collagen by prior enzymatic treatment (glycoprotein-enriched) were prepared from human lungs (normal adult, infant, adult emphysema) according to methods previously described by one of us (11). The glycoprotein-"enriched" fractions contained 60% collagen, 25% glycoprotein and 15% elastin. Total protein was 85% of the weight of the fractions, the rest was composed of nonprotein materials (unidentified). To obtain a fraction further enriched in elastin, the glycoprotein-enriched samples were extracted with 85% phenol:15% acetic acid, washed thoroughly, and the resultant residues were then extracted with 1% sodium dodecylsulfate (SDS). The treatment with phenol:acetic acid and SDS removed additional collagen and glycoprotein leaving a protein residue with a relative 3-fold enrichment in elastin. Specifically, the final residue was composed of 40% elastin, 20% glycoprotein and 40% collagen. These proteins again constituted 85% of the total residue, the balance being unidentified, nonprotein materials. Finally, SDS was removed by washing with 95% ethanol.

In addition to the human lung fractions described above, whole lung powders were made from mouse organs in the following manner. Animals were anesthetized with sodium pentobarbital, and their lungs were perfused with cold, normal saline via the right ventricle or postcaval vein until tissues were free of visible blood. The perfused tissue was finely diced with scissors and rinsed in cold normal saline followed by cold distilled water to remove excess salt. Diced lung was then dried overnight in a vacuum desiccator at 22° and subsequently ground in a mortar and pestle. Some of the tissues were also extracted with 1 *M* saline as described below to remove lung inhibitor substances, prior to drying and powdering. (Excess salt was washed from the tissue as before.)

[1] Supported in part by grants from the National Heart and Lung Institute (HE 14262 and HE 09458), the Tuberculosis and Respiratory Disease Association of New York and the New York Chapter of the Arthritis Foundation Inc.

TABLE I. Proteolysis of Lung Connective Tissue by Enzymes and Cell Fractions.[a]

Type of substrate	Amount of substrate (mg)	Enzyme or cell fraction	Protein (μg) added	Buffer (see Methods)	Incub. time (hr)	Supernatant analysis: Protein released (mg)[b,c]	Supernatant analysis: Corrected O.D. Ninhydrin assay[b]	Supernatant analysis: Amino acids recovered Res/1000[d]: HO-proline	Supernatant analysis: Amino acids recovered Res/1000[d]: q-desmosines[e]
Crude connective tissue, normal adult lung (human)	3.0	PE	16	Tris	4	1.53	0.288	—	—
	3.0	Th	5	Tris	4	1.17	0.123	—	—
	3.0	PMN-g	270	Tris	4	1.08	0.197	—	—
Glycoprotein-enriched fraction, normal adult lung (human)	3.0	PE	16	Tris	4	0.99	0.170	—	—
	3.0	Th	5	Tris	4	0.78	0.081	—	—
	3.0	PMN-g	270	Tris	4	1.23	0.255	—	—
Glycoprotein-enriched fraction, infant lung (human)	3.0	PE	16	Tris	4	2.05	0.280	—	—
	3.0	Th	5	Tris	4	1.34	0.227	—	—
	3.0	PMN-g	270	Tris	4	0.06	0.100	—	—
Glycoprotein-enriched fraction, adult emphysema lung (human)	3.0	PE	16	Tris	4	0.54	0.258	—	—
	3.0	Th	5	Tris	4	0.84	0.238	—	—
	3.0	PMN-g	270	Tris	4	0.42	0.178	—	—
Elastin-enriched fraction, normal adult lung (human)	5.0	PE	25	Tris	4.5	3.10 (2.94)	—	40	4
	5.0	Th	10	Tris	4.5	3.30 (3.05)	—	41	4
	5.0	T	25	Tris	4.5	0.60 (0.80)	—	74	0
	5.0	PMN-g	586	Tris	4.5	0.46 (0.90)	—	35	3
Whole mouse lung powder, unwashed	3.0	PE	16	Phosphate	3	0.41	—	—	—
	3.0	Th	5	Phosphate	3	0.41	—	—	—
	3.0	PMN-g	270	Phosphate	3	0.16	—	—	—
Whole mouse lung powder, salt-washed	3.0	PE	16	Phosphate	3	0.53	—	—	—
	3.0	Th	5	Phosphate	3	0.43	—	—	—
	3.0	PMN-g	270	Phosphate	3	0.36	—	—	—

[a] PE = pancreatic elastase, Th = thermolysin, T = trypsin, PMN-g = human PMN granule extract.

[b] All numbers shown are averages of duplicate assays. Values for whole mouse lung powder are average results of two digestion experiments.

[c] Based on Lowry assay; (values) based on amino acid analysis.

[d] Supernatant of substrate incubated alone and solutions of enzymes and cell fractions contained undetectable amounts of HO-proline, desmosine and isodesmosine.

[e] Sum of quarter-desmosines and isodesmosines.

Lung inhibitor substances. Fresh mouse lungs and postmortem specimens of human lungs were perfused and diced as described above and were then vigorously shaken in 1 *M* saline at room temperature for 1 hr. The supernatants obtained by centrifugation of the tissue suspensions at 3000*g* for 15 min were stored at —20° and used in inhibition experiments.

Enzymes. Thermolysin (a bacterial elastase) was obtained 3× crystallized from Calbiochem, Los Angeles, CA. Crystallized trypsin (EC 3.4.4.4) and electrophoretically purified pancreatic elastase (EC 3.4.4.7) were both purchased from Worthington Biochemical Corp., Freehold, NJ. Extracts of human PMN granules were prepared by previously described methods (12).

Assays. Incubation of whole lung powders and of lung connective tissue fractions with enzymes or granule extracts was performed in one of two neutral buffers. These were: 0.05 *M* Tris–HCl containing 0.1 *M* NaCl and 0.001 *M* $CaCl_2$ (pH 7.6 at 37°) or 0.075 *M* sodium phosphate (pH 7.0). Incubation was generally carried out in 1 ml volumes at 37° with gentle shaking for periods ranging between 3 and 4.5 hr. (Amounts of substrates and enzymes are given under *Results.*) After incubation, the contents were centrifuged at 3000 to 4000*g* for 10 to 15 min at 4° to remove insoluble (undigested) material and the supernatants were analyzed for total soluble protein using Lowry and co-worker's method (13) with crystallized bovine serum albumin as standard. Separate, 0.1 ml aliquots of the digestion supernatants were analyzed for their relative contents of low molecular weight peptide fragments and free amino acids by performing Ninhydrin reactions according to Moore and Stein (14). Finally, identification of elastin and collagen breakdown products was based on amino acid analysis of digestion supernatants (lyophilized and acid hydrolyzed) in the Spinco and Technicon automated amino acid analyzers. Appropriate corrections were made in all three assays (Lowry, Ninhydrin, amino acid) for soluble proteins of the substrates, enzymes and granule fractions alone.

The suppression of enzyme activity by lung inhibitor substance(s) was measured using the hydrolysis of *tert*-butyloxycarbonyl (*t*-BOC)-l-alanine-*p*-nitrophenol according to methods previously described (15).

Results and Discussion. Table I shows that, at neutral pH, extracts of human PMN granules released protein from most of the lung substrates tested. The specific activity of leukocyte granules against crude connective tissue and both of the glycoprotein-enriched connective tissue fractions from adult human lung was about 5% of the specific activity of crystalline pancreatic elastase. [Previously, one of us had found that human PMN granules also possessed about 5% of the specific elastolytic activity of the same preparation of purified pancreatic enzyme (12).] However, PMN granules did not release nearly as much protein from an infant lung glycoprotein fraction, although the soluble protein which was present in the digestion supernatant of the latter appeared to be extensively degraded (see Table I).

An elastin-enriched preparation from normal, adult human lung was well digested by the purified elastases and was also attacked, albeit to a lesser extent, by human PMN granules. Against this substrate, leukocyte granules showed 1 to 2% of the specific activity of the pancreatic enzyme (see Table I). It should be noted that desmosine and isodesmosine (amino acid residues specific to elastin) were only detected in the digestion products of the leukocyte granules or crystalline elastases and not in the products of trypsin digestion.

In the experiments with powdered, whole, mouse lung an increase in susceptibility to digestion by PMN granules was noted after washing the lung in 1 *M* NaCl. Similar results were obtained in experiments with two different mouse lung preparations (average values are shown in Table I) and in one experiment with human lung (not shown). On the other hand, salt washing did not significantly affect proteolytic breakdown of the powdered lung by pancreatic elastase or thermolysin. In conjugation with this, it was observed that salt washings from mouse and hu-

TABLE II. Inhibition of Esterolysis[a] by Lung Wash.[b]

Source of wash	Vol tested (ml)	Protein tested (μg)	Inhibition (%) of PE	Inhibition (%) of PMN-g
Mouse lung	0.25	—	0	75
Human lung	0.25	180	0	80

[a] Hydrolysis of *t*-BOC-*l*-alanine-*p*-nitrophenyl ester (0.2 m*M*, pH 6.5, 25°).

[b] 1 *M* NaCl wash of diced lung previously freed of grossly visible blood by perfusion (see Methods). 0.25 ml of 1 *M* NaCl alone was not inhibitory in the assay system. PE = pancreatic elastase; PMN-g = human leukocyte granules. Activities of each were adjusted to provide equal rates of esterolysis in the absence of lung wash.

man lung tissues inhibited the esterolytic activity of PMN granules but not that of pancreatic elastase (see Table II).

Hydrolysis of *t*-BOC-*l*-alanine-*p*-nitrophenol by pancreatic elastase as well as by PMN granules is inhibited by alpha-1-antitrypsin (16). Thus, the failure of lung wash to inhibit breakdown of this substrate by the pancreatic enzyme suggests that the suppression of PMN esterolytic activity by the tissue wash was not solely due to alpha-1-antitrypsin in the wash fluid. Instead, lung tissue may contain other agents capable of blunting the neutral protease activity of human PMN, in addition to adsorbed alpha-1-antitrypsin. Alpha-2-macroglobulin is not excluded as a contributing factor in view of the report that complexes of this inhibitor with pancreatic elastase retain esterase activity against low molecular weight synthetic substrates (17). We plan to examine salt washings from a larger series of human lungs, including those of emphysema patients and patients with alpha-1-antitrypsin deficiency, for variations in capacity to inhibit leukocyte proteases.

Summary. Human PMN granules were found to release protein, at neutral pH, from insoluble connective tissue fractions prepared from human lungs and from whole, powdered, mouse lungs. The leukocyte granules also attacked human lung elastin as shown by release of desmosine and isodesmosine from a lung fraction enriched in this component. The proteolytic activity of leukocyte granules upon mouse lung powder was selectively enhanced if the lung tissue was first washed with 1 *M* NaCl. An inhibitor of PMN alanine-*p*-nitrophenyl esterase (PMN elastase) was detected in the salt wash of mouse and human lung tissue, which is different from alpha-1-antitrypsin.

The authors are grateful to Joanne Blondin for valuable technical assistance.

1. Laurell, C. B., and Eriksson, S., Scand. J. Clin. Lab. Invest. **15**, 132 (1963).

2. Eriksson, S., Acta Med. Scand. **175**, 197 (1964).

3. Gross, P., Pfitzer, E. A., Tolker, E., Babyak, M. A., and Kaschak, M., Arch. Environ. Health **11**, 50 (1965).

4. Goldring, I. P., Greenburg, L., and Ratner, I. M., Arch. Environ. Health **16**, 59 (1968).

5. Eriksson, S., Acta Med. Scand. **177** (Suppl. 432), 1 (1965).

6. Kueppers, F., and Bearn, A. G., Proc. Soc. Exp. Biol. Med. **121**, 1207 (1966).

7. Lieberman, J., and Gawad, M. A., J. Lab. Clin. Med. **77**, 713 (1971).

8. Marco, V., Mass, B., Meranze, D. R., Weinbaum, G., and Kimbel, P., Amer. Rev. Resp. Dis. **104**, 595 (1971).

9. Janoff, A., "Symposium on Proteolysis and Pulmonary Emphysema." Harper and Row, New York, in press.

10. Janoff, A., Amer. Rev. Resp. Dis. **105**, 121 (1972).

11. Fitzpatric, M., and Hospelhorn, V., Amer. Rev. Resp. Dis. **92**, 792 (1965).

12. Janoff, A., and Scherer, J., J. Exp. Med. **128**, 1137 (1968).

13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

14. Moore, S., and Stein, W. H., J. Biol. Chem. **176**, 367 (1948).

15. Janoff, A., Biochem. J. **114**, 157 (1969).

16. Rosenberg, R., Sandhaus, R., and Janoff, A., Amer. Rev. Resp. Dis., in press.

17. Steinbuch, M., Rev. Fr. Transfus. **14**, 61 (1971).

Received Nov. 8, 1971. P.S.E.B.M., 1972, Vol. 140.