5. L. Du Pasquier, X. Chardonnens, V. C. Miggiano, *Immunogenetics* **1**, 482 (1975).
6. J. Kompf, S. Bissbort, S. Gussmann, H. Ritter, *Humangenetik* **27**, 141 (1975).
7. J. Kompf and S. Bissbort, *Hum. Genet.* **32**, 197 (1976); K. Bender and K.-H. Grzeschik, *Cytogenet. Cell Genet.* **16**, 93 (1976); E. R. Giblett and M. Lewis, *ibid.*, p. 313; M. Lewis *et al.*, *ibid.*, p. 317; P. Meera Khan, W. S. Volkers, B. A. Doppert, A. B. Bijnen, J. Schreuder, J. J. van Rood, *ibid.*, p. 328; L. R. Weitkamp and S. A. Guttormsen, *ibid.*, p. 364.
8. L. R. Weitkamp, *Tissue Antigens* **7**, 273 (1976).
9. D. C. Shreffler, *Transplant. Reviews* **32**, 140 (1976).
10. T. H. Hansen, T. N. Krasteff, D. C. Shreffler, *Biochem. Genet.* **12**, 281 (1974).
11. T. Meo, T. Krasteff, D. C. Shreffler, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4536 (1975).
12. G. E. Edwards, R. G. Miller, R. A. Phillips, *J. Immunol.* **105**, 719 (1970).
13. L. Uotila and M. Koivusalo, *Eur. J. Biochem.* **52**, 493 (1975).
14. A. Gropp, J. Olert, R. Maurizio, *Experientia* **27**, 1226 (1971); B. M. Cattanach and H. Moseley, *Cytogenet. Cell Genet.* **12**, 264 (1973).
15. D. Michie, *Proc. R. Soc. London Ser. B* **114**, 241 (1955).
16. R. A. Fisher and F. Yates, *Statistical Tables* (Oliver and Boyd, Edinburgh, 1963).
17. F. H. Ruddle and R. P. Creagen, *Annu. Rev. Genet.* **9**, 407 (1975).
18. K. Bender and K.-H. Grzeschik, *Hum. Genet.* **31**, 341 (1976).
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Elastase Release from Human Alveolar Macrophages: Comparison Between Smokers and Nonsmokers

Abstract. *Alveolar macrophages from smokers, in contrast to those of nonsmokers, release elastase into serum-free culture medium. Since enzymes that digest elastin produce pulmonary emphysema in experimental animals, release of elastase by alveolar macrophages from smokers suggests that these cells are important in the pathogenesis of emphysema of smokers.*

Preparations that digest elastin, including papain (*1*), polymorphonuclear leukocyte homogenates (*2*), and pancreatic elastase (*3*), alter the connective tissue framework of the lung in experimental animals, yielding lesions similar to those of emphysema. It has been speculated that elastase activity is important in the pathogenesis of human emphysema. Alveolar macrophages (AM) and polymorphonuclear leukocytes (PMN) are the most likely sources of elastase which may affect human lung tissues.

The presence of elastase (E.C. 3.4.21.–) in PMN's is well established (*4*), but studies to demonstrate elastase in alveolar macrophages have been inconclusive (*5–7*). Most reports of alveolar macrophage elastase have relied on measurements of whole cell homogenates or cell components. Recently, however, Werb and Gordon (*8*) demonstrated that peritoneal macrophages in culture release an enzyme with elastolytic activity. Their finding of elastase as a secretory product is consistent with other data indicating that macrophages are capable of secreting a variety of substances (*9*). In the present investigation we have observed elastase release from human alveolar macrophages in culture. Notably, elastase activity was detected in cell cultures from smokers, but was not detected in cultures from nonsmokers.

Alveolar macrophages were obtained by lung lavage from 12 healthy volunteers (seven males and five females) whose ages ranged from 24 to 33 years. Five of the subjects smoked more than 30 cigarettes daily; the others did not smoke. Lung lavages were performed through a fiber-optic bronchoscope placed in a right lower lobe subsegmental bronchus. Five or six equal volumes of warm (37°C) sterile saline (0.9 percent NaCl, weight to volume) were instilled through the bronchoscope from a sterile 50-ml syringe. The fluid was removed by gentle suction.

The yield of alveolar macrophages ranged from 1 to 2.2×10^7 in nonsmokers and 15 to 28×10^7 in smokers. As noted by others, the material from smokers was dark brown in contrast to the ivory-colored material obtained from nonsmokers. The fluid recovered (approximately 60 percent of the instilled volume) was placed in sterile, plastic, conical centrifuge tubes, chilled to 4°C, and centrifuged at 500*g* for 10 minutes. The supernatant was decanted. The cells were washed twice in an equal volume of Neuman and Tytell medium (serum free); a portion of this cell suspension was counted in a hemocytometer, and the remaining cells were suspended in the same medium to a final concentration of 3×10^6 cells per milliliter. Portions (1 ml) of the cell suspension were placed in 35-mm culture dishes (Falcon). After 2 hours of incubation at 37°C in a humidified incubator under 5 percent carbon dioxide and 95 percent air, the culture medium was removed. The cultures were washed three times with fresh medium to remove nonadherent cells and then 1 ml of fresh medium was added to each dish. The cells were reincubated for 48 hours. Cell viability was assessed by examining representative cells for exclusion of supravital dye and by measuring the distribution of lactic acid (LDH; E.C. 1.1.1.27) dehydrogenase and β-glucuronidase (E.C. 3.2.1.31) activities between the media and the cells. In some experiments the culture period was extended for as long as 14 days. In the prolonged experiments, the medium was removed and replaced at various intervals.

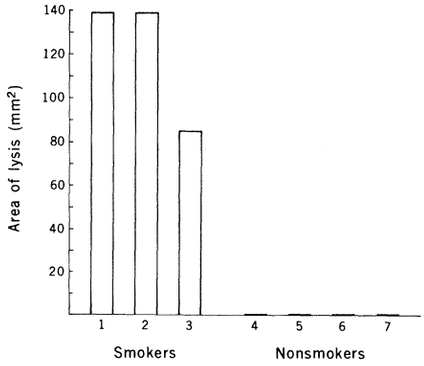

Fig. 1. The effects of reconstituted culture media upon elastin agarose. The activity was determined by calculating the area of lysis.

Medium removed from the cultures was centrifuged at 500*g* for 10 minutes at 4°C and dialyzed for 24 to 48 hours against cold 10 m*M* tris-HCl, *p*H 7.6, containing 1 m*M* $CaCl_2$. This solution was lyophilized and the product stored at −70°C. Prior to enzymatic analysis, dialyzed lyophilized medium was reconstituted with cold distilled water to 0.5 percent of its original volume.

Cell lysates were prepared by exposing freshly washed cultures to 1 percent Triton X-100 for 20 minutes. These cells were scraped from the plates and subjected to sonication at 2500 amp for 15 seconds. Complete cell disruption was confirmed by light microscopy.

Elastolytic activity was measured by solubilization of elastin particles (< 400 mesh) suspended in agarose (*8*), and by the hydrolysis of succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide (SLAPN), a synthetic substrate with a high degree of specificity for elastases (*10*).

The medium from three of the smokers' cultures showed easily detectable activity against elastin suspended in agarose, whereas culture medium from four of the nonsmokers' AM failed to show activity even after prolonged incubation (Fig. 1). At 48 hours, more than 80 percent of the cells excluded dye. Enzyme partition studies demonstrated that 15 percent of the total β-glucuronidase and 50 percent of the total LDH activity was extracellular. Elastolytic activity was demonstrable in the media of

smokers' AM (Fig. 2) for as long as 2 weeks; however, after 72 hours the majority of the cells showed morphological and biochemical deterioration, suggesting that the cells were no longer viable. Table 1 summarizes the activities of the media from three of the smokers and four of the nonsmokers against SLAPN. Medium from smokers hydrolyzed the substrate, whereas medium from nonsmokers failed to show any activity within the standard 30-minute assay or with prolonged incubation up to 24 hours. Similar results were obtained from the culture media of two additional smokers' and three additional nonsmokers whose AM were sampled and assayed at different times and at concentrations from those in Fig. 1 and Table 1. Elastolytic activity was not affected by 1 percent Triton X-100.

Leukocytes were rare contaminants of the bronchial lavage fluids even from smokers. Moreover, the majority of nonadherent cells were removed after the initial 2-hour culture period. The fact that PMN's do not survive in culture past 48 hours, and that fresh media introduced more than a week after bronchial lavage contained elastase argues strongly against the presence of leukocytes being the source of the elastase in the cultures. The present data do not, however, distinguish between elastase of macrophage and leukocyte origins. Thus, it is possible that the elastase activity released by the macrophages could represent internalized leukocyte elastase (*11*).

Elastase was not detectable in cell homogenates from either smokers or nonsmokers. These results suggest that elastase is not stored. The possible presence of cytoplasmic inhibitors of elastase, such as alpha-1-antitrypsin, may influence measurement of elastase on cell homogenates (*5, 12*).

Trace amounts of elastolytic activity might not be detected by the methods currently available. We cannot, therefore, exclude the possibility that some elastolytic activity is released by AM from the nonsmokers. This study indicates, however, that more elastolytic activity is released by the alveolar macrophages of smokers than by those of nonsmokers. This difference becomes even more impressive when one considers that the lungs of cigarette smokers contain a much greater number of AM than the lungs of nonsmokers. The quantity of elastase released in the lungs of smokers might exceed that of nonsmokers by 20-fold or more. Variations in the amount of elastase between the smokers may reflect differences between the subjects' smoking habits, environments, or other factors.

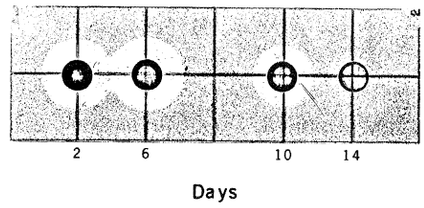

Fig. 2. Elastolytic activity in lyophilized, reconstituted culture media at various times during 14 days of culture (subject 2). Each 5-mm well was filled with 50 μl of culture medium, and the plate was incubated for 4 days at 37°C in a sealed container. The wells contained portions of the media from the time periods as shown. The media had been changed between each period.

These data may implicate the AM in the pathogenesis of emphysema related to smoking. Indeed, several lines of evidence have already associated macrophages with the chain of events linking smoking and emphysema. Smokers' macrophages have significantly greater levels of esterase and proteases than do those of nonsmokers (*5*). The increased numbers of AM in the sputum of heavy smokers appears to be related to the number of cigarettes smoked (*13*). Histological studies have shown collections of macrophages in the respiratory bronchioles of young smokers, in precisely those sites which commonly develop emphysematous lesions (*14*). Macrophages also appear in early lesions of emphysema (*13*).

Table 1. Cells recovered by lavage and elastase activity of cell culture media. Elastase activity is expressed in terms of nanomoles of *p*-nitroanilide released from SLAPN per 50 μl of medium in 30 minutes of incubation. The cells were cultured at a density of 3×10^6/ml for 48 hours at 37°C in a humidified incubator (5 percent CO_2). The media were then removed, dialyzed, and lyophilized. Assays for elastase activity were routinely conducted for 30 minutes on 50-μl of reconstituted medium. For comparison, 0.02 unit of pancreatic elastase will give an absorbance of 0.6 at 410 nm of hydrolyzed SLAPN. This is equivalent to 64 nmole of *p*-nitroanilide.

Subject	Cells obtained by lavage ($\times 10^{-6}$)	Elastase activity of media
	Smokers	
1	150	117
2	158	261
3	286	48
	Nonsmokers	
4	18	0
5	28	0
6	22	0
7	15	0

Phagocytosis of nondigestible particles increases the release of elastase from peritoneal macrophages (*8*). Macrophages from smokers contain inorganic crystalline residues including kaolinite, that can also be identified in cigarette tobacco (*15*). We postulate that the nondigestible residues of cigarette smoke act as a stimulus to AM for the production and release of elastase. These events and the subsequent destruction and remodeling of the lung elastin network may be important factors in the pathogenesis of emphysema.

RAFAEL J. RODRIGUEZ*
Pulmonary Disease and Respiratory Care Division, Department of Medicine, Jewish Hospital of St. Louis, Washington University School of Medicine, St. Louis, Missouri 63110
RICHARD R. WHITE†
Department of Pathology, Washington University School of Medicine
ROBERT M. SENIOR
Pulmonary Disease and Respiratory Care Division, Department of Medicine, Jewish Hospital of St. Louis
EDITH A. LEVINE
Pulmonary Division, Department of Medicine, St. Louis University School of Medicine, St. Louis, Missouri 63104

References and Notes

1. P. Gross, E. A. Pfitzer, E. Tolker, M. A. Babyak, M. Kaschak, *Arch. Environ. Health* **11**, 50 (1965).
2. V. Marco, B. Mass, D. R. Meranze, G. Weinbaum, P. Kimbel, *Am. Rev. Respir. Dis.* **104**, 595 (1971).
3. P. D. Kaplan, C. Kuhn, J. A. Pierce, *J. Lab. Clin. Med.* **82**, 349 (1973).
4. A. Janoff, *Lab. Invest.* **29**, 458 (1973); K. Ohlsson and I. Olsson, *Eur. J. Biochem.* **42**, 519 (1974).
5. J. O. Harris, G. N. Olsen, J. R. Castle, A. S. Maloney, *Am. Rev. Respir. Dis.* **111**, 579 (1975).
6. E. A. Levine, R. M. Senior, J. V. Butler, *ibid.* **113**, 25 (1976).
7. A. Janoff, R. Rosenberg, M. Galdston, *Proc. Soc. Exp. Biol. Med.* **136**, 1054 (1971); N. R. Ackerman and J. R. Beebe, *Chest* **66** (Suppl.), 21 (1974).
8. Z. Werb and S. Gordon, *J. Exp. Med.* **142**, 361 (1975).
9. E. R. Unanue, *Am. J. Pathol.* **83**, 396 (1976).
10. J. Bieth, B. Spiess, C. G. Wermuth, *Biochem. Med.* **11**, 350 (1974).
11. A. Janoff, B. Sloan, G. Weinbaum, V. Damiano, R. A. Sandhaus, J. Elias, P. Kimbel, *Am. Rev. Respir. Dis.* **115**, 461 (1977).
12. A. B. Cohen, *J. Clin. Invest.* **52**, 2793 (1973); A. Janoff and J. Blondin, *Lab. Invest.* **25**, 565 (1971); G. N. Olsen, J. O. Harris, J. R. Castle, R. H. Waldman, H. J. Karmgard, *J. Clin. Invest.* **55**, 427 (1975).
13. R. F. McLaughlin and E. E. Tueller, *Chest* **59**, 592 (1971).
14. D. M. Spain, H. Siegel, V. A. Bradess, *J. Am. Med. Assoc.* **224**, 322 (1973); D. E. Niewoehner, J. Kleinerman, D. B. Rice, *N. Engl. J. Med.* **291**, 755 (1974).
15. A. R. Brody and J. E. Craighead, *Lab. Invest.* **32**, 125 (1975).
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* Present address: Department of Medicine, University of Puerto Rico, Medical Sciences Campus, San Juan 00936.
† Present address: Department of Pathology, State University of New York at Stony Brook, Stony Brook 11794.

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