THE INHIBITORY COMPLEX OF HUMAN α_1-PROTEINASE INHIBITOR AND HUMAN LEUKOCYTE ELASTASE IS A NEUTROPHIL CHEMOATTRACTANT

BY MICHAEL J. BANDA,* AUDIE G. RICE,* GAIL L. GRIFFIN,‡ AND ROBERT M. SENIOR‡

*From the *Laboratory of Radiobiology and Environmental Health, University of California, San Francisco, California 94143; and the ‡Respiratory and Critical Care Division, Department of Medicine, Jewish Hospital at Washington University Medical Center, St. Louis, Missouri 63110*

α_1-Proteinase inhibitor (α_1-PI)[1] is a member of the serpin group of serine proteinase inhibitors (1, 2). Based on inhibitory kinetics, the principal target of α_1-PI is human leukocyte elastase (HLE) (3). The metalloproteinase elastase secreted by mouse macrophages inactivates α_1-PI (4) by hydrolyzing the Pro-357-Met-358 bond (5), which results in M_r 47,800 and M_r 4,200 fragments. We recently observed that α_1-PI proteolytically inactivated by mouse macrophage elastase becomes a potent chemotactic factor for human neutrophils (6). After proteolysis, the 47,800 and 4,200 fragments of α_1-PI remain associated with each other in a bimolecular complex, with the chemotactic activity residing in a newly exposed region of the 4,200 fragment (6).

When HLE is inactivated by α_1-PI, the Met-358-Ser-359 bond is hydrolyzed, resulting in a COOH-terminal cleavage fragment very similar to that generated by mouse macrophage elastase. In contrast to mouse macrophage elastase, which does not form an inhibitory complex with α_1-PI, HLE remains tightly associated with α_1-PI. Therefore, the HLE–α_1-PI complex actually consists of three molecules: HLE and two fragments of α_1-PI. Because it is likely that the orientation of the two fragments of α_1-PI in the HLE–α_1-PI complex is similar to that of the two α_1-PI fragments generated after proteolysis by mouse macrophage elastase, we considered the possibility that the HLE–α_1-PI complex possesses chemotactic activity similar to that of the proteolytically inactivated α_1-PI. In the study reported here, using homologous reagents, we examined the ability of complexes of HLE and α_1-PI to stimulate directed migration of human neutrophils.

Materials and Methods

Materials. Chemicals were purchased from the following sources: human α_1-PI from CalBiochem-Behring Corp., La Jolla, CA; human leukocyte elastase from Elastin Prod-

This work was supported by the Office of Health and Environmental Research, U.S. Department of Energy (contract no. DE-AC03-76-SF01012) and the National Institutes of Health (grant HL-29594). Address correspondence to M. J. Banda, Laboratory of Radiobiology and Environmental Health, Box 0750, University of California, San Francisco, CA 94143.

[1] *Abbreviations used in this paper:* α_1-PI, α_1-proteinase inhibitor; HLE, human leukocyte elastase (also known as human neutrophil elastase and human granulocyte elastase).

 J. EXP. MED. © The Rockefeller University Press · 0022-1007/88/05/1608/08 $2.00
Volume 167 May 1988 1608–1615

ucts Co., Pacific, MO; rabbit anti-α_1-PI IgG and anti-α_2-macroglobulin IgG from Sigma Chemical Co., St. Louis, MO; prestained protein standards for SDS-PAGE from Bethesda Research Laboratories, Gaithersburg, MD; and all reagents for electrophoresis from Bio-Rad Laboratories, Richmond, CA. Sheep anti-HLE IgG was a gift of D. Burnett and R. Stockley, The General Hospital, Birmingham, England.

HLE–α_1-PI Complexes. Reaction mixtures containing HLE and α_1-PI (1:1, mol/mol) were incubated at 37°C in a 10 mM phosphate buffer, pH 6.8, for 15 min. The reaction was stopped either by addition of PMSF ($\leq$1 mM) or by injection onto an anion-exchange column.

Electrophoresis. SDS-PAGE was performed according to the method of Laemmli (7) using 12% resolving gels on a Mini-slab apparatus from Idea Scientific (Corvallis, OR). After electrophoresis the protein bands were stained for 15 min with 0.5% (vol/vol) Coomassie Blue R250 in acetic acid/isopropyl alcohol/water (1:3:6), destained with 50% methyl alcohol, and silver stained according to Wray et al. (8).

Cells. Neutrophils were separated on Ficoll-Paque gradients from peripheral blood obtained from healthy volunteers (9).

Chemotaxis. Chemotaxis was determined in modified Boyden chambers, as previously described (10, 11). Briefly, 1.2×10^6 neutrophils/ml were placed in the upper compartment and separated from chemoattractants in the lower compartment by a 2-μm pore size filter (Nucleopore Corp., Pleasanton, CA) overlaying a 0.45-μm pore size filter (Millipore Corp., Bedford, MA). After 1 h the chambers were disassembled and the membranes were stained with hematoxylin. Chemotaxis was quantified by counting at high dry magnification ($\times$ 400) the number of cells that migrated to the interface between the membranes (expressed as cells per high power grid). Five fields were counted for each experiment, and the results were corrected for the number of cells migrating in control chambers, which contained cells in the upper compartment and medium alone in the lower compartment. Experiments were performed in triplicate.

Purification of HLE–α_1-PI Complexes. Chromatography was performed on a liquid chromatograph (model 1090; Hewlett-Packard Co., Palo Alto, CA) equipped with an HP1040 photodiode array detector. Elutions were monitored at 280 nm. Samples of HLE–α_1-PI reaction mixtures were injected onto an AX-300 anion-exchange column (4.6 $\times$ 250 mm) (Brownlee Labs, Santa Clara, CA) equilibrated with 5 mM Tris-HCl, pH 8.0 (solvent A), and developed with a gradient to 0.8 M NaCl in solvent A (solvent B). The gradient was delivered at 0.5 ml/min, as follows: 0–5 min, 0% B; 5–10 min, 0–20% B; 10–20 min, 20–25% B; 20–25 min, 25% B; 25–65 min, 25–65% B.

α_1-PI Fragment. The M_r 4,200 fragment of α_1-PI was generated by hydrolysis of the Pro-357-Met-358 bond by mouse macrophage elastase and purified as described previously (6).

Antibodies. Purified HLE–α_1-PI complex (100 μl, 10^{-6} M) was incubated with 14 μl of stock anti-α_1-PI IgG, 2 μl of anti-HLE IgG, or 24 μl of anti-α_2-macroglobulin IgG before use in a chemotactic assay. Controls were HLE–α_1-PI complex incubated in assay medium alone and FMLP incubated in assay medium alone or with anti-α_1-PI IgG. All incubations were at ambient temperature for 60 min.

Preexposure to Competitive Ligands. Neutrophils were incubated in 10 nM purified HLE–α_1-PI complex or 10 nM purified M_r 4,200 fragment of α_1-PI for 30 min at ambient temperature, then washed three times with medium. The cells were then assayed for a chemotactic response to 1 nM HLE–α_1-PI complex, 1 nM 4,200 fragment, or 10 nM FMLP.

Results

Equal molar amounts of HLE and human α_1-PI were incubated together at 37°C for 15 min, resulting in the formation of a stable HLE–α_1-PI inhibitory complex. Dilutions of this reaction mixture were assayed for their ability to stimulate migration of human neutrophils in modified Boyden chambers. Migration

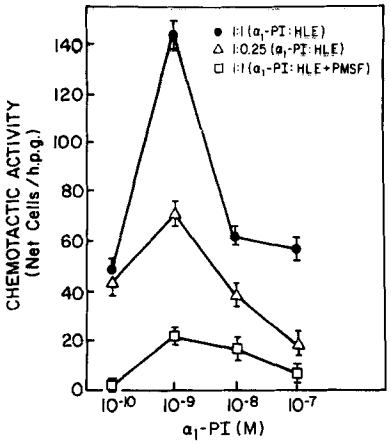

FIGURE 1. Stimulation of neutrophil chemotaxis by reaction mixtures containing HLE–α_1-PI complexes. α_1-PI was incubated for 15 min with HLE, 1:1 mol/mol (●); HLE, 1:0.25 mol/mol (△); or HLE (1:1 mol/mol) that had been inactivated with PMSF before incubation (□). Dilutions of the reaction mixtures were then assayed for their ability to stimulate chemotaxis of human neutrophils. The points represent the average of triplicate determinations, and the error bars represent the standard error of the mean. h.p.g., high power grid. Migration stimulated by 10^{-8} M FMLP (the positive control) was 153 ± 4.2 cells.

was equivalent to that stimulated by 10 nM FMLP (the positive control). Maximal stimulation of migration was achieved at a dilution equivalent to 1 nM α_1-PI initially added to the reaction mixture (Fig. 1). Chemotaxis was reduced when the proportion of HLE in the reaction mixture was reduced four-fold, suggesting that the chemotactic activity was proportional to the HLE–α_1-PI complex in the reaction mixture. Neither native α_1-PI nor HLE alone stimulated migration of neutrophils.

In our previous study (6), proteolytically modified α_1-PI was shown to be chemotactic for neutrophils. In that study the metalloproteinase macrophage elastase was used to degrade α_1-PI because it would not form an inhibitory complex (4, 5). In the present study, a stable inhibitory complex was formed between

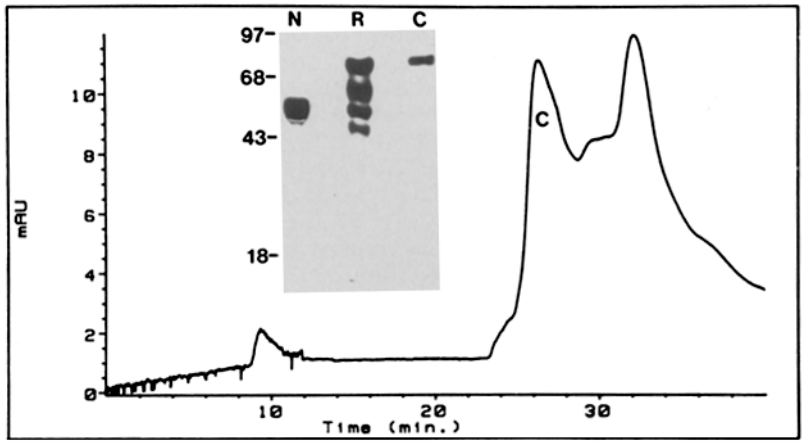

FIGURE 2. Purification of HLE–α_1-PI complex. α_1-PI was incubated with an excess of HLE, then applied to an AX-300 anion-exchange column equilibrated with 5 mM Tris-HCl, pH 8.0. Absorbance was monitored at 280 nm and expressed as milliabsorbance units (mAU). The column was developed with a gradient of NaCl as described in Materials and Methods. *(Inset)* Silver-stained SDS-polyacrylamide electrophoretic gel containing native α_1-PI (*N*), HLE–α_1-PI reaction mixture (*R*), and an aliquot of a fraction corresponding to peak C containing HLE–α_1-PI complex (*C*). Molecular weight markers ($M_r \times 10^{-3}$) are shown at the left.

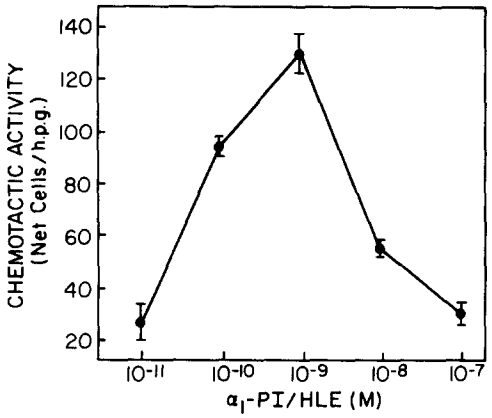

FIGURE 3. Stimulation of neutrophil chemotaxis by purified HLE–α_1-PI complex. Purified HLE–α_1-PI complex obtained by anion-exchange chromatography was assayed for its ability to stimulate chemotaxis of human neutrophils. The points represent the average of triplicate determinations, and the error bars represent the standard error of the mean. Migration stimulated by 10^{-8} M FMLP was 127 ± 6.6 cells.

HLE and α_1-PI. When HLE and α_1-PI were incubated under conditions of excess HLE, proteolytically modified complex and α_1-PI were detected in addition to the classical inhibitory complex. To determine if the inhibitory complex itself stimulated migration, the complex was purified. Equal molar amounts of HLE and α_1-PI were incubated together to form the inhibitory complex. The reaction mixture was then fractionated on an AX-300 anion-exchange column, and peak fractions were analyzed by electrophoresis. The HLE–α_1-PI complex eluted as a single band with an apparent M_r of 80,300 (Fig. 2). Various concentrations of the purified complex were assayed for their ability to stimulate migration of neutrophils. Again, migration was equivalent to that stimulated by the FMLP positive control (Fig. 3). The optimal concentration of purified HLE–α_1-PI complex was 1 nM, the same concentration that stimulated maximal migration when the crude reaction mixture was used (Fig. 1).

To distinguish chemotactic migration from chemokinetic migration, various concentrations of purified HLE–α_1-PI complex were placed in the upper and lower compartments of modified Boyden chambers during the migration assay. A "checkerboard" analysis verified that HLE–α_1-PI complex stimulated chemotaxis, because cells migrated only when there was a concentration gradient (Table I).

TABLE I
"Checkerboard" Analysis of Chemotaxis to HLE–α_1-PI Complex

		HLE–α_1-PI (M) in upper compartment			
		0	10^{-11}	10^{-10}	10^{-9}
HLE–α_1-PI (M) in lower compartment	10^{-11}	34* ± 4.2	12 ± 2.4	2 ± 5.3	12 ± 3.6
	10^{-10}	74 ± 4.2	14 ± 4.9	8 ± 2.8	17 ± 2.6
	10^{-9}	168 ±5.7	102 ± 3.6	64 ± 6.6	26 ± 1.7

* Data are expressed as mean number of cells ± SE per high power grid.

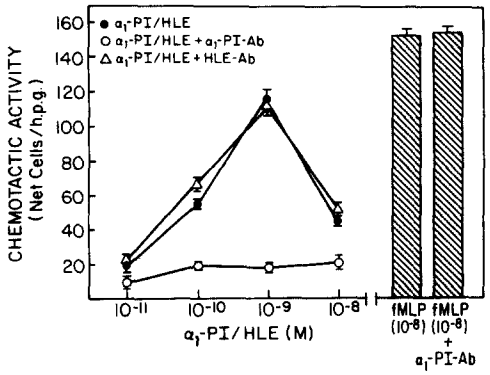

FIGURE 4. Effect of specific antibodies on neutrophil chemotaxis. Purified HLE–α_1-PI complex was incubated for 60 min at ambient temperature in assay medium (●); with antibody against α_1-PI (○); or with antibody against HLE (△) before assay for the chemotactic response. The points represent the average of triplicate determinations, and the error bars represent the standard error of the mean. *(Histogram)* Before assay, FMLP was incubated for 60 min at ambient temperature with medium alone or with antibody against α_1-PI. h.p.g., high power grid.

Because the HLE–α_1-PI complex contains elastase as well as α_1-PI, it was possible that the chemotactic signal was associated with the elastase portion of the complex. To test this possibility, purified HLE–α_1-PI complex was separately incubated with anti–α_1-PI, anti-HLE, and anti–α_2-macroglobulin antibodies before use in the migration assay. The antibody against α_1-PI inhibited chemotaxis, whereas antibody against HLE did not (Fig. 4). Therefore, the chemotactic signal was associated with α_1-PI and not the elastase portion of the complex. The lack of inhibition by anti–α_2-macroglobulin antibody (data not shown) demonstrated that the inhibition of chemotaxis by anti–α_1-PI antibody was not due to nonspecific protein binding. Moreover, because antibody to α_1-PI did not inhibit chemotaxis stimulated by FMLP, it was evident that inclusion of anti–α_1-PI antibody in the reaction mixture did not generally interfere with the ability of neutrophils to migrate.

The chemotactic activity of proteolytically inactivated α_1-PI resides in the M_r 4,200 fragment (6), which is the result of hydrolysis of the Pro-357-Met-358 peptide bond (5). When HLE forms an inhibitory complex with α_1-PI, the Met-358-Ser-359 bond is broken, resulting in a similar fragment. In both cases the cleavage fragment is not released as a free molecule but remains associated with the remainder of the α_1-PI molecule in a rearranged position (6, 12). To deter-

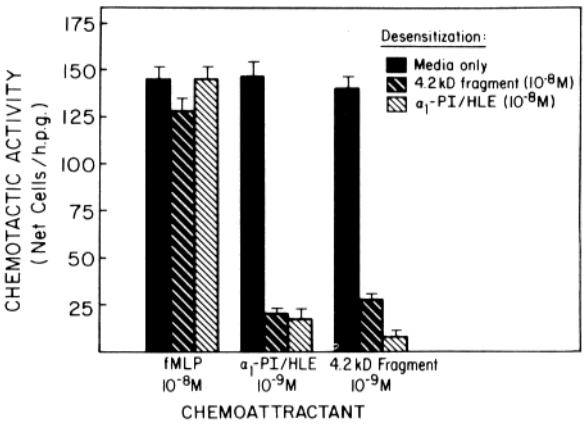

FIGURE 5. Neutrophil chemotaxis before and after exposure to competitive ligands. Neutrophils were incubated for 30 min at ambient temperature with 10 nM purified HLE–α_1-PI complex *(light striped bars)*, 10 nM purified M_r 4,200 fragment of α_1-PI *(dark striped bars)*, or assay medium alone *(solid bars)*, then washed three times with assay medium. The cells were then assayed for a chemotactic response to 1 nM HLE–α_1-PI complex, 1 nM 4,200 fragment, or 10 nM FMLP. The data represent an average of triplicate determinations, and the error bars represent the standard error of the mean.

mine if the source of the activity of the HLE–α_1-PI inhibitory complex could be localized to the region of the 4,200 fragment, the following experiments were carried out. Neutrophils were incubated with 10 nM 4,200 fragment isolated from proteolytically inactivated α_1-PI and then washed free of unbound fragment before they were tested for their chemotactic response to 1 nM HLE–α_1-PI complex. This pretreatment eliminated chemotaxis (Fig. 5). Similarly, when neutrophils were preincubated with 10 nM HLE–α_1-PI complex, migration toward 1 nM 4,200 fragment was eliminated. Therefore, the source of the chemotactic activity of the HLE–α_1-PI complex was the same as that of the 4,200 fragment. Preincubation with either 4,200 fragment or HLE–α_1-PI complex had no effect on migration toward 10 nM FMLP, indicating that the preincubations did not produce a generalized decrease in the chemotactic responses of these cells.

Discussion

The function of α_1-PI is usually considered solely in light of its ability to inactivate serine proteinases, especially HLE. The present study suggests that a consequence of α_1-PI–mediated inhibition of HLE is the generation of a chemotactic signal for neutrophils. The inhibitory complex therefore becomes an inflammatory mediator. This newly described characteristic of α_1-PI is not limited to proteinase–inhibitor complexes, because proteolytically inactivated α_1-PI also seems to act as an inflammatory mediator by a similar mechanism (6). The data suggest that an identical site with chemotactic potential is exposed in both HLE–α_1-PI complex and α_1-PI proteolytically inactivated by hydrolysis of the Pro-357-Met-358 bond.

The local regulation of α_1-PI–mediated chemotaxis may be quite complicated. α_1-PI from the circulation may diffuse into tissue and form inhibitory complexes or become proteolytically inactivated. In addition, α_1-PI may be made locally by human monocytes (13) and macrophages (14). It is unclear whether human monocytes or macrophages secrete metalloproteinases that could proteolytically inactivate α_1-PI. However, neutrophil collagenase can proteolytically inactivate α_1-PI (15). Human monocytes have been reported to produce a serine elastase similar to HLE (16) and may generate HLE–α_1-PI complexes (14). Therefore, local production of α_1-PI, HLE, and α_1-PI–degrading proteinases may initiate or alter the inflammatory response.

Evidence presented in this study suggests that a single neutrophil surface receptor binds both proteolytically inactivated α_1-PI and the α_1-PI in the inhibitory complex. The site responsible for recognition appears to reside in a domain carboxyl to the Met-358-Ser-359 peptide bond; exposure of this area is probably the result of the severe rearrangement of modified α_1-PI (12). Saturation of the receptor by preincubation with HLE–α_1-PI complex prevents further chemotaxis toward modified α_1-PI but does not interfere with chemotaxis toward an unrelated molecule, FMLP. Therefore, this as yet uncharacterized receptor must be different from the FMLP receptor. Whether it is a receptor for other known neutrophil chemotactic stimulants, such as C5a or LTB4, remains to be determined.

Little is known about the fate of HLE–α_1-PI complexes. α_2-Macroglobulin–

proteinase complexes are cleared by a macrophage cell surface receptor (17, 18). Although it has been suggested that HLE is transferred from α_1-PI to α_2-macroglobulin (19), this is unlikely because of the nature of the HLE–α_1-PI bond. Furthermore, because binding of HLE–α_1-PI to neutrophils is directly mediated by α_1-PI, transfer to α_2-macroglobulin and subsequent binding to the α_2-macroglobulin receptor does not appear likely. The putative receptor for modified α_1-PI may provide a clearance mechanism for HLE–α_1-PI complexes as well as for proteolytically inactivated α_1-PI. If so, turnover of native α_1-PI would also involve this receptor.

Summary

An inhibitor–proteinase complex consisting of human α_1-PI and human leukocyte elastase is chemotactic for human neutrophils. The chemotactic activity is optimal at 1 nM and is associated only with the α_1-PI portion of the complex. Neither HLE in the complex, free HLE, nor native α_1-PI possesses chemotactic activity for human neutrophils. α_1-PI in complex is hydrolyzed at the Met-358-Ser-359 bond. The chemotactic activity is associated with the M_r 4,200 fragment of α_1-PI that has Ser-359 as its NH_2 terminus. The region of the HLE–α_1-PI complex that stimulates chemotaxis appears to be the same as that of the M_r 4,200 fragment generated by hydrolysis of the Pro-357-Met-358 bond during proteolytic inactivation of α_1-PI. The data suggest the presence of a neutrophil surface receptor bound by α_1-PI after the formation of a complex with HLE or after proteolytic degradation. This receptor may play a role in clearance of these modified α_1-PI molecules.

Received for publication 11 January 1988.

References

1. Travis, J., and G. S. Salvesen. 1983. Human plasma proteinase inhibitors. *Annu. Rev. Biochem.* 52:655.
2. Carrell, R., and J. Travis. 1985. α_1-Antitrypsin and the serpins: variation and countervariation. *Trends Biochem. Sci.* 10:20.
3. Beatty, K., J. Bieth, and J. Travis. 1980. Kinetics of association of serine proteinases with native and oxidized α-1-proteinase inhibitor and α-1-antichymotrypsin. *J. Biol. Chem.* 255:3931.
4. Banda, M. J., E. J. Clark, and Z. Werb. 1980. Limited proteolysis by macrophage elastase inactivates human α_1-proteinase inhibitor. *J. Exp. Med.* 152:1563.
5. Banda, M. J., E. J. Clark, S. Sinha, and J. Travis. 1987. Interaction of mouse macrophage elastase with native and oxidized human α_1-proteinase inhibitor. *J. Clin. Invest.* 79:1314.
6. Banda, M. J., A. G. Rice, G. L. Griffin, and R. M. Senior. 1988. α_1-Proteinase inhibitor is a neutrophil chemoattractant after proteolytic inactivation by macrophage elastase. *J. Biol. Chem.* In press.
7. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
8. Wray, W., T. Boulikas, V. P. Wray, and R. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* 118:197.
9. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21(Suppl. 97):77.

10. Deuel, T. F., R. M. Senior, D. Chang, G. L. Griffin, R. L. Heinrikson, and E. T. Kaiser. 1981. Platelet factor 4 is chemotactic for neutrophils and monocytes. *Proc. Natl. Acad. Sci. USA.* 78:4584.
11. Deuel, T. F., R. M. Senior, J. S. Huang, and G. L. Griffin. 1982. Chemotaxis of monocytes and neutrophils to platelet-derived growth factor. *J. Clin. Invest.* 69:1046.
12. Loebermann, H., R. Tokuoka, J. Deisenhofer, and R. Huber. 1984. Human α_1-proteinase inhibitor. Crystal structure analysis of two crystal modifications, molecular model and preliminary analysis of the implications for function. *J. Mol. Biol.* 177:531.
13. Van Furth, R., J. A. Kramps, and M. M. C. Diesselhof-Den Dulk. 1983. Synthesis of α_1-anti-trypsin by human monocytes. *Clin. Exp. Immunol.* 51:551.
14. Perlmutter, D. H., F. S. Cole, P. Kilbridge, T. H. Rossing, and H. R. Colten. 1985. Expression of the α_1-proteinase inhibitor gene in human monocytes and macrophages. *Proc. Natl. Acad. Sci. USA.* 82:795.
15. Vissers, M. C. M., P. M. George, I. C. Bathurst, S. O. Brennan, and C. C. Winterbourn. 1987. Specific cleavage and inactivation of $\alpha_1$proteinase inhibitor by human neutrophil collagenase. *Fed. Proc.* 46:1390.
16. Senior, R. M., E. J. Campbell, J. A. Landis, F. R. Cox, C. Kuhn, and H. S. Koren. 1982. Elastase of U-937 monocytelike cells: comparisons with elastases derived from human monocytes and neutrophils and murine macrophagelike cells. *J. Clin. Invest.* 69:384.
17. Kaplan, J., and M. L. Nielsen. 1979. Analysis of macrophage surface receptors. I. Binding of α-macroglobulin · protease complexes to rabbit alveolar macrophages. *J. Biol. Chem.* 254:7323.
18. Kaplan, J., and M. L. Nielsen. 1979. Analysis of macrophage surface receptors. II. Internalization of α-macroglobulin · trypsin complexes by rabbit alveolar macrophages. *J. Biol. Chem.* 254:7329.
19. Ohlsson, K., and C.-B. Laurell. 1976. The disappearance of enzyme-inhibitor complexes from the circulation of man. *Clin. Sci. Mol. Med.* 51:87.