INFECTION AND IMMUNITY, Feb. 1985, p. 555–560
0019-9567/85/020555-06$02.00/0

Vol. 47, No. 2

In Vitro Evidence that Human Airway Lysozyme is Cleaved and Inactivated by *Pseudomonas aeruginosa* Elastase and Not by Human Leukocyte Elastase

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Received 17 July 1984/Accepted 30 October 1984

The in vitro effects of *Pseudomonas aeruginosa* elastase (*P. aeruginosa* E) and of human leukocyte elastase on human airway lysozyme (HAL) were investigated. *P. aeruginosa* E inactivated and cleaved HAL, whereas human leukocyte elastase had no effect. Total inactivation of HAL by *P. aeruginosa* E was observed after 120 min of incubation at 37°C, for an elastase-to-lysozyme molar ratio of 1:5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of reaction mixtures containing HAL and *P. aeruginosa* E in an elastase-to-lysozyme molar ratio of 1:10 showed a progressive disappearance of the HAL band upon increasing the incubation time with *P. aeruginosa* E. Gel filtration chromatography indicated that HAL was cleaved into at least three peptide fragments. The cleavage of HAL by *P. aeruginosa* E was accompanied by parallel losses of its bacteriolytic activity and its immunoreactive property.

Lysozyme is an enzyme present in high concentration in airway secretions from patients with chronic pulmonary diseases (3, 25). Surface epithelium, human submucosal tracheal glands, and pulmonary alveolar macrophages are the major contributors for most of the lysozyme found in vivo in human airway secretions (24). Human monocytes and leukocytes have been shown to contain lysozyme (12, 23). The physiological role of human lysozyme remains unclear, and several functions have been reported in recent years. Because of its bacteriological activity (muramidase activity) on cell walls of sensitive microorganisms such as *Micrococcus luteus* (35) and some strains of staphylococci or streptococci (8), lysozyme is generally considered as an antibacterial agent implicated in the local pulmonary defense mechanisms. However, it has been suggested that most pathogenic bacteria are insensitive to lysozyme in the absence of antibodies, complement, or other enzymes (10, 40). The possible role of human airway lysozyme (HAL) in controlling the rheological properties of airway secretions has also been suggested. According to Jenssen et al. (18), the interaction between positive charges of lysozyme and negative charges of acidic mucins may be responsible for building up a macromolecule network, giving mucuses their gel-like properties which are necessary for efficient mucociliary transport (36). In a recent study, Gordon et al. (11) postulate that human lysozyme may also function in a negative feedback system to modulate the inflammatory response. They reported that in vitro lysozyme from urine origin inhibited the leukocyte chemotactic motility and the production of toxic oxygen radicals by stimulated leukocytes.

In purulent and infected bronchial secretions, proteolytic enzymes are released from bacteria and leukocytes (6, 39). These proteases are able to degrade and inactivate some proteins implicated in the defense of lungs against invading bacteria. For example, *Pseudomonas aeruginosa*, a pathogen that often causes very severe lung infections (4, 15) secretes an elastase which can inactivate immunoglobulin G antibodies (7), several human complement factors (37), and the two major inhibitors of serine proteinases present in lungs, the alpha 1 proteinase inhibitor (33) and the bronchial inhibitor (20). An in vitro study (5) showed that *P. aeruginosa* elastase (*P. aeruginosa* E) was also able to cleave human immunoglobulin A and secretory immunoglobulin A, the latter being considered as the major immunoglobulin of the respiratory tract. The elastase released by human leukocytes has also been shown to degrade in vitro human immunoglobulins (9) and the C3 and C5 component of human complement (21).

Therefore, during in vivo infection with purulent airway secretions, the increased concentration of proteolytic enzymes released from bacteria and leukocytes should induce an irreversible degradation or inactivation (or both) of locally produced proteins, such as lysozyme, which are potentially important in the lung microbial defense. Such a situation may be commonly encountered in patients with cystic fibrosis, which is a genetic disease characterized by the colonization of the airways with *P. aeruginosa* (14, 26).

The purpose of the present work was to examine the in vitro effects of *P. aeruginosa* E and human leukocyte elastase (HLE) on the bacteriolytic activity and molecular properties of lysozyme isolated from human airway secretions.

MATERIALS AND METHODS

Purification of proteins. The purification of HAL was adapted from the method of Konstan et al. (24). A 24-h volume of sputum was collected from patients with chronic bronchitis. The sputum samples were pooled and stored at −25°C until used. After thawing at 4°C, 2 liters of sputum was adjusted in 0.5 M NaCl and stirred gently for 1 h. Then, the pH of the mixture was adjusted to 3.0 with 1.0 N HCl. Trichloracetic acid was added to a final concentration of 4%, and the mixture was centrifuged at 30,000 × *g* for 30 min. The supernatant was buffered with 0.05 M Tris, neutralized with 10 N NaOH, and dialyzed against 0.02 M phosphate buffer (pH 6.2), using a Spectrapor 3 membrane (Spectrum Medical Industries, Inc., Los Angeles, Calif.). The dialyzed sputum extract (1.3 liters) was mixed with 30.0 g of CM

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Sephadex C25 resin (Pharmacia Fine Chemicals, Uppsala, Sweden) previously equilibrated in 0.02 M phosphate buffer, pH 6.2. After gentle stirring for 2 h at 4°C, the resin was extensively washed with 0.02 M phosphate buffer (pH 6.2), then packed in a chromatographic column (5.0 by 20 cm) and equilibrated in 0.02 M phosphate buffer (pH 6.2) until the absorbance at 280 nm returned to the base line. The lysozyme was further eluted in the same buffer with a linear gradient of NaCl from 0 to 1.0 M. Lysozyme was eluted between NaCl concentrations of 0.25 and 0.55 M. The fractions containing lysozyme were pooled and concentrated on an Amicon concentrator with a YM_2 membrane (Amicon Corp., Lexington, Mass.) and applied onto a Sephadex G 75 column (2.5 by 96 cm) (Pharmacia) equilibrated and eluted with 0.05 M phosphate buffer–0.15 M NaCl (pH 6.2). HAL was pure as judged by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method from King and Laemmli (22), and we estimated an apparent molecular weight of 15,000 for HAL. The specific activity of HAL was about 3.1 times higher than that of the hen egg-white lysozyme (Sigma Chemical Co., St. Louis, Mo.).

HLE was prepared from purulent sputum samples according to the method of Martodam et al. (29). The purification procedure involved NaCl extraction, affinity chromatography on Sepharose-bound Trasylol, and ion exchange chromatography on CM Sephadex C50 (Pharmacia). The enzyme was pure as judged by polyacrylamide gel electrophoresis at pH 4.0, and 98% was active, based on active site titration using turkey ovomucoid (42).

Crystalline *P. aeruginosa* elastase was supplied by Nagase Biochemicals Ltd., Tokyo, Japan. The molar concentrations of HAL, HLE and *P. aeruginosa* E were calculated by using molecular weights of 15,000, 29,000 (1), and 23,000 (32) and absorbance coefficients ($E^{1\%}_{280\ nm}$) of 22.41 (determined from weighing lyophilized salt-free HAL), 9.85 (1), and 14.52 (33), respectively.

Assay of lysozyme lytic activity. The lytic activity of HAL was evaluated by measuring the initial rate of lysis of an *M. luteus* cell wall suspension (Worthington Biochemicals Corp., Freehold, N.J.) used as substrate according to a modified procedure of Shugar (38). A 0.1-ml sample of test enzyme solution was added to a 2.9-ml substrate (0.15 mg/ml) in 0.07 M phosphate buffer (pH 7.0). The change in absorbance (ΔA) at 450 nm was followed spectrophotometrically for 3 min at 25°C, and the initial velocity was measured to give ΔA/min.

Effects of *P. aeruginosa* E and leukocyte elastase on lysozyme lytic activity. To assess the effects of *P. aeruginosa* E and HLE on the HAL lytic activity, mixtures containing various molar concentration ratios of *P. aeruginosa* E to HAL (1:5 to 1:40) and HLE to HAL (1:1 to 1:40) were incubated at 37°C. *P. aeruginosa* E-to-HAL molar ratios of 1:5, 1:10, 1:20, and 1:40 were obtained by mixing 13.0, 6.5, 3.2, and 1.6 μg, respectively, of *P. aeruginosa* E with 42.4 μg of HAL in a total volume of 2.4 ml of 0.1 M Tris buffer (pH 8.0) containing 2 mM calcium chloride. HLE-to-HAL molar ratios of 1:1, 1:5, 1:10, 1:20, and 1:40 were obtained by mixing 81.9, 16.4, 8.2, 4.1, and 2.0 μg, respectively, of HLE with 42.4 μg of HAL in a total volume of 2.4 ml of 0.1 M Tris buffer (pH 8.0) containing 2 mM calcium chloride. From each of the above reaction mixtures, a 200-μl sample was removed at different incubation times over a 4-h period. *P. aeruginosa* E and HLE were inhibited by adding 100 μl of EDTA (final concentration, 10 mM) and 100 μl of $alpha_1$ proteinase inhibitor (final concentration, 1.0 mg/ml; Sigma), respectively. Then, the HAL lytic activity was measured as mentioned above and expressed as a percentage of HAL control incubated and assayed in the same conditions. All incubation assays were realized at least in triplicate, and results were expressed as the mean arithmetic values.

Polyacrylamide gel electrophoresis. Incubation mixtures containing *P. aeruginosa* E and HAL in an elastase-to-lysozyme molar ratio of 1:10 were analyzed by electrophoresis on 15% polyacrylamide gel in 0.04 M Tris-acetate buffer (pH 7.4) in the presence of 0.2% SDS by the method of King and Laemmli (22). The reaction mixtures were prepared by mixing 400 μg of HAL, 61.3 μg of *P. aeruginosa* E in a total volume of 450 μl of 0.1 M Tris buffer containing 2 mM calcium chloride. At the selected incubation periods, *P. aeruginosa* E was inhibited by adding 100 μl of EDTA (final concentration, 10 mM) and the solutions were treated with 5% β-mercaptoethanol–1% SDS at 100°C for 10 min. A 30-μl sample of this mixture was applied to the gel, and the electrophoresis was run for 3 h with a current of 45 mA per gel. The gel slabs were stained for protein with Coomassie blue R-250 and scanned in a photometer Integraph gel scanner apparatus (Vernon, Paris, France). The amount of intact HAL was calculated from the relative intensity of the Coomassie blue stain as the area under the scanned peaks and plotted versus the HAL lytic activity of the corresponding incubation mixture. Molecular weight markers were phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and alpha lactalbumin (14,400) (Pharmacia).

Rocket immunoelectrophoresis. Incubation mixtures containing *P. aeruginosa* E and HAL in an elastase-to-lysozyme molar ratio of 1:10 were also analyzed by rocket immunoelectrophoresis as described by Laurell (27) and modified by Johansson and Malmquist (19). The reaction mixtures were prepared as indicated above for polyacrylamide gel electrophoresis. After the selected incubation period, a 10-μl sample of each reaction mixture was removed and tested for HAL immunoreactivity. Rocket immunoelectrophoresis was performed in 0.75% agarose gel (Indubiose A 37; IBF, Villeneuve la Garenne, France) in 0.02 M barbital buffer (pH 8.6) containing 2 mM calcium chloride. Anti-human lysozyme antiserum was obtained from Behring-Werke, Marburg, Federal Republic of Germany. The electrophoresis was carried out at 10 V/cm for 4 h in the same barbital buffer. Because of the high isoelectric point of lysozyme (pI ≃ 11) (28), the gel plate was arranged for cathodic migration. The rocket immunoprecipitates were stained with Coomassie blue R-250.

Gel filtration chromatography. Incubation mixtures containing *P. aeruginosa* E and HAL in an elastase-to-lysozyme molar ratio of 1:10 were also analyzed by gel filtration on Ultrogel AcA 202 (IBF). The reaction mixture was prepared as indicated above for polyacrylamide gel electrophoresis. After the selected incubation periods, each incubated mixture (550 μl) was applied to the Ultrogel AcA 202 column (1.6 by 90 cm) that was equilibrated and eluted with 0.1 M Tris buffer–0.17 M NaCl (pH 8.0) at a flow rate of 6.0 ml/h. All gel filtration experiments were performed at 4°C. Peptide fragments were detected at 280 nm.

RESULTS

Effects of *P. aeruginosa* E and leukocyte elastase on lysozyme lytic activity. HAL was progressively inactivated by *P. aeruginosa* E (Fig. 1). The decrease of HAL lytic activity towards the substrate *M. luteus* was found to be both time and molar ratio dependent. For instance, the half-time of inactivation was 240 min for a *P. aeruginosa* E-to-HAL

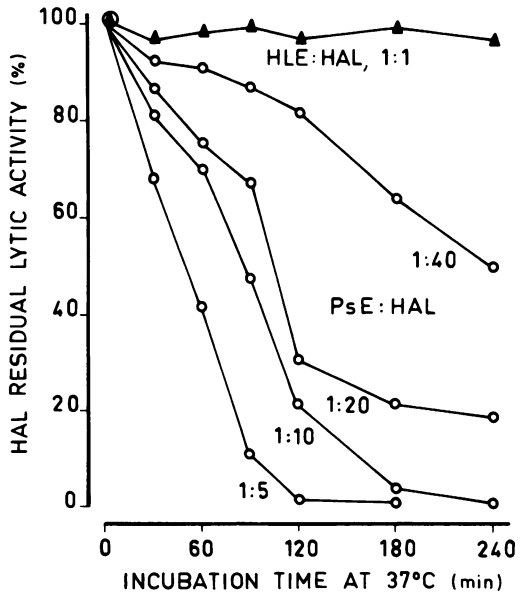

FIG. 1. Effect of *P. aeruginosa* E (PsE) and of HLE on HAL lytic activity toward *M. luteus* substrate. The experimental conditions are described in the text. Symbols: ○, molar ratios of *P. aeruginosa* E to HAL of 1:5 to 1:40; ▲, molar ratio of HLE to HAL of 1:1. The lytic activity of HAL is reported as the percentage of remaining activity relative to a similarly incubated control of HAL. The values represent the means of three replicate experiments. Standard errors of the means were less than or equal to 10% for all assays.

molar ratio of 1:40 and 45 min for a *P. aeruginosa* E-to-HAL molar ratio of 1:5. Total inhibition of the HAL lytic activity was observed within 120 min of incubation at 37°C for a *P. aeruginosa* E-to-HAL molar ratio of 1:5. The inactivation could be completely prevented by 10 mM EDTA. A control of pure HAL, incubated and assayed under the same conditions, underwent no change in its lytic activity.

In separate experiments, HAL was incubated with HLE in the same conditions and with molar concentration ratios for HLE to HAL of 1:1 to 1:40. In each case, no significant variation of HAL lytic activity could be detected, even in the presence of a high concentration of HLE (1:1 molar ratio) (Fig. 1) and after a long period of incubation up to 15 h.

Proteolysis. To determine whether the inactivation of HAL by *P. aeruginosa* E occurred by proteolysis, incubation mixtures with a *P. aeruginosa* E-to-HAL molar concentration ratio of 1:10 were analyzed by SDS-polyacrylamide gel electrophoresis and by gel filtration chromatography. Gel electrophoresis patterns (Fig. 2A) indicate that the band corresponding to HAL progressively disappeared during incubation with *P. aeruginosa* E. After 180 min of incubation (Fig. 2A, lane 6), no material remained at the undegraded HAL position. The gel slabs were scanned and the relative amount of the remaining HAL band was plotted versus the corresponding HAL lytic activity. As seen in Fig. 2B, the degradation of HAL strongly correlated with the loss of its lytic activity ($r = 0.99$, $P < 0.001$). The effect of incubation time on the cleavage of HAL by *P. aeruginosa* E was further analyzed by gel filtration chromatography on Ultrogel AcA 202. The elution profiles of incubation mixtures containing *P. aeruginosa* E and HAL in an elastase-to-lysozyme molar concentration ratio of 1:10 showed that HAL was progressively cleaved into several lower molecular weight fragments (Fig. 3). After 180 min of incubation,

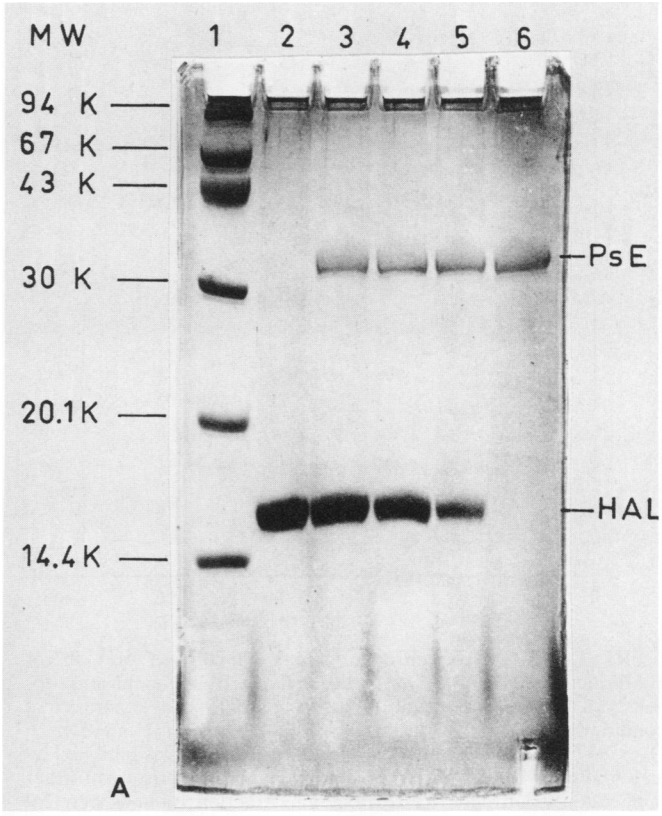

FIG. 2. (A) Effect of the incubation time on the degradation of HAL by *P. aeruginosa* E (PsE) observed by 15% SDS-polyacrylamide gel electrophoresis. The experimental conditions are described in detail in the text (molar ratio of *P. aeruginosa* E to HAL of 1:10). Incubation for 30, 60, 120, and 180 min corresponds to lanes 3, 4, 5, and 6, respectively. Lane 2, HAL alone; lane 1, low-molecular-weight markers (MW). K, $\times 10^3$. The anode was at the bottom. (B) Relationship between the HAL residual lytic activity and the amount of remaining HAL band as determined by densitometric scanning of polyacrylamide gel. Point 2 (HAL alone). Points 3, 4, 5, and 6 are HAL incubated with *P. aeruginosa* E for 30, 60, 120, and 180 min, respectively.

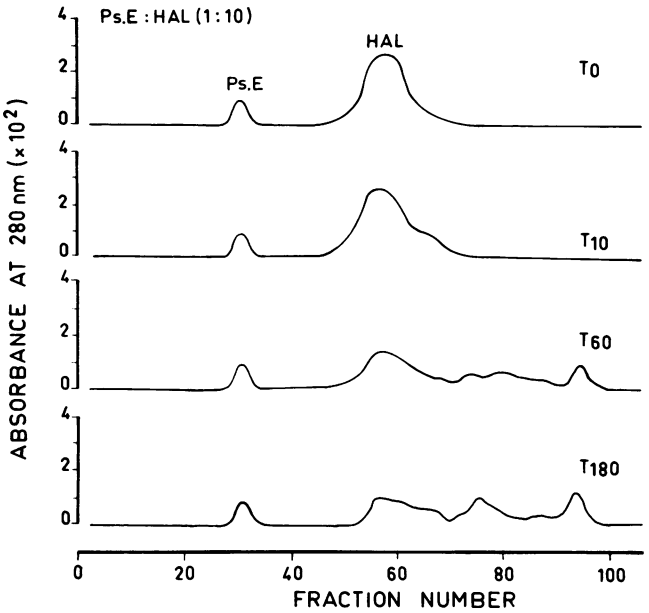

FIG. 3. Gel filtration chromatography on Ultrogel AcA 202 of HAL incubated with *P. aeruginosa* E (Ps.E) in an elastase-to-lysozyme molar concentration ratio of 1:10. The experimental conditions are described in the text. At zero time (T_0) and after 10 (T_{10}), 60 (T_{60}), and 180 min (T_{180}), *P. aeruginosa* E was inhibited by an addition of 10 mM EDTA. Each incubation mixture was further applied to the Ultrogel AcA 202 column. Fraction volumes were 2.0 ml.

which corresponded to a 100% inactivation of HAL lytic activity, the elution profile showed at least three major peak areas of peptide fragments detected at 280 nm.

Immunoreactivity. The effect of incubation time on the cleavage of HAL by *P. aeruginosa* E in a *P. aeruginosa* E-to-HAL molar concentration ratio of 1:10 was also studied by rocket immunoelectrophoresis (Fig. 4). It appeared that the surface of HAL immunoprecipitate decreased with the duration of incubation with *P. aeruginosa* E. After 60 min of incubation, the surface of HAL immunoprecipitate was 60% less than that of the control. After 120 min of incubation, no HAL immunoprecipitate was observed. Similar to gel electrophoresis patterns, the loss in the immunoreactive property of HAL correlated strongly with the loss of its lytic activity ($r = 0.99$, $P < 0.001$).

DISCUSSION

High concentrations of leukocyte elastase have been observed in infected bronchial secretions from chronic bronchitis (39) and cystic fibrosis patients (41). *P. aeruginosa*, a dominant pathogen often isolated in sputa from cystic fibrosis patients (14, 26), is well known to produce extracellular proteases such as alkaline protease and elastase (30, 31, 34). The latter is considered as an important virulence factor (2) involved in the pathogenesis of *P. aeruginosa* infections (32, 43). However, the interaction of the elastases released by leukocytes and *P. aeruginosa* with HAL has still not been investigated. The present work is, to our knowledge, the first to demonstrate clearly that *P. aeruginosa* E markedly inactivates HAL in vitro. On the other hand, it is surprising that HLE which has been shown to degrade human immunoglobulins (9) and complement components in vitro (21), has no effect on HAL lytic activity, even in a high concentration of HLE (1:1 molar ratio).

The loss of HAL functional activity by *P. aeruginosa* E is accompanied by proteolytic cleavage of HAL. Inactivation and breakdown of HAL were demonstrated both by functional assays (Fig. 1) and by polyacrylamide gel electrophoresis, gel filtration chromatography and rocket immunoelectrophoresis, using specific human lysozyme antiserum (Fig. 2, 3, and 4). The degraded fragments of HAL were not observed on polyacrylamide gels at 15% acrylamide concentration, suggesting that *P. aeruginosa* E had degraded HAL into low-molecular-weight polypeptides not detected by the gel system used. On the other hand, these degraded products have been detected by gel filtration chromatography on Ultrogel AcA 202, showing and confirming that *P. aeruginosa* E cleaved HAL into low-molecular-weight fragments in a time-dependent reaction (Fig. 3). Additional studies are under consideration to characterize these peptide fragments and the sites of the HAL molecule susceptible to hydrolysis by *P. aeruginosa* E.

According to our in vitro findings, we suggest that HAL might be inactivated and degraded in *P. aeruginosa*-infected bronchial secretions. However, we have recently shown (17) that there is no significant difference in immunological concentrations of HAL in cystic fibrosis sputa regardless of their *P. aeruginosa* numeration. This may be due to either a HAL release secondary to an increased granulocyte turnover observed in chronic bacterial infection of the respiratory tract (16) or to an inactivation of *P. aeruginosa* E. Recently, Döring et al. (6) have suggested that specific antibodies to *P. aeruginosa* E might neutralize the proteolytic activity of this elastase in vivo. According to these authors, *P. aeruginosa* E was only detected in bronchial secretions from cystic fibrosis patients when antibodies to this elastase were lacking in sera and bronchial secretions. It has also been demonstrated that plasma alpha-2 macroglobulin completely inhibits *P. aeruginosa* E (13). In a recent study, Tournier et al. (41) suggest that alpha-2 macroglobulin may also be a potential factor limiting *P. aeruginosa* E activity in vivo in cystic fibrosis bronchial secretions. They

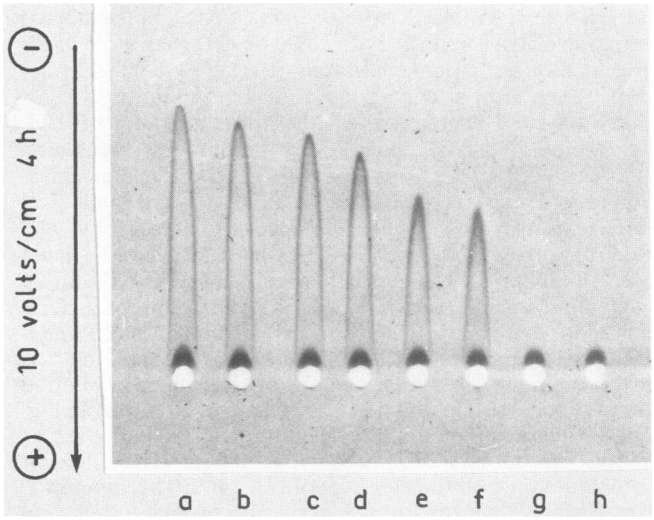

FIG. 4. Rocket immunoelectrophoresis of HAL incubated with *P. aeruginosa* E in an elastase-to-lysozyme molar concentration ratio of 1:10. At zero time (a), 10 (b), 20 (c), 30 (d), 60 (e), 90 (f), 120 (g), and 180 min (h), *P. aeruginosa* E was inhibited by an addition of 10 mM EDTA. Reaction mixtures were tested for HAL immunoreactivity as reported in the text.

showed that alpha-2 macroglobulin concentrations were negatively correlated with *P. aeruginosa* E activities in sputa from cystic fibrosis patients which were contaminated by *P. aeruginosa*. These results suggest that *P. aeruginosa* E-induced inactivation of HAL may be restricted in vivo by specific antibodies to *P. aeruginosa* E or alpha-2 macroglobulin or both.

In summary, the present work demonstrates that HAL is cleaved and inactivated by *P. aeruginosa* E and not by HLE in vitro. Further studies should be undertaken to investigate whether *P. aeruginosa* E may actually degrade HAL in vivo and therefore limit its potential antibacterial role in defense of the lung.

ACKNOWLEDGMENTS

This work was supported by grants from the Commission des Communautés Européennes (7248.12.003) and the Association Francaise de Lutte contre la Mucoviscidose (2.81/3).

We express sincere appreciation to Jean-Marc Egly (INSERM Unité 184, Strasbourg, France) and to Guy Linden and Jean-Yves Le Deaut (Université Nancy I, Nancy, France) for critical reading and for excellent suggestions. We also acknowledge Yves Meckler for technical assistance and Patricia Ulmer and Marie-Claude Rohrer for preparing the manuscript.

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