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Inactivation of Human Gamma Interferon by *Pseudomonas aeruginosa* Proteases: Elastase Augments the Effects of Alkaline Protease Despite the Presence of α_2-Macroglobulin

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Pseudomonas aeruginosa **alkaline protease (AP) has recently been shown to produce limited proteolysis of human gamma interferon (IFN-γ) and thereby destroy the antiviral and macrophage-activating activities of the lymphokine. In the present study we describe some of the characteristics of *Pseudomonas* elastase (E) with regard to inactivation of human IFN-γ. The inhibitory effect of E on IFN-γ bioactivity differed from that of AP in that the direct effects of E were reduced in the presence of human serum. That this property of human serum was in large part attributable to the protease inhibitor α_2-macroglobulin (α_2-M) was suggested by the following observations: (i) methylamine treatment of serum reduced its effect on E, (ii) E interacted directly with α_2-M to induce a characteristic conformational change in the protease inhibitor, and (iii) preformed E–α_2-M complexes lacked IFN-γ-degrading activity. Despite these findings, anti-E antiserum partially neutralized the effect that a *Pseudomonas* filtrate showed on IFN-γ, suggesting that E contributes to the activity of bacterial filtrates. Treatment of IFN-γ with E in the presence of a suboptimal concentration of AP resulted in an E dose-dependent inactivation of the lymphokine. Preformed E–α_2-M complexes, although ineffective by themselves at cleaving IFN-γ, degraded the lymphokine, providing AP was also present in the reaction mixture. These data demonstrate that the destruction of small, biologically significant peptides by *Pseudomonas* proteases can involve protease-protease synergy that acts even in the presence of the serum protease inhibitor α_2-M.**

A number of studies have shown that the opportunistic pathogen *Pseudomonas aeruginosa* can nonspecifically inhibit T-lymphocyte-mediated immunity in vitro (14, 15, 26, 32, 36) and in vivo (5, 14, 30). Patients infected with *P. aeruginosa* often show evidence of impaired cell-mediated immunity (28, 33, 34). In support of these clinical findings, Neely et al. (25) demonstrated that infection with *P. aeruginosa* predisposed burned mice to lethal infections with *Candida albicans*. Two independent groups, Blackwood et al. (5) and Petit et al. (30), found that mice injected with *P. aeruginosa* were more susceptible to infection with the intracellular pathogen *Listeria monocytogenes* and showed a decrease in delayed-type hypersensitivity to *Listeria* and erythrocyte antigens.

Gamma interferon (IFN-γ) is a T-cell-derived lymphokine with potent antiviral activity that undoubtedly plays an important role in cell-mediated immunity. We recently observed that the *Pseudomonas* exoenzyme alkaline protease (AP) reduced the antiviral and immunomodulatory activities of human IFN-γ by limited proteolytic degradation of the lymphokine (15). Thus, AP caused a minor reduction in the mass of IFN-γ, which resulted in a significant decline in the bioactivity of the lymphokine. These findings provide a plausible explanation for some of the observations cited above regarding the immunosuppressive properties of *P. aeruginosa* and suggest a general mechanism whereby pathogenic microorganisms may interfere with host immune responses.

Since many clinical isolates of *P. aeruginosa* produce both AP and a second enzyme, elastase (E) (16, 17, 23), we were interested in determining what effects E might have on IFN-γ. The action of the two proteases differed in at least one fundamental respect. Whereas the effect of AP on IFN-γ was undiminished by the presence of human serum, the direct action of E was markedly reduced by serum components. This serum effect was primarily attributable to α_2-macroglobulin (α_2-M), a protease inhibitor found in high concentration (1 to 3 mg/ml) in human serum. Inactivation of E by α_2-M appeared to result from the characteristic binding and trapping of the protease by α_2-M, and E–α_2-M complexes lacked the ability to degrade IFN-γ directly. Nonetheless, E appeared to augment the effects AP had on human IFN-γ, even in the presence of serum. Presented here is a mechanism for this unexpected finding that suggests that the regulation of protease effects during *Pseudmonas* infections may not be as simple as previously assumed.

MATERIALS AND METHODS

P. aeruginosa **and *Pseudomonas* proteases.** *P. aeruginosa* GoM has been characterized previously as an AP- and E-producing strain (15, 27, 29) and was used in the present study as a representative of the majority of clinical isolates we have tested. Filtrates from this strain were prepared as previously described (15). Crystalline preparations of *Pseudomonas* AP (lots 9356012 and 9442009) and E (lot 9442008) were purchased from Nagase Co., Ltd. (Tokyo, Japan) and have been characterized previously (15, 27). Although both enzyme preparations contained lipopolysaccharide, cross contamination with the heterologous protease was less than 0.1% as judged by enzyme-linked immunosorbent assay, radioimmunoassay, or Western immunoblot assays with rabbit antisera to either protease or by stimulation of protease-specific T-cell clones (27).

Anti-AP and anti-E antisera. Antisera to the *Pseudomonas*

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proteases were prepared by immunizing rabbits as previously described (15). Anti-AP and anti-E antisera reacted on Western immunoblots with proteins of 57 and 33 kilodaltons, respectively, and specifically neutralized proteolysis of casein induced only by the appropriate enzyme.

Human serum and α_2-M. Normal human sera were collected from seronegative donors (donors lacking antibodies to *Pseudomonas* proteases detected by enzyme-linked immunosorbent assay) and were either used fresh or stored at −70°C. The α_2-M content of serum samples ranged from 1.0 to 3.0 mg/ml as determined by radial immunodiffusion with an antiserum to α_2-M (Chemicon, El Segundo, Calif.) and purified, biologically active (electrophoretically slow) α_2-M (Biodesign, Kennebunkport, Maine) as a standard. Where indicated, serum or α_2-M was pretreated for 2 h at 37°C with 0.1 M methylamine–0.1 M sodium phosphate, pH 8, to inactivate α_2-M (2, 7). The enzymatic activity of *Pseudomonas* E was unaffected by exposure to methylamine.

Protease assay. Proteolytic activity was measured by a colorimetric assay by using hide blue powder substrate (Calbiochem-Behring, La Jolla, Calif.) in the procedure of Rinderknecht et al. (31). Briefly, 20 mg of hide blue powder substrate was suspended in 1 ml of 0.1 M phosphate buffer, pH 8, containing 2 μg of E. In some experiments 25 to 50 mM EDTA was added to inhibit AP-mediated proteolysis. Incubation was accompanied by occasional mixing at 37°C until an intense blue appeared in the supernatant fluids. Reactions were stopped by the addition of ice-cold buffer, and supernatant A_{595} was measured. Data represent duplicate measurements and are expressed as absorbance units per hour per microgram of enzyme.

IFN-γ and antiviral assay. Unpurified natural IFN-γ was prepared by stimulating human mononuclear blood leukocytes from tuberculin-reactive donors with tuberculin purified protein derivative and collecting supernatant culture fluids on day 5. Human recombinant IFN-γ (rIFN-γ) (lot G11026-01) was kindly provided by Michael Shepard, Genentech, Inc. (South San Francisco, Calif.). The antiviral activities of both natural and rIFN-γ were completely neutralized by a monoclonal antibody (69B) specific for human IFN-γ (kindly provided by S. Pestka, [18]).

Antiviral activity was determined as described previously (15) by measuring inhibition of the cytopathic effect of vesicular stomatitis virus on the human amniotic cell line WISH. Results are expressed as units per milliliter, where 1 U of activity was defined as the amount of sample that inhibited cytopathic effect by 50% under assay conditions standardized to a National Institutes of Health IFN-γ standard (Gg 23-901-530).

Polyacrylamide gel electrophoresis and Western immunoblot assays. Electrophoresis of protease–α_2-M incubation mixtures was performed on 5% native polyacrylamide minigels (Bio-Rad Laboratories, Richmond Calif.) by using the discontinuous buffer system described by Van Leuven et al. (37). Following electrophoresis of native gels, proteins were electrophoretically transferred (100 V/15 min) to nitrocellulose in 25 mM Tris–192 mM glycine, pH 8, containing 5% methanol. After blocking with 1% bovine serum albumin in phosphate-buffered saline, α_2-M was visualized with a specific polyclonal antiserum and peroxidase-conjugated anti-immunoglobulin antibody. Western transfers were then developed with 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo.) prepared by dissolving the substrate in methanol and adding Tris-buffered saline (pH 7.5) that contained 0.02% hydrogen peroxide.

For Western immunoblots of IFN-γ, samples were diluted in sodium dodecyl sulfate (SDS)-containing sample buffer, heated to 95°C for 3 min, and applied to 14% polyacrylamide minigels containing SDS but lacking urea and mercaptoethanol (19). Nitrocellulose transfers were prepared as described above and were developed with an antiserum to human IFN-γ obtained from Genzyme (Boston, Mass.). Similar results were obtained with an antiserum from Chemicon produced by immunization of goats with a synthetic peptide of IFN-γ. A peroxidase-conjugated second antibody was used as described above to visualize immunoprecipitates.

To verify that both E and α_2-M were present within complexes formed between the protease and human serum, 100 μl of human serum was incubated with 20 μg of E at 37°C for 1 h. The sample was diluted with an equal volume of sample buffer and electrophoresed under nondissociating conditions on a 5% polycarylamide gel. Control lanes were stained with Coomassie blue to visualize protein bands. Material from the region of the nonstained portion of the gel that corresponded to the electrophoretically fast migrating α_2-M band was eluted at 4°C overnight in 0.1 M phosphate buffer, pH 7. This band was clearly resolvable from the region of the gel containing free E. The eluted sample was diluted in sample buffer containing SDS and 2-mercaptoethanol and then heated to 95°C for 5 min. The sample was then applied to several lanes of a 9 to 14% polyacrylamide gradient gel containing SDS, electrophoresed, and transferred to nitrocellulose. Western immunoblots were developed as described above by using either anti–α_2-M or anti-E to detect the two components.

RESULTS

Figure 1 shows the effects of purified *Pseudomonas* AP and E on the antiviral activity of human rINF-γ. Samples of rIFN-γ were incubated with the proteases, and then residual bioactivity was assessed in the WISH cell antiviral assay. Both proteases reduced IFN-γ bioactivity in a dose-dependent fashion. Of note was the reduction in inhibitory activity associated with E when enzyme treatment was performed in the presence of 10% human serum. Approximately 10 times more protease was required in the presence of serum to achieve the effect seen without serum. By contrast, this concentration of human serum had little effect on the activity of AP.

The serum used for this experiment was devoid of antiprotease antibodies detectable by enzyme-linked immunosorbent assay. For this reason we postulated that α_2-M or a similar serum protease inhibitor might be responsible for the effect. To test this hypothesis, E was incubated with either human serum or methylamine-treated human serum for 2 h at 37°C. Methylamine is known to inactivate α_2-M by inducing a conformational change in the molecule that prevents its binding to proteases (2, 7, 35). The mixtures were then assayed for residual protease activity or their ability to inactivate IFN-γ.

Human serum (1.5 to 2%) inhibited E proteolytic activity, and methylamine treatment of the serum prevented this effect (Fig. 2A). Addition of as much as 22% methylamine-treated serum had no significant effect on E proteolysis of hide blue powder substrate. Similarly, the effect that E had on rIFN-γ (Fig. 2B) was prevented by 4 to 5% serum, whereas concentrations approaching 20% methylamine-treated serum were required for a comparable effect.

These data suggested that serum α_2-M interacted directly with E to reduce the effect of the enzyme on IFN-γ. α_2-M is

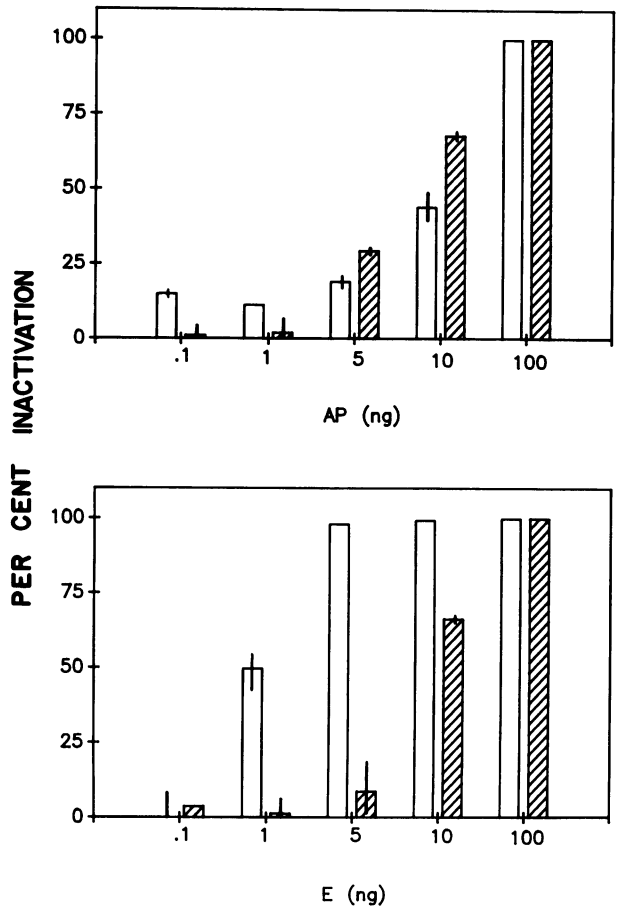

FIG. 1. The effect of 10% human serum on the inactivation of human rIFN-γ by *Pseudomonas* proteases. Samples of rIFN-γ (200 ng) were treated with AP or E (0.1 to 100 ng) in a total volume of 10 μl for 24 h at 4°C in the absence (□) or presence (▨) of 10% human serum. Following dilution in medium, the samples were assayed for residual antiviral activity. Data are expressed as percent inactivation of IFN-γ, in which the activity of the untreated control was 8.2 × 10^5 antiviral units per ml. A portion of the data in this figure has been published previously (15) and is presented here for comparison. Error bars indicate 1 standard deviation. The data are representative of three such experiments.

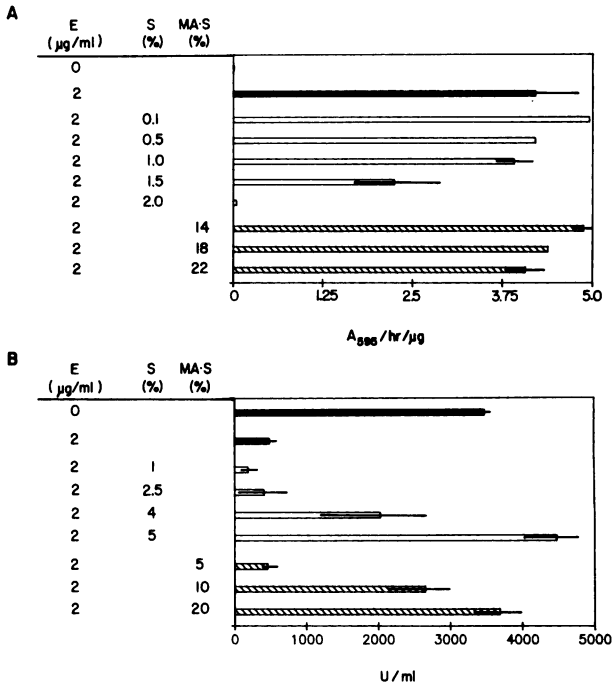

FIG. 2. Methylamine prevents the effects that human serum has on E. (A) A 2-μg/ml solution of E was treated with various concentrations of serum (S) or methylamine-treated serum (MA-S) for 2 h at 37°C, and residual proteolytic activity was measured. (B) A 2-μg/ml solution of E was similarly treated with serum or methylamine-treated serum. Treated protease samples were then combined with equal volumes of rIFN-γ (2,000 U/ml) in RPMI 1640–1% bovine serum albumin and incubated for 5 h at 37°C. Residual antiviral activity of the lymphokine was then measured. Error bars indicate 1 standard derivation. These data are representative of three such experiments.

known to regulate the activity of a number of circulating proteases and does so by forming a complex with the protease, thereby sterically inhibiting its enzymatic activity. To determine whether enzymatically inactive complexes were formed between α_2-M and *Pseudomonas* E, the protease was incubated with serum or purified α_2-M and the mixtures were examined by immunoblots of native or SDS-polyacrylamide gels (Fig. 3). Figure 3A shows the effects of treating human serum with E and monitoring the electrophoretic mobility of α_2-M with an anti–α_2-M antibody. In serum, α_2-M showed a slow electrophoretic mobility (lane 2) that corresponded to the migration of biologically active purified α_2-M (lane 1). This slow form was converted to a fast form with E treatment (lanes 3 through 6). The conversion was similar to the effect that has been reported following trypsin treatment (2, 5) or to that seen with methylamine treatment (lane 7). As is typical of other protease-α_2-M complexes, complete conversion to the fast form occurred when protease/α_2-M molar ratios approximated 1:1. A similar effect was seen when purified α_2-M (instead of serum) was treated with E (data not shown).

Consistent with these findings was the observation that both E and α_2-M could be recovered from complexes formed between the protease and serum. This was shown by incubating E with serum, electrophoresing the mixture under nondissociating conditions on a 5% polyacrylamide gel, and eluting the material that migrated in the region of the gel corresponding to the fast α_2-M band. When this eluted material was then electrophoresed on a reducing SDS-polyacrylamide gel, transferred to nitrocellulose, and developed with either anti-E or anti–α_2-M, both components were detected (data not shown).

To test the biologic effects of such complexes, samples of rIFN-γ were incubated with either E or α_2-M-treated E and then inspected on immunoblots for evidence of proteolytic cleavage. Treatment of IFN-γ with E led to a progressively greater fragmentation of the lymphokine as enzyme concentrations were increased (Fig. 3B). Samples of rIFN-γ treated with 10 ng of E (lane 4) were completely devoid of antiviral activity (data not shown). By contrast, E pretreated with α_2-M (lanes 5 through 7) lacked proteolytic activity in the hide blue powder assay (data not shown) and failed to cleave IFN-γ, even at approximately equal molar ratios of E and IFN-γ (lane 7). Viewed together, these data argue that *Pseudomonas* E, when exposed to serum α_2-M, forms a complex with the macroglobulin that prevents direct proteolysis of human IFN-γ by the enzyme.

The effects of the purified *Pseudomonas* proteases on the antiviral activity of a preparation of natural IFN-γ are shown

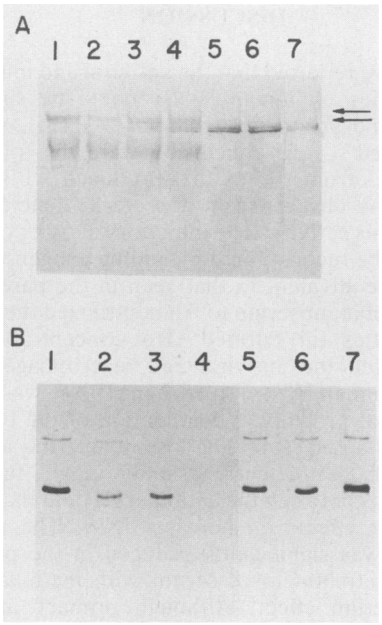

FIG. 3. Western immunoblots show that *Pseudomonas* E interacts directly with α_2-M, resulting in a reduction in E proteolytic activity. (A) E converts the α_2-M in human serum from a slow- to a fast-migrating form. The following samples were electrophoresed on a native 5% polyacrylamide gel, transferred to nitrocellulose, and developed with anti-α_2-M antiserum. Lanes: 1, α_2-M; 2 through 6, 1 μl of human serum (2 μg of α_2-M) combined with 0, 10, 20, 40, or 80 ng of E, respectively, in 20 μl of phosphate-buffered saline and incubated for 1 h at 37°C; 7, methylamine-treated human serum. The serum used in this experiment contained 2.0 mg of α_2-M per ml. The arrows indicate the positions of slow and fast α_2-M. (B) Effects of E and α_2-M-treated E on human rIFN-γ. A total of 1 μg of E was treated with 25 μg of purified α_2-M in 0.1 M PO_4, pH 8, for 1 h at 37°C. Treated and untreated E were then diluted and used to further treat 100-ng samples of rIFN-γ (37°C for 2 h; total volume, 20 μl), and evidence for proteolysis was assessed by Western immunoblots. Lanes: 1, 100 ng of rIFN-γ; 2 through 4, 100 ng of rIFN-γ treated with 0.1, 1, and 10 ng of E, respectively; 5 through 7, 100 ng of rIFN-γ treated with 1, 10, and 100 ng of α_2-M-treated E, respectively. Both monomeric and nondissociated dimeric rIFN-γ are visualized here. Each of these experiments was repeated at least once, with essentially the same results.

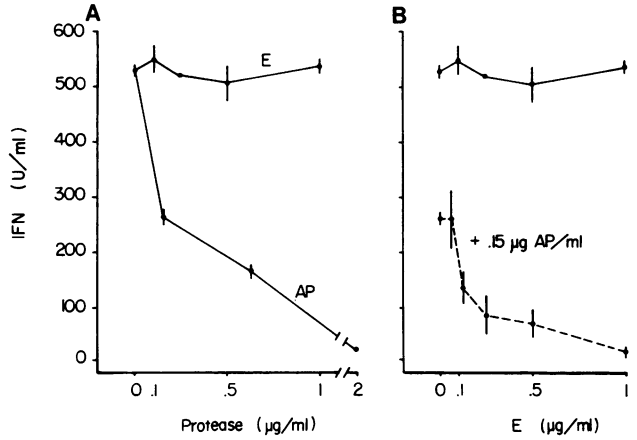

FIG. 4. Effects of purified *Pseudomonas* AP or E or both on human IFN-γ. (A) An unpurified sample of natural IFN-γ contaning 10% human serum was incubated at 37°C for 24 h with the indicated concentrations of one of the two proteases, and residual antiviral activity was measured. (B) IFN-γ was incubated with E at the indicated concentrations at 37°C for 24 h either in the absence or presence of 0.15 μg of AP per ml. Then the residual antiviral activities of the preparations were determined.

in Fig. 4A. The IFN-δ sample used here was a culture supernatant fluid from antigen-stimulated T cells and contained 10% human serum. As with rIFN-γ, natural IFN-γ was susceptible to AP at protease concentrations of 0.1 to 2 μg/ml but was resistant to E at similar concentrations. An unexpected finding is shown in Fig. 4B. When a suboptimal concentration of AP was used with E, the latter protease showed a potent concentration-dependent inhibitory effect, reducing the antiviral activity of the IFN-γ sample more than 80% at an E concentration of 250 ng/ml. This implied that a synergistic relationship existed between the two proteases despite the presence of serum α_2-M.

On the basis of these data, one would predict that the ability of certain *Pseudomonas* culture filtrates to inactivate IFN-γ could be reduced by pretreating the filtrates with an antiserum specific for E. The results of such an experiment are shown in Fig. 5. *Pseudomonas* strain GoM produces both AP and E (27), and filtrates prepared from the strain inactivate IFN-γ bioactivity (15). Pretreatment of the filtrate with either an antiserum specific for AP or an antiserum specific for E partially reversed the effect of the filtrate on IFN-γ. Normal rabbit serum had no such effect, indicating that the effect of the antiserum at these concentrations was not due simply to α_2-M or to a similar protease inhibitor. These results indicate that both proteases contributed to the overall effects of the filtrate, even in the presence of human serum.

Since E was shown to readily form complexes with α_2-M when added to human serum and these complexes lacked proteolytic effects on IFN-γ, it was of interest to determine whether E–α_2-M complexes degraded IFN-γ in the presence of AP. rIFN-γ was susceptible to partial degradation by AP (Fig. 6, lane 2). Once again, E–α_2-M complexes had no such proteolytic effect (lane 3). Incubation of rIFN-γ with both AP and E–α_2-M resulted in significantly greater proteolytic degradation of the lymphokine than was seen with AP alone

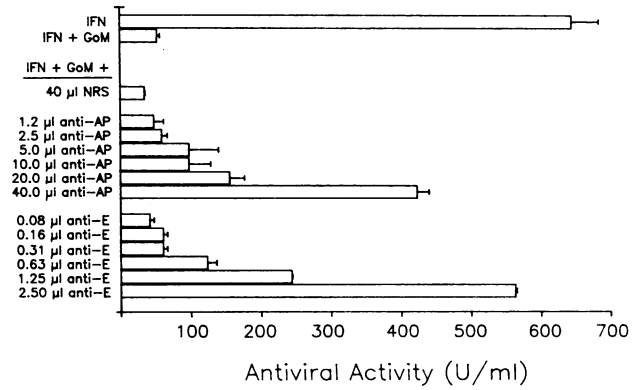

FIG. 5. Neutralization of the activity of *Pseudomonas* strain GoM filtrate by preincubation with antisera to AP or E. Samples (25 μl) of *Pseudomonas* strain GoM filtrate were mixed with the indicated volumes of antisera or normal rabbit serum (NRS) and diluted to a total volume of 1 ml. These mixtures were incubated at 25°C for 2 h and then added to an equal volume of natural IFN-γ. Incubation was continued at 37°C for 24 h before residual antiviral activity was measured. Error bars indicate standard deviations. The various rabbit sera had no effect on the antiviral assay.

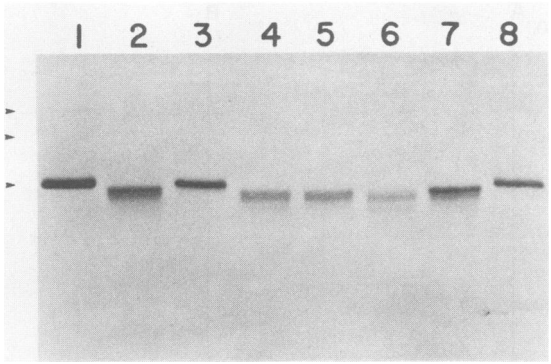

FIG. 6. α_2-M prevents the degradation of human rIFN-γ, but not AP-treated human rIFN-γ, by E. E–α_2-M complexes were prepared at a ratio of 1:30 (100 μg of E and 3 mg of α_2-M in 1 ml) as described in Materials and Methods. Human rIFN-γ (1 μg) was incubated in a reaction volume of 10 μl with each enzyme preparation for 1 h at 37°C. Each reaction mixture was then electrophoresed on an SDS-polyacrylamide gel and stained with Coomassie blue stain. Lanes: 1, no proteases; 2, 10 ng of AP; 3, 10 ng of E–α_2-M; 4, 5 ng of AP plus 0.1 ng of E–α_2-M; 5, 5 ng of AP plus 1 ng of E–α_2-M; 6, 5 ng of AP plus 10 ng of E–α_2-M; 7, 10 ng of AP plus 10 ng of E–α_2-M in 1 mM phosphoramidon; 8, no proteases. Indicated quantities of E–α_2-M are based on E content. The arrows denote the migration of molecular size markers of 39, 27, and 17 kilodaltons (top to bottom). These results are representative of four experiments.

(lanes 4 through 6). That this latter effect was due to the action of E was shown by inhibiting the effect of E–α_2-M with phosphoramidon, an active-site inhibitor of E (24) (lane 7). Therefore, E degraded IFN-γ, even in the presence of α_2-M, provided that AP was also present in the reaction mixture.

These results could be explained by postulating that AP inactivated α_2-M and released enzymatically active E from the complex. To test this hypothesis, E–α_2-M complexes were exposed to AP under conditions similar to those described for Fig. 6 to determined whether release of proteolytic activity from the complex was observed. The hide blue powder assay was modified by inclusion of 25 to 50 mM EDTA, which inhibits the action of AP but not E. The inhibition of E activity caused by preincubation with α_2-M was not reversed by treatment of the complex with AP (Table 1). These results suggest that the effects of AP shown in Fig. 6 are on the substrate, rIFN-γ, not on the protease inhibitor.

TABLE 1. Effect of AP on E–α_2-M complexes

Incubation mixtures (protease concn [μg/ml])	Activity[a] (A_{595}/h per μg)
None	0.000
E (2)	0.968 ± 0.012
AP (2)[b]	0.776 ± 0.030
AP (2)	0.047 ± 0.016
E–α_2-M complex[c] (2)	0.012 ± 0.009
E–α_2-M complex (2) + AP (2)	0.014 ± 0.012

[a] Data presented here are mean values ± standard deviations for an experiment in which 25 mM EDTA was included in the protease assay buffer; a second experiment using buffer containing 50 mM EDTA yielded comparable results.

[b] Without 25 mM EDTA in the protease assay buffer.

[c] E–α_2-M complexes were prepared in the absence of EDTA by combining 2 μg E and 60 μg α_2-M in 40 μl as described in Fig. 6. The complexes were then treated with 2 μg of AP (37°C for 1 h) in the absence of EDTA. This incubation mixture was then diluted in assay buffer containing 25 mM EDTA.

DISCUSSION

Previously we reported that the antiviral and immunomodulatory activities of human IFN-γ could be substantially reduced by exposure to *Pseudomonas* AP (15). Among the findings that led to this conclusion were the following. (i) Culture filtrates from strains that produced AP inactivated IFN-γ, whereas filtrates from a protease-deficient mutant strain lacked this activity. The inhibitory activity of the latter filtrates could be reconstituted by adding exogenous AP at a concentration equivalent to that seen in the parental-strain filtrate. (ii) Rabbit antiserum to AP neutralized the activity of inhibitory filtrates. (iii) Purified AP at concentrations of 1 to 2 μg/ml reduced the antiviral and macrophage-activating properties of human IFN-γ. (iv) When rIFN-γ was incubated with AP, partial proteolytic degradation of the lymphokine occurred, with a corresponding loss in antiviral activity.

It is clear from the current study that a fundamental difference exists between the action of AP and that of E with regard to their effects on human IFN-γ. The proteolytic activity of E was significantly reduced in the presence of serum, and pretreatment of serum with methylamine prevented the serum effect. Although primary amines can modify other serum proteins, these results were consistent with a role for α_2-M in mediating serum effects on E. It should also be noted that the effect of methylamine can be incomplete. Therefore, the degree to which a methylamine-treated serum preparation prevents protease action can depend on the protease assay used. Assays requiring longer incubation periods between a protease and its substrate may show greater α_2-M effects (less methylamine inactivation) than those with short incubation periods (e.g., Fig. 2B versus A, respectively). Alternatively, IFN-γ may simply be a poorer substrate for E than certain proteins found in serum. As serum concentration is increased, greater competition for E activity is seen and less IFN-γ inactivation results.

Several previous reports have suggested that α_2-M may inhibit the proteolytic activity of *Pseudomonas* E in vitro (9, 11, 12) or in vivo (12, 13), but evidence for a direct interaction between α_2-M and *Pseudmonas* E has, until now, been lacking. The interaction of E with α_2-M resulted in changes in the protease inhibitor similar to those seen when α_2-M binds other proteolytic enzymes. As initially proposed by Barrett and Starkey (3), α_2-M inactivates proteases by a capture mechanism that requires protease attack at a particular region of the macroglobulin (4, 10), followed by a conformational change in the protease inhibitor (2, 4, 21, 37). This results in the trapping of the enzyme (8) and steric inhibition of its action on large substrates. The enzyme can retain proteolytic activity for smaller substrates that can penetrate the complex (6, 20, 22). The more compact, or fast, form of the macroglobulin shows altered migration on native polyacrylamide gels and reduced protease-binding activity. Both of these properties can also be induced by treatment of α_2-M with primary amines (2, 4, 7).

Several findings indicate that α_2-M forms a complex with *Pseudomonas* E. First, E converted electrophoretically slow α_2-M to the fast form, whose migration on native gels was indistinguishable from that of methylamine-treated α_2-M. The migration of E-treated α_2-M could not be further affected by subsequent methylamine treatment or vice versa (data not shown), suggesting that E induced a conformational change in α_2-M similar to that induced by methylamine. Second, when serum and E were incubated together and electrophoresed on native polyacrylamide gels, material

could be eluted from the region of the gel corresponding to fast α_2-M, and this eluted material reacted with both anti-E and anti-α_2-M antisera. This region of the native gel was otherwise uncontaminated by free E (i.e., free E showed a slower migration than α_2-M on native gels). Third, and consistent with these biochemical findings, α_2-M prevented the degradation of IFN-γ by E. Taken together these data argue that α_2-M forms a complex with *Pseudomonas* E that prevents direct proteolytic degradation and inactivation of human IFN-γ.

Certain *Pseudomonas* filtrates reduced IFN-γ antiviral activity in the presence of serum, and antibody to E neutralized the effects of these filtrates. This latter finding did not at first seem consistent with the α_2-M effect described above. One would have predicted that the action of E in such filtrates would have been prevented by its complexing with serum α_2-M. The finding that AP and E acted synergistically, even in the presence of serum, suggested an explanation for these seemingly disparate observations. Although α_2-M inhibits the proteolytic activity of *Pseudmonas* E, it may do so only for certain substrates. These include native human IFN-γ. However, in the presence of AP, E–α_2-M complexes appeared to further degrade rIFN-γ beyond what was seen with AP alone.

Although there are several potential explanations for this phenomenon, recent studies by Borth and Teodorescu (6) suggest a likely mechanism. These investigators have reported that the ability of trypsin to inactivate interleukin-2 (IL-2) was inhibited by pretreating the enzyme with α_2-M. However, trypsin–α_2-M complexes were not completely devoid of proteolytic activity for IL-2 and degraded the lymphokine at a rate one-sixth that of free trypsin. They postulated that this finding reflected the fact that the relatively small substrate (15,500 daltons) gained access to the active site of the bound protease, an interpretation that is also consistent with results from similar studies with proinsulin (22) and certain synthetic peptides (1). Thus, the same mechanism may explain the action of *Pseudomonas* E. We would suggest that α_2-M binds E and inactivates its direct proteolytic attack on native human IFN-γ. However, in the presence of AP, IFN-γ is modified in such a manner that it is accessible to the enzymatic site of E within the complex and is further degraded by the second protease. Besides the partial digestion of IFN-γ by AP shown on immunoblots, it is also possible that the protease promotes dissociation of the dimeric form of the lymphokine and thereby reduces its molecular dimensions. Thus, even in the presence of serum, the two proteases together yielded a much higher level of IFN-γ inhibitory activity than was seen with either enzyme alone. Minor modifications of IFN-γ by AP, visualized on gels, were further enhanced by E–α_2-M complexes. Alternative mechanisms, including proteolytic activation of one enzyme by the other, have not as yet been entirely ruled out. However, AP and E do not show a similar synergistic effect on IFN-α, IFN-β, or casein. Likewise, we were unable to show that AP inactivates α_2-M, thereby releasing active E from a preformed E–α_2-M complex.

The cleavage of cytokines by bacterial proteases may provide an important means of modulating host immune responses during infections. Theander et al. (36) have reported that *Pseudomonas* AP and E reduced mitogen-induced T-cell proliferation by a process that appeared to involve degradation of IL-2. The protease concentrations used by these investigators, while typical of those found in stationary-phase bacterial culture filtrates, were 10 to 50 times higher than those we found to be adequate to inactivate human IFN-γ (Fig. 4). In experiments performed earlier (15), AP at a concentration of 2 μg/ml was sufficient to completely eliminate the antiviral activity produced in antigen-stimulated T-cell cultures, whereas IL-2-dependent proliferation of the cells was unaffected by this protease concentration. Since the experiments of Theander et al. were also performed in the presence of serum, we can only attribute their ability to inactivate IL-2 to the substantially higher protease concentrations they used.

If the effects reported here occur in vivo, the outcome for the host will depend on both the nature and quantity of proteases produced during an infection and on the concentrations of protease inhibitors found in infection sites. For example, during initial *Pseudomonas* infections in the lungs (i.e., prior to extensive exudation), the pulmonary airways have very low concentrations of α_2-M. One would expect that both AP and E would be fully active under these conditions. By contrast, patients with chronic *Pseudomonas* pulmonary infections, as is seen in cystic fibrosis, have significant concentrations of α_2-M in their lungs. Goldstein and Döring (9) have reported mean concentrations in sputum for a group of cystic fibrosis patients of 29 μg of α_2-M per ml and slightly elevated levels in serum averaging 4.8 mg/ml. This would be expected to result in a significant reduction in *Pseudomonas* E activity within the lungs even at protease concentrations of 1 μg/ml. The current study demonstrates that even this relationship between protease production and α_2-M concentration is not a simple one, since the concomitant production of AP by the bacterium permits the expression of E proteolytic effects on some important substrates.

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