Pseudomonas aeruginosa Alkaline Protease Degrades Human Gamma Interferon and Inhibits Its Bioactivity

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This study was performed to determine the effect of Pseudomonas aeruginosa on gamma interferon (IFN-γ) production by antigen-stimulated human T-cell clones. Crude bacterial filtrates prepared from certain strains of P. aeruginosa inhibited IFN-γ production by T cells and reduced the antiviral activity of preformed IFN-γ. Bacterial filtrates prepared from mutant strains that did not produce the exoenzyme alkaline protease (AP) did not inhibit IFN-γ activity. The inhibitory activity of bacterial filtrates was heat and trypsin sensitive and was neutralized by an antiserum to AP. Crystalline AP mimicked the effects of the bacterial filtrates, and an inactive filtrate from a protease-deficient mutant strain was reconstituted by the addition of AP. AP-treated recombinant IFN-γ showed altered migration on Western blots (immunoblots) of polyacrylamide gels, and this modification correlated with a dose-dependent loss of antiviral activity. The ability of recombinant IFN-γ to elevate the expression of Fc receptors on cells of the U-937 histiocytic cell line was also diminished by AP treatment. These results indicate that the Pseudomonas protease AP can inhibit the antiviral and immunomodulatory activities of IFN-γ.

Classical models of immune protection against Pseudomonas aeruginosa require a central role for opsonizing antibodies. While antibodies to P. aeruginosa undoubtedly play a role, recent evidence suggests that P. aeruginosa-specific T cells can also limit bacterial growth. In studies reported by Markham et al. (28, 31, 32), murine T cells were protective against Pseudomonas bacterial challenge when adoptively transferred into nonimmune recipients. Immune protection occurred despite the absence of detectable antibody production in the recipients. Numerous reports have shown that P. aeruginosa also elicits T-cell immunity in humans (22, 24, 30, 36). Moreover, the finding that T cells from individuals suffering from chronic Pseudomonas infections (e.g., cystic fibrosis) often fail to respond to Pseudomonas antigens in vitro (23, 36, 37) supports the hypothesis that T-cell-mediated immunity may play an important antibacterial role.

Several studies have indicated that P. aeruginosa or its products can inhibit cellular immune functions (3, 11, 21, 25, 27, 35), and it has been postulated that this immunosuppression could favor the persistence of the organism in the host. Blackwood et al. (3) and Petit et al. (27) have shown that injection of P. aeruginosa into mice suppresses both immunity to an unrelated intracellular pathogen, Listeria monocytogenes, and delayed-type hypersensitivity to L. monocytogenes or sheep erythrocytes. These studies imply that P. aeruginosa or substances derived from it can nonspecifically impair cellular immune functions. Because the T-cell-derived lymphokine gamma interferon (IFN-γ) is known to play a central role in cellular immunity (4, 6, 8, 13, 19, 29, 34, 41), in the present study we investigated the effects of P. aeruginosa on IFN-γ activity derived from antigen-stimulated human T lymphocytes.

Previously, we derived a series of Pseudomonas-specific human T-cell clones (22, 24) which, when activated in vitro, proliferated and secreted IFN-γ. Exposure of these cells to certain crude bacterial-antigen preparations also stimulated lymphoproliferation but failed to elicit production of detectable IFN-γ antiviral activity. Described here is an explanation for these observations that suggests a mechanism whereby certain Pseudomonas strains down-regulate the activity of IFN-γ. This model may explain some immunosuppressive effects of the microorganism.

MATERIALS AND METHODS

Cell preparations. Mononuclear cells (MC) were isolated from the peripheral blood of healthy human donors by centrifugation over Ficoll-Hypaque. After the cells were washed twice in RPMI 1640, the MC were irradiated (3,000 rad) and used as antigen-presenting cells (APC). The isolation and characterization of the human T-cell clones used in this study have been reported in detail in previous publications (22, 24). All clones were specific for Pseudomonas proteases.

Lymphocyte cultures. Antigenic or mitogenic stimulation of T-cell clones was performed by the procedures described previously (22, 24), with the following modifications. As a source of APC, irradiated MC (10^6 per well) were allowed to adhere to 96-well flat-bottom plates in RPMI 1640 containing 30% serum from healthy Pseudomonas-seronegative donors. After 2 h, nonadherent cells were removed by washing, and culture medium containing 10% human serum was added to the adherent APC. Antigen or phytohemagglutinin (Wellcome Diagnostics, Research Triangle Park, N.C.) was added to APC 18 to 24 h before the addition of the T-cell clones. Each of the antigens, Pseudomonas elastase (E) and Pseudomonas alkaline protease (AP), was used at a concentration of 30 ng/ml. Phytohemagglutinin (Wellcome) was diluted according to the instructions of the manufacturer and was used at a concentration of 5 μg/ml. For the measurement of IFN-γ production, culture fluids were collected after 24 h, centrifuged to remove cells, and stored at −70°C until antiviral assays were performed. Proliferation of T cells was measured on day 3 by labeling cultures with 1 μCi of [3H]thymidine per well for 8 h. The contents of each well were recovered on fiber glass filters and counted by scintillation spectrophotometry. Data are expressed as mean counts per minute ± 1 standard deviation.

Assay for antiviral activity. Antiviral activity was mea-
sured by assessing inhibition of the cytopathic effect of vesicular stomatitis virus on cultured WISH cells. IFN-γ samples were diluted twofold in 96-well plates and incubated with WISH cells (5 × 10⁶ per well) for 18 to 24 h. Monolayers were then infected with vesicular stomatitis virus at a multiplicity of infection of 0.1. Cytopathic effect was determined 40 to 48 h later by staining the cells with 0.5% crystal violet. The dye was eluted with ethylene glycol, and the A₅₂₀ of the eluted dye was measured by using a Titertek Multiskan Platereadter. The interferon titer was estimated from a linear regression function of the log of percentage of maximum absorbance versus the log interferon dilution. All results are expressed as units per milliliter and represent the means of two replicate titrations of each IFN-γ sample. One unit of activity was defined as the amount of interferon that inhibited cytopathic effect by 50% under assay conditions standardized to a National Institutes of Health IFN-γ standard. Thus, an IFN-γ laboratory standard, prepared by stimulating MC with tuberculin purified protein derivative (PPD), was standardized against a National Institutes of Health IFN-γ standard (Gg 23-901-530) and was titrated in each assay. This preparation, as well as culture fluids from stimulated T-cell clones, typically contained 100 to 1,500 U of IFN-γ per ml. Antiviral activity was due to IFN-γ, as evidenced by monoclonal antibody neutralization (clone 69B, kindly supplied by Sidney Pestka [26]) and pH 2 sensitivity.

Assay for IFN-γ immunomodulatory activity. The human histiocytic cell line U937 was used to measure the Fc receptor (FcR)-inducing activity of recombinant IFN-γ (rIFN-γ). Before assay, cells were maintained overnight in RPMI 1640 containing 1% fetal calf serum to minimize FcR expression on uninduced cells. rIFN-γ (G11026-01) was provided by Michael Shepard of Genentech, Inc., South San Francisco, Calif., and had a specific activity of 4 × 10⁷ U/mg in our antiviral assay. One million U937 cells were then incubated for 24 h at 37°C with rIFN-γ or AP-treated rIFN-γ in RPMI 1640 containing 10% fetal calf serum. The percentage of cells expressing high-affinity FcR was then determined by one of two techniques. Cells were allowed to form rosettes with rabbit immunoglobulin G (IgG) antibody-sensitized sheep erythrocytes and scored positive if more than 5 sheep erythrocytes bound per U937 cell. Standard deviations associated with the data reported for these experiments averaged 22% of the mean values. Alternatively, a flow immunocytometry assay similar to that used by Arend et al. (2) was used. U937 cells were treated with saturating amounts of murine IgG (Coulter Immunology, Hialeah, Fla.) and then were exposed to fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Coulter) which had been degagregated by centrifugation at >100,000 × g for 20 min before use to eliminate background fluorescence. After fixation with 1% paraformaldehyde, the percentage of FcR-positive cells was determined by flow immunocytometry (Epics V; Coulter) by using the Immuno-statistical analysis program (Coulter). Analysis was performed after gating by light scatter to eliminate nonviable cells, and results were expressed as the net increase in the frequency of receptor-bearing cells over that seen for cell populations not exposed to IFN-γ. (See Fig. 4B for single datum points for each rIFN-γ concentration, representative of two experiments of this type.)

Pseudomonas products. The mucoid Pseudomonas GoM strain used in this study was originally isolated from a cystic fibrosis patient. This strain secretes both AP and E into its culture fluids (22). The Pseudomonas strains PAO-1 (10) and PAO-1-1641 were kindly provided by B. H. Iglewski. Strain PAO-1 produces both AP and E, whereas the mutant strain PAO-1-1641 fails to produce detectable quantities of either protease. Sterile culture filtrates from these strains were prepared by growing the respective bacteria in tryptic soy broth for 24 to 48 h, centrifuging the cultures at 10,250 × g for 30 min to remove organisms, and filtering with a membrane filter (Millipore Corp., Bedford, Mass.) (0.22-µm pore size). These bacterial filtrates were added directly to lymphocyte cultures. In preliminary experiments designed to determine the effects of pyocin pigments (21, 35), fresh filtrates were compared with filtrates that were dialyzed against phosphate-buffered saline and sterilized. Dialysis did not affect the results, and all experiments reported here were performed with nondialyzed filtrates. The inhibitory activity of these filtrates was expressed as units of activity per milliliter. One unit was arbitrarily defined as the amount of Pseudomonas filtrate necessary to reduce IFN-γ activity by 85%. A typical inhibitory filtrate contained 500 to 1,500 inhibitory units per ml.

Pseudomonas AP (lot 9442009) was purchased as a crystalline enzyme from Nagase Co., Ltd. (Tokyo, Japan) and was judged to be more than 99% pure by densitometric scans of Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels. The enzyme preparation also contained less than 0.1% contamination with Pseudomonas E as determined by enzyme-linked immunosorbent assays with specific rabbit antiserum and T-cell clones stimulated by a 1,000-fold excess of the heterologous protein.

Anti-AP serum. Antiserum to AP was produced by immunizing a rabbit subcutaneously and intramuscularly with 1 mg of crystalline AP emulsified in complete Freund adjuvant. Booster immunizations were given every 3 weeks with 0.5 mg of AP in incomplete Freund adjuvant, and the animal was bled 1 week after each immunization. On Western blots (immunoblots), this antiserum detected a major band of approximately 57 to 60 kilodaltons as well as several smaller components both in the crystalline AP and in crude Pseudomonas culture filtrates. The antiserum failed to react with purified E in Western blot assays but did show reactivity with Pseudomonas lipopolysaccharide. In assays for proteolytic activity in which casein was used as a substrate, anti-AP serum neutralized proteolysis by AP but not by E.

Protease assay. Protease activity was assayed by a slight modification of the procedure described by Rinderknecht et al. (33) with hide blue powder substrate (Sigma Chemical Co., St. Louis, Mo.).

Immunoblots. Samples of human rIFN-γ were treated with Pseudomonas AP in the absence or presence of 10% human serum from seronegative donors (i.e., the donors lacked antiprotease antibodies detectable by enzyme-linked immunosorbent assay). Samples (20 µl) of human rIFN-γ (80 µg/ml) were treated with equal volumes of Pseudomonas AP at concentrations ranging from 40 ng/ml to 40 µg/ml. Samples were then incubated for 24 h at 4°C. After a sample of the rIFN-γ was retained for testing antiviral activity, the remaining sample was diluted 1:2 in electrophoresis sample buffer and heated at 95°C for several minutes. Samples were then applied to 13 to 18% polyacrylamide gradient gels containing sodium dodecyl sulfate by using a minigel apparatus (Bio-Rad Laboratories, Richmond, Calif.) and electrophoresed. Proteins were electrophoretically transferred to nitrocellulose (Bio-Rad) with a transblot apparatus (Bio-Rad) at 100 V. The nitrocellulose was then blocked for 60 min with 1% bovine serum albumin in phosphate-buffered saline. The transfers were incubated overnight with a poly-
RESULTS

Inhibition of antiviral activity of IFN-γ produced by human T-lymphocyte clones by addition of a Pseudomonas culture filtrate. Human T-cell clones specific for Pseudomonas proteases were challenged with antigen in the presence of autologous, irradiated APC, and lymphoproliferation and IFN-γ production were measured as described above. The response of a typical clone is shown in Fig. 1. In this case the clone was specific for Pseudomonas E, which was used at an antigen concentration of 50 ng/ml. In the presence of antigen (Fig. 1A), the cells proliferated and produced IFN-γ. This clone also responded to E in the crude bacterial culture filtrate of strain GoM (22) as assessed by proliferation.

However, in contrast to challenge with purified E, IFN-γ antiviral activity was markedly reduced when cells were stimulated instead with the GoM filtrate. The filtrate also reduced the level of detectable antiviral activity produced in cultures stimulated with purified E. This implied the presence of a component in the bacterial filtrate that selectively inhibited IFN-γ antiviral activity without altering T-lymphocyte proliferation.

Inhibition did not require antigenic stimulation of the clone as revealed by similar experiments with phytohemagglutinin-stimulated cultures (Fig. 1B). Similarly, the GoM bacterial filtrate inhibited IFN-γ production by a variety of Pseudomonas-specific T-cell clones as well as uncloned T cells specific for non-Pseudomonas antigens (e.g., tuberculin PPD; data not shown). These findings suggested that a substance(s) produced by P. aeruginosa suppressed the IFN-γ antiviral activity normally produced by activated T cells.

Antiviral activity produced in cultures of T-cell clones was inhibited whether the filtrate was added at the onset of culture (0 h) or up to 1 h before recovery of culture fluids (23 h). The effects of the GoM filtrate during the final 2 h of culture are shown in Fig. 2. Three doses of the filtrate were added to antigen-stimulated T-cell cultures at various times before IFN-γ activity was measured. The inhibitory activity was both time and dose dependent, i.e., at higher doses of the filtrate less time was required for inhibition (Fig. 2A). On the basis of these data it seemed probable that the inhibitory activity of the filtrate was not dependent on an effect on IFN-γ-producing cells, but that the inhibitor(s) acted directly on the lymphokine itself.

Two additional observations supported this interpretation. Addition of the filtrate directly to a sample of preformed IFN-γ (a supernatant fluid from PPD-stimulated MC) inhibited antiviral activity in a similar dose- and time-dependent fashion (Fig. 2B). Second, pretreatment of T cells or APC or both with the bacterial filtrate failed to inhibit antigen-induced IFN-γ production assessed in the antiviral assay (data not shown).

Identification of the inhibitory component(s) in the bacterial filtrate. Several observations (data not shown) suggested the identity of the inhibitor in bacterial culture filtrates. First, inhibitory activity was both heat (55 to 60°C for 30 min) and trypsin sensitive. Second, strains that failed to secrete significant protease activity in their culture broth did not inhibit IFN-γ activity. Of the 20 Pseudomonas strains isolated from clinical specimens that have been tested to date, 12 have shown significant IFN-γ inhibitory activity. These inhibitory strains were also the highest AP-producing strains studied, as determined by radioimmunoassay (data not shown). Third, ion exchange chromatography (17, 20) of inhibitory filtrates reproducibly yielded fractions that were greater than 100-fold enriched in inhibitory activity and greatly enriched for AP.

Since the IFN-γ inhibitory activity showed characteristics of the exoprotease AP, rabbit antiserum to the enzyme was tested for its ability to reverse inhibition. (The specificity of this antiserum is discussed in Materials and Methods.) Table 1 shows the effects of anti-AP serum on the inhibitory activity of a bacterial filtrate prepared from strain GoM. Anti-AP treatment of the filtrate restored its inhibition of IFN-γ antiviral activity in a dose-dependent fashion, whereas normal rabbit serum had no significant effect.

Two experimental approaches were used to verify that the exoprotease AP was in part responsible for the inhibition of IFN-γ antiviral activity. First, a culture filtrate from a
and tested for antiviral activity. The protease-deficient *Pseudomonas* mutant (PAO-1-1641) was tested for inhibitory activity (Table 2). Compared with a filtrate prepared from the parental protease-producing strain, PAO-1, the mutant filtrate lacked inhibitory activity. Addition of crystalline AP to the PAO-1-1641 filtrate restored the inhibitory activity that the parental strain exhibited, confirming the hypothesis that the presence of AP in *Pseudomonas* bacterial filtrates can result in inhibition of IFN-γ activity.

The second approach involved treating IFN-γ-rich T-cell culture fluids directly with purified AP (Table 2). Exposure to pure AP significantly reduced antiviral activity in a dose-dependent fashion. Although the IFN-γ in this experiment was treated with AP for 24 h, similar results were obtained after only 1 h of treatment with the protease (2 μg/ml), and in this sense the results are consistent with the observations reported in Fig. 2.

To ascertain whether AP interfered with the antiviral

**TABLE 2.** Effects of *Pseudomonas* mutant filtrates and purified AP on IFN-γ antiviral activity

<table>
<thead>
<tr>
<th>Filtrate or protease</th>
<th>Dose concn*</th>
<th>IFN-γ titer (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>742 ± 48</td>
</tr>
<tr>
<td>PAO-1</td>
<td>2</td>
<td>758 ± 11</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>363 ± 18</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>170 ± 25</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>46 ± 1</td>
</tr>
<tr>
<td>PAO-1-1641</td>
<td>2</td>
<td>773 ± 53</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>748 ± 28</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>756 ± 31</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>788 ± 110</td>
</tr>
<tr>
<td>PAO-1-1641 + 20 μg of AP/ml</td>
<td>2</td>
<td>611 ± 6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>482 ± 5</td>
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<td></td>
<td>25</td>
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</tr>
<tr>
<td></td>
<td>100</td>
<td>151 ± 19</td>
</tr>
<tr>
<td>AP</td>
<td>0.031</td>
<td>759 ± 58</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>415 ± 32</td>
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<tr>
<td></td>
<td>0.5</td>
<td>219 ± 35</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>96 ± 13</td>
</tr>
</tbody>
</table>

* Samples of an IFN-γ-rich T-cell culture fluid (MC + tuberculin PPD) were incubated with bacterial filtrates or purified *Pseudomonas* proteases or both for 24 h at 37°C before residual antiviral activity was measured. Data are expressed as means ± 1 standard deviation.

* *Samples of a filtrate prepared from *Pseudomonas* GoM were diluted to a concentration of 25 μg/ml in RPMI 1640 containing the indicated final concentrations of normal rabbit serum (NRS) or anti-AP serum. These were incubated at room temperature for 2 h. Samples were then added to an equal volume of preformed IFN-γ (a PPD-stimulated T-cell culture fluid) and incubated for 24 h before residual antiviral activity was measured. Data are expressed as means ± 1 standard deviation. Neither the antisera nor the normal rabbit serum had any effect on the antiviral assay.

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**FIG. 2.** Effect of GoM filtrate on antiviral activity. (A) *Pseudomonas* filtrate at concentrations of 0 (■), 0.1 (□), 0.5 (●), and 2.5 (○) μg/ml was added to activated T cells at various times during the final 2 h of culture. All supernatant fluids were collected at the end of culture and tested for antiviral activity. (B) The same doses of bacterial filtrate were added to a standard sample of preformed IFN-γ for the same time periods. All samples were then simultaneously tested for antiviral activity. Data presented here are representative of three individual experiments.
assay, WISH cells were preincubated with several concentrations of pure AP or the bacterial GoM filtrate. After being washed, the cells were challenged in an antiviral assay with preformed IFN-γ. Neither AP nor the filtrate adversely affected the ability of preformed IFN-γ to protect WISH cells from viral infection. This suggested that Pseudomonas AP acted directly on the IFN-γ protein rather than by interfering with the bioassay by blocking or destroying IFN-γ receptors on the indicator cells.

Since IFN-γ inhibitory activity in Pseudomonas filtrates was associated with the presence of AP, proteolytic cleavage of the IFN-γ protein seemed a likely mechanism. To test this hypothesis, samples of human rIFN-γ were incubated with the protease and then examined for modified antiviral activity and migration on polyacrylamide gels. rIFN-γ was used in these experiments because it was available in pure form and could be used at concentrations adequate for visualization on Western blots by using a polyclonal antiserum to IFN-γ.

Figure 3A and B show the effects of incubating rIFN-γ with various concentrations of AP. Treating 200 ng of rIFN-γ with 0.1 or 1 ng of AP had no apparent effect on its antiviral activity or appearance on Western blots. When the quantity of AP was increased to 5 or 10 ng, a band corresponding to a slightly lower-molecular-weight species of IFN-γ was visualized together with what appeared to be the parent form of the protein. This smaller species is presumed to be a degradation product caused by AP cleavage of rIFN-γ protein. No loss of bioactivity was detected at 5 ng of AP, but treatment with 10 ng of the protease (i.e., an enzyme-to-substrate molar ratio of approximately 1:60) caused a 44% reduction in antiviral activity. When rIFN-γ was treated with 100 ng of AP, the parent IFN-γ species was no longer visualized, but two distinct degradation products were seen. The larger peptide was similar in migration to the band seen when rIFN-γ was digested with 5 or 10 ng of AP, while the other peptide had an apparent Mr approximately 10% less than that of the first. With the disappearance of the parent species of rIFN-γ, the corresponding antiviral activity was reduced by 99%. This indicated that proteolysis of rIFN-γ accompanied reduction in the antiviral activity of the lymphokine when it was exposed to increasing concentrations of AP.

Because our preparations of natural IFN-γ contained 10% human serum and since serum contains a number of protease inhibitors (7, 15, 38, 39), the effects of serum on AP degradation of rIFN-γ were studied. Human serum at 10% did not alter AP effects on rIFN-γ (Fig. 3C and D). Here, too, rIFN-γ treated with 5 to 100 ng of AP appeared as two distinct bands on Western blots and showed a corresponding dose-dependent loss of antiviral activity. Only 1 to 3% of the original activity remained in samples of AP-treated rIFN-γ in which complete degradation of the parent form of the protein was observed. Thus, AP degraded rIFN-γ even in the presence of serum.

Inhibition of the immunomodulatory activity of human rIFN-γ by AP. Figure 4 shows the results obtained when U937 cells were treated with either rIFN-γ or AP-treated rIFN-γ and assayed for the expression of FcR. Treatment of the cells with 0.3 ng of rIFN-γ per ml (12 U/ml) for 24 h resulted in an increase in antibody-sensitized sheep erythrocyte rosette formation over that seen with uninduced cells by a factor of 3 (Fig. 4A). Similar levels of FcR expression could be induced by AP-treated rIFN-γ, but only with concentrations greater than 10 ng/ml. It should be noted that rIFN-γ was treated with the protease for only 3 h in this experiment.

Similar results were obtained when FcR expression was
DISCUSSION

The results reported here suggest that bacterial filtrates prepared from some Pseudomonas strains inhibited IFN-γ antiviral and immunomodulatory activity. This property could apparently be attributed to the presence of AP, which partially degraded the lymphokine. This conclusion is based on the following observations. (i) The inhibitor in the GoM bacterial filtrate acted directly on preformed IFN-γ, rather than requiring a primary effect on T lymphocytes. (ii) The inhibitory activity of the Pseudomonas filtrate was nondialyzable, sensitive to heat treatment at 55 to 65°C, and inactivated by trypsin. These findings suggested that the inhibitory component was macromolecular and, at least in part, proteinaceous in nature. This is in contrast to many other known immunoregulatory bacterial substances like lipopolysaccharide (18) or pyocin pigments (21, 35). Partial purification of the inhibitory activity from a GoM filtrate yielded an active fraction with several characteristics of AP. The fraction contained a major component detectable on Western blots with an anti-AP serum that had a molecular weight similar to that of AP and exhibited proteolytic activity that could be completely destroyed by heating to 65°C for 30 min (data not shown). (iv) Rabbit antisera to AP neutralized the inhibitory activity of the bacterial filtrate. (v) Purified AP, alone or added to a Pseudomonas filtrate devoid of protease activity, destroyed the antiviral activity of preformed IFN-γ in a dose-dependent fashion. AP-treated rIFN-γ also lacked FcR-inducing activity. (vi) rIFN-γ treated with the protease evidenced a reduction in apparent molecular weight, and this modification paralleled its loss in antiviral activity. Other purified Pseudomonas products, such as toxin A, lipopolysaccharide, or purified outer membrane proteins, had no significant effect on the antiviral activity of IFN-γ (data not shown). However, recent evidence indicates that E, the second principal protease produced by some Pseudomonas strains, can also degrade IFN-γ, although it is much less active than AP if human serum is present. Although we have not excluded the participation of still other non-AP components of P. aeruginosa in the inhibition of IFN-γ in T-cell culture fluids, we favor the interpretation that the principal inhibitory component is the exoenzyme AP.

A number of the T-cell clones used in this study were specific for Pseudomonas AP. Given that AP inhibits IFN-γ, it seems paradoxical that these protease-specific T cells produced IFN-γ when challenged with the enzyme. However, the antigenic dose of AP used to activate the cells (50 ng/ml) was lower than the minimum dose required to significantly inhibit IFN-γ bioactivity (125 ng/ml). AP used as antigen for stimulating T cells was routinely stored at 4°C for several weeks. These preparations were generally devoid of both proteolytic and IFN-γ inhibitory activity.

A number of studies investigating structure-function relationships have indicated that certain synthetic peptides of IFN-γ produced by recombinant techniques or peptides derived by chemical or proteolytic cleavage of intact rIFN-γ exhibit modified bioactivity in vitro (1, 16, 40). Studies by Arakawa et al. (1) and, more recently, Leinikki et al. (16) have demonstrated that cleavage of approximately 10% of the C-terminal portion of human rIFN-γ by trypsin, pronase, or other proteases with broad substrate specificities markedly inhibits antiviral activity. Although our results are consistent with these reports, we have not, as yet, identified the site at which AP cleaves IFN-γ. However, ours is the first report that brings potential in vivo relevance to this type of proteolytic modification of IFN-γ by associating such activity with a bacterial pathogen known for its immunosuppressive effects.

Since IFN-γ has been implicated as the principal T-cell product which mediates macrophage activation (19, 29, 34), its destruction by a soluble product of an infectious bacterium could result in suppression of a number of important antimicrobial defenses. Recognition of bacterial antigens by T lymphocytes is restricted by class II histocompatibility complex molecules on the surfaces of APCs (6, 43), and IFN-γ has been shown to increase expression of these cell surface structures on a variety of cell types (29, 41). The expression of immunoglobulin FcR on phagocytic cells is influenced by IFN-γ (8), as is the transcription of interleukin-1, urokinase-type plasminogen activator, and tumor necrosis factor (5) in murine macrophages. Recent studies by Jensen et al. (13) have demonstrated that human rIFN-γ is capable of stimulating human pulmonary macrophages to inhibit the growth of an intracellular bacterial parasite, Legionella pneumophila. Buchmeier and Schreiber (4) neutralized IFN-γ in vivo with monoclonal antibodies and prevented the in vivo clearance of L. monocytogenes from infected mice. These and other studies have clearly established that IFN-γ plays a major role in activating macrophages and promoting antimicrobial immunity in vivo as well as in vitro.

Two independent groups, Blackwood et al. (3) and Petit et al. (27), reported that injection of P. aeruginosa bacteria into mice depressed cell-mediated immunity to L. monocytogenes. In the present study we showed that a protease produced by a variety of P. aeruginosa strains can modify the immunoenhancing activity of IFN-γ. This property may explain some of the immunosuppressive effects that have
been attributed to the organism. Although both Pseudomonas exoproteases are thought to contribute to the virulence properties of the organism in a nonspecific fashion (9, 12, 14, 42), to date little attention has been given to their effects on specific immune responses. The current study implicates AP as a Pseudomonas product that selectively down-regulates cellular immunity by inhibiting IFN-γ bioactivity. Because IFN-γ is known to increase the antimicrobial activity of phagocytic cells, our observations suggest that a closer inspection of the role of T-cell-mediated immunity in chronic Pseudomonas infections is warranted.

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