

# Small Molecular Weight Secretory Factors from *Pseudomonas aeruginosa* Have Opposite Effects on IL-8 and RANTES Expression by Human Airway Epithelial Cells

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*Pseudomonas aeruginosa* is an opportunistic human pathogen that causes both an acute lung disease in patients with hospital-acquired pneumonia and a chronic lung disease in individuals with cystic fibrosis. Many of the pathophysiologic effects of *P. aeruginosa* infection are due to factors secreted by the bacterium. Conditioned media from cultures of *P. aeruginosa* increased interleukin-8 expression and decreased regulated on activation, normal T cells expressed and secreted (RANTES) expression by human airway epithelial cells. Both of these activities were present in heat-treated, protease-treated, small molecular weight fractions. The activities were not inhibited by polymyxin B and were not extracted into ethyl acetate, suggesting that they were not due to endotoxin or autoinducer. Conversely, results from chloroform extractions and studies with a phenazine-minus mutant suggested that the blue pigment pyocyanin contributes to these activities when present. In addition to the effects of small molecular weight factors on cytokine expression, proteases in bacterial-conditioned media further decreased levels of RANTES. By altering expression, release, and/or activity of inflammatory cytokines, secretory factors from *P. aeruginosa* could disrupt the delicate balance that constitutes the immune response to bacterial infection and thus could contribute to the lung damage that occurs in *P. aeruginosa*-infected airways.

*Pseudomonas aeruginosa* is the most common bacterial pathogen associated with hospital-acquired (nosocomial) pneumonia (1). Acute lung infections result in mortality rates as high as 70%, even with appropriate antibiotic therapy. Additionally, *P. aeruginosa* is commonly associated with the chronic lung disease observed in individuals with cystic fibrosis (CF) (2). The clinical course that follows the initial colonization of the CF airway by this bacterium is characterized by recurrent exacerbations and remissions that eventually lead to progressive pulmonary failure (3). Although patients with CF may temporarily respond to antibiotic therapy, clearance of the bacterium from the lung is rarely achieved. Lung disease is currently the leading cause of morbidity and mortality in CF.

*P. aeruginosa* is a highly adaptable microorganism that survives in a variety of limiting environments (4). Among

the features that make it so successful is the production of a large array of secretory factors, including proteases, exotoxins, phospholipases, and pigments (5, 6). Many of these secretory factors have been shown to have biologic effects on host cells that may contribute to the pathogenesis of *P. aeruginosa*-associated lung disease. Among these effects are changes in expression and/or activity of cytokines.

For example, the blue phenazine derivative pyocyanin inhibits expression of both interleukin (IL)-2 and its receptor by T cells (7), as well as expression of regulated on activation, normal T cells expressed and secreted (RANTES) by airway epithelial cells (8). Additionally, several cytokines (interferon [IFN]- $\gamma$ , tumor necrosis factor [TNF]- $\alpha$ , and IL-2) are sensitive to *P. aeruginosa* metalloproteases (9–11). Proteolysis of these cytokines leads to loss of biologic activity.

In contrast to these inhibitory effects, previous reports by our laboratory and others demonstrate that multiple *P. aeruginosa* factors increase expression of the potent neutrophil chemokine IL-8 by human airway epithelial cells. These factors include pilin, flagellin, autoinducer, elastase, nitrite reductase, and pyocyanin (8, 12–14). In addition to purified factors, bacterial-conditioned medium increases IL-8 release both *in vitro* (15) and *in vivo* (16). The factor responsible for this stimulatory activity has yet to be identified.

To extend these latter studies and to examine the effect of conditioned medium on RANTES release, we used conditioned media from wild-type and mutant strains of *P. aeruginosa* (PA01 and PA14) and measured the effect of these media on expression of IL-8 and RANTES by several human airway epithelial cell lines as well as by primary epithelial cells. Our data suggest the presence of both stimulatory and inhibitory factors that alter the expression of these important inflammatory cytokines and identify pyocyanin as one of these factors.

## Materials and Methods

### Airway Epithelial Cell Culture

The human alveolar type II cell line A549 (American Type Culture Collection, Rockville, MD) and the human airway epithelial cell line Calu-3 (American Type Culture Collection) were cultured as previously described (8) in Dulbecco's modified Eagle's medium: Ham's F12 (1:1) (GIBCO BRL, Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM glutamine, and 500 U/ml each of penicillin and streptomycin (GIBCO BRL). The human alveolar type II cell line NCI-H441 (American Type Culture Collection) was cultured in RPMI supplemented with 10% FBS and antibiotic/antimycotic supplement (penicillin, streptomycin, and amphotericin B; GIBCO BRL). Normal human bronchial epithelial cells (NHBE) were purchased from Clonetics (BioWhit-

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**Abbreviations:** cystic fibrosis, CF; enzyme-linked immunosorbent assay, ELISA; glycerol-alanine-conditioned medium, GACM; HEPES-buffered saline, HBS; high phosphate-conditioned medium, HPCM; interferon, IFN; interleukin, IL; lipopolysaccharide, LPS; supplemented M9-conditioned medium, MCM; messenger RNA, mRNA; nuclear factor, NF; normal human bronchial epithelial cells, NHBE; nitric oxide, NO; regulated on activation, normal T cells expressed and secreted, RANTES; RNase protection assay, RPA; standard deviation, SD; tumor necrosis factor, TNF.

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taker Inc., Walkersville, MD) and cultured in bronchial epithelial growth medium (Clonetics) according to the manufacturer's recommendations.

### Bacterial Culture

Wild-type PA01 was generously provided by Dr. Charles Cox (Department of Microbiology, University of Iowa, Iowa City, IA). Wild-type PA14 and mutants were generously provided by Dr. Frederick Ausubel (Department of Molecular Biology, Massachusetts General Hospital, Boston, MA): 3E8, phenazine-minus mutant; 36A4, mutation in an *hrp* homolog that codes for an enzyme involved in the biosynthesis of periplasmic glycans; 23A2, mutation in *mexA* that codes for a subunit of a non-adenosine triphosphatase efflux pump (17). Stock bacterial suspensions were kept in distilled water. Stocks were also maintained by weekly streaking onto tryptic soy broth agar plates. Plates were then incubated overnight at 37°C and were subsequently stored at 4°C.

### Preparation of Bacterial-Conditioned Medium

Three bacterial culture media were used: supplemented M9 medium (15) (M9 salts, 0.2% glucose, 2 mM MgSO<sub>4</sub>, 100 μM CaCl<sub>2</sub>, 100 μM L-glutamic acid, 15 g/liter succinic acid); high phosphate medium (18) (4 mM potassium phosphate buffer, pH 7.4, 10 mM 3-(*N*-morpholino)propanesulfonic acid, 20 mM sodium succinate, 40 mM NH<sub>4</sub>Cl, 2 mM K<sub>2</sub>SO<sub>4</sub>, 0.4 mM MgCl<sub>2</sub>, 1 μM each of MnCl<sub>2</sub>, CaSO<sub>4</sub>, ZnCl<sub>2</sub>, and FeCl<sub>3</sub>); and glycerol-alanine medium (19) (1% glycerol, 0.6% L-alanine, 0.2% MgSO<sub>4</sub>, 0.01% K<sub>2</sub>HPO<sub>4</sub>, 0.001% FeSO<sub>4</sub>). To prepare conditioned medium, 10 ml of culture medium was inoculated with bacteria from an agar plate and grown overnight at 37°C with shaking. Subsequently, 5 ml of the growth culture was added to 100 ml of culture medium and cultures were incubated at 37°C with shaking for 72 h. Bacteria were removed by centrifuging at 100,000 × *g* for 30 min and filtering the supernatant fraction through a 0.2-μm filter. All nonconditioned and conditioned media were stored at 4°C. Protein measurements were made using the Micro BCA Assay (Pierce, Rockford, IL). Pyocyanin was measured in these media by extracting into CHCl<sub>3</sub> and reading absorbance at 690 nm as previously described (18); the limit of detection by this method is approximately 1 μM.

### Treatment of Conditioned Medium

To test heat stability, nonconditioned and bacterial-conditioned media were placed in a boiling water bath for 30 min. Size fractionation was done using Centricon filters (Amicon, Inc., Beverly, MA) with a molecular weight cutoff of 3 kD. To generate the organic and aqueous phases of a CHCl<sub>3</sub> extraction, 5 ml of nonconditioned and bacterial-conditioned media were extracted three times with 2 ml of CHCl<sub>3</sub>. Phases were separated by centrifuging (2,500 × *g*, 5 min). The lower CHCl<sub>3</sub> phases were pooled, dried under a stream of nitrogen, and reconstituted to 5 ml with the appropriate bacterial culture medium. Nitrogen was bubbled through the aqueous phase to remove residual CHCl<sub>3</sub>. A similar procedure was done using ethyl acetate: upper phase represents organic phase.

### Enzyme-Linked Immunosorbent Assay

Cells were cultured in 48-well tissue culture plates until they were confluent. Nonconditioned and bacterial-conditioned media were placed on the cells (250 μl/well), and cultures were incubated for the indicated time. Studies of conditioned medium-dependent stimulation of IL-8 expression were measured using both untreated and TNF-treated epithelial cells. Conversely, because airway epithelial cells do not constitutively express RANTES, all studies to measure inhibition of RANTES expression and release were done using cells treated with host cytokines. At the end of the incubation period, the culture medium was recovered and

stored frozen at -20°C until assay. Cytokine levels were determined by enzyme-linked immunosorbent assay (ELISA) using matched antibodies purchased from R&D Systems, Inc. (Minneapolis, MN) as previously described (8). Standard curves for both IL-8 and RANTES were in the range of 15 to 1,000 pg/ml.

### RNase Protection Assay

A549 cells were seeded into T75 tissue culture flasks, grown to confluence, and exposed to nonconditioned or conditioned medium for the indicated times. At the end of the incubation period, total RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH) and steady-state levels of cytokine messenger RNA (mRNA) were determined using a nonradioactive RNase protection assay (RPA) as previously described (8) with 40 μg of total RNA per assay.

### Assay for Cell Viability

To mimic conditions used to study cytokine release, cells in 48-well plates were exposed to the indicated concentration of nonconditioned or conditioned medium for 0 to 30 h. At the end of the incubation period, the cells were washed twice with HEPES-buffered saline (135 mM NaCl, 5 mM KOH, 10 mM HEPES, 1.2 mM each CaCl<sub>2</sub> and MgCl<sub>2</sub>, pH 7.4) supplemented with 10 mM glucose and 0.1% bovine serum albumin (HBS). Calcein-AM from the Molecular Probes LIVE/DEAD assay kit (Eugene, OR) for mammalian cells was diluted to 2 μM in HBS and the resulting solution was placed in the wells (200 μl/well). Fluorescence changes over time (0 to 40 min) at 37°C were measured using the FluoStar microplate fluorometer (BMG Lab Technologies, Inc., Durham, NC) (ex/em λs, 485/538). To generate minimum fluorescence values (min em<sub>538</sub>), cells were exposed for 10 min to 70% ethanol, washed twice with HBS, and used in the assay. Values for cells treated with nonconditioned medium were defined as maximum fluorescence values (max em<sub>538</sub>). Values from the linear portion of the time course (routinely 30 min) were used to calculate the percentage of live cells defined as (sample em<sub>538</sub> - min em<sub>538</sub>) / (max em<sub>538</sub> - min em<sub>538</sub>) × 100.

### Assays for Detecting Proteolysis of RANTES

To determine whether bacterial-conditioned medium contained factors that could degrade RANTES, purified recombinant human RANTES (500 pg/ml) was combined with nonconditioned medium or with increasing concentrations of bacterial-conditioned media and samples were incubated at 37°C for 24 h. At the end of the incubation period, RANTES in the samples was measured using ELISA.

To determine whether purified *P. aeruginosa* proteases (elastase and alkaline protease) could degrade RANTES released by A549 cells, TNF-treated A549 cells were incubated with increasing concentrations of bacterial proteases for 24 h. At the end of the incubation period, RANTES was measured in the culture medium using ELISA.

### Statistical Analysis

Raw data (triplicates) were analyzed using Student's *t* test. Differences were considered statistically significant if *P* < 0.05.

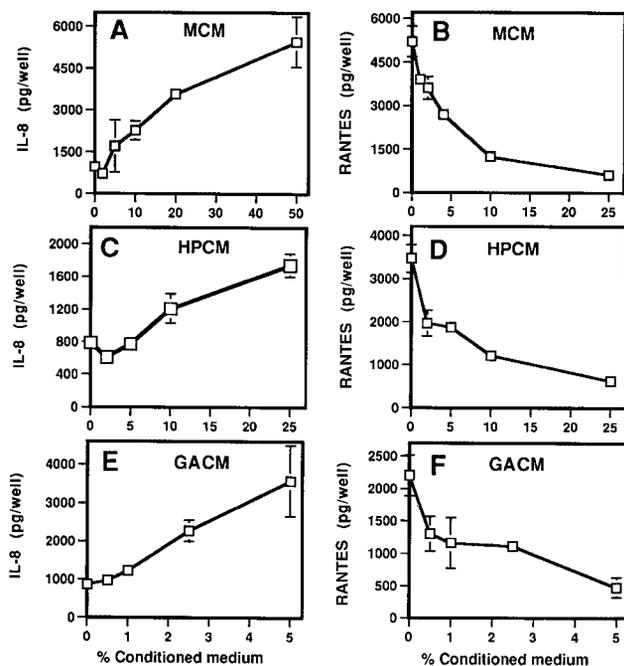
## Results

### Effects of Conditioned Medium on Cytokine Release by A549 Cells

Previous studies demonstrated that supplemented M9-conditioned medium (MCM) from PA01 stimulates expression of IL-8 both *in vitro* (15) and *in vivo* (16). Although several purified factors have been shown to stimu-

late IL-8 release (8, 12, 13), the identity of the stimulatory factor present in this medium has yet to be determined. In addition, studies by our laboratory show that *P. aeruginosa* pyocyanin stimulates IL-8 expression while inhibiting TNF-dependent increases in steady-state levels of mRNA for the chemokine RANTES (8). Based on these studies, we wished to examine further the effect of secreted factors from *P. aeruginosa* on the release of IL-8 and RANTES by human airway epithelial cells and to assess the contribution of pyocyanin to these effects.

To test the effect of PA01 MCM on release of these cytokines, A549 cells were treated for 30 h with increasing concentrations of MCM, and cytokine levels in the culture medium were measured using ELISA. As previously reported (15), MCM increased basal IL-8 release in a dose-dependent manner (Figure 1A). These increases were similar to those observed with other bacterial agonists. Additionally, MCM further increased IL-8 release by cells treated with 10 ng/ml TNF- $\alpha$  (data not shown), suggesting synergy/additivity with host factors. In contrast to the IL-8 results, PA01 MCM inhibited TNF-dependent RANTES release (Figure 1B). Inhibition of RANTES release was not studied in the absence of host cytokines because human airway epithelial cells do not constitutively express this cytokine. Increasing concentrations of nonconditioned medium had no effect in either case (data not shown).



**Figure 1.** Effect of bacterial-conditioned medium on cytokine release by A549 cells. A549 cells were incubated with increasing concentrations of the indicated bacterial-conditioned medium for 30 h and IL-8 or RANTES was measured in the medium using ELISA: supplemented MCM (A and B); HPCM (C and D); GACM (E and F). TNF- $\alpha$  (10 ng/ml) was present in samples used for RANTES measurements. Values are expressed as mean  $\pm$  SD for triplicate samples. Similar results were seen in three or more independent experiments.

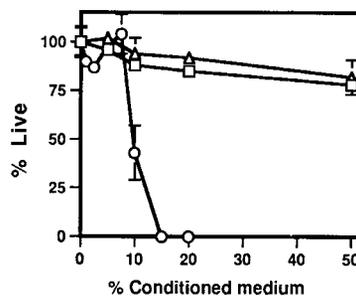
Previous studies indicate that the bacterial growth medium used has a significant impact both on the type and the amount of factors secreted by this organism. For this reason, we tested two additional media, high phosphate and glycerol-alanine media. Of note, glycerol-alanine medium stimulates the synthesis of several pigments, including the blue redox active compound pyocyanin (19). The growth rate of the bacterium was similar in each medium (data not shown). Both high phosphate (HPCM) and glycerol-alanine-conditioned (GACM) media increased basal IL-8 release (Figures 1C and 1E) and TNF-dependent IL-8 release (data not shown) while reducing TNF-dependent RANTES release (Figures 1D and 1F). As with MCM, increasing concentrations of nonconditioned medium had no effect in either case (data not shown).

Although recent reports indicate that *P. aeruginosa* lipopolysaccharide (LPS) stimulates IL-8 release by airway epithelial cells (20), previous studies provided evidence that the IL-8 stimulatory activity in MCM is not due to LPS (15). To determine whether LPS contributed to the observed effect on RANTES, studies were done in the presence and absence of the LPS inhibitor polymyxin B (20  $\mu$ g/ml). Polymyxin B had no effect on conditioned medium-dependent changes in IL-8 or RANTES (data not shown).

#### Cytotoxicity of Conditioned Medium

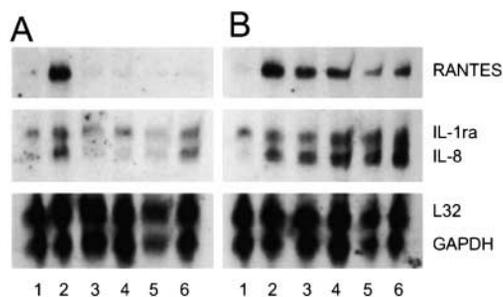
Under most conditions, when the same samples from TNF-treated A549 cells were assayed for both IL-8 and RANTES, IL-8 was increased, whereas RANTES was decreased. This suggested that the inhibitory effect on RANTES release observed in these samples was not due to nonspecific effects on cell viability and/or on protein biosynthesis. However, in some experiments, treatment with higher concentrations of bacterial-conditioned media resulted in a decline in IL-8 levels relative to the maximal levels observed. These data suggested possible nonspecific inhibitory effects. Consistent with this, cellular detachment was observed under some conditions.

To determine directly whether bacterial-conditioned media were cytotoxic at higher concentrations, the effect of increasing concentrations of conditioned media on cell viability was measured using the reagent calcein-AM (Molecular Probes). Representative results from these experiments are shown in Figure 2. No effect was seen with any nonconditioned medium over the range of concentrations



**Figure 2.** Effect of bacterial-conditioned medium on cell viability. A549 cells were incubated with increasing concentrations of the indicated bacterial-conditioned medium for 30 h, and cell viability was assayed using calcein-AM as described in MATERIALS AND METHODS: supplemented MCM (squares); HPCM (triangles); GACM (circles). Values are expressed as % live and represent the mean  $\pm$  SD of triplicate samples. Similar results were seen in three independent experiments.

Similar results were seen in three independent experiments.



**Figure 3.** Effect of bacterial-conditioned medium on steady state-levels of cytokine mRNA. (A) A549 cells were incubated for 6 h with nonconditioned high phosphate medium (lane 1), 10 ng/ml TNF- $\alpha$  alone (lane 2), or 2.5, 7.5, 15, and 25% of HPCM (lanes 3 to 6, respectively). (B) A549 cells were incubated for 6 h with nonconditioned high phosphate medium (lane 1), 10 ng/ml TNF- $\alpha$  alone (lane 2), or TNF- $\alpha$  in combination with 2.5, 7.5, 15, and 25% HPCM (lanes 3 to 6, respectively). At the end of the incubation period, total RNA was isolated and mRNA levels were determined using a nonradioactive multiprobe RPA as described in MATERIALS AND METHODS. To verify sample recovery, the mRNAs of the housekeeping genes L32 (ribosomal protein) and glycerol aldehyde phosphate dehydrogenase (GAPDH) were measured.

tested (0 to 50%) (data not shown). Both MCM and HPCM showed no or minimal effects on cell viability up to 50% (higher concentrations were not tested), whereas GACM was cytotoxic at concentrations  $\geq 10\%$ . Effects on viability were observed within 2 h after addition of conditioned medium (data not shown). Results with this assay correlated with visual observations of cellular detachment. These data suggest that GACM contains factor(s) that decrease cell viability. To simplify data interpretation, reported studies were performed under conditions where negligible cytotoxicity was observed.

#### Effect of Conditioned Medium on Steady-State Levels of Cytokine mRNA

The reduced levels of RANTES release by A549 cells exposed to bacterial-conditioned media could be due to blocking of RANTES release from the cells. To test this possibility, the culture media were collected from A549 cells exposed to nonconditioned or bacterial-conditioned

medium for 24 h, then the cells were lysed (phosphate-buffered saline, 1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride) to release intracellular cytokine pools. Finally, both the culture media and the cell extracts were assayed by ELISA. In a representative experiment, extracellular RANTES levels from nonconditioned medium- and GACM-treated cells were  $68 \pm 7$  and  $11 \pm 6$  ng/well, respectively (mean  $\pm$  standard deviation [SD] for triplicate samples,  $P < 0.001$ ), whereas the corresponding intracellular levels were  $3.1 \pm 0.3$  and  $2.3 \pm 0.3$  ng/well, respectively. Similar results were seen with HPCM (data not shown). These data suggest that the observed decreases in RANTES in the extracellular medium were not due to accumulation of cytokines within the epithelial cells.

A second possible mechanism by which bacterial-conditioned medium could increase/decrease levels of cytokine released is by regulating gene expression. To determine whether changes in cytokine protein levels reflected changes in steady-state mRNA levels, cells were treated under varying conditions, and mRNA levels were measured using a nonradioactive, multiprobe RPA as previously described (8). Bacterial-conditioned medium alone increased IL-8 mRNA levels in a dose-dependent manner (Figure 3A, lanes 3 to 6). Consistent with ELISA results, RANTES mRNA levels were below the level of detection under these conditions. In contrast, the positive control TNF- $\alpha$  increased mRNA levels for both cytokines (Figure 3A, lane 2). Exposure of TNF-treated cells to increasing concentrations of bacterial-conditioned medium (Figure 3B, lanes 3 to 6) further increased IL-8 mRNA levels relative to TNF- $\alpha$  alone (Figure 3B, lane 2), while in the same cells decreasing steady-state levels of RANTES mRNA. These data suggest that the increased/decreased levels of cytokines were due, at least in part, to effects on steady-state levels of cytokine mRNA.

#### Effect of Size Fractionation and Heat Treatment on the Biologic Activities of Bacterial-Conditioned Medium

To examine the molecular weight of the factors involved, bacterial-conditioned media were spun through Centricon filters with a molecular weight cutoff of 3 kD, and the effect of the filtrate on cytokine levels was determined (Table 1). As previously reported for MCM (15), the small molecular weight fraction from each type of bacterial-con-

TABLE 1  
*Effect of size fractionation and heat treatment*

	IL-8 (pg/well)			RANTES (pg/well)		
	MCM	HPCM	GACM	MCM	HPCM	GACM
I. Nonconditioned medium	620 $\pm$ 20	65 $\pm$ 20	45 $\pm$ 20	2,240 $\pm$ 320	8,530 $\pm$ 220	8,200 $\pm$ 230
Complete conditioned medium	4,230 $\pm$ 640	250 $\pm$ 15	1,330 $\pm$ 210	15 $\pm$ 5	2,490 $\pm$ 590	500 $\pm$ 230
< 3 kD	4,940 $\pm$ 1,870	160 $\pm$ 20	1,270 $\pm$ 350	475 $\pm$ 50	6,740 $\pm$ 1,100	1,260 $\pm$ 610
II. Nonconditioned medium	190 $\pm$ 60	785 $\pm$ 58	870 $\pm$ 120	3,100 $\pm$ 335	5,700 $\pm$ 720	3,620 $\pm$ 100
Untreated conditioned medium	1,210 $\pm$ 160	1,210 $\pm$ 180	3,570 $\pm$ 920	840 $\pm$ 50	1,640 $\pm$ 110	350 $\pm$ 160
Heat-treated	1,460 $\pm$ 290	1,160 $\pm$ 140	3,330 $\pm$ 790	1,400 $\pm$ 80	3,700 $\pm$ 690	580 $\pm$ 130

Treated and untreated nonconditioned medium and bacterial-conditioned medium (MCM, HPCM, and GACM) were prepared as described in MATERIALS AND METHODS. Cultures of A549 cells in 48-well plates were incubated for 30 h with the indicated CM (20% MCM and HPCM, 5% GACM), the culture medium was collected, and samples were assayed for the indicated cytokine by ELISA. Samples for RANTES measurements were all done in the presence of 10 ng/ml TNF- $\alpha$ . Values are presented as mean  $\pm$  SD for triplicate samples. Similar results in each case were seen with two or more preparations of each conditioned medium in three or more independent experiments.

TABLE 2

Effect of the < 3 kD fractions of bacterial-conditioned media on cytokine release by human airway epithelial cells

	IL-8 (pg/well)			RANTES (pg/well)		
	H441	Calu-3	NHBE	H441	Calu-3	NHBE
None	580 ± 66	4,500 ± 1,000	840 ± 20	620 ± 30	133 ± 19	448 ± 26
HPCM	1,660 ± 61	10,300 ± 1,900	1,530 ± 240	60 ± 9	66 ± 9	163 ± 20
GACM	1,560 ± 40	7,400 ± 300	2,380 ± 70	270 ± 20	78 ± 10	88 ± 22

Cultures of the indicated airway epithelial cell type in 48-well plates were incubated for 30 h without conditioned medium or with < 3 kD fraction of bacterial-conditioned medium (20% HPCM and 10% GACM). The culture medium was collected, and samples were assayed for the indicated cytokine by ELISA. Samples for RANTES measurements were all done in the presence of 10 ng/ml TNF- $\alpha$  without (H441) or with 200 U/ml IFN- $\gamma$  (Calu-3 and NHBE). Values are presented as mean  $\pm$  SD for triplicate samples. Similar results in each case were seen in three independent experiments.

ditioned medium significantly increased basal IL-8 release ( $P < 0.001$  in each case) as well as TNF-dependent IL-8 release (data not shown). Similarly, this fraction significantly inhibited TNF-dependent RANTES release relative to nonconditioned medium controls ( $P < 0.001$  in each case). Moreover, the small molecular weight fraction increased IL-8 and decreased RANTES mRNA levels as measured by RPA (data not shown), suggesting that the observed changes in cytokine release were due, at least in part, to changes in transcription and/or mRNA stability. Of note, in contrast to IL-8 where values for the < 3 kD fraction were comparable to values for complete conditioned medium, the < 3 kD fraction was consistently less able than complete conditioned medium to reduce RANTES ( $P < 0.01$  for MCM and HPCM;  $P < 0.05$  for GACM relative to complete conditioned medium).

Previous studies also report that the stimulatory effect on IL-8 release by MCM is heat stable (15). To determine whether the effect on RANTES release was heat stable or heat labile, nonconditioned and bacterial-conditioned media were boiled for 30 min and the effect of this treatment on cytokine release by A549 cells was determined (Table 1). All heat-treated conditioned media significantly increased IL-8 release ( $P < 0.001$  in each case) and decreased RANTES release ( $P < 0.001$  in each case) relative to untreated nonconditioned medium controls: heat-treating nonconditioned medium had no effect (data not shown). For IL-8, the values for heat-treated conditioned media were comparable to untreated controls, whereas inhibition of RANTES release was partially heat labile. Taken together, these data suggest that small molecular weight, heat-stable factors present in bacterial-conditioned medium were responsible for all of the observed increases in IL-8 release but for only part of the observed decreases in RANTES release.

#### Effect of Bacterial-Conditioned Medium on Cytokine Release by Human Airway Epithelial Cell Cultures

To determine whether the small molecular weight factors present in bacterial-conditioned medium had similar effects on cytokine release by other airway epithelial cells, we used two additional airway epithelial cell lines (H441, Calu-3) as well as NHBE (Clonetics). Representative results from these experiments are shown for HPCM and GACM (Table 2). As with A549 cells, the < 3 kD fractions from bacterial-conditioned media increased IL-8 release ( $P < 0.01$  in each case) and decreased RANTES release ( $P < 0.01$  in each case) by each cell type. Also, as

with A549 cells, effects on RANTES expression were studied in cytokine-treated cells because no constitutive release of RANTES was observed for any cell type tested, including primary cells (data not shown). Both Calu-3 and NHBE required stimulation by a combination of TNF- $\alpha$  and IFN- $\gamma$ . A requirement by bronchial epithelial cells for a combination of cytokines is consistent with previous reports by other laboratories (21, 22).

#### Potential Contribution of Proteases to the Effects on RANTES

Although some of the inhibitory effects on RANTES appeared to be due to small molecular weight, heat-stable factors, additional inhibitory activity was observed using untreated bacterial-conditioned media (Table 1). Based on previous studies showing that several host cytokines are sensitive to *P. aeruginosa* proteases, we hypothesized that proteases could account, at least in part, for this additional inhibitory activity. Several pieces of data support this hypothesis.

When purified human recombinant RANTES was combined with bacterial-conditioned medium in cell-free assays, there was a concentration-dependent decrease in RANTES as measured by ELISA (Figure 4A) as well as a time-depen-

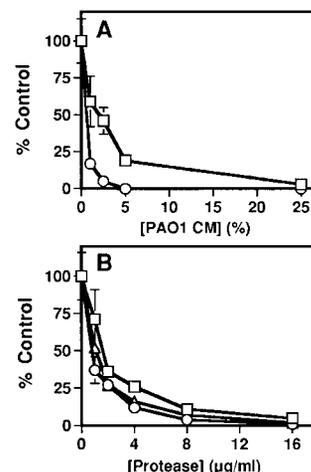


Figure 4. Effect of proteases on RANTES protein levels. (A) Cell-free studies: Purified recombinant human RANTES (500 pg/ml) was combined with 25% nonconditioned medium (control) or with the indicated concentration of HPCM (squares), or GACM (circles). Samples were incubated for 24 h. At the end of the incubation period, RANTES was measured in the samples using ELISA. (B) TNF-treated A549 cells: Cells were incubated for 24 h without (control) or with the indicated concentration of purified *P. aeruginosa* elastase (circles), alkaline protease (squares) or equal amounts of each protease (triangles). At the end of the incubation period, RANTES was measured in the culture medium using ELISA. Values are expressed as % control and represent the mean  $\pm$  SD for triplicate samples. Similar results were seen in three separate independent experiments.

dent decrease as measured by Western blot analysis (data not shown). In addition, we found that metalloprotease inhibitors (ethylenediaminetetraacetic acid [EDTA] and desferoximine) prevented this degradation in the cell-free assays (data not shown). Unfortunately, these inhibitors could not be used in cell culture studies to determine whether proteases contributed to the effects on RANTES release by epithelial cells: EDTA promoted epithelial cell detachment, and desferoximine alone potentially inhibited TNF-dependent RANTES release (data not shown). As an alternative approach, we added increasing amounts of purified *P. aeruginosa* metalloproteases (elastase and alkaline protease) to cultures of A549 cells treated with TNF- $\alpha$ . We found that each protease alone or a combination of both together decreased RANTES in a concentration-dependent manner (Figure 4B). Together, these data are consistent with the conclusion that bacterial metalloproteases degrade human RANTES and thus, they suggest that proteases in bacterial-conditioned medium were responsible, at least in part, for reduced RANTES levels. They do not rule out the possibility, however, that additional high molecular weight, heat-labile bacterial factors were present that contributed to the observed changes in RANTES expression and release.

#### Effect of Organic Solvent Extraction on the Biologic Activities of Bacterial-Conditioned Media

The factors in MCM that stimulate IL-8 release are found in the aqueous phase after extraction with CHCl<sub>3</sub>/MeOH (15). To characterize further the physical properties of factors that affect levels of IL-8 and RANTES, nonconditioned and bacterial-conditioned media were extracted with CHCl<sub>3</sub>, as described in MATERIALS AND METHODS. Based on our earlier studies (8), we hypothesized that pyocyanin, present in the CHCl<sub>3</sub> phase of extracted GACM, would contribute to the observed activities. Representative results from these experiments are shown in Table 3: MCM was not tested on RANTES release. Values reported for nonconditioned medium controls are for media not exposed to CHCl<sub>3</sub> because both the aqueous and CHCl<sub>3</sub> phases from CHCl<sub>3</sub> extractions of nonconditioned medium gave similar results (data not shown). Pyocyanin concentrations

were < 1  $\mu$ M for all MCM and HPCM and ranged from 1.0 to 1.5 mM for GACM preparations (5% GACM ~ 50 to 75  $\mu$ M). Only the aqueous phase from MCM and HPCM stimulated IL-8 release ( $P < 0.001$  in each case). In contrast, significant stimulatory activity was observed in the CHCl<sub>3</sub> phase from GACM ( $P < 0.001$ ).

Representative results from RANTES measurements are also shown in Table 3. As with IL-8, only the aqueous phase from HPCM inhibited RANTES release ( $P < 0.001$ ), whereas both phases from GACM had this effect (aqueous,  $P < 0.001$ , CHCl<sub>3</sub>  $P < 0.01$ ). Taken together, these data are consistent with the presence of at least two factors in GACM that affect both IL-8 and RANTES release. Moreover, our conclusion that pyocyanin represents the activity in the CHCl<sub>3</sub> phase of GACM is consistent with our earlier studies showing that 5 to 100  $\mu$ M of purified pyocyanin both increase IL-8 and decrease RANTES expression (8).

One of the purified factors shown to increase IL-8 is *P. aeruginosa* autoinducer (12). To determine whether autoinducer contributed to the observed activities in our conditioned media, nonconditioned and bacterial-conditioned media were extracted with ethyl acetate, and the effects of the aqueous and ethyl acetate phases on IL-8 and RANTES release were determined (Table 3). Autoinducer is purified by extraction into ethyl acetate (23). As mentioned previously, the values reported for nonconditioned medium controls are for media not exposed to ethyl acetate because both the aqueous and ethyl acetate phases of ethyl acetate extracted nonconditioned medium gave similar results (data not shown). We observed no effect by the ethyl acetate phase from each bacterial-conditioned medium on either IL-8 or RANTES release and there was no loss of activity from the corresponding aqueous phase. These data suggest that autoinducer does not account for these activities. Note that these studies do not rule out a role for autoinducer *in vivo* where its concentration may be considerably higher or where the microenvironment may modulate its effects.

#### Time Course Studies

To determine the time course of the effects on IL-8 release, cells were exposed for increasing times to noncondi-

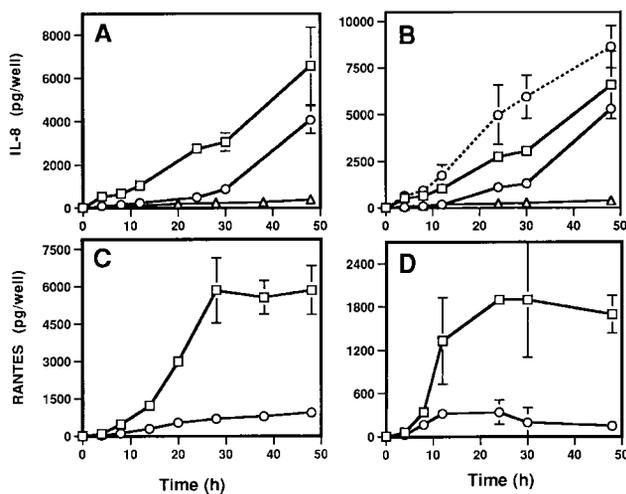
TABLE 3  
Effect of extraction with organic solvents

	IL-8 (pg/well)			RANTES (pg/well)		
	MCM	HPCM	GACM	MCM	HPCM	GACM
I. Nonconditioned medium	830 $\pm$ 80	343 $\pm$ 102	100 $\pm$ 10			
Complete conditioned medium	5,790 $\pm$ 830	2,020 $\pm$ 140	6,681 $\pm$ 1,380			
Aqueous	5,130 $\pm$ 1,430	2,160 $\pm$ 360	2,900 $\pm$ 1,060			
CHCl <sub>3</sub>	690 $\pm$ 120	430 $\pm$ 60	1,940 $\pm$ 695			
II. Nonconditioned medium	820 $\pm$ 50	343 $\pm$ 102	560 $\pm$ 45	7,770 $\pm$ 1,100	7,980 $\pm$ 1,140	6,200 $\pm$ 1,010
Complete conditioned medium	1,830 $\pm$ 30	2,020 $\pm$ 140	2,390 $\pm$ 150	1,780 $\pm$ 580	4,200 $\pm$ 1,000	490 $\pm$ 120
Aqueous	1,390 $\pm$ 150	1,550 $\pm$ 650	2,800 $\pm$ 130	1,210 $\pm$ 290	4,320 $\pm$ 370	400 $\pm$ 70
Ethyl acetate	520 $\pm$ 30	270 $\pm$ 80	730 $\pm$ 70	7,630 $\pm$ 750	7,370 $\pm$ 1,600	5,290 $\pm$ 860

Aqueous and organic phases of bacterial-conditioned medium (MCM, HPCM, and GACM) for CHCl<sub>3</sub> (I) and ethyl acetate (II) extractions were prepared as described in MATERIALS AND METHODS. Cultures of A549 cells in 48-well plates were incubated for 30 h with the indicated conditioned medium (20% MCM and HPCM, 5% GACM), the culture medium was collected, and samples were assayed for the indicated cytokine by ELISA. Samples for RANTES measurements were all done in the presence of 10 ng/ml TNF- $\alpha$ . Values are presented as mean  $\pm$  SD for triplicate samples. Similar results in each case were seen with two or more preparations of each conditioned medium in three independent experiments.

tioned or bacterial-conditioned medium, and IL-8 release was measured using ELISA. A total of 5% GACM (Figure 5A) and 20% HPCM (Figure 5B) from PA01 cultures increased IL-8 release relative to nonconditioned medium controls ( $P < 0.01$  for times  $> 12$  h). Interestingly, the kinetics for each bacterial-conditioned medium was different from those for the positive control TNF- $\alpha$ . Specifically, TNF- $\alpha$  stimulated a rapid increase at early times and what appeared to be a secondary increase at times between 30 and 48 h. In contrast, there was a significant lag ( $> 12$  h) before bacterial-conditioned media increased IL-8 release. If cells were treated with both TNF- $\alpha$  and bacterial-conditioned medium together (Figure 5B, dotted line), release of IL-8 was enhanced at all times tested. The enhancement of the response to TNF- $\alpha$  at early times ( $\leq 12$  h) suggests that bacterial-conditioned media activated signaling pathways at these times, but the lag that is observed with bacterial-conditioned medium alone suggests that activation of these pathways was not sufficient to increase IL-8 release.

The kinetics of the effect on RANTES release was also examined (Figures 5C and 5D). The  $< 3$  kD fractions were used in these studies to exclude the possibility that proteases contributed to the effect. TNF- $\alpha$  stimulated an early increase in RANTES that reached maximum values at or before 24 h. The  $< 3$  kD fractions of both PA01 GACM (Figure 5C) and PA14 HPCM (Figure 5D) inhibited increases in RANTES expression at all times tested. This suggests that the inhibitory effects of the small molecular weight factors were immediate and prolonged.



**Figure 5.** Time course of conditioned medium-dependent changes in cytokine release. (A) A549 cells were incubated for increasing times with nonconditioned medium (triangles), 10 ng/ml TNF- $\alpha$  (squares), or 5% GACM (circles). (B) A549 cells were incubated for increasing times with nonconditioned medium (triangles), TNF- $\alpha$  alone (squares), 20% HPCM (circles), or TNF- $\alpha$  and HPCM together (circles, dotted line). (C and D) TNF-treated A549 cells were incubated with nonconditioned medium (squares), with 5% GACM (C, circles), or with 20% HPCM (D, circles). At the end of the incubation period, the media were assayed for IL-8 or RANTES using ELISA. Values are expressed as mean  $\pm$  SD for triplicate samples. Similar results were seen in three independent experiments.

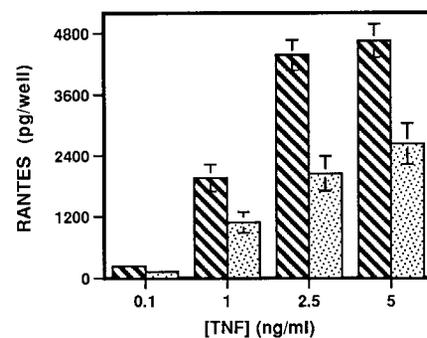
### Effect of Conditioned Medium on RANTES Release in Response to Increasing Concentrations of TNF- $\alpha$

Combined data from studies using bacterial-conditioned medium preparations presumably lacking protease activity ( $< 3$  kD or heat-treated) indicated that these preparations inhibited TNF-dependent RANTES release approximately  $60 \pm 3\%$  (mean  $\pm$  standard error of the mean,  $n = 34$ ). Because the TNF- $\alpha$  concentration used in these studies (10 ng/ml) was supramaximal, we hypothesized that these fractions might be more effective if submaximal concentrations of TNF- $\alpha$  were used. To test this hypothesis, we incubated cells for 30 h with increasing concentrations of TNF- $\alpha$  with and without 5% of the  $< 3$  kD fraction from PA01 GACM and measured RANTES release into the medium. Surprisingly, bacterial-conditioned medium inhibited the response  $\sim 40$  to 60%, regardless of the TNF- $\alpha$  concentration tested (Figure 6). Values for 0.1 ng/ml TNF- $\alpha$  alone and with GACM were  $230 \pm 50$  and  $120 \pm 10$  pg/ml, respectively ( $P < 0.01$ ). Values for cells not exposed to TNF- $\alpha$  were below the limit of detection by ELISA (15 pg/ml).

### Studies with PA14 Wild-Type and Mutant Strains

The observation that the CHCl<sub>3</sub> phase from GACM contained both the stimulatory and inhibitory activities (Table 3) suggested to us that pyocyanin or other phenazine derivatives might be responsible for these activities. To test this hypothesis further, we used *P. aeruginosa* PA14 wild-type and mutant strains, one of which, 3E8, is a phenazine-minus mutant (17). Results similar to those described previously using PA01-conditioned media were seen in selected experiments with PA14-conditioned media, suggesting that similar factors were released by this strain (data not shown).

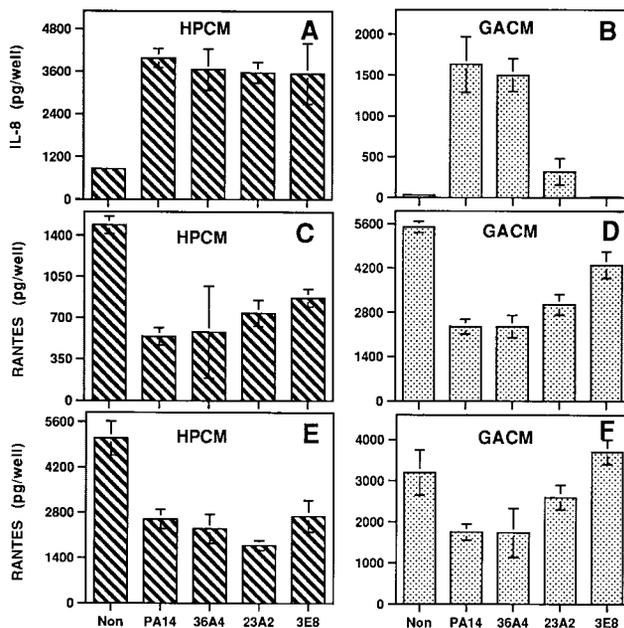
To assess the potential contribution of phenazine derivatives to the biologic activities,  $< 3$  kD fractions of both HPCM and GACM from PA14 wild-type and mutant cultures were prepared. The  $< 3$  kD fractions were used in these studies to avoid proteolysis of RANTES. Bacterial growth of wild-type and mutant strains was comparable



**Figure 6.** Effect of bacterial-conditioned media on the dose response of TNF-dependent RANTES release. A549 cells were exposed for 30 h to the indicated concentration of TNF- $\alpha$  in the absence (striped bars) or presence (dotted bars) of 5% of the  $< 3$  kD fraction of PA01 GACM. RANTES release into the medium was determined using ELISA. Values are expressed as mean  $\pm$  SD for triplicate samples. Similar results were seen in two separate independent experiments.

for each medium, as was total bacterial protein released into the conditioned medium (data not shown). Only very low or nondetectable levels of pyocyanin ( $\leq 1 \mu\text{M}$ ) were present in any HPCM tested and in GACM from cultures of 3E8. In contrast, pyocyanin concentrations in GACM from PA14 wild-type, 36A4, and 23A2 cultures were 150, 100, and 40  $\mu\text{M}$ , respectively; 5% GACM was 7.5, 5, and 2  $\mu\text{M}$  pyocyanin, respectively.

HPCM from PA14 wild-type and mutant cultures was equally potent in stimulating IL-8 release (Figure 7A). In contrast, GACM from the phenazine-minus mutant 3E8 and from 23A2 (lowest pyocyanin concentration) were less potent than conditioned media from wild-type and 36A4 (Figure 7B). Similarly, HPCM from all strains inhibited TNF-dependent RANTES release equally well (Figure 7C), whereas GACM with low (23A2) or negligible (3E8) pyocyanin concentrations was less effective than wild-type- or 36A4-conditioned medium (Figure 7D). These data suggest that factors other than pyocyanin/phenazines account for the biologic activities in HPCM but that phenazine derivatives significantly contribute to these activities in GACM.



**Figure 7.** Effect of bacterial-conditioned media from PA14 wild-type and mutant cultures on cytokine release by A549 cells. Bacterial-conditioned media were prepared using wild-type and mutant strains of PA14 as described in MATERIALS AND METHODS. (A and B) A549 cells were incubated for 30 h with nonconditioned medium (Non) or with the < 3 kD fraction of HPCM (20%) (A) or GACM (5%) (B) from the indicated bacterial strain. (C and D) A549 cells treated with 10 ng/ml TNF- $\alpha$  were incubated for 30 h with nonconditioned medium or with the < 3 kD fraction of HPCM (C) or GACM (D). (E and F) A549 cells treated with 10 ng/ml of IL-1 $\alpha$  were incubated for 30 h with nonconditioned medium or with the < 3 kD fraction of HPCM (E) or GACM (F). At the end of the incubation period, the indicated cytokine was measured in the culture medium using ELISA. Values are expressed as mean  $\pm$  SD for triplicate samples. Similar results were seen in two separate independent experiments.

### Effect of Conditioned Medium on RANTES Release in Response to IL-1

The other “early response” cytokine released upon bacterial infection is IL-1. Like TNF- $\alpha$ , IL-1 increases expression of RANTES in airway epithelial cells (24). To determine whether bacterial-conditioned medium inhibits IL-1-dependent RANTES release, cells were treated for 30 h with 10 ng/ml IL-1 $\alpha$  or IL-1 $\beta$  and with nonconditioned medium or the < 3 kD fractions from PA14 wild-type- and mutant-conditioned media. RANTES release into the culture medium was then determined. Representative results are shown for experiments using IL-1 $\alpha$  (Figures 7E and 7F). Similar results were seen with IL-1 $\beta$  (data not shown). As with TNF-treated cells, inhibition of IL-1-dependent RANTES release by the < 3 kD fractions was only partial. Also as observed for TNF- $\alpha$ , HPCM from PA14 wild-type and mutant cultures inhibited RANTES release to a similar extent (Figure 7E), whereas GACM from 23A4 and 3E8 cultures were relatively less effective (Figure 7F).

### Discussion

A summary of our findings is shown in Table 4. Thus far, our data suggest that effects on both IL-8 and RANTES release may be mediated by at least two small molecular weight, heat-stable factors, one of which in GACM is pyocyanin. Similar to previous reports (15), these small molecular weight factors were also found to be protease resistant (data not shown). Moreover, a comparison of the physical properties suggests that the same small molecular weight factors may affect both IL-8 and RANTES release. This is consistent with our observation that purified pyocyanin can both increase IL-8 and decrease RANTES expression by A549 cells (8). Studies with polymyxin B and ethyl acetate extraction suggest that these factors are not LPS or autoinducer. Preliminary high performance liquid chromatography analysis of the < 3 kD fractions identified a peak in both HPCM and GACM that stimulates IL-8 release (data not shown). This peak is distinct from pyocyanin and 1-hydroxy-phenazine. Further characterization of the compound is currently under way.

TABLE 4  
*Properties of secreted factors from P. aeruginosa*

MCM/HPCM	GACM
I. Noncytotoxic at $\leq 50\%$ conditioned medium	Cytotoxic at $\geq 10\%$ conditioned medium > 3 kD, heat-stable factor(s)
II. RANTES proteolysis > 3 kD, heat-stable factor(s)	RANTES proteolysis > 3 kD, heat-labile factor(s)
III. Increased IL-8; decreased RANTES < 3 kD, heat-stable, protease-resistant factor(s) present only in the aqueous phase of $\text{CHCl}_3$ extractions activity of PA14 wild-type = 36A4 = 23A2 = 3E8	Increased IL-8; decreased RANTES < 3 kD, heat-stable, protease-resistant factor(s) present in both the aqueous and $\text{CHCl}_3$ phases of $\text{CHCl}_3$ extractions activity of PA14 wild-type = 36A4 > 23A2 > 3E8

In addition to its effects on IL-8 and RANTES expression, GACM was cytotoxic at concentrations  $\geq 10\%$ . Cytotoxicity was not present in the  $< 3$  kD fractions (data not shown). This observation suggests that factors  $> 3$  kD are required but does not rule out the possibility that both small and large molecular weight factors act in concert. In addition, the cytotoxicity was largely heat stable (data not shown). The identity of factors that mediate this cytotoxicity remains to be determined.

Studies shown in Table 1 suggested the presence of higher molecular weight ( $> 3$  kD), heat-labile factors that decreased RANTES levels. Data from several approaches suggest that bacterial proteases contributed to this inhibitory effect. Further characterization of this proteolytic activity is currently under way.

These studies extend previous studies focused on the regulation of IL-8 by *P. aeruginosa* factors. The mechanism by which these factors regulate IL-8 expression has been partially characterized for only two of these factors, nitrite reductase and pyocyanin. Studies by Mori and co-workers (25) suggest that nitrite reductase activates the transcription factor nuclear factor (NF)- $\kappa$ B and that this activation mediates increased IL-8 expression. With respect to pyocyanin, studies by our laboratory indicate that pyocyanin-dependent increases in IL-8 expression are mediated by signaling pathways that include oxidants, protein tyrosine kinases, and the mitogen-activated protein kinases extracellular regulated kinase and p38 (8). IL-8 expression is regulated by activation of three transcription factors, activator protein-1, NF- $\kappa$ B, and NF-IL-6 (26). Our time-course studies suggest differences between TNF- $\alpha$  and bacterial-conditioned medium in the magnitude and/or timing of transcription factor activation.

Regulation of RANTES expression is considerably less well characterized than regulation of IL-8 expression. In the case of epithelial cells, RANTES expression is increased by cytokines such as TNF- $\alpha$  and IL-1 (24), by viral infection (27, 28), and by protein overload in the kidney (29). For primary airway epithelial cells, a combination of IFN- $\gamma$  and IL-1 $\beta$  or TNF- $\alpha$  appears to be required (21, 22). RANTES expression appears to require activation of NF- $\kappa$ B (27). In contrast to IL-8 where bacterial-conditioned media enhanced the response to host cytokines, these media inhibited RANTES expression and release. The use of cytokine-stimulated cells in these studies is physiologically relevant as multiple proinflammatory factors are likely to be present in the inflamed airway and will contribute to cytokine expression and release. Interestingly, inhibition was approximately 50%, regardless of the concentration of TNF- $\alpha$  used. The simplest interpretation of these data is that there are at least two pathways by which TNF- $\alpha$  increases RANTES expression and that only one of these pathways is affected by bacterial-conditioned medium.

Currently, little is known about agonists and conditions that inhibit RANTES. Studies in rat glomeruli suggest that nitric oxide (NO) downregulates RANTES in endotoxin-treated kidney epithelial cells (30). However, increased NO production by A549 cells requires a mixture of cytokines (IFN- $\gamma$ , TNF- $\alpha$ , and IL-1), and no other airway epithelial cell lines express NO (data not shown). Thus, it seems unlikely that NO is increased under the conditions

of our study and thus unlikely that it contributes to the inhibitory effects of bacterial-conditioned medium on RANTES release. Interestingly, heat shock inhibits TNF-dependent RANTES expression by A549 cells (31). This inhibition appears to be due to protection of phosphorylated I $\kappa$ B- $\alpha$  from degradation and hence prevention of NF- $\kappa$ B activation. Because pyocyanin increases oxidant stress in A549 cells (32, 33), it is tempting to speculate that it acts through a mechanism similar to heat-related stress. However, if pyocyanin and/or bacterial-conditioned medium inhibit RANTES expression by inhibiting NF- $\kappa$ B activation, then it would follow that NF- $\kappa$ B activation could not account for a simultaneous increase in IL-8 expression in response to these agonists.

With respect to the biologic relevance of our results, cytotoxic effects by *P. aeruginosa* secretory factors could lead to epithelial cell death and loss of barrier function. Furthermore, secreted factors that increase IL-8 release, either alone or in combination with host inflammatory factors, could contribute to a vigorous neutrophilic response that could lead to neutrophil-mediated tissue damage (34, 35). Conversely, secreted factors that reduce levels of certain cytokines, either by proteolysis (IL-2, IFN- $\gamma$ , TNF- $\alpha$ , and RANTES) (9–11) or by effects on expression (IL-2 and RANTES) (7, 8), would compromise aspects of both the innate and acquired immune responses. Together, these effects could significantly contribute to the pathophysiology of *P. aeruginosa*-associated lung disease.

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## References

- Horan, T., D. Culver, W. Jarvis, G. Emori, S. Banerjee, W. Martone, and C. Thornsberry. 1988. Pathogens causing nosocomial infections. *Antimicrobial Newsletter*. 5:65–67.
- Gilligan, P. 1991. Microbiology of airway disease in patients with cystic fibrosis. *Clin. Microbiol. Rev.* 4:35–51.
- Fick, R. B. J. 1989. Pathogenesis of the *Pseudomonas* lung lesion in cystic fibrosis. *Chest* 96:158–164.
- Fick, R. J. 1993. *Pseudomonas aeruginosa*—the microbial hyena and its role in disease: an introduction. In *Pseudomonas aeruginosa the opportunist: Pathogenesis and Disease*. R. J. Fick, editor. CRC Press, Ann Arbor, MI. 1–6.
- Clarke, P. H. 1990. Introduction: *Pseudomonas aeruginosa*, an opportunistic pathogen. In *Pseudomonas Infection and Alginates*. P. Gacesa and N. J. Russell, editors. Chapman and Hall, New York. 1–12.
- Buret, A., and A. W. Cripps. 1993. The immunoevasive activities of *Pseudomonas aeruginosa*: relevance for cystic fibrosis. *Am. Rev. Respir. Dis.* 148:793–805.
- Nutman, J., M. Berger, P. Chase, D. Dearborn, K. Miller, R. Waller, and R. Sorensen. 1987. Studies on the mechanism of T cell inhibition by the *Pseudomonas aeruginosa* phenazine pigment pyocyanine. *J. Immunol.* 138: 3481–3487.
- Denning, G., L. Wollenweber, M. Railsback, C. Cox, L. Stoll, and B. Britigan. 1998. *Pseudomonas* pyocyanin increases interleukin-8 expression by human airway epithelial cells. *Infect. Immun.* 66:5777–5784.
- Horvat, R., and M. Parmely. 1988. *Pseudomonas aeruginosa* alkaline protease degrades human gamma interferon and inhibits its bioactivity. *Infect. Immun.* 56:2925–2932.
- Theander, T., A. Kharazmi, B. Pedersen, L. Christensen, N. Tvede, L. Poulsen, N. Odum, M. Svenson, and K. Bendtzen. 1988. Inhibition of human lymphocyte proliferation and cleavage of interleukin-2 by *Pseudomonas aeruginosa* proteases. *Infect. Immun.* 56:1673–1677.
- Parmely, M., A. Gale, M. Clabaugh, R. Horvat, and W.-W. Zhou. 1990. Proteolytic inactivation of cytokines by *Pseudomonas aeruginosa*. *Infect. Immun.* 58:3009–3014.
- DiMango, E., H. Zar, R. Bryan, and A. Prince. 1995. Diverse *Pseudomonas*

- aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. *J. Clin. Invest.* 96:2204–2210.
13. Oishi, K., B. Sar, A. Wada, Y. Hidaka, S. Matsumoto, H. Amano, F. Sonoda, S. Kobayashi, T. Hirayama, T. Nagatake, and K. Matsushima. 1997. Nitrite reductase from *Pseudomonas aeruginosa* induces inflammatory cytokines in cultured respiratory cells. *Infect. Immun.* 65:2648–2655.
  14. Shibata, Y., H. Nakamura, S. Kato, and H. Tomoike. 1996. Cellular detachment and deformation induce IL-8 gene expression in human bronchial epithelial cells. *J. Immunol.* 156:772–777.
  15. Massion, P. P., H. Inoue, J. Richman-Eisenstat, D. Grunberger, P. G. Jorens, B. Housset, J. F. Pittet, J. P. Wiener-Kronish, and J. A. Nadel. 1994. Novel *Pseudomonas* product stimulates interleukin-8 release by airway epithelial cells in vitro. *J. Clin. Invest.* 93:26–32.
  16. Inoue, H., M. Hara, P. Massion, K. Grattan, J. Lausier, B. Chan, T. Kaneko, K. Isono, P. Jorens, I. Ueki, and J. Nadel. 1995. Role of recruited neutrophils in interleukin-8 production in dog trachea after stimulation with *Pseudomonas* in vivo. *Am. J. Respir. Cell Mol. Biol.* 13:570–577.
  17. Mahajan-Miklos, S., M. -W. Tan, L. Rahme, and F. Ausubel. 1999. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa*-*Caenorhabditis elegans* pathogenesis model. *Cell* 96:47–56.
  18. Cox, C. 1986. Role of pyocyanin in the acquisition of iron from transferrin. *Infect. Immun.* 52:263–270.
  19. MacDonald, J. C. 1963. Biosynthesis of pyocyanine. *Can. J. Microbiol.* 9: 809–819.
  20. Koyama, S., E. Sato, H. Nomura, K. Kubo, M. Miura, T. Yamashita, S. Nagai, and T. Izumi. 2000. The potential of various lipopolysaccharides to release IL-8 and G-CSF. *Am. J. Physiol.* 278:L658–L666.
  21. Terada, N., K. -I. Maesako, N. Hamano, T. Ikeda, M. Sai, T. Yamashita, S. Fukuda, and A. Konno. 1996. RANTES production in nasal epithelial cells and endothelial cells. *J. Allergy Clin. Immunol.* 98:S230–S237.
  22. Stellato, C., L. Beck, G. Gorgone, D. Proud, T. Schall, S. Ono, L. Lichtenstein, and R. Schleimer. 1995. Expression of the chemokine Rantes by a human bronchial epithelial cell line. *J. Immunol.* 155:410–418.
  23. Pearson, J., K. Gray, L. Passador, K. Tucker, A. Eberhard, B. Iglewski, and E. Greenberg. 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc. Natl. Acad. Sci. USA* 91: 197–201.
  24. Deckers, J., F. Van der Woude, S. Van der Kooij, and M. Daha. 1997. Synergistic effect of IL-1 $\alpha$ , IFN- $\gamma$ , and TNF- $\alpha$  on RANTES production by human renal tubular epithelial cells in vitro. *J. Am. Soc. Nephrol.* 9:194–202.
  25. Mori, N., K. Oishi, B. Sar, N. Mukaida, T. Nagatake, K. Matsushima, and N. Yamamoto. 1999. Essential role of transcription factor nuclear factor- $\kappa$ B in regulation of interleukin-8 gene expression by nitrite reductase from *Pseudomonas aeruginosa* in respiratory epithelial cells. *Infect. Immun.* 67:3872–3878.
  26. Ben-Baruch, A., D. Michiel, and J. Oppenheim. 1995. Signals and receptors involved in recruitment of inflammatory cells. *J. Biol. Chem.* 270:11703–11706.
  27. Thomas, L., J. Friedland, M. Sharland, and S. Becker. 1998. Respiratory syncytial virus-induced RANTES production from human bronchial epithelial cells is dependent on nuclear factor- $\kappa$ B nuclear binding and is inhibited by adenovirus-mediated expression of I $\kappa$ B $\alpha$ . *J. Immunol.* 161:1007–1016.
  28. Matsukura, S., F. Kokubu, H. Noda, H. Tokunaga, and M. Adachi. 1996. Expression of IL-6, IL-8, and RANTES on human bronchial epithelial cells, NCI-H292, induced by influenza virus A. *J. Allergy Clin. Immunol.* 98:1080–1087.
  29. Zoja, C., R. Donadelli, S. Colleoni, M. Figliuzzi, S. Bonazzola, M. Morigi, and G. Remuzzi. 1998. Protein overload stimulates RANTES production by proximal tubular cells depending on NF- $\kappa$ B activation. *Kidney Int.* 53:1608–1615.
  30. Haberstroh, U., K. Stilo, J. Pocock, G. Wolf, U. Helmchen, U. Wenzel, G. Zahner, R. Stahl, and F. Thaiss. 1998. L-arginine suppresses lipopolysaccharide-induced expression of RANTES in glomeruli. *J. Am. Soc. Nephrol.* 9:203–210.
  31. Ayad, O., J. Stark, M. Fiedler, I. Menendez, M. Ryan, and H. Wong. 1998. The heat shock response inhibits RANTES gene expression in cultured human lung epithelium. *J. Immunol.* 161:2594–2599.
  32. Denning, G., M. Railsback, G. Rasmussen, C. Cox, and B. Britigan. 1998. *Pseudomonas* pyocyanine alters calcium signaling in human airway epithelial cells. *Am. J. Physiol.* 274:L893–L900.
  33. Gardner, P. R. 1996. Superoxide production by mycobacterial and *Pseudomonas* quinoid pigments phthiocol and pyocyanine in human lung. *Arch. Biochem. Biophys.* 333:267–274.
  34. Miller, R. A., and B. E. Britigan. 1995. The formation and biologic significance of phagocyte-derived oxidants. *J. Invest. Med.* 43:39–49.
  35. Wilson, R., R. Dowling, and A. Jackson. 1996. The effects of bacterial products on airway cells and their function. *Am. J. Respir. Crit. Care Med.* 154(Suppl.):S197–S201.