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Role of Interleukin-8 (IL-8) and an Inhibitory Effect of Erythromycin on IL-8 Release in the Airways of Patients with Chronic Airway Diseases

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To evaluate of the role of interleukin-8 (IL-8), a chemotactic cytokine, in the continuous neutrophil accumulation in the airways of patients with chronic airway disease (CAD) and persistent *Pseudomonas aeruginosa* infection, we investigated the cell population, IL-8 levels, IL-1β levels, tumor necrosis factor (TNF) activities, and neutrophil elastase (NE) activities of bronchoalveolar lavage (BAL) fluids in 17 CAD patients (with *P. aeruginosa* infections [CAD+PA], $n = 9$; without any bacterial infections [CAD−PA], $n = 8$) and 8 normal volunteers. We found significant elevations of neutrophil numbers, IL-8/albumin ratios, and NE/albumin ratios in BAL fluids from CAD patients, in the following rank order: CAD+PA > CAD−PA > normal volunteers. IL-1β/albumin ratios were elevated only in CAD+PA, while no TNF bioactivity was detected in BAL fluids. The neutrophil numbers correlated significantly with the IL-8/albumin ratios and NE/albumin ratios in the BAL fluids of CAD patients. When anti-human IL-8 immunoglobulin G was used for neutralizing neutrophil chemotactic factor (NCF) activities in BAL fluids, the mean reduction rate of NCF activities in CAD+PA patients was significantly higher than that in CAD−PA patients. We also evaluated the effects of low-dose, long-term erythromycin therapy in BAL fluids from three CAD+PA and two CAD−PA patients. Treatment with erythromycin caused significant reductions of neutrophil numbers, IL-8/albumin ratios, and NE/albumin ratios in BAL fluids from these patients. To elucidate the mechanism of erythromycin therapy, we also examined whether erythromycin suppressed IL-8 production by human alveolar macrophages and neutrophils in vitro. We demonstrated a moderate inhibitory effect of erythromycin on IL-8 production in *Pseudomonas*-stimulated neutrophils but not in alveolar macrophages. Our data support the view that persistent *P. aeruginosa* infection enhances IL-8 production and IL-8-derived NCF activity, causing neutrophil accumulation in the airways and the progressive lung injuries observed in patients with CAD. The clinical efficacy of erythromycin therapy for CAD patients might be partly mediated through a reduced IL-8 production, diminishing neutrophil accumulation and NE release in the airways.

The importance of *Pseudomonas aeruginosa* as an opportunistic pathogen of the lower respiratory tract is well established (5, 34). Given the predisposing alterations in host defense and airway milieu of chronic airway diseases (CAD), including diffuse panbronchiolitis (DPB) (10, 11), mucoid *P. aeruginosa* may arise as a chronic infection and worsen the prognosis of these diseases (9, 49).

Inflammation in the airways of patients with CAD is characterized by dense neutrophil infiltrations (14, 16, 25, 35). By using electron microscopy, neutrophil accumulation in sputum samples from CAD patients with *P. aeruginosa* infection was recently reported (1). Neutrophils cause progressive airway damage by the release of oxygen metabolites and proteolytic enzymes, including neutrophil elastase (NE) (5, 42). This destructive enzyme also interferes with host defense (2, 6, 7). Various neutrophil chemotactic factors (NCFs) have been demonstrated in the respiratory tract and are thought to be responsible for neutrophil infiltration into the airways of patients with CAD and other pulmonary diseases (7, 12, 13, 23, 33, 46, 52). Recently, interleukin-8 (IL-8), a chemotactic and activating cytokine for human neutrophils, was isolated, purified, and cloned (24, 56). This cytokine is produced in vitro by a variety of cells, including human peripheral blood monocytes, human alveolar macrophages (AMs), fibroblasts, hepatocytes, epithelial cells, and neutrophils, in response to lipopolysaccharide (LPS) and proinflammatory cytokines such as IL-1β and tumor necrosis factor alpha (TNF-α) (24, 29, 36, 37, 43, 47, 51). The participation of IL-8 has been demonstrated in both local and systemic bacterial infections in humans (8, 18). IL-8 also participates in neutrophil-mediated airway inflammation in CAD, including cystic fibrosis (CF) (25, 33, 35). However, the influence of persistent *P. aeruginosa* infection on IL-8-related airway inflammation in CAD has not been fully understood.

On the other hand, it has already been shown that low-dose, long-term erythromycin therapy is effective in CAD, including DPB (21, 28, 39, 55). It seems likely that erythromycin reduces neutrophil accumulation (16) and elastase-like activity in bronchoalveolar lavage (BAL) fluids of patients with DPB (14). In this regard, treatment with erythromycin might suppress in vivo IL-8 release in the airway of patients with CAD.

This study was designed to evaluate the role of IL-8 in the mechanism of continuous accumulation of neutrophils, based on the cytokine-network system, in the airways of CAD patients, especially as associated with persistent *P. aeruginosa* infection. Furthermore, we also evaluated whether the treat-

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ment with erythromycin reduced the neutrophil number, IL-8 level, and NE activity in BAL fluids of patients with CAD.

MATERIALS AND METHODS

Reagents. Recombinant human IL-1β was provided by Genzyme (Boston, Mass.). Erythromycin was a kind gift from Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. Erythromycin was dissolved at a concentration of 10 mg/ml in dimethyl sulfoxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and subsequently diluted in serum-free culture medium for testing AMs or neutrophils. Cycloheximide was obtained from Sigma Chemical Co., St. Louis, Mo.

Bacteria and LPS. *P. aeruginosa* 5276 with a mucoid phenotype was isolated from a patient with DPB. This organism was grown overnight in brain heart infusion broth and harvested in sterile normal saline, and then formalin-killed bacteria were prepared as described previously (32). LPS from strain 5276 was extracted by the hot phenol-water method of Johnson and Perry (15).

Subjects. Three groups of subjects were studied: nine (two male and seven female) patients with CAD and persistent *P. aeruginosa* infection (CAD+PA), eight (six male and two female) CAD patients without any bacterial infection (CAD−PA), and eight male normal volunteers (NV). The CAD+PA patients had *P. aeruginosa* isolated repeatedly from their sputa for 2 months (at least) to 10 years. No pathogenic organism, including *P. aeruginosa*, was isolated from their sputa, which was obtained from the CAP−PA patients before the performance of BAL, and their BAL fluid. Two patients had DPB, one had bronchiectasis, and six had chronic bronchitis. The average age of the CAD+PA patients was 57.9 ± 4.8 years, and all were nonsmokers. Among CAD-PA patients, one had DPB, one had bronchiectasis, and six had chronic bronchitis. Their average age was 62.0 ± 7.0 years, and only one was a smoker. DPB was diagnosed by clinical symptoms, physical examination, and chest radiography as well as pulmonary function tests and lung biopsy, according to the diagnostic criteria of Japanese Ministry of Health and Welfare (11). Chronic bronchitis was diagnosed by using the clinical criteria of persistent cough and sputum for more than 2 years and pulmonary function test showing a decreased forced expiratory volume in 1 s (FEV_1) of less than 70% predicted (3). Bronchiectasis was diagnosed by symptoms and bronchographic examinations. There were no clinical signs or radiographic findings suggesting pneumonia or acute exacerbation of the diseases. None of the patients had been treated with glucocorticoids. The NV subjects were volunteers without symptoms or abnormal findings by chest radiography or pulmonary function tests. Their average age was 23.1 ± 1.2 years, and four were smokers. Three patients with CAD+PA (two with DPB and one with bronchiectasis) and two patients with CAD−PA (one with DPB and one with bronchiectasis) had received low-dose oral erythromycin therapy (600 mg/day, every 8 h) for approximately 3 months after the performance of the first BAL. The effects of this drug on the findings in BAL fluids were evaluated after treatment in each case. No patients were treated with antibiotics other than erythromycin. Informed consent was obtained from all patients and volunteers.

BAL fluid samples. BAL was performed with a fiber-optic bronchoscope (BF-1T20; Olympus Co., Tokyo, Japan) in a subsegmental bronchus of the right lung of NV and patients with CAD as described previously (33). Fifty milliliters of sterile saline was introduced gently by syringe through the bronchoscope and recovered. This procedure was repeated three times, and BAL fluids were pooled and filtered through three layers of cotton gauze. The cells of the BAL fluids were counted in a hemocytometer. The supernatants of the BAL fluids were obtained by centrifugation at 200 × *g* for 10 min at 4°C and stored at −80°C until used. The cell pellets were prepared with a Cytospin 2 (Shandon Southern Products, Ltd., Astmoor, England) and stained with May-Giemsa stain. Differential cell counts were determined for 200 cells.

IL-8 assay. The IL-8 levels were determined by an enzyme-linked immunosorbent assay (ELISA), using monoclonal antibody WS 4 as the capturing antibody and polyclonal rabbit anti-IL-8 antibody as the secondary antibody, both of which were raised against human recombinant IL-8 of 72 amino acids as described previously (19). The IL-8 content of BAL fluid or serum was measured directly. The detection limit of this assay was 31.3 pg of IL-8 per ml. Albumin concentrations in BAL fluids and serum were determined by laser nephelometry to compare the IL-8/albumin ratios in BAL fluids with those in sera.

IL-1β and TNF assays. IL-1β levels in BAL fluids were measured by a commercially available ELISA kit (Otsuka Pharmaceutical Co., Tokushima, Japan). The detection limit of IL-1β was 2 pg/ml. TNF bioactivities in BAL fluids were measured as cytolysis of actinomycin D-sensitized L-929 murine fibroblasts (American Type Culture Collection, Rockville, Md.), determined by crystal violet staining by the procedure described by Ruff and Gifford (38). This end point corresponded to 20 pg/ml when a recombinant human TNF-α standard was used.

NE activity. NE activities in BAL fluid were determined with the substrate methoxy-succinyl-alanyl-alanyl-prolyl-valyl *p*-nitroanilide (Sigma) as described previously (52). Purified NE (Elastin Product Co., Inc., Pacific, Mo.) was used as a standard. This assay for NE activity did not detect purified *P. aeruginosa*-derived elastase (Nagase Biochemicals, Kyoto, Japan).

NCF activity. The NCF activities in BAL fluids from eight CAD patients (CAD+PA, $n = 4$; CAD−PA, $n = 4$) and four NV subjects were measured by using the membrane filter method and a 48-well microchemotactic chamber (Neuro Probe, Inc., Bethesda, Md.) as described previously (33). These BAL fluids from CAD patients and NV subjects were selected at random from each group. Neutrophils were purified from the peripheral blood of a normal volunteer by dextran sedimentation and Ficoll-Hypaque density gradient centrifugation and suspended at a concentration of 2×10^6 cells per ml in Hanks' balanced salt solution containing 1% human serum albumin (Miles, Inc., Kankakee, Ill.). Both the purity and the cell viability of neutrophils were determined to be >99%. Twenty-five microliters of BAL fluid sample or 10^{-7} M *N*-formylmethionylleucyl phenylalanine (Sigma) was placed in quadruplicate in the bottom wells of the chamber separated by a membrane filter with pores 3 μm in diameter, and the upper wells were filled with 50 μl of neutrophil suspension. The chemotaxis chamber assembly was incubated at 37°C in humidified 95% air–5% CO_2 for 1 h, and then the filter was removed, fixed in methanol, and stained with Diff-Quick stain (Kokusaisiyaku, Kobe, Japan). The cells that migrated through the filter were counted under oil immersion in five fields (magnification, ×1,000). In neutralization studies, 100 μl of BAL fluid sample was incubated with the same volume of anti-IL-8 polyclonal rabbit immunoglobulin G (IgG; 100 μg/ml; Endogen, Inc., Boston, Mass.) or control rabbit IgG (100 μg/ml; Zymed Laboratories, Inc., San Francisco, Calif.) for 30 min at 4°C and then employed in the NCF assay. Anti-IL-8 polyclonal rabbit IgG at 100 μg/ml completely neutralizes neutrophil chemotaxis of recombinant IL-8 at levels up to 100 ng/ml. The levels of IL-8 in BAL fluids were all less than 50 ng/ml. Thus,

TABLE 1. Characteristics of BAL cells from patients with CAD and from NV[a]

Patient group	Log (total cells/ml)	% AMs	% Neutrophils	% Lymphocytes
CAD+PA (n = 9)	6.1 ± 0.2**	22.4 ± 20.7**	72.4 ± 9.6**	4.7 ± 9.4
CAD−PA (n = 8)	5.5 ± 0.1	53.3 ± 26.6*	37.8 ± 9.6*	6.2 ± 8.1
NV (n = 8)	5.2 ± 0.1	91.9 ± 6.2	2.4 ± 0.7	6.0 ± 5.6

[a] *, $P < 0.05$; **, $P < 0.01$ (compared with NV group by Kruskall-Wallis test and Tukey's multiple comparison).

anti-IL-8 polyclonal rabbit IgG at 100 μg/ml was expected to neutralize NCF activities of IL-8 in the BAL fluids completely. NCF activities were expressed as the mean values of four individual experiments.

AM and neutrophil culture. Human AMs were collected from three healthy nonsmokers by BAL. Recovered cells were resuspended in Eagle minimum essential medium (Nissui, Tokyo, Japan) containing 1 mM L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 1% nonessential amino acids (GIBCO, Grand Island, N.Y.). The BAL fluid cell differential counts revealed 96.0% AMs, 1.6% neutrophils, and 2.3% lymphocytes. The lavage cells were >95% viable by the trypan blue dye exclusion method. Freshly isolated human AMs were plated on 96-well polystyrene plates (Corning Laboratory Science Co., Corning, N.Y.) at a concentration of 2×10^5 cells per ml. Both the purity and the cell viability of neutrophils plated from the peripheral blood of NV as described above were determined to be >99%. Purified neutrophils were resuspended in sterile RPMI 1640 (Nissui) containing 1 mM L-glutamine and 25 mM HEPES. The cells were plated in 96-well polystyrene plates at a concentration of 4×10^6 cells per ml. AMs or neutrophils were stimulated at time zero with formalin-killed bacteria (10^7 CFU/ml), LPS (1 μg/ml) of *P. aeruginosa* 5276, or IL-1β (10 ng/ml) at 37°C in humidified 95% air–5% CO_2. Cell-free supernatants of conditioned media were collected at 24 h poststimulation. To determine whether erythromycin suppresses AM- or neutrophil-derived IL-8 production, AMs or neutrophils were stimulated with formalin-killed *P. aeruginosa* 5276 (10^7 CFU/ml) or IL-1β (10 ng/ml) and simultaneously incubated with erythromycin at various concentrations (0.1, 1.0, 5.0, and 10 μg/ml), diluted in dimethyl sulfoxide. Erythromycin (0.1 to 10 μg/ml) did not alter the cell viability of AMs or neutrophils under the same stimulation during 24 h of incubation. The final concentrations of dimethyl sulfoxide alone (0.001, 0.01, 0.05, and 0.1%) did not suppress AM or neutrophil-derived IL-8 production when stimulated with formalin-killed *P. aeruginosa* 5276 (10^7 CFU/ml) or IL-1β (10 ng/ml). Cell-free supernatants of culture media were harvested at 24 h poststimulation. All supernatants of culture media were stored at −80°C until tested with the IL-8 ELISA.

Statistical analysis. All data were expressed as the mean ± standard deviation. Differences in total cell numbers, percentages of AMs and neutrophils, IL-8/albumin ratios, IL-1β/albumin ratios, and NE/albumin ratios among three subgroups were determined by a Kruskall-Wallis test and Tukey's multiple comparison. The NCF activity in BAL fluids and neutrophil numbers, IL-8/albumin ratios, and NE/albumin ratios before and after erythromycin therapy were analyzed by the paired Student t test. IL-8 levels of culture supernatants were compared with a single control without erythromycin by Dunnett's multiple comparison. Data were considered statistically significant if P values were less than 0.05.

RESULTS

Cells in BAL fluid. The total cell numbers in BAL fluids of patients with CAD+PA were significantly higher than those of NV subjects ($P < 0.01$) (Table 1). The neutrophil fraction in BAL fluids of patients with CAD+PA or CAD−PA was higher than that of NV subjects ($P < 0.01$ for CAD+PA; $P < 0.05$ for CAD−PA). Consequently, the AM fraction in BAL fluids of patients with CAD+PA or CAD−PA was smaller than that of NV subjects ($P < 0.01$ for CAD+PA; $P < 0.05$ for CAD−PA). No significant difference between CAD+PA and CAD−PA patients was seen in the total cell numbers or the fractions of AMs and neutrophils.

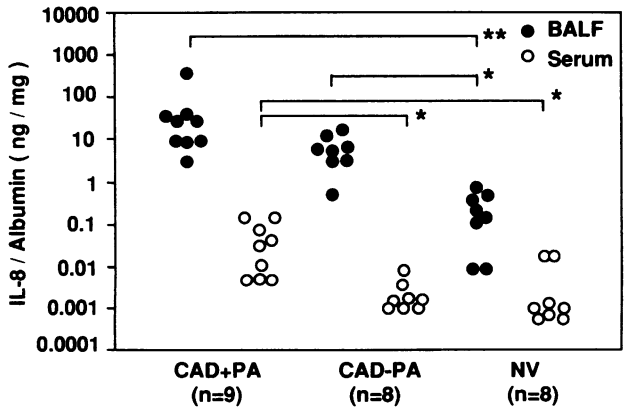

FIG. 1. Comparative analysis of IL-8/albumin ratios in serum and BAL fluids (BALF) among three groups: patients with CAD+PA, patients with CAD−PA, and NV. *, $P < 0.05$; **, $P < 0.01$ (by Kruskall-Wallis test and Tukey's multiple comparison).

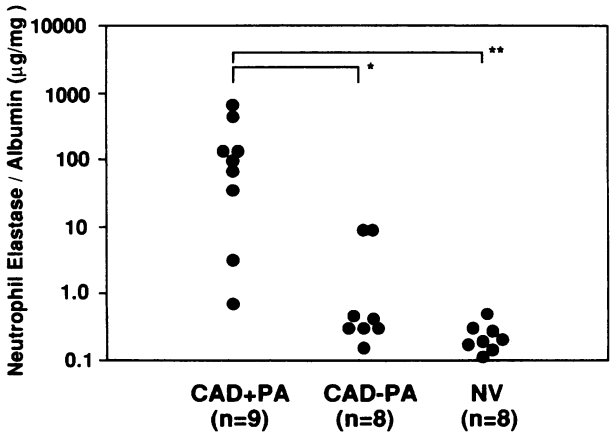

FIG. 2. Comparative analysis of NE/albumin ratios in BAL fluids among three groups: patients with CAD+PA, patients with CAD−PA, and NV. *, $P < 0.05$; **, $P < 0.01$ (by Kruskall-Wallis test and Tukey's multiple comparison).

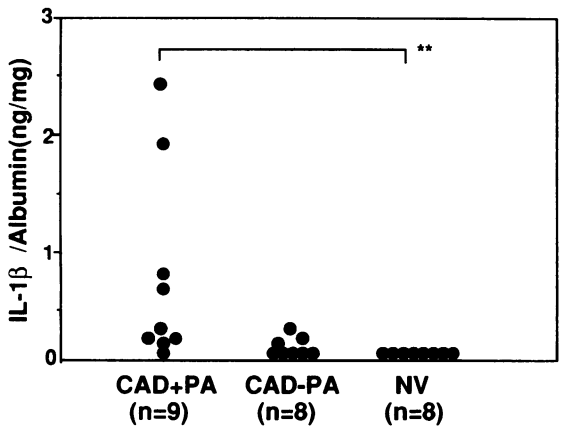

FIG. 3. Comparative analysis of IL-1β/albumin ratios in BAL fluids among three groups: patients with CAD+PA, patients with CAD−PA, and NV. **, $P < 0.01$ (by Kruskall-Wallis test and Tukey's multiple comparison).

IL-8 in serum and BAL fluids. In each subgroup of CAD+PA or CAD−PA, the IL-8/albumin ratio in BAL fluids was significantly higher than that in the serum samples ($P < 0.01$ in CAD+PA; $P < 0.05$ in CAD−PA) (Fig. 1). These data indicate local production of IL-8 in the airways of CAD patients. In BAL fluids, IL-8/albumin ratios of patients with CAD+PA (65.5 ± 132.4 ng/mg) or CAD−PA (7.6 ± 2.2 ng/mg) were significantly higher than those of NV subjects (0.3 ± 0.1 ng/mg; $P < 0.01$ for CAD+PA; $P < 0.05$ for CAD−PA). However, no significant difference was seen in the IL-8/albumin ratio between CAD+PA and CAD−PA patients. In serum samples, a significant elevation of IL-8/albumin ratios was demonstrated in patients with CAD+PA (51.2 ± 20.0 pg/mg) compared with patients with CAD−PA (2.7 ± 2.7 pg/mg) or NV subjects (5.3 ± 3.0 pg/mg; $P < 0.05$).

NE activity in BAL fluids. NE/albumin ratios in BAL fluids were significantly elevated in patients with CAD+PA (124.6 ± 167.1 μg/mg) compared with CAD−PA patients (2.8 ± 1.7 μg/mg; $P < 0.05$) or NV subjects (0.2 ± 0.1 μg/mg; $P < 0.01$) (Fig. 2). No significant difference was noted in the NE/albumin ratio between patients with CAD−PA and NV subjects.

IL-1β and TNF levels in BAL fluids. As shown in Fig. 3, IL-1β/albumin ratios were much lower than IL-8/albumin ratios or NE/albumin ratios in BAL fluids of patients with CAD. Only IL-1β/albumin ratios in BAL fluids of patients with CAD+PA (0.63 ± 0.68 ng/mg) were significantly higher than those of NV subjects (0.01 ± 0.01 ng/mg; $P < 0.01$). There was no significant difference in the IL-1β/albumin ratio between patients with CAD+PA and CAD−PA (0.07 ± 0.10 ng/mg). TNF bioactivities were not detected in BAL fluids from patients with CAD+PA or CAD−PA or NV subjects.

Relationship between neutrophil numbers and IL-8, NE, and IL-1β levels. As shown in Fig. 4A, a significant correlation between IL-8/albumin ratios and neutrophil numbers in BAL fluids from patients with CAD was observed ($r = 0.70$; $P < 0.01$). The ratios of NE/albumin also correlated well with neutrophil numbers in BAL fluids of patients with CAD ($r = 0.82$; $P < 0.001$; Fig. 4B). A weaker correlation between IL-1β/albumin ratios and neutrophil numbers in BAL fluids from CAD patients was observed ($r = 0.54$; $P < 0.01$). Consequently, IL-8/albumin ratios in BAL fluids from CAD patients also correlated well with NE/albumin ratios and IL-1β/albumin ratios in BAL fluid ($r = 0.75$ and $P < 0.01$ for NE/albumin; $r = 0.63$ and $P < 0.01$ for IL-1β/albumin).

NCF activity in BAL fluid. We examined NCF activities in BAL fluids from eight patients with CAD (CAD+PA, $n = 4$; CAD−PA, $n = 4$) and four NV subjects and also performed neutralization experiments to determine how much IL-8 contributed to NCF activities of these samples, using anti-human IL-8 or control polyclonal IgG. As shown in Fig. 5, preincubation with anti-IL-8 IgG resulted in a significant reduction of NCF activities in BAL fluids of both CAD+PA (control IgG, 74.4% ± 8.6%, versus anti-IL-8 IgG, 47.9% ± 11.2%; $P < 0.01$) and CAD−PA (control IgG, 68.2% ± 10.4%, versus anti-IL-8 IgG, 53.6% ± 8.2%; $P < 0.01$) patients. The reduction of NCF activities of CAD+PA patients ($P < 0.05$) (Fig. 6A) was significantly higher than that of CAD−PA patients (36.3% ± 8.1% versus 20.9% ± 6.6%; $P < 0.05$). These data suggest a significant participation of IL-8 in neutrophil accumulation in the airways of CAD patients, especially as associated with persistent *P. aeruginosa* infection.

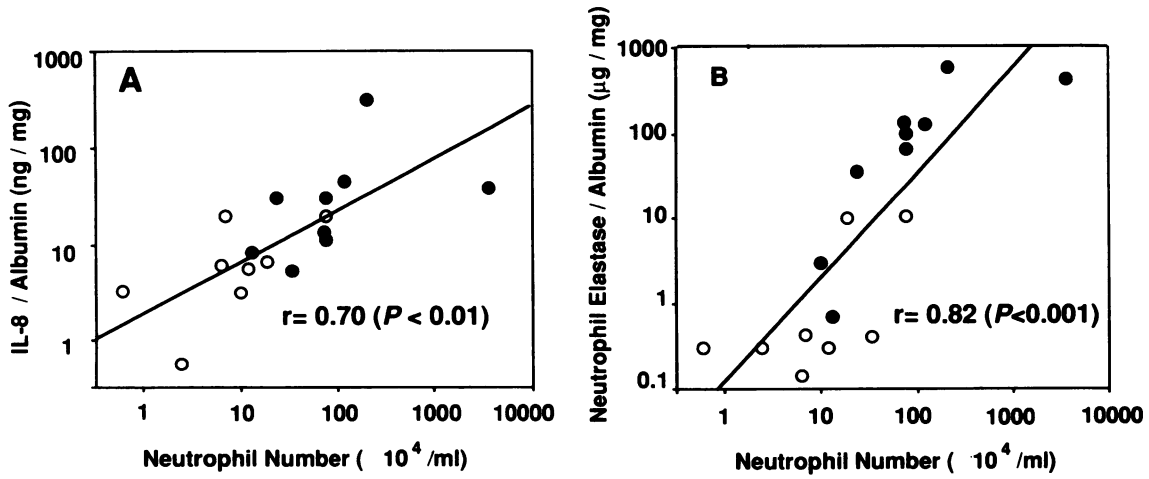

FIG. 4. Correlation between neutrophil numbers and IL-8/albumin ratios (A) and NE/albumin ratios (B) in BAL fluids from patients with CAD+PA ($n = 9$) (closed circles) or patients with CAD−PA ($n = 8$) (open circles).

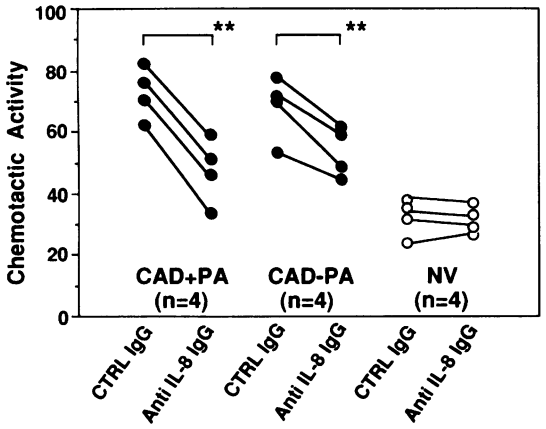

FIG. 5. The NCF activities of BAL fluids from four patients with CAD+PA, four patients with CAD−PA, and four NV. The BAL fluids were treated with anti-IL-8 polyclonal rabbit IgG (100 μg/ml) or control rabbit IgG (100 μg/ml) for 30 min at 4°C. The NCF activity is expressed as a percentage of the positive control (10^{-7} M *N*-formylmethionylleucyl phenylalanine). Each value is the mean of quadruplicate determinations. **, $P < 0.01$ (by Student's *t* test).

Effects of erythromycin therapy in CAD patients. The low-dose, long-term treatment with erythromycin significantly decreased the $\log_{10}$ (neutrophil numbers per milliliter) (before, 6.0 ± 1.0, versus after, 4.7 ± 0.7; $P < 0.05$) (Fig. 6A), the IL-8/albumin ratios (before, 12.0 ± 7.5, versus after, 2.2 ± 1.4 ng/mg; $P < 0.05$) (Fig. 6B), and the NE/albumin ratios (before, 41.1 ± 65.7, versus after, 0.5 ± 0.4 μg/mg; $P < 0.05$) (Fig. 6C) in BAL fluids from three CAD+PA patients and two CAD−PA patients.

AM and neutrophil-derived IL-8. LPS (1 μg/ml) and formalin-killed bacteria (10^7 CFU/ml) of *P. aeruginosa* 5276 stimulated human AM to produce IL-8 at 52.8 ± 11.2 and 95.5 ± 5.0 ng/ml, respectively, at 24 h poststimulation. IL-1β was selected as a stimulator of both AMs and neutrophils, since IL-1β, but not TNF-α, was found to be elevated in BAL fluids from patients with CAD+PA in this study. Addition of IL-1β (10 ng/ml) induced AM-derived IL-8 production to a maximum of 40.4 ± 11.0 ng/ml, while unstimulated human AMs produced 16.0 ± 3.7 ng/ml of IL-8 at 24 h postincubation. Stimulation of human neutrophils with strain 5276 LPS (1 μg/ml) induced low-level production of IL-8 (5.2 ± 0.2 ng/ml) at 24 h poststimulation. In contrast, stimulation of human neutrophils with formalin-killed strain 5276 (10^7 CFU/ml) induced high levels of IL-8 production (81.1 ± 4.4 ng/ml) at the same incubation time. IL-1β (10 ng/ml) stimulated human neutrophils to produce IL-8 at maximal levels of 9.1 ± 1.7 ng/ml at 24 h poststimulation. IL-8 production in AM and neutrophil culture was dependent on protein synthesis, because the addition of cycloheximide at a concentration of 5 μg/ml completely inhibited IL-8 release in both AM and neutrophil cultures in the presence or absence of each stimulant (data not shown).

Effects of erythromycin on AM and neutrophil-derived IL-8. We examined whether erythromycin suppresses IL-8 production induced by AMs or neutrophils stimulated with formalin-killed *P. aeruginosa* 5276 or IL-1β. Erythromycin suppressed *Pseudomonas*-induced, neutrophil-derived IL-8 in a dose-dependent manner (Fig. 7). Significant inhibitions were observed at erythromycin concentrations of 5 μg/ml (percent maximum IL-8, 81.0% ± 5.0%; $P < 0.05$) and 10 μg/ml (percent maximum IL-8, 71.1% ± 5.2%; $P < 0.01$). However, erythromycin did not suppress IL-1β-induced neutrophil-derived IL-8 production (data not shown). Erythromycin at a range of 0.1 to 10.0 μg/ml showed no inhibitory effects on the IL-8 production of AMs stimulated with formalin-killed *P. aeruginosa* 5276 or IL-1 β (data not shown).

DISCUSSION

In patients with CAD, the present study first demonstrated that persistent *P. aeruginosa* infection caused a significant increase in NE levels of BAL fluids, whereas neutrophil recruitment and the release of IL-8 and IL-1β into the airways of patients with CAD were not significantly increased. No TNF bioactivity was detected in the airways of patients with CAD. Shaw and Fick similarly reported that TNF-α levels were not elevated in the airway fluids from CF patients with chronic *P. aeruginosa* infections, while IL-1β levels were found to be high (41). The authors suggested that TNF-α was inactivated in vitro by serine proteases identified in the airway fluid from CF patients with chronic *P. aeruginosa* infection. Taken together, these data indicate that TNF-α may not be essential for the pathogenesis of CAD with persistent *P. aeruginosa* infection.

The large IL-8 production by human AMs stimulated with formalin-treated bacteria or LPS of *P. aeruginosa* 5276, as shown in vitro, supports the higher levels of IL-8 in the airway of patients with CAD+PA. More importantly, our in vitro data from human neutrophil culture in the presence of formalin-killed bacteria or LPS of *P. aeruginosa* 5276 suggested that the

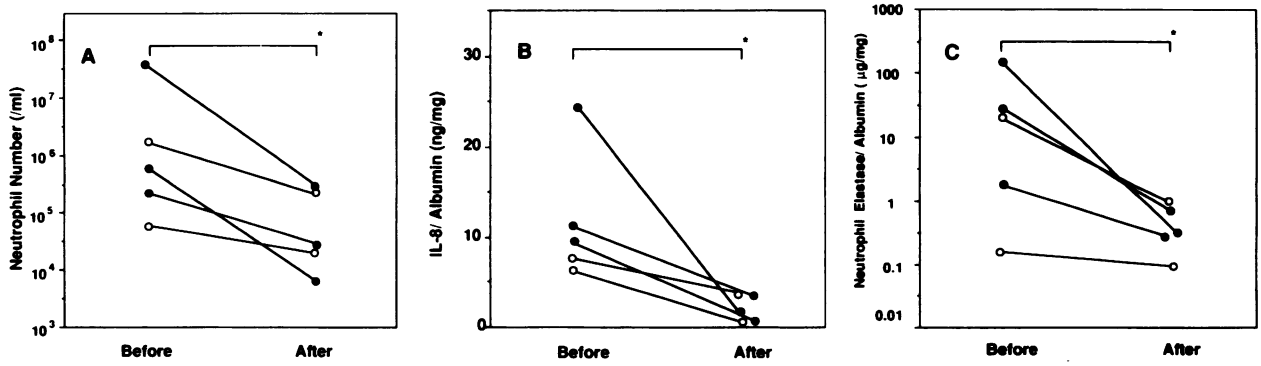

FIG. 6. Comparison of neutrophil numbers (A), IL-8/albumin ratios (B), and NE/albumin ratios in BAL fluids from three patients with CAD+PA (closed circles) or two patients with CAD−PA (open circles) before and after erythromycin therapy. *, $P < 0.05$ (by Student's *t* test).

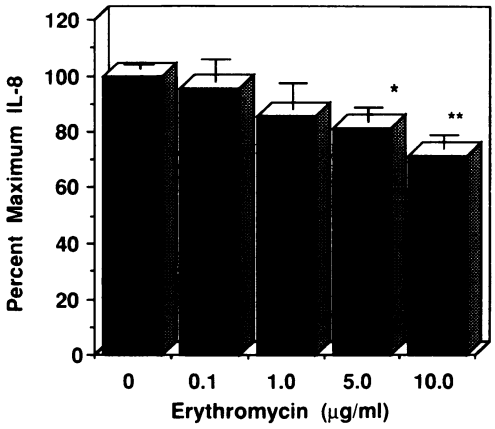

FIG. 7. Effects of erythromycin on neutrophil-derived IL-8 production. Neutrophils were stimulated with formalin-killed *P. aeruginosa* 5276 at a concentration of 10^7 CFU/ml and simultaneously incubated with erythromycin at concentrations of 0.1, 1.0, 5.0, and 10.0 μg/ml. Cell-free supernatants were harvested at 24 h poststimulation. The results were expressed as the percentage of maximum IL-8 production by stimulated neutrophils. Each value represents the mean ± standard deviation of three determinations. *, $P < 0.05$; **, $P < 0.01$ (compared with no erythromycin treatment by Dunnett's multiple comparison).

large amount of neutrophil-derived IL-8 might augment the inflammatory response through additional recruitment of neutrophils. The IL-8/albumin ratios and NE activity/albumin ratios correlated well with neutrophil numbers in BAL fluids of patients with CAD. Therefore, local IL-8 production could directly mediate neutrophil migration into the air space of patients with CAD and stimulate neutrophils to release NE (50). Recently, McElvaney et al. (25) found a similar correlation between IL-8 and NE levels in BAL fluids of CF patients. Nakamura and his colleagues have shown that NE itself induces IL-8 gene expression and IL-8-like NCF activity in a bronchial epithelial cell line (29). In preliminary immunohistochemical studies, we found antigenic IL-8 localized to bronchial epithelial cells of autopsied lung tissue from two cases of DPB (31). These data suggest that a vicious cycle of IL-8 production and neutrophil accumulation induces progressive tissue damage in the airways of patients with CAD, which is mediated by neutrophil-derived lysosomal enzymes and superoxide anions (5, 40). Furthermore, neutrophil proteases, elastase, and cathepsin G were found to stimulate airway gland serous cell secretion in airway diseases associated with neutrophil infiltrations (43).

We demonstrated a significant correlation between IL-1β levels and neutrophil numbers in BAL fluid in CAD patients, as previously shown in children with bacterial infections (54). This proinflammatory cytokine is capable of inducing IL-8 production in various types of respiratory cells, such as AM (36, 45), bronchial and pulmonary epithelial cells (29, 44), and pulmonary fibroblasts (37). In this article, we have shown that the level of human AM-derived IL-8 production induced by IL-1β was comparable to that induced by *P. aeruginosa* LPS. Therefore, airway IL-1β appears to mediate neutrophil accumulation through IL-8 release from IL-1β-stimulated human AM and other respiratory cells.

Recent literature has shown that IL-8 is a major NCF in patients with CAD, idiopathic pulmonary fibrosis, or CF (33, 35). Neutralization of IL-8 with anti-IL-8 antibody-coupled agarose gel in BAL fluids from these patients resulted in a 20 to 30% decrease of NCF activity in patients with CAD or idiopathic pulmonary fibrosis. In the present study, anti-IL-8 polyclonal IgG inhibited NCF activities in BAL fluids of CAD+PA patients by 28 to 47% and of CAD−PA patients by 16 to 30%. Richman-Eisenstat et al. showed that anti-IL-8 monoclonal antibody neutralized 75 to 98% of NCF activities in sputa from patients with CF, bronchiectasis, and chronic bronchitis (35). However, this might be due to differences in the methods of retrieving the mediators or in the specificities of the anti-IL-8 antibodies used.

The literature considering the possible inhibitors of IL-8 production is increasing (26, 27, 36, 45). It has been shown that glucocorticoids suppress IL-8 mRNA transcripts expressed from LPS-stimulated AM (45) and peripheral blood mononuclear cells (27). Amiloride, a pyrazinoylguanidine compound, suppressed steady-state levels of mRNA for IL-8 expressed in LPS-stimulated AM (36). Recently, nebulized amiloride was shown to reduce the decline of pulmonary function in patients with severe CF (17). The authors suggested that the effect of amiloride was due to an alteration of the water content of respiratory secretions. However, an additional mechanism, involving a suppressive effect of amirolide on AM-derived IL-8 production, may be operative. $1,25(OH)_2$-vitamin D_3 is another potential inhibitor of IL-1-induced IL-8 production in peripheral blood mononuclear cells, keratinocytes, and fibroblasts, but not in endothelial cells (22). Topical application of a $1,25(OH)_2$-vitamin D_3 analog, calcipotriol, has been used successfully in the treatment of patients with psoriasis, in which the dermal level of IL-8 is high (20). These reports strongly support the possible clinical application of IL-8 inhibitors against IL-8-related inflammatory diseases.

Previous studies have shown the clinical usefulness of low-dose, long-term erythromycin therapy for patients with CAD, including DPB (21, 39, 55). Erythromycin has been suggested to provide an anti-inflammatory rather than antimicrobial effect (21, 30). A recent report showed that erythromycin suppressed neutrophil numbers and elastase-like activities in BAL fluids of DPB patients (14). Other investigators demonstrated that erythromycin reduced NCF activities in BAL fluids of DPB patients (16). In addition, erythromycin inhibited the intrapulmonary influx of neutrophils in mice intratracheally challenged with LPS. These clinical and experimental data suggest that erythromycin suppresses the release of NCFs in the airways. In this study, we documented significant in vivo inhibitory effects of erythromycin therapy on neutrophil numbers, IL-8 levels, and NE levels in BAL fluids of five patients with CAD+PA or CAD−PA. Therefore, we hypothesized that erythromycin might inhibit the release of IL-8 from AMs or neutrophils. To test our hypothesis, we determined whether erythromycin suppressed production of IL-8 by human AMs or neutrophils under stimulation by mucoid *P. aeruginosa* 5276 or IL-1β in vitro. We found a dose-dependent inhibitory effect of erythromycin on IL-8 production by *Pseudomonas*-stimulated neutrophils but not by IL-1β-stimulated neutrophils or by *Pseudomonas*- or IL-1β-stimulated AMs. The diluent dimethyl sulfoxide at concentrations of 0.001 to 0.1% failed to alter IL-8 production by *Pseudomonas*- or IL-1β-stimulated AMs or neutrophils, in contrast to a recent report (4). Erythromycin at levels of 1 to 10 μg/ml provided approximately a 15 to 30% reduction in IL-8 production by *Pseudomonas*-stimulated neutrophils. The range of erythromycin concentration in serum or sputum samples from patients with DPB has been reported to be 0.29 to 1.75 μg/ml and 0.17 to 1.86 μg/ml, respectively (28). The clinically achievable level of erythromycin is expected to induce up to a 20% reduction of neutrophil-derived IL-8 production. Therefore, continuous oral administration of erythromycin might partly provide in vivo suppression of IL-8

release in the airways of CAD patients. However, this in vitro observation does not explain the drastic suppression of IL-8 production by erythromycin in CAD patients, especially in CAD−PA patients. An indirect mechanism of erythromycin in vivo to regulate IL-8 production remains to be explored.

In summary, persistent *P. aeruginosa* infection tended to enhance IL-8, IL-1β, and NE activity and induced higher IL-8-derived NCF activities and dense neutrophil infiltrations in the airways of CAD patients. Moreover, persistent *P. aeruginosa* infections may stimulate neutrophils in the airways to release the additional IL-8. Therefore, a perpetual cycle of IL-8 production and neutrophil accumulation plays an important role in the pathogenesis of airways of CAD patients, especially as associated with persistent *P. aeruginosa* infections. We also demonstrated that the low-dose, long-term erythromycin therapy resulted in significant reductions of the neutrophil numbers and IL-8 and NE levels in BAL fluids of CAD+PA or CAD−PA patients. Reduction of two important inflammatory mediators, IL-8 and NE, by erythromycin might break the vicious cycle of IL-8-neutrophil accumulation in the airways of patients with CAD.

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