Erythromycin Modulates IL-8 Expression in Normal and Inflamed Human Bronchial Epithelial Cells

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Erythromycin (EM) and its 14-member macrolide analogues have attracted attention for its effectiveness in a variety of airway diseases, including diffuse panbronchiolitis (DPB), sinobronchial syndrome, and chronic sinusitis. However, its mechanisms of action remain unelucidated. We evaluated the effects of several antibiotics on IL-8 expression by normal and transformed human bronchial epithelial cells, an important source of this potent chemokine involved in cell recruitment into the airways. EM and clarithromycin (CAM) uniquely suppressed mRNA levels as well as the release of IL-8 at the therapeutic and noncytotoxic concentrations (% inhibition of IL-8 protein release: 25.0 $\pm$ 5.67% and 37.5 $\pm$ 8.99%, respectively, at 10^{-6} M). The other antimicrobes, including a 16-member macrolide josamycin, showed no effect. Bronchial epithelial cells from very peripheral airways as well as from main bronchi were obtained from patients with chronic airway inflammatory diseases, and EM and CAM inhibited IL-8 release from these cells. Among five patients who underwent bronchoscopy before and after macrolide treatment, four showed decreased levels of IL-8 expression in airway epithelium as assessed by reverse transcription and polymerase chain reaction. Our findings showed these 14-member macrolides had inhibitory effect on IL-8 expression in human bronchial epithelial cells, and this new mode of action may have relevance to their clinical effectiveness in airway diseases.
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Erythromycin (EM) is a macrolide antibiotic widely used for the treatment of upper and lower respiratory tract infections. Recent reports showed that EM is effective for the treatment of chronic airway diseases such as diffuse panbronchiolitis (DPB), bronchial asthma, and chronic sinusitis (1–3). Its precise mechanisms, however, remain unclear. Several cytokines, including IL-8, have been reported to be elevated in bronchoalveolar lavage fluids from patients with such airway inflammatory diseases and to be decreased after appropriate therapy, suggesting roles in airway inflammatory processes (2, 4, 5). IL-8, a potent neutrophil chemoattractant and activating factor (6), is known to be released by monocytes (6), macrophages (7), and fibroblasts (8), and recent data showed that airway epithelial cells are an important source of this CXC-type chemokine (9, 10). Here, we evaluated the effect of several antimicrobes, including EM, on IL-8 expression by normal airway epithelial cells and by those from patients with chronic airway inflammatory disease. We further studied the changes in IL-8 mRNA in airway epithelial cells before and after macrolide therapy by quantitative reverse transcription and polymerase chain reaction (RT-PCR) technique.

METHODS

The study was planned according to the ethical guidelines following the declaration of Helsinki and given the institutional approval, and an informed consent was obtained from each patient.

Preparation of Normal Human Bronchial Epithelial Cells

Normal human bronchial epithelial cells were prepared by the method reported previously (10–12). Briefly, a piece of macroscopically and microscopically normal human lobar or segmental bronchus (averaged 7-mm width) was obtained either at the time of the resection of lung tumor or at autopsy. The bronchus was rinsed in sterile Hanks' balanced salt solution (HBSS) (GIBCO, Grand Island, NY) without calcium and magnesium and incubated in Ham's F12 medium (GIBCO) containing 0.1% protease (Sigma Chemical Co., St. Louis, MO) at 4° C overnight. The bronchus was rinsed with Ham's F12 medium supplemented with 10% fetal calf serum (FCS, heat inactivated;

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GIBCO), and the recovered cells were washed twice in HBSS. The number of the cells was counted by a standard hemocytometer, and cell viability was assessed by trypan blue dye exclusion technique.

Culture of Bronchial Epithelial Cells

The cells were plated onto collagen-coated 24-well flat-bottom tissue culture plates (Koken, Tokyo, Japan) at the density of 5×10^4 cells/well in hormonally defined Ham's F12 medium (HD-F12), as reported (10, 11). HD-F12 contained 1% penicillin-streptomycin, 5 μg/ml insulin (GIBCO), 5 μg/ml transferrin (GIBCO), 25 ng/ml epidermal growth factor (Collaborative Research Corp., Lexington, MA), 15 μg/ml endothelial cell growth supplement (Collaborative Research Corp.), 2×10^{-10} M triiodothyronin (GIBCO), and 10^{-7} M hydrocortisone (GIBCO). The cells were incubated in a humidified atmosphere at 37° C and 5% CO_2. The medium was changed at day 1 and subsequently every 2 d. Confluent monolayers of epithelial cells were stained with anti-keratin (KL-1; Immunotech, Marseille Cedex, France) or anti-vimentin (DAKO-Vimentin; DAKOPatts, Glostrup, Denmark), or with control IgG1 monoclonal antibodies using the avidin-biotin complex method (12, 13). We used primary and secondary passage cells for the experiments. In all preparations of primary and secondary passage bronchial epithelial cells, no less than 98% of the cells were positive to keratin but negative to vimentin, indicating that the cells were of epithelial cell origin as described (12, 13). A human bronchial epithelial cell line BEAS-2B (14) (a kind gift from Drs. J. F. Lechner and C. C. Harris, National Cancer Institute, Betheseda, MD) was cultured in HD-F12, as reported (15). For the evaluation of effects of antibiotics, EM, clarithromycin (CAM), and josamycin (JM) (a kind gift from Dr. S. Omura, Kitasato Institute, Tokyo) were dissolved in methanol as stock solutions and further diluted in sterile physiological saline. Aminobenzyl penicillin (ABPC), cefazolin (CEZ), and tetracycline (TC) were dissolved in physiological saline and were used for the experiments.

Northern Blot Analysis for IL-8 mRNA Expression in Human Bronchial Epithelial Cells

Northern blot analysis was performed to study the effect of the drugs on IL-8 mRNA expression in human bronchial epithelial cells by the method described previously (15, 16). Briefly, total cellular RNA was extracted by the method of Chomszynski and Sacchi (17) and electrophoresed on formaldehyde denatured agarose gel (10 μg/lane) followed by capillary transfer onto Biodyne nylon membrane. RNA integrity and equivalency of loading were routinely evaluated by ethidium bromide fluorescence. Blots were baked, prehybridized, and hybridized with a ^{32}P 5′ end-labeled oligonucleotide probes specific for human IL-8 and β-actin. The probes used in this study were reported previously (16). Blots were stringently washed after hybridization and exposed to X-ray film.

Isolation of Airway Epithelial Cells from Patients with Chronic Airway Inflammatory Disease and Effect of Antimicrobes on IL-8 Release from These Cells

To assess the effect of macrolide antibiotics on IL-8 production by inflamed airway epithelium, bronchial epithelial cells were obtained from 10 patients (3 with DPB, 5 with sinobronchial syndrome, 1 with nonatopic asthma associated with chronic sinusitis, and 1 with diffuse bronchiectasis; mean age of 54.8, all were non- or ex-smokers) under fiberoptic bronchoscopy as previously reported (18, 19). All the patients received no treatment except for oral ambroxol or carbocysteine for at least 1 mo. Briefly, under local anesthesia a fiberoptic bronchoscope (Olympus BF-20, Tokyo, Japan) was inserted transorally. A sheath-covered brush was introduced via the sampling channel, and the epithelial surface of bilateral main bronchi was brushed several times. In the other group of two cases with asthma and four cases with DPB, it was possible to obtain the very peripheral airway epithelial cells by the method reported previously (19). Briefly, under fluoroscopic guidance an ultrathin fiberscope (BF-2.7T, the outer diameter of 2.7 mm with a biopsy channel of 0.8 mm; Olympus) was inserted through a 2.8-mm diameter biopsy channel of BF-20. A BC-0.7T brush was then inserted to collect cells by brushing the airway mucosal surfaces. The epithelial cells were harvested by vortexing the brush in the media containing 10% FCS. The number of the cells was counted by a standard hemocytometer, and the cell viability was assessed by trypan blue dye exclusion technique. The cells were routinely stained by antikeratin antibody by a method described above, and only samples with more than 95% positive were utilized in the study. The number of harvested cells was $1.94 \pm 0.21 \times 10^6$, and the cell viability was 67 ± 11% for the cells from main bronchi (n = 10). The cell number was $0.97 \pm 0.33 \times 10^6$, and the viability was 60.2 ± 9.98% for the cells from very peripheral airways (n = 6). The cells were suspended at a density of 1×10^5/ml in Ham's F12 media and incubated with and without a variety of antibiotics for 24 h at 37° C, and supernatants were harvested and stored at −80° C until assay.

Cytokine Assay

Specific immunoreactivity for IL-8 in culture supernatants were measured by ELISA kits (R&D Systems, Inc., Minneapolis, MN) (10). Each sample was assayed in duplicates as recommended by the manufacturer. This assay was specific for human IL-8 and did not cross react with IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, RANTES, or GM-CSF. The sensitivity was 1.0 pg/ml and intra- and interassay variations were less than 5%. The samples obtained from airway epithelial cells before and after therapy were assayed simultaneously.

Reverse Transcription-polymerase Chain Reaction (RT-PCR) for IL-8 mRNA

To compare IL-8 mRNA levels before and after the macrolide treatment in human airway epithelial cells from five patients with chronic airway inflammatory disease (Table 1), a quantitative assay utilizing RT-PCR previously reported (20) was performed. Briefly, the cells were routinely stained by antikeratin antibody by a method described above, and only samples with more than 95% positive were utilized in the study. Total RNA was isolated by RNeasy (Qiagen, Inc., Chatsworth, CA), and the equivalent amount of RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (Clontech Laboratories, Inc., Palo Alto, CA). Then the PCR was done with the IL-8 primers with the sequences of (1)5′ primer: 5′ ATGACTTCCAAGCTGGCCGTGCT3′ (2)3′ primer: 5′ TCTCAGCCCTCTTCAAAAACTTCTC 3′, and with the β-actin primers: (1)5′ primer: 5′ ATGGATGATGATATCGCCGCG3′ (2) 3′ primer: 5′ CTAGAAGCATTTGCGGTGGGACGATGGAGGGGCC3′ (Clontech Laboratories). The predicted sizes of the amplified IL-8 and β-actin DNA products were 289 and 1,126 bp, respectively. The PCR cycle was determined by preliminary experiments showing linear relationship between PCR cycles and intensity of signals on ethidium bromide–stained agarose gels. For quantitative evaluation of IL-8 and β-actin, 35 and 30 cycles were chosen, respectively. The RNA isolated from samples before and after therapy were reverse-transcribed on the day of isolation and stored at −80° C. Then each pair of samples were measured and identical dose of DNA was then amplified simultaneously. Intensity of IL-8 mRNA levels were corrected by β-actin transcripts calculated by a densitometer.

Statistics

The results were analyzed by Student's *t* test for comparison between the two groups and by nonparametric equivalents of analysis of variance (ANOVA) for multiple comparison, as reported (10–12).

RESULTS

EM and CAM Suppressed IL-8 Production by Normal Cultured Bronchial Epithelial Cells

As previously reported (10), normal human bronchial epithelial cells spontaneously released immunoreactive IL-8, and this process appeared to require protein synthesis as assessed by the effect of cycloheximide (10 μg/ml) (data not shown). Proinflammatory cytokines such as IL-1α, IL-1β, and TNFα stimulated IL-8 release in a dose-dependent fashion. Northern blot analysis showed that the epithelial cells expressed constitutive IL-8 mRNA, which was significantly unregulated by the cytokines listed above (data not shown). A human bronchial epithelial cell line BEAS-2B also expressed and released IL-8 as reported (9, 10) (data not shown).

TABLE 1

CLINICAL CHARACTERISTICS OF PATIENTS WITH CHRONIC AIRWAY INFLAMMATORY DISEASE TREATED WITH MACROLIDE THERAPY

	Age/Sex	Disease	Smoking	Therapy	Other Therapy	Clinical Effectiveness
1	40/M	SBS	(−)	EM 600 mg/d, 3mo	Theophylline, ambroxol	Yes
2	56/M	Mild asthma	Ex-smoker	EM 600 mg/d, 4 mo	Theophylline, ambroxol	Yes
3	49/F	DPB	(−)	CAM 400 mg/d, 6 mo	Ambroxol	Yes
4	65/M	Bronchiectasis	(−)	CAM 400 mg/d, 5 mo	Carbocysteine	Yes
5	64/F	SBS	Ex-smoker	EM 600 mg/d, 12 mo	Theophylline, carbocysteine	No change

Definition of abbreviations: SBS = sinobronchial syndrome; asthma = nonatopic bronchial asthma associated with chronic sinusitis; DPB = diffuse panbronchiolitis.

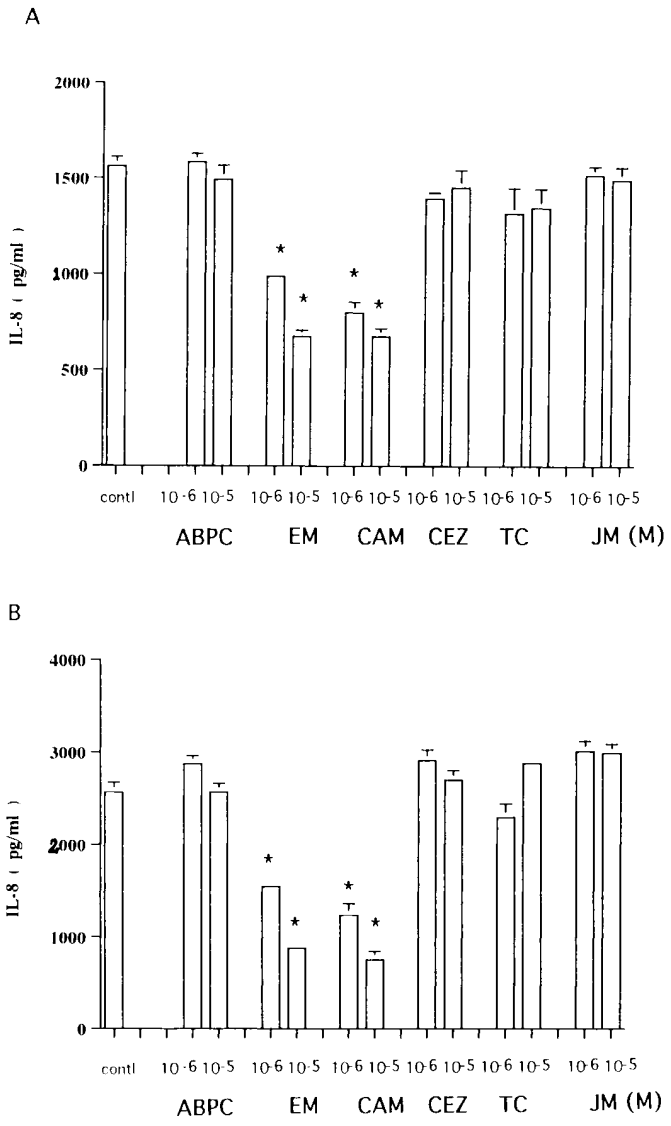

Figure 1. Effect of a variety of antimicrobial agents on IL-8 production by transformed human bronchial epithelial cells BEAS-2B. EM and CAM, but not the other antibiotics, significantly inhibited IL-8 release from human bronchial epithelial cells in culture. (*A*) Effect of antibiotics (10^{-6} and 10^{-5} M) on baseline release of IL-8 after 24 h. (*B*) Effect of antibiotics on IL-8 release from IL-1α (10 ng/ml)-stimulated bronchial epithelial cells in culture. The antibiotics were added simultaneously with IL-1α, and the supernatants were harvested after 24 h. EM and CAM uniquely suppressed IL-8 release. The data were shown as mean + SEM from three representative experiments. *p Value < 0.01 compared with controls (ANOVA).

Among the antimicrobes tested, only 14-member macrolides EM and CAM showed an inhibitory action on IL-8 release by unstimulated and IL-1α-stimulated human bronchial epithelial cells BEAS-2B (Figure 1). These two drugs also showed a significant inhibitory effect on IL-8 release from IL-1β and TNFα-stimulated cells (data not shown). LDH release assay and the trypan blue dye exclusion test, as well as a colormetric MMT assay (15), showed that this effect was not due to cytotoxicity (data not shown). EM and CAM showed a dose-dependent inhibitory effect on IL-8 release by primary and secondary passage bronchial epithelial cells as well as BEAS-2B cells (Figure 2). The percentage of inhibition of IL-8 protein release in human primary bronchial epithelial cells was 25.0 ± 5.67% and 37.5 ± 8.99%, respectively, at 10^{-6} M. Northern blot analysis showed that both drugs, but not ABPC, CEZ, TC, or a 16-member macrolide JM, decreased the steady state levels of IL-8 mRNA in IL-1α (10 ng/ml)-stimulated BEAS-2B cells (Figure 3). EM showed a dose-dependent inhibition on IL-8 mRNA levels as corrected by β-actin signals (% inhibition: 15.2 ± 3.11% at 10^{-7} M, 23.0 ± 7.23%* at 10^{-6} M, and 38.8 ± 10.2%* at 10^{-5} M, *$p < 0.01$ compared with control, ANOVA). EM and CAM also suppressed IL-8 mRNA levels in primary and secondary passage cells (data not shown).

EM and CAM Inhibited IL-8 Release by Airway Epithelial Cells Obtained from Patients with Airway Inflammatory Disease

Spontaneous IL-8 release by airway epithelial cells from inflamed airways was significantly suppressed with the addition of EM and CAM, but not with ABPC (Figure 4). In the epithelial cells obtained from very peripheral airways, the IL-8 release was also reduced by EM after 24 h (% inhibition: 24.3 ± 4.21% in 6 samples at 10^{-6} M, $p < 0.002$, Student's *t* test).

Changes in IL-8 mRNA Levels and IL-8 Protein Release by Airway Epithelial Cells before and after Macrolide Therapy

In five patients with chronic airway disease, it was possible to obtain airway epithelial cells before and after macrolide therapy (Table 1). All the patients received oral EM or CAM therapy for more than 3 mo with no side effects. The other prescribed drugs listed in Table 1 were unchanged during these periods. Clinical signs and symptoms such as dyspnea on exertion, daily amount of sputa, chest radiographic findings, and arterial blood gas analysis were improved in four patients as shown in Table 1. In accordance with these clinical changes, IL-8 mRNA levels corrected by β-actin transcripts were decreased in the four patients (patients 1 through 4) by RT-PCR (Figure 5). We repeated the semiquantitative PCR with the

same cDNA samples and could show that the difference of the signals between pre- and posterythromycin periods was consistently demonstrated (data not shown). Spontaneous IL-8 release from epithelial cells was also decreased by macrolide therapy in the four patients (before therapy: 442 ± 34.5 pg/24 h/10^5 cells; after therapy: 209 ± 18.0 pg/24 h/10^5 cells, n = 4, $p < 0.05$, Student's *t* test), but not in patient 5.

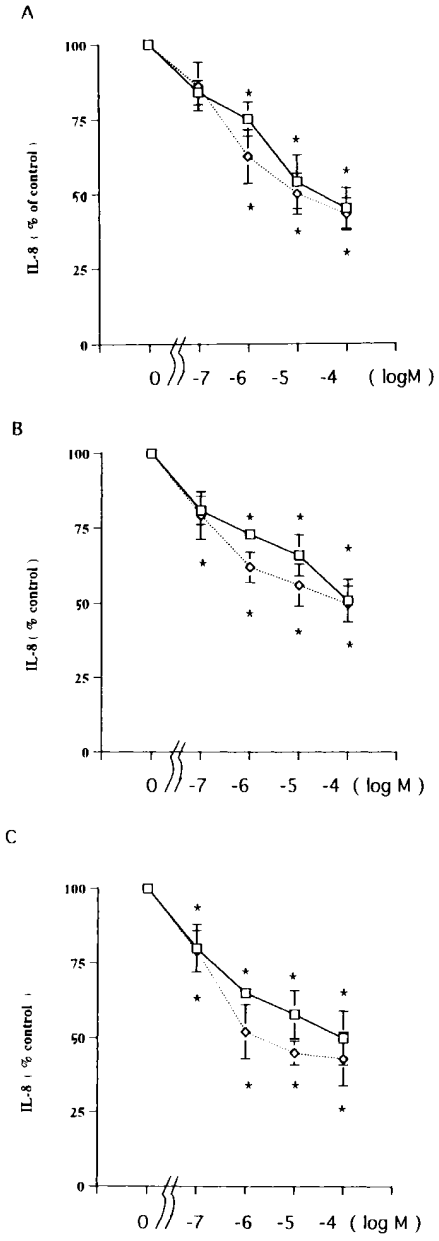

Figure 2. Dose-dependent inhibitory effect of EM (*open squares*) and CAM (*open diamonds*) on IL-8 release by (*A*) primary, (*B*) second-passage bronchial epithelial cells, and (*C*) BEAS-2B transformed human bronchial epithelial cells. Each kind of cell was treated simultaneously with IL-1α (10 ng/ml) and the antibiotics. The supernatants were harvested after 24 h for IL-8 measurement. Both antibiotics showed a dose-dependent inhibitory effect on IL-8 release from the three kinds of bronchial epithelial cells (n = 3 in each figure). *p Value < 0.01 compared with controls (ANOVA). The data were shown as mean ± SEM from three representative experiments.

DISCUSSION

IL-8 is one of the potent chemokines that are important in airway inflammation (2, 4, 5, 7). In fact, increased levels of this cytokine in airway lining fluids have been reported in several airway inflammatory diseases (2, 4, 5), which were decreased with successful therapy (2, 4). Appropriate modulation of overproduced cytokines, such as IL-8, may therefore be one mechanism that leads to the attenuation of airway inflammation. In DPB, there is a local accumulation of neutrophils, which are important effector cells in chronic airway inflammation. Kadota and associates (2) demonstrated an increase of neutrophil chemotactic activity (NCA) in bronchoalveolar lavage fluids, which showed a clear correlation with neutrophil numbers. EM treatment caused decline in both neutrophil numbers and in NCA. Therefore, it is probable that EM attenuates airway inflammatory responses by decreasing the local chemokine levels and thus decreasing the recruitment of inflammatory cells such as neutrophils. Airway epithelial cells

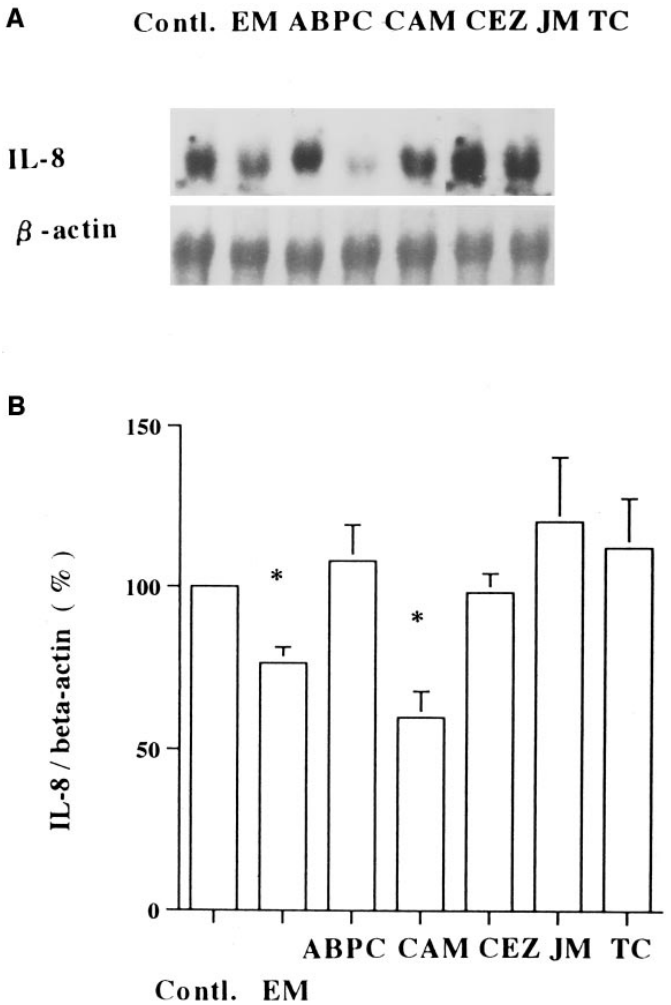

Figure 3. Northern blot analysis showing effect of EM, CAM, and the other antibiotics on IL-8 mRNA expression by IL-1α (10 ng/ml)-stimulated BEAS-2B cells in culture. (*A*) EM and CAM, but not ABPC, CEZ, TC, or JM, showed an inhibition at 10^{-6} M. (*B*) Measurement of densitometric signals of IL-8 corrected by actin transcripts showed a significant inhibition by EM and CAM (10^{-6} M). *p Value < 0.01 compared with controls (ANOVA). The data were shown as mean + SEM from three representative experiments.

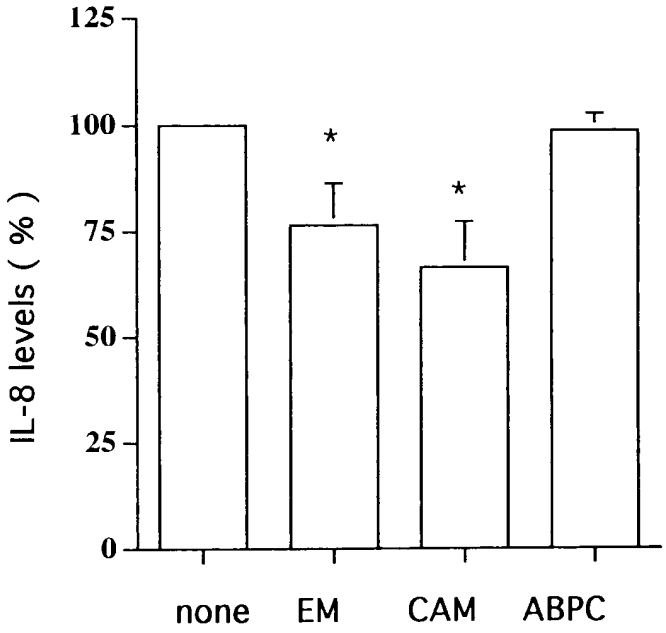

Figure 4. Effect of EM and CAM on IL-8 protein release by epithelial cells obtained from patients with chronic airway inflammatory disease. Spontaneous release of IL-8 was evaluated after 24 h with or without EM, CAM, or ABPC (10^{-6} M). EM and CAM, but not ABPC, showed a significant inhibitory effect on IL-8 release. *p Value < 0.01 compared with controls (ANOVA, n = 10).

are one of the potent sources of this chemokine (9, 10), and their anatomical location suggests their proviral role in the regulation of cell recruitment into the airway (21).

EM has been shown to modulate neutrophil migration (22), lymphocyte proliferation (23), and monocyte differentiation (24). EM has been shown to inhibit tumor necrosis factor α release from human monocytes (25). Konno and colleagues (26) showed that another 14-ring member macrolide antibiotic, roxithromycin, suppressed lymphokine expression by lymphocytes. We previously reported that EM and CAM uniquely inhibited IL-6 expression by normal bronchial epithelial cells (27). Recently, Khair and coworkers (28) reported that EM inhibited release of IL-8 as well as IL-6 release from *Haemophilus influenzae* endotoxin-stimulated normal bronchial epithelial cells. In the present study, EM at the range of therapeutic concentration (10^{-6} M) reduced IL-8 expression at mRNA levels as well as at protein levels in human normal and transformed bronchial epithelial cells. This action appeared to be unique, because other antibiotics, including a 16-member macrolide, JM, did not show any effect. We further obtained airway epithelial cells from patients with chronic airway disease and showed the inhibitory effect of EM and CAM on IL-8 release from the inflamed epithelium *in vitro*. In six patients, it was possible to obtain epithelial cells from so-called small airways, which are the initial lesions of DPB, and we found a clear decline of IL-8 release by EM *in vitro*. Finally, we harvested airway epithelial cells before and after long-term macrolide therapy from patients with chronic airway inflammation. In four patients whose clinical findings improved with the therapy, the magnitude of IL-8 mRNA expression corrected by β-actin transcripts, as assessed by RT-PCR as well as IL-8 protein release, decreased. Among antimicrobial agents available in clinical practice, only 14-member macrolides, such as EM, CAM, and roxithromycin, have been reported to be clinically effective for the treatment of chronic airway inflammatory diseases (1, 2). In the present study, EM and CAM uniquely suppressed IL-8 expression in human bronchial epithelial cells. Therefore, it is probably one mechanism of the clinical beneficial effect of these macrolide antibiotics.

EM also has a motilin-like stimulating activity on gastrointestinal smooth muscles (29). Therefore, inhibitory effect on cytokine expression in human cells reported here may be a

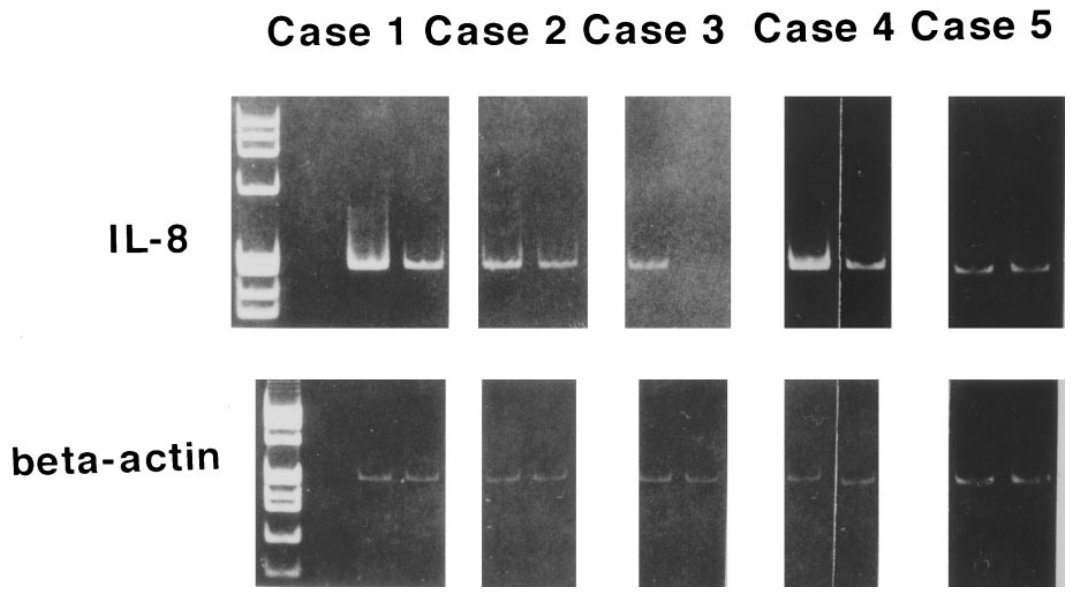

Figure 5. Evaluation of IL-8 mRNA levels in human bronchial epithelial cells before (*left*) and after (*right*) macrolide therapy by RT-PCR in five patients (Table 1). In comparison with the magnitude of β-actin, macrolide therapy resulted in decline of IL-8 gene levels in four cases (patients 1 through 4) but in no change in patient 5.

third bioactivity of this macrolide antibiotic. Characterization of the chemical structure responsible for its potential would be important to pursue, and further investigation for the molecular mechanism would be necessary for a possible new type of antiinflammatory agent.

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