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Inhibitory Effect of Erythromycin on Interleukin 8 Production by 1α,25-Dihydroxyvitamin D3-Stimulated THP-1 Cells

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We have recently reported that long-term administration of erythromycin at a low dose reduced the number of neutrophils and concentrations of interleukin 8 (IL-8) in bronchoalveolar lavage fluid in patients with chronic lower respiratory tract disease. To investigate the mechanism of action of erythromycin, we evaluated its effect on IL-8 production in the 1α,25-dihydroxyvitamin D3-stimulated human monocytic cell line THP-1. Erythromycin at a concentration of 10 μg/ml significantly reduced IL-8 production by THP-1 cells stimulated with lipopolysaccharide (10 ng/ml) and 1% normal human serum compared with the amount produced by untreated cells (untreated cells, 2,448 pg/ml; erythromycin-treated cells, 872 pg/ml). Our results suggest that erythromycin may impair IL-8 production by alveolar macrophages, ultimately reducing neutrophil accumulation in the airspace.

Several Japanese studies have recently demonstrated the therapeutic benefits of low-dose and long-term administration of erythromycin and other macrolides for the treatment of diffuse panbronchiolitis (DPB) (4, 9, 11, 18). The accumulation of neutrophils in the airspace may contribute to lung damage through the release of proteases, free oxygen radicals, and other degradative enzymes (12, 14). The participation of interleukin 8 (IL-8) in this process has been demonstrated in patients with neutrophil-mediated airway inflammation that occurs as part of a variety of chronic airway diseases, including DPB (9, 20) and cystic fibrosis (15, 22). We have also described a significant correlation between the accumulation of neutrophils and IL-8 levels in bronchoalveolar lavage fluid (BALF) obtained from patients with DPB (23). In addition, our results demonstrated a reduction in the levels of these two elements in BALF associated with clinical improvement following long-term treatment with low doses of erythromycin or roxithromycin (23). Since IL-8 is mainly produced by monocytes and macrophages (3), it is possible that erythromycin impairs IL-8 production by alveolar macrophages and ultimately reduces the level of neutrophil accumulation in the airspace, leading to clinical improvement. Several other investigators also suggested that erythromycin may act as an anti-inflammatory agent rather than as an bactericidal antibiotic (2, 7, 9, 16–20, 23).

Recent work has shown that the release of IL-8, tumor necrosis factor alpha, and interleukin 1β from monocytic cells is facilitated by lipopolysaccharide (LPS), through LPS-binding protein (LBP) complexes that interact with CD14 (25). The CD14-LBP-dependent pathway is operative under physiological conditions and controls cell activation when nanomolar concentrations of LPS, observed in BALF from DPB patients (19), were used (24, 26). In the present study, we investigated the ability of erythromycin to inhibit IL-8 production by monocytic cells through their CD14 molecules using the THP-1 cell.

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1α,25-Dihydroxyvitamin D3 (1,25-D3) was purchased from BIOMOL (Plymouth Meeting, Pa.) and was stored at −20°C in ethanol. LPS (*Pseudomonas aeruginosa* serotype 10; Sigma Chemical Co., St. Louis, Mo.) was reconstituted in sterile water and was stored at −80°C. Erythromycin (Dainippon Pharmaceutical Co., Osaka, Japan), roxithromycin (Nihon Roussel Co., Tokyo, Japan), josamycin (Yamanouchi Pharmaceutical Co., Tokyo, Japan), midecamycin acetate (Meiji Seika Kaisha, Tokyo, Japan), piperacillin sodium (Toyama Chemical Co., Tokyo, Japan), and clindamycin (Nihon Upjohn, Tokyo, Japan) were prepared at concentrations of 10 mg/ml in methanol. Clarithromycin (Taishou Pharmaceutical Co., Tokyo, Japan) was prepared at a concentration of 3.0 mg/ml in methanol. Ciprofloxacin hydrochloride (Bayer Pharmaceutical Co., Osaka) was dissolved at a concentration of 1.0 mg/ml in NaOH and was subsequently diluted in RPMI 1640 (GIBCO/BRL, Life Technologies, Inc., Gaithersburg, Md.) for testing. Mouse anti-CD14 monoclonal antibody (MAb) MY4 (immunoglobulin G2b [IgG2b] conjugated with fluorescein isothiocyanate [FITC]) was purchased from Coulter Immunology (Hialeah, Fla.).

Normal human serum, collected in endotoxin-free tubes from a healthy donor, was prepared from platelet-poor plasma by the addition of a 2% fluid volume of 1.0 M $CaCl_2$; this was followed by incubation for 3 h at 56°C to allow for clot formation. The serum was heated at 56°C for 30 min to inactivate the complement system and was then stored at −80°C until use.

THP-1 cells were cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum (GIBCO/BRL) in a 5% CO_2 humidified atmosphere at 37°C. For the induction of surface CD14, THP-1 cells (2×10^5 cells per well) were cultured in 24-well plates (Falcon Plastics, Oxnard, Calif.) in the presence of 100 nM 1,25-D3 for 72 h as described previously (25). After pretreatment with 1,25-D3, LPS at final concentrations of between 0.1 and 10 ng/ml and various antibiotics at final concentrations ranging from 0.1 to 10 μg/ml were added in the absence or presence of 1% human serum. After an additional incubation for 72 h, the culture supernatant was collected and was stored at −80°C until measurement of IL-8 levels. After each incubation, the cell viability was confirmed to be >95% by the trypan blue dye exclusion method.

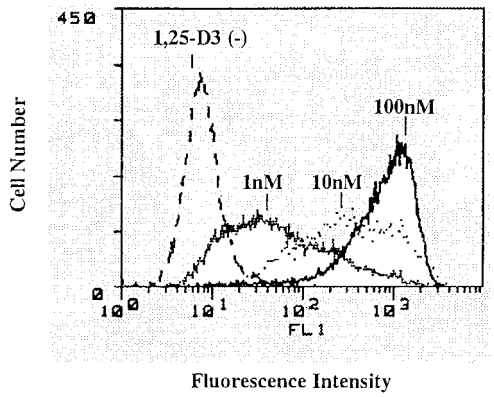

FIG. 1. Flow cytometric analysis of THP-1 cells cultured in the presence of 1,25-D3. 1,25-D3 was added at 0, 1, 10, and 100 nM to THP-1 cells, and after 72 h of incubation, CD14 expression was measured with a FACScan flow cytometer by using anti-CD14 MAb MY4.

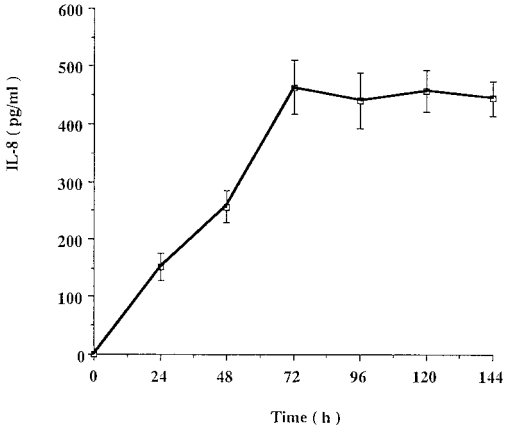

FIG. 2. Time course of IL-8 production by THP-1 cells. THP-1 cells were treated for 24, 48, 72, 96, 120, and 144 h with 100 nM 1,25-D3. The concentration of IL-8 in the culture supernatant was measured by ELISA. Datum points represent the means ± SEMs of three independent experiments.

Fluorescence-activated cell sorter analysis was performed by collecting 1,25-D3-induced THP-1 cells. The cells were washed twice with phosphate-buffered saline (PBS), and their numbers were adjusted to 5×10^6 cells per ml. A total of 10 μl of anti-CD14 MAb MY4-FITC was placed into a polystyrene tube (Falcon Plastics), and 100 μl of the cell suspension was added. The cells were incubated for 30 min in darkness, washed twice in cold PBS, and then resuspended in cold PBS containing 0.5% paraformaldehyde. Stained cells were analyzed on a FACSscan flow cytometer (Becton Dickinson & Co., Mountain View, Calif.), and a Consort 30 computer system (Becton Dickinson) was used for data acquisition and analysis. Mouse IgG2b conjugated with FITC was purchased from Coulter Immunology and was used as a control antibody. Detection of IL-8 in cell culture supernatants was performed with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (CLB, Amsterdam, The Netherlands). Data were expressed as means ± standard errors of the means (SEMs). Statistical differences were determined by the Student *t* test. Data were considered statistically significant when the *P* value was less than 0.05.

To verify that 1,25-D3-stimulated THP-1 cells express CD14 and to define optimal conditions, 1,25-D3 was added to THP-1 cells at concentrations of 0, 1, 10, and 100 nM for 72 h; this was followed by measurement of the level of CD14 expression on the cells by flow cytometry with anti-CD14 MAb MY4. As shown in Fig. 1, the maximum level of expression of CD14 was observed at 100 nM 1,25-D3. This concentration was used in all subsequent experiments.

In the next step, we examined the effect of erythromycin on the expression of CD14 on THP-1 cells. THP-1 cells were preincubated with 10 μg of erythromycin per ml for 7 or 14 days. The cells were washed at the end of this period and were stimulated for 72 h with 100 nM 1,25-D3. Erythromycin treatment did not influence the expression of CD14 on THP-1 cells (mean ± SEM of fluorescence intensity; control cells, 1,212 ± 225; cells treated with erythromycin for 7 days, 1,277 ± 153; cells treated with erythromycin for 14 days, 1,200 ± 156). In addition, no change in the level of CD14 expression was observed following a 3-day treatment with erythromycin by using a concentration of 100 nM, even after the induction of expression by 1,25-D3 (fluorescence intensity, 1,208 ± 125).

As shown in Fig. 2, 1,25-D3 stimulated THP-1 cells to produce IL-8. The concentration of IL-8 in the culture supernatant increased gradually between 24 and 72 h but reached a plateau thereafter. In these experiments, the concentration of endotoxin (Sumitomo Kinzoku Bioscience Co., Tokyo, Japan) in the medium was less than 10 pg/ml. This concentration does not enhance the production of IL-8 by THP-1 cells, as described below.

Gallay and colleagues (5) have recently reported that the mean concentration of LBP is 18.1 ± 4.0 μg/ml in normal human serum. It is also reported that the presence of 100 ng of LBP per ml dramatically enhances IL-8 or tumor necrosis factor alpha responses to LPS (24, 25). Accordingly, we used a serum concentration of 1% in the present study. As shown in Fig. 3, IL-8 production by 1,25-D3-induced THP-1 cells was significantly enhanced by 10 ng of LPS per ml for 72 h in the presence of 1% serum but not in the absence of serum. IL-8 release by both LPS and serum was inhibited by the addition of anti-CD14 MAb (suppressed from 2,154 to 1156 pg/ml). Furthermore, when THP-1 cells were not pretreated with 1,25-D3, both LPS (0.1 to 1,000 ng/ml) and 1% serum failed to release IL-8. These results indicated that the expression of CD14 and

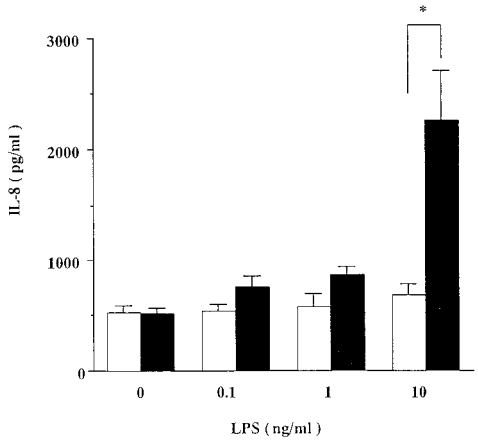

FIG. 3. Comparison of IL-8 production by 1,25-D3-induced THP-1 cells stimulated with 0, 0.1, 1, and 10 ng of LPS per ml in the absence or presence of 1% normal human serum for 72 h. The IL-8 concentration in the culture supernatant was measured by ELISA. Values are the means ± SEMs of three independent experiments. Closed bars, 1% normal human serum; open bars, no serum. *, $P < 0.01$.

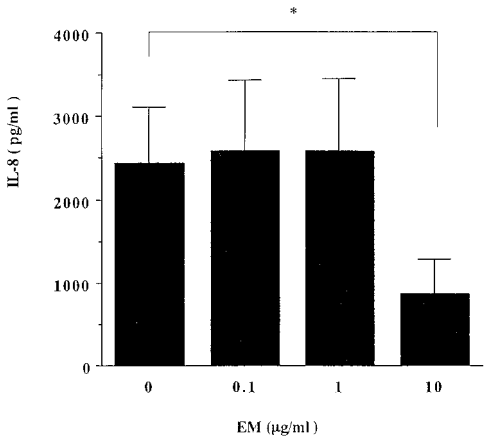

FIG. 4. Effect of erythromycin (EM) on IL-8 production by 1,25-D3-induced THP-1 cells. THP-1 cells were stimulated with 10 ng of LPS per ml and 1% normal human serum and were simultaneously incubated with erythromycin at concentrations of 0.1, 1, and 10 μg/ml. The IL-8 concentration in the culture supernatant was measured by ELISA. The values are the means ± SEMs of six independent experiments. *, $P < 0.01$.

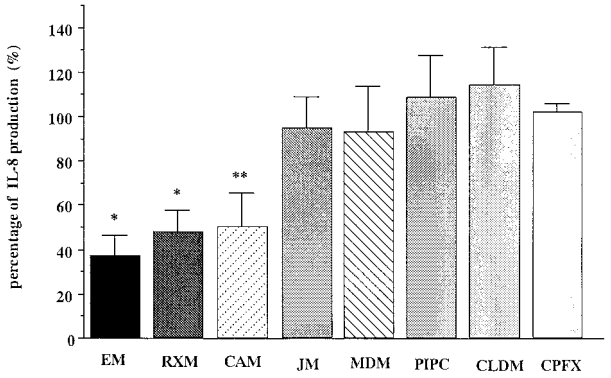

FIG. 5. Effects of various antibiotics on IL-8 production by 1,25-D3-stimulated THP-1 cells. THP-1 cells were stimulated with 10 ng of LPS per ml and 1% normal human serum and were simultaneously incubated with 10 μg of the test antibiotic per ml. The IL-8 concentration in the culture supernatant was measured by ELISA, and the results were expressed as the percentage of IL-8 production relative to that by the control cells. Values represent the means ± SEMs of six independent experiments. *, $P < 0.01$; **, $P < 0.05$ compared with the control. EM, erythromycin; RXM, roxithromycin; CAM, clarithromycin; JM, josamycin; MDM, midecamycin acetate; PIPC, piperacillin sodium; CLDM, clindamycin; CPFX, ciprofloxacin hydrochloride.

a functional LBP-dependent pathway play key roles in enhancing the induction of IL-8 production in response to LPS.

On the basis of the results depicted in Fig. 3, LPS and serum were used at concentrations of 10 ng/ml and 1%, respectively, in this set of experiments. When 1,25-D3-stimulated THP-1 cells were cultured with 10 ng of LPS per ml and 1% serum, the addition of 10 μg of erythromycin per ml for 72 h significantly inhibited IL-8 production by these cells compared with that by nontreated cells (control cells, 2,448 ± 667 pg/ml; erythromycin-treated cells, 872 ± 411 pg/ml; $P < 0.01$) (Fig. 4). Erythromycin at concentrations of 0.1 to 10 μg/ml did not influence THP-1 cell viability throughout the incubation period. After a shorter exposure period (24 h), there was a trend toward inhibition, but the difference was not statistically significant (control cells, 1,345.9 ± 314.2 pg/ml; after erythromycin treatment for 24 h, 985.6 ± 261.7 pg/ml; $n = 3$; $P = 0.90$).

We also examined the effects of roxithromycin, clarithromycin, josamycin, midecamycin acetate, piperacillin sodium, clindamycin, and ciprofloxacin hydrochloride on IL-8 production by stimulated THP-1 cells. As shown in Fig. 5, roxithromycin and clarithromycin (10 μg/ml), macrolides with a 14-membered macrocyclic ring structure similar to that of erythromycin, were inhibitory to IL-8, with no influence on cell viability in a manner similar to that of erythromycin. However, josamycin and midecamycin acetate, macrolides with a 16-membered macrocyclic ring structure, and other nonmacrolide drugs (piperacillin sodium, clindamycin, and ciprofloxacin hydrochloride) did not influence IL-8 production. At lower concentrations (0.1 to 1.0 μg/ml), none of the drugs tested inhibited IL-8 production or altered cell viability.

The major finding in the present study is that 10 μg of erythromycin, roxithromycin, and clarithromycin per ml significantly suppressed IL-8 production by 1,25-D3-stimulated THP-1 cells. The concentrations of these drugs used in the present experiment were approximately 10 times higher than the effective therapeutic concentrations in plasma. However, since the concentrations of these antibiotics in phagocytes are actively maintained at levels 10 times higher than those in the extracellular fluid (1, 21), the concentrations used in the present experiments may be effectively similar to those in the alveolar macrophages of patients on long-term and low-dose erythromycin, roxithromycin, or clarithromycin therapy. Our results are similar to those of other studies demonstrating a modulating effect for erythromycin on the synthesis of the proinflammatory mediators. Khair et al. (10) recently reported that erythromycin significantly blocked *Haemophilus influenzae* endotoxin-induced release of IL-6, IL-8, and sICAM-1 by cultured human bronchial epithelial cells. Furthermore, Oishi et al. (20) also demonstrated that erythromycin has an inhibitory effect on IL-8 production in pseudomonas-stimulated human neutrophils in vitro. The results of the present study add further importance to these early findings since we demonstrated an inhibitory effect of erythromycin on IL-8 production by human monocytic cells. In this regard, the accumulation of foamy macrophages in the pulmonary interstitium (6, 8) is thought to play an important part in the pathological process of DPB, and erythromycin acts mainly on alveolar macrophages.

We also demonstrated in the present study that erythromycin did not alter the level of CD14 expression of 1,25-D3-stimulated THP-1 cells. Furthermore, erythromycin failed to antagonize the response to the anti-CD14 MAb in 1,25-D3-treated THP-1 cells. These findings suggest that the mechanism of IL-8 inhibition by erythromycin does not involve CD14 itself. Aoshiba et al. (2) have recently reported that erythromycin and other macrolide antibiotics shortened the period of neutrophil survival, at least in part through an increase in intracellular cyclic AMP levels. Mitsuyama et al. (16) have also recently reported that the inhibitory effects of erythromycin on fMLP-induced O_2^- production were probably mediated by activating cyclic AMP-dependent protein kinase. Although the molecular mechanisms of erythromycin were not investigated in the present study, it is possible that the signal transduction system involving activation of protein kinase may be involved in the erythromycin-induced suppression of IL-8 production.

Recent studies have focused on the important role of serum, and more precisely of LBP, in mediating the presentation of LPS to CD14 on monocytes and macrophages (5, 24–26). LBP is a 60-kDa glycoprotein with a binding site for lipid A, binding with high affinity to LPS (25), and the mean concentration of LBP in serum was 18.1 μg/ml in 60 healthy subjects and 40 to 60 μg/ml in the serum of patients with septic shock (5). LBP

was also detected at 204.4 ng/ml in the BALF of patients with adult respiratory distress syndrome, at 15.4 ng/ml in patients with interstitial lung disease, and at 4.6 ng/ml in healthy volunteers (13). In the present study, 1,25-D3 was used to induce a high level of CD14 expression in THP-1 cells, resulting in an enhanced response of these cells to LPS in the presence of serum and a reduced amount of LPS (10 ng/ml) necessary to induce IL-8 release. We have previously demonstrated that the concentration of LPS in the BALF of patients with DPB is 1,500 ± 140 pg/ml (19). Thus, the LPS concentration of 10 ng/ml used in the present study was thought to be a clinically achievable level.

In conclusion, we demonstrated that erythromycin inhibited IL-8 production by 1,25-D3-induced THP-1 cells when they were stimulated with LPS and serum through their CD14 molecules. Although our observations are limited to one human monocytic cell line in vitro, these results suggest that erythromycin may impair IL-8 production by alveolar macrophages, ultimately reducing neutrophil accumulation in the interstitium and improving chronic lower respiratory tract infections. Further experiments with human blood monocytes or alveolar macrophages are necessary to confirm the findings of the study.

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