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Erythromycin Inhibits Interleukin-6 and Interleukin-8 Expression and Promotes Apoptosis of Activated Human Neutrophils *in Vitro*

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Diffuse panbronchiolitis (DPB) is a pulmonary disease characterized by chronic inflammation of the bronchioles and chronic infiltration of inflammatory cells in the lungs. Macrolides are effective therapeutic agents for chronic respiratory tract diseases, such as DPB. However, the mechanisms by which macrolides modulate the immune responses in patients with DPB remain unclear. To understand clinical efficacy for the treatment of DPB by macrolides, the effects of erythromycin (EM) on the expression of pro-inflammatory cytokines such as interleukin-6 (IL-6) and interleukin-8 (IL-8) by human neutrophils were examined. Pre-treatment with EM significantly decreased the expression of IL-6 and IL-8 transcripts by lipopolysaccharide (LPS)-stimulated human neutrophils. EM also reversed the enhanced survival of human neutrophils by LPS. These data indicate that EM has achieved therapeutic effect for patients with DPB, in part, through decreasing the expression of pro-inflammatory cytokines and the survival of neutrophils.

Key Words: Erythromycin, Macrolide, Anti-inflammation, Neutrophils, Lipopolysaccharides, Diffuse panbronchiolitis

INTRODUCTION

Macrolide antibiotics are widely used for the treatment of acute airway infections. Recent reports showed that they are also effective anti-inflammatory agents (Everard et al, 1997; Tamaoki et al, 2004). However, the precise mechanisms underlying the anti-inflammatory action remain unclear. Studies by Khair et al. (1995) have suggested that EM may exert an anti-inflammatory effect by blocking *H. influenzae* endotoxin-induced release of IL-6, IL-8, and sICAM-1 in cultured human bronchial epithelial cells. In contrast, Criqui et al. (2000) showed that sputum induction by 0.2 ppm ozone gave no significant differences in per cent neutrophils, IL-6 and IL-8 between azithromycin and placebo conditions in the post- minus pre-exposure value. Azthromycin have been shown to reduce airway neutrophilia in patients with bronchiolitis obliterans syndrome (Verleden et al, 2006). However, pretreatment with azithromycin significantly changed neither total cell number nor neutrophil percentage in bronchoalveolar lavage fluid in comparison to the vehicle-treated and LPS-challenged group in mice (Ivetić et al, 2006).

In this study, we have hypothesized that macrolide antibiotics may exert its anti-inflammatory effect by modulating the expression of proinflammatory cytokines, from human neutrophils. To test this hypothesis, we have investigated the effect of EM on LPS-stimulated expression of IL-6 and IL-8 mRNA in human neutrophils by quantitative reverse transcription and polymerase chain reaction (RT-PCR) technique. Additionally, we have studied the effect of this agent on LPS-induced enhancement of neutrophil survivals *in vitro*.

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METHODS

Reagents

Erythromycin, LPS from *Escherichia coli* and Histopaque-1077 were purchased from Sigma (St. Louis, MO, USA). Culture medium RPMI 1640 was purchased from Gibco (Grand Island, NY, USA). TRIzol agent was purchased from Invitrogen. Dextran 500 was purchased from Amersham Biosciences (Arlington Heights, USA). Cell counting kit-8 (CCK-8) obtained from Dojindo Laboratories (Kumamoto, Japan). All chemicals were of analytical grades.

Isolation of neutrophils

Human neutrophils were prepared as previously described (Ernens et al, 2006). 50 ml of Heparinized blood was obtained by venipuncture from healthy volunteers, mixed 1:1 with layered on Ficoll gradient. After centrifugation at 2,500 rpm for 30 min, layers containing red blood cells and neutrophils were collected and further purified by means of 3 % dextran gradient. The residual red blood cells were lysed by hypotonic shock, and the cells were suspended in

ABBREVIATIONS: BAL, bronchoalveolar lavage; DPB, diffuse panbronchiolitis; EM, erythromycin; IL, Interleukin; LPS, lipopolysaccharides.

1×PBS (pH 7.4). The final preparation contained more than 95 % neutrophils and cell viability was more than 95 % as determined by trypan blue exclusion technique. Purified neutrophils were resuspended in RPMI 1640 supplemented with 5% fetal bovine serum at a final concentration of 1×10^6 cells/ml and incubated at 37°C in a 5 % CO_2 humidified atmosphere.

Assay for mRNA expression

Messenger RNA expression was examined by means of real-time reverse transcriptase-polymerase chain reaction. EM (10 μg/ml) was added to 1×10^6 neutrophils and was incubated for 1 h; then, LPS (1 μg/ml) was added and incubated for various times. Total RNA was extracted from neutrophils with TRIzol reagent according to the manufacturer's instructions. The first-strand cDNA synthesis from 1 μg of mRNA was performed with a 25 μl reaction mixture containing 0.5 μg of oligo dT 15 primer and 30 units of AMV-reverse transcriptase was incubated at 70°C for 5 min, 25°C for 15 min and then 37°C for 60 min. The expression of IL-6 and IL-8 genes was analyzed with a Rotor-gene 3000 PCR using the CYBR green method. The following specific primers were purchased from Qiagen (QIAGEN, Inc., Valencia, CA, USA); IL-6 (QT00083720), IL-8 (QT00000322) and GAPDH (QT01192646). A real-time RT-PCR assay was performed on a 20 μl reaction mixture containing 60 ng of sample cDNA 0.5 μM primer and 10 μl of QuantiTect SYBR Green PCR Master Mix (QIAGEN Inc). The PCR was carried out for 40 cycles of 15 min at 95°C, 15 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C. To normalize the amplified products in each sample, we used GAPDH as a quantitative internal control. The mRNA expression levels of each of the 2 targeted genes were presented as a ratio to that of GAPDH, and the relative expression levels were calculated.

Neutrophil viability assay

The cell viability was assessed CCK-8 to count living cells by adding WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] and 1-methoxy PMS [1-methoxy-phenazine methosulfate]. After the medium was removed, 10 μl of CCK-8 solution was added to each well of the plate. The plate was incubated for 1 h in the humidified incubator (at 37°C, 5 % CO_2). After incubation, the number of live neutrophils was determined colorimetrically at 450 nm using ELISA.

Determination of apoptosis and necrosis

Isolated human neutrophils (1×10^6 cells in 1 ml of medium) were incubated for 3 h with LPS in the presence or absence of EM for 1 h at 37°C in a humidified 5% CO_2, 95% air incubator. For the flow cytometric procedure, apoptosis was investigated using annexinV - fluorescein isothiocyanate (FITC) (BD Biosciences, San Jose, CA, USA) and propidium iodide (PI) labeling. The fluorescence of each cells were measured using a flow cytometry (Guava EasyCyte, Guava Technologies, Hayward, CA, USA). Results were expressed as percentage of apoptotic cells. For each measurement 3,000 events were collected.

Statistical analysis

In all cases, data were expressed as mean ± SEM. Statistical differences were analysed by Student's *t*-test. A probability value <0.05 was considered statistically significant.

RESULTS and DISCUSSION

The mechanism of the anti-inflammatory activity of macrolide antibiotics is unclear. One of these mechanisms may be the inhibition of the expression of proinflammatory cytokines in neutrophils. In this study, the ability to reduce the expression of proinflammatory cytokines of a macrolide antibiotic, EM, has been investigated in human neutrophils. Expression of IL-6 and IL-8 transcripts in human neutrophils was determined by quantitative real-time PCR.

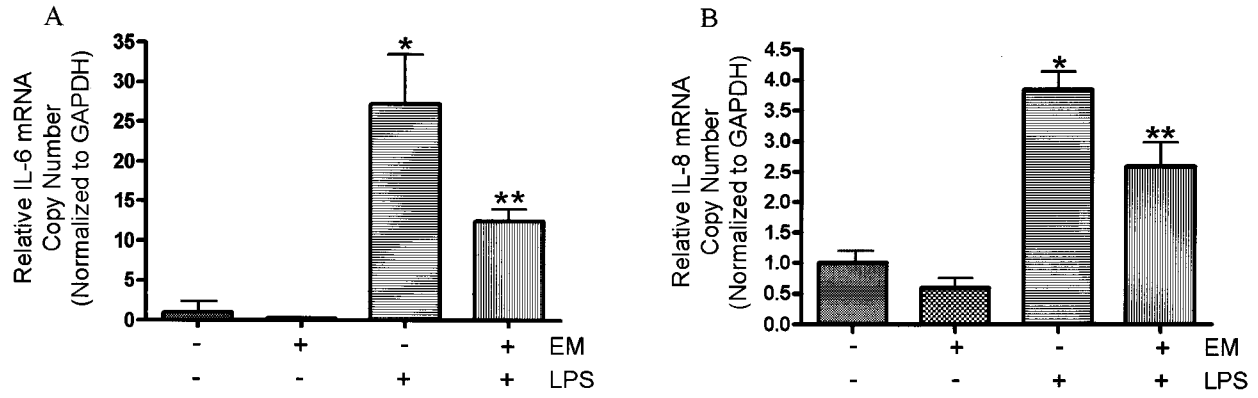

Fig. 1. Inhibitory effects of erythromycin on LPS-induced expression of IL-6 and IL-8 genes in human neutrophils. Human neutrophils were treated with EM (10 μg/ml) or vehicle, and then they were treated 1 h later with LPS (1 μg/ml) or vehicle. Two (IL-8) or 6 h (IL-6) after LPS treatment, RNA was isolated from neutrophils, and SYBR Green real-time PCR was performed for IL-6 (A), and IL-8 (B). The bars represent fold changes compared with baseline (left bar), which was arbitrarily set to 1. The asterisk(*) indicates statistical significance ($p < 0.05$) compared with control and the double asterisk(**) significance ($p < 0.05$) comparing LPS and LPS/EM treated neutrophils. The data in this panel indicate the mean ± SEM of three separate experiments.

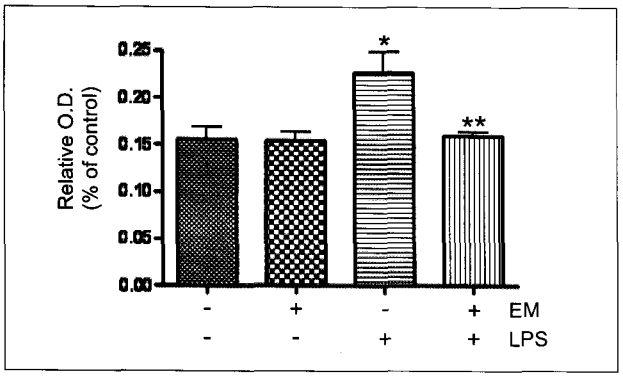

Fig. 2. Effect of EM on the viability of human neutrophils. Neutrophils were pre-treated with EM (10 μg/ml) for 2 h and then the neutrophils were cultured in the presence or absence of LPS (1 μg/ml) for 3 h. Relative cell viability was determined by WST-8 and 1-Methoxy PMS and is shown as relative O.D. Data are shown as means ± SEM of three independent experiments. The asterisk(*) indicates statistical significance ($p<0.05$) compared with control and the double asterisk(**) significance ($p<0.05$) comparing LPS and LPS/EM treated neutrophils.

We found that mRNA for IL-6 and IL-8 increased after stimulation of neutrophils with LPS (1 μg/ml), the expression peaking at 6 h for IL-6 (3.2-fold increase ($p<0.05$)) and 2 h for IL-8 following LPS (data not shown). We then examined the effect of EM, a macrolide antibiotic, under conditions in which expression of IL-6 and IL-8 is induced. EM significantly attenuated IL-6 and IL-8 transcript accumulation after 6 h and 2 h of stimulation with LPS, respectively, also treatment of neutrophils with EM had similar effects (Fig. 1). Our studies suggest that macrolides are likely to act indirectly by modulating the synthesis of the proinflammatory mediators, such as IL-6 and IL-8, which affect the activity of neutrophils, the key effector cell in the pathogenesis of diffuse panbronchiolitis. IL-6 and IL-8 are known to be involved in several types of airway inflammation, including airway inflammation in mice (Fujimaki et al., 2006) and human chronic inflammatory airway diseases, particularly DPB which is inhibited by macrolides (Tamaoki et al, 2004).

To evaluate the effect of EM on the viability of neutrophils, human neutrophils were incubated with EM for 2 h and then in the presence or absence of LPS for 3 h and viability was determined by CCK-8 assay. As shown in Fig. 2, incubation of neutrophils with LPS for 3 h significantly

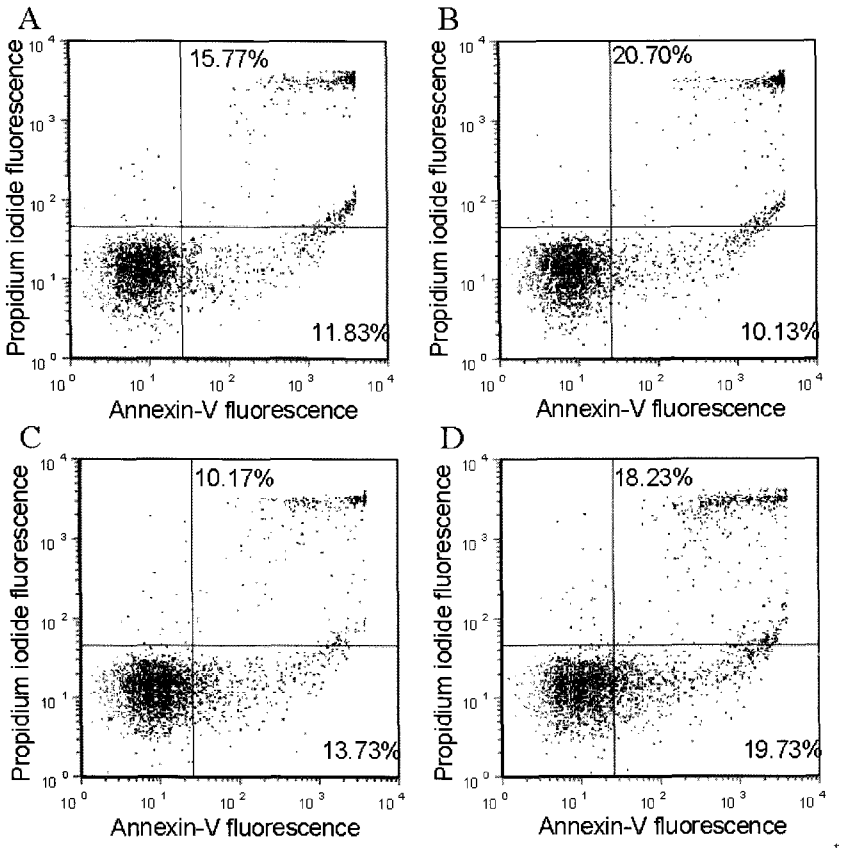

Fig. 3. Double staining of neutrophils with annexin and propidium iodide. Neutrophils were incubated with medium alone (A), with 10 μg/ml of EM (B), with 1 μg/ml of LPS (C) for 4 h. Some wells with neutrophils were pretreated with 10 μg/ml EM for 1 h and then 1 μg/ml LPS was added into the culture for further 3 h (D). Subsequently, cells were resuspended in binding buffer and stained with FITC-labeled annexin-V and PI in binding buffer, and their fluorescence was determined by flow cytometry. A fluorescence dot plot and the percentage of positive cells are shown. The results are representative of three separate experiments using neutrophils isolated from three different healthy donors.

delayed the spontaneous cell death of neutrophils. In contrast, pre-treatment of EM suppressed the viability of LPS-stimulated neutrophils. The cell surface expression of phosphatidylserine (PS) is considered to be one of the early characteristics of apoptosis (Andree et al, 1990). Annexin-V has a high affinity for PS and is used in conjunction with PI to distinguish apoptotic cells from necrotic cells. The basis of this method is that PI selectively penetrates the plasma membrane of necrotic cells, but not the apoptotic cells (Vitale et al, 1993). As shown in Fig. 3A, neutrophils incubated with medium alone for 4 h exhibited apoptotic changes with positive staining for annexin-V (right panel). Treatment of neutrophils with 1 μg/ml LPS for 4 h (Fig. 3C) decreased the extent of apoptotic change. On the other hand, pre-treatment of neutrophils with 10 μg/ml EM for 1 h and then with 1 μg/ml LPS for 3 h significantly promoted the apoptosis of cells with nuclear staining by PI (Fig. 3D). This result that EM could decrease LPS-induced delay in spontaneous apoptosis of neutrophils after pre-treatment of neutrophils with EM, is in agreement with previous reports showing that patients with DPB had increased number of neutrophils in their bronchoalveolar lavage (BAL) fluid than did healthy volunteers and that a significant reduction of the number of neutrophils in BAL fluid was observed after treatment with EM (Ichikawa et al, 1992; Kadota et al 1993; Aoshiba et al, 1995). These effects of macrolides on LPS-induced delay in spontaneous apoptosis of neutrophils may shorten the lifespan of neutrophils and fasten the elimination of neutrophils from the inflammation sites. Thus, this process is beneficial to the resolution of inflammation.

Taken together, the present study shows that macrolide antibiotics may exert their anti-inflammatory activities at least partly by preventing the LPS-stimulated expression of IL-6 and IL-8 transcripts and also increasing the apoptosis of neutrophils.

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