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The effects of erythromycin on human peripheral neutrophil apoptosis*

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SUMMARY

Erythromycin is reported to have an anti-inflammatory action, which may account for its clinical effectiveness in treating chronic inflammatory diseases of the respiratory tract such as diffuse panbronchiolitis (DPB) and chronic sinusitis.

To evaluate the anti-inflammatory action of erythromycin, we examined apoptosis of isolated neutrophils incubated with and without erythromycin. As a result, erythromycin augmented neutrophil apoptosis in a dose-dependent manner, with a maximal effect at 10 μg/ml and above. The percentage of neutrophil apoptosis at 12 h was 79.2±2.3% in medium with 10 μg/ml of erythromycin compared with 51.2±4.1% in control medium ($p<0.005$). In a manner similar to that of erythromycin, another macrolide antibiotic, roxithromycin, also increased neutrophil apoptosis. However, there was no effect on apoptosis induced by treatment with josamycin (macrolide antibiotic), ampicillin (β-lactam.) and cefazolin (cephalosporin antibiotic), or gentamycin (aminoglycoside). These findings suggest that erythromycin shortens neutrophil survival by accelerating neutrophil apoptosis.

Key words: apoptosis, erythromycin, neutrophils

INTRODUCTION

Erythromycin is a broad-spectrum antibiotic that has recently been commonly used to treat patients with diffuse panbronchiolitis (DPB) and chronic sinusitis in Japan (Nagai et al., 1991; Yamamoto et al., 1990). DPB is a clinicopathological disease entity characterized by chronic inflammation localized in the respiratory bronchioles (Homma et al., 1983). In the past, patients with DPB developed repeated pulmonary infections and often died of respiratory failure (Kudoh et al., 1984; Kudoh et al., 1987). Since the effectiveness of low-dose, long-term erythromycin treatment was established, the disease has become easily controllable and is now curable (Nagai et al., 1991; Yamamoto et al., 1990; Kudoh et al., 1984; Kudoh et al., 1987). Patients with DPB often have chronic sinusitis as well. Low-dose, long-term erythromycin treatment is also effective for chronic sinusitis with DPB (Suzuki et al., 1990). Recently this treatment has been applied to patients with chronic sinusitis without DPB and has been shown to be highly effective in improving clinical symptoms and radiographic findings of patients with chronic sinusitis (Hashiba et al., 1992).

While the mechanisms that underlie the efficacy of erythromycin for these diseases remain unknown, clinical findings suggest that its efficacy depends on an activity other than its bactericidal activity. Recent reports indicated that neutrophils may be involved in the deterioration of symptoms in patients with these diseases by producing persistent and excessive oxidants and proteolytic enzymes, which ultimately injure infected or inflamed tissue (Whyte et al., 1993; Savill et al., 1989). Recent reports also indicated that erythromycin may inhibit various neutrophil functions such as chemotaxis, superoxide production and phagocytosis (Ichikawa et al., 1990; Ichikawa et al., 1992; Shirai et al., 1995; Nelson et al., 1987; Kadota et al., 1993). These effects have been considered to contribute to its clinical effectiveness in treating DPB and chronic sinusitis.

The life span of neutrophils is short compared with that of other hemopoietic cells (Takeda et al., 1993). These cells eventually die through an apoptotic process and the dead cells are phagocytozed by macrophages (Savill et al., 1989). Apoptosis, which is one type of cell death, is considered to play an important role in regulating the number of neutrophils (Tuchida et al., 1995).

However, relatively little attention has been paid to the possible effects of erythromycin on neutrophil apoptosis. It is proposed that apoptosis decreases the ability of neutrophils to damage tissue by directly inhibiting the capacity of neutrophils to generate potentially injurious products or by making senescent neutrophils available for phagocytosis and degradation by macropha-

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ges (Takei et al., 1996). Thus, if erythromycin accelerates neutrophil apoptosis, this induction may account for the anti-inflammatory action and clinical effectiveness of erythromycin in the treatment of DPB and chronic sinusitis (Sendo et al., 1995). To clarify this point, the effect of erythromycin on neutrophil apoptosis was examined.

MATERIALS AND METHODS

Reagents

Erythromycin, roxithromycin, ampicillin, cefazolin, gentamycin, cycloheximide, RNase and proteinaseK were purchased from Sigma Chemical Co.(St. Louis, MO, USA). These reagents were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml and stored at -40°C until use. Josamycin was generously donated by Yamanouchi Phamacoceutical Co., Ltd.(Tokyo, Japan). Other reagents and culture media used in the present study were obtained as follows: Mono-poly resolving medium (Dainippon Phamaceutical Co., Ltd. Tokyo, Japan), RPMI 1640 (GIBCO, Grand Island, NY, USA), fetal calf serum (Whittaker, M.A. Bioproducts, MD, USA), as molecular standards, λ-Hind III (Bio-Lad Laboratories) and 1 kb DNA Ladder (Hercules, CA, USA).

Neutrophil Preparation

Heparinized venous blood obtained from healthy volunteers was centrifuged at 400 g for 30 min at room temperature on mono-poly resolving medium. The granulocyte pellet was washed in RPMI 1640 medium containing 10% FCS, and contaminating erythrocytes were eliminated by hypotonic shock using 0.2% NaCl. Preparations obtained in this manner contained more than 95% neutrophils.

Cell Culture

Freshly isolated neutrophils (2×10^6/ml) were seeded into 24 well flat-bottom plates (Falcon 3407, Becton Dickinson, Oxford, CA, USA) and incubated with or without the following antibiotics or agents at 37°C for various periods of time under 5% CO_2 and 95% air. Cell suspensions were then incubated at 37°C for various periods of time under 5% CO_2 and 95% air. These reagents were dissolved in DMSO, which was diluted to a level that did not affect neutrophil apoptosis during incubation. Therefore control media that did not contain DMSO were used.

1) Examination of Apoptosis

Light Microscopic Examination

Neutrophils were cultured under various conditions and smears were made of these cells, which were stained with May-Giemsa solution. The number of apoptotic cells in a total of at least 200 neutrophils /slide /patient was counted under a light microscope.

Agarose Gel Electrophoresis of DNA

Neutrophils treated under the various conditions described above were suspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at a concentration of 5×10^7 /ml. Cells were lysed with a lysis buffer consisting of 0.5% SDS, 1 mM EDTA, 10 mM Tris-Hcl, pH 8.0. The lysate was incubated with DNAase-free RNase (20 mg/ml) for 1 h at 37°C for preparation of genomic DNA. Then, proteinase K (100 mg/ml) was added. The lysates were placed in a water bath for 3 h at 50°C. The resulting lysates were extracted with phenol/chloroform. DNA was ethanol-precipitated and then collected by centrifugation at 13,000 g for 20 min at 4°C. The pellet was washed with 70% ethanol, dried superficially and redissolved in Tris/EDTA buffer. To detect DNA fragments, purified DNA was separated on a 1.5% agarose gel for 14 h at 30 V. The gel was then stained with ethidium bromide.

TUNEL assay

To stain apoptotic cells, DNA strand breaks were labeled by means of TUNEL. After washing and fixation, cells were processed with an in situ apoptosis detection kit (Apop Tag) according to the instructions of the manufacture (Appligene/Oncor, Illkirch, France). Apoptosis was morphologically confirmed by microscopy.

2) Effects of dose and time courses of neutrophil apoptosis accelerated by erythromycin

Apoptosis of neutrophils cultured in the presence or absence of erythromycin. Neutrophils were incubated with various concentration of erythromycin for 12 h. To examine the time courses of neutrophil apoptosis accelerated by erythromycin, neutrophils were incubated with or without erythromycin for various periods. After neutrophils were incubated in erythromycin for various exposure periods, cells were washed and incubated in RPMI medium containing 10% FCS. The total incubation period was 12 h.

3) Effects of other antibiotics on neutrophil apoptosis

To compare the effect of erythromycin on neutrophil apoptosis with those of other antibiotics, neutrophils were treated with various types of antibiotics for 12 h. Concentrations of antibiotic were erythromycin (14-membered ring macrolide): 1, 10 μg/ml, roxithromycin (14-membered ring macrolide): 1, 10 μg/ml, josamycin (16-membered ring macrolide): 1 μg/ml, ampicillin: 30 μg/ml, cefazolin: 40 μg/ml and gentamycin: 4 μg/ml. The concentrations (1 μg/ml) of erythromycin and roxithromycin were equivalent to the concentration of a low-dose treatment without antibacterial effect.

The concentrations of josamycin, ampicillin, cefazolin and gentamycin were equivalent to the concentration of a therapeutic dose with antibacterial effect.

Statistical Analysis

Values are expressed as mean±SE. Statistical analysis was performed with the Mann-Whitney test.

RESULTS

1) Effects of erythromycin on neutrophil apoptosis

As shown in Figure 1, when neutrophils were treated with erythromycin (Figure 1b) for 12 h, condensed and fragmented nuclei, relatively intact plasma membranes, and decreases in cell size were observed compared to those of control neutro-

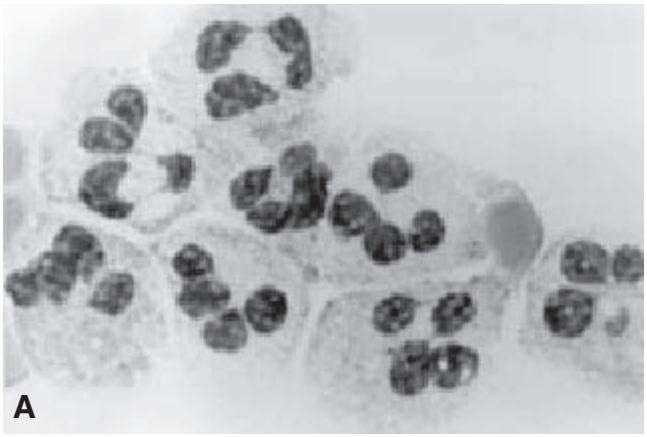

Figure 1a.

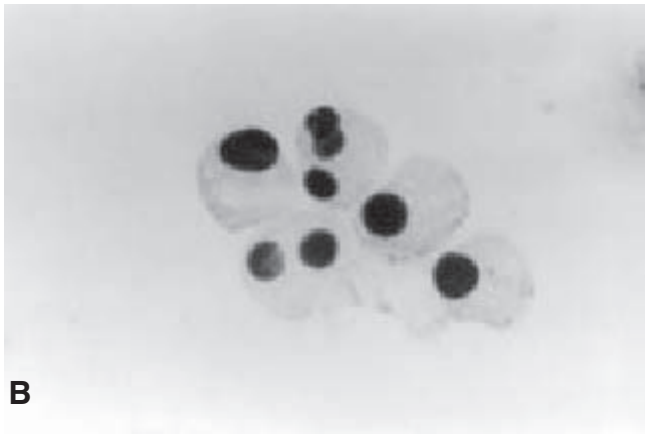

Figure 1b.

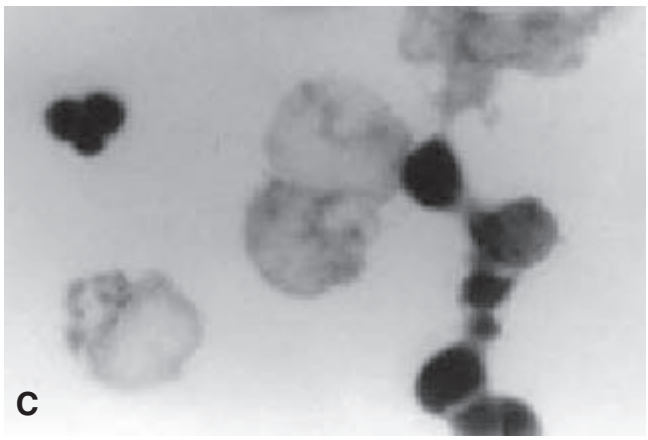

Figure 1c. Control neutrophils and apoptotic neutrophils.
Microscopic appearances of control neutrophils (a) and apoptotic neutrophils (b) induced by incubation with 10 μg/ml of erythromycin for 12 h. Note the nuclear fragmentation and condensation of apoptotic neutrophils (May-Giemsa stain, original magnification ×1000).
Neutrophils were also examined by TUNEL assay. DNA strand breaks of apoptotic neutrophils were stained with this assay (c).

phils (Figure 1a). Nucleus of apoptotic neutrophils were also stained with TUNEL assay (Figure 1c).

Figure 2 clearly shows that DNA extracted from erythromycin-treated neutrophils for 12 h exhibited typical ladder formation on agarose gel electrophoresis. Taken together, these results suggest that erythromycin clearly accelerates neutrophil apoptosis.

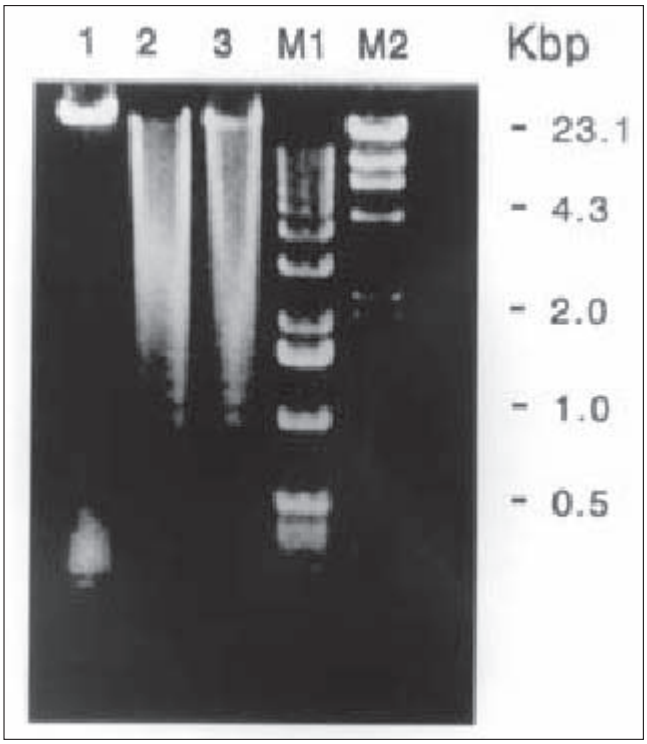

Figure 2. Argarose gel electrophoresis of neutrophil DNA.
Argarose gel electrophoresis of DNA isolated from neutrophils incubated for 12 h in either the presence or absence of 10 μg/ml of erythromycin. Each lane contains 5 μg of DNA.
(1: Control, 2: Erythromycin, 3: TNF + Cycloheximide, M1: 1kb DNA Ladder, M2: λHind III digest).

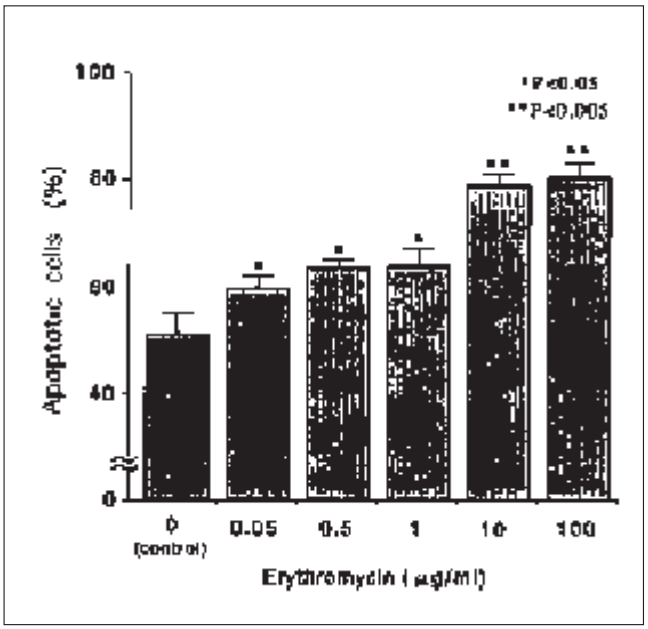

Figure 3. Apoptosis of neutrophils cultured with various concentrations of erythromycin.
Apoptosis of neutrophils cultured in the presence or absence of erythromycin. Neutrophils were incubated with various concentration (0.05, 0.5, 1, 10 and 100 μg/ml) of erythromycin for 12 h. After incubation at various concentrations, apoptotic neutrophils were counted on May-Giemsa-stained smears under light microscopy. Data are expressed as the mean±SE. n=3. *P<0.05, **P<0.005 compared with the control.

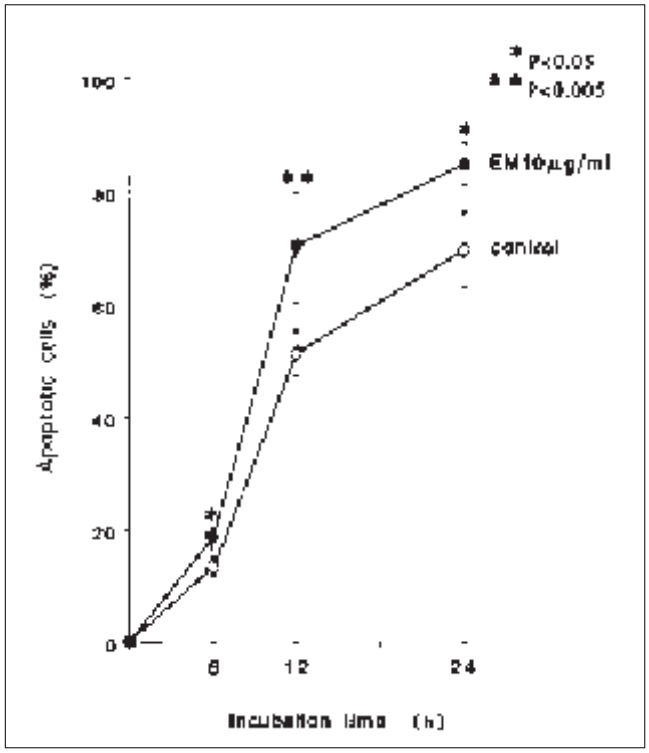

Figure 4. Time course of neutrophil apoptosis.
Neutrophils were incubated with or without erythromycin (10 μg/ml) for various periods (0~24 h). Data are expressed as the mean±SE. n=5. *P<0.05 compared with the control.

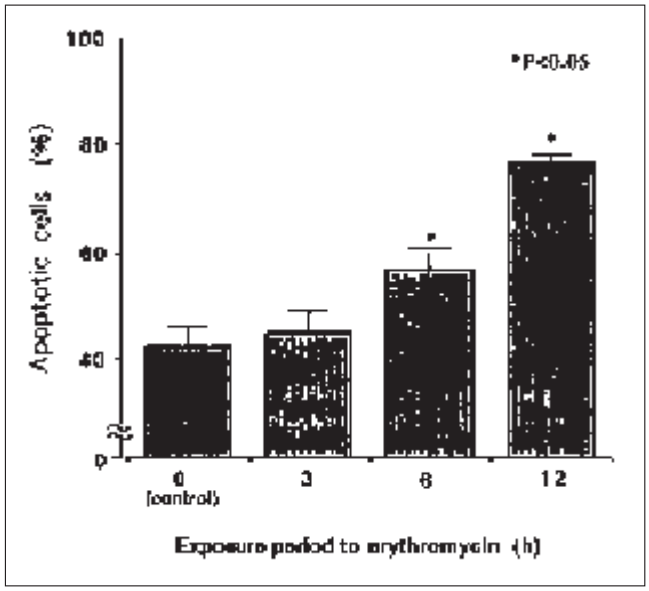

Figure 5. Various exposure times to erythromycin.
After neutrophils were incubated in erythromycin (10 μg/ml) for various exposure periods (3, 6 and 12 h), cells were washed and incubated in RPMI medium containing 10% FCS. After 12h incubation, apoptotic neutrophils were counted. Data are expressed as the mean±SE. n=3. *P<0.05 compared with the control.

2) Effects of dose and time courses of neutrophil apoptosis accelerated by erythromycin

When neutrophils were incubated with varying concentrations of erythromycin, neutrophil apoptosis was significantly enhanced by erythromycin in a dose-dependent manner (Figure 3). The maximal effect was obtained at 10 mg/ml and above in our investigation. The percentage of neutrophil apoptosis at 12 h was 79.2±2.3% in medium with 10 μg/ml of erythromycin compared with 51.2±4.1% in control medium (P<0.005). Augmentation of neutrophil apoptosis by erythromycin was observed after 6 h incubation, and increased thereafter (Figure 4). When neutrophils were incubated in erythromycin for various exposure times, the percentage of neutrophil apoptosis increased with the duration of exposure to erythromycin (Figure 5). Augmentation of neutrophil apoptosis was observed for more than 6 h of erythromycin exposure in comparison to that observed with control medium (P<0.05).

3) Effects of other antibiotics on neutrophil apoptosis

In a manner similar to that of erythromycin, other macrolide antibiotics were tested at the doses used for low-dose, long-term treatment. Roxithromycin also augmented neutrophil apoptosis in comparison with that by control medium (P<0.05). The percentage of neutrophil apoptosis at 12 h was 53.7±1.1% in 1 μg/ml of roxithromycin. In contrast, josamycin did not affected neutrophil apoptosis. Other antibiotics (ampicillin, cefazolin and gentamycin) were tested at therapeutic doses showing antibacterial effects, but did not affect neutrophil apoptosis (Figure 6).

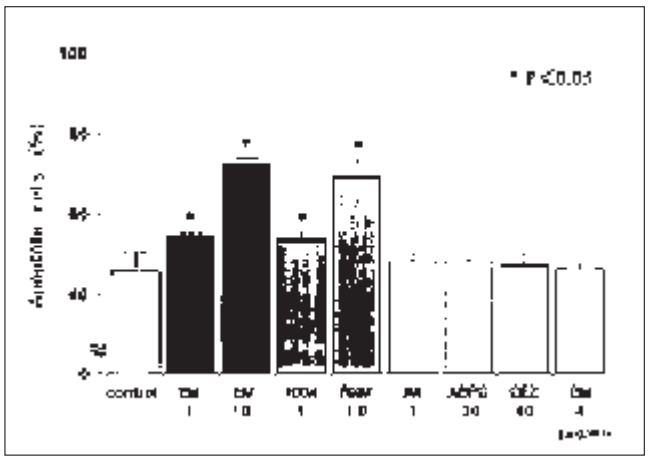

Figure 6. Effects of various types of antibiotics on neutrophil apoptosis.
Neutrophils were treated with various types of antibiotics for 12 h. Data are expressed as the mean±SE. n=3. *P<0.05 compared with the control. (EM: erythromycin, RXM: roxithromycin, JM: Josamycin ABPC: ampicillin, CEZ: cefazolin, GM: gentamycin).

DISCUSSION

In 1984 Kudoh first reported that low-dose and long-term treatment with erythromycin was effective for chronic lower respiratory tract infections including diffuse panbronchiolitis (DPB) (Kudoh et al., 1984). Recently several investigators have also shown similar clinical benefits using roxithromycin and clarithromycin, two new erythromycin derivatives (14-membered ring macrolide antibiotics) (Hashiba et al., 1992; Shirai et al., 1995; Anderson, 1989). Previously, the prognosis of DPB was poor. However, the long-term administration of low-dose erythromycin occasionally improved clinical symptoms along with

findings on chest radiograph, respiratory function tests and blood gas analysis (Kudoh et al., 1984; Kudoh et al., 1987). This treatment rarely eradicates pathogens from the lower respiratory tract, unlike conventional doses used to treat acute exacerbation. Erythromycin is effective even in cases showing colonization by *Pseudomonas aeruginosa,* which is insensitive to this antibiotic (Homma et al., 1983; Kudoh et al., 1984; Kudoh et al., 1987). These findings indicate that the efficacy of erythromycin in DPB may correlate with mechanisms unrelated to its microbicidal activity. We sought to clarify the potential mechanism of clinical effectiveness in this therapy.

It was previously reported that larger percentages of neutrophils were observed in the bronchoalveolar lavage fluid of DPB patients than in that of healthy volunteers and that a significant reduction in the neutrophil concentration in bronchoalveolar fluid was observed after treatment with erythromycin and roxithromycin (Ichikawa et al., 1990; Ichikawa et al., 1992; Labro et al., 1989). However, it was reported that josamycin (16-membered ring macrolide) was ineffective in treating DPB and chronic sinusitis (Shirai et al., 1995; Eyraud et al., 1986).

Clinical evidence suggests that erythromycin and roxithromycin are effective in treating DPB and chronic sinusitis, whose pathogenesis is likely related to the presence of neutrophils and neutrophil toxic products in the respiratory tract (Nagai et al., 1991; Yamamoto et al., 1990; Ichikawa et al., 1992; Kadota et al., 1993). Thus, it is postulated that the clinical effectiveness of erythromycin in treating of disease is related to a reduction in the intralesional burden of neutrophils. In fact, recent studies have shown that erythromycin inhibits neutrophil chemotaxis, phagocytosis and superoxide production in vitro, although conflicting results have also been reported (Nelson et al., 1987; Anderson et al., 1989; Esterly et al., 1978; Plewig et al., 1975). It has also been reported that erythromycin treatment significantly reduced BALF levels of IL-1β and IL-8 in patients with DPB (Sakito et al., 1996). IL-8 is a potent neutrophil chemoattractant. The decreased IL-8 level in BALF may reduce the volume of neutrophils migrating to the inflammation site.

Our findings demonstrate that erythromycin induced a significant increase in neutrophil apoptosis in a dose-dependent manner. Whether the increased apoptosis of neutrophils observed in the present study occurred at a level high enough to be clinical relevant in vivo remains to be tested. A similar effect was observed by another macrolide antibiotic roxithromycin, but not by b-lactams, ampicilin and cefazolin or an aminoglycoside, gentamycin. Moreover, because the number of neutrophils in tissue reflects the balance between the rates of influx and efflux, our finding that erythromycin induces neutrophil apoptosis may account for the reduced neutrophil burden after erythromycin treatment. Furthermore, apoptotic neutrophils, unlike necrotic cells, retain their membrane integrity and are efficiently phagocytosed by macrophages before final disintegration and the release of histotoxic contents can occur (Savill et al., 1989; Takei et al., 1996). In addition, apoptotic neutrophils display specific deficits in functions, including chemotaxis, degranulation and respiratory burst on stimulation (Whyte et al., 1993). Thus, accelerated neutrophil apoptosis caused by erythromycin may limit tissue injury by making senescent neutrophils available for phagocytosis and degradation by macrophages and by directly inhibiting the capacity of neutrophils to produce potentially injurious responses to inflammatory mediators (Sendo et al., 1995). The erythromycin-mediated changes in neutrophil apoptosis and survival described above may be the mechanism of clinical effectiveness of erythromycin in inflammatory diseases. Recently, it was reported that cAMP played an important role in intracellular control of neutrophils, since cAMP and cAMP-dependent protein kinases delayed human neutrophil apoptosis thereby promoting neutrophil survival (Rossi et al., 1995; Parvathenani et al., 1998). Therefore, modulation of cAMP levels is an important factor in treating inflammatory conditions. We assume that erythromycin-mediated changes in human neutrophil apoptosis may be related to the modulation of cAMP levels.

In future studies, it may be necessary to investigate cAMP levels, enzyme activation and cytokine production in relation to human neutrophil apoptosis in order to analyze their regulatory mechanisms.

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