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Erythromycin Shortens Neutrophil Survival by Accelerating Apoptosis

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Erythromycin is reported to have an anti-inflammatory action, which may account for its clinical effectiveness in the treatment of chronic inflammatory diseases such as diffuse panbronchiolitis. To evaluate the anti-inflammatory action of erythromycin, we examined the survival of isolated neutrophils with and without erythromycin. Erythromycin shortened neutrophil survival in a dose-dependent fashion, with a maximum effect at 10 mg/ml and above. Survival at 24 h was 63.4% in medium with 10 mg of erythromycin per ml compared with 82.7% in control medium ($P < 0.01$). This shortening of survival was brought about by acceleration of apoptosis, as evidenced by transmission electron microscopy. In a manner similar to that of erythromycin, other macrolide antibiotics, i.e., clarithromycin, roxithromycin, and midecamycin, also shortened neutrophil survival, but neither the β-lactams ampicillin and cefazolin nor the aminoglycoside gentamicin affected their survival. Erythromycin increased intracellular levels of cyclic AMP (cAMP) to 150% of control levels in neutrophils. Forskolin, rolipram, and dibutyryl-cAMP, which are known to increase intracellular cAMP levels, also shortened neutrophil survival. H-89, an inhibitor of cAMP-dependent protein kinase A, partially blocked the survival-shortening effect of erythromycin. Our findings suggest that erythromycin shortens neutrophil survival at least in part through elevation of intracellular cAMP levels.

Erythromycin has been used worldwide for about 30 years, particularly in the treatment of infections by gram-positive bacteria and facultative intracellular pathogens such as *Mycoplasma pneumoniae* and *Legionella pneumophila*. The pharmacokinetics and direct effects of erythromycin on pathogens have been studied extensively, while recent observations indicate that this antibiotic may profoundly suppress inflammatory reactions and resultant tissue injury (14, 15, 22, 25). This anti-inflammatory action of erythromycin may contribute to its clinical effectiveness in treatment of such inflammatory diseases as diffuse panbronchiolitis (14, 15) and certain dermatologic disorders (4).

The proposed mechanisms of the anti-inflammatory action of erythromycin include inhibition of various neutrophil functions (14, 15). Although crucial to antibacterial defense, neutrophils and neutrophil products, such as oxygen radicals and proteolytic enzymes, may in turn damage infected or inflamed tissue (18, 31). Erythromycin has been found to be 10- to 25-fold concentrated in neutrophils and to remain in its active form (10, 30). It is postulated that this high concentration of erythromycin within the neutrophil cytoplasm may alter neutrophil functions. In fact, erythromycin-mediated inhibition of neutrophil chemotaxis (7, 17, 25), oxidant generation (1, 9, 17, 21), and phagocytosis (8, 17) has been studied intensively. However, possible alterations in apoptosis and subsequent death of neutrophils treated with erythromycin have been largely neglected. It is proposed that apoptosis limits the ability of neutrophils to damage tissue by directly inhibiting the capacity of the cells to release potentially injurious products (32) or by marking senescent neutrophils for phagocytosis and degradation by macrophages (26). Thus, if erythromycin is capable of accelerating the apoptosis of neutrophils, this capacity may account for the anti-inflammatory action of erythromycin and the clinical effectiveness of erythromycin in treatment of chronic inflammatory diseases.

The present study was undertaken to evaluate the effects of erythromycin and other antibiotics on the apoptosis and survival of neutrophils and to determine what mechanism underlies this effect.

MATERIALS AND METHODS

Isolation and culture of neutrophils. (i) Neutrophil isolation. Neutrophils were prepared by using sterile procedures and discontinuous plasma-Percoll gradients, as previously described (11). In brief, fresh blood was drawn by venipuncture from healthy volunteers and immediately supplemented with 1/10 volume of EDTA. The leukocyte population was separated from blood cells by sedimentation in 1.5% dextran for 45 min at room temperature. The upper layer of leukocyte-enriched plasma was then gently layered over 42%/51% plasma-Percoll gradients (Pharmacia Chemicals AB, Uppsala, Sweden) and centrifuged at 400 × *g* for 10 min at 20°C. The neutrophil-rich layer was collected, washed once in excess *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (Gibco Laboratories, Grand Island, N.Y.) containing 1 mg of albumin per ml, and resuspended in RPMI 1640 without serum at the concentrations indicated below. The final preparation contained more than 95% neutrophils, and no platelets were visible. Cell viability exceeded 98% as determined by trypan blue dye exclusion.

(ii) Treatment of neutrophils. The cell culture medium used was RPMI 1640 without serum, and cells were cultured in 1.5-ml polypropylene tubes at 37°C in 5% CO_2. First, we incubated the neutrophils (10^6 cells per ml) in medium with or without the following antibiotics: the macrolides erythromycin (1 to 20 mg/ml, 1.3×10^{-6} to 2.6×10^{-5} M; Dai-nippon Pharmaceutical Co. Ltd., Osaka, Japan), clarithromycin (1.3×10^{-5} M; Dai-nippon Pharmaceutical Co. Ltd.), roxithromycin (1.3×10^{-5} M; Eisai Co. Ltd., Tokyo, Japan), and midecamycin (1.3×10^{-5} M; Meiji Seika Kaisha, Ltd., Tokyo, Japan); the β-lactams ampicillin (30 mg/ml; Meiji Seika Kaisha) and cefazolin (40 mg/ml; Fujisawa Pharmaceutical Co. Ltd., Tokyo, Japan); and the aminoglycoside gentamicin (4 mg/ml; Sigma Chemical Co., St. Louis, Mo.). Second, we incubated neutrophils in medium with and without cyclic AMP (cAMP)-raising agents. They included the adenylate cyclase activator forskolin (10^{-5} M) (Sigma), the phosphodiesterase IV-specific inhibitor rolipram (10^{-5} and 10^{-4} M) (28) (Meiji Seika Kaisha), and the cell-permeable cAMP analog dibutyryl-cAMP (10^{-5} and 10^{-4} M) (Sigma). Third, we incubated neutrophils with erythromycin (10 mg/ml), in the presence

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or absence of H-89 (10^{-5} M), a potent inhibitor of cAMP-dependent protein kinase A. Stock solutions of all water-insoluble compounds were prepared in methanol (final concentrations, <0.2% [vol/vol]), dimethyl sulfoxide (final concentrations, <0.01% [vol/vol]), or acetone (final concentration, 0.01% [vol/vol]). Aliquots were diluted in RPMI 1640 before each experiment. Preliminary experiments confirmed that these concentrations of solvents did not increase cell necrosis or modulate the rate of neutrophil apoptosis (data not shown).

Analysis of neutrophil survival. After 24, 36, and 48 h of culture, neutrophils were counted with a hemocytometer and cell viability was determined by the trypan blue dye exclusion method. Neutrophil survival was calculated by the following formula: survival = % viable × (number of cells remaining/original number of cells seeded). To exclude the possibility that cell loss in culture was due to cell attachment to the plastic wells, we stained the wells with Diff-Quick stain (Kokusai-shiyaku Co., Ltd., Kobe, Japan) after counting the cells and found that there was no cell attachment to the wells.

Estimation of apoptotic neutrophils. Cultured neutrophils were centrifuged, fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed in 1% osmium tetroxide–0.1 M cacodylate buffer, and processed for analysis by routine transmission electron microscopic techniques (final magnification, ×2,500). All samples were coded, and at least 200 cells per sample were counted. Apoptotic cells were identified according to the criteria of chromatin aggregation, nucleolar prominence, and cytoplasmic vacuolation (33).

DNA electrophoresis. DNA fragmentation on gel electrophoresis was analyzed as described previously (5), with a slight modification. Neutrophils (10^7 cells) were incubated at 56°C for 1 h in 500 ml of lysing buffer (10 mM Tris [pH 8.0], 20 mM EDTA, 2% Triton X, 1 mg of proteinase K per ml). The DNA was extracted with phenol and an equal volume of chloroform by centrifugation at 15,000 × *g* for 5 min at 0°C and precipitated with 1 volume of cold ethanol and 0.06 volume of 5 M NaCl by incubation for 20 min at −20°C. Each sample was resuspended in 1 ml of buffer (10 mM Tris [pH 8.0], 1 mM EDTA, 1% sodium dodecyl sulfate) and electrophoresed on 1% agarose gels with 40 mM Tris-acetate buffer containing 0.5 mg of ethidium bromide per ml. DNA was visualized by UV examination and photographed with a Polaroid camera.

cAMP assay. After 5 h of incubation in control medium or medium containing 10 mg of erythromycin per ml, neutrophils (3.8×10^6 cells) were quickly sonicated in ice-cold 10% trichloroacetic acid. After trichloroacetic acid was extracted with ether, the residue was dissolved in acetone-water. The cAMP levels were measured in duplicate by [^{3}H] cyclic AMP (New England Nuclear, Boston, Mass.) and normalized for protein content of the cells by the Lowry method with bovine serum albumin as a standard (6).

LDH release assay. After 24 h of neutrophil incubation in control medium or medium containing 1 to 20 mg of erythromycin per ml, aliquots of medium were collected. Sonicated cell samples were used to determine the total lactate dehydrogenase (LDH) activity. LDH activity was determined by an LDH assay kit which contains the reagents lactate and NAD. LDH catalyzes the oxidation of lactate and concurrent reduction of NAD. The change in A_{340} as a result of the formation of NADH was measured with a spectrophotometer (model Du-62; Beckman, Fullerton, Calif.). LDH activity was obtained by using a standardized enzyme solution. The results were expressed as a percentage of the total LDH activity per well.

Statistical analysis. Results are shown as means ± standard errors of the mean (SEM) for five experiments. Statistical significance was tested by Student's *t* test or analysis of variance with Scheffe's test, as appropriate. A *P* value of <0.05 was judged to be statistically significant.

RESULTS

Erythromycin significantly shortened neutrophil survival in a dose-dependent fashion (Fig. 1). The survival-shortening effect of erythromycin reached a maximum at 10 mg/ml (1.3×10^{-5} M) and above: survival rates at 24 h were 82.7% ± 0.6% for the control and 72.5% ± 1.0%, 67.2% ± 1.3%, 63.4% ± 0.9%, and 63.5% ± 2.4% at 1, 5, 10, and 20 mg of erythromycin per ml, respectively ($P < 0.01$ versus the control). These findings were supported by results of the LDH release assay: rates of LDH release at 24 h were 49.0% ± 4.2% for the control and 43.1% ± 1.6%, 49.0% ± 4.2%, 79.0% ± 10.5%, and 86.5% ± 6.1% at 1, 5, 10, and 20 mg of erythromycin per ml, respectively ($P < 0.05$ versus the control) (Fig. 2).

After 24 h of culture, a considerable number of neutrophils already showed the characteristics of apoptosis (Fig. 3A). When neutrophils were cultured in control medium, the percentage of apoptotic cells was 66.0% ± 0.6% at 24 h. In contrast, culture of neutrophils with erythromycin resulted in a dose-dependent increase in the percentage of apoptotic cells (Fig. 3B). The maximal effect was obtained at 10 mg/ml and above: the percentage of apoptotic cells was 85.7% ± 0.6% at 10 mg/ml. The induction of apoptosis in neutrophils, assessed morphologically, was confirmed by gel electrophoresis (Fig. 4). Figure 4 shows the characteristic DNA ladder fragmentation, having an approximately 200-bp multiple pattern, that oc-

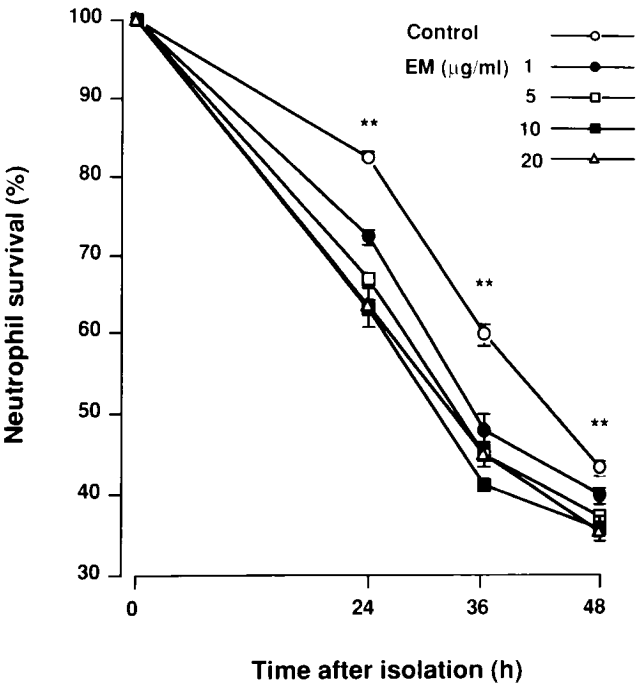

FIG. 1. Time course of the survival of freshly isolated neutrophils cultured in control medium and medium supplemented with 1 to 20 mg of erythromycin per ml. Erythromycin shortened neutrophil survival in a dose-dependent fashion, with a maximal effect at 10 mg/ml. Data are shown as means ± SEM (error bars); $n = 5$. **, $P < 0.01$ for the control versus cells cultured with 1, 5, 10, and 20 mg of erythromycin per ml. EM, erythromycin.

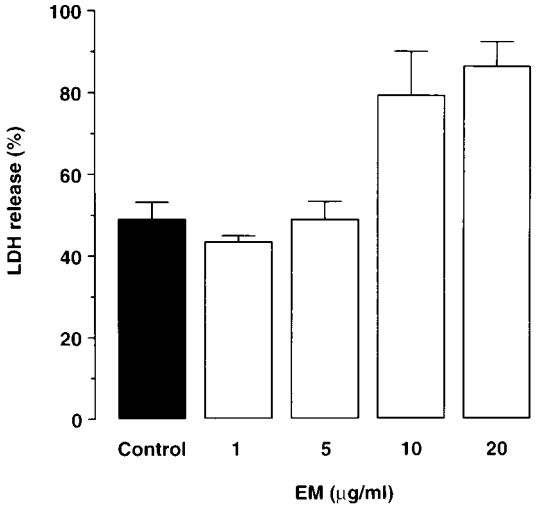

FIG. 2. LDH release from neutrophils cultured in control medium and medium supplemented with 1 to 20 mg of erythromycin per ml. After 24 h of incubation, aliquots of medium were collected and LDH activity was measured. The results are expressed as percentages of total LDH activity per well. Erythromycin increased LDH release from neutrophils in a dose-dependent fashion ($P < 0.05$ by analysis of variance). Data are shown as means ± SEM (error bars); $n = 4$. EM, erythromycin.

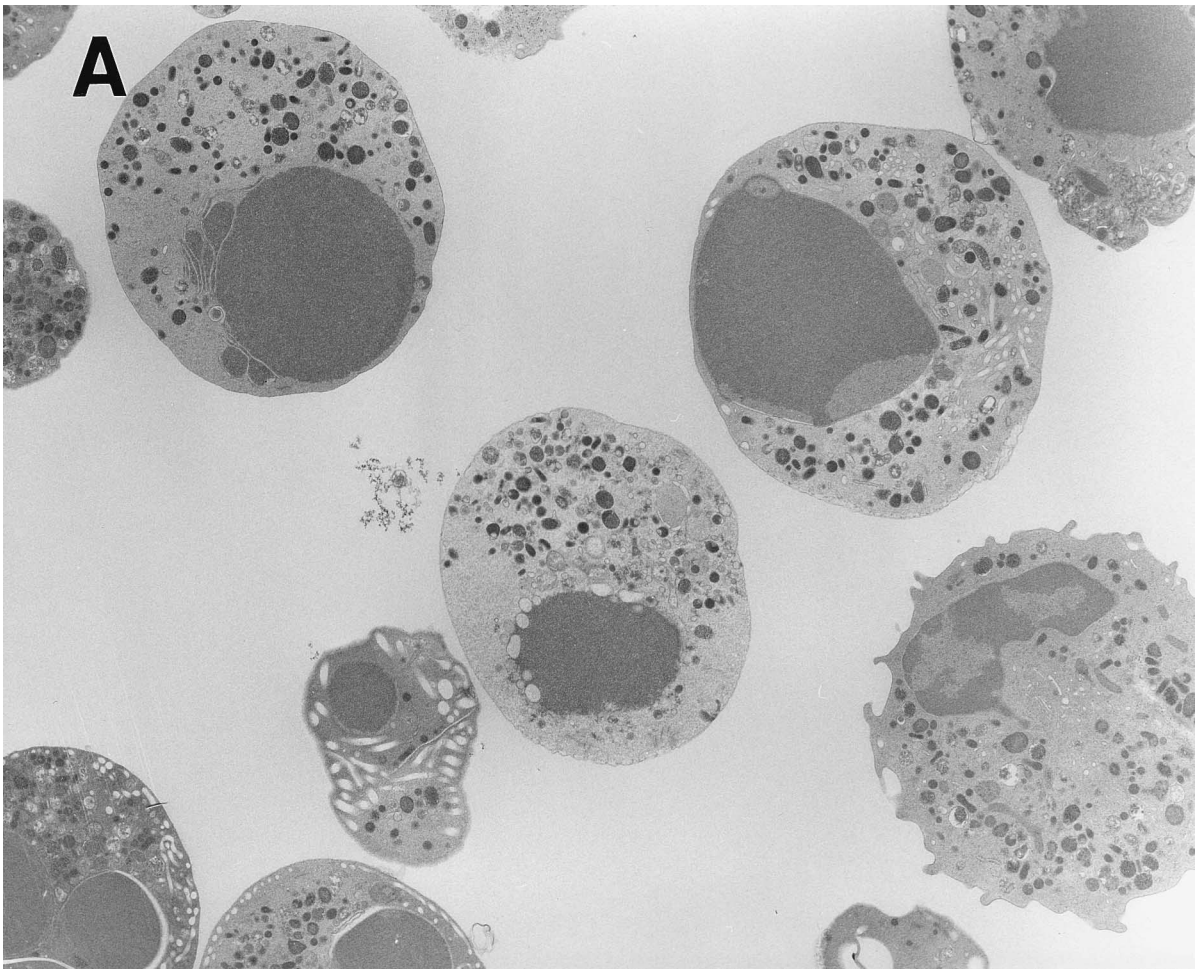

FIG. 3. (A) Transmission electron micrograph of apoptotic neutrophils cultured in control medium for 24 h, showing chromatin aggregation, nuclear prominence, and cytoplasmic vacuolation, a triad of featuresthat are highly characteristic of apoptosis, with intact cell membrane and organelles. Magnification, ×12,000. (B) Percentage of apoptotic neutrophils, assessed morphologically, in the presence and absence of erythromycin after 24 h of culture. Data are shown as means ± SEM (error bars); $n = 5$. *, $P < 0.05$ versus the control. EM, erythromycin.

curred at 24 h in both control medium and medium with 10 mg of erythromycin per ml. The appearance of low-molecular-weight DNA confirms the induction of apoptosis in neutrophils, being indicative of the activation of an endogenous endonuclease that cleaves at internucleosomal sites.

In a manner similar to that of erythromycin, the other macrolide antibiotics tested at 1.3×10^{-5} M, that is, clarithromycin, roxithromycin, and midecamycin, also decreased neutrophil survival (68.7% ± 0.7%, 65.1% ± 0.8%, and 65.6% ± 0.9%, respectively, versus 83.1% ± 1.6% for the control; $P < 0.01$) (Fig. 5). In contrast, neither the β-lactams, ampicillin and cefazolin, nor the aminoglycoside gentamicin affected survival.

After the neutrophils had been exposed to 10 mg of erythromycin per ml for 5 h, intracellular levels of cAMP showed a corresponding increase in response to 10^{-5} M forskolin, an adenylate cyclase activator (6.7 ± 0.6, 10.3 ± 1.0, and 10.6 ± 0.8 pmol/mg of protein for control, erythromycin-treated, and forskolin-treated cells, respectively; $P < 0.05$ versus the control). Each of the cAMP-raising agents, that is, forskolin, the cAMP-selective phosphodiesterase inhibitor rolipram, and the cell-permeable cAMP analog dibutyryl-cAMP, shortened neutrophil survival (Fig. 6). The survival-shortening effect of forskolin was found to be associated with an increased percentage of apoptotic cells (38.5% ± 1.6% and 56.8% ± 0.8% after 15

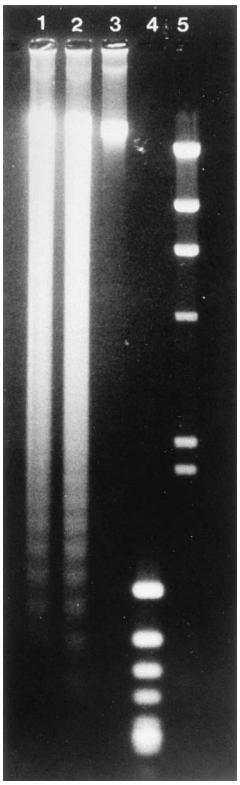

FIG. 4. Gel electrophoresis demonstrating DNA fragmentation. Lane 1, cells cultured with 10 mg of erythromycin per ml at 24 h; lane 2, control cells at 24 h; lane 3, control cells at time zero; lane 4, fX174-*Hin*cII digest DNA size markers; lane 5, λ DNA-*Hin*dIII digest DNA size markers.

h of culture for control and 10^{-5} M forskolin-treated cells, respectively; $P < 0.01$). Furthermore, the addition of H-89, an inhibitor of cAMP-dependent protein kinase A, partially blocked the survival-shortening effect of erythromycin: survival rates at 24 h were 79.6% ± 2.0%, 58.2% ± 2.9%, and 66.9% ± 1.0% for control cells, cells treated with 10 mg of erythromycin per ml, and cells treated with erythromycin and 10^{-5} M H-89, respectively ($P < 0.05$ for cells treated with erythromycin versus cells treated with erythromycin and H-89).

DISCUSSION

Our findings show that erythromycin significantly shortens neutrophil survival in a dose-dependent fashion by accelerating apoptosis. A similar survival-shortening effect was provided by the other macrolide antibiotics, clarithromycin, roxithromycin, and midecamycin, but not by the β-lactams ampicillin and cefazolin or the aminoglycoside gentamicin. Our findings also showed that erythromycin increased cAMP levels within the cytoplasm of neutrophils and that forskolin, rolipram, and dibutyryl-cAMP, which are known to increase intracellular cAMP levels, shortened neutrophil survival. In addition, we found that H-89, an inhibitor of cAMP-dependent protein kinase A, partially blocked the survival-shortening effect of erythromycin. These findings suggest that the survival-shortening effect of erythromycin described above resulted, at least in part, from increased cAMP levels within the neutrophils.

We found that erythromycin caused about a 20% decrease in neutrophil survival after 24 h of culture (82.7% in the control medium versus 63.5% in medium containing 10 mg of erythromycin per ml). This effect of erythromycin was significant at 1 mg/ml, the lowest concentration tested, which is achieved in serum clinically after the oral ingestion of 500 mg of erythromycin (2). Moreover, tissue drug concentrations may be much higher than the serum drug concentration. Thus, our results suggest that the survival-shortening effect of erythromycin occurs in vitro at clinically relevant concentrations.

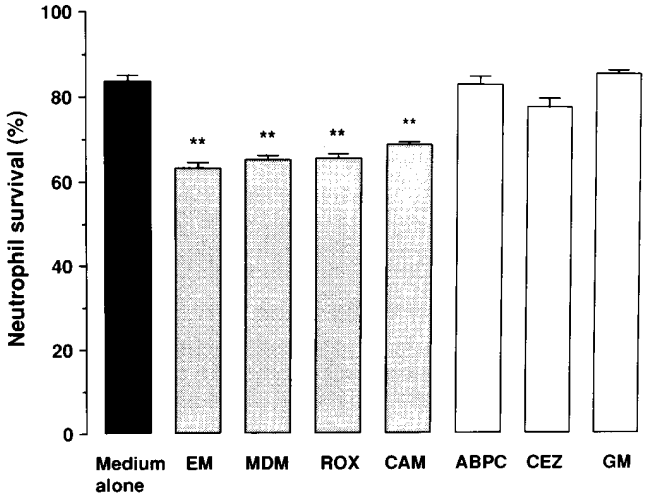

FIG. 5. Effects of various types of antibiotics on neutrophil survival. Neutrophils were incubated for 24 h in control medium and medium supplemented with erythromycin (EM) (1.3×10^{-5} M [10 mg/ml]), midecamycin (MDM) (1.3×10^{-5} M), roxithromycin (ROX) (1.3×10^{-5} M), clarithromycin (CAM) (1.3×10^{-5} M), ampicillin (ABPC) (30 mg/ml), cefazolin (CEZ) (40 mg/ml), or gentamicin (GM) (4 mg/ml). Data are shown as means ± SEM (error bars); $n = 5$. **, $P < 0.05$ versus the control.

Our findings indicate that the shortening of neutrophil survival by erythromycin may be secondary to increased cAMP

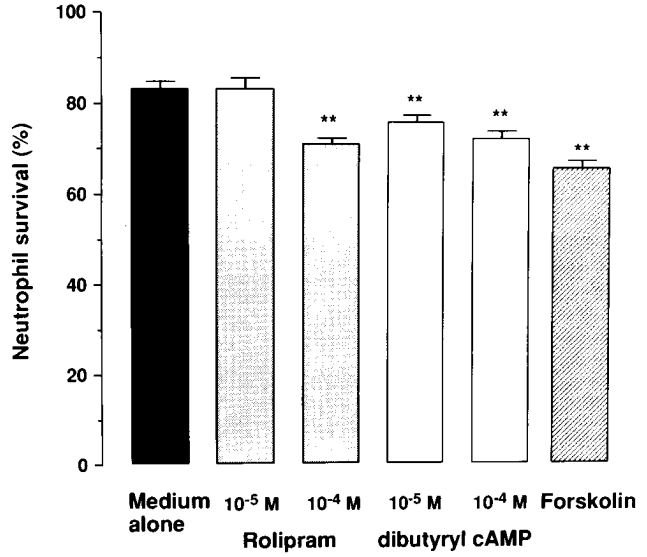

FIG. 6. Effects of cAMP-raising agents on neutrophil survival. Neutrophils were incubated for 24 h in control medium and medium supplemented with rolipram (10^{-5} and 10^{-4} M), a phosphodiesterase IV-specific inhibitor; dibutyryl-cAMP (10^{-5} and 10^{-4} M), a cell-permeable cAMP analog; and forskolin (10^{-5} M), an adenylate cyclase activator. Data are shown as means ± SEM (error bars); $n = 5$. **, $P < 0.01$ versus the control.

levels in neutrophils brought about by erythromycin. We found that erythromycin increased intracellular cAMP levels to approximately 150% of control levels in neutrophils. A similar finding has been reported by previous investigators, who demonstrated increased intracellular levels of cAMP in airway epithelial cells treated with the macrolide roxithromycin (29). We also found that all of the agents which increase intracellular cAMP levels shortened neutrophil survival to a degree similar to that induced by erythromycin and that the cAMP-dependent protein kinase A inhibitor H-89 abrogated the survival-shortening effect of erythromycin. cAMP is regarded as a second messenger regulating neutrophil functions. In fact, numerous investigators have reported that elevation of intracellular cAMP levels inhibits such neutrophil functions as chemotaxis (3, 12), O_2^- generation (22, 23), and enzyme release (19, 27). In contrast to the situation for the inhibitory effect of cAMP on neutrophil functions, relatively little information is available for the effect of cAMP on apoptosis and death in neutrophils. In thymocytes and lymphocytes, on the other hand, elevation of cAMP levels has generally been considered to induce apoptosis and cell death (16, 20). Thus, our results suggest that cAMP-induced apoptosis also occurs in neutrophils.

Clinical evidence suggests that erythromycin is effective in the treatment of diffuse panbronchiolitis, whose pathogenesis is likely related to the presence of neutrophils and neutrophil toxic products in the lower respiratory tract (13–15). It was previously reported that patients with diffuse panbronchiolitis had larger percentages of neutrophils in their bronchoalveolar lavage fluid than did healthy volunteers and that they showed a significant reduction in bronchoalveolar lavage fluid neutrophil percentages after treatment with erythromycin (14, 15). Thus, it is postulated that the clinical effectiveness of erythromycin for treatment of this disease is related to a reduction in the intrapulmonary burden of neutrophils. In fact, earlier studies have shown that erythromycin inhibits neutrophil chemotaxis in vitro (7, 25), although conflicting results have been reported (1). Moreover, because the number of neutrophils in tissue reflects a balance between rates of influx and removal, our finding that erythromycin shortens neutrophil survival by accelerating 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 release of histotoxic contents occur (26). In addition, apoptotic neutrophils display specific deficits in function, including chemotaxis, degranulation, and respiratory burst on stimulation (32). Thus, accelerated neutrophil apoptosis caused by erythromycin may limit tissue injury by marking senescent neutrophils for phagocytosis and degradation by macrophages and by directly inhibiting the capacity of neutrophils to make potentially injurious responses to inflammatory mediators. The erythromycin-mediated changes in neutrophil apoptosis and survival described above may provide a mechanism for the clinical effectiveness of erythromycin in inflammatory diseases. Whether the increased apoptosis of neutrophils we observed in the present study is at a level high enough for clinical relevance in vivo remains to be tested.

ACKNOWLEDGMENTS

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