Macrolide Antibiotics Directly Reduce Active Oxygen Generation by Neutrophils in Human Peripheral Blood

EIICHIRO SUGIHARA*,**, TAKESHI KOYANAGI***, TAKASHI NIIZEKI***, NAOTOSHI HIROTA**, MASAKO NAGAFUCHI***, KAZUHIKO YAMADA*, YASUKO KIDO*, NORIYUKI ONO**, TORU RIKIMARU* AND HISAMICHI AIZAWA*

*Department of Medicine, Kurume University School of Medicine, Kurume 830-0011 and
**Department of Internal Medicine, Chikugo City Hospital, Chikugo 833-0041, Japan

Summary: Since a “low-dose and long-term” administration of erythromycin (EM) was reported to be effective in patients with chronic respiratory diseases, including diffuse panbronchiolitis (DPB), the modulation of host defense responses by EM has attracted much attention. Despite considerable controversy, it was recently demonstrated that macrolides reduced neutrophil function. In this study, we investigated the effects of EM, a 14-membered ring macrolide, azithromycin (AZM), a 15-membered ring macrolide, and rokitamycin (RKM), a 16-membered ring macrolide, on neutrophil function in terms of active oxygen generation of neutrophils in the absence and presence of mononuclear cells in vitro. EM and AZM significantly suppressed active oxygen generation by neutrophils in the absence of mononuclear cells at low concentration (0.5 μg/ml, p<0.05). At the next step, to confirm that EM and AZM directly reduced active oxygen generation by neutrophils, we investigated whether mononuclear cells affected this effect of EM and AZM. In the presence of mononuclear cells pretreated with EM or AZM, both antibiotics suppressed active oxygen generation at concentrations ranging from 0.5 to 20 μg/ml. However, the inhibition rates induced by EM and AZM at low concentrations were not so different between the absence and the presence of mononuclear cells. These results indicated that EM and AZM have direct effects on the active oxygen generation by neutrophils and those effects that were not influenced by mononuclear cells. This inhibitory effect may be responsible for the therapeutic efficacy of these 14-membered and 15-membered ring macrolides in patients with DPB.

Key words neutrophils, superoxide, neutrophil function, macrolide antibiotics, diffuse panbronchiolitis

INTRODUCTION

Diffuse panbronchiolitis (DPB) was first reported as an entity distinct from other bronchiolitis in 1969 in Japan [1,2]. The disease is characterized by chronic inflammation with infiltration of inflammatory cells predominantly localized in the respiratory bronchioles and clinical features are chronic or continuous sinopulmonary infection and inflammation. Since, a "low-dose and long-term" erythromycin (EM) treatment has been reported as being effective in chronic lower respiratory tract diseases, including DPB, the potential of macrolide antibiotics to modulate the host defense responses has attracted much attention. However, the mechanism by which EM modulates the immune responses is not clear as yet. Accordingly, the immunomodulatory activity of EM has been extensively investigated. Many investigators suggested from the results of in vitro and in vivo observations that this drug might act as an anti-inflammatory agent rather than as an antimicrobial agent.
On the other hand, DPB is a disease characterized by marked accumulation of neutrophils [3] and excess mucus production in the bronchioles. Usually, neutrophils, which migrate to and are activated at the infectious site, protect the tissues from destruction by killing and eliminating the causative organisms. Neutrophils which accumulate in excess and remain in the lesions for long periods in an activated state, are often hazardous to the tissues because of the production and release of harmful substances such as active oxygen molecules [4] and lytic enzymes [5]. Thus, neutrophils may play a crucial role in the pathogenesis of DPB. To elucidate the mode of action through which EM exerts its efficacy in patients with DPB, we examined the effects of EM, and azithromycin (AZM), on active oxygen production by neutrophils in vitro as compared with rokitamycin (RKM), and the influence of mononuclear cells. EM and AZM, but not RKM, are effective against DPB.

MATERIALS AND METHODS

Preparation of macrolide solutions

Erythromycin (EM: Wako Pure Chemicals Co.), azithromycin (AZM: Pfizer Pharmaceutical.) and rokitamycin (RKM: Asahi Chemical Co.) are 14-membered, 15-membered and 16-membered ring macrolides, respectively, and 4 mg of each drug was dissolved in 1.0 ml of 99.5% ethanol and stored at −20°C until use. The stock solution was diluted with phosphate buffered saline, pH 7.4, to final concentrations of 0.05, 0.1, 0.5, 1.0, 10.0 and 20.0 μg/ml. The final concentration of the solvent (99.5% ethanol) used in the reaction mixture was adjusted to less than 1% so that it would not affect reaction.

Preparation of neutrophils and mononuclear cells

Neutrophils and peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation against a Ficoll-Conray density from heparinized blood of healthy volunteers and washed twice with phosphate buffered saline (PBS, pH 7.4). Each cell preparation contained more than 95% of neutrophils or PBMC as determined by Wright staining. Neutrophils were suspended in the buffer at cell density of 2×10⁶ cell/ml. PBMC were suspended in it at cell density of 2×10⁵ and 2×10⁶ cell/ml.

Assay of reactive oxygen production by neutrophils in the absence of mononuclear cells

The production of active oxygen by neutrophils was measured using the cytochrome C method. Neutrophils were isolated from heparinized peripheral blood from a healthy volunteer. EM, AZM or RKM was added to final concentrations of 0.05, 0.1, 0.5, 1.0, 10.0 and 20.0 μg/ml in the suspension of neutrophils. As a control study, only the solvent (ethanol) was added to the suspension of neutrophils. Then, 40 μl of 12.5 mg/ml of cytochrome C (Wako Pure Chemical), and 100 μl of 2 μg/ml of phorbol 12-myristate 13-acetate (PMA: Sigma) were added to the neutrophil suspensions (1000 μl) and the reaction mixture was adjusted to 2.0 ml by addition of PBS. Reduction of cytochrome C during incubation of the reaction mixture at 37°C was measured by recording optical density at 550 nm using a spectrophotometer (Hitachi 220A) and the amount of active oxygen formed was calculated.

Assay of reactive oxygen production by neutrophils in the presence of mononuclear cells

Neutrophils and mononuclear cells were isolated from heparinized peripheral blood from a healthy volunteer. To each the suspension of neutrophils and mononuclear cells, EM, AZM and RKM was added to final concentrations of 0.05, 0.1, 0.5, 1.0, 10.0 and 20.0 μg/ml. Then these pretreated-PBMC suspensions were added to the pretreated-neutrophils suspensions (2×10⁶ cells/ml), at the rate of 1% (2×10⁴ cells/ml) or 10% (2×10⁵ cells/ml) to the neutrophils. After they were mixed, 100 μl of a 2 μg/ml solution of PMA and 40 μl of a 12.5 mg/ml solution of cytochrome C were added to the suspensions containing pretreated PBMC and pretreated neutrophils. Then, active oxygen generation by neutrophils in the presence of mononuclear cells was measured by the same method described above.

Statistical analysis

All data are expressed as the mean±SD. Data analyses were performed using the Wilcoxon signed rank test for matched pairs of with a paired t test using the Excel software package (Microsoft Co, Oakland, CA). P values <0.05 were considered significant.

RESULTS

Effect of EM on the generation of active oxygen by neutrophils

Figure 1 illustrates the effect of EM on active oxygen production by neutrophils in the absence of mononuclear cells.
oxygen generation by neutrophils. In the absence of mononuclear cells pretreated with EM, the active oxygen production by neutrophils was suppressed by EM at the concentration of 0.5 μg/ml. O.D. at 550 nm at 0 μg/ml vs 0.211±0.019 at 0.5 μg/ml p<0.05). In the presence of mononuclear cells pretreated with EM at the rate of 1% of the neutrophils, active oxygen production by neutrophils

Fig. 1. Effect of EM on active oxygen generation by neutrophils. (A) The active oxygen production by neutrophils in the absence of mononuclear cells is shown. (B) and (C) The active oxygen production by neutrophils in the presence of mononuclear cells at the rate of 1% and 10% of the neutrophils, respectively, is shown. *p<0.05, **p<0.01 versus control. Each column and bar represent the mean±SD.

Fig. 2. Effect of AZM on active oxygen generation by neutrophils. (A) The active oxygen production by neutrophils in the absence of mononuclear cells is shown. (B) and (C) The active oxygen production by neutrophils in the presence of mononuclear cells at the rate of 1% and 10% of the neutrophils, respectively, is shown. *p<0.05, **p<0.01 versus control. Each column and bar represent the mean±SD.
was suppressed by EM at the concentration of 0.5 μg/ml and above. (0.225±0.016 O.D. at 550 nm at 0 μg/ml vs 0.190±0.016 at 0.5 μg/ml, p<0.01, 0.019±0.026 at 1.0 μg/ml, p<0.05, 0.167±0.022 at 10.0 μg/ml, p<0.01. 0.142±0.022 at 20.0 μg/ml, p<0.01). In the presence of mononuclear cells at the rate of 10%, the active oxygen production by neutrophils was suppressed by EM at concentrations from 0.1 to 20.0 μg/ml. (0.239±0.034 O.D. at 550 nm at 0 μg/ml vs 0.208±0.025 at 0.1 μg/ml, 0.192±0.024 at 0.5 μg/ml, 0.184±0.022 at 1.0 μg/ml, 0.163±0.032 at 10.0 μg/ml, 0.126±0.027 at 20.0 μg/ml, p<0.01).

Effect of EM on the generation of active oxygen by neutrophils

In the presence of mononuclear cells at the rate of 10%, the active oxygen production by neutrophils was suppressed by EM at concentrations from 0.1 to 20.0 μg/ml. (0.230±0.019 at 0 μg/ml vs 0.207±0.023 at 0.5 μg/ml, p<0.05 and 0.189±0.030 at 0.5 μg/ml, 0.176±0.030 at 1.0 μg/ml, 0.145±0.021 at 10.0 μg/ml, 0.118±0.021 at 20.0 μg/ml, p<0.01).

Effect of AZM on the generation of active oxygen by neutrophils

Figure 2 illustrates the effect of AZM on active oxygen generation of neutrophils. In the absence of mononuclear cells pretreated with AZM, the active oxygen production by neutrophils was suppressed by AZM at the concentrations of 0.5 and 20 μg/ml. (0.258±0.0.4 O.D. at 550 nm at 0 μg/ml vs 0.214±0.0.34 at 0.5 μg/ml, p<0.01, 0.213±0.0.28 at 20 μg/ml, p<0.05). In the presence of mononuclear cells pretreated with AZM at the rate of 1% and 10% of the neutrophils, active oxygen production by neutrophils was suppressed at concentrations from 0.1 to 20 μg/ml. (0.215±0.019 O.D. at 550 nm at 0 μg/ml vs 0.201±0.017 at 0.1 μg/ml, 0.184±0.016 at 0.5 μg/ml, 0.177±0.014 at 1.0 μg/ml, 0.159±0.009 at 10.0 μg/ml, p<0.01, mononuclear cells at the rate of 1%). (0.230±0.019 at 0 μg/ml vs 0.207±0.023 at 0.5 μg/ml, p<0.05 and 0.189±0.030 at 0.5 μg/ml, 0.176±0.030 at 1.0 μg/ml, 0.145±0.021 at 10.0 μg/ml, 0.118±0.021 at 20.0 μg/ml, p<0.01).

Effect of RKM on the generation of active oxygen by neutrophils

In the absence of mononuclear cells, the active oxygen generation was not significantly suppressed by RKM at any concentration. In the presence of mononuclear cells (rates of 1% and 10%), RKM suppressed the active oxygen generation, but only at the highest concentrations of 10 and 20 μg/ml (Fig. 3).

Inhibition rates of active oxygen generation by neutrophils induced by macrolides (EM, AZM, RKM) in the absence and presence of mononuclear cells

Figures 4 and 5 shows the inhibition rates of active oxygen generation by neutrophils induced by EM and AZM. At low doses (0-0.5 μg/ml), the inhibition rates induced by EM and AZM were not affected by the presence and absence of mononuclear cells pretreated with EM or AZM. However at high doses (≥1.0 μg/ml), the inhibition rates became more prominent in the presence of mononuclear cells.

![Graph](https://example.com/graph.png)

**Fig. 3.** Effect of RKM on active oxygen generation by neutrophils. (A) The active oxygen production by neutrophils in the absence of mononuclear cells is shown. (B) and (C) The active oxygen production by neutrophils in the presence of mononuclear cells at the rate of 1% and 10% of the neutrophils, respectively, is shown. *p<0.05, **p<0.01 versus control. Each column and bar represent the mean±SD.
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Figure 6 shows the inhibition rates induced by RKM. In the presence of mononuclear cells, the inhibition rates became more prominent at the highest doses (10 and 20 μg/ml).

DISCUSSION

Since a “low-dose and long-term” EM treatment was reported to be effective in patients with chronic inflammatory diseases of the lower respiratory tracts, including DPB, the potential of EM to modulate the host defense response has attracted much attention. In spite of the comprehensive investigations on the in vitro and in vivo immunomodulatory activities of EM, the mechanism remains obscure.

Kudoh et al. [6] suggested that EM treatment normalized the hyperimmune reactivity in the bronchioles of patients with DPB and that this might be the cause of chronic intractable inflammation of the bronchioles. In accordance with this hypothesis, it has been reported that EM might influence the function of immune competent cells such as macrophages, lymphocytes, and neutrophils. Meanwhile, remarkable increases both in count and percentage of neutrophils in bronchoalveolar lavage fluids (BALFs) from patients with DPB have been shown to be normalized by the treatment with EM [3,7,8], in association with an apparent reduction in elastolytic-like activity [5]. These results of clinical and in vitro observations strongly suggested that the agent might alter the function of neutrophils in the bronchioles of patients with DPB. Therefore, we investigated whether EM modified neutrophils functions at low concentrations of 1.0 μg/ml and below which were achievable at therapeutic doses, and the influence of mononuclear cells. We observed the effect of a 14-membered, 15-membered and 16-membered ring macrolide (EM, AZM and RKM, respectively) on active oxygen generation by neutrophils in the presence and absence of mononuclear cells. From clinical observations, both EM and AZM have already been shown to be effective in patients with DPB whereas RKM is not effective.

The present in vitro study demonstrated that at a low concentration (0.5 μg/ml) both EM and AZM reduced active oxygen generation by neutrophils in the absence of mononuclear cells. In contrast, RKM showed no statistically significant inhibition of active oxygen generation by neutrophils. At the next step, we examined the effect of mononuclear cells on active oxygen production by neutrophils. At low
concentrations of EM (0.05-0.5 µg/ml) and AZM (0.05-0.5 µg/ml), pretreated-mononuclear cells had no effect on active oxygen production by neutrophils. At high concentrations (≥1.0 µg/ml), however, the inhibition rates of active oxygen production became more prominent in the presence of mononuclear cells pretreated with EM and AZM. RKM reduced active oxygen generation of neutrophils in the presence of mononuclear cells pretreated with RKM, but only at high concentrations which are not achievable at therapeutic doses.

With regard to the pattern of EM-induced alteration of neutrophil functions, however, there are considerable controversies; some investigators reported no substantial changes [9], while others mentioned a reduction [10-12] or conversely, an enhancement [13]. But it should be noted that the reported reduction in neutrophils function by in vitro treatment with EM could be demonstrated only when neutrophils were treated with very high concentrations of EM (more than 10 µg/ml), which were not achievable at therapeutic doses. The serum concentrations of EM in patients with DPB under “low-dose and long-term” therapy were demonstrated to be within a range of 0.5 to 1.0 µg/ml. Therefore, it was considered unlikely that EM would benefit the outcome of patients with DPB by acting directly on neutrophils and changing their functions. However, the present study clearly demonstrated that some macrolides are able to directly inhibit active oxygen generation by neutrophils even at low concentrations which are easily achieved at therapeutic doses without the influence of the mononuclear cells. The inhibitory effect on active oxygen generation by neutrophils was clearly demonstrated in vitro when EM or AZM was used, both of which are known to be effective in patients with DPB. In contrast, when RKM was employed, no significant inhibition of active oxygen generation was demonstrated at therapeutic doses.

Furthermore, our present experimental results suggest that the mechanism by which active oxygen production is inhibited in neutrophils treated with EM or AZM differs from that of macrolides in the serum. It is suggested that neutrophils are stimulated by some unknown mediator secreted from PMA-stimulated mononuclear cells, but not by PMA. From our results that inhibitory rates of active oxygen production by neutrophils were not significantly different at low concentration between presence and absence of mononuclear cells pretreated with EM or AZM, these macrolides seem to directly inhibit active oxygen generation by neutrophils at low concentrations, regardless of the presence or absence of pretreated mononuclear cells. However, at high concentrations their direct effect on neutrophils is reduced. And we postulate that mononuclear cells activated by PMA produce and secrete some unknown substance and that the synthesis of that substance is suppressed by treatment with EM or AZM at high concentrations.

In conclusion, we suggest that EM and AZM, at low concentrations which are within therapeutic doses, act not only as antibiotics, but also as anti-inflammatory agents by directly reducing active oxygen production by neutrophils, regardless of the presence of mononuclear cells or their secreted mediator. The direct in vitro effect of EM and AZM on active oxygen generation by neutrophils, may be responsible at least in part, for the therapeutic efficacy of these 14-membered and 15-membered ring macrolides in patients with DPB.

REFERENCES


