Suppressive activity of a macrolide antibiotic, roxithromycin, on pro-inflammatory cytokine production in vitro and in vivo

H. Suzaki1, K. Asano2, CA, S. Ohki1, K. Kanai1, T. Mizutani1 and T. Hisamitsu2

1Department of Otolaryngology; and 2Department of Physiology, School of Medicine, Showa University, 1–5-8 Hatanodai, Shinagawa-ku, Tokyo 142–8555, Japan

CA Corresponding Author
Tel: (+81) 3 3784 8110
Fax: (+81) 3 3784 5368

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Introduction

Sinobronchial syndrome involves the coexistence of chronic rhinosinusitis, and chronic lower airway inflammation such as chronic bronchitis, bronchiectasis and diffuse panbronchiolitis. In the treatment and management of the diseases, we use macrolide antibiotics such as erythromycin, clarithromycin and roxithromycin (RXM), and satisfactory results are obtained when the patients are given the agents in one-half the recommended therapeutic doses for more than 6 weeks.1 Although investigation of the mechanisms of improvement has suggested that it is not due to the anti-microbiological effects of the drugs,2,3 the precise mechanisms are not well understood.

Many reports clearly demonstrated that the macrolide antibiotics, such as erythromycin and RXM, strongly inhibit chemotaxis and generation of oxygen radicals by polymorphonuclear leukocytes when the cells were cultured in vitro in the presence of the macrolides.4–6 It is generally recognized that T cells, especially helper T cells, play an important role in the initiation and development of inflammatory diseases through their secretion of several types of cytokines. Recently, we have reported that RXM strongly inhibits the production of inflammatory cytokines, interleukin (IL)-3 and IL-4, from human peripheral blood T cells in response to in vitro stimulation with concanavalin A (Con A).7 This inhibitory action of RXM on T cell cytokine production was also observed in vivo:8 the ability of spleen cells to produce inflammatory T cell cytokines such as IL-5 was significantly suppressed when the cells were prepared from RXM-pretreated mice and cultured in vitro with Con A. From these reports, it is reasonable to speculate that orally administered macrolide antibiotics inhibit inflammatory responses via suppression of T cell cytokine production and results in an improvement of the clinical status of the patients with sinobronchial syndrome.

Macrophages play an important role in modulating the inflammatory response, by acting as scavenger cells for damaged tissue components. These cells also produce several inflammatory cytokines, especially IL-1β and tumor necrosis factor (TNF)-α.9 However, the effect of RXM on macrophages remains unclear.
We therefore performed the present study to answer unresolved questions regarding the favorable effects of macrolide antibiotics on inflammatory diseases by examining the influence of RXM on macrophage cytokine production in vitro and in vivo.

Materials and methods

Mice
Specific pathogen-free, 5-week-old, male BALB/c mice were purchased from Charles River Japan Inc. (Atsugi, Japan). To prevent bacterial and fungal airway infection, they were housed in filter-barriered cages and given autoclaved food and tap water ad libitum throughout the experiments.

Drugs
RXM was kindly donated from Hoechst Marion Roussel Co., Ltd., as a water-insoluble pure powder. The agent was dissolved in methyl alcohol at 50 mg/ml and diluted with phosphate buffered-saline (PBS), so as to give a concentration of 1.0 mg/ml. This solution was then filtered through a 0.22 μm filter (Nihon Millipore Corp., Yonezawa, Japan) and stored as a stock solution at 4°C until use. For in vitro use, all dilutions were prepared from this stock solution by diluting with RPMI-1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES, 2 × 10^{-5} M 2ME (RPMI-FCS). Metabolized RXM, RU28111, RU39001, RU44981 and RU45179 were also kindly supplied by Hoechst Marion Roussel Co., Ltd., as water-insoluble pure powders. They were dissolved in RPMI-FCS, as in the case of RXM.

Preparation and purification of human peripheral blood monocytes
Heparinized peripheral blood was obtained from six healthy donors who gave written informed consent. The blood was diluted an equal amount of 0.9% NaCl and overlayers on Lymphocytes Separation Medium (Organon Teknika, Durham, NC, USA). After centrifugation at 1500 rpm for 30 min at 25±2°C, the medium–plasma interface, which contained mononuclear cells, was collected. The cells located in this fraction were washed five times at 600 rpm at 25±2°C to remove platelets. The resultant cell suspension was transferred into plastic culture plates and allowed to attach for 1 h at 37°C. The plates were then washed vigorously three times with RPMI-FCS to remove non-adherent cells. The adherent cells were collected by scraping with a rubber policeman, washed once, resuspended in RPMI-FCS at a concentration of 2 × 10^6 cells/ml, and used as human peripheral blood monocytes (PBMC). The viability of cell population exceeded 98% as judged by trypan blue exclusion, and peroxidase staining revealed that more than 95% of cells were monocytes.

Preparation of culture supernatant
An aliquot of cells (1.0 ml) was introduced into each well of 24-well plates containing 1.0 ml of various concentrations of agents and 2.0 μg/ml lipopolysaccharide (LPS) extracted from Klebsiella pneumoniae (Sigma Pure Chemicals, St. Louis, MO, USA). After incubation for 24 h, the supernatant was collected after pelleting the cells by centrifugation at 2000 rpm for 10 min at 25±2°C. The supernatant was stored frozen at −40°C until assayed for cytokine concentration.

Treatment of mice with RXM
Mice were given 2.5 mg/kg RXM once a day for 5, 7, 9, and 12 weeks via a stomach tube. In this experiment, the daily dose was one-half of the recommended therapeutic dose in a patient weighing 60 kg. The control mice orally received PBS alone.

Treatment of mice with LPS
LPS (Sigma Pure Chemicals) was dissolved in PBS and sterilized by passing through a 0.22 μm filter. Mice were lightly anesthetized intraperitoneally with 0.5 mg pentobarbital, and a middle incision was performed above the sternum. The trachea was exposed by blunt dissection, a 28-gauge needle was inserted into the trachea above the carina, and 0.1 ml PBS containing various concentrations of LPS was injected. Control mice received a similar volume of sterile PBS alone.

Preparation of aqueous airway and lung extract
Mice were administered an intraperitoneal injection of pentobarbital at a dose of 60 mg/kg body weight, 24 h after LPS injection. Airway and lungs were removed and inflated with 1.0 ml PBS and homogenized in 2.0 ml PBS using a glass tissue homogenizer for 60 s in an ice-cold water bath. The homogenates were then centrifuged at 2000×g for 60 min at 4°C. The supernatants were collected and used for aqueous airway and lung extract (ALE).

Assay for cytokines
The activity of IL-1β and TNF-α in both culture supernatants and ALE were examined using commercially available mouse cytokine enzyme-linked immunosorbent assay (ELISA) Test Kits (GenZyme Corp., Cambridge, MA, USA). The ELISA was performed in duplicate according to the manufacturer’s recommended procedure.
Statistical analysis

A statistical evaluation of the data was made with the Mann–Whitney U test.

Results

Inhibitory effects of RXM on cytokine secretion from PBM in vitro

To examine the influence of RXM on cytokine secretion from PBM in response to LPS stimulation in vitro, PBM were cultured in the presence of various concentrations of RXM. As shown in Fig. 1, RXM caused a dose-dependent reduction of both IL-1β (A) and TNF-α (B) secretion from PBM in response to LPS stimulation in vitro. This suppression was first observed when the cells were cultured in the presence of RXM at a concentration of as little as 0.05 μg/ml. Since it is reported that RXM administered orally into animals analyzed into four different metabolized materials, the next experiments were designed to examine whether metabolized RXM could also suppress cytokine secretion from PBM. The data in Fig. 2 clearly show that metabolized RXM could suppress cytokine secretion from PBM in a dose-dependent manner. It is also indicated that suppressive activity of metabolized RXM on IL-1β secretion (Fig. 2A) is stronger than that on TNF-α secretion (Fig. 2B).

FIG. 1. Influence of RXM on pro-inflammatory cytokine production from human PBM in response to LPS stimulation in vitro. The cells (2×10^6/ml) were cultured in the presence of various concentrations of RXM. The culture supernatants stimulated with 1.0 μg/ml LPS were harvested 24 h later. IL-1β (A) and TNF-α (B) concentrations were assayed by ELISA. Each data is the mean percent of control (without agent)±SD of six different subjects.

FIG. 2. Influence of metabolized RXM on pro-inflammatory cytokine production from human PBM in response to LPS stimulation in vitro. The cells (2×10^6/ml) were cultured in the presence of various concentration of metabolized RXM. The culture supernatants stimulated with 1.0 μg/ml LPS were harvested 24 h later. IL-1β (A) and TNF-α (B) concentrations were assayed by ELISA. Each data is the mean percent of control (without agent) of six different subjects. □: RU28111, ■: RU39001, ○: RU44981, ●: RU45179.
Influence of RXM on cytokine appearance in vivo

This study was designed to examine the in vivo effects of RXM on cytokine secretion. To do this, the first set of experiments was carried out to investigate the influence of intratracheal infusion of LPS on cytokine levels in ALE. The optimal concentration of LPS for secretion of cytokines into ALE was first examined by quantitation of IL-1β. The data in Fig. 3 show that as the concentration of LPS instilled is increased, the level of IL-1β in ALE also increases. The maximum level of IL-1β in ALE was observed with the use of 1.0 μg/ml LPS, and the higher concentration was inhibitory (Fig. 3). The kinetics of IL-1β appearance in ALE was examined in the next experiments. Mice were instilled intratracheally with 1.0 μg/ml LPS in a volume of 0.1 ml. ALE was prepared from five individual mice various hours post-instillation. Control ALE from five PBS-instilled mice was similarly prepared. LPS instillation caused a rapid increase in IL-1β level, reaching a maximum 24 h later, followed by a slow decrease (Fig. 4). The final set of experiments was undertaken to examine the influence of RXM on cytokine appearance in ALE. Mice were orally administered with 2.5 mg/kg RXM once a day for various periods. During the course of RXM treatment, mice were intratracheally instilled with 1.0 μg/ml LPS, and ALE was prepared 24 h after LPS infusion.

As shown in Fig. 5A, short-term (within 5 weeks) administration of RXM into mice did not influence the appearance of IL-1β in ALE induced by LPS stimulation. On the other hand, long-term (more than 7 weeks) administration of RXM dramatically suppressed the appearance of IL-1β in ALE. The data in
Fig. 5B clearly show that the influence of RXM administration on TNF-α appearance in ALE is similar to that in IL-1β: the level of TNF-α in ALE was markedly suppressed when donor mice were treated orally with RXM for more than 7 weeks, but not within 5 weeks.

**Discussion**

The present results clearly demonstrate that RXM and metabolized RXM strongly inhibit cytokine secretion from human PBM in response to mitogenic stimulation *in vitro*, and that the minimum concentration of agents that cause inhibition of cytokine secretion is as little as 0.05 μg/ml (Figs 1 and 2).

Pharmacological studies revealed rapid and complete absorption of RXM administered orally, resulting in high plasma levels and in prolonged half-life, since the macrocyclic of RXM is locally modified to prevent inactivation in gastric juice. It is also reported that, after oral administration, plasma levels of RXM were gradually increased and attained a plateau at a concentration of approximately 10.0 μg/ml. RXM in plasma was then analyzed into four different metabolized materials, and metabolized materials except for two types, RU44981 and RU45179, are excreted into urine and feces, suggesting that an increase in unmetabolized and hardly excreted metabolized RXM may occur progressively. Together with the present results, it may be suggested that RXM inhibits human monocyte activation, especially cytokine secretion, and helps to favorably modify the clinical conditions of patients with sinonasal syndrome. However, before drawing the conclusion that the ability of RXM to inhibit cytokine secretion is responsible for the beneficial effect of macrolides on sinonasal syndrome, it is necessary to examine whether suppressive activity of RXM on cytokine secretion could also be observed *in vivo*.

Endotoxins, LPS components of gram-negative bacteria, are known to be important stimuli in the development of inflammatory responses. Pauwels *et al.* reported that inhalation of aerosolized endotoxin causes pulmonary inflammatory responses in rats. It has also been reported that endotoxins can stimulate various inflammatory cells, including macrophages, and may induce the release of a variety of inflammatory mediators, such as IL-1β and TNF-α. Therefore, we used an endotoxin/mouse system and carried out the next experiments. The data in Figs 3–5 clearly demonstrate that intratracheal injection of LPS causes the appearance of inflammatory cytokines in airways and lungs, which was inhibited by pretreatment of mice with RXM. These results may suggest that RXM orally administered into mice inhibits inflammatory cytokine secretion in airways and results in attenuating effects on sinonasal syndrome.

Although the present results suggest that macrolide antibiotics inhibit macrophage activation, especially cytokine secretion, and result in a favorable modification of the clinical status of patients with sinonasal syndrome, the mechanisms by which RXM inhibits cytokine secretion *in vitro* and *in vivo* are not clear at present. It is possible that RXM binds to receptors such as immunophilin, which is the intracellular receptor of immunosuppressive agents FK-506 and rapamycin, and the complexes interfere with cytokine gene transcription, resulting in inhibition of cytokine secretion. This suggestion may be supported by our previous experiments showing that RXM could suppress mRNA expression of TNF-α in human peripheral blood monocytes when the cells
were cultured in vitro with the agent. Furthermore, our unpublished data also revealed the inhibitory action of RXM on macrophage growth and maturation from its precursors in vitro, suggesting that inhibition of in vivo macrophage cytokine secretion by RXM may be due to, at least partly, its suppressive activity on macrophage growth. In any case, more in-depth analysis of the relationship between inhibition of secretion of inflammatory mediators by macrolides and inflammatory responses should allow us to identify the precise mechanisms involved in the therapeutic mode of action of macrolides on sino-bronchial syndrome.

References


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