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Shin-ichi Konno^a
Kazuhito Asano^b
Masatsugu Kurokawa^a
Kazuhito Ikeda^b
Ken-ichi Okamoto^b
Mitsuru Adachi^a

^a 1st Department of Internal Medicine, and

^b Department of Medical Biology,
School of Medicine, Showa University,
Tokyo, Japan

Antiasthmatic Activity of a Macrolide Antibiotic, Roxithromycin: Analysis of Possible Mechanisms in vitro and in vivo

Key Words

Aqueous lung extract
Bronchial responsiveness
Cytokine secretion
Endotoxin
Human peripheral blood
Leukocytes
Roxithromycin

Abstract

This study was designed to examine the possible mechanisms by which macrolide antibiotics favorably influence the clinical course of asthmatic patients. In the first set of experiments, we investigated the effect of roxithromycin (RXM), a newly synthesized macrolide antibiotic, on in vitro cytokine secretion by mitogen-activated human peripheral blood leukocytes. RXM suppressed the secretion of T cell cytokine interleukins (IL) 2-4 and monocyte cytokine tumor necrosis factor alpha. This inhibitory effects on cytokine secretion was dose dependent and firstly noted at a concentration of as little as 0.5 µg/ml which is much lower than therapeutic blood levels. In the second part of experiments, we examined the influence of RXM on cytokine appearance in mouse lung extract induced by lipopolysaccharide (LPS) inhalation and on bronchial responsiveness to methacholine in LPS-treated mice. As compared with mice pretreated with phosphate-buffered saline, RXM administered orally at a single dose of 5 mg/kg once a day for 21 days inhibited the appearance of IL-3, IL-4, IL-5, and tumor necrosis factor alpha in aqueous lung extracts. Pretreatment with RXM also decreased the bronchial responsiveness to methacholine induced by intratracheal injection of LPS. We conclude that the attenuating effect of macrolide antibiotics on asthmatic syndromes might be explained partially by their inhibitory effects on cytokine secretion from leukocytes.

Introduction

There is much evidence that airway hyperresponsiveness is a characteristic feature of bronchial asthma and that airway inflammation with predominant infiltration of mast cells and eosinophils plays an important role in the development of asthma [1-3]. Antiallergic drugs such as disodium cromoglycate [4], azelastine [5] and ketotifen [6] are

well known to have favorable effects on asthma. Beneficial effects of these drugs are considered to be dependent partially on anti-inflammatory actions on airway inflammations [7]. Several studies have shown that macrolide antibiotics such as erythromycin and troleandomycin can favorably influence the clinical status of certain patients with asthma [8-12]. Although investigation of the mechanisms of improvement has suggested that it is not due to the anti-

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Correspondence to: Dr. Shin-ichi Konno
1st Department of Internal Medicine
Showa University, School of Medicine
1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142 (Japan)

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microbiological effects of the drug [8, 12], the precise mechanisms are not well understood.

Systemic administration of erythromycin have been shown to be effective in the treatment of inflammatory diseases such as acne, rosacea, and dermatitis herpetiformis [13, 14]. The efficacy of this therapy of these inflammatory dermatoses is believed generally to be due to its anti-inflammatory effects [13, 14]. Furthermore, a lot of reports clearly demonstrated that the macrolide antibiotics erythromycin, troleandomycin, and RU 28965 (roxithromycin; RXM) strongly inhibit chemotaxis and generation of inflammatory mediators (O_2 and H_2O_2 , etc.) by polymorphonuclear leukocytes when the cells were cultured *in vitro* in the presence of macrolides [15–17]. From these reports, it is reasonable to speculate that orally administered macrolide antibiotics inhibit airway inflammation and result in an improvement of the clinical status in certain asthmatic patients. However, there is little information about a relationship among anti-inflammatory actions of macrolide antibiotics and the mechanisms of a favorable influence on the clinical status of asthmatic patients.

Inflammatory responses with eosinophilia, mast cell replenishment, and IgE production observed in asthma patients are now known to be mediated by helper T lymphocytes and their products, so-called cytokines: helper T lymphocytes, upon contact with appropriate antigens, activate and then release cytokines that control establishment and regulation of inflammatory responses. It is also well known that macrophages, through their secretion of interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF- α) are important in the mediation of inflammatory responses.

Therefore, we performed the present study to answer unresolved questions regarding the favorable effects of macrolide antibiotics on asthma by examining the effects of RXM on cytokines, which are known to influence the pathogenesis of asthma, and on the secretion from human peripheral blood leukocytes *in vitro*. Furthermore, we evaluated effect of RXM on the broncheal responsiveness (BR) to methacholine (Mch) in mice which inhaled endotoxin, since this agent in experimental animals caused airway inflammation which is considered to be closely linked to the development of BR [18–20].

Materials and Methods

Mice

Specific pathogen-free 5-week-old male BALB/c mice were purchased from Charles River Japan (Atsugi, Japan). After arrival at the Department of Medical Biology, they were housed in filter-barriered cages, and autoclaved food (Oriental Kobo Kogyo, Tokyo, Japan) and

tap water were given *ad libitum* to prevent fungal and bacterial airway infection.

Drugs

RXM was kindly supplied by Morishita-Roussel (Osaka, Japan) as a water-insoluble pure powder. The agent was dissolved in methyl alcohol at 50 mg/ml and diluted with RPMI-1640 (Flow Laboratories, Irvine, UK) supplemented with 10 mM HEPES, 2×10^{-5} M 2ME, 10% fetal calf serum (Flow Laboratories, North Ride, Australia), 100 U/ml penicillin, and 100 μ g/ml streptomycin (RPMI-FCS), so as to give a concentration of 1.0 mg/ml. This solution was then filtered through a 0.22- μ m filter (Nihon Millipore, Yonezawa, Japan) and stored as a stock solution at 4°C until use. All dilutions used in this study were prepared from this stock solution by diluting with RPMI-FCS. Metabolized RXM, RU 28111, RU 39001, RU 44981, and RU 45179 were kindly supplied by Morishita-Roussel, as water-insoluble pure powders. They were dissolved in RPMI-FCS as in the case of RXM. The chemical structures of these agents are shown in figure 1.

Preparation and Purification of Human Peripheral Blood Leukocytes (PBL)

Heparinized peripheral blood was obtained from 5 healthy donors who gave written informed consent. The blood was diluted twice with 0.9% NaCl and overlaid on lymphocyte separation medium (Organon Technika, Durham, N.C., USA). After centrifugation at 1,500 rpm for 30 min at $25 \pm 2^\circ\text{C}$, the medium-plasma interface, which contained mononuclear cells, was collected. The cells located in this fraction were washed five times at 600 rpm for 10 min at $25 \pm 2^\circ\text{C}$ to remove platelets. The resultant cells were suspended to RPMI-FCS at a concentration of 2×10^6 cells/ml and used as PBL. To purify PBL, the cell suspensions were transferred to plastic culture plates (No. 1-43982; Nunc, Denmark) and allowed to attach for 1 h at 37°C . The plates were then washed thoroughly three times with RPMI-FCS to remove nonadherent cells. The adherent cells were collected by scraping with rubber policeman, washed once, resuspended in RPMI-FCS at a concentration of 2×10^6 cells/ml, and used as monocytes. Peroxidase staining revealed that more than 95% of the adherent cells were monocytes. Nonadherent cells were fractionated into T and B cells by panning [21]. The cells (2×10^6 /ml) were placed into plastic Petri dishes (No. 150326; Nunc) coated with monoclonal antibody to CD22 (Immunotech, France; final volume 5.0 ml). After incubation at 37°C for 30 min, the dishes were gently rotated for 30 s, and nonadherent cells were then poured off. The cells were washed once and reincubated in a second set of antibody-coated dishes for 30 min at 37°C in order to deplete adherent cells. Nonadherent cells from the second set of dishes were removed, washed, resuspended in RPMI-FCS at a concentration of 2×10^6 cells/ml, and used as purified T cell population. The viability of the cell population exceeded 98%, as judged by trypan blue exclusion. The purity of the cells was checked by incubating separated cells with monoclonal antibody to CD7 (Immunotech) followed by fluoresceinated antimouse IgG antibody (Immunotech). Fluorescence microscopic examination revealed that more than 98% of the nonadherent cells were CD7 positive (T cells).

Cell Culture

An aliquot of PBL (0.1 ml) was introduced into each well of a 96-well flat-bottomed microculture plate (Nunc) that contained 0.1 ml of various concentration of agents and 20.0 μ g/ml concanavalin A (ConA; Sigma Chemicals, St. Louis, Mo., USA). The plate was maintained at 37°C , 5% CO_2 in air, and 90% humidity. After 40 h, $1.0 \mu\text{Ci}$

pressure. The respiratory resistance was determined by the method of Konzett and Rossler as described by Burden and Parkes [22] from the transpulmonary pressure, air flow, and respiratory volumes measures. The BR to Mch (Sigma) was measured by intravenous administration of increasing quantities of Mch (10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} , and 10^{-2} mol) in a volume of 0.1 ml.

Assay for Cytokines

IL-2, IL-3, IL-4, and TNF- α activities in culture supernatants were assayed using a commercially available human cytokine enzyme-linked immunosorbent assay test kit (Amersham). IL-3, IL-4, IL-5, and TNF- α in ALE were also assayed by a mouse cytokine enzyme-linked immunosorbent assay test kit purchased from Genzyme. This assay was performed in duplicate according to the method described previously [23]. The cytokine activities in culture supernatant and in ALE were expressed as percent of control and as picograms per milliliter, respectively.

Statistical Analysis

Statistical differences between the data were established by employing the Mann-Whitney U test.

Results

Inhibitory Effects of RXM on PBL Activation

To examine influence of RXM on PBL activation, cells were cultured in the presence or absence of RXM. The cell activation was assayed by examining ^3H -TdR uptake into DNA and by quantitation of cytokine levels in culture supernatants. Typical results of 2 out of 5 different subjects are shown in figure 2 and demonstrate that RXM and metabolized RXM could not inhibit cell activation as assessed by ^3H -TdR uptake which was nearly identical in experimental and in control animals ($p < 0.05$). Although the activation of T cells by antigens or mitogens has been measured routinely by assays that measure the rate of incorporation of radiolabeled precursors into newly synthesized DNA, there is much evidence that quantitation of cytokines is a more sensitive indicator of T cell activation than measuring the precursor uptake [23–25]. We, therefore, examined the influence of RXM on cytokine secretion from PBL in response to mitogenic stimulation. The data shown in figure 3 clearly indicate that RXM caused a reduction in cytokine secretion. This suppression was observed when the cells were cultured in the presence of RXM at a concentration as little as 0.5 $\mu\text{g}/\text{ml}$. The fourth experiment was carried out to examine whether metabolized RXM could also inhibit cytokine secretion from PBL. As also shown in figure 3, metabolized RXM strongly inhibited the cytokine secretion. This inhibition occurred in a dose-dependent manner, most significant at 10.0 $\mu\text{g}/\text{ml}$.

Influence of RXM on Cytokine Appearance in vivo

To examine the in vivo effect of RXM on cytokine secretion, BALB/c mice which were treated with RXM once a day for 21 days were intratracheally injected with 1.0 $\mu\text{g}/\text{ml}$ LPS, and ALE was prepared 24 h after LPS injection. As shown in table 1, ALE prepared from noninjected mice contained undetectable levels of IL-3, IL-4, and IL-5 (< 3 , < 15 , and < 5 pg/ml, respectively). The TNF- α level in control ALE was detectable only (table 1). Intratracheal injection of LPS resulted in the appearance of high levels of cytokines in ALE. The most striking effects of LPS injection on cytokine appearance was observed in the TNF concentration (table 1). The results in table 1 also indicate that oral administration of RXM into mice significantly suppressed the appearance of cytokines in ALE in response to LPS injection ($p < 0.05$).

Influence of RXM on the BR to the Mch in Mice Injected Intratracheally with LPS

To examine the influence of RXM on BR, mice orally administered with RXM were injected intratracheally with 1.0 $\mu\text{g}/\text{ml}$ LPS, and BR to Mch was assayed 24 h after LPS injection. As shown in figure 4, LPS injection caused significant changes in the BR to Mch as compared with PBS-injected mice: the minimum concentration of Mch to cause a significant increase in air overflow in experimental mice was 10^{-4} mol Mch and that in control mice was 10^{-3} mol Mch ($p < 0.05$). Pretreatment of mice with RXM strongly inhibited airway changes to 10^{-4} mol Mch: the volume of air overflow in nontreated mice was 0.14 ± 0.01 ml and that in RXM-treated mice was 0.03 ± 0.01 ml (table 2).

Effect of Anti-TNF- α Antibody Injection on Airway Changes in Mice following LPS Injection

Since intratracheal injection of mice with LPS causes an increase in both several types of cytokines in ALE and BR to Mch, the present study was undertaken to examine type of cytokine(s) responsible for BR to Mch. The results in table 3 clearly indicate that TNF- α is an important mediator in BR to Mch: pretreatment of LPS-injected mice with anti-TNF antibody completely blocked LPS-induced increase in Mch responsiveness.

Discussion

Macrolide antibiotics are used therapeutically in the chronic management and the treatment of asthma [8–11]. However, the mechanisms by which macrolide antibiotics modify the clinical status of asthma are not well understood.

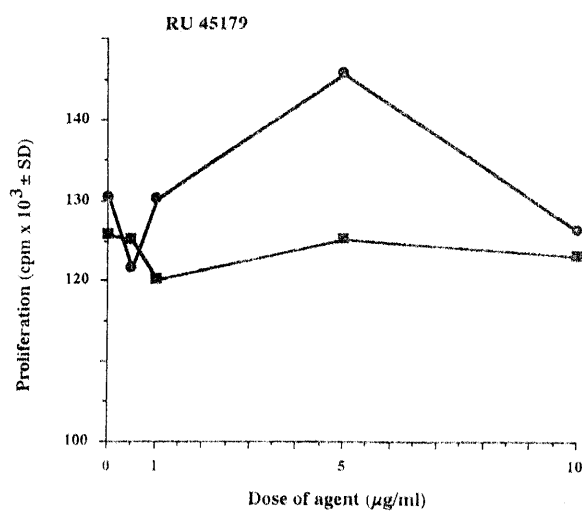
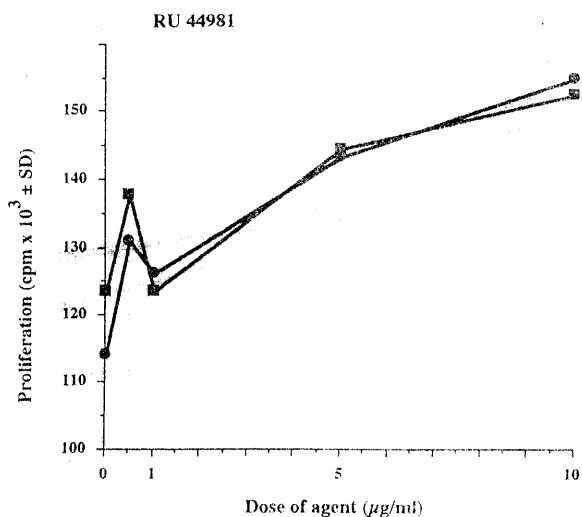
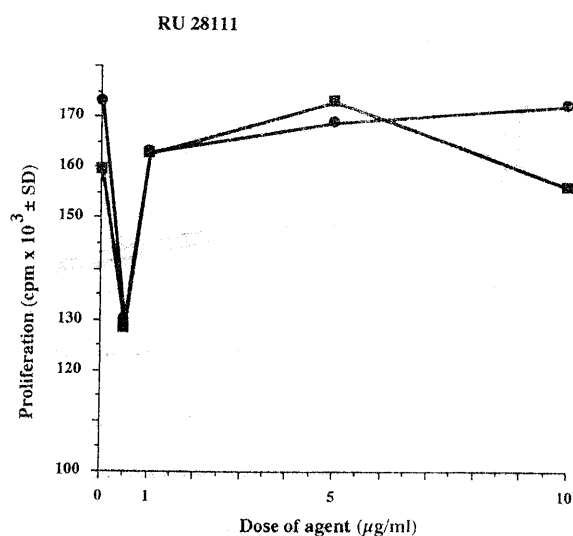
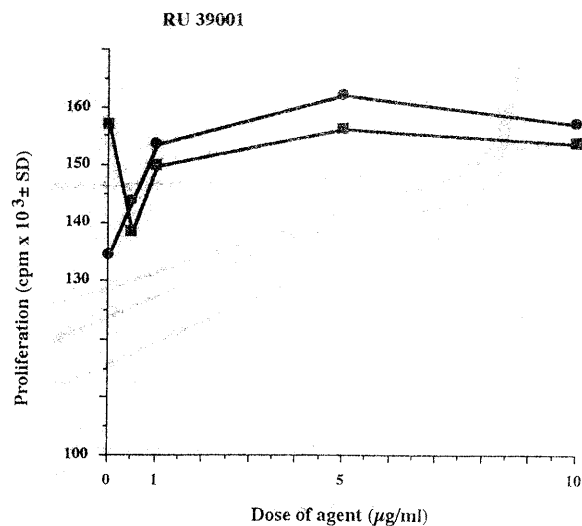
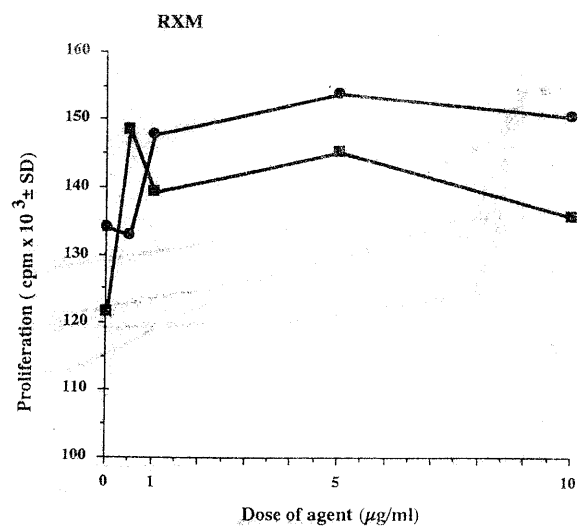


Fig. 2. Influence of RXM on ConA-induced proliferation of human PBL in vitro. PBL ($2 \times 10^5/0.1$ ml) were cultured in the presence of various concentrations (indicated) of RXM and metabolized RXM. The cells were pulsed with $1.0 \mu\text{Ci } ^3\text{H}$ -thymidine for the last 8 h of the 48-hour culture. Mean values $\pm$ SD from triplicate cultures of 2 out of 5 different subjects are shown. Vertical bars showing the SD are omitted to clarify. ● = subject no. 1, ■ = subject no. 2.

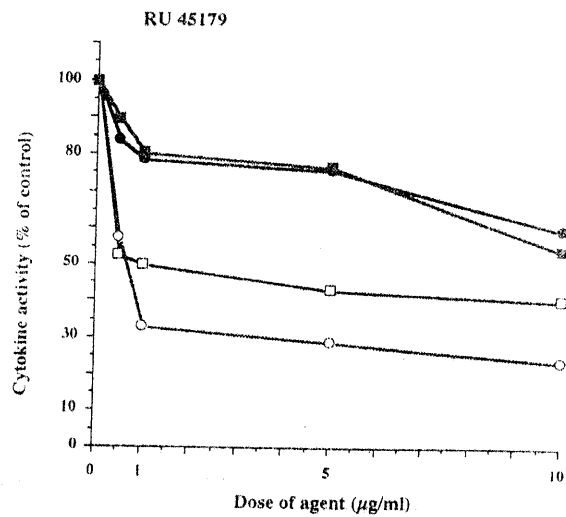
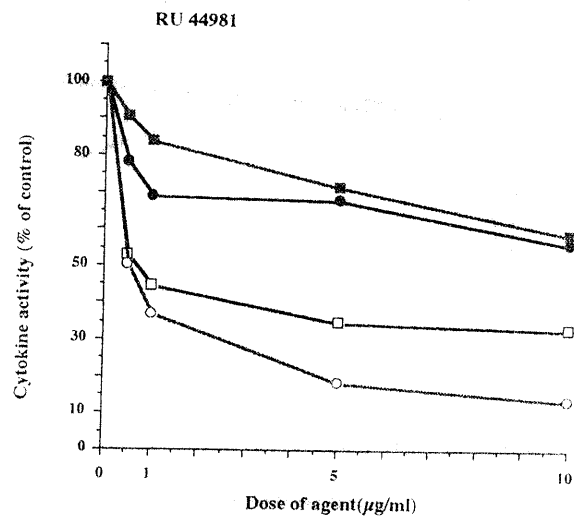
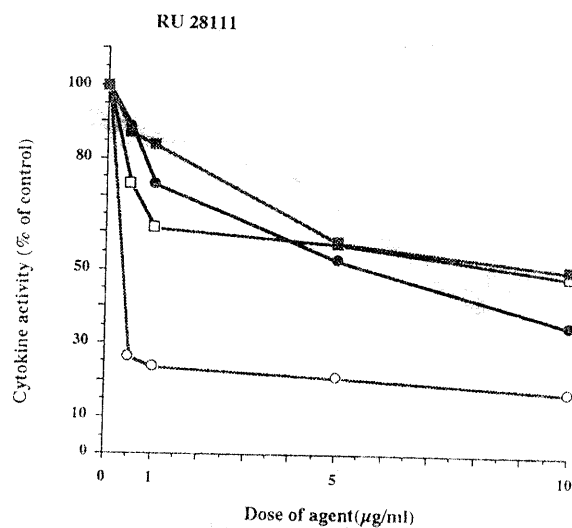
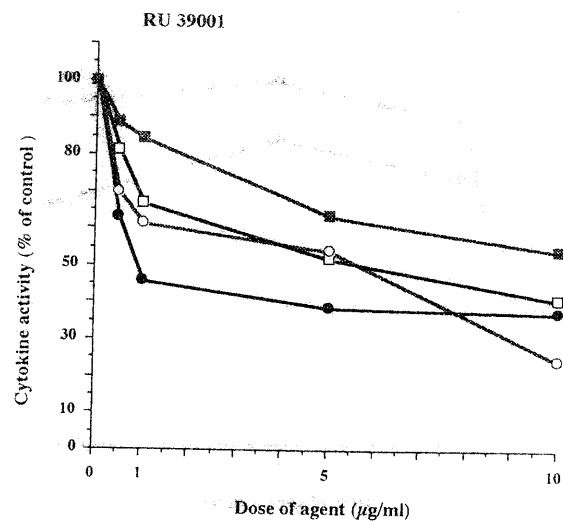
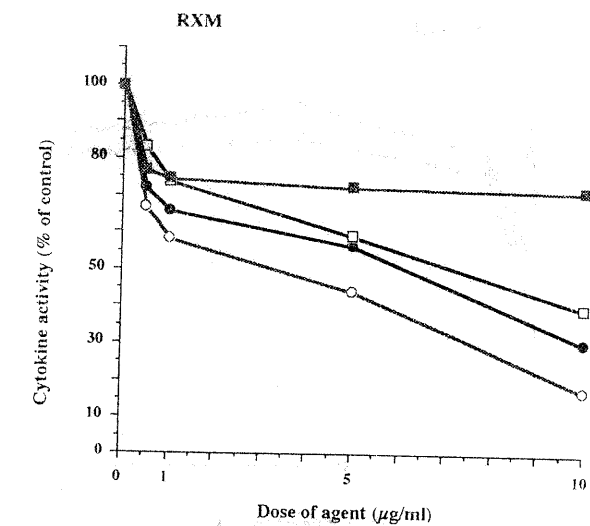


Fig. 3. Influence of RXM on mitogen-induced cytokine secretion from human peripheral blood monocytes and T cells. The cells ($2 \times 10^6/\text{ml}$) were cultured in the presence of various concentrations (indicated) of RXM and metabolized RXM. T cell culture supernatants stimulated with ConA were harvested at 48 h for interleukin IL-2 (●), IL-3 (□), and IL-4 (○) assays. Monocyte culture supernatants stimulated with LPS were harvested at 24 h for TNF- α (■) assay. Data are mean of % of control (without agents) of 5 different subjects.

Table 1. Inhibition of cytokine appearance in aqueous lung extract prepared from BALB/c mice orally treated with RXM (mean $\pm$ SD)

Group	Cytokine activity, pg/ml			
	IL-3	IL-4	IL-5	TNF- α
Nontreated	ND	ND	ND	15.57 $\pm$ 13.9
LPS	8.48 $\pm$ 2.07	100.63 $\pm$ 3.15	20.00 $\pm$ 6.99	5,316.30 $\pm$ 652.0
LPS + RXM	2.91 $\pm$ 0.79	75.65 $\pm$ 6.62	7.50 $\pm$ 1.27	2,229.00 $\pm$ 409.30

Each group consisted of 6 animals; ND = not determined.

Table 2. Influence of RXM on bronchial changes to 10^{-4} mol Mch in BALB/c mice following LPS injection

Group	Air overflow, ml
Normal controls	0.02 $\pm$ 0.01
LPS	0.14 $\pm$ 0.01
LPS + RXM	0.03 $\pm$ 0.01

Each group was consisted of 6 animals.

Table 3. Influence of anti-TNF- α antibody injection on airway changes in BALB/c mice following LPS injection (mean $\pm$ SD)

Group	TNF- α^a , pg/ml	Air overflow, ml
PBS-injected	ND	0.05 $\pm$ 0.01
Anti-TNF-injected	ND	0.04 $\pm$ 0.01
LPS	5,781.20 $\pm$ 313.9	0.14 $\pm$ 0.01
LPS + anti-TNF- α	1,650.40 $\pm$ 621.0	0.03 $\pm$ 0.01
LPS + RXM	2,532.50 $\pm$ 520.0	0.06 $\pm$ 0.01

One representative experiment of two.

Each group consisted of 5 animals; ND = not determined.

^a TNF- α concentration in aqueous lung extracts.

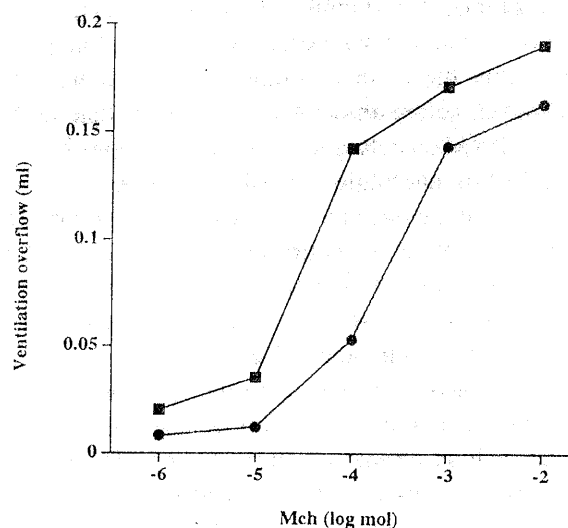


Fig. 4. BR to Mch in BALB/c mice. The BR was measured after intravenous injection of various concentrations (indicated) of Mch. Mean values $\pm$ SD of 5 individual mice of one of two different experiments which gave reproducible results. Vertical bars showing SD are omitted to clarify. $\bullet$ = PBS-injected mice; $\blacksquare$ = LPS-injected mice.

It is accepted that T cells (especially helper T cells) associated with the development of asthma directly respond to allergens and elaborate cytokines of IL-3 and IL-4, among others [26, 27]. These cytokines and IL-2 are believed to be involved in severity and length of the diseases [28, 29]. Recently, RXM was analyzed and used therapeutically, since its effects are quite similar to that of erythromycin. We previously reported that oral administration of RXM into mice once a day for 1 week inhibits their capacity of splenic T cells to produce IL-5 when the cells were stimulated in vitro with ConA [9]. This report may suggest that macrolide anti-

biotics administered orally into asthmatic patients inhibit cytokine secretion from T cells and result in favorable effects on asthma. In the first experiments, therefore, we examined the influence of RXM on in vitro cytokine secretion from human peripheral blood leukocytes. The present results clearly demonstrate that RXM and metabolized RXM strongly inhibit cytokine secretion from human monocytes and T cells in response to mitogenic stimulation in vitro and that the minimum amount of agents that cause inhibition of cytokine secretion is as little as 0.5 μ g/ml. Pharmacokinetics of RXM are characterized by rapid and complete absorp-

tion after oral administration, 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 of RXM, plasma levels of agent was gradually increased and attained a plateau at a concentration of about 10.0 µg/ml [30]. RXM in plasma were then metabolized into four different metabolized materials (fig. 1) and metabolized materials except for two types of them such as RU 44981 and RU 45179, are excreted into urine and feces [30], suggesting that increase in unmetabolized and hardly excreted metabolized RXM may occur progressively. Judging from these reports and the present results, it is reasonable to speculate that macrolide antibiotic, RXM inhibits human monocyte and T cell activation, especially cytokine secretion, and helps to favorably modify the clinical conditions of patients with asthma. However, before drawing the conclusion that inhibitory action of RXM on cytokine secretion is responsible for beneficial effect of macrolides on asthma, it is necessary to examine the following questions: (1) whether inhibitory effect of RXM on cytokine secretion could be also observed *in vivo*, and (2) whether this inhibitory effect might be contribute to therapeutic mode of action of macrolides on asthma.

Endotoxin, LPS components of gram-negative bacteria are known to be one of important stimuli in the development of asthma. Potential sources of airborne endotoxin in our environment include domestic water, air humidifiers, and house dusts [18–20]. Organic dusts have also been shown to be contaminated by endotoxin [18, 20]. Both in normal and asthmatic subjects, inhalation of endotoxin has been reported to cause an increase in BR [31]. It is also reported that local administration of endotoxin to airways in experimental animals causes pulmonary inflammatory responses with an increase in BR [18–20]. Therefore, we used an endotoxin-in-mouse system and carried out a second part of experiments to answer the questions described above. The data shown in table 1 clearly demonstrate that intratracheal injection of LPS results in the appearance of inflammatory cytokines in airways and lungs which was inhibited by pretreatment with RXM. An increase in BR to Mch observed in LPS-injected mice is also diminished by pretreatment with RXM (table 2). These results strongly suggest that RXM orally administered into mice inhibits inflammatory cytokine production in airways and results in a therapeutic effect on LPS-induced increase in BR to Mch. LPS is known to be a very potent stimulus of TNF synthesis and release from macrophages and monocytes. TNF- α has especially have been proposed as a major mediators of endotoxin-induced airway changes [19, 20]. In the present study, pretreatment with RXM partially blocked not only appearance of TNF in

ALE, but also an increase in BR to Mch (tables 1 and 2). Likewise, pretreatment with anti-TNF antibody inhibits the LPS-induced increase in Mch responsiveness (table 3). These results strongly indicate that the protective effect of RXM on BR may be due to its suppressive effect on TNF- α release and that TNF- α mediates at least partly the increase in responsiveness occurring after LPS administration.

Although the present results suggest that macrolide antibiotics inhibit lymphocyte and macrophage activation especially cytokine secretion and result in favorable modification of clinical status of asthmatic patients, more in-depth analysis of the relationship between inhibition of secretion of inflammatory mediators by macrolides and airway changes should allow us to identify the precise mechanisms involved in the therapeutic mode of action of macrolides on asthma. Furthermore, 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 immunophilin, intracellular binding protein, and the complexes interferes cytokine gene transcription, resulting in inhibition of cytokine secretion. Because FK-506 and rapamycin, macrolide antibiotics extracted from the fermentation broth of *Streptomyces* spp, exert their immunosuppressive effects by formation of complex between immunophilin and agent which inhibits cytokine gene transduction [32, 33]. Anyway, further experimentation is needed to clarify this point.

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