**Fourteen-Membered Ring Macrolides Inhibit Vascular Cell Adhesion Molecule 1 Messenger RNA Induction and Leukocyte Migration**

*Role in Preventing Lung Injury and Fibrosis in Bleomycin-Challenged Mice*

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**Background and objective:** Although the pathogenesis of interstitial pneumonia and pulmonary fibrosis are not well understood, it has been reported that inflammatory cells, especially neutrophils, and the injurious substances produced by them play important roles in the progression of interstitial pneumonia and subsequent fibrosis. Erythromycin and other 14-membered ring macrolides (14-MRMLs) have been reported to improve the survival of patients with diffuse panbronchiolitis by antineutrophil and several other anti-inflammatory mechanisms. The present study was undertaken to investigate the effects of 14-MRMLs on an experimental model of bleomycin-induced acute lung injury and subsequent fibrosis in mice.

**Methods:** Bleomycin was administered IV to ICR mice. At 28 days after bleomycin injection, fibrotic foci were histologically observed in left lung tissues, and hydroxyproline content in right lung tissues was chemically analyzed. The inhibitory effects of 14-MRMLs were assessed by overall comparison between control (normal saline solution [NS] alone), untreated (bleomycin alone), and treated (bleomycin plus 14-MRMLs) groups. For evaluation of early-phase inflammation, cell populations in BAL fluid and induction of messenger RNA (mRNA) of adhesion molecules (E-selectin, P-selectin, intercellular adhesion molecule 1 [ICAM-1], and vascular cell adhesion molecule 1 [VCAM-1]) in lung tissues were examined at 0 to 13 days after bleomycin treatment. These parameters were also compared with those for the control (NS alone), 14-MRML untreated (bleomycin alone), and 14-MRML pretreated (bleomycin plus 14-MRML pretreated) groups.

**Results:** Bleomycin-induced pulmonary fibrosis was inhibited by erythromycin and other 14-MRMLs on day 28 after bleomycin injection in ICR mice, especially those pretreated with 14-MRMLs. Hydroxyproline content in lung tissues was also decreased in the 14-MRML-pretreated groups. The number of neutrophils in BAL fluid significantly increased, with two peaks at 1 day and 9 days (from 6 to 11 days) after bleomycin administration. 14-MRMLs significantly inhibited both peaks of neutrophil infiltration into the airspace. Changes in mRNA expression of adhesion molecules (E-selectin, P-selectin, ICAM-1, VCAM-1) were associated with leukocyte migration into the airspace. 14-MRMLs clearly inhibited the induction of VCAM-1 mRNA, and tended to attenuate that of ICAM-1 mRNA, but inhibited the induction of neither E-selectin mRNA nor P-selectin mRNA.

**Conclusion:** These findings indicate that attenuation of inflammatory cell migration into the airspace by 14-MRMLs, especially of neutrophils and macrophages, resulted in inhibition of lung injury and subsequent fibrosis. 14-MRMLs clearly attenuated the expression of VCAM-1 mRNA during the early phase of bleomycin-induced lung injury, and this might be one mechanism of inhibition of neutrophil and macrophage migration into the airspace by 14-MRMLs. This may be one mechanism of the anti-inflammatory and antifibrotic effects of 14-MRMLs. These findings suggest that prophylactic administration of 14-MRMLs may be clinically efficacious in preventing acute exacerbation of interstitial pneumonia and acute lung injury.

**Key words:** bleomycin; lung fibrosis; macrolide; vascular cell adhesion molecule 1

**Abbreviations:** AG = arabic gum; BALF = BAL fluid; cDNA = complementary DNA; G6PD = glucose-6-phosphate dehydrogenase; ICAM-1 = intercellular adhesion molecule 1; IFP = idiopathic pulmonary fibrosis; MMP = matrix metalloproteinase; 14-MRMLs = 14-membered ring macrolides; mRNA = messenger RNA; NS = normal saline solution; PCR = polymerase chain reaction; RT = reverse transcriptase; VCAM-1 = vascular cell adhesion molecule 1
bleomycin-induced lung injury and subsequent fibrosis in animals is a widely used experimental model of acute lung injury and fibrosis in humans.1–3 The mechanisms of interstitial pneumonia and pulmonary fibrosis are not understood in detail, but it has been found that neutrophils and the injurious substances they produce play important roles in the progression of pulmonary fibrosis.4–7 We found that E-selectin, acting as a key molecule in the initial phase of neutrophil adhesion to vascular endothelial cells, played an essential role in the progression of pulmonary fibrosis.8 Adhesion molecules, including selectins and integrins, are required for neutrophil migration into lung tissues. The pathologic features of acute lung injury include prominent accumulation of neutrophils and macrophages in the lung parenchyma.9,10

In patients with diffuse panbronchiolitis receiving oral erythromycin, increase in number of neutrophils in BAL fluid (BALF) was significantly reduced.11 Erythromycin and other 14-membered ring macrolides (14-MRMLs) have been reported to improve the survival of patients with diffuse panbronchiolitis by several anti-inflammatory mechanisms.11,12

We previously reported that the number of neutrophils and the amount of neutrophil elastase in BAL played important roles in the acute lung injury induced by bleomycin, and that both were decreased by treatment with erythromycin.7 In this study, we examined the inhibitory effects of 14-MRMLs including erythromycin, clarithromycin, and roxithromycin on bleomycin-induced pulmonary fibrosis following acute lung injury. We further investigated the role of adhesion molecules in neutrophil adhesion to endothelial cells, subsequent migration of neutrophils into lung tissue and progression of pulmonary fibrosis, and elucidated the mechanisms by which 14-MRMLs exhibit anti-inflammatory effects and oppose the fibrosis that follows inflammation in bleomycin-challenged mice.

**MATERIALS AND METHODS**

**Animals**

Male, 7-week-old ICR mice (Nippon CLEA; Tokyo, Japan), weighing 30 g each on average, were randomly classified into groups.

**Materials**

Bleomycin (Nippon Kayaku; Tokyo, Japan) was dissolved in normal saline solution (NS) and administered IV to ICR mice at a dosage of 100 mg/kg (0.3 mL per mouse). Erythromycin, 50 mg/kg (Dainabott; Osaka, Japan); clarithromycin, 8.9 mg/kg (Dainabott); and roxithromycin, 10 mg/kg (Aventis Pharma; Tokyo, Japan) were suspended in 5% arabic gum (AG) [Wako Pure Chemical Industries; Tokyo, Japan]. Solutions, 0.3 mL per mouse, were orally administered by force with a microtube daily to ICR mice.

**Schedule and Process of Evaluation of Late-Phase Fibrosis**

The bleomycin-Unpretreated Groups included the NS-treated group (group 1), and 14-MRML-alone-treated groups (erythromycin, clarithromycin, roxithromycin) [group 2].

The bleomycin-Treated Groups included the 14-MRML-unpretreated group (bleomycin alone) [group 3]; 14-MRML-pretreated groups (day –3 to day 13) [bleomycin plus pretreatment erythromycin, bleomycin plus pretreatment clarithromycin, and bleomycin plus pretreatment roxithromycin] [group 4]; and 14-MRMLs-posttreated groups (day 3 to day 19) [bleomycin plus posttreatment erythromycin, bleomycin plus posttreatment clarithromycin, and bleomycin plus posttreatment roxithromycin] [group 5].

NS was administered IV to bleomycin-unpretreated mice (day 0). Bleomycin was administered IV to bleomycin-treated mice (day 0); AG was orally administered every day to mice of groups 1 and 3 (day –3 to day 13). 14-MRML was orally administered daily to mice of groups 2 and 4 (day –3 to day 13) and mice of group 5 (day 3 to day 19). Mice in all groups were killed under ether anesthesia at day 28 after bleomycin or NS injection. Fibrotic foci were assessed histologically in left lung tissues, Ashcroft scores were compared among the groups, and hydroxyproline content in right lung tissues was chemically analyzed.

**Schedule and Evaluation of Early-Phase Inflammation**

NS was administered IV to NS-treated group mice (day 0). Bleomycin was administered IV to bleomycin-treated group mice (day 0). AG was orally administered daily to mice of the NS-alone and bleomycin-alone-treated groups from day –3 until the time of death. 14-MRMLs were administered daily to the 14-MRML-pretreated groups from day 3 until the time of death. Mice of the NS-alone and bleomycin-alone groups were killed under ether anesthesia at 0, 6 h, or 12 h, or 1, 2, 3, 5, 6, 7, 9, 11, or 13 days after bleomycin or NS injection. All groups were examined for cell population in BALF and induction of messenger RNA (mRNA) of adhesion molecules (E-selectin, P-selectin, intercellular adhesion molecule 1 [ICAM-1], and vascular cell adhesion molecule 1 [VCAM-1]) in lung tissues by reverse transcriptase (RT)-polymerase chain reaction (PCR) at 0 to 13 days after bleomycin or NS injection. Mice of the 14-MRML-pretreated groups were killed under ether anesthesia at 1 day and 9 days after bleomycin injection and underwent the same experiment. We repeated the same experiment three times in early-phase evaluation, and examined the cell population in BALF. The total number of mice was 10 in each group for each time point. RT-PCR examination was repeated three times.

**Histologic Analysis**

For histologic examination, we added 0.5 mL of 10% formalin with 10 cm H2O of pressure to the left lobe of every mouse; 10% formalin-fixed lung tissues were embedded in paraffin. Paraffin-
sections were stained with either hematoxylin-eosin or Masson trichrome stain, and systematically scanned with a light microscope using a 20 × 10 objective. We compared severities of interstitial fibrosis among the groups by the score of Ashcroft et al.13

Hydroxyproline Measurement

The right lung was freeze-dried for 3 days using a Tray Dryer (FTS Systems; Stoneridge, NY) as soon as possible after the animals were killed. The total collagen content of the right lung was determined by hydroxyproline assay;14 After acid hydrolysis of the right lung with 12 N HCl at 100°C for 20 h in a sealed glass tube (Iwaki; Tokyo, Japan), hydroxyproline content was determined by high-performance liquid chromatography.

Analysis of BALF

BALF was obtained by injection of 1 mL of saline solution (three times; total, 3 mL) followed by gentle aspiration of the fluid from the right and left lungs after securing the intratracheal catheter within a main bronchus. With this catheter, recovery ratios of lavage fluids ranged from 65 to 75% and did not significantly differ among the groups. Total cell numbers in BALF were counted with a hemocytometer. For differential counts of leukocytes in BALF, cytospin (Labsystems; Tokyo, Japan) smear slides were prepared. The smears were stained with Giemsa solution (Merck; Tokyo, Japan). Differential cell counts of leukocytes in BALF, cytospin (Labsystems; Tokyo, Japan) smear slides were prepared. The smears were stained with Giemsa solution (Merck; Tokyo, Japan). Differential cell counts were performed on 200 cells per smear.

Quantitative Analysis of Adhesion Molecule mRNA by RT-PCR

Total RNA was extracted from each specimen of lung tissue using ISOGEN (Nippon GENE; Tokyo, Japan). Single-strand complementary DNA (cDNA) was synthesized using RT (Gibco BRL; Rockville, MD) and oligo (dT)12–18 primer. To measure gene expression, we performed the PCR method, with cDNA amplified using a TaKaRa PCR Thermal Cycler 480 and TaKaRa Ex Taq (Takara Shuzo; Otsu, Japan). For amplification of the desired cDNA, the following gene-specific primers were used:15 E-selectin, sense (EL-11) 5’-gattgga-cactcaatgtcag-3’, antisense (EL-9) 5’-eetggatcttgtaaagggc-3’; P-selectin, sense 5’-aegagtgaatgacgccg-3’, antisense 5’-gget-ggctcaatttaacag-3’; ICAM-1, sense 5’-tcggag gatcacaaacgaagc-3’, antisense 5’-scctttgaaaccgatcaac-3’. The PCR products underwent electrophoresis on 2% agarose gels, stained with ethidium bromide, and observed with ultraviolet transillumination. Intensity analysis of the bands was performed with Adobe Photoshop 5.0 (Adobe Systems; Tokyo, Japan), and expression of adhesion molecule messenger RNA was determined by high-performance liquid chromatography.

Statistical Analysis

Differences between two groups (grade of fibrosis, hydroxyproline, leukocyte counts) were tested for significance using the Mann-Whitney U test. Repeated-measures analysis of variance was used to evaluate differences in leukocyte counts among groups over time.

Results

Histopathologic Assessment of Late-Phase Pulmonary Fibrosis:

Bleomycin-induced lung fibrosis was significantly inhibited by treatment with erythromycin, clarithromycin, and pretreatment with roxithromycin at day 28 after bleomycin injection in ICR mice. Comparison of Ashcroft scores revealed significant difference between pretreatment and posttreatment with 14-MRMLs (Fig 1). A typical picture of attenuation of fibrosis is shown in Figure 2. Administration of 14-MRMLs alone resulted in no remarkable changes in results of histopathologic assessment of lung tissue (data not shown).

Hydroxyproline Content in Lung Tissue:

The concentration of hydroxyproline at day 28 after bleomycin injection was significantly higher in the bleomycin-alone group than in the NS-alone group. Of the groups pretreated with 14-MRML, hydroxyproline was significantly reduced only then in the bleomycin-alone group. Hydroxyproline was not significantly attenuated in the groups posttreated with 14-MRMLs. Comparison of hydroxyproline revealed a significant difference between pretreatment and posttreatment with 14-MRMLs (Fig 3).

Time Course of Changes in Cell Number in BALF After Treatment With Bleomycin

Total cell number in BALF peaked at 6 to 13 days after bleomycin injection (Fig 4, top, A, a). The

![Figure 1. Histopathologic assessment of late-phase pulmonary fibrosis in bleomycin-challenged mice; comparison of Ashcroft score. Significantly different from bleomycin alone-treated group by the Mann-Whitney U test, *p < 0.05, **p < 0.01. Significantly different from 14-MRML-pretreated groups by the Mann-Whitney U test, #p ≤ 0.05. Values are means; bars = SD, n = 10. N = NS-treated group; B = bleomycin-alone-treated group; BE = bleomycin- and erythromycin-treated group; BC = bleomycin- and clarithromycin-treated group; BR = bleomycin and roxithromycin-treated group.](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21985/ on 06/27/2017)
number of neutrophils in BALF peaked twice, from 12 h to 2 days and from 5 to 11 days after bleomycin injection, and returned to control level at 13 days after bleomycin injection (Fig 4, top, A, b). The number of macrophages in BALF was maximal at 6 to 11 days after bleomycin injection (Fig 4, top, A, c). The number of lymphocytes in BALF was significantly increased at 9 to 13 days after bleomycin injection (Fig 4, top, A, d).

Effects of 14-MRMLs on Leukocyte Number in BALF

14-MRMLs significantly decreased the number of neutrophils in BALF at 1 day and 9 days after injection of bleomycin (Fig 4, bottom, B, a). The increase in number of macrophages in BALF at 9 days after bleomycin injection was significantly attenuated by erythromycin and roxithromycin, and slightly attenuated by clarithromycin (Fig 4, bottom, B, b). The number of lymphocytes in BALF at 9 days after bleomycin injection was significantly attenuated by erythromycin and clarithromycin, and slightly attenuated by roxithromycin (Fig 4, bottom, B, c).

Time Course of Changes in mRNA Expression of Adhesion Molecules in Lung Tissue After Treatment With Bleomycin

E-selectin (Fig 5, top, A, a), P-selectin (top, A, b), and ICAM-1 (top, A, c) mRNAs were induced at 6 to 12 h, decreased to control levels at 1 day, and were induced for the second time 2 to 13 days after bleomycin injection. VCAM-1 mRNA (Fig 5, top, A, d) was induced 2 to 13 days after bleomycin injection.

Inhibitory Effect of 14-MRML on mRNA Expression of VCAM-1 and ICAM-1

14-MRMLs clearly inhibited the induction of VCAM-1 mRNA (Fig 5, bottom, B, d) and tended to attenuate ICAM-1 mRNA expression (Fig 5, bottom, B, c) at day 9 after bleomycin. However, 14-MRMLs markedly inhibit the induction of neither E-selectin (bottom, B, a) nor P-selectin mRNAs (Fig 5, bottom, B, b).
Figure 4. Top, A: Time course of changes in cell numbers in BALF (upper left, a: total cell number; upper right, b: No. of neutrophils; lower left, c: No. of macrophages; lower right, d: No. of lymphocytes). Significantly different from NS-treated group at each time point by the Mann-Whitney U test, $p \leq 0.0004$, $p < 0.01$. Significantly different from the first time point (0 h) after bleomycin (BLM) administration by repeated-measures analysis of variance, $***p < 0.0001$, $**p < 0.01$, $*p < 0.05$. Values are means; bars = SE, n = 10. Bottom, B: Effects of 14-MRMLs on leukocyte number in BALF on day 1 and day 9 after injection of bleomycin (top, a: No. of neutrophils in BALF on days 1 and 9 after injection of bleomycin; bottom left, b: No. of macrophages in BALF on day 9 after injection of bleomycin; bottom right, c: No. of lymphocytes in BALF on day 9 after injection of bleomycin). Significantly different from bleomycin alone-treated group by the Mann-Whitney U test, $**p < 0.01$, $*p < 0.05$. Values are means; bars = SE, n = 10. See Figure 1 for expansion of abbreviations.
Figure 5. Top, A: Time course of E-selectin (upper left, a), P-selectin (upper right, b), ICAM-1 (lower left, c), and VCAM-1 (lower left, d) mRNA inductions in lung tissue after bleomycin administration. Bottom, B: Effects of 14-MRMLs on expression of adhesion molecules such as E-selectin (upper left, a), P-selectin (upper right, b), ICAM-1 (lower left, c), and VCAM-1 (lower right, d) mRNA in lung tissue on day 9 after bleomycin administration. These photographs show typical results. Each density of PCR products was measured using Adobe Photoshop 5.0; G6PD was measured as an internal control. The ratio of each adhesion molecule against G6PD is shown by histogram. bp = base pair; see Figure 1 legend for expansion of abbreviations.
**DISCUSSION**

Idiopathic pulmonary fibrosis (IPF) is the prototype of many other interstitial lung disorders in which parenchymal inflammation is a typical feature; such disorders number > 100. Although the pathogenesis of pulmonary fibrosis remains unclear, many investigators have found that neutrophil-mediated lung injury occurs prior to initiation and progression of the fibrogenic process.\(^{20,21}\) Neutrophil adhesion to vascular endothelial cells is an important process in cell-mediated lung injury, along with release of proteases and free-radical formation.\(^4,7\) Inhibition of production of injurious substances (e.g., neutrophil elastase by ONO5046),\(^5\) other proteases by truncated secretary leukocyte protease inhibitor,\(^9\) and by α-1 protease inhibitor\(^10\) is a promising method for prevention of pulmonary fibrosis. The initial phase of neutrophil migration prior to activation in lung parenchyma is an important point of intervention in strategies that are effective in preventing tissue injury and subsequent fibrosis. We previously reported that E-selectin played an essential role in neutrophil adhesion to vascular endothelial cells and subsequent lung fibrosis.\(^8\)

We initially investigated bleomycin-induced histopathologic changes in lung tissues. Bleomycin-induced pulmonary fibrosis at day 28 was significantly inhibited by pretreatment with 14-MRMLs after bleomycin administration (Figs 1–3). The effectiveness of pretreatment with 14-MRMLs (beginning 3 days before bleomycin injection) is significantly higher than that of posttreatment with 14-MRMLs (beginning 3 days after bleomycin injection) [Figs 1–3]. The difference of potency for inhibitory effects between pretreatment and posttreatment with 14-MRML may have depended on the difference of their inhibitory effects of neutrophil recruitment in lung tissue, especially to the first peak (from 12 h to 2 days after bleomycin injection) of neutrophil migration. Development of pulmonary fibrosis is thought to include two phases: an acute inflammatory phase and a sequential fibrotic phase.\(^22\) To determine the mechanisms of the prophylactic effect of 14-MRMLs on bleomycin-induced pulmonary fibrosis in mice, we further investigated the mechanisms of the early inflammatory (i.e., up-stream) phase.

We found two peaks of neutrophil concentration in BALF. The two peaks of neutrophil recruitment paralleled but were slightly delayed compared with induction of E-selectin, P-selectin, and ICAM-1 mRNAs. The second peak and the induction of VCAM-1 mRNA were also parallel with slight delay (Fig 4, top, A, b, and Fig 5, top, A).

α4/β1 integrin, acting as a ligand for VCAM-1, is believed to contribute to migration of macrophages, eosinophils, and lymphocytes into inflamed tissues.\(^18,23\) Some investigators have reported that neutrophil adhesion to vascular endothelial cells depends principally on α9/β1-VCAM-1 interaction.\(^24\) In the setting of leukocyte-endothelial interactions, there are diverse mechanisms that modulate leukocyte integrin avidity. Crosslinking of the E-selectin and P-selectin counter-structures, CD15 (sialyl Le\(^a\)), or its sialylated form on neutrophils caused activation.\(^25\) Therefore, we considered the possibility that neutrophils bound selectins in the initial phase, and that simultaneously α9/β1 integrin was activated by selectins, with tight binding of neutrophils to VCAM-1, which might have induced the second neutrophil peak. We also considered the possibility that 14-MRMLs attenuated induction of VCAM-1 on vascular endothelial cells binding to neutrophils due to inhibition of the second neutrophil peak.

We also investigated the inhibitory effects of 14-MRMLs on bleomycin-induced lung injury to evaluate the early inflammatory phase in the same experimental model in mice. 14-MRMLs clearly inhibited the expression of VCAM-1 mRNA (Fig 5, bottom, B, d), and significantly inhibited the second neutrophil peaks after injection (Fig 4, bottom, B, a) in bleomycin-induced lung injury. We therefore considered the possibility that 14-MRMLs attenuated induction of VCAM-1 on vascular endothelial cells binding to neutrophils due to inhibition of the second neutrophil peak.

14-MRMLs tended to attenuate the expression of ICAM-1 mRNA (Fig 5, bottom, B, c). We therefore considered the possibility that attenuation by 14-MRMLs of the expression of ICAM-1 mRNA was related to inhibition of the two peaks of neutrophil recruitment. Lung injury may have depended on macrophage antigen 1 and lymphocyte function-associated antigen 1 integrin on neutrophils acting as ligands for ICAM-1 on vascular endothelial cells. This might be one mechanism of inhibition of neutrophil migration into the airspace by 14-MRMLs.

The number of macrophages changed in parallel with slight delay for the induction of VCAM-1 mRNA in the present study (Fig 4, top, A, c, and Fig 5, top, A, d). VCAM-1 mRNA induction was mainly associated with movement of macrophages into the airspace. We considered the possibility that 14-MRMLs inhibited binding of α4/β1 on macrophages to VCAM-1 on vascular endothelial cells due to inhibition of movement of macrophages of ligand expression, which requires further examination.

These findings suggested that attenuation of inflammatory cell migration by 14-MRMLs, especially of neutrophils and macrophages, resulted in inhib-
tion of lung injury and subsequent fibrosis. Inhibition by 14-MRMLs of VCAM-1 mRNA expression might be one mechanism of inhibition of neutrophil and macrophage migration into the airspace by 14-MRMLs, and subsequent fibrotic effects of 14-MRMLs. Inhibition of adhesion of leukocytes to endothelial cells may be useful for prevention of leukocyte-mediated lung injury and subsequent fibrosis, including IPF in humans. 14-MRMLs are thus promising agents for acute exacerbation of interstitial pneumonia, acute lung injury, and prophylaxis of progression of IPF.

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