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Macrolide Antibiotics Protect Against Immune Complex-Induced Lung Injury in Rats: Role of Nitric Oxide from Alveolar Macrophages

Jun Tamaoki, Mitsuko Kondo, Kazuhiro Kohri, Kazutetsu Aoshiba, Etsuko Tagaya, and Atsushi Nagai

Macrolide antibiotics have unique immunomodulatory actions apart from antimicrobial properties. We studied the effects of macrolides on IgG immune complex (IgG-ICx)-induced lung injury in rats in vivo and in vitro. Intrapulmonary deposition of IgG-ICx produced a time-dependent increase in the concentration of NO in exhaled air. There were corresponding increases in the number of neutrophils accumulated into alveolar spaces, and lung wet-to-dry weight ratio. All of these changes were inhibited by pretreatment with erythromycin or josamycin, but not by amoxicillin or cephalor. Incubation of cultured pulmonary alveolar macrophages caused up-regulation of NO production and expression of inducible NO synthase mRNA, an effect that was dose dependently inhibited by erythromycin, roxithromycin, or josamycin. The macrolides also reduced IgG-ICx-induced release of IL-1β and TNF-α, but did not alter the release of NO induced by exogenously added IL-1β and TNF-α. These results suggest that macrolide antibiotics specifically inhibit immune complex-induced lung injury presumably by inhibiting cytokine release and the resultant down-regulation of inducible NO synthase gene expression and NO production by rat pulmonary alveolar macrophages. The Journal of Immunology, 1999, 163: 2909–2915.

Nitrergic is generated from the amino acid l-arginine by NO synthase (NOS), which exists in both constitutive and inducible isoforms. In the respiratory tract, endogenous NO plays a role in the regulation of airway and vascular smooth muscle tone, microvascular permeability, neurotransmission, and host defense (1). Apart from these regulatory actions, NO also exerts deleterious effects when it is inappropriately generated or overproduced, and excessive amounts of NO and its metabolites such as peroxynitrite anion and hydroxyl radical may contribute to the pathophysiology of inflammation and the resultant tissue damage (1–3). Constitutive form of NOS is localized to several cell types, including vascular endothelial cells, nerve cells, and airway epithelial cells (4), indicating that these cells are constitutively generating NO. On the other hand, the production of NO is up-regulated in pulmonary inflammatory diseases (5–7), in which the cell sources of NO may be pulmonary alveolar macrophages (PAM) and airway epithelial cells that contain inducible NOS (iNOS).

There is increasing evidence that macrolide antibiotics have a variety of biological actions in addition to their antimicrobial properties. For instance, erythromycin and roxithromycin inhibit cytokine production and chloride secretion from airway epithelial cells (8, 9), expression of adhesion molecules on neutrophils (10), and neurotransmission in vagal nerve fibers (11). However, it is unknown whether macrolides affect NO generating system. Because lung injury induced by immune complex may depend on l-arginine (12), we hypothesized that, if macrolides could inhibit the release of NO, then the drugs might protect against lung injury in this model. Therefore, objective of the present study was to determine the effects of macrolide antibiotics on immune complex-induced lung injury and, if so, to elucidate whether the effect is associated with alterations in NO generation. To accomplish this goal, we studied lung histopathology and exhaled NO concentration in the rats following intrapulmonary deposition of IgG immune complex (IgG-ICx) in vivo, and the release of NO and expression of iNOS mRNA by the rat PAM incubated with IgG-ICx in vitro.

Materials and Methods

Reagents

Except where noted, all reagents were purchased from Sigma (St. Louis, MO). Rabbit polyclonal IgG rich in anti-BSA was obtained from Organon Teknika (Westchester, PA). Anti-iNOS mAb was obtained from Transduction Laboratories (Lexington, KY). All antibiotics were generous gifts from pharmaceutical companies, as follows: erythromycin (DAINABOT, Tokyo, Japan); josamycin (Yamamoto Pharmaceutical, Tokyo, Japan); roxithromycin (Hoechst Marion Rousell Pharmaceutical, Tokyo, Japan); amoxicillin (Takeda Pharmaceutical Industries, Osaka, Japan); and cefaclor (Shionogi Pharmaceutical, Osaka, Japan).

Animal model

All experimental procedures were approved by the Animal Research Committee of Tokyo Women’s Medical University. Pathogen-free male Sprague Dawley rats, weighing 200–220 g, were obtained from SLC Japan (Hamamatsu, Japan) and used in this study. The rats were given a macrolide antibiotic, erythromycin, or josamycin at a dose of 50 mg/kg, or its vehicle (0.9% sterile saline) 3 h before induction of lung injury. Freshly prepared diluted macrolide or vehicle was administered in 0.5 ml bol by oral gavage with a 21-gauge feeding tube fitted to a 1-ml syringe. To test whether other antibiotics can also affect lung injury, the rats were given...
amoxicillin, a penicillin derivative, or cephacar, a cephalosporin derivative, at a dose of 50 mg/kg in a similar manner.

The rats were anesthetized i.p. with sodium pentobarbital (40 mg/kg) and were given supplemental doses as needed. The blood samples were taken from right femoral vein, and the plasma concentration of erythromycin was measured by a HPLC (15). The larynx and upper trachea were exposed, and the trachea just below the larynx was incised. To induce IgG-ICx-induced lung injury, the rats were given intratracheal instillation of rabbit polyclonal IgG rich in anti-BSA (2.5 mg in 0.3 ml saline) and the subsequent i.v. injection of BSA (10 mg in 0.1 ml saline), as has been described previously (12). In the control group, the same volumes of saline alone were consecutively given. An intubation tube was then inserted into the trachea, and the rats were artificially ventilated (frequency, 60 breaths/min; tidal volume, 10 ml/kg) with a volume-cycled ventilator (model SN-480-7; Shinano, Tokyo, Japan).

Measurement of exhaled NO concentration

At 1, 3, 6, 12, 24, and 36 h after the application of IgG-ICx, exhaled air was collected over a 10-min period by connecting the expiratory port of the ventilator to a 3-L nondiffusing gas collection bag (Sasaki Medical, Tokyo, Japan). To measure the concentration of NO in the exhaled air, 50-ml aliquots of the collected gas were introduced into a chemiluminescence analyzer (model 200-A; Riken Keiki, Tokyo, Japan) through a modified purging chamber, which was continuously flushed with nitrogen at a flow rate of 50 ml/min. The electrical signals were amplified and integrated to measure the area under the curve with an integration time of 2 s. The NO analyzer was calibrated by injecting different volumes of a certified NO gas (10 ppm balanced nitrogen; Yamato Sanki, Tokyo, Japan) with establishment of a linear calibrated scale (range, 2-100 pmol of NO; r² = 0.99) of NO amount (pmol) vs analyzer output. The NO concentration in the exhaled air was expressed as ppb and calculated as the quotient of the NO amount (pmol) and the volume of exhaled air (pmol/100 ml).

Lung inflammation and injury

To assess lung inflammation, 24 h after administration of IgG-ICx, the rats were anesthetized with sodium pentobarbital, and the right cranial lobe was fixed by inflating with 10% Formalin. Sagittal sections were cut for embedding in paraffin. Sections 5 μm thick were taken from the blocks and stained with hematoxylin-eosin, and the number of neutrophils was counted in the effect of macrolides, we measured the cytokine concentration in the medium bathing cultured PAM. The concentration of IL-1β was determined using a murine IL-1β ELISA kit (BioSource, Camarillo, CA), and TNF-α activity was determined using a standard LM cell cytotoxicity assay, as previously described (18).

To assess NO release, the concentration of NO in the medium was determined by an NO meter (Model NO-501; Inter Medical, Tokyo, Japan) by measuring a redox current between the following two electrodes (19). A counter electrode was made of carbon fiber, and an NO-selective working electrode was made of PtIr alloy (0.2 mm in diameter; Pt 90%, Ir 10%) coated with a three-layered membrane that consisted of KCl, NO-selective resin, and normal silicone membranes. The KCl membrane was electrochemically deposited on the PtIr wire to suppress overvoltage in the discharge. The NO-selective resin was made of puroxyline lacquer and coated by immersion of the wire in 0.8% nitrocellulose solution and dried under air for 12 h. The silicon membrane was affixed as the outermost membrane to avoid a nonspecific ionic effect and electrochemical reactions. The working electrode was supplied with 0.4–0.8 M for the electrochemical oxidation of NO. The NO diffusing through the membrane was detected as an electrical current based on the following reaction: NO + 2H₂O → NO₃⁻ + 4H⁺ + 3e⁻. The current flow was proportional to the rate of diffusion through the membrane, which was, in turn, proportional to the concentration of NO at the outer surface of the membrane.

Cytokine production and NO generation by PAM

Previous study has shown that immune complex can stimulate both PAM and type II alveolar epithelial cells to produce NO, in which the former cells may be a predominant source of NO (17). We thus focused on PAM as a predominant source of NO (17). We thus focused on PAM as a predominant source of NO (17). The cytokines IL-1β and TNF-α may play a role in immune complex-induced NO generation by PAM (17). Therefore, to assess possible involvement of IL-1β and TNF-α in the effect of macrolides, we measured the cytokine concentration in the medium bathing cultured PAM. The concentration of IL-1β was determined using a murine IL-1β ELISA kit (BioSource, Camarillo, CA), and TNF-α activity was determined using a standard LM cell cytotoxicity assay, as previously described (18).

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Cytokine production and NO generation by PAM

All values are expressed as means ± SEM and analyzed on a Macintosh computer using the Statview 4.5 statistical software package (Abacus Concepts, Berkeley, CA). Student’s t test was performed by a t-test assuming a normal distribution, and analysis was performed by Jonckheere’s test for multiple comparison. Student’s t test was used for comparison between two groups. A p value of less than 0.05 was considered statistically significant.
Results

Plasma concentration of erythromycin

Immediately before intrapulmonary deposition of IgG-ICx, the concentration of erythromycin in the rat plasma was $4.4 \pm 0.8 \times 10^{-6} \text{ M}$ ($n = 58$).

Exhaled NO concentration

The changes in NO concentration in the exhaled air of six groups of rats are shown in Fig. 1. In the control group, exhaled NO concentration remained unchanged at least for 36 h from the baseline value of $9.1 \pm 0.5 \text{ ppb}$ ($n = 12$). In the animals subjected to immune complex, exhaled NO concentration increased 3-fold after 6 h of IgG-ICx deposition, which reached a plateau level of $43.3 \pm 4.9 \text{ ppb}$ ($p < 0.001$ vs control, $n = 12$) after 12 h and remained elevated thereafter. Oral administration of erythromycin (50 mg/kg) reduced the IgG-ICx-induced increase in exhaled NO concentration, the maximal level of NO concentration being only $16.9 \pm 2.1 \text{ ppb}$ ($p < 0.001$ vs IgG-ICx alone, $n = 12$). Josamycin at a same dose likewise attenuated the effect of IgG-ICx on exhaled NO, whereas amoxicillin and cephaclor were without effect.

Lung inflammation and injury

Lung histopathology and W/D weight ratio were assessed 24 h after IgG-ICx. As shown in Fig. 2, the typical features of IgG-ICx-induced lung reaction were margined neutrophils in blood vessels and the presence of neutrophils, red cells, macrophages, and strands of fibrin in alveolar spaces. The number of neutrophils present in alveoli was increased in the IgG-ICx-treated rats. The rats pretreated with erythromycin or josamycin had fewer neutrophils in the alveoli than the IgG-ICx alone animals did, but pretreatment with amoxicillin or cephaclor did not significantly alter the IgG-ICx-induced neutrophil accumulation (Fig. 3, upper panel). The lung W/D weight ratio was increased by IgG-ICx from $5.31 \pm 0.19$ to $6.78 \pm 0.23$ ($p < 0.01$, $n = 8$). Administration of erythromycin and josamycin each reduced the IgG-ICx-induced increase in W/D weight ratio to $5.52 \pm 0.30$ and $5.44 \pm 0.19$, respectively ($p < 0.05$, $n = 8$ vs IgG-ICx alone), but amoxicillin and cephaclor did not (Fig. 3, lower panel).

Release of cytokines from cultured PAM

As shown in Fig. 4, incubation of PAM for 24 h with IgG-ICx (3 $\mu$g/ml) caused increases in the concentrations of IL-1β and TNF-α in the medium. Coincubation of cells with erythromycin ($10^{-4}$ M) inhibited the IgG-ICx-induced release of IL-1β by $71 \pm 9\%$ and TNF-α by $64 \pm 5\%$ ($p < 0.01$, $n = 8$ for each), whereas amoxicillin ($10^{-4}$ M) had no effect.
Release of NO from cultured PAM

The output current of the NO-selective electrode in the medium bathing rat-cultured PAM is demonstrated in Fig. 5. Immersion of the electrode in the medium did not produce electrical current in PAM that had been incubated for 24 h with the vehicle (saline) alone even in the presence of L-arginine (10^{-3} M) (Fig. 5A). When the cells were incubated with IgG-ICx (3 mg/ml), the baseline current was not detected, but application of L-arginine caused an increase in the current within 10 min. Between 15 and 30 min after the addition of L-arginine, the response showed the maximal value of 116 ± 23 pA (p < 0.001, n = 11), which corresponds to the medium NO concentration of 92 ± 18 nM, and the subsequent electrical current remained elevated for at least next 1 h (Fig. 5B).

To assess whether the observed response was associated with iNOS-mediated release of NO, after the response of electrical current reached a plateau, aminoguanidine (10^{-5} M), a specific inhibitor of NO synthesis through iNOS (23), was added to the medium. As shown in Fig. 5C, aminoguanidine rapidly decreased the current by 94 ± 5% (p < 0.001, n = 8) in IgG-ICx-treated PAM.

Then, the effects of antibiotics on the IgG-ICx-induced NO release were determined. As shown in Fig. 5, D and E, the NO-selective electrical current induced by IgG-ICx (3 μg/ml, 24 h) was greatly reduced by erythromycin (10^{-4} M), but not by amoxicillin (10^{-4} M). The inhibitory effects of macrolide antibiotics on the NO release were dose dependent, the concentration of the drug required to reduce 50% of NO contents being 9.1 ± 1.3 × 10^{-6} M for erythromycin, 8.7 ± 0.6 × 10^{-6} M for roxithromycin, and 1.2 ± 1.1 × 10^{-5} M for josamycin (n = 8 for each) (Fig. 6).

Incubation of PAM for 24 h with a mixture of IL-1β (1500 pg/ml) and TNF-α (3000 pg/ml) increased the release of NO to the same degree as did IgG-ICx. However, this effect was not inhibited by 10^{-4} M erythromycin or roxithromycin (Fig. 7).

**iNOS gene expression**

To assess the expression of iNOS gene, PAM were incubated for 24 h with IgG-ICx (3 μg/ml) in the absence or presence of each...
antibiotic agent or dexamethasone, and total RNA was then extracted and analyzed by Northern blotting with iNOS and β-actin cDNA probes. Three distinct bands were observed in the hybridization for iNOS, as reported previously (24, 25). The most prominent 4.4-kb transcript is shown in Fig. 8. When the cells were treated with IgG-ICx, a marked increase in the steady state level of iNOS mRNA was seen. This induction of iNOS mRNA expression was inhibited by coincubation with 10^{-2} M erythromycin (percentage of inhibition: 68%), 10^{-4} M roxithromycin (77%), 10^{-4} M josamycin (75%), and 10^{-7} M dexamethasone (97%) (Fig. 8A), and the inhibitory effect of erythromycin was concentration dependent (Fig. 8B). Coincubation of PAM with amoxicillin or cefaclor at 10^{-4} M did not alter IgG-ICx-induced iNOS mRNA expression (Fig. 8C).

Discussion

The main findings of the present in vivo and in vitro studies are: 1) intrapulmonary deposition of IgG-ICx in the rats elicited lung injury and increased NO concentration in the exhaled air; 2) incubation of cultured rat PAM with IgG-ICx stimulated the release of NO and induced iNOS mRNA expression; and 3) all of these effects of IgG-ICx were inhibited by macrolide antibiotics. Our results indicate for the first time that macrolides protect against immune complex-induced lung injury presumably by inhibiting PAM to generate iNOS-mediated NO.

In the in vivo model of lung injury, intratracheal instillation of anti-BSA Ab followed by i.v. injection of BSA increased the number of neutrophils in the alveolar compartment and the lung W/D weight ratio, indicating the presence of neutrophilic lung inflammation and vascular hyperpermeability induced by IgG-ICx. There is indirect evidence that acute lung injury occurring after immune complex deposition may be related to NO generated by PAM. Previous studies have shown that pretreatment of rats with NG-monomethyl-L-arginine, a NOS inhibitor, reduces IgG-ICx-induced lung hemorrhage (12) and that IgG-ICx stimulates the production of nitrite and nitrate, which are metabolites of NO, from PAM (17). In the present study, involvement of NO was confirmed by a direct measurement of exhaled NO in mechanically ventilated rats. In our experimental condition, basal NO concentration was low, because the animals were ventilated through tracheal cannulae to exclude contribution of large amounts of NO released from nasal mucosa (26). The rise in exhaled NO concentration was observed at 6 h after the deposition of IgG-ICx and thereafter. This delayed response is consistent with the finding in endotoxin-induced lung injury (27), and may reflect induction of iNOS within the cells of lower respiratory tract.

The concentration of NO in exhaled air is increased in patients with asthma (5, 6) and bronchiectasis (7), and it has been reported that long-term administration of erythromycin, roxithromycin, or clarithromycin is effective in the treatment of these airway diseases (28, 29). Although the mechanism for the efficacy is uncertain, it may not be accounted for by antimicrobial properties of macrolides, but rather by immunomodulatory and/or anti-inflammatory...
actions (8–10). In our study, pretreatment of rats with erythromycin, a 14-membered ring macrolide, and josamycin, a 16-membered ring macrolide, each inhibited the accumulation of neutrophils into alveoli and reduced the increase in lung W/D weight ratio induced by IgG-ICx, and these effects were accompanied by the corresponding reductions in exhaled NO concentration. We thus speculate that the suppressive action of antibiotics on lung inflammation and injury is specific for macrolides, and that the effect might be associated with the inhibition of NO generation. Additionally, the potency of the inhibition of IgG-ICx-induced increase in exhaled NO concentration was not different between erythromycin and josamycin, implying that macrolide antibiotics may possess inhibitory actions against NO production regardless of their structures of the lactone ring.

It is known that NO and its metabolite peroxinitrite cause injury to endothelial cells, leading to vascular leakage in the lung. On the other hand, NO does not stimulate, but rather inhibits neutrophil chemotaxis in vitro (30). Thus, the observed inhibition of lung injury in the macrolide-treated rats, as assessed by the lung W/D weight ratio, is most likely attributable to the reduced NO production, but the inhibition of neutrophil accumulation into alveoli may not be related to NO. One possible mechanism for the inhibition of neutrophil accumulation would be macrolides’ inhibition of the expression of adhesion molecule for neutrophil (10) and/or the release of neutrophil chemoattractant cytokines such as IL-8 (8).

Warner et al. (17) have shown that, in the rat model of IgG-ICx-induced lung injury, the percentage of iNOS-positive cells was much greater in PAM than type II alveolar epithelial cells. Therefore, to characterize the effects of macrolides on NO generation, we studied the response of isolated PAM to IgG-ICx in vitro. Direct measurement of NO using an amperometric NO sensor showed that incubation with IgG-ICx caused a release of NO into the culture medium. We also found that the subsequent addition of aminoguanidine, a specific inhibitor of iNOS (23), abolished the NO-selective electrical current. This implies that the observed production of NO was most likely mediated by iNOS, and this notion was further supported by the corresponding induction of iNOS mRNA after stimulation with IgG-ICx.

The release of NO from PAM was inhibited by macrolides in a dose-dependent manner, supporting our in vivo findings that macrolides decreased NO generation in inflamed lung. In this regard, a discrepancy seems to exist in the concentrations of erythromycin required to exert its in vivo and in vitro effects. The plasma concentration of erythromycin at the time of IgG-ICx deposition was

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FIGURE 7. Representative tracing of the current detected by an NO-selective electrode in RPMI medium containing rat PAM. After equilibration, L-arginine (L-Arg, 10^{-3} M) was added to the medium (arrows). A, Response of electrical current in the medium containing unstimulated PAM. B, PAM were incubated for 24 h with a mixture (Cytomix) of IL-1β (1500 pg/ml) and TNF-α (3000 pg/ml). C and D, PAM were incubated for 24 h with Cytomix in the presence of erythromycin (EM, 10^{-4} M) or roxithromycin (RXM, 10^{-4} M).

FIGURE 8. Effects of antibiotics and steroid on iNOS mRNA expression by rat PAM stimulated with IgG-ICx (3 μg/ml). In the control experiment, PAM were treated with saline alone. Total RNA was isolated after a 24-h incubation period, and analyzed by Northern blotting with iNOS and β-actin cDNA probes. A, Effects of erythromycin (EM), roxithromycin (RXM), and josamycin (JM) at 10^{-4} M, and dexamethasone (DEX) at 10^{-7} M on IgG-ICx-induced iNOS mRNA expression. B, Effects of various concentrations of EM on IgG-ICx-induced iNOS mRNA expression. C, Effects of erythromycin (EM), amoxicillin (AMPC), and cefaclor (CCL) at 10^{-4} M on IgG-ICx-induced iNOS mRNA expression.
the release of IL-1 was 35 times greater than the plasma concentration. It has been shown that tracheal instillation of IgG-ICx stimulates the release of IL-1β and TNF-α into bronchoalveolar lavage fluid (18), and that these cytokines are necessary for IgG-ICx-induced expression of PAM iNOS in the rats (17). In our in vitro experiments, IgG-ICx increased the release of IL-1β and TNF-α from PAM, and incubation of the cells with a mixture of these cytokines caused NO production. Pretreatment with erythromycin inhibited the cytokine release, but had little effect on the cytokine-induced NO release. These findings suggest that macrolides’ inhibition of immune complex-mediated NO production may be derived from the inhibition of cytokine generation. Indeed, macrolides are capable of down-regulating the release of IL-6 and IL-8 from human bronchial epithelial cells. In conclusion, we assessed the expression of iNOS mRNA and made a direct measurement of NO release from rat PAM, and found that macrolide antibiotics inhibited iNOS gene expression and the consequent production of NO induced by IgG-ICx. Therefore, it is possible that macrolides could favorably affect immune complex-mediated airway inflammation and lung injury.

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References