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Anti-inflammatory effects of a novel non-antibiotic macrolide, EM900, on mucus secretion of airway epithelium

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ABSTRACT

Objective: Low-dose, long-term use of 14-membered macrolides is effective for treatment of patients with chronic airway inflammation such as diffuse panbronchiolitis or chronic rhinosinusitis. However, long-term use of macrolides can promote the growth of drug-resistant bacteria, and the development of anti-inflammatory macrolides that lack antibiotic effects is desirable. Previously, we developed EM900, a novel 12-membered erythromycin A derivative, which has potent anti-inflammatory and immunomodulatory activities and lacks any antibacterial activity. We examined the anti-inflammatory effects of EM900 on mucus secretion from airway epithelial cells.

Methods: To examine the *in vivo* effects of EM900 on airway inflammation, we induced hypertrophic and metaplastic changes of goblet cells in rat nasal epithelium *via* intranasal instillation of lipopolysaccharides. *In vitro* effects of EM900 on airway epithelial cells were examined using cultured human airway epithelial (NCI-H292) cells. Mucus secretion was evaluated *via* enzyme-linked immunosorbent assays with an anti-MUC5AC monoclonal antibody.

Results: Oral administration of EM900 or clarithromycin (CAM) significantly inhibited LPS-induced mucus production from rat nasal epithelium. EM900, CAM, or erythromycin significantly inhibited MUC5AC secretion induced by tumor necrosis factor-α from NCI-H292 cells. MUC5AC mRNA expression was also significantly lower in EM900-treated cells.

Conclusion: These results indicated that a novel non-antibiotic macrolide, EM900 exerted direct inhibitory effects on mucus secretion from airway epithelial cells, and that it may have the potential to become a new anti-inflammatory drug for the treatment of chronic rhinosinusitis.

1. Introduction

Low-dose, long-term use of a 14- or 15-membered macrolide such as clarithromycin (CAM), erythromycin (EM), or azithromycin (AZM) is a very effective treatment for patients with a chronic airway disease such as diffuse panbronchiolitis [1], chronic bronchitis [2,3], cystic fibrosis [4], or chronic rhinosinusitis (CRS) [5,6]. These useful clinical effects may depend on the anti-inflammatory activity of macrolides rather than the antibacterial activity. The anti-inflammatory activity includes the immunomodulatory effect on inflammatory cells, the modulation of cytokine production from epithelial cells, and the inhibition of bacterial functions and biofilm formation.

Mucus hypersecretion is an important feature of airway inflammation, and macrolide therapy results in a significant reduction of the amount of secreted mucus; sputum and rhinorrhea. In previous studies, we demonstrated that macrolides inhibit hypersecretion of mucus in airways [7,8]. Intranasal instillation of lipopolysaccharides (LPS) causes inflammation of rat nasal epithelium, and oral administration of CAM, EM, or AZM significantly inhibits the LPS-induced hypertrophic and metaplastic changes of goblet cells in this rat model of airway inflammation. CAM, EM, or AZM also exerts direct inhibitory effects on mucus secretion from cultured airway epithelial (NCI-H292) cells or human nasal epithelial cells. These results indicate that low-dose, long-term macrolide therapy can be useful for the treatment of hypersecretory conditions associated with chronic airway inflammation.

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However, long-term use of macrolides can promote the growth of drug-resistant bacteria, and non-antibiotic macrolide derivatives with anti-inflammatory activities are desirable. Recently, Sunazuka and coworkers developed a novel 12-membered erythromycin A (EMA) derivative, (8R,9S)-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A (EM900) that has potent anti-inflammatory and immunomodulatory activities, but apparently lacks antibacterial activity [9,10]. In that study, the anti-inflammatory activities of EMA derivatives were evaluated by the THP-1 assay system, which examined the promotion of the differentiation of monocytes to macrophages.

Here, we examined the anti-inflammatory effects of EM900 on mucus secretion from airway epithelial cells; specifically, we evaluated (1) the *in vivo* effects of EM900 on LPS-induced mucus production in rat nasal epithelium and (2) the *in vitro* effects of EM900 on tumor necrosis factor-α (TNF-α)-induced MUC5AC secretion and MUC5AC mRNA expression in cultured human airway epithelial (NCI-H292) cells.

2. Materials and methods

2.1. Mucus hypersecretion in rat nasal epithelium

All experiments were approved by the Committee for the Care and Use of Laboratory Animals of Shiga University of Medical Science. LPS instillation was performed with rats as described previously [11]. Male Fischer 344 rats (6 weeks old) were anesthetized with ether, and 0.1 mL saline containing 0.1 mg LPS from *Escherichia coli* 0111:B4 (Sigma) or 0.1 mL saline control was intranasally instilled once daily for three consecutive days.

EM900 was a gift from T. Sunazuka (Kitasato Institute for Life Sciences and Graduate School of Infection Control Sciences, Kitasato University, Tokyo, Japan). EM900 (1–10 mg/kg) or CAM (10 mg/kg, Taisho Pharmaceutical, Tokyo) in 0.5% carboxymethyl cellulose sodium salt was administered orally exactly 1 h before the intranasal instillation of LPS on each of the three instillation days. Each rat was sacrificed 24 h after its last intranasal instillation; the nasal cavity was then transversely sectioned at the level of the incisive papilla. Paraffin sections were stained with alcian blue-periodic acid-Schiff (AB-PAS) or hematoxylin and eosin (H-E).

2.2. Morphometry

The amount of AB-PAS-stained mucosubstance in the surface epithelium was determined with an image analyzer (Image-Pro Plus, Medical Cybernetics, Maryland) as described previously [11]. The area of nasal epithelium was outlined, and the image analyzer determined the area of AB-PAS-stained mucosubstances within this reference area. The percent area of stored mucosubstance per surface area was calculated over 2 mm (1 mm each side of nasal septum) of the basal lamina at the center of the septal cartilage.

2.3. Cell cultures

A human mucoepidermoid carcinoma cell line, NCI-H292, was grown on plastic dishes in RPMI 1640 medium containing 10% fetal bovine serum, penicillin streptomycin (50 U/mL–50 μg/mL), and HEPES (25 mM). EM900, CAM, or EM was dissolved in *N,N*-dimethylformamide at a concentration of 10^{-1} M, and each of these stock solutions was stored at 4 °C until use; each stock solution was diluted with the appropriate medium to result in a final concentration within the range between 10^{-4} and 10^{-6} M in each experiment. When the NCI-H292 cells become confluent, TNF-α, and EM900 or CAM was added to the culture medium for 20 h, then the culture medium was collected and total RNA was extracted from each culture.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Each subsample of culture medium and serial dilution of "standard" purified human nasal mucin [12] were incubated in one well of a 96-well plate at 40 °C until dry. Wells were then incubated with 2% bovine serum albumin for 1 h, and then with 50 μL of mouse monoclonal MUC5AC antibody (1:100, Thermo Scientific, Massachusetts) for 1 h. Wells were then incubated with 100 μL of horseradish peroxidase-goat anti-mouse IgG conjugate (1:10,000) for 1 h. Color reaction was developed using 3,3′,5,5′-tetramethylbenzidine peroxidase solution. Absorbance was read at 450 nm. Data were expressed as the percent above the control vehicle (RPMI-1640) as described previously [7,8].

2.5. Reverse transcription-polymerase chain reaction (RT-PC)

Total RNA was extracted from cultured cells, reverse transcribed, then the cDNA was amplified by PCR using the Superscript preamplification system kit (Gibco, Grand Island, NY). The MUC5AC cDNA was amplified using the sense primer 5′-CACCAA ATACGCCAACAAGAC-3′ and the antisense primer 5′-CAGGGC-CACGCAGCCAGAGAA-3′. The GAPDH cDNA was amplified using the sense primer 5′-CCACCCATGGCAAATTCCATGGCA-3′ and the antisense primer 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. These steps were described previously [7].

2.6. Statistics

All data are expressed as mean ± standard error of the mean (SEM). Differences between variables were assessed *via* the Mann–Whitney *U* test. Probability values of $p < 0.05$ were considered significant.

3. Results

3.1. In vivo *effects of macrolides on LPS-induced mucus production*

Intranasal instillation of LPS induced hypertrophic and metaplastic changes of goblet cells in rat nasal septal epithelium within 24 h after the last instillation (Fig. 1). Only a few goblet cells were observed in untreated or saline-instilled control rats. Oral administration of EM900 (1–10 mg/kg) or CAM (10 mg/kg) inhibited LPS-induced hypertrophic changes of goblet cells, and quantitative measurement of the area of epithelial mucosubstance revealed a significant inhibition of intraepithelial mucus production in EM900- or CAM-treated rats (Fig. 2).

The number of infiltrating neutrophils in nasal septal mucosa was significantly higher in LPS-treated rats than in saline-treated rats. Oral administration of EM900 or CAM slightly inhibited LPS-induced neutrophil infiltration, although these changes are statistically insignificant (Fig. 3).

3.2. In vitro *effects of macrolides on TNF-α-induced MUC5AC secretion*

TNF-α (20 ng/ml) stimulated the secretion of MUC5AC mucin from cultured NCI-H292 cells. At concentrations from 10^{-4} to 10^{-6} M, EM900 significantly inhibited TNF-α-induced MUC5AC secretion in a dose-dependent manner. CAM and EM showed inhibitory effects on TNF-α-induced MUC5AC secretion similar to those of EM900 (Fig. 4).

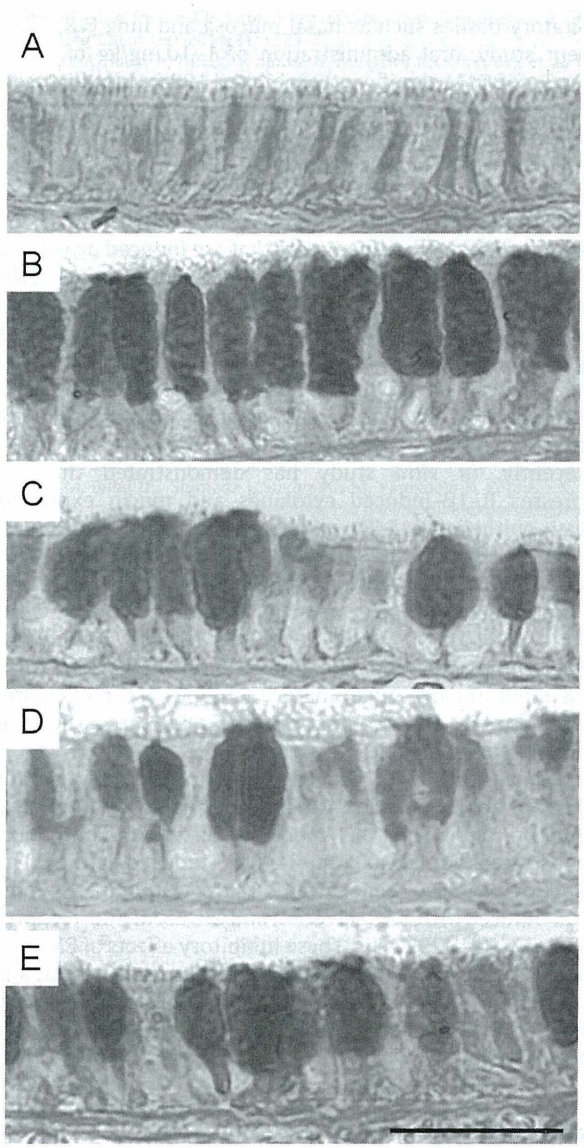

Fig. 1. Light micrographs illustrating the nasal septal epithelium of rats. Bar = 30 μm. (A) Saline-instilled control. (B) LPS-instilled rats (LPS rats). (C) EM900 (1 mg/kg)-treated, LPS-instilled rats. (D) EM900 (10 mg/kg)-treated, LPS-instilled rats. (E) CAM (10 mg/kg)-treated, LPS-instilled rats. Hypertrophic and metaplastic changes of goblet cells were induced by LPS instillation. Oral administration of EM900 or CAM inhibited hypertrophic and metaplastic changes of goblet cells.

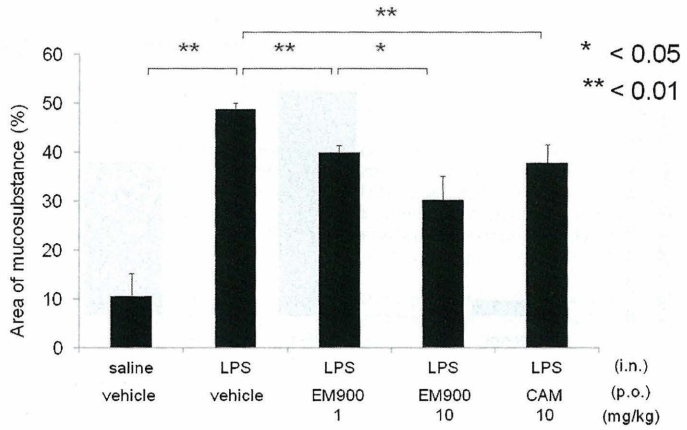

Fig. 2. Effects of EM900 (1–10 mg/kg) or CAM (10 mg/kg) on LPS-induced mucus production in rat nasal epithelium ($n = 6$). LPS instillations resulted in a significant increase in intraepithelial mucosubstance within 24 h after three consecutive days of intranasal instillations. Oral administration of EM900 or CAM significantly inhibited LPS-induced mucus production. Data are shown as mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$. i.n., intranasal; p.o., per os.

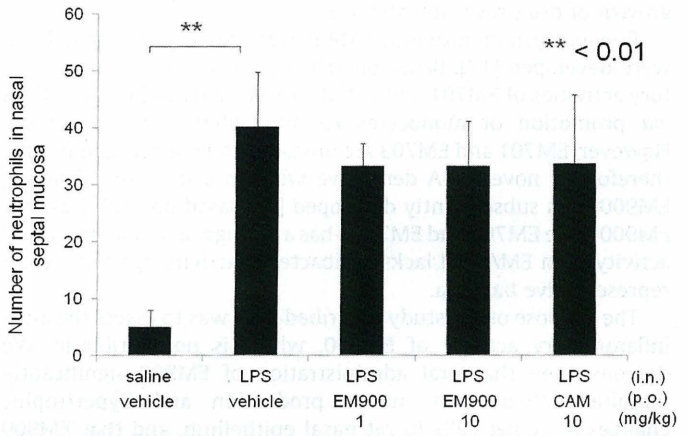

Fig. 3. Effects of EM900 (1–10 mg/kg) or CAM (10 mg/kg) on neutrophil infiltration in rat nasal epithelium. The number of infiltrating neutrophils in nasal septal mucosa was significantly higher in LPS-treated rats than in saline-treated rats. Oral administration of EM900 or CAM slightly inhibited LPS-induced neutrophil infiltration, although these changes are statistically insignificant. Data are shown as mean ± SEM. $n = 6$, $^{**}p < 0.01$. i.n., intranasal; p.o., per os.

Changes of MUC5AC gene expression were evaluated by RT-PCR; EM900 (10^{-4} M) significantly inhibited TNF-α-induced MUC5AC mRNA expression in cultured NCI-H292 cells (Fig. 5).

4. Discussion

Low-dose, long-term use of 14-membered macrolides, EM or CAM [macrolide therapy], has been widely used for the treatment of patients with CRS in Japan [13]. Macrolide therapy is effective for hypersecretory conditions and for neutrophilic chronic inflammation of nasal cavity and paranasal sinuses. However, long-term use of macrolides can promote the growth of drug-resistant bacteria. A number of international and national surveillance studies have documented high levels of macrolide resistance in respiratory tract infection [14,15], and more than 70% of *Streptococcus pneumoniae* are resistant to EM in Japan [16]. Therefore, macrolide derivatives

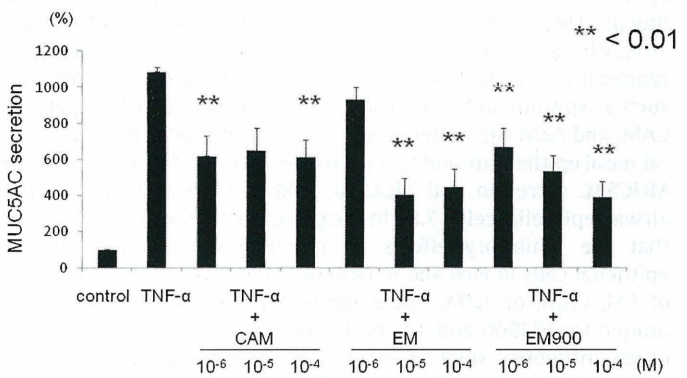

Fig. 4. Effects of CAM, EM, or EM900 on TNF-α (20 ng/mL)-induced MUC5AC secretion from NCI-H292 cells ($n = 8$). At concentrations from 10^{-4} to 10^{-6} M, EM900 significantly inhibited TNF-α-induced MUC5AC secretion in a dose-dependent manner. CAM and EM showed inhibitory effects on TNF-α-induced MUC5AC secretion similar to those of EM900. Data are expressed as the percent above the control vehicle (RPMI-1640), and are shown as mean ± SEM. $^{**}p < 0.01$.

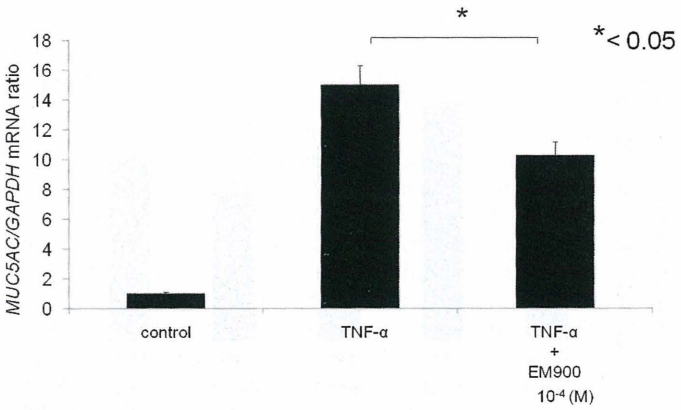

Fig. 5. Effects of EM900 on TNF-α (20 ng/mL)-induced MUC5AC mRNA expression from NCI-H292 cells ($n = 6$). Total RNA was isolated and analyzed for MUC5AC and GAPDH mRNA expression by RT-PCR. EM900 significantly inhibited TNF-α-induced MUC5AC mRNA expression at 10^{-4} M as demonstrated by the MUC5AC/GAPDH ratio. Data are shown as mean ± SEM. $^{*}p < 0.05$.

that have anti-inflammatory activity, but lack antibacterial activity, would be highly useful because they should not promote growth of drug-resistant bacteria.

Previously, non-antibiotic EMA derivatives, EM701 and EM703, were developed [17]. Based on THP-1 assays, the anti-inflammatory activities of EM701 and EM703 are stronger than that of EMA *via* promotion of monocytes to macrophages differentiation. However, EM701 and EM703 are unstable under acidic conditions; therefore, a novel EMA derivative with an acid-stable skeleton, EM900, was subsequently developed [9]. Based on THP-1 assays, EM900—like EM701 and EM703—has a stronger anti-inflammatory activity than EMA and lacks antibacterial activity against several representative bacteria.

The purpose of the study described here was to assess the anti-inflammatory activity of EM900, which is non-antibiotic. We demonstrated that oral administration of EM900 significantly inhibited LPS-induced mucus production and hypertrophic changes of goblet cells in rat nasal epithelium, and that EM900 directly suppressed TNF-α-induced MUC5AC secretion and MUC5AC mRNA expression in cultured airway epithelial cells. This is the first report demonstrating *in vivo* that EM900 has anti-inflammatory activity in nasal epithelium, and that EM900 exerts direct inhibitory effect on mucus secretion from airway epithelial cells.

Hypertrophy and metaplasia of secretory cells in surface epithelium and submucosal glands result in hypersecretion of mucus. These are major factors in the pathogenesis of chronic airway inflammation. Clinical effectiveness of macrolide therapy is represented by the inhibition of the hypersecretory symptoms, such as sputum and rhinorrhea. We previously reported that EM, CAM, and AZM each attenuates LPS-induced mucus production in rat nasal epithelium, and that each directly inhibits TNF-α-induced MUC5AC secretion and MUC5AC mRNA expression in cultured airway epithelial cells [7,8]. In the present study, we demonstrated that the inhibitory effects on mucus secretion from airway epithelial cells *in vivo* and *in vitro* of EM900 were similar to those of EM, CAM, or AZM. These inhibitory actions appeared to be unique for EM900 and 14- or 15-membered macrolides because other antibiotics such as josamycin (16-membered macrolides) and ampicillin showed no such effect [7,8]. These results indicate that EM900 possesses anti-inflammatory activities similar to those of EM, CAM, or AZM, which are each effectively used for macrolide therapy.

Reportedly, macrolide antibiotics reach higher concentrations in tissues and cells than in blood, and they diffuse extensively into respiratory tissues such as nasal mucosa and lung [18,19]. In the present study, oral administration of 1–10 mg/kg of EM900 or 10 mg/kg of CAM significantly inhibited LPS-induced intraepithelial mucus production. These doses are similar to the clinical CAM dose, and are comparable with tissue concentrations of 10^{-4} to 10^{-5} M in rat airways [20]. EM900, CAM, or EM significantly inhibited TNF-α-induced MUC5AC secretion from cultured airway epithelial cells at concentrations from 10^{-4} to 10^{-5} M. TNF-α is one of the proinflammatory mediators that are induced by exposure to LPS in human airways. LPS stimulation enhances TNF-α/β production in rat lung [21], and a TNF-α antagonist inhibits LPS-induced mucus hypersecretion in rat nasal epithelium [22]. These results indicate that *in vivo* effects of EM900 or CAM on LPS-induced mucus production are caused in some part by a direct inhibitory effect on mucus secretion from airway epithelial cells.

Recently, *in vitro* study has demonstrated that EM900 suppresses IL-1β-induced cytokines and mucin expression in airway epithelial (A549) cells; specifically, EM900 (10^{-5} M) inhibits IL-1β-induced expression of IL-8, TNF-α, IL-1β, and MUC5AC mRNAs [23]. A NFκB-regulated transcriptional pathway is reportedly very important for IL-1β-induced MUC5AC expression [24]. At 10^{-5} M, EM900 or EM significantly inhibited IL-1β-induced NFκB activation. The inhibition of NFκB activation is one of the important anti-inflammatory activities of the 14-membered macrolides, CAM and EM; importantly, EM900 has a similar effect on airway epithelial cells.

In conclusion, we induced hypertrophic and metaplastic changes of goblet cells in rat nasal epithelium by intranasal LPS instillation, and using this model of airway inflammation, we demonstrated that EM900 inhibited LPS-induced epithelial mucus production. We also demonstrated that EM900 directly inhibited TNF-α-induced MUC5AC secretion and MUC5AC mRNA expression in cultured NCI-H292 cells. These inhibitory effects of EM900 were similar to those of CAM or EM. These results indicate that a novel, non-antibiotic EMA derivative, EM900, has the potential to become a new anti-inflammatory drug for the treatment of chronic rhinosinusitis.

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References

[1] Kudoh S, Azuma A, Yamamoto M, Izumi T, Ando M. Improvement of survival in patients with diffuse panbronchiolitis treated with low-dose erythromycin. Am J Respir Crit Care Med 1998;157:1829–32.
[2] Fujita K, Shimizu T, Majima Y, Sakakura Y. Effects of macrolides on interleukin-8 secretion from human nasal epithelial cells. Eur Arch Otorhinolaryngol 2000;257:199–204.
[3] Yamada T, Fujieda S, Mori S, Yamamoto H, Saito H. Macrolide treatment decreased the size of nasal polyps and IL-8 levels in nasal lavage. Am J Rhinol 2000;14:143–8.
[4] Cai Y, Chai D, Wang R, Bai N, Liang BB, Liu Y. Effectiveness and safety of macrolides in cystic fibrosis patients: a meta-analysis and systematic review. J Antimicrob Chemother 2011;66:968–78.
[5] Shirai T, Sato A, Chida K. Effect of 14-membered ring macrolide therapy on chronic respiratory tract infections and polymorphonuclear leukocyte activity. Intern Med 1995;34:469–74.
[6] Oishi K, Sonoda F, Kobayashi S, Iwagaki A, Nagatake T, Matsushita K, et al. Role of interleukin-8 [IL-8] and an inhibitory effect of erythromycin on IL-8 release in the airways of patients with chronic airway diseases. Infect Immun 1994;62:4145–52.
[7] Shimizu T, Shimizu S, Hattori R, Gabazza E, Majima Y. *In vivo* and *in vitro* effects of macrolide antibiotics on mucus secretion in airway epithelial cells. Am J Respir Crit Care Med 2003;168:581–7.
[8] Shimizu T, Shimizu S. Azithromycin inhibits mucus hypersecretion from airway epithelial cells. Mediators Inflamm 2012;2012:265714.
[9] Sugawara A, Sueki A, Hirose T, Nagai K, Gouda H, Hirono S, et al. Novel 12-membered non-antibiotic macrolides from erythromycin A; EM900 series

as novel leads for anti-inflammatory and/or immunomodulatory agents. Bioorg Med Chem Lett 2011;21:3373–6.
[10] Sugawara A, Sueki A, Hirose T, Shima H, Akagawa KS, Omura S, et al. Novel 12-membered non-antibiotic macrolides, EM900 series with anti-inflammatory and/or immunomodulatory activity; synthesis, structure–activity relationships and in vivo study. J Antibiot (Tokyo) 2012;65:487–90.
[11] Shimizu T, Takahashi Y, Kawaguchi S, Sakakura Y. Hypertrophic and metaplastic changes of goblet cells in rat nasal epithelium induced by endotoxin. Am J Respir Crit Care Med 1996;153:1412–8.
[12] Kishioka C, Shimizu T, Fujita K, Ito Y, Majima Y, Sakakura Y. Monoclonal antibody-detectable carbohydrate epitopes of human nasal secretions are differentially expressed in tissue and diseases. Am J Rhinol 1999;13:37–43.
[13] Majima Y, Kurono Y, Hirakawa K, Ichimura K, Haruna S, Suzuki H, et al. Efficacy of combined treatment with S-carboxymethylcysteine (carbocisteine) and clarithromycin in chronic rhinosinusitis patients without nasal polyp or with small nasal polyp. Auris Nasus Larynx 2012;39:38–47.
[14] Felmingham D, Cantón R, Jenkins SG. Regional trends in beta-lactam, macrolide, fluoroquinolone and telithromycin resistance among *Streptococcus pneumoniae* isolates 2001–2004. J Infect 2007;55:111–8.
[15] Felmingham D, White AR, Jacobs MR, Appelbaum PC, Poupard J, Miller LA, et al. The Alexander Project: the benefits from a decade of surveillance. J Antimicrob Chemother 2005;56(Suppl. 2):ii3–21.
[16] Hotomi M, Billal DS, Shimada J, Suzumoto M, Yamauchi K, Fujihara K, et al. Increase of macrolide-resistant *Streptococcus pneumoniae*-expressing mefE or ermB gene in the nasopharynx among children with otitis media. Laryngoscope 2005;115:317–20.
[17] Yoshida K, Sunazuka T, Nagai K, Sugawara A, Cho A, Nagamitsu T, et al. Macrolides with promotive activity of monocyte to macrophage differentiation. J Antibiot (Tokyo) 2005;58:79–81.
[18] Honeybourne D, Kees F, Andrews JM, Baldwin D, Wise R. The levels of clarithromycin and its 14-hydroxy metabolite in the lung. Eur Respir J 1994; 7:1275–80.
[19] Fraschini F, Scaglione F, Pintucci G, Maccarinelli GS, Demartini G. The diffusion of clarithromycin and roxithromycin into nasal mucosa, tonsil and lung in humans. J Antimicrob Chemother 1991;27(Suppl. A):61–5.
[20] Yoshida H, Furuta T. Tissue penetration properties of macrolide antibiotics – comparative tissue distribution of erythromycin-stearate, clarithromycin, roxithromycin and azithromycin in rats. Jpn J Antibiot 1999;52: 497–503.
[21] Ermert M, Pantazis C, Duncker HR, Grimminger F, Seeger W, Ermert L. In situ localization of TNFalpha/beta, TACE and TNF receptors TNF-R1 and TNF-R2 in control and LPS-treated lung tissue. Cytokine 2003;22:89–100.
[22] Kim DH, Jeon EJ, Park SN, Park KH, Park YS, Yeo SW. Effects of a tumor necrosis factor-α antagonist on experimentally induced rhinosinusitis. J Biomed Biotechnol 2011;2011:360457.
[23] Otsu K, Ishinaga H, Suzuki S, Sugawara A, Sunazuka T, Omura S, et al. Effects of a novel nonantibiotic macrolide, EM900, on cytokine and mucin gene expression in a human airway epithelial cell line. Pharmacology 2011;88: 327–32.
[24] Fujisawa T, Velichko S, Thai P, Hung LY, Huang F, Wu R. Regulation of airway MUC5AC expression by IL-1beta and IL-17A; the NF-kappaB paradigm. J Immunol 2009;183:6236–43.