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Effects of long-term administration of erythromycin on cytokine production in rat alveolar macrophages

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Effects of long-term administration of erythromycin on cytokine production in rat alveolar macrophages. Y. Sugiyama, K. Yanagisawa, S-I. Tominaga, S. Kitamura. ©ERS Journals Ltd 1999.

ABSTRACT: Low-dose long-term erythromycin treatment has recently been reported to be very effective in patients with chronic respiratory infection and inflammation. This effect of erythromycin was thought to be not antibacterial but anti-inflammatory. However, the exact mechanism of the effect of erythromycin has not yet been clarified. The aims of this study were to investigate the effects of erythromycin on cytokine production and its mechanisms of actions in rat alveolar macrophages.

Using rats with or without administration of erythromycin for 3 months, the production of the cytokines tumour necrosis factor-α (TNF-α), cytokine-induced neutrophil chemoattractant (CINC)-1 and CINC-2α by enzyme-linked immunosorbent assay and the expression of TNF-α and CINC-1 messenger ribonucleic acid (mRNA) by Northern blotting in rat alveolar macrophages were analysed. CINC-1 is the rat counterpart of human interleukin-8, and CINC-2α of human macrophage inflammatory peptide-2.

Erythromycin reduced cytokine production and secretion when cytokines was induced by lipopolysaccharide treatment. Conversely, erythromycin slightly upregulated the expression of cytokine mRNA.

These results suggest that erythromycin inhibits cytokine production and exhibits anti-inflammatory effects by means of a translational and/or posttranslational mechanism.

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Low-dose, long-term erythromycin treatment has recently been reported to be very effective in patients with chronic respiratory infection and inflammation, such as diffuse panbronchiolitis and bronchiectasis [1, 2]. This effect of erythromycin is thought to be due to an anti-inflammatory action rather than an antibacterial effect. Multiple biological actions of erythromycin, including inhibition of neutrophil chemotaxis [3], interleukin (IL)-8 generation [4], submucosal gland secretion and chloride-secretion by tracheal epithelial cells [5, 6], and anti-lymphocytic activity [7], have been reported. Although, in view of these pharmacological actions, erythromycin may function as an anti-inflammatory or immunomodulatory agent, the precise mechanism of the effects of erythromycin has not been clarified. TAKIZAWA *et al.* [8] reported that erythromycin decreased the steady-state levels of IL-6 messenger ribonucleic acid (mRNA) expression in bronchial epithelial cells.

Cytokine-induced neutrophil chemoattractant (CINC)-1 is the rat counterpart of human IL-8 and CINC-2α is the chemokine related to macrophage inflammatory peptide-2. Both chemokines are chemoattractants for neutrophils. In this study, the effects of long-term administration of erythromycin on the production and secretion of the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α) and the chemokines CINC-1 and CINC-2α in rat alveolar macrophages were evaluated.

Methods

Animals

Specific-pathogen-free male Wistar rats of mean weight 250 g were used for these studies. They were housed in an antigen- and virus-free environment and maintained on standard rat food (CE-II) (control group) or special food containing erythromycin (CE-II-based) (erythromycin group) and water *ad libitum* for 3 months. The special rat food was produced at <70°C (Nihon Kurea, Tokyo, Japan), and contained 160 mg erythromycin·kg CE-II^{-1}. The rats consumed ~12 mg erythromycin·kg body weight^{-1}. They were bred in the Animal Care Facility of Jichi Medical School. Experimental planning was carried out with the approval of the management committee of the Jichi Medical Laboratory of Experimental Medicine, based upon the School's "Guide for Laboratory Animals". Four rats were used for TNF-α experiments, six for C1NC-1 and six for CINC-2α. Rats of the same experimental group were housed in the same cages and ate the same food.

Isolation and culture of bronchoalveolar lavage cells

Alveolar macrophages were harvested by lavaging the lungs of the rats as described previously [9]. In brief, rats were anaesthetized with sodium pentobarbital (Abbott, Osaka, Japan) and sacrificed by exsanguination *via* a

section of the abdominal aorta. After tracheotomy, bronchoalveolar lavage (BAL) was performed by repeated instillation with a total of 50 mL sterile phosphate-buffered saline (PBS) without magnesium or calcium (Gibco BRL, Grand Island, NY, USA) through a vinyl catheter. Each lavage was treated separately.

The recovered BAL fluid was filtered through sterile gauze. A total cell count was carried out using a haematacytometer. Cell differentials were performed on cytocentrifuge slides stained with Diff-Quick, and 500 cells were counted. Cell viability was determined by means of trypan blue exclusion, and recovered cells were >95% viable. Cells were washed in PBS and resuspended in RPMI 1640 containing penicillin (100 $U \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$) at 1×10^5 $cells \cdot well^{-1} \cdot mL^{-1}$ in 12-well flat-bottomed tissue culture plates (Corning, NY, USA) and cultured for 2 h at 37°C. After culture, nonadherent cells were removed by gentle washing of the wells with medium, and >95% of adherent cells were found to be alveolar macrophages.

Fresh RPMI 1640 medium was added to each well and cultured for 24 h with or without various concentrations of lipopolysaccharide (LPS). The experiments were performed in duplicate. After culture, supernatant was collected and stored at -70°C. The amounts of cytokines secreted into the supernatant was measured using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

Correction of cell number by adhesion rate

Since the adhesion rate of alveolar macrophages might have been different between the erythromycin and the control group, the number of adhesive alveolar macrophages in each experiment was calculated by counting the nonadhesive cells in the decanted medium. The number of adhesive alveolar macrophages was then corrected.

Ribonucleic acid analysis

Cytokine gene expression was analysed by means of Northern blotting analysis. Alveolar macrophages from the erythromycin and control groups were cultured in the presence (0.1 and 0.01 $\mu g \cdot mL^{-1}$ for 2 and 12 h) or absence (0 h) of LPS. Total ribonucleic acid (RNA) was prepared from alveolar macrophages using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Equal amounts (5 $mg \cdot lane^{-1}$) of RNA were then electrophoresed in 1% agarose gels containing formaldehyde after uniformity of loading was confirmed by examination of the 28 and 18 S RNA bands in separate minigels. RNA was transferred to Hybond-N nylon filters (Amersham, Tokyo, Japan). After overnight transfer onto the filters, the gels were baked, pre-hybridized and then hybridized with antisense oligonucleotide probes for TNF-α and CINC-1. Complementary deoxyribonucleic acid (cDNA) inserts were labelled with α-^{32}p-deoxycytidine triphosphate by means of random priming. In order to obtain the probes for rat TNF-α and CINC-1, total RNA was reverse transcribed as described previously [10], and the polymerase chain reaction (PCR) performed with rat CINC-1 sense (base pair (bp) -29–6-8, 5′-AGACTCCAGCCACACTCCAACA-3′) and antisense (bp303–325,5′-AGACGCCATCGGTGCAATCTATC-3′) primers [11], or with rat TNF-α sense (bp 4–26, 5′-AGCACAGAAAGCATGATCCGAGA-3′) and antisense (bp 658–682,5′-CCTGCCCGGACTCCGTGATGTCTAA-3′) primers [12]. The PCR products were cloned in the Bluescript II vector (Stratagene, La Jolla, CA, USA), then the inserts released, using *Xanthomonas holcicola* 1 and *Streptomyces achromogenes* I for TNF-α or *Haemophilus influenzae* Rd III and *Xanthomonos badrii* I for CINC-1, and purified. Human glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA, which cross-hybridizes with rat GAPD, was purchased from Clontech Laboratories (Palo Alto, CA, USA).

The results were analysed using a BAS 2000 system (Fuji Film, Tokyo, Japan). Ribosomal RNA (28 S) stained with methylene blue on a nylon membrane was used as internal standard.

Reagents

Commercial kits for quantitative ELISA of rat TNF-α (Cytoscreen; limit of detection 4 $pg \cdot mL^{-1}$) were purchased from BioSource (Camarillo, CA, USA), and of rat CINC-1 and CINC-2α (limit of detection 50 $pg \cdot mL^{-1}$) were purchased from Immuno-Biological Laboratories (Fujioka, Japan). ELISA assays were performed in duplicate. Erythromycin and LPS (*Escherichia coli* 0111:B4) were purchased from Sigma (St. Louis, MO, USA).

Statistical analysis

All data are expressed as mean±SD. Analysis of data was performed using the unpaired Student's t-test. A p-value <0.05 was considered significant.

Results

Bronchoalveolar lavage fluid cell differentiation and adhesion rate

The total cell count was lower in the erythromycin group than in the control group, but this difference was not significant (table 1). The pattern of differentiation of BAL fluid (BALF) cells in the erythromycin group was not different from that of the control group (data not shown). In both groups, >98% of BALF cells were alveolar macrophages. The adhesion rate was higher in the erythromycin group than in the control group (85.1±6.1% *versus* 79.3±5.8%), but this difference was not significant (table 1).

Cytokine production

Basal and LPS-stimulated rat TNF-α secretion by alveolar macrophages tended to be lower in the erythromycin group than in the control group. TNF-α secretion was

Table 1. – Body weight and bronchoalveolar lavage fluid (BALF) cell count and adhesion rate in the erythromycin and control group

	Subjects n	Body weight g	BALF	
			Total cell count $\times 10^5$	Adhesion rate %
Erythromycin	6	427±64	70.9±13.4	85.1±6.1
Control	6	418±64	88.9±27.2	79.3±5.8
p-value		NS	NS	NS

Data are presented as mean±SD. NS: nonsignificant.

significantly decreased in the erythromycin group compared with control when the cells were stimulated with 0.01 and 0.1 pg·mL^{-1} of LPS (227±71 *versus* 367±70 pg·mL^{-1}, p<0.05 and 310±92 *versus* 547±106 pg·mL^{-1}, p<0.02) (fig. 1). Similarly, basal and LPS-induced CINC-1 and CINC-2α secretion by alveolar macrophages tended to be lower in the erythromycin group, and, at some concentrations of LPS, the differences were statistically significant (CINC-1: LPS 0.01 µg, 746±131 *versus* 1,062±264 pg·mL^{-1}, p< 0.05; LPS 0.1 mg, 1,667±437 *versus* 2,329±259 pg·mL^{-1}, p<0.02 and CINC-2α: LPS 0.1 µg, 159±74 *versus* 328± 45 pg·mL^{-1}, p<0.01) (figs. 2 and 3).

Northern blotting analysis

The expression of rat TNF-α mRNA was not suppressed in the absence or presence (0.1 µg·mL^{-1} for 2 h or 0.1 µg·mL^{-1} for 12 h) of LPS. In fact, the mRNA, in these conditions, was slightly upregulated, rather than suppressed, in the erythromycin group (fig. 4). In the case of CINC-1, the results were unaltered.

Discussion

The present study demonstrated that cytokine production in alveolar macrophages of rats receiving low-dose long-term erythromycin was significantly lower than that of control rats. Conversely, the expression of the mRNA of these cytokines was slightly upregulated rather than suppressed.

Many investigations have revealed multiple biological actions of erythromycin, other than its essential antibacterial action, such as inhibition of neutrophil chemotaxis, IL-8 generation [3, 4], submucosal gland secretion and chloride-secretion by tracheal epithelial cells [5, 6], and antilymphocytic activity [7]. Recently, some investigators have reported prophylactic effects of erythromycin on acute lung injury induced by bleomycin [13] and influenza virus [14], the former through inhibition of neutrophil-derived elastase and the latter through inhibition of inflammatory cell responses and nitric oxide overproduction.

In the present study, the adhesion rate of alveolar macrophages in the erythromycin group was increased compared with that in the control group. Keicho *et al.* [15] reported that erythromycin significantly increased the number of adherent monocyte-derived macrophages, and promoted the differentiation of a human monocyte/macrophage lineage, which is in accordance with the present results.

Erythromycin suppressed the release of IL-6 in human bronchial epithelial cells [8]. Also, Khair *et al.* [16] reported that erythromycin significantly blocked the *Haemophilus influenzae* endotoxin-induced release of IL-6, IL-8 and soluble intercellular adhesion molecule-1 from airway epithelial cells. In human monocytes stimulated with LPS, 24 h culture with erythromycin inhibited TNF-α release [17]. All of these results show the suppressive effects of erythromycin on cytokine production, and are compatible with the present results.

As for mRNA, in contrast to the previously published report of Takizawa *et al.* [8], a decrease in the expression of cytokine mRNA was not demonstrated. In the present

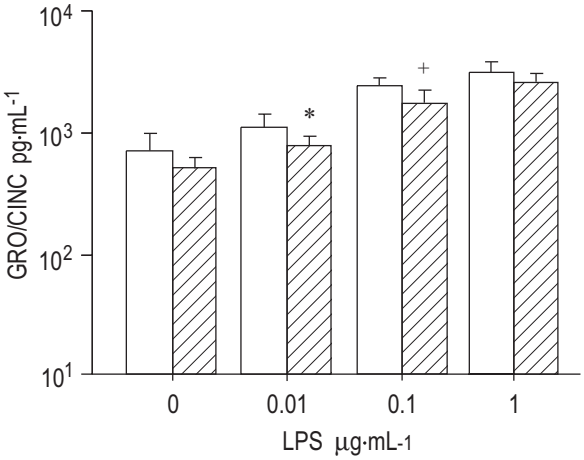

Fig. 2. – Cytokine-induced neutrophil chemoattractant (CINC)-1 production in rat alveolar macrophages after 3 months without (□) or with (▨; ~12 mg·kg body weight^{-1}·day^{-1}) erythromycin. CINC-1 production significantly decreased in the erythromycin group stimulated with 0.01 and 0.1 µg·mL^{-1} lipopolysaccharide (LPS). *: p<0.05, $^{+}$: p<0.02 *versus* control.

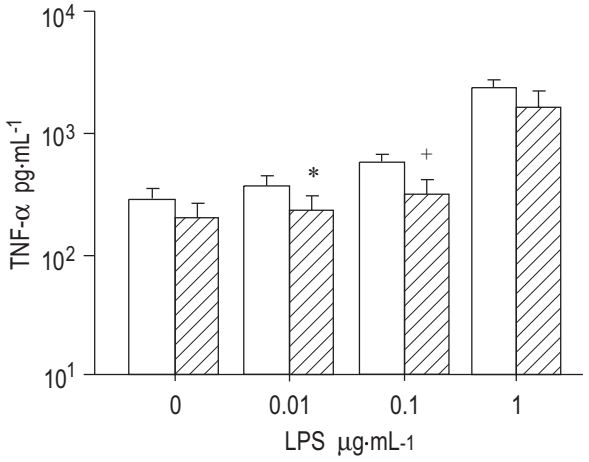

Fig. 1. – Tumour necrosis factor-α (TNF-α) production in rat alveolar macrophages after 3 months without (□) or with (▨; ~12 mg·kg body weight^{-1}·day^{-1}) erythromycin. TNF-α production significantly decreased in the erythromycin group stimulated with 0.01 and 0.1 µg·mL^{-1} lipopolysaccharide (LPS). *: p<0.05, $^{+}$: p<0.02 *versus* control.

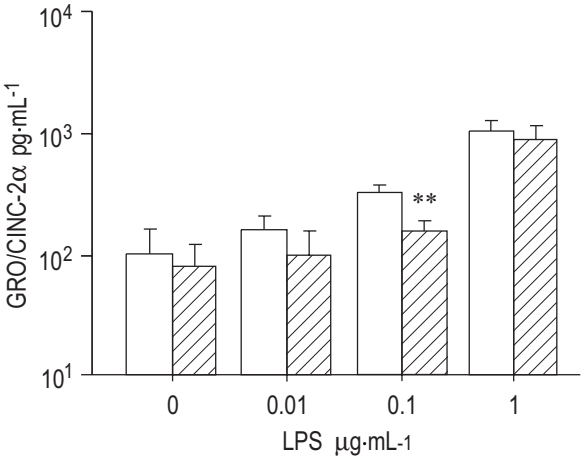

Fig. 3. – Cytokine-induced neutrophil chemoattractant (CINC)-2α production in rat alveolar macrophages after 3 months without (□) or with (▨; ~12 mg·kg body weight^{-1}·day^{-1}) erythromycin. CINC-2α production significantly decreased in the erythromycin group stimulated with 0.1 µg·mL^{-1} lipopolysaccharide (LPS). **: p<0.01 *versus* control.

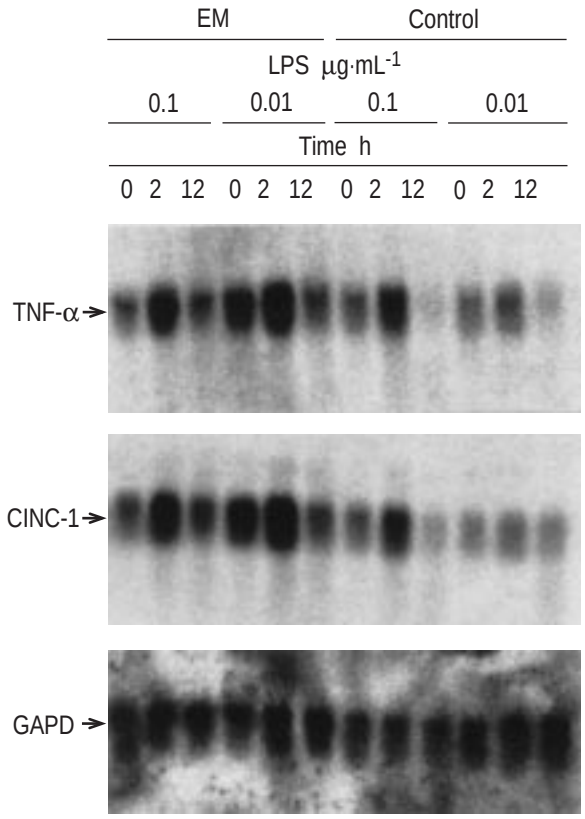

Fig. 4. – Northern blotting analysis of tumour necrosis factor-α (TNF-α) and cytokine-induced neutrophil chemoattractant (CINC)-1 messenger ribonucleic acid (mRNA). Alveolar macrophages from the erythromycin (EM) and control groups were cultured in the presence (0.1 and 0.01 μg·mL^{-1} for 2 h and 12 h) or absence (0 h) of lipopolysaccharide (LPS). As a control for ribonucleic acid loading, expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPD) gene was analysed. mRNA expression was slightly upregulated in the EM group. A representative experiment from a single animal is shown.

study, mRNA levels were slightly increased rather than decreased. In the work of TAKIZAWA *et al.* [8], a different cell types was used (bronchial epithelial cells), and the culture period with erythromycin was only 24 h. It was important that the administration of erythromycin was performed *in vivo* and for a long period of time in the present study. This is an essential difference between the study of TAKIZAWA *et al.* [8] and the present one. The phenomenon in which the expression of mRNA is slightly upregulated instead of the inhibition of protein synthesis occurring is known as "superinduction" [18]. The increase in mRNA expression might be the result of inhibition of protein production. This suggests that cytokine production was suppressed at the level of protein synthesis and/or process that followed. A more precise understanding of the mechanism of erythromycin action at the molecular level would allow the clinical use of erythromycin to be extended. The long-term administration of antibiotics like erythromycin may cause induction of bacterial resistance even at low doses. There is little information on this subject, and it should be clarified in the future.

In summary, erythromycin was administered to rats for 3 months, and the alveolar macrophages from these rats showed decreased production of a pro-inflammatory cytokine (tumour necrosis factor-α) and two chemokines (cytokine-induced neutrophil chemoattractant-1 and cytokine-induced neutrophil chemoattractant-2α) without decreasing the messenger ribonucleic acid levels. This anti-inflammatory action of erythromycin may occur *via* the mechanism of its regulatory effects on chronic respiratory infection and inflammation, such as in diffuse panbronchiolitis.

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