

Erythromycin inhibits Cl secretion across canine tracheal epithelial cells

J. Tamaoki, K. Isono, N. Sakai, T. Kanemura, K. Konno

Erythromycin inhibits Cl secretion across canine tracheal epithelial cells. J. Tamaoki, K. Isono, N. Sakai, T. Kanemura, K. Konno.

ABSTRACT: We studied the effect of the macrolide antibiotic erythromycin on bioelectrical properties of canine cultured tracheal epithelium under short-circuit conditions *in vitro*.

Addition of erythromycin to the submucosal but not to the mucosal side dose-dependently decreased short-circuit current (I_{sc}), the maximal decrease from the baseline value and the concentration required to produce a half-maximal effect (IC_{50}) being $5.6 \pm 1.0 \mu A \cdot cm^{-2}$ (mean \pm SE, $p < 0.001$) and $18 \mu M$, respectively. In contrast, other antibiotics including ampicillin, cephalosporin and tetracycline were without effect. The erythromycin-induced decrease in I_{sc} was not altered by amiloride, but it was abolished by bumetanide, diphenylamine-2-carboxylate₂, and substitution of Cl in the bathing medium with gluconate ($p < 0.001$, in each case). The effect of erythromycin on epithelial I_{sc} was attenuated by pretreatment of cells with indomethacin but not with AA-861 a lipoxygenase inhibitor. Incubation of cells with erythromycin inhibited the release of prostaglandins E_2 and $F_{2\alpha}$ from tracheal epithelial cells.

These results indicate that erythromycin may selectively inhibit Cl secretion across airway epithelium through the inhibition of prostaglandin synthesis and suggest that this action possibly reflects its clinical efficacy in the treatment of airway hypersecretion.

Eur Respir J, 1992, 5, 234-238.

Pulmonary Division
First Department of Medicine
Tokyo Women's Medical College
Tokyo, Japan.

Correspondence: J. Tamaoki
First Department of Medicine
Tokyo Women's Medical College
8-1 Kawada-Cho
Shinjuku, Tokyo 162, Japan.

Keywords: Airway secretion; antibiotics; adenosine monophosphate; epithelium; ion transport.

Received: March 12, 1991; accepted after revision August 16, 1991.

This study was supported in part by a grant for Scientific Research No. 63770524 from the Ministry of Education, Science and Culture, Japan.

Bronchial hypersecretion is one of the characteristic features of chronic bronchitis, asthma and bronchiectasis, and a large amount of sputum may cause airflow limitation and recurrent respiratory infection due to the impaired mucociliary transport functions [1]. It has generally been accepted that airway secretions are mainly composed of the mucus synthesized and released by submucosal glands and the water which is transported across airway mucosa [2] in relation to the active ion transport process of airway epithelial cells [3].

The macrolide antibiotic erythromycin has been shown to be effective in the treatment of patients with acute bronchitis and chronic airway diseases including asthma and chronic bronchitis [4, 5]. The efficacy of this antibiotic can be attributed not only to its antimicrobial activities but also to its immunomodulatory action on neutrophils and macrophages [6, 7]. In addition, many patients receiving erythromycin report the reduction of sputum volume and the dryness of sputum, the mechanism of which may partly be associated with the direct inhibition of airway glycoprotein secretion by this drug [8]. However, the effect of erythromycin on ion transport across airway mucosa is unknown. Therefore, to determine the influence of erythromycin on airway epithelial ion transport and the

possible mechanism of its action, we measured bioelectric properties of canine cultured tracheal epithelium under short-circuit conditions *in vitro*.

Material and methods

Cell preparation

Mongrel dogs weighing 19-42 kg were anaesthetized with intravenous pentobarbital sodium ($35 \text{ mg} \cdot \text{kg}^{-1}$), and the trachea was removed. After dissecting connective tissue and blood vessels, tracheal epithelial strips were obtained and enzymatically digested by 0.1% protease type XIV (Sigma Chemicals, St. Louis, MO, USA) at 4°C for 24 h. In this preparation of cells, fibroblasts and other nonepithelial cells constituted less than 1.5% of the total, and the viability was 85-95% as assessed by trypan blue exclusion. These cells were then concentrated by centrifugation ($800 \times g$) and resuspended in Ham's nutrient F12 medium containing 5% fetal calf serum, $5 \mu\text{g} \cdot \text{ml}^{-1}$ insulin, $5 \mu\text{g} \cdot \text{ml}^{-1}$ transferrin, and $10 \text{ ng} \cdot \text{ml}^{-1}$ epidermal growth factor. We plated cells at a density of $1.5 \times 10^6 \cdot \text{cm}^{-2}$ per Linbro tissue culture multi-well plate (Flow Lab Inc., McLean, VA, USA).

and cultured on nucleopore polycarbonate filters (13 mm diameter, 0.45 μ m pore size) at 37°C in a CO₂ incubator (5% CO₂ - 95% air) for 10 days, when cells became confluent as observed with phase contrast microscopy. Our separate studies on transmission electron microscopy showed that this preparation was monolayer or bilayer culture in which non-ciliated epithelial cells constituted more than 99% of the total, and that typical tight junctions separated two distinct membranes: the membrane facing the overlying medium contained microvilli and glycocalyx, whereas the membrane facing the filter surface was relatively unspecialized.

Measurement of bioelectric properties

The short-circuit technique for measuring electrical properties of cultured airway epithelium had been described previously [9]. Briefly, the filter on which cells were grown was mounted between Ussing chambers (0.5 cm² surface area) and bathed with oxygenated Krebs-Henseleit solution. The calomel half-cells were paired to within 0.2 mV of each other. The short-circuit current (I_{sc}) was recorded continuously except for 3 s every 3 min when the voltage clamp (Nihon Kohden, CEZ-9100, Tokyo, Japan) was turned off and the spontaneous transepithelial potential difference (PD) was recorded. Cell conductance (G) in mS·cm² was calculated by dividing the I_{sc} per surface area (μ A·cm²) by the PD (mV).

The cells were allowed to equilibrate for 20 min to establish a baseline I_{sc}, and erythromycin (10⁻⁴ M) was added to either the mucosal or submucosal solution. In control experiments, the solvent of erythromycin alone was added. To assess whether the effect of erythromycin is reversible, after the response of I_{sc} to erythromycin reached a plateau, cells were washed with Krebs-Henseleit solution and I_{sc} was measured continuously. For the assessment of a dose-response relationship, erythromycin at a concentration of 10⁻⁷ to 10⁻⁴ M was cumulatively added to the chamber, while the maximal I_{sc} response to each concentration was determined. Because no changes of I_{sc} were seen when erythromycin was added to the mucosal solution in this study, it was added only to the submucosal solution in the subsequent experiments.

To evaluate the effects of other antibiotics on bioelectric properties of tracheal epithelium, we likewise measured I_{sc} in response to ampicillin, cephalosporin and tetracycline, at concentrations from 10⁻⁷ to 10⁻³ M.

To assess whether the erythromycin-induced changes in I_{sc} were associated with Cl secretion and/or Na absorption by tracheal epithelium, the responses of I_{sc} to 10⁻⁴ M erythromycin were determined after the treatment of cells for 30 min with each of the following substances: amiloride (10⁻⁴ M), a Na channel blocker [10]; bumetanide (10⁻⁴ M), a Cl transport inhibitor [11]; diphenylamine-2-carboxylate (DPC, 10⁻⁴ M), a Cl channel blocker [12]; and Cl-free medium, in which Cl was substituted with gluconate that is not transported across canine tracheal epithelium [13].

Intracellular arachidonic acid metabolites have been known to play an important role in the regulation of airway epithelial bioelectric properties [14]. Thus, to assess a possible contribution of this process to the action of erythromycin, the effects of preincubation of cells with indomethacin (3 \times 10⁻⁶ M), a cyclooxygenase inhibitor, or AA-861 (10⁻⁶ M), a lipoxygenase inhibitor [15], on the erythromycin (10⁻⁴ M)-induced changes in I_{sc} were examined.

Prostaglandin release

Canine tracheal epithelial cells release various arachidonic acid metabolites, among which prostaglandin (PG) E₂ and PGF_{2 α} are major products [16] and can stimulate epithelial Cl secretion [17, 18]. Thus, to determine whether erythromycin changed the rate of PG release from tracheal epithelium, concentrations of PGE₂ and PGF_{2 α} in the medium were determined before and 20 min after the addition of 10⁻⁴ M erythromycin to either the mucosal or submucosal solution. In control experiments, the solvent of erythromycin was added to both sides of the chamber. Aliquots (100 μ l) from each sample were analysed for PGE₂ and PGF_{2 α} contents in duplicate by ¹²⁵I-radio-immunoassay (New England Nuclear, Boston, MA, USA).

Drugs

The following drugs were used: erythromycin as erythromycin base (Shionogi Pharmaceutical, Osaka, Japan), amiloride, bumetanide, indomethacin (Sigma), DPC (Nakarai Inc., Tokyo, Japan), AA-861, ampicillin (Takeda Chemicals, Osaka, Japan), cephalosporin (Fujisawa Pharmaceutical, Osaka, Japan), tetracycline (Nihon Lederle, Tokyo, Japan). Erythromycin was dissolved in dimethyl sulphoxide (10⁻¹ M), subsequently diluted in Krebs-Henseleit solution, and the pH was adjusted to 7.4.

Statistical analysis

All values are expressed as means \pm SE. Statistical analysis was performed by one-way analysis of variance or the Newman-Keuls multiple comparison test, n refers to the number of dogs from which tissues were obtained, and a p value <0.05 was considered significant.

Results

Electrical properties

As demonstrated in figure 1, the solvent (0.1% dimethyl sulphoxide) alone did not change I_{sc} of canine tracheal epithelium. By contrast, addition of erythromycin (10⁻⁴ M) to the submucosal bath but not to the mucosal bath gradually decreased I_{sc}. This decrease in

Isc was reversed by the subsequent wash of cells by Krebs-Henseleit solution, implying that erythromycin may not be toxic to tracheal epithelium in culture. The decrease in Isc induced by the submucosal application of erythromycin (8.2 ± 0.8 to $2.5 \pm 0.4 \mu\text{A}\cdot\text{cm}^{-2}$, $p < 0.001$, $n=12$) was accompanied by the significant decrease in PD and G (table 1).

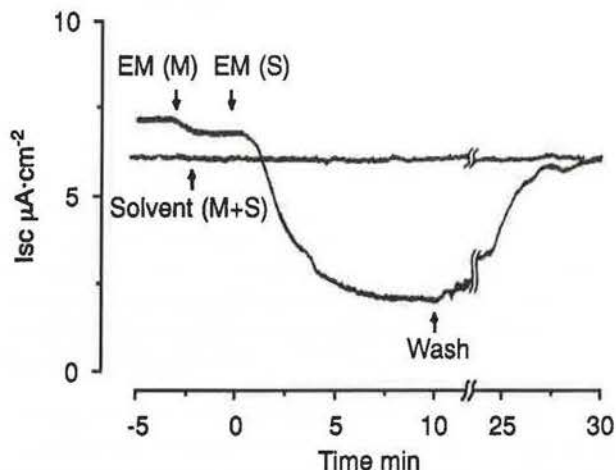


Fig. 1. — A representative tracing showing the time course of the effects of erythromycin (EM) and its solvent on short-circuit current (Isc) of canine tracheal epithelium. EM (10^{-4} M) was added to the mucosal (M) and then submucosal (S) solutions in Ussing chamber. After the Isc response reached a plateau, cells were washed with Krebs-Henseleit solution.

Table 1. — Effect of erythromycin on bioelectric properties of canine tracheal epithelium in culture

	Isc $\mu\text{A}\cdot\text{cm}^{-2}$	PD mV	G $\text{mS}\cdot\text{cm}^{-2}$
Baseline	8.2 ± 0.8	2.6 ± 0.4	3.2 ± 0.5
Erythromycin (M)	8.5 ± 1.2	2.4 ± 0.4	3.5 ± 0.5
Erythromycin (S)	$2.5 \pm 0.4^{***}$	$1.2 \pm 0.3^{**}$	$2.1 \pm 0.3^*$

Isc: short-circuit current; PD: potential difference; G: conductance. Erythromycin (10^{-4} M) was added to the mucosal (M) or submucosal (S) solution in Ussing chamber. Values are means \pm SE; $n=12$. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, significantly different from baseline values.

The inhibitory effect of the submucosal erythromycin on Isc was dose-dependent, the maximal decrease from the baseline value and the concentration required to produce a half-maximal effect (IC_{50}) being $5.6 \pm 1.0 \mu\text{A}\cdot\text{cm}^{-2}$ ($p < 0.001$, $n=9$) and $18 \mu\text{M}$, respectively (fig. 2). In contrast, Isc was not significantly altered by the mucosal erythromycin at a concentration of up to 10^{-3} M.

The effects of other antibiotics including ampicillin, cephalosporin and tetracycline on epithelial bioelectric properties were also examined in multiple dose-response experiments. None of these drugs at concentrations ranging from 10^{-7} to 10^{-3} M caused any significant changes in Isc of canine cultured tracheal epithelium (data not shown).

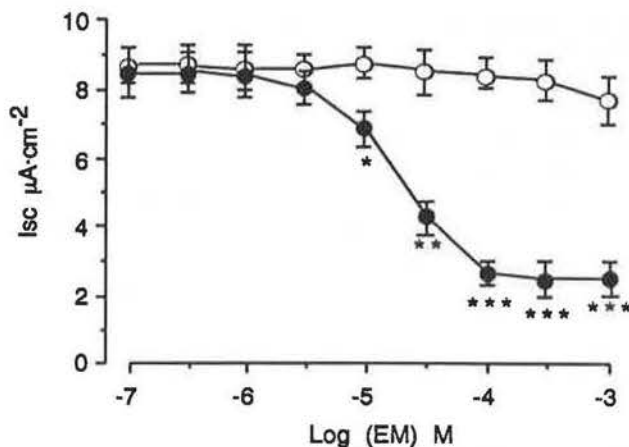


Fig. 2. — Dose-dependent effect of erythromycin (EM) on short-circuit current (Isc) of canine cultured tracheal epithelium. EM was cumulatively added to either the mucosal (open circles) or submucosal (closed circles) solution. Data are means \pm SE; $n=17$ for mucosal addition, and $n=9$ for submucosal addition. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, significantly different from baseline values.

Pretreatment of cells with amiloride, bumetanide, DPC, and Cl-free medium decreased the baseline Isc by 2.5 ± 0.4 , 4.2 ± 1.0 , 4.3 ± 0.9 , and $4.7 \pm 0.8 \mu\text{A}\cdot\text{cm}^{-2}$, respectively ($n=9$ in each case). In amiloride-treated cells, subsequent addition of 10^{-4} M erythromycin produced a similar decrease in Isc as observed in cells without pretreatment. On the other hand, each of bumetanide, DPC and Cl-free medium abolished the erythromycin-induced decrease in Isc ($p < 0.001$, fig. 3).

The decrease in Isc induced by 10^{-4} M erythromycin was not affected by pretreatment of cells with 10^{-6} M AA-861, but it was inhibited by that with 3×10^{-6} M indomethacin ($p < 0.05$, $n=10$, fig. 4).

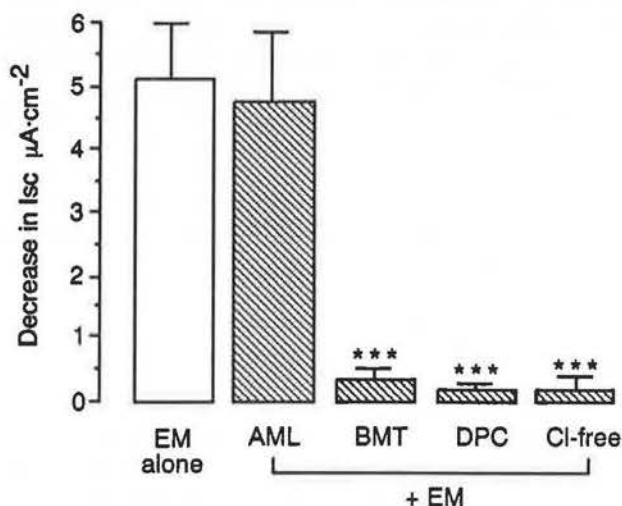


Fig. 3. — Effects of amiloride (AML, 10^{-4} M), bumetanide (BMT, 10^{-4} M), diphenylamine-2-carboxylate (DPC, 10^{-4} M), and substitution of Cl in the medium with gluconate (Cl-free) on the decrease in short-circuit current (Isc) induced by erythromycin (EM, 10^{-4} M) in canine cultured tracheal epithelium. Response are expressed as means \pm SE; $n=9$ for each group. ***: $p < 0.001$, significantly different from the response to EM alone.

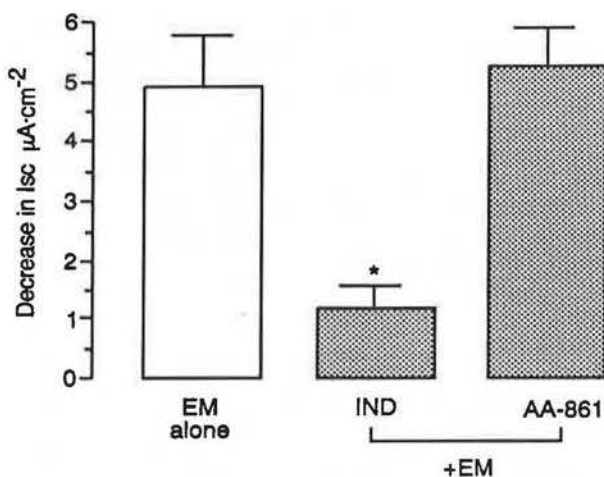


Fig. 4. — Effects of indomethacin (IND, 3×10^{-6} M) and AA-861 (10^{-6} M) on the decrease in short-circuit current (Isc) induced by erythromycin (EM, 10^{-4} M) in canine cultured tracheal epithelium. Responses are expressed as means \pm SE; $n=10$ for each group. *: $p<0.05$, significantly different from the response to EM alone.

Prostaglandin release

Addition of erythromycin (10^{-4} M) to the submucosal solution decreased the rate of PG release by tracheal epithelium from 970 ± 130 to 430 ± 100 $\text{pg}\cdot\text{h}^{-1}$ for PGE_2 ($p<0.05$, $n=6$), and 280 ± 50 to 70 ± 20 $\text{pg}\cdot\text{h}^{-1}$ for $\text{PGF}_{2\alpha}$ ($p<0.05$, $n=6$), whereas the mucosal erythromycin was without effect (Fig. 5).

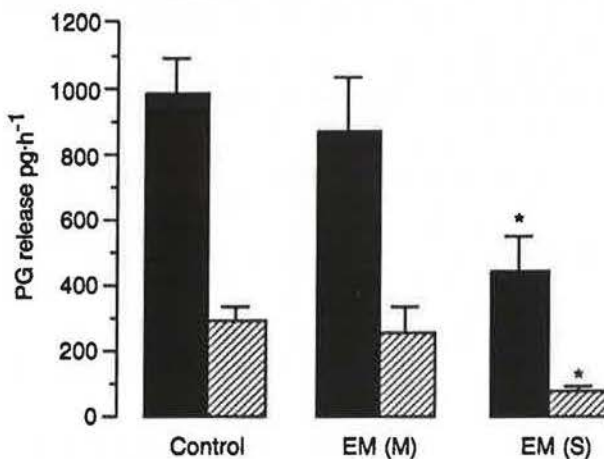


Fig. 5. — Effects of the mucosal (M) and submucosal (S) addition of erythromycin (EM, 10^{-4} M) on the release of prostaglandin (PG) E_2 (solid bars) and $\text{PGF}_{2\alpha}$ (shaded bars) from canine cultured tracheal epithelium. Values are means \pm SE; $n=6$ for each bar. *: $p<0.05$, significantly different from corresponding control values.

Discussion

Our *in vitro* studies on epithelial bioelectric properties provide the indirect evidence that the macrolide erythromycin may selectively inhibit Cl secretion across canine cultured tracheal epithelial cells. This notion is based on the findings that the decrease in Isc induced by erythromycin was abolished by the substitution of Cl in the bathing medium with gluconate, an anion that is not transported by airway epithelium [13], or by the

preincubation of cells with each bumetanide, an inhibitor of Cl transport [11], and DPC, an inhibitor of Cl conductance at the apical membrane [12] but not by the Na channel blocker amiloride [10]. In the presence of amiloride, the Isc appears to be generated entirely by Cl secretion, which was further decreased by erythromycin. Thus, the effect of this drug is Cl-dependent. On the other hand, cells treated with bumetanide, DPC, or Cl-free medium, in which Isc can be attributable to Na absorption, did not respond to erythromycin, implying that Na movement may not be influenced by erythromycin. To confirm our conclusion, the direct measurement of ion fluxes would be valuable.

In contrast to the effect of the submucosal application of erythromycin, mucosal erythromycin, even at high concentrations, did not alter the Isc of tracheal epithelium. The reason for this difference is unknown, but one possible explanation would be that the erythromycin binding sites might be localized to the submucosal but not to the mucosal membrane of airway epithelial cells.

A wide variety of antibiotics are used in the treatment of patients with acute bronchitis, chronic bronchitis and bronchiectasis, who have copious sputum production. These drugs include erythromycin, ampicillin, cephalosporin and tetracycline, among which only erythromycin was found to possess modulatory actions on airway epithelial ion transport in the present experiments. The erythromycin-induced inhibition of Cl secretion theoretically leads to the decrease in water movement toward the airway lumen [3], thus possibly implicating its clinical efficacy to reduce the amount of sputum production [5]. A discrepancy seems to exist in the concentrations of erythromycin required to exert its *in vivo* and *in vitro* effects. The mean serum concentration following the ingestion of 500 mg erythromycin by the adult volunteers has been reported to be 1.6×10^{-6} M [19], whereas in our *in vitro* experiments the decrease in Isc was observed only with concentrations of at least 10^{-5} M. However, because of the species difference, these findings may not necessarily negate its clinical significance. In addition, the serum concentrations of erythromycin does not accurately reflect the local concentration since this drug has been shown to concentrate intracellularly more than tenfold [20].

Among arachidonic acid metabolites synthesized and released from canine tracheal epithelium, PGE_2 and $\text{PGF}_{2\alpha}$ are main products [16], and both PGs are known to stimulate Cl secretion [17, 18]. Because erythromycin has been shown to inhibit the PG release from mononuclear and polymorphonuclear leucocytes [21], we examined whether the observed effect of erythromycin on tracheal epithelium was likewise associated with the inhibition of arachidonic acid metabolism. We found that the cells, in which cyclooxygenase activity had already been blocked by indomethacin, showed less Isc response to erythromycin, and that pretreatment of cells with the lipooxygenase inhibitor AA-861 [15] was without effect. Therefore, the inhibitory effect of erythromycin on airway bioelectric properties may be

accomplished by the inhibition of arachidonic acid conversion to its metabolites *via* the cyclooxygenase pathway. This notion is further supported by the finding that the submucosal but not mucosal erythromycin decreased the release of PGE₂ and PGF_{2α} from tracheal epithelium.

In addition to its antimicrobial and immunomodulatory actions [6, 21], erythromycin has a direct inhibitory action on the secretion of glycoprotein from airway submucosal glands [8]. This function seems compatible with the empirical clinical experience and a previous study [5] that erythromycin is effective in reducing excessive accumulation of mucus in the respiratory tract. On the other hand, active transport of Cl⁻ by airway epithelium is correlated to the secretion of water [3], thereby affecting the depth of sol phase of airway surface fluid and the rheological properties of mucus that interacts with ciliary beating to perform mucociliary transport [2]. Therefore, inhibition of Cl⁻ secretion by erythromycin might result in the decrease in the amount of water in the airway lumen, which could influence airway mucociliary transport function.

Acknowledgements: The authors thank M. Shino and Y. Sugimura for their technical assistance.

References

1. Wanner A. - State of the art: clinical aspects of mucociliary transport. *Am Rev Respir Dis*, 1977; 116: 73-125.
2. Nadel JA, Widdicombe JH, Peatfield AC. - Regulation of airway secretions, ion transport, and water movement. In: *The respiratory system*. A.P. Fishman ed., American Physiological Society, Bethesda, 1985; pp. 419-445.
3. Welsh MJ, Widdicombe JH, Nadel JA. - Fluid transport across the canine tracheal epithelium. *J Appl Physiol: Respirat Environ Exercise Physiol*, 1980; 49: 905-909.
4. Willey RF, Gould JC, Grant WB. - A comparison of ampicillin, erythromycin and erythromycin with sulphametopyrazine in the treatment of infective exacerbations of chronic bronchitis. *Br J Dis Chest*, 1978; 72: 12-20.
5. Suez D, Szefer SJ. - Excessive accumulation of mucus in children with asthma: a potential role for erythromycin? A case discussion. *J Allergy Clin Immunol*, 1986; 77: 330-334.
6. Roche Y, Gougerot-Pocidalo M-A, Fay M, Forest N, Pocidalo J-J. - Macrolides and immunity: effects of erythromycin and spiramycin on human mononuclear cells proliferation. *J Antimicrob Chemother*, 1986; 17: 195-203.
7. Anderson R. - Erythromycin and roxithromycin potentiate human neutrophil locomotion *in vitro* by inhibition of leukoattractant-activated superoxide generation and auto-oxidation. *J Infect Dis*, 1989; 159: 966-973.
8. Goswami SK, Kivity S, Marom Z. - Erythromycin inhibits respiratory glycoconjugate secretion from human airways *in vitro*. *Am Rev Respir Dis*, 1990; 141: 72-78.
9. Coleman DL, Tuet IK, Widdicombe JH. - Electrical properties of dog tracheal epithelial cells grown in monolayer culture. *Am J Physiol*, 1984; 246: C355-C359.
10. Al-Bazzaz FJ, Zevin R. - Ion transport and metabolic effects of amiloride in canine tracheal mucosa. *Lung*, 1984; 162: 357-367.
11. Widdicombe JH, Nathanson IT, Highland E. - Effect of "loop" diuretics on ion transport by dog tracheal epithelium. *Am J Physiol*, 1983; 245: C388-C396.
12. DiStefano A, Wittner M, Schlatter E, Lang HJ, Englert H, Greger R. - Diphenylamine-2-carboxylate, a blocker of the Cl⁻ conductive pathway in Cl⁻-transporting epithelia. *Pflügers Arch*, 1985; 405: S95-S100.
13. Widdicombe JH, Welsh MJ. - Anion selectivity of the chloride-transport process in dog tracheal epithelium. *Am J Physiol*, 1980; 239: C112-C117.
14. Langridge-Smith JE, Rao MC, Field M. - Chloride and sodium transport across bovine tracheal epithelium: effects of secretagogues and indomethacin. *Pflügers Arch*, 1984; 402: 42-47.
15. Schulz R, Seeger W. - Release of leukotrienes into the perfusate of calcium-ionophore stimulated rabbit lungs: influence of 5-lipoxygenase inhibitors. *Biochem Pharmacol*, 1986; 35: 183-193.
16. Leikauf GD, Ueki IF, Nadel JA, Widdicombe JH. - Release of cyclooxygenase products from cultured epithelium derived from human and dog trachea [Abstract]. *Fed Proc*, 1985; 44: 1920.
17. Al-Bazzaz FJ, Yadava VP, Westenfelder C. - Modification of Na and Cl transport in canine tracheal mucosa by prostaglandins. *Am J Physiol*, 1981; 240: F101-F105.
18. Smith PL, Welsh MJ, Stoff JS, Frizzell RA. - Chloride secretion by canine tracheal epithelium. I. Role of intracellular cAMP levels. *J Membr Biol*, 1982; 70: 217-226.
19. Fernandes AC, Anderson R, Theron AJ, Jooné G, Vanrensburg CEJ. - Enhancement of human polymorphonuclear leucocyte motility by erythromycin *in vitro* and *in vivo*. *S Afr Med J*, 1984; 66: 173-177.
20. Johnson JD, Hand WL, Francis JB, King-Thompson N, Corwin RW. - Antibiotic uptake by alveolar macrophages. *J Lab Clin Med*, 1980; 95: 429-439.
21. Anderson R, Fernandes AC, Eftychis HE. - Studies on the effects of ingestion of a single 500 mg oral dose of erythromycin stearate on leucocyte motility and transformation and on release *in vitro* of prostaglandin E₂ by stimulated leucocytes. *J Antimicrob Chemother*, 1984; 14: 41-50.