Am J Physiol Lung Cell Mol Physiol 289: L565–L573, 2005;
doi:10.1152/ajplung.00316.2004.

Erythromycin increases bactericidal activity of surface liquid in human airway epithelial cells

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Submitted 23 August 2004; accepted in final form 20 May 2005

Ishizawa, Kota, Tomoko Suzuki, Mutsuo Yamaya, Yu Xia Jia, Seiichi Kobayashi, Shiro Ida, Hiroshi Kubo, Kiyohisa Sekizawa, and Hidetada Sasaki. Erythromycin increases bactericidal activity of surface liquid in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 289: L565–L573, 2005; doi:10.1152/ajplung.00316.2004.—Macrolide antibiotics have clinical benefits in patients with diffuse panbronchiolitis and in patients with cystic fibrosis. Although many mechanisms have been proposed, the precise mechanisms are still uncertain. We examined the effects of erythromycin on bactericidal activity of airway surface liquid secreted by cultured human tracheal epithelial cells. Airway surface liquid was collected by washing the surface of human tracheal epithelial cells with a sodium solution (40 meq/l). Methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* were incubated with airway surface liquid, and the number of surviving bacteria was examined. The number of bacteria in airway surface liquid from the cells cultured in medium alone was significantly lower than that in the sodium solution. Furthermore, the number of bacteria in airway surface liquid from the cells treated with erythromycin was significantly lower than that in airway surface liquid from the cells treated with solvent alone. The production of mRNA and protein of human β-defensin-1 and human β-defensin-2 was significantly increased by erythromycin. Bactericidal activity of airway surface liquid was observed at low concentrations (40 meq/l) of sodium but not at higher concentrations (≥80 meq/l). Airway surface liquid did not contain significant amounts of antibiotics supplemented in the culture medium. Erythromycin at the levels in airway surface liquid and in culture medium did not inhibit bacterial growth. These results suggest that erythromycin may increase bactericidal activity of airway surface liquid in human airway epithelial cells through human β-defensins production and reduce susceptibility of the airway to bacterial infection.

defensin; airway surface liquid

MACROLIDE ANTIBIOTICS IMPROVE survival in patients with diffuse panbronchiolitis (DPB) (30) and have clinical benefits in patients with cystic fibrosis (CF) (13). Many mechanisms have been proposed, including the effects on neutrophil function (26), IL-8 production (27), sputum rheology (12), goblet cell hypersecretion (44), the alginate biofilm produced by *Pseudomonas aeruginosa* (28), and direct antipseudomonal activity (45). However, the precise mechanisms are still uncertain (23).

Defensins, one of the most intensively studied classes of antimicrobial peptides, are identified in a wide distribution of animals, including birds, rodents, and humans (46). The main function of defensins is believed to be to kill bacteria and fungi either on the surfaces of the epithelial cells or within phagolysosomes of phagocytes. Defensins are small cationic peptides containing 29–47 arginine-rich amino acids with three disulfide bonds, which can be divided into the α- and β-defensin subfamilies in human subjects (46). The α-defensins are produced by neutrophils and intestinal Paneth cells, whereas β-defensins are mainly produced by epithelial cells. Of the β-defensins, airway epithelial cells produce human β-defensin (HBD)-1, HBD-2, and HBD-3 (11, 17, 18, 24, 40, 46). mRNA for HBD-1 is constitutively expressed, whereas mRNA expression for HBD-2 and HBD-3 is induced by proinflammatory cytokines such as tumor necrosis factor (TNF)-α (19) and lipopolysaccharide (LPS) (2). Recent studies have demonstrated that human airway epithelial cells produce sodium-sensitive antimicrobial peptides into the apical side of surface liquid and that the bactericidal activity of HBD decreases in CF, suggesting a major role of HBD in the host defense against bacterial infections (17, 40).

Erythromycin has a variety of biological functions other than antibacterial activities in the airway epithelial cells (27, 42). However, the effects of erythromycin on the bactericidal factors secreted by the human airway epithelium are not well understood. We therefore examined the bactericidal activity of airway surface liquid (ASL) and HBD production in cultured human tracheal epithelial cells.

MATERIALS AND METHODS

Experiments in the present study. Experiments performed in the present study are listed in Table 1.

Media components. Reagents for cell culture media were obtained as follows: DMEM, Ham's F-12 medium, and FCS were from GIBCO BRL (Life Technologies, Palo Alto, CA); dithiothreitol, Sigma type XIV protease, penicillin, streptomycin, gentamicin, amphotericin B, and erythromycin were from Sigma (St. Louis, MO); vitrogen solution was from Cohesion (Palo Alto, CA); and Ultroser G serum substitute (USG) was from BioSepra (Cergy-Saint-Christophe, France).

Culture of human tracheal epithelial cell and lung epithelial cell line. The tracheae were obtained 3–6 h after death from 28 patients (mean age, 74 ± 9 yr; range, 58–90 yr). A protocol for cell culture from tracheae removed from deceased patients and other procedures used in this study were passed by the Tohoku University Ethics Committee. There was no history of pulmonary disease in these patients, including lung cancer, lung fibrosis, chronic obstructive pulmonary disease, pneumoconiosis, or bronchial asthma. Isolation and culture of human tracheal surface epithelial cells were performed as previously described (49). In brief, the surface epithelium was scored into longitudinal strips and pulled off the submucosa. The tracheal surface epithelial cells were isolated by digestion with pro-

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Table 1. *List of experiments*

Materials	Experiments
Human tracheal epithelial cells	Cell viability
	mRNA expression of HBD-1 and HBD-2
Airway surface liquid	Bactericidal activity of ASL
	Salt sensitivity of bactericidal activity of ASL
	HBD protein level in ASL
	LTF production and Lyz activity in ASL
	Residual content of antibiotics in ASL
A549 cells and BEAS-2B cells	mRNA expression of HBD-1 and HBD-2
Eyrthromycin solution	Antimicrobial activity of erythromycin

HBD, human β-defensin; ASL, airway surface liquid; LTF, lactoferrin; Lyz, lysozyme.

tease (0.4 mg/ml, Sigma type XIV) dissolved in PBS at 4°C overnight. The cells were pelleted (200 *g*, 10 min) and suspended in a mixture of DMEM-Ham's F-12 (DF-12) medium (50/50, vol/vol) containing 5% FCS. The cells were plated at 10^6 viable cells/cm^2 onto filter Millicell-CM inserts (0.45-μm pore size and 0.6-cm^2 area; Millipore Products Division; Bedford, MA). This medium was replaced by DF-12 medium containing 2% USG on the first day after the cells were plated. Cells were grown with an air interface (i.e., no medium added to the apical surface). Cell culture medium was supplemented with penicillin (10^5 U/l), streptomycin (50 mg/l), gentamicin (50 mg/l), and amphotericin B (2.5 mg/l). Millicell inserts were coated with vitrogen gels. To make the vitrogen gels, 10-fold minimum essential medium, 0.1 N NaOH, and vitrogen solution were mixed at 4°C (10/10/80, vol/vol/vol). After being mixed, 0.4 ml/cm^2 of this solution was added to the Millicell inserts.

We also cultured the BEAS-2B human bronchial epithelial cell line and the A549 human type II lung cell line (34) in T-25 flasks (Costar Corning, Cambridge, MA) in an immersed condition (i.e., medium added to the apical surface). The BEAS-2B cells (1×10^6 cells) were cultured in T-25 flasks in 5 ml of serum-free medium consisting of DF-12 medium (50/50, vol/vol) and the following growth factors: 10 μg/ml insulin, 5 μg/ml transferrin, 25 ng/ml epidermal growth factor, 7.5 μg/ml endothelial cell growth supplement, 20 ng/ml triiodothyronine, 0.36 μg/ml hydrocortisone, and 20 ng/ml cholera toxin. The A549 cells were cultured in T-25 flasks in 5 ml of DMEM supplemented with 8% FCS. When A549 cells made confluent sheets in T-25 flasks, the cells were rinsed with PBS and further cultured in 5 ml of the DF-12 with growth factors. Cell culture medium was supplemented with 10^5 U/l penicillin, 50 mg/l streptomycin, and 50 mg/l gentamicin.

Preparation of ASL and treatment of human tracheal epithelial cells with erythromycin: effects of erythromycin on bactericidal activity of ASL, salt sensitivity of bactericidal activity of ASL, HBD protein level, lactoferrin production, and lysozyme activity in ASL, and residual content of antibiotics in ASL. Preparation of ASL from cultured human tracheal epithelial cells was performed as described elsewhere (35, 40). On *days 6–8* of culture, the DF-12 medium containing 2% USG and antibiotics in the basolateral side was replaced by antibiotics-free medium five times over 48 h, and the apical surface was washed three times over 48 h with antibiotics-free PBS. Thereafter, the antibiotics-free DF-12 medium containing 2% USG was changed in the basolateral side at 2- to 3-day intervals. To treat the cells with erythromycin, the apical surface was washed with antibiotics-free PBS three times, the antibiotics-free DF-12 medium containing 2% USG and erythromycin (10 μM) was added to the basolateral side, and cells were cultured for 24 h, since the maximum erythromycin concentrations in the serum and bronchial secretions become higher than 10 μM after an intravenous drip infusion of erythromycin (35). The apical cell surface and basolateral side were then rinsed with antibiotics-free PBS three times, and the DF-12 medium containing 2% USG and erythromycin in the basolateral side was replaced by antibiotics-free and erythromycin-free DF-12 medium containing 2% USG. Cells were further incubated for 24 h, and the crude ASL was then collected by washing the apical surface of the cells with 350 μl of sodium solution (40 meq/l). The collected solution was spun down at 200 *g* for 10 min, and the supernatant was recovered as ASL. Because bactericidal activity of HBD-1 and HBD-2 is reported to be salt sensitive (17, 40), ASL was diluted with saline and water to adjust the sodium concentration at 40 meq/l.

Bacterial stocks and quantification of bacteria: effects of erythromycin on bactericidal activity of ASL, and salt sensitivity of bactericidal activity of ASL and antimicrobial activity of erythromycin. Bacteria were prepared as previously described (7, 9, 10). Single colonies of two strains of methicillin-resistant *Staphylococcus aureus* (MRSA), isolated from patients with pneumonia, were incubated on mannitol salt agar plates (Eiken, Tokyo, Japan) (7, 9) and cultured overnight at 37°C with methods as previously described. Minimum inhibitory concentration (MIC) for two strains of MRSA was >8 μg/ml in penicillin G and gentamicin and >4 μg/ml in erythromycin. MRSA used in the present study was resistant to erythromycin. Single colonies of a wild strain and a mucoid type strain of *P. aeruginosa* were inoculated on MacConkey agar plates (10) (Eiken) and cultured overnight at 37°C. MIC for two strains of *P. aeruginosa* was >16 μg/ml in penicillin G, 8 μg/ml in gentamicin, and >4 μg/ml in erythromycin. The MRSA and *P. aeruginosa* were then collected from the colonies and washed once in 10 mM PBS and twice in water. To make bacterial stocks, a suspension of MRSA or *P. aeruginosa* with turbidity equivalent to a McFarland standard (BD, Franklin Lakes, NJ) of 1.0 [corresponding to 3×10^8 colony-forming units (CFU) per ml] was prepared. The concentrations of MRSA and *P. aeruginosa* were also measured by counting the colonies on mannitol salt agar plates and MacConkey agar plates after serial dilution of the bacteria. To study the bactericidal activity of ASL, MRSA and *P. aeruginosa* were further diluted in double-distilled water to an appropriate density. Unless otherwise described, 500 bacteria in 2 μl of double-distilled water were mixed with 30 μl of ASL or double-distilled water and used for the study. The concentrations of MRSA and *P. aeruginosa* are expressed as CFU per ml. We also studied the bactericidal activity of ASL in brain-heart infusion agar that does not contain high salt (6, 41). We used an average value of triplicate cultures from the same bacteria suspension.

Bactericidal activity assay of ASL: effects of erythromycin on bactericidal activity of ASL and on salt sensitivity of bactericidal activity of ASL. The bactericidal activity of ASL was examined as previously described (17, 40). In the preliminary study, we found that bactericidal activity of ASL was most significant in ASL eight times diluted in sodium solution with 40 meq/l of sodium concentration between cells treated with erythromycin and cells treated with a solvent of erythromycin (0.1% ethanol). Therefore, to examine the effects of erythromycin on the bactericidal activity of ASL, a suspension of MRSA or *P. aeruginosa* in 2 μl of double-distilled water (500 bacteria/2 μl) was mixed with ASL (30 μl) eight times diluted in sodium solution (40 meq/l) in 96-well microplates. After this solution containing the bacteria and ASL was incubated for 2 h at 37°C and seeded on mannitol salt agar plates for MRSA and MacConkey agar plates for *P. aeruginosa*, the agar plates were incubated overnight at 37°C, and the number of colonies on the plates was counted. Furthermore, the bactericidal activity of ASL was examined with microdilution assays as described elsewhere (1, 35, 38) with some modification. To examine the bactericidal activity of ASL with microdilution assays (1, 35, 38), 30 μl of ASL were twofold diluted with sodium solution with 40 meq/l of sodium concentration in 96-well plates. Two microliters of suspension of MRSA or *P. aeruginosa* (500 bacteria/2 μl) were added to the diluted ASL in 96-well plates. The bactericidal activity of ASL is expressed as the minimum inhibitory dilution ratio to inhibit the growth of MRSA and *P. aeruginosa*. We measured the minimum inhibitory dilution ratio that is the minimum ratio in which diluted ASL showed significant inhibitory effects on bacterial growth

compared with sodium solution (40 meq/l) alone. In contrast, in further diluted ASL, the number of bacteria surviving after mixing with diluted ASL did not differ from that after mixing with the sodium solution alone. The dilution ratio was calculated by the volume of ASL compared with the volume of sodium solution used to dilute ASL.

To study the concentration-response effects of erythromycin on the growth of MRSA and *P. aeruginosa*, the cells were treated with erythromycin, with concentrations ranging from 10^{-9} to 10^{-5} M, or solvent of erythromycin (0.1% ethanol) for 24 h. A suspension of MRSA and *P. aeruginosa* in 2 μl of double-distilled water (500 bacteria/2 μl) was mixed with 30 μl of ASL, collected from the cells, and eight times diluted with sodium solution with 40 meq/l of sodium concentration.

To study the concentration-response effects of sodium on the bactericidal activity of ASL with methods as previously described (35), ASL was diluted with a sodium solution and water with sodium concentrations ranging from 40 to 110 meq/l. Later, 2 μl of MRSA or *P. aeruginosa* (500 bacteria/2 μl) were mixed with 30 μl of the diluted ASL with various sodium concentrations in 96-well plates. After this solution containing the bacteria and ASL was incubated for 2 h at 37°C and seeded on the agar plates, the agar plates were incubated overnight at 37°C, and the number of colonies on the plates was counted.

Real-time quantitative RT-PCR for HBD-1 and HBD-2: effects of macrolide antibiotics on mRNA expression of HBD-1 and HBD-2. To quantify the mRNA of HBD-1, HBD-2, and β-actin expression in the human tracheal epithelial cells, BEAS-2B cells, or A549 cells after treatment with erythromycin, real-time quantitative RT-PCR by using the *Taq*man technology (Applied Biosystems, Foster City, CA) was performed as previously described (32). The effects of azithromycin (10 μg/ml), a new 15-member ring macrolide (37), were also studied in the human tracheal epithelial cells. Azithromycin also has a variety of biological functions other than antibacterial activities, such as reduced mucus secretion, like erythromycin, in the airway epithelial cells (43), and has also clinical benefits in patients with CF (13). Total RNA from the cells 24 h after treatment with erythromycin (10 μM) or solvent of erythromycin (0.1% ethanol) was isolated with RNAzol B (TEL-TEST, Friendswood, TX) according to the manufacturer's protocol. We used the PrimerExpress program (Applied Biosystems) to design the probe and primers according to the guidelines for the best performance of the PCR. The fragment of mRNA for HBD-1 and HBD-2 was reverse transcribed into cDNA (30 min at 48°C) and amplified by PCR for 40 cycles (15 s at 95°C and 1 min at 60°C). Briefly, 100 ng of RNA dissolved in 10 μl of water from each aliquot of the cells were denatured at 90°C for 90 s. Each RNA sample (100 ng/10 μl of water) was mixed in 40 μl of buffer containing the reagents for the one-step RT-PCR reaction as previously described (32), including 100 nM each primer pair: HBD-1, 5′-CCTGAAATCCTGAGTGTTGC-3′ and 5′-GCGTCATTTCTTCTGGTCAC-3′; HBD-2, 5′-CCAGCCATCAGCCATGAGGGT-3′ and 5′-GGAGCCCTTTCTGAATCCGCA-3′ (19); 100 nM *Taq*man probe HBD-1, 5′-(FAM) CCAGTCGCCATGAGAACTTCCTACCT (TAMRA)-3′; HBD-2, 5′-(FAM) TGGCACCTGTGGTCTCCCTGGAACA (TAMRA)-3′. The minimum PCR cycle to detect the fluorescent signal was defined as the cycle threshold (C_t), which is predictive of the quantity of an input target fragment (20). The standard curve between the fluorescence emission signals and C_t was obtained by means of duplicated serial dilutions of the total RNA from human tracheal epithelial cells cultured in medium alone. Real-time quantitative RT-PCR for β-actin was also performed using the same PCR products. mRNA expression of HBD-1 and HBD-2 was normalized to a constitutive expression of β-actin mRNA.

To treat the A549 cells and BEAS-2B cells with erythromycin, cells in the T-25 flasks were washed with antibiotics-free PBS three times, 5 ml of antibiotics-free DF-12 medium containing growth factors and erythromycin (10 μM) were added to T-25 flasks, and the cells were cultured for 24 h.

Western blot analysis of HBDs: effects of erythromycin on HBD protein levels in ASL. Western blot analysis of HBDs was performed as previously described (1, 35) with some modification. To perform Western blot analysis, ASL was collected before and 24 h after exposure to erythromycin (10 μM) or solvent of erythromycin (0.1% ethanol). ASL (20 μl) was electrophoresed on a 16.5% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P^{SQ}, Millipore). Recombinant HBD-1 or HBD-2 peptide (Sigma) was also loaded in the same gel. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween 20 and then incubated in a 1:100 dilution of polyclonal goat anti-HBD-1 or -HBD-2 (Santa Cruz Biotechnology, Santa Cruz, CA). After being rinsed, the membrane was incubated in a 1:1,000 dilution of horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) and developed using an enhanced chemiluminescence detection system (ECL Plus; Amersham Pharmacia Biotech, Piscataway, NJ).

ELISAs for lactoferrin and enzyme activity assay for lysozyme: effects of erythromycin on lactoferrin production and lysozyme activity in ASL. To examine the effects of erythromycin on the production of antimicrobial lactoferrin (LTF) by the cells, we performed specific ELISA for LTF (35). The sensitivity of the assay for the LTF ELISA kit (OxisResearch, Portland, OR) was 1.6 ng/ml. The enzyme activity for lysozyme (Lyz), which induces the lysis of *Micrococcus lysodeikticus*, is commonly measured in human fluids by turbidimetric techniques (35, 51). The sensitivity of the enzyme activity assay for Lyz was 1.0 μg/ml.

Antibiotic concentrations in ASL: residual content of antibiotics in ASL. The content of penicillin G and amphotericin B in ASL was measured with high-performance liquid chromatography (4, 33), the content of streptomycin was measured with spectrophotometric methods (3), and the content of gentamicin was measured with fluorescence polarization immunoassay (25), to examine whether antibiotics were left on the cultured cells. We also measured the content of erythromycin in ASL with high-performance liquid chromatography with electrical detection methods (8).

The detection limit was 5 ng/ml for penicillin G, 25 ng/ml for streptomycin, 50 ng/ml for gentamicin, 40 ng/ml for amphotericin B, and 0.003 μg/ml for erythromycin.

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed using one-way analysis of variance, and multiple comparisons were made using Bonferroni's method. Significance was accepted at $P < 0.05$; n is the number of donors from which cultured epithelial cells were used.

RESULTS

Effects of erythromycin on cell viability. Cell viability, assessed by the exclusion of trypan blue, was consistently >96% in the erythromycin-treated culture as previously described (42). To examine whether erythromycin induced cytotoxic effects on the cultured cells after the cells made a confluent sheet, we counted the cell numbers after the treatment with erythromycin. The cell numbers were constant in the confluent epithelial cells in the control medium, and the coefficient of variation was small (7.5%). Erythromycin treatment (10 μM; 3 days) did not have any effect on the cell numbers. Cell numbers after erythromycin treatment ($1.9 \pm 0.1 \times 10^6$, $P > 0.50$, $n = 7$) were not significantly different from those after treatment with the solvent alone ($1.8 \pm 0.2 \times 10^6$, $n = 7$).

Effects of erythromycin on the bactericidal activity of ASL. The number of MRSA and *P. aeruginosa*, estimated by counting the colonies on the agar plates, was 202 ± 10 CFU ($n = 3$, means ± SE) and 84 ± 5 CFU ($n = 3$), when the suspension of MRSA or *P. aeruginosa* in 2 μl of water (500 bacteria/2 μl)

was mixed with 30 μl of double-distilled water eight times diluted in a sodium solution. The number of MRSA and *P. aeruginosa* surviving after being mixed with ASL from human tracheal epithelial cells was significantly lower than that after being mixed with sodium solution alone (Fig. 1). Furthermore, the number of MRSA and *P. aeruginosa* after being mixed with ASL from the cells treated with erythromycin was lower than that after being mixed with ASL from the cells treated with a solvent of erythromycin (0.1% ethanol; Fig. 1).

The number of another strain of MRSA (78 ± 11 CFU, $n = 3$) and a mucoid type of *P. aeruginosa* (31 ± 15 CFU, $n = 3$) surviving after being mixed with ASL from human tracheal epithelial cells was significantly lower than that after being mixed with sodium solution alone (208 ± 11 CFU, $n = 3$, $P < 0.05$ in MRSA, and 87 ± 6 CFU, $n = 3$, $P < 0.05$ in mucoid type *P. aeruginosa*). Furthermore, the number of MRSA (14 ± 11 CFU, $n = 5$, $P < 0.05$) and mucoid type *P. aeruginosa* (8 ± 5 CFU, $n = 5$, $P < 0.05$) after being mixed with ASL from the cells treated with erythromycin was significantly lower than that after being mixed with ASL from the cells treated with a solvent of erythromycin (0.1% ethanol).

In brain-heart infusion agar, the number of MRSA (82 ± 12 CFU, $n = 3$) and *P. aeruginosa* (34 ± 15 CFU, $n = 3$) surviving after being mixed with ASL from human tracheal epithelial cells was also significantly lower than that after being mixed with sodium solution alone (223 ± 12 CFU, $n = 3$, $P < 0.05$ in MRSA, and 92 ± 5 CFU, $n = 3$, $P < 0.05$ in *P. aeruginosa*). Furthermore, the number of MRSA (15 ± 10 CFU, $n = 3$, $P < 0.05$) and *P. aeruginosa* (9 ± 5 CFU, $n = 3$, $P < 0.05$) after being mixed with ASL from the cells treated with erythromycin was significantly lower than that after being mixed with ASL from the cells treated with a solvent of erythromycin (0.1% ethanol).

We also studied the antimicrobial effects of nonmacrolide antibiotics in the cells treated with either penicillin G (10^5 U/l, 24 h), streptomycin (50 mg/l, 24 h), gentamicin (50 mg/l, 24 h), or amphotericin B (2.5 mg/l, 24 h) after human tracheal epithelial cells were cultured for 48 h in antibiotics-free DF-12 medium containing 2% USG. The number of MRSA and *P. aeruginosa* after being mixed with ASL from the cells treated with either penicillin G (203 ± 10 CFU, $n = 3$, $P > 0.50$ in MRSA, and 86 ± 5 CFU, $n = 3$, $P > 0.50$), streptomycin (200 ± 9 CFU, $n = 3$, $P > 0.50$ in MRSA, and 85 ± 5 CFU, $n = 3$, $P > 0.50$ in *P. aeruginosa*), gentamicin (204 ± 10 CFU, $n = 3$, $P > 0.50$ in MRSA, and 82 ± 5 CFU, $n = 3$, $P > 0.50$ in *P. aeruginosa*), or amphotericin B (205 ± 10 CFU, $n = 3$, $P > 0.50$ in MRSA, and 81 ± 5 CFU, $n = 3$, $P > 0.50$ in *P. aeruginosa*) did not differ from that in the cells treated with vehicle alone (PBS, 201 ± 10 CFU, $n = 3$, in MRSA, and 85 ± 5 CFU, $n = 3$, in *P. aeruginosa*).

In the microdilution assays, the number of MRSA and *P. aeruginosa* surviving after being mixed with ASL was significantly lower than that after being mixed with sodium solution alone at the dilution ratios of 1, 1:2, 1:4, and 1:8 in MRSA and at the dilution ratio of 1, 1:2, 1:4, 1:8, and 1:16 in *P. aeruginosa* (Fig. 2). The minimum inhibitory dilution ratio of ASL with microdilution assays was 1:8 for MRSA and 1:16 for *P. aeruginosa* after being mixed with ASL from control cells treated with a solvent of erythromycin (Fig. 2). Furthermore, erythromycin significantly increased the bactericidal activity of ASL. The number of MRSA and *P. aeruginosa* was lower after being mixed with ASL from the cells treated with erythromycin (10 μM) than that after being mixed with ASL from the cells treated with the solvent alone at the dilution ratios of 1:8, 1:16, 1:32, and 1:64 in MRSA and *P. aeruginosa* ($P < 0.05$) (Fig. 2).

ASL had no bactericidal activity when ASL was collected immediately after being rinsed with fresh medium and PBS. The effects of erythromycin on the bactericidal activity of ASL were dose dependent, and maximum effects were observed at 10 μM (Fig. 3).

Salt sensitivity of bactericidal activity of ASL. The inhibitory effects of ASL on the growth of MRSA and *P. aeruginosa* were dependent on the sodium concentrations as described previously (35, 40). At a 40 meq/l sodium concentration, ASL inhibited the growth of MRSA and *P. aeruginosa* after being mixed with ASL in the cells treated with a solvent of erythromycin (68 ± 8 CFU, $n = 3$ in MRSA and 26 ± 3 CFU, $n = 3$ in *P. aeruginosa*; $P < 0.05$). In contrast, the number of MRSA and *P. aeruginosa* increased in ASL with sodium

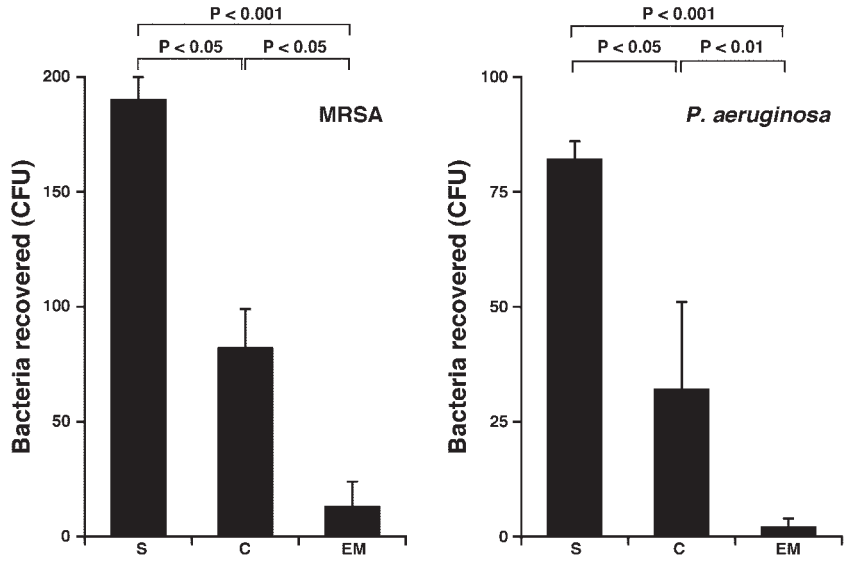

Fig. 1. Growth of methicillin-resistant *Staphylococcus aureus* (MRSA) (*left*) and *Pseudomonas aeruginosa* (*right*) is measured by counting the number of colonies in the absence [sodium solution (S), 40 meq/l] or presence of airway surface liquid (ASL) from the cultured human tracheal epithelial cells treated with erythromycin (EM) or solvent of EM (C, 0.1% ethanol). Results are reported as means ± SE from 3 samples. CFU, colony-forming units.

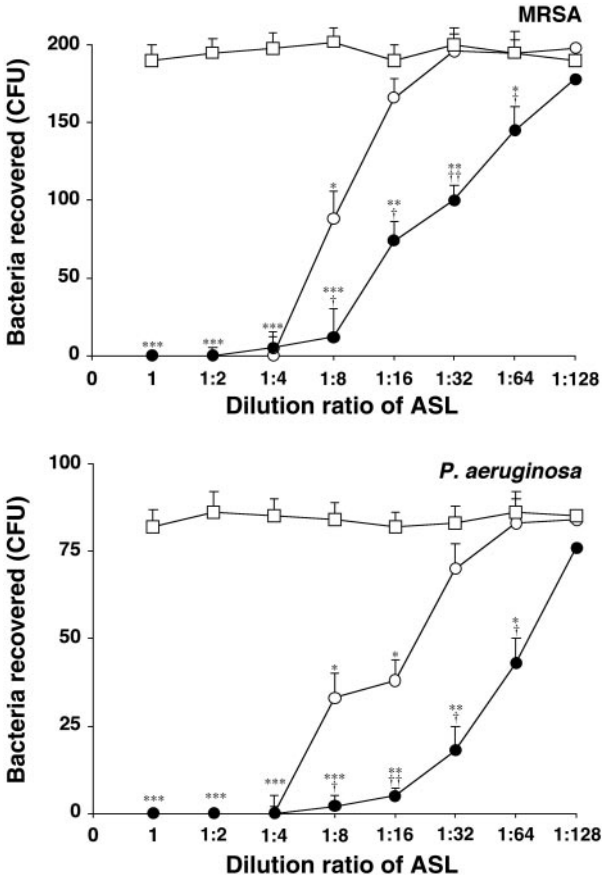

Fig. 2. Growth of MRSA (*top*) and *P. aeruginosa* (*bottom*) is measured with microdilution assays in the ASL from cultured human tracheal epithelial cells treated with EM (10 μM, ●) or solvent of EM (0.1% ethanol, ○) or in distilled sodium solution alone (□). Results are reported as means ± SE from 3 samples. Significant differences from sodium solution alone (□) are indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Significant differences from ASL of the cells treated with solvent alone (○) are indicated by $\dagger P < 0.05$ and $\dagger\dagger P < 0.01$.

concentrations of 80 meq/l (185 ± 11 CFU, $n = 3$ in MRSA and 78 ± 5 CFU, $n = 3$ in *P. aeruginosa*) and 110 meq/l (182 ± 10 CFU, $n = 3$ in MRSA and 81 ± 8 CFU, $n = 3$ in *P. aeruginosa*). On the other hand, the number of MRSA and *P. aeruginosa* was significantly lower, at a 40 meq/l sodium concentration, after being mixed with ASL in the cells treated with erythromycin (13 ± 2 CFU, $n = 3$, $P < 0.05$ in MRSA and 3 ± 1 CFU, $n = 3$, $P < 0.001$ in *P. aeruginosa*) than that after being mixed with ASL in the epithelial cells treated with a solvent of erythromycin. In contrast, at a 110 meq/l sodium concentration, the number of MRSA and *P. aeruginosa*, after being mixed with ASL in the cells treated with erythromycin (171 ± 9 CFU, $n = 3$, $P > 0.50$ in MRSA and 77 ± 6 CFU, $n = 3$, $P > 0.50$ in *P. aeruginosa*), did not differ from that after being mixed with ASL in the cells treated with a solvent of erythromycin (177 ± 10 CFU, $n = 3$ in MRSA and 79 ± 7 CFU, $n = 3$ in *P. aeruginosa*).

Real-time quantitative RT-PCR for HBDs. Treatment with erythromycin consistently increased the amount of mRNA expression of HBD-1 and HBD-2 in human tracheal epithelial cells. mRNA extracted from the cells at 24 h after treatment with erythromycin (10 μg/ml) revealed significant increases in the amount of mRNA expression of HBD-1 and HBD-2 in the cells (Fig. 4, *A* and *B*). The increases in mRNA expression of HBD-2 were higher than those of HBD-1.

Human tracheal epithelial cells, grown in the T-25 flasks in an immersed condition, produced HBD-1 but not HBD-2 (data not shown). Furthermore, erythromycin (10 μg/ml) did not increase mRNA expression of HBD-1 (1.01 ± 0.01/β-actin ratio compared with control cells, $n = 3$, $P > 0.50$).

Azithromycin (10 μg/ml) increased the amount of mRNA expression of HBD-1 (1.40 ± 0.01/β-actin ratio, $n = 3$, $P < 0.05$) and HBD-2 (1.28 ± 0.01/β-actin ratio, $n = 3$, $P < 0.05$) compared with control cells. IL-1β (10 ng/ml) (19, 38) also increased the amount of HBD-1 and HBD-2 mRNA expression (Fig. 4).

We also studied the effects of erythromycin on the production of HBDs in the lung and airway epithelial cell line. Because A549 and BEAS-2B cells were difficult to culture with an air interface on Millicell-CM inserts, studies were performed in the cells cultured in T-25 flasks. A549 cells expressed HBD-1 mRNA, but erythromycin (10 μg/ml) did not increase the amount of HBD-1 mRNA expression (1.1 ± 0.2 mRNA of HBD-1/β-actin ratio compared with that of control cells treated with a solvent of erythromycin, $n = 3$, $P > 0.20$). A549 cells did not express mRNA of HBD-2. BEAS-2B cells expressed mRNA of HBD-1 and HBD-2, but erythromycin (10 μg/ml) did not increase the amount of HBD-1 mRNA expression (1.2 ± 0.2 mRNA of HBD-1/β-actin ratio compared with that of control cells treated with a solvent of erythromycin, $n = 5$, $P > 0.20$) or HBD-2 mRNA expression (1.1 ± 0.1 mRNA of HBD-1/β-actin ratio compared with that of control cells treated with a solvent of erythromycin, $n = 5$, $P > 0.20$). When A549 and BEAS-2B cells could be grown with an air interface ($n = 2$), the cells expressed mRNA of HBD-1 and HBD-2, but erythromycin did not increase HBD-1 and HBD-2 mRNA expression (data not shown).

Effects of erythromycin on HBD protein level in ASL. The protein level of HBD-1 was more abundant than that of HBD-2 in ASL from the human tracheal epithelial cells. The protein level of HBD-1 and HBD-2 in ASL was stable for at least 24 h after being treated with a solvent of erythromycin (0.1% ethanol; data not shown). In contrast, the protein level of HBD-2 in ASL was increased 24 h after being treated with erythromycin (10 μM), although the protein level of HBD-1 in ASL showed only a small amount of increase after being treated with erythromycin (Fig. 5). The treatment of the cells with IL-1β (10 ng/ml) also increased the protein level of HBD-2 (Fig. 5). The magnitude of HBD-1 mRNA expression did not correlate with the protein expression (Figs. 4 and 5).

Effects of erythromycin on LTF production and Lyz activity in ASL. The LTF protein level in ASL 24 h after treatment with erythromycin (10 μM) did not differ from that in ASL 24 after treatment with a solvent of erythromycin (0.1% ethanol) in the human tracheal epithelial cells (Fig. 6). Likewise erythromycin treatment (10 μM, 24 h) did not change the Lyz activity in ASL (Fig. 6). The treatment of the cells with IL-1β (10 ng/ml) (19, 39) did not increase the LTF protein level and the Lyz activity in ASL (Fig. 6).

Antimicrobial activity of erythromycin. To examine the antimicrobial activity of erythromycin, which was treated with the cells, 10 μg/ml of erythromycin were added to the bacterial solution, and bacterial growth was examined. Erythromycin

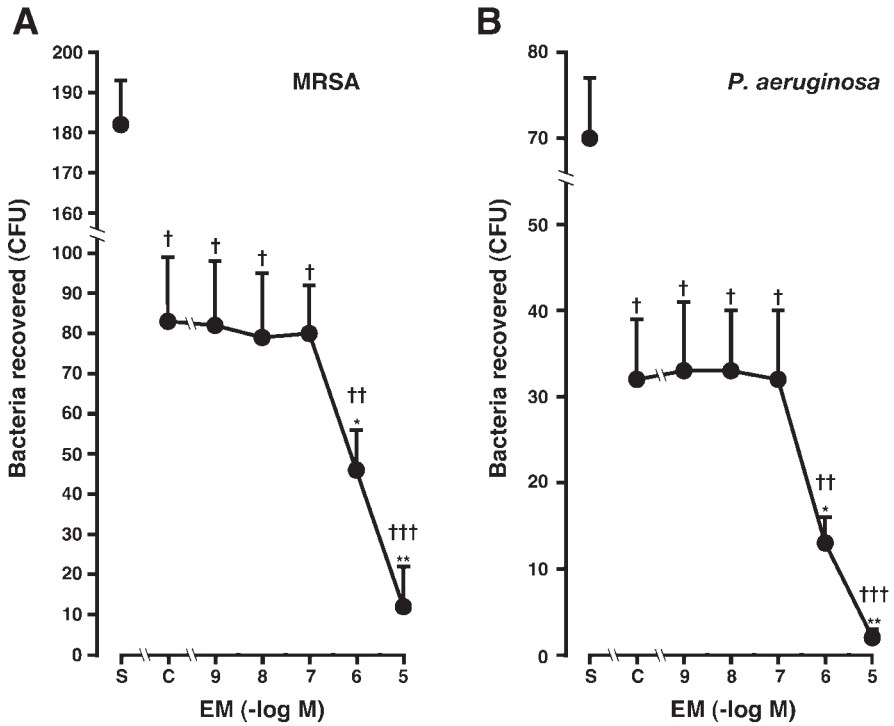

Fig. 3. Shown are concentration-response effects of EM on the growth of MRSA (*A*) and *P. aeruginosa* (*B*) measured by counting the number of colonies after being mixed with ASL from cultured human tracheal epithelial cells treated with EM or a solvent of EM (C, 0.1% ethanol) or after being mixed with sodium solution (S, 4 meq/l). Results are reported as means ± SE from 3 samples. Significant differences from ASL of the cells treated with solvent alone (C) are indicated by $*P < 0.05$ and $**P < 0.01$. Significant differences from the number of bacteria after being mixed with sodium solution (S) are indicated by $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, and $\dagger\dagger\dagger P < 0.001$.

(10 μg/ml) did not inhibit the growth of MRSA and *P. aeruginosa* compared with sodium solution alone. The number of MRSA was 198 ± 8 CFU and that of *P. aeruginosa* was 87 ± 8 CFU after being mixed with erythromycin, and the number of MRSA was 192 ± 11 CFU and that of *P. aeruginosa* was 85 ± 8 CFU after being mixed with sodium solution ($n = 5$, $P > 0.50$).

Residual content of antibiotics in ASL. The content of penicillin G, streptomycin, gentamicin, and amphotericin B in ASL was below the levels detectable (5 ng/ml in penicillin G, 25 ng/ml in streptomycin, 50 ng/ml in gentamicin, and 40 ng/ml in amphotericin B). The content of erythromycin in ASL was 0.0031 ± 0.0001 μg/ml ($n = 5$).

DISCUSSION

The present study suggests that erythromycin may increase the bactericidal activity of ASL from primary cultures of human tracheal epithelial cells via the production of HBDs. These conclusions are made on the basis of the observation that the number of two strains of MRSA and a wild strain and a mucoid type strain of *P. aeruginosa* surviving after being mixed with ASL from the cells treated with medium was significantly lower than that after being mixed with sodium solution alone. Furthermore, the number of MRSA and *P. aeruginosa* surviving after being mixed with ASL from the cells treated with erythromycin was significantly lower than

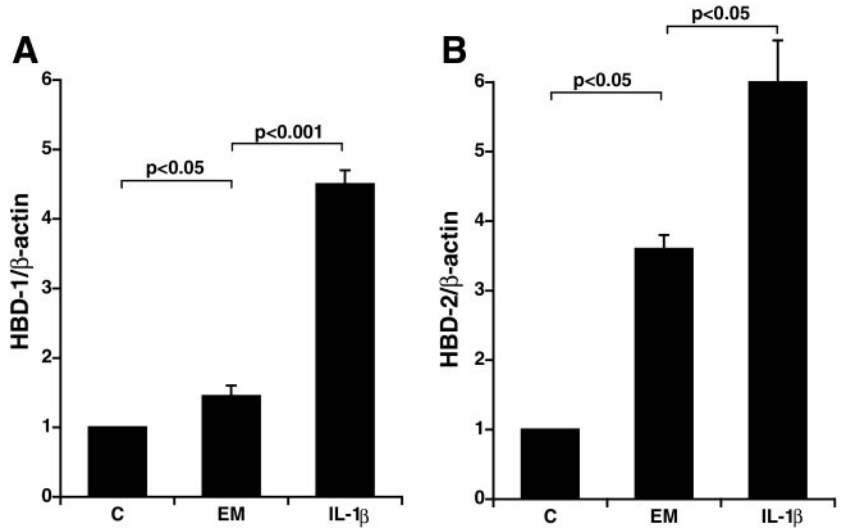

Fig. 4. Expression of human β-defensin-1 (HBD-1; *A*) or HBD-2 (*B*) mRNA in human tracheal epithelial cells 24 h after treatment with EM (10 μM), IL-1β (10 ng/ml), or solvent of EM (C, control, 0.1% ethanol) as detected by real-time quantitative RT-PCR is shown. The mRNA expression for HBDs was standardized by the expression of the β-actin mRNA. Results are expressed as the relative ratio compared with that of control cells treated with a solvent of EM and are reported as means ± SE from 3 samples.

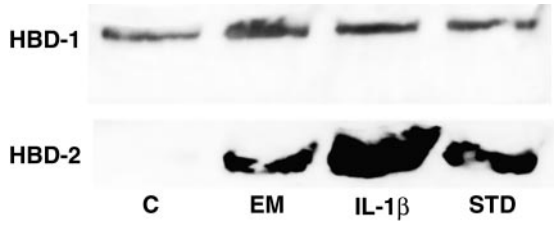

Fig. 5. Western blot analysis demonstrates protein levels of HBD-1 and HBD-2 in ASL from human tracheal epithelial cells 24 h after treatment with EM (10 μM), IL-1β (10 ng/ml), or a solvent of EM (C, control, 0.1% ethanol). Recombinant HBD-1 or HBD-2 peptide was also loaded (STD). Data are representative of 3 different experiments.

that after being mixed with ASL from the cells treated with a solvent of erythromycin, suggesting that the bactericidal activity of ASL for MRSA and *P. aeruginosa* may further increase in cells after erythromycin treatment. The effects of erythromycin on bactericidal activity were dose dependent. Erythromycin dramatically increased the expression of mRNA of HBD-2 in the cells and the protein levels of HBD-2 in ASL. HBD-1 was slightly increased by erythromycin. Bactericidal activity of ASL was observed at low concentrations (40 meq/l) of sodium but not at higher concentrations (≥80 meq/l) of sodium, being consistent with sodium sensitivity of HBD (1, 17, 40). Penicillin G, streptomycin, gentamicin, or amphotericin B were not detectable in ASL, and none of these antibiotics increased the antimicrobial activity of ASL. Although ASL contained a small amount of erythromycin, this amount of erythromycin did not inhibit the growth of MRSA or *P. aeruginosa*. ASL had no bactericidal activity when ASL was collected immediately after being rinsed with fresh medium and PBS as described previously (35). Therefore, observed bactericidal activities might be newly secreted on the apical surface during the incubation after being rinsed. Another kind of macrolide antibiotic, azithromycin, also increased the expression of mRNA of HBD-2, although the potency of the stimulating effect was smaller than that of erythromycin. These findings suggest that erythromycin may increase the production of bactericidal molecules, including defensins in the airway epithelial cells, thereby increasing the bactericidal activity of ASL.

Erythromycin did not change the activity of Lyz, one of the bactericidal factors secreted from airway epithelial cells (48). Although erythromycin slightly increased the LTF levels, the increases in LTF levels were not significant. IL-1β increased the production of protein and mRNA expression of HBD-1 and HBD-2. However, IL-1β did not increase the content of Lyz and LTF, as shown in nasal epithelial cells (50). The LTF levels were 1–4 ng/ml and much lower than those in pulmonary secretions (5). Therefore, HBDs, rather than LTF, might be responsible for antimicrobial activity of the ASL observed in the present study. Furthermore, bactericidal activity of ASL was observed at low concentrations of sodium but not at high concentrations of sodium. These findings suggest that HBD-1 and HBD-2 with sodium sensitivity (17, 40) are responsible for antimicrobial activity of the ASL observed in the present study. In addition, ASL without erythromycin (treated with solvent) had baseline antimicrobial activity (Fig. 1), and this activity was attenuated by a higher concentration of sodium. As seen in Fig. 5, HBD-1 was present in ASL treated with solvent, but HBD-2 was not. HBD-2 dramatically increased in ASL after erythromycin treatment (Fig. 5). These findings suggest that HBD-1 plays a role in the baseline antimicrobial activity. In contrast, the increased bactericidal activity in ASL after erythromycin treatment is mainly brought by HBD-2.

Recently, another sodium-sensitive and inducible defensin was discovered and named HBD-4 (15). Although we did not evaluate the expression of HBD-4 in the present study, because an anti-HBD-4 antibody does not exist and proving the presence of HBD-4 is virtually impossible, HBD-4 may be another candidate for erythromycin-induced bactericidal activity.

In patients with DPB and with CF, various bacteria, including *S. aureus* and *P. aeruginosa*, are isolated (16). Of the bacteria isolated from patients with DPB, *S. aureus* are initially colonized, and *P. aeruginosa* are then colonized, as a disease process (16). *Haemophilus influenzae* is also important in the pulmonary exacerbations in patients with CF (22), although we did not test this in the present study. Long-term, low-dosage erythromycin improves symptoms and increases survival in patients with DPB (30). Since the clinically achievable concentrations of macrolide antibiotics are below their minimum inhibitory concentrations, many mechanisms other than antimicrobial activities have been suggested for the improvement of the survival and clinical benefit in patients with DPB (30) and with CF (13), including effects on neutrophil function (26),

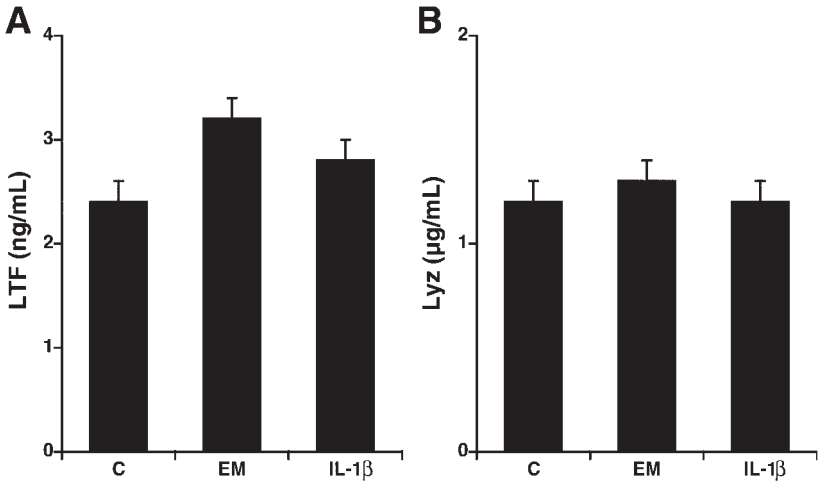

Fig. 6. The levels of lactoferrin (LTF; *A*) and lysozyme (Lyz; *B*) are shown in ASL from human tracheal epithelial cells 24 h after treatment with EM (10 μM), IL-1β (10 ng/ml), or a solvent of EM (C, 0.1% ethanol). Results are expressed as means ± SE from 5 samples.

IL-8 production (27), sputum rheology (12), goblet cell hypersecretion (44), and the alginate biofilm produced by *P. aeruginosa* (28).

In contrast, chronic infection of *P. aeruginosa* was reported to be associated with a poor prognosis in CF (36). Furthermore, serum antibody titer to alginate, which is associated with the biofilm formation by mucoid type *P. aeruginosa*, was higher in *Pseudomonas*-positive patients with DPB than that in *Pseudomonas*-negative patients, and the serum titer decreased in patients who obtained favorable prognosis after clarithromycin treatment (28). These findings suggest that long-term and low-dosage macrolide antibiotics may reduce the growth of *P. aeruginosa* as well as the biofilm formation. In the present study, 1 and 10 μM erythromycin, a clinically achievable concentration of erythromycin (31), increased the production of HBDs in human tracheal epithelial cells and bactericidal activity of ASL from the cells. Ten micromolars of erythromycin did not inhibit the growth of MRSA and *P. aeruginosa* used in the present study. Therefore, antimicrobial activity of HBDs produced in the airway epithelial cells may also be associated with the beneficial effects of macrolide antibiotics in patients with DPB and CF.

An essential amino acid, including L-isoleucin, induces enteric β-defensin in Madin-Darby bovine kidney epithelial cells through the activation of NF-κB (14). Airway epithelial cells produce HBD-1 and HBD-2 (11, 17, 24, 40, 46). mRNA for HBD-1 is constitutively expressed, whereas various stimuli induce HBD-2 production in airway, lung, and intestinal epithelial cells, including IL-1β (19, 39), TNF-α (19), LPS (2), bacterial lipopeptide (21), and lipoteichoic acid (47), through the activation of NF-κB (2, 19, 47), as observed in the present study in the human tracheal epithelial cells treated with IL-1β. In contrast, erythromycin reduces NF-κB activation in the human tracheal epithelial cells (42). Krisanaprakornkit et al. (29) reported HBD-2 induction in gingival epithelial cells involved in mitogen-activated protein kinase pathways, but not in the NF-κB transcription factor family. Therefore, mechanisms other than NF-κB, including these pathways, might be associated with the increased production of HBDs by erythromycin, although we did not study them in the present study.

In the present study, both MRSA and *P. aeruginosa* showed good growth in mannitol salt agar and MacConkey agar, which contain high salt, as previously reported (7, 9, 10). Inhibitory effects of ASL on bacterial growth studied in mannitol salt agar and MacConkey agar were also similar to that in previous reports (17, 40) measured in other kinds of agar such as Luria agar. On the other hand, ASL inhibited bacterial growth in brain heart infusion agar with low salt (6, 41). However, the inhibitory effects of ASL on bacterial growth studied in mannitol salt agar and MacConkey agar were similar to those of ASL in brain heart infusion agar. Thus brain-heart infusion agar that does not contain high salt did not further enhance the effects of the ASL fluid. Although precise reasons are uncertain, both bacteria might be killed rapidly by antimicrobial peptides in ASL, including defensins as reported previously (40), when these bacteria are mixed with ASL before being plated on the agar plates.

Erythromycin (10 μg/ml) did not inhibit the growth of MRSA and *P. aeruginosa* compared with sodium solution alone, and erythromycin concentration in ASL was 0.003 μg/ml. Therefore, erythromycin in either culture medium or ASL had no antimicrobial activity for MRSA and *P. aeruginosa*.

The A549 cells cultured in T-25 flasks in an immersed condition expressed HBD-1 mRNA, but erythromycin did not increase HBD-1 mRNA expression. Likewise, the BEAS-2B cells expressed mRNA of HBD-1 and HBD-2. However, erythromycin did not increase the expression of HBD-1 and HBD-2 mRNA in BEAS-2B cells in T-25 flasks. When A549 and BEAS-2B cells were grown with an air interface, the cells expressed mRNA of HBD-1 and HBD-2, but erythromycin did not increase mRNA expression of HBD-1 and HBD-2. On the other hand, primary cultures of the human tracheal epithelial cells grown in immersed feeding in T-25 flasks produced HBD-1 but not HBD-2, and erythromycin did not increase HBD-1 mRNA expression. Production of HBD-1 and HBD-2 in primary cultures of human tracheal epithelial cells, grown on the filter membrane with an air interface, in this study is consistent with that reported previously (35, 40). Therefore, the loss of stimulating effects of erythromycin on HBD mRNA expression in A549 and BEAS-2B cells might be associated with cell species differences, although the precise reasons are uncertain. Factors in culture media for primary cultures of human tracheal epithelial cells also differed from those for A549 and BEAS-2B. Culture conditions such as an air interface, an immersed condition, and factors in culture media might also alter the expression of HBDs.

In summary, we demonstrated in the present study that erythromycin may increase the bactericidal activity of ASL from human airway epithelial cells via, in part, the induction of HBDs. Increased production of antimicrobial peptide, including HBDs, may be associated with the clinical benefit of macrolide antibiotics in the treatment of DPB and CF.

ACKNOWLEDGMENTS

The authors thank Grant Crittenden for reading the manuscript and Hirokazu Andoh for technical assistance.

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