RESEARCH LETTER

Fosfomycin suppresses RS-virus-induced *Streptococcus pneumoniae* and *Haemophilus influenzae* adhesion to respiratory epithelial cells via the platelet-activating factor receptor

Shin-ichi Yokota[1], Tamaki Okabayashi[1], Yuko Yoto[2], Tsukasa Hori[2], Hiroyuki Tsutsumi[2] & Nobuhiro Fujii[1]

[1]Department of Microbiology, Sapporo Medical University School of Medicine, Sapporo, Japan; and [2]Department of Pediatrics, Sapporo Medical University School of Medicine, Sapporo, Japan

Correspondence: Nobuhiro Fujii, Department of Microbiology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan. Tel.: +81 11 611 2111, ext. 2710; fax: +81 11 612 5861; e-mail: fujii@sapmed.ac.jp

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bacterial adhesion; fosfomycin; *Haemophilus influenzae*; human respiratory syncytial virus; platelet-activating factor receptor; *Streptococcus pneumoniae*.

Abstract

Human respiratory syncytial virus (RSV) sometimes causes acute and severe lower respiratory tract illness in infants and young children. The platelet-activating factor (PAF) receptor, which is a receptor for *Streptococcus pneumoniae* and *Haemophilus influenzae*, is upregulated by RSV infection in the pulmonary epithelial cell line A549. Fosfomycin, an antimicrobial agent, significantly suppressed PAF receptor induction by RSV infection at the mRNA and cell surface expression levels. Fosfomycin also suppressed RSV-induced adhesion of fluorescence-labeled *S. pneumoniae* and *H. influenzae* cells, as determined by flow cytometry and fluorescence microscopy. The RSV-induced bacterial adhesion was suggested to be host-PAF-receptor and bacterial-phosphocholine mediated. Fosfomycin, which has been shown to exhibit antimicrobial and immunomodulatory activities, was found here to suppress adhesion by disease-causing bacteria. Thus, fosfomycin might prevent secondary bacterial infection during RSV infection.

Introduction

Human respiratory syncytial virus (RSV) is one of the most important infectious agents causing acute lower respiratory tract illness, such as bronchiolitis and pneumonia, in infants and young children. Frequently, there are multiple infections with respiratory viruses, including RSV, and bacteria that cause community-acquired respiratory diseases, such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. There is evidence for a positive correlation between infections with *S. pneumoniae* and RSV in the pathogenesis of otitis media, pneumonia, and meningitis (Kim *et al.*, 1996; Andrade *et al.*, 1998; Hament *et al.*, 1999; Chonmaitree & Heikkinen, 2000).

Streptococcus pneumoniae and *H. influenzae* colonize to host respiratory epithelium via host cell surface receptors, such as the platelet-activating factor (PAF) receptor (Cundell *et al.*, 1995, 1996; Swords *et al.*, 2000). These bacteria interact with the PAF receptor via phosphocholine, which is a component of the bacterial cell surface. *Haemophilus influenzae* lipooligosaccharides contain phosphocholines in their carbohydrate chain (Swords *et al.*, 2000). An enhanced adherence of live and heat-killed *S. pneumoniae* cells is observed in human epithelial cells infected with RSV (Hament *et al.*, 2004). The upregulation of PAF receptor expression induced by infection with respiratory viruses, including RSV, results in the enhanced adherence of *S. pneumoniae* and *H. influenzae* to respiratory epithelial cells (Ishizuka *et al.*, 2003; Avadhanula *et al.*, 2006). The PAF receptor expression and *S. pneumoniae* cell adhesion are also upregulated by exposure to acid, which cause tissue injury and an inflammatory response (Ishizuka *et al.*, 2001).

An antimicrobial agent, fosfomycin, has various applications and indications, including upper and lower respiratory infectious diseases, in Japan, European countries, and other countries, whereas the current indication is limited to urinary tract infections in the United States. Fosfomycin inhibits the biosynthesis of *N*-acetyl-neuraminic acid, which is an early step of peptidoglycan synthesis. Fosfomycin shares broad-spectrum antibacterial activities and synergistic activities with various antibiotics including β-lactams

(reviewed in Popovic *et al.*, 2009). In addition to its antibacterial activities, fosfomycin is suggested to have immunomodulatory properties, such as the suppression of proinflammatory cytokine production, as shown by *in vitro* and *in vivo* experimental evidence (Morikawa *et al.*, 1993a, b, 1996, 2003; Matsumoto *et al.*, 1997, 1999; Honda *et al.*, 1998; Ishizaka *et al.*, 1998; Okabayashi *et al.*, 2009). A mechanism for the suppression of proinflammatory cytokines is indicated to be inhibition of transcription factor NF-κB activity, which plays a key role in inflammatory responses (Yoneshima *et al.*, 2003; Okabayashi *et al.*, 2009). PAF receptor expression is also regulated by NF-κB (Mutoh *et al.*, 1994; Shimizu & Mutoh, 1997). Indeed, an NF-κB-specific inhibitor, pyrrolidine dithiocarbamate (PDTC), suppresses acid-induced PAF-receptor-mediated *S. pneumoniae* adhesion to respiratory epithelial cells (Ishizuka *et al.*, 2001). In the present study, we examine the effect of fosfomycin on PAF receptor expression and bacterial adhesion to epithelial cells induced by RSV infection.

Materials and methods

Viruses, cell lines, bacteria, and reagents

RSV strains Long and A2, human type II pulmonary epithelial cell line A549, *S. pneumoniae* strain R6, and *H. influenzae* strain Rd (KW20) were obtained from the American Type Culture Collection (Manassas, VA). Clinical isolates of *S. pneumoniae* and *H. influenzae* were described previously (Yokota *et al.*, 2004; Ohkoshi *et al.*, 2008). RSV was grown in HEp-2 cells. The virus titer of RSV was determined by a plaque-forming assay using HEp-2 cells as an indicator (Okabayashi *et al.*, 2009). Fosfomycin was obtained from Meiji Seika Kaisha (Tokyo, Japan). A PAF receptor antagonist, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phospho(*N*,*N*,*N*,-trimethyl)- hexanolamine, was purchased from Calbiochem-Merck KGaA (Darmstadt, Germany). An NF-κB inhibitor, PDTC, was purchased from Sigma-Aldrich (St. Louis, MO).

Isolation of a phosphocholine-deficient *S. pneumoniae* mutant

A phosphocholine-deficient mutant was isolated by serial passage of *S. pneumoniae* strain R6 in a chemically defined medium (CDM) containing decreasing concentrations of ethanolamine with each passage according to Yother *et al.* (1998). Briefly, approximately 10^6 cells were cultured in 2 mL of CDM containing 200 μg mL^{-1} ethanolamine for 12 h at 37 °C and then diluted 100-fold into the same medium. Following five 12-h passages in CDM containing 200 μg mL^{-1} ethanolamine, similar passages were performed in successively lower concentrations of ethanolamine (20, 2,

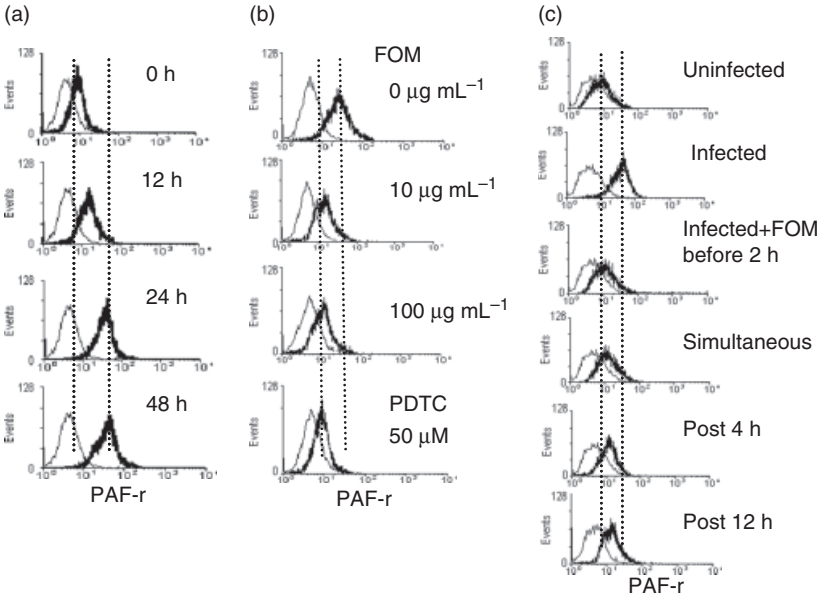

Fig. 1. Upregulation of the PAF receptor (PAF-r) by RSV infection in A549 cells, as determined by flow cytometry (a). Suppression of RSV-induced PAF-r expression by fosfomycin (b and c). (a) A549 cells were infected with the RSV strain Long at MOI 1. After infection at the time indicated, the cells were collected and then stained with an anti-PAF-r antibody and a phycoerythrin-labeled anti-mouse IgG antibody (thick lines). The stained cells were analyzed by flow cytometry. Thin lines indicate cells stained with an unrelated isotype control antibody instead of the anti-PAF-r antibody. (b) Dose dependence of fosfomycin. Two hours before RSV infection, fosfomycin or PDTC was added at the indicated concentration. After a 24-h infection, the expression levels of the PAF-r were determined by flow cytometry as in (a). PDTC, an NF-κB inhibitor, was used as a control. (c) Fosfomycin treatment schedule. Two hours before, simultaneously with, or 4 or 12 h after RSV infection, fosfomycin was added at a concentration of 100 μg mL^{-1}. After 24 h of infection, the expression level of the PAF-r was analyzed by flow cytometry as in (a).

0.2, and then 0 μg mL^{-1}). The resulting mutant was capable of growth in CDM without choline or ethanolamine. The cell wall fraction was prepared as follows: cells grown to the mid-log phase were harvested and immediately boiled with saline containing 4% SDS for 20 min. The boiled cells were disrupted by sonication and then centrifuged at 20 000 ***g*** for 15 min. The pellet was washed extensively with saline, and then used as a cell wall fraction. The content of choline in the cell wall preparation was determined using an enzymatic method (Assmann & Schriewer, 1985). The choline contents of cell wall fractions from R6 and the mutant were 435 nmol mg^{-1} and undetectable, respectively.

Flow cytometry

Cell surface expression of the PAF receptor was examined by flow cytometry. A549 cells were harvested from culture flasks using a cell scraper, and then incubated with 2.5 μg mL^{-1} of mouse anti-PAF receptor monoclonal antibody [11A4 (clone 21); Cayman Chemical, Ann Arbor, MI] or mouse IgG2a,κ isotype control antibody (eBioscience, San Diego, CA). After incubation at 4 °C for 30 min, cells were collected by centrifugation and washed once with Dulbecco's phosphate-buffered saline [PBS(–)]. Cell suspensions were incubated with a phycoerythrin-conjugated goat anti-mouse IgG F(ab)$_2$ fragment antibody (1 : 100 dilution) (Abcam, Cambridge, UK) at 4 °C for 30 min, and the stained cells were assessed with a FACSCalibur (BD Bioscience, San Jose, CA).

Fluorescein isothiocyanate (FITC) labeling of bacteria

A bacterial suspension in 0.1 M NaCl–50 mM sodium carbonate buffer (pH 9.5) at 1×10^8 CFU mL^{-1} was prepared. FITC isomer-I (Dojindo Laboratories, Kumamoto, Japan) was added at a concentration of 1 mg mL^{-1}, and the mixture was incubated at 4 °C for 1 h. The cells were washed three times with PBS(–).

Adhesion assay

A monolayer of A549 cells infected with RSV at a multiplicity of infection (MOI) of 1 for 48 h or of uninfected A549 cells was incubated with FITC-labeled *S. pneumoniae* or *H. influenzae* cells at MOI 10 at 37 °C for 30 min. In some experiments, 20 μg mL^{-1} 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phospho(*N*,*N*,*N*,-trimethyl)-hexanolamine or 10 μg mL^{-1} mouse anti-PAF receptor monoclonal antibody [11A4(clone 21)] was added 2 h before the addition of FITC-labeled bacteria. The cell monolayer was washed three times with PBS(–) and observed by fluorescence microscopy. Alternatively, the cells were harvested with a cell scraper and then assessed by flow cytometry (FACSCalibur).

Semi-quantitative reverse transcription (RT)-PCR

Total RNA was prepared from cells using the QuickGene SP kit RNA cultured cell HC with the QuickGene-800 system (Fujifilm, Tokyo, Japan). RT-PCR was performed using the One-Step RT-PCR kit (Qiagen, Hilden, Germany) as described previously (Okabayashi *et al.*, 2006, 2009). The quantitative nature of the RT-PCR was validated by the linearity of the determination curve obtained with various concentrations of RNA. Detection of PAF receptor mRNA was carried out with the primer set: 5′-ATGGAGCCACATGACTCCTC-3′ and 5′-GAGCCAGCACTGTCGGGCACTGTG-3′.

Statistical analysis

The results between two groups were compared using unpaired Student's *t*-test.

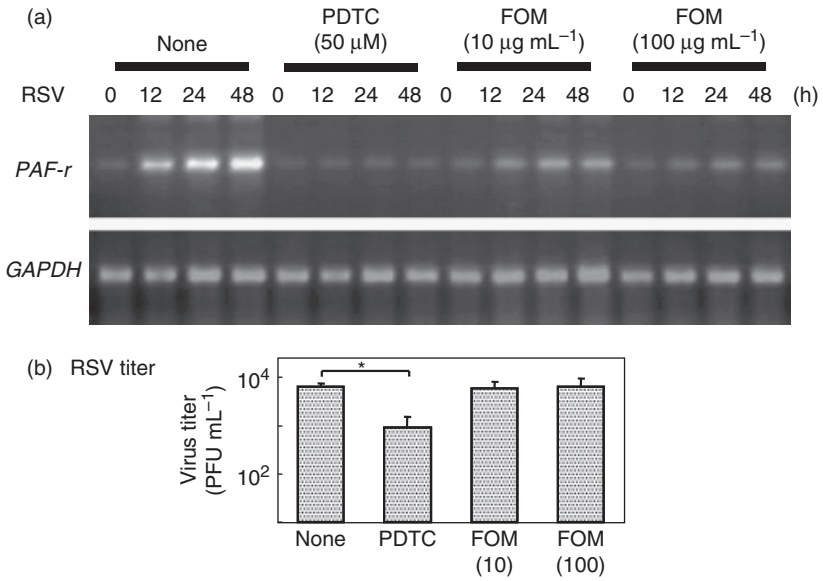

Fig. 2. Suppression of RSV-induced PAF receptor mRNA expression by fosfomycin (FOM) in A549 cells, as determined by RT-PCR. (a) RT-PCR. Two hours before RSV infection, FOM or PDTC was added to cultured A549 cells. PDTC, an NF-κB inhibitor, was used as a positive control for suppression. The cells were infected with RSV strain Long at MOI 1. Total RNA was isolated from cells collected at the indicated times after infection. PAF receptor mRNA (*PAF-r*) levels were determined by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels were determined as controls. (b) Virus titer of RSV. Culture supernatants were collected at 24 h after RSV infection as above. The RSV titer in the culture supernatant was determined by a plaque-forming assay.

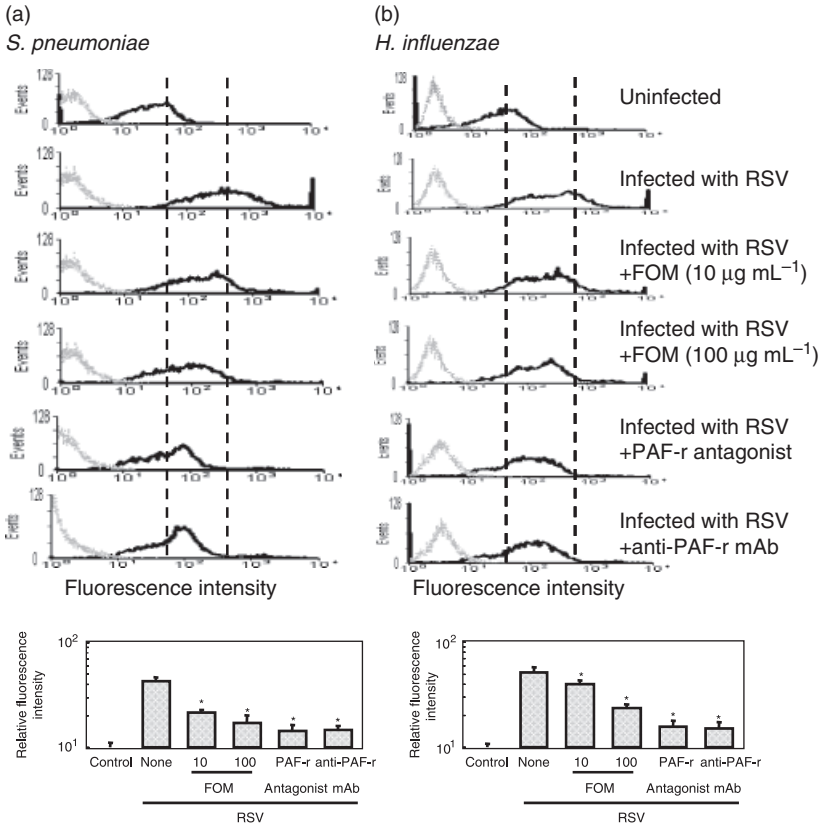

Fig. 3. Suppression by fosfomycin (FOM) of RSV-induced adhesion to A549 cells of FITC-labeled *Streptococcus pneumoniae* (a) and *Haemophilus influenzae* (b), as determined by flow cytometry. Two hours before RSV infection, FOM (10 or 100 μg mL^{-1}), PAF receptor (PAF-r) antagonist (20 μg mL^{-1}), or anti-PAF-r monoclonal antibody (10 μg mL^{-1}) was added to cultured A549 cells. The cells were infected with RSV strain Long at MOI 1. After a 24-h infection, FITC-labeled bacterial cells were added to the cell monolayer at MOI 10, and incubation was continued at 37 °C for 30 min. The adhered labeled bacteria were detected by flow cytometry (black lines). Gray lines indicate cells incubated with unlabeled bacteria. Each experiment was performed in triplicate. The mean fluorescence intensity was estimated and the relative value to the mean fluorescence intensity of RSV-uninfected cells incubated with FITC-labeled bacteria was calculated. Graph presents the mean of relative fluorescence intensity ± SD. *Statistically significant ($P < 0.01$) from RSV-infected cells without the addition of any inhibitory agents (none).

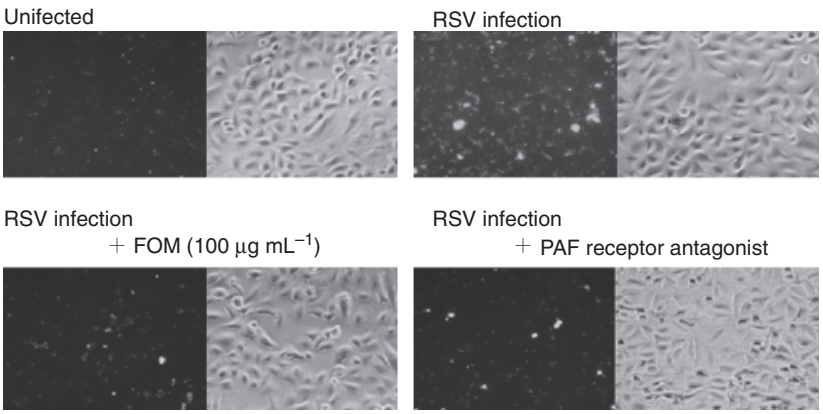

Fig. 4. Suppression by fosfomycin (FOM) of RSV-induced adhesion to A549 cells of FITC-labeled *Streptococcus pneumoniae* adhesion, as observed by fluorescence microscopy. RSV infection, treatment with inhibitors, and incubation with FITC-labeled bacteria were the same as in Fig. 3. Bacteria adhering to the A549 cell monolayer were visualized by fluorescence microscopy. Left photo in each image pair, fluorescence microscopy; right photo, phase-contrast microscopy.

Results

When A549 cells were infected with RSV at MOI 1, the expressions of the PAF receptor were upregulated as detected by flow cytometry (Fig. 1a) and RT-PCR (Fig. 2a). In the presence of fosfomycin during RSV infection, the RSV-induced upregulation of the PAF receptor was significantly suppressed in a dose-dependent manner (Figs 1b and 2a). The degree of suppression by fosfomycin was slightly less than that by an NF-κB inhibitor, PDTC. Whereas fosfomycin did not influence RSV replication, PDTC significantly suppressed RSV replication (Fig. 2b). Suppression of PAF receptor expression was also observed when A549 cells were post-treated with fosfomycin (4 or 12 h after RSV infection) (Fig. 1c).

We examined the adhesion of FITC-labeled *S. pneumoniae* and *H. influenzae* cells to A549 cells by flow cytometry (Fig. 3). RSV infection significantly enhanced *S. pneumoniae* and *H. influenzae* adhesion to A549 cells, and this enhancement was suppressed by the addition of the PAF receptor antagonist or the anti-PAF receptor monoclonal antibody. This result indicated that the RSV-induced bacterial adhesion was via the PAF receptor on A549 cells. The bacterial adhesion was more strongly suppressed by 100 μg mL^{-1} fosfomycin than by 10 μg mL^{-1} fosfomycin (Fig. 3). Suppression of *S. pneumoniae* adhesion by fosfomycin was stronger than that of *H. influenzae* adhesion. A similar observation was made by fluorescence microscopic analysis of *S. pneumoniae* (Fig. 4) and *H. influenzae* (data not shown) adhesion.

Phosphocholine on *S. pneumoniae* cell surface is the ligand for the PAF receptor. Thus, we examined the cell adhesion activity of a phosphocholine-deficient *S. pneumoniae* mutant (Fig. 5). The adherence of the phosphocholine-deficient mutant was slightly upregulated by RSV infection compared to the parent strain R6. The upregulation by RSV infection in R6 was significantly suppressed in the presence of fosfomycin, whereas the adhesion of the mutant to A549 cells was not significantly altered by fosfomycin treatment. These results indicated that fosfomycin suppressed *S. pneumoniae* and *H. influenzae* adhesion in a PAF receptor-dependent manner via bacterial phosphocholine.

Several clinical isolates of *S. pneumoniae* and *H. influenzae* were also assessed. Similar RSV-induced bacterial adhesion and significant suppression by fosfomycin, as well as PAF receptor antagonist occurred (Fig. 6). Furthermore, both strains of RSV, Long and A2, yielded comparable results for upregulation of the PAF receptor and the inhibitory effect of fosfomycin on PAF receptor induction (data not shown). These lines of evidence confirm that the expression of the PAF receptor is induced by RSV infection and indicate that this induction is suppressed by fosfomycin treatment.

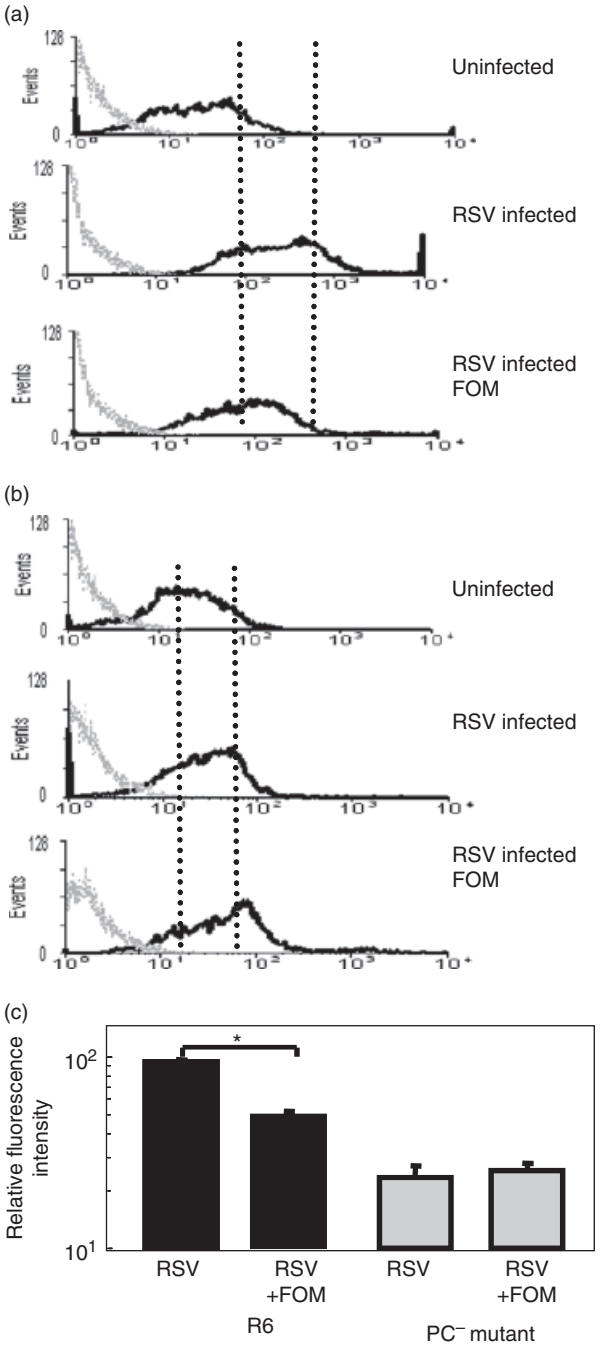

Fig. 5. Effect of fosfomycin (FOM) of RSV-induced adhesion to A549 cells of FITC-labeled *Streptococcus pneumoniae* cells of strain R6 (a) and its phosphocholine-deficient (PC$^-$) mutant, as determined by flow cytometry. Two hours before RSV infection, FOM (100 μg mL^{-1}) was added to cultured A549 cells. The cells were infected with RSV strain Long at MOI 1. After a 24-h infection, FITC-labeled bacterial cells were added to the cell monolayer at MOI 10, and incubation was continued at 37 °C for 30 min. The adhered labeled bacteria were detected by flow cytometry (black lines). Gray lines indicate cells incubated with unlabeled bacteria. Each experiment was performed in triplicate. The mean fluorescence intensity was estimated and the relative value to the mean fluorescence intensity of RSV-uninfected cells incubated with FITC-labeled bacteria was calculated. Graph presents the mean of relative fluorescence intensity ± SD. $^*P < 0.01$.

Discussion

Recently, we reported that fosfomycin suppressed the RSV-induced production of chemokines, such as interleukin-8 (IL-8) and 'regulated on activation, normal T-cell expressed and secreted' (RANTES), in the respiratory epithelial cell line A549, but that it did not affect virus replication (Okabayashi *et al.*, 2009). The suppression of chemokine induction by RSV is due to the downregulation of NF-κB

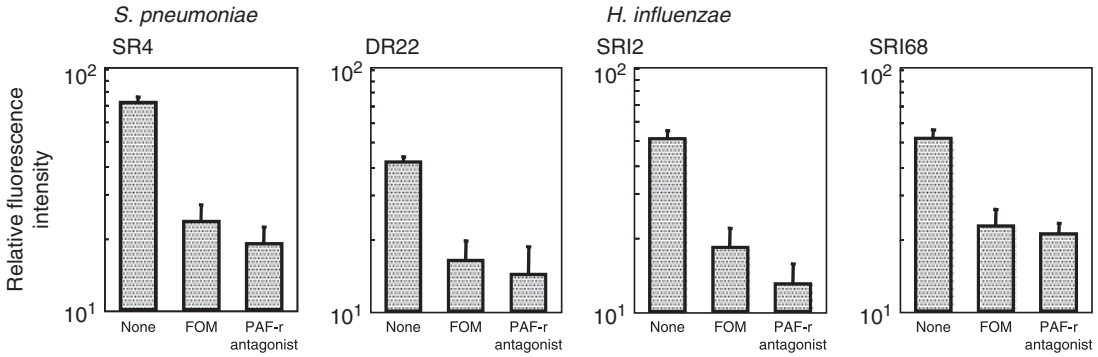

Fig. 6. Suppression by fosfomycin (FOM) of RSV-induced adhesion to A549 cells of FITC-labeled cells of *Streptococcus pneumoniae* (strain SR4 and DR22) (a) and *Haemophilus influenzae* (strain SRI2 and SRI68) (b) clinical isolates, as determined by flow cytometry. Two hours before RSV infection, FOM (100 μg mL^{-1}) or the PAF receptor (PAF-r) antagonist (20 μg mL^{-1}) was added to cultured A549 cells. The cells were infected with RSV strain Long at MOI 1. After a 24-h infection, FITC-labeled bacterial cells were added to the cell monolayer at MOI 10, and incubation was continued at 37 °C for 30 min. The adhered labeled bacteria were detected by flow cytometry (black lines). Gray lines indicate cells incubated with unlabeled bacteria. Each experiment was performed in triplicate. The mean fluorescence intensity was estimated and the relative value to the mean fluorescence intensity of RSV-uninfected cells incubated with FITC-labeled bacteria was calculated. Figure shows the mean of relative fluorescence intensity ± SD. *Statistically significant ($P < 0.01$) from RSV-infected cells without the addition of any inhibitory agents (none).

activation (Okabayashi *et al.*, 2009). Yoneshima *et al.* (2003) also reported the suppression of NF-κB activation by fosfomycin in the human monocytic cell line U937 and in the T-cell line Jurkat stimulated with Gram-negative bacterial lipopolysaccharides. The PAF receptor is a receptor for *S. pneumoniae* and *H. influenzae* (Cundell *et al.*, 1995; Swords *et al.*, 2000). Transcription of the PAF receptor gene is controlled by NF-κB (Mutoh *et al.*, 1994; Shimizu & Mutoh, 1997). Ishizuka *et al.* (2001) showed that the specific NF-κB inhibitor PDTC suppressed acid-induced *S. pneumoniae* adhesion to A549 cells via the suppression of PAF receptor induction. Hence, the suppression of PAF receptor expression by fosfomycin seems to be due to the suppression of NF-κB activation. Respiratory viruses, including RSV, induce PAF receptors and bacterial adhesion via it (Ishizuka *et al.*, 2003; Avadhanula *et al.*, 2006). In the present study, we showed that fosfomycin suppressed *S. pneumoniae* and *H. influenzae* adhesion to RSV-infected A549 cells. The bacterial adhesion was suppressed by the PAF antagonist and the anti-PAF receptor antibody. On the other hand, the phosphocholine-deficient *S. pneumoniae* mutant did not show RSV-induced adhesion, which was suppressed by fosfomycin. These results indicated that the PAF receptor (host)/phosphocholine (bacteria) interaction, which is enhanced by RSV infection, was suppressed by fosfomycin through the inhibition of PAF receptor induction.

Fosfomycin efficiently suppressed PAF receptor expression and RSV-induced PAF receptor-dependent bacterial adhesion at a concentration of 10 μg mL^{-1} (Figs 1 and 2). Goto *et al.* (1981) reported that the peak serum levels of fosfomycin after a rapid intravenous administration of 20 and 40 mg kg^{-1} were 132.1 ± 31.8 and 259.3 ± 32.5 μg mL^{-1}, respectively. Also, the peak serum levels of fosfomycin after oral administration were 7.1 ± 1.6 and 9.4 ± 3.6 μg mL^{-1} for the 20 and 40 mg kg^{-1} doses, respectively. Thus, fosfomycin is expected to suppress the enhanced bacterial adhesion to the RSV-induced PAF receptor by both an intravenous and an oral administration of clinically appropriate doses. Upregulation of PAF receptor expression and the enhanced adhesion of *S. pneumoniae* and *H. influenzae* to respiratory epithelial cells are considered to be major risk factors for secondary bacterial infections after a respiratory virus infection. We propose that fosfomycin efficiently suppresses RSV-induced PAF receptor expression and the enhanced adhesion of disease-causing bacteria.

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