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Role of phosphorylcholine in *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* adherence to epithelial cells☆

Hiroyuki Iuchi *, Junichiro Ohori, Takayuki Kyutoku, Kotoko Ito, Yuichi Kurono

Department of Otolaryngology, Head and Neck Surgery, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan

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ABSTRACT

Objective: Phosphorylcholine (PC) is a structural component of *Streptococcus pneumoniae* (Spn) and nontypeable *Haemophilus influenzae* (NTHi), and is known to be associated with adherence through the platelet activating factor receptor (PAF-R). Furthermore, high PC expression is considered to be involved in Spn and NTHi virulence. In this study, we examined the influence of PC expression on the adherence of Spn and NTHi to epithelial cells in order to clarify the potential effectiveness of a vaccine targeting PC.

Methods: Twenty-seven strains of Spn and twenty-two strains of NTHi were used, cultured overnight, and PC expression was evaluated by fluorescence activated cell sorting; the strains were divided into two groups: PC low expression (PC-low) and PC high expression (PC-high) groups. Bacterial adherence was then examined using Detroit 562 cells and BALB/c mice. Bacterial invasion was then examined in Detroit 562 cells.

Results: The adherence of Spn and NTHi and invasion of NTHi in the PC-high group was significantly reduced by pretreatment with a monoclonal anti-PC antibody (TEPC-15), PAF-R antagonist (ABT-491), and PC-keyhole limpet hemocyanin (PC-KLH). However, such findings were not observed in the PC-low group.

Conclusion: The present study suggests that PC is involved in the mucosal adhesion of Spn and NTHi, and the mucosal invasion of NTHi with PC-high strains, but not PC-low strains. These results suggest that a PC-targeting mucosal vaccine only affects PC-high Spn and NTHi strains and does not disturb commensal bacterial flora in the upper respiratory tract, which comprises nonpathogenic PC-low bacteria.

1. Introduction

Seven- and thirteen-valent pneumococcal conjugate vaccines are well acknowledged to be highly effective in reducing the burden of invasive respiratory infections caused by *Streptococcus pneumoniae* (Spn) in children and the prevalence of antibiotic-resistant strains [1,2]. However, the actual prevention rate for the onset of acute otitis media (AOM) is as low as 6–8% [1,3], and the

☆ This work was performed at Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Kagoshima, Japan.

* Corresponding author at: Department of Otolaryngology, Head and Neck Surgery, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima, 890-8520, Japan.
E-mail address: hamoshun@m.kufm.kagoshima-u.ac.jp (H. Iuchi).

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increases in serotype replacement and AOM caused by non-vaccine type Spn have become a problem [2]. Similarly, routine immunization of infants with a conjugated vaccine against *Haemophilus influenzae* type b (Hib) has greatly reduced the incidence of invasive Hib disease [4]; however, the vaccine is not effective against upper respiratory tract infections including AOM caused by nontypeable *Haemophilus influenzae* (NTHi) that lacks a polysaccharide capsule. Consequently, a marked change in the predominant invasive serotype from Hib to NTHi has occurred [5]. These results indicate the necessity of developing a broad-spectrum vaccine that is effective against most Spn and NTHi strains.

There are several candidates for a protein-based broad-spectrum vaccine including pneumococcal surface protein A (PspA), which is a protein antigen present on the cell surface of Spn; P6, which is the outer membrane protein of NTHi; and phosphorylcholine (PC), a structural component of many pathogens including Spn and NTHi. However, the effectiveness of a vaccine containing PspA or P6 is limited to Spn or NTHi strains, respectively. In contrast, a vaccine containing PC would be effective against both Spn and NTHi as PC is a common pathogen-associated molecular pattern and presents on the outer cell membrane of both Gram-positive and negative bacteria [6]. Furthermore, high PC expression is considered to increase Spn and NTHi virulence. For instance, it is reported that Spn with high PC expression causes more invasive infection than that with low PC expression [7]. Moreover, PC expression in NTHi is associated with prolonged colonization of the nasopharynx in patients with otitis media with effusion [8,9]. Bacterial invasion and colonization is initially induced by bacterial adhesion and fixation onto host mucosal cells, and the binding of PC with platelet activating factor receptor (PAF-R) expressed on the cell surface is known to play a role in the adherence of Spn [10] and NTHi [11]. Bacterial adherence can be inhibited by the secretory IgA induced by mucosal vaccination such as oral and intranasal immunization [12]. Intranasal immunization with protein-linked PC is reported to protect mice against a lethal intranasal challenge with Spn and enhances the clearance of several different Spn and NTHi strains from the nasal cavity [13,14]. These findings indicate the potency of PC as a broad-spectrum mucosal vaccine, which inhibits the adherence of almost all bacteria including Spn and NTHi to the respiratory mucosa. However, there are concerns that a mucosal vaccine including PC might disturb the commensal nonpathogenic bacterial flora, even though these strains have low PC expression.

In the present study, the influence of PC expression and the inhibitory effects of PC-specific IgA on bacterial adherence to cultured human pharyngeal epithelial cells and murine nasal mucosa were examined using several different Spn and NTHi strains. Based on the results, the effectiveness and safety of a mucosal vaccine targeting PC are discussed.

2. Materials and methods

2.1. Bacteria

Five laboratory strains of Spn (BG7322, EF3030, TIGR4, D39, and L82016, provided by Professor Hotomi, University of Wakayama, Wakayama, Japan), sixteen clinical strains of Spn, and eighteen clinical strains of NTHi isolated from the nasopharynx of patients with otitis media with effusion [15]; four clinical strains each of Spn and NTHi isolated from the blood and spinal fluid of patients with meningitis (provided by Professor Nishi, University of Kagoshima, Kagoshima, Japan); and two ATCC strains of Spn (6303 and 6312) were used in the study. All bacteria were stored in skim milk with glycerol at −80 °C until use. An aliquot of each bacterial stock was thawed and cultured on sheep blood agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) or chocolate II agar plates (Nippon Becton Dickinson Company, Tokyo, Japan), as appropriate, overnight at 37 °C in a 5% CO_2 incubator. After washing with 0.5% bovine serum albumin-phosphate buffered saline (PBS), the bacteria were used for cell adhesion assays. Bacterial concentrations of Spn and NTHi were adjusted to 1.0×10^8 colony forming units (CFU)/ml at an absorbance of 580 nm.

2.2. Cell culture

Detroit 562 cells (CCL-138; American Type Culture Collection, Manassas, VA, USA), a human pharyngeal carcinoma epithelial cell line, were grown to confluence in minimal essential medium (Nacalai Tesque, Kyoto, Japan) supplemented with sodium pyruvate (1 mM) (Nacalai Tesque), 10% fetal bovine serum (Invitrogen, San Diego, CA, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Nacalai Tesque) at 37 °C in a 5% CO_2 incubator. The cells were then harvested using trypsin (final concentration, 0.02%) and ethylenediaminetetraacetic acid (EDTA) (final concentration, 0.02%) (Nacalai Tesque).

2.3. Mice

Six-week-old female BALB/c mice were purchased from CLEA Japan Inc. (Shizuoka, Japan) and maintained in the experimental animal facility of Kagoshima University under specific pathogen-free conditions. All mice used in this study were 6-7 weeks of age. The experimental protocol was approved by the Ethics Board of the Institute of Laboratory Animal Sciences of Kagoshima University (Approval Number: 16110 and 17110).

2.4. PC expression on Spn and NTHi

PC expression levels on bacterial surfaces were quantified using fluorescence activated cell sorting (FACS; CytoFLEX, Beckman Coulter, Tokyo, Japan). Bacteria were cultured overnight on blood agar or chocolate II agar plates. The next day, bacteria were collected, suspended in PBS, and incubated at 4 °C for 1–4 h with TEPC-15, a monoclonal IgA antibody specific to PC (1:100 dilution; Sigma–Aldrich, St. Louis, MO, USA), or with a purified mouse IgA antibody (1:50 dilution; BD Bioscience, San Jose, CA, USA) used as an isotype control. The bacteria were then rinsed in PBS and incubated with a fluorescein isothiocyanate (FITC-labeled) goat anti-mouse antibody (1:50 dilution; KPL, Gaithersburg, MD, USA) for 30 min at 20 °C before analysis.

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2.5. PAF-R expression on Detroit 562 cells

FACS analysis was used to determine the PAF-R expression on Detroit 562 cell surfaces. The cells were incubated for 1 h at room temperature with mouse anti-human PAF-R monoclonal antibody (1:1000 dilution; Cayman Chemical, Ann Arbor, MI, USA) or mouse IgG2a antibody (1:1000 dilution; DakoCytomation, Glostrup, Denmark) as an isotype control, followed by incubation with FITC-labeled goat anti-mouse antibody (1:20 dilution; DakoCytomation, Glostrup, Denmark) for 30 min at 4 °C. The mean fluorescence intensity of PAF-R was compared to that of the isotype control.

2.6. Adherence assay (in vitro)

Detroit 562 cells were adhered to the wells of a 96-well plate (Thermo Fisher Scientific Nunc A/S, Roskilde, Denmark). One hundred microliters of each Spn strain (1.0×10^7 CFU/ml) and NTHi strain (1.0×10^5 CFU/ml) was added to the cells and allowed to adhere at 37 °C in a 5% CO_2 incubator for 2 h. Each well was then washed 10 times with 200 μl of PBS and treated with 100 μl of saponin at 37 °C in a 5% CO_2 incubator for 15 min. Subsequently, 100 μl of solution from each well was placed on sheep blood agar or chocolate II agar plates, and the number of colonies formed was measured.

To investigate the effects of PC-specific IgA on bacterial adherence, bacterial cells were treated with TEPC-15 (1 μg/ml) at 37 °C in a 5% CO_2 incubator for 1 h, and then the adherence assay was performed. In addition, PAF-R expressed on Detroit 562 cell surfaces was blocked with 100 μM of a PAF-R antagonist (ABT-491; Cayman Chemical) or 200 μg/ml PC-keyhole limpet hemocyanin (PC-KLH; Biosearch, San Rafael, CA, USA) at 37 °C in a 5% CO_2 incubator for 1 h, and the influence on bacterial adherence was then examined. The number of adherent bacteria were counted by two blind observers in each strain and the mean number of adherent bacteria was calculated for each PC expression group.

2.7. Adherence assay (in vivo)

In order to examine the adherence of Spn and NTHi *in vivo*, the mice were pretreated intranasally for 1 h with TEPC (1 μg/ml), ABT-491 (100 μM), and PC-KLH (100 μg/ml). Next, 10 μl of Spn and NTHi suspended in PBS (10^6 CFU/ml) was administered intranasally. The mice were sacrificed at 12 h after bacterial inoculation and nasal passages were obtained as reported previously [14]. Nasal passage samples were homogenized with 1 ml of culture medium and filtered through a stainless-steel screen. The samples were then spread on agar plates in the same manner as nasal wash samples. After overnight incubation at 37 °C with 5% CO_2, the number of colonies was counted by two blind observers.

2.8. Intracellular invasion assay

One hundred microliters each of the NTHi strains (1.0×10^7 CFU/ml) were added to Detroit 562 cells cultured in a 96-well plate and allowed to adhere at 37 °C in a 5% CO_2 incubator for 6 h. Each well was then treated with gentamicin (200 μg/ml) at 37 °C in a 5% CO_2 incubator for 1 h. After washing five times with 200 μl of PBS, the cells were treated with 100 μl of saponin at 37 °C in a 5% CO_2 incubator for 15 min. Further, 100 μl of the samples from each well were placed on chocolate II agar plates, cultured overnight, and the number of colonies formed was counted. Furthermore, the effects of TEPC-15, ABT-491, and PC-KLH on intracellular invasion were investigated as described for the adherence assay. The number of intracellular invading bacteria were counted by two blind observers in each strain and the mean number of invasive bacteria was calculated for each PC expression group.

2.9. Statistical analysis

The median values of PC expression levels were measured and statistically analyzed using the Mann–Whitney *U* test. Other data are expressed as means ± standard deviations and statistically analyzed using the unpaired Student's *t*-test. Differences were considered significant when the probability value was less than 5%.

3. Results

3.1. PC expression on Spn and NTHi

FACS analysis of PC expression on Spn and NTHi strains showed that all the strains examined expressed PC. However, the level of PC expressed on the bacterial cell surfaces, as assessed by mean fluorescence intensity (MFI), differed in each strain. Thus, we divided the Spn and NTHi strains into two groups according to the median value of MFI in the total strains of Spn and NTHi, respectively, as the PC low expression (PC-low) and PC high expression (PC-high) groups. When the median values of MFI were compared between the PC-low and PC-high groups, a significant difference was observed in both Spn (4243.28 ± 387.14 *vs.* $13{,}196.23 \pm 1571.48$; $p < 0.05$) and NTHi ($17{,}971.93 \pm 1530.46$ *vs.* $59{,}985.28 \pm 8509.81$; $p < 0.05$). (Fig. 1) Further, a significantly positive correlation between PC expression and the number of bacteria adhering to Detroit 562 cells was observed in both Spn ($r = 0.83$, $p < 0.05$) and NTHi ($r = 0.81$, $p < 0.05$). (Fig. 2)

3.2. PAF-R expression

PAF-R expression on Detroit 562 cells was confirmed by FACS. The MFI indicating PAF-R expression was significantly increased by staining with PAF-R monoclonal antibody ($55{,}133.43 \pm 1167.79$) compared with the isotype control ($47{,}689.2 \pm 192.35$) ($p < 0.05$).

3.3. Bacterial adherence and the effects of TEPC-15, ABT-491, and PC-KLH (in vitro)

In this experiment, it was concerned that the numbers of Spn and NTHi adhering to Detroit 562 cells might be contaminated by intracellularly invading bacteria. Therefore, in order to clarify when those bacteria start to invade into Detroit cells, the cells were treated with gentamicin after incubation with Spn and NTHi for 1–6 h to kill the adherent bacteria. Then the cells

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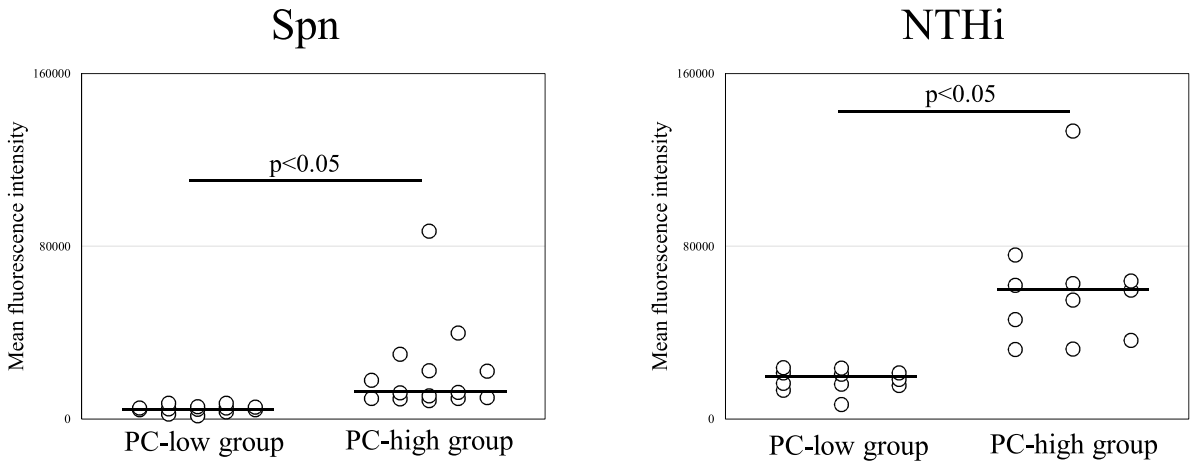

Fig. 1. PC expression on Spn and NTHi.
Spn and NTHi were divided into PC-high and PC-low groups by the median value of MFI of the total strains of Spn and NTHi, respectively. There was a significant difference in the median values of MFI between the two groups in both Spn and NTHi.

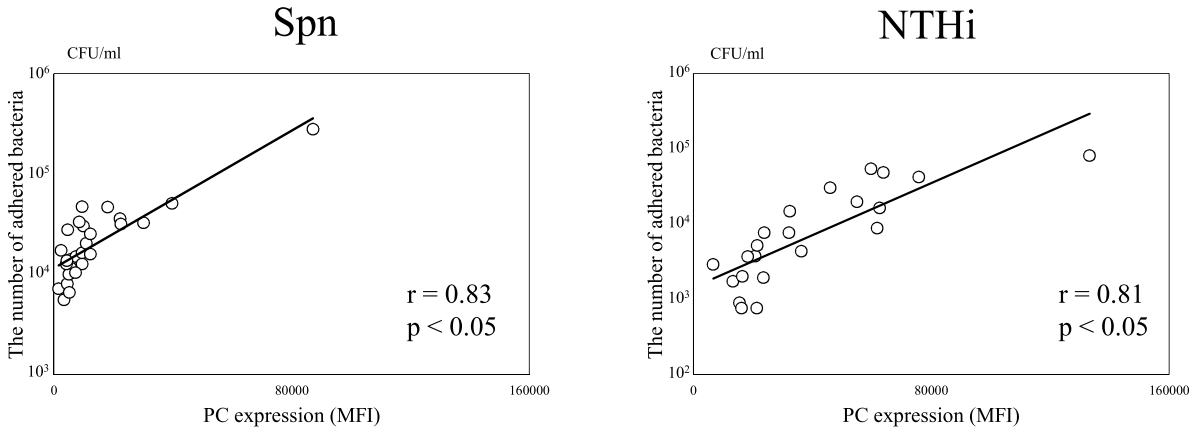

Fig. 2. Relationship between PC expression and bacterial adhesion.
PC expression showed high positive correlation with the adhesive ability of Spn (r = 0.83, p = 0.007) and NTHi (r = 0.81, p = 0.01).

were treated with saponin to collect Spn and NTHi invading into the cells and the numbers of bacteria were determined after culture the samples on agar plates. The results showed that Spn do not invade into Detroit cells regardless of culture time and that NTHi start to invade into Detroit cells after incubation for 4 h, suggesting that the number of NTHi invading into Detroit cells is negligible when the samples were cultured for 2 h. According to the results, adherence assay *in vitro* was performed after culturing the cells for 2 h.

In the PC-high Spn, adherence was significantly suppressed by pretreatment with TEPC-15 (20,410.87 ± 7574.35 CFU/ml; $p < 0.05$), ABT-491 (16,366.40 ± 3593.00 CFU/ml; $p < 0.05$), and PC-KLH (16,324.11 ± 4242.32 CFU/ml; $p < 0.05$), respectively, compared with the control without any inhibitors (48,363.54 ± 18,072.66 CFU/ml). (Fig. 3) Conversely, the adherence of PC-low Spn was not affected by pretreatment with TEPC-15 (8468.65 ± 1361.13 CFU/ml), ABT-491 (8277.32 ± 1195.62 CFU/ml), and PC-KLH (7858.79 ± 2122.81 CFU/ml) compared with the control (12,007.26 ± 1622.05 CFU/ml).

Similar findings were observed in the adherence of NTHi. The adherence of PC-high NTHi was remarkably suppressed by pretreatment with TEPC-15 (10,442.27 ± 1607.42 CFU/ml; $p < 0.05$), ABT-491 (9498.79 ± 1372.70 CFU/ml; $p < 0.05$), and PC-KLH (12,735.45 ± 2204.76 CFU/ml; $p < 0.05$), respectively, compared with the control without any inhibitors (29,768.25 ± 7246.12 CFU/ml). (Fig. 3) In the PC-low NTHi, pretreatment with these inhibitors did not reduce the adherence (TEPC-15: 1959.37 ± 382.96 CFU/ml, ABT-491: 2206.06 ± 480.65 CFU/ml, and PC-KLH: 2504.86 ± 1236.32 CFU/ml) when compared with the control (2831.16 ± 637.67 CFU/ml).

3.4. Bacterial adherence and the effects of TEPC-15, ABT-491, and PC-KLH (in vivo)

In PC-high Spn, adherence was significantly suppressed by pretreatment with TEPC-15 (12,4857.14 ± 44,141.16 CFU/ml; $p < 0.05$), ABT-491 (10,8225.00 ± 14,028.44 CFU/ml; $p < 0.05$), and PC-KLH (62,196.43 ± 11,022.30 CFU/ml; $p < 0.05$), respectively, compared with the control without any inhibitors (490,232.14 ± 220,827.65 CFU/ml). (Fig. 4), Conversely, the adherence of PC-low Spn was not affected by pretreatment with TEPC-15 (198,704.62 ± 34,959.61 CFU/

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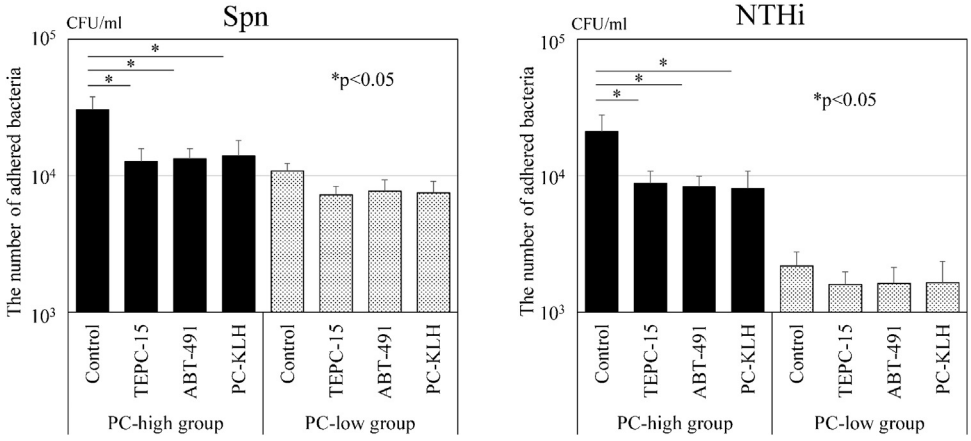

Fig. 3. Effects of TEPC-15, ABT-491, and PC-KLH on the adherence of Spn and NTHI *in vitro*.
Bacterial adherence assay *in vitro* showed significant suppression in both Spn and NTHi upon pretreatment with TEPC-15, ABT-491, and PC-KLH in the PC-high group compared to the control, but not in the PC-low group. *p < 0.05.

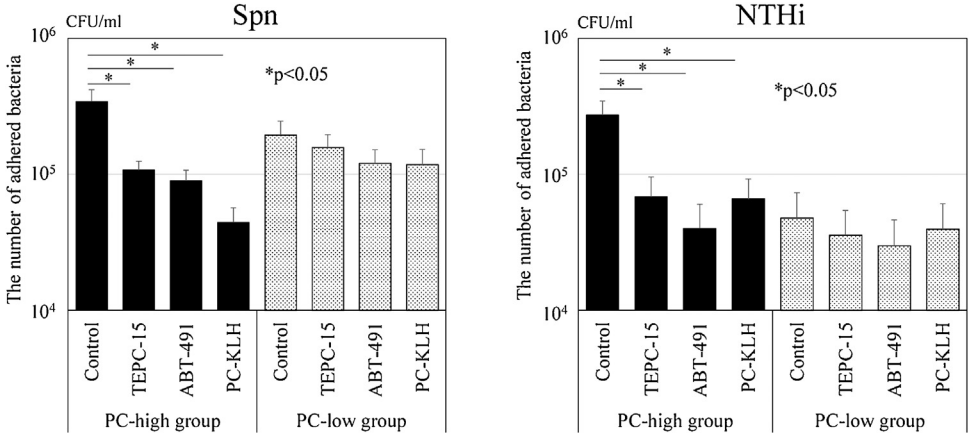

Fig. 4. Effects of TEPC-15, ABT-491, and PC-KLH on the adherence of Spn and NTHi *in vivo*.
Bacterial adherence assay *in vivo* showed significant suppression of adherence in both Spn and NTHi by pretreatment with TEPC-15, ABT-491, and PC-KLH in the PC-high group compared to the control, but not in the PC-low group. *p < 0.05.

ml), ABT-491 (156,510.77 ± 33424.99 CFU/ml), and PC-KLH (151,442.31 ± 25,016.19 CFU/ml) compared with the control (245,052.05 ± 44141.16 CFU/ml).

Similar findings were observed in the adherence of NTHi. In PC-high NTHi, was significantly suppressed by pretreatment with TEPC-15 (106,240.91 ± 26,343.81 CFU/ml; $p < 0.05$), ABT-491 (92,081.82 ± 24,398.56 CFU/ml; $p < 0.05$), and PC-KLH (105,097.73 ± 29,539.64 CFU/ml; $p < 0.05$), respectively, compared with the control without any inhibitors (363,077.27 ± 97,377.45 CFU/ml). (Fig. 4) In PC-low NTHi, pretreatment with these inhibitors did not reduce the adherence (TEPC-15: 59,759.09 ± 13,812.83 CFU/ml, ABT-491: 54,809.09 ± 14,126.73 CFU/ml, and PC-KLH: 73,022.73 ± 21,979.24 CFU/ml) compared with the control (83,205.45 ± 20,498.93 CFU/ml).

3.5. Intracellular invasion of NTHi and the effects of TEPC-15, ABT-491, and PC-KLH

Since Spn did not invade into Detroit cells as described above, intracellular assay was performed only with NTHi. The results showed that the invasion of NTHi was significantly enhanced in the PC-high group compared with the PC-low group (4010.85 ± 1156.00 *vs.* 1086.60 ± 264.81 CFU/ml; $p < 0.05$). (Fig. 5) In PC-high NTHi, intracellular invasion was significantly suppressed by pretreatment with TEPC-15 (1298.15 ± 464.05 CFU/ml; $p < 0.05$), ABT-491 (1591.92 ± 534.22 CFU/ml; $p < 0.05$), and PC-KLH (1629.69 ± 461.94 CFU/ml; $p < 0.05$), respectively, compared with the control (4010.85 ± 1156.00 CFU/ml). Conversely, the intracellular invasion of PC-low NTHi was not influenced by pretreatment with these inhibitors (TEPC-15: 755.83 ± 175.67 CFU/ml, ABT-491: 855.33 ± 214.74 CFU/ml, and PC-KLH: 780.63 ± 213.25 CFU/ml) compared with the control (1298.15 ± 464.05 CFU/ml).

4. Discussion

The presence of PC was first discovered in Spn by Tomasz in 1967 [16]. The present study demonstrates that the level of PC expressed on the cell surface of Spn as well as NTHi differs in each strain as assessed by fluorescence intensity in flow

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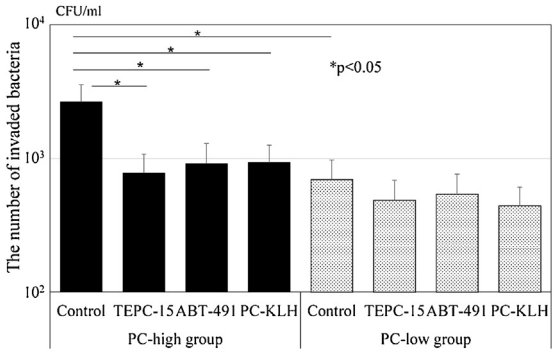

Fig. 5. Effects of TEPC-15, ABT-491, and PC-KLH on intracellular invasion of NTHI in vitro.
Intracellular invasion of PC-high group was significantly enhanced compared with the PC-low group. Intracellular invasion of NTHi was significantly suppressed by pretreatment with TEPC-15, ABT-491, and PC-KLH in the PC-high group compared to the control, but not in the PC-low group. *$p < 0.05$.

cytometry. Thus, we divided Spn and NTHi strains into two groups, PC-low and PC-high, in order to investigate the role of PC in the adherence of these bacteria. The results obtained from *in vitro* and *in vivo* experiments clearly showed that the adherence of PC-high Spn and NTHi was significantly higher than that of respective PC-low strains. Several investigators have reported the importance of adhesive activity as a virulence factor in Spn and NTHi. Andersson et al. found that the adhesive capacity of Spn was highest among the strains isolated from patients with AOM, and suggested that the capacity to attach to the pharyngeal mucosa is a virulence factor in Spn causing AOM [17]. Shimamura et al. demonstrated that the adherence of NTHi, as well as Spn, had a higher affinity to epithelial cells in children than to those in adults and that adherence was significantly greater in patients with otitis media with effusion than in normal subjects [18]. PC expression is also known to be involved in Spn and NTHi infection. A mouse antibody against PC can protect mice from lethal infection with mouse-virulent human Spn isolates [7]. In a mouse model of pulmonary infection induced by NTHi, serial passage of NTHi increased both the PC content and bacterial resistance to pulmonary clearance, and no such increases were observed in NTHi mutants lacking PC [19]. These findings suggest that PC expression in Spn and NTHi is associated with their adhesive activity to mucosal surfaces, which might be one of the reasons that PC-expressing strains exhibit increased virulence.

Pretreatment of bacteria with TEPC-15, a monoclonal PC-specific IgA antibody, significantly reduced the adherence of PC-high Spn and NTHi in *vitro* as well as *in vivo*, possibly by inhibiting the binding between the PC expressed on these bacteria and the receptors present on the cultured epithelial cell surfaces. We previously demonstrated that the adherence of Spn and NTHi was remarkably reduced by treating bacteria with nasopharyngeal secretions with secretory IgA antibody activity against these bacteria [15]. Furthermore, intranasal immunization of mice with PC induced PC-specific IgA in mucosal secretions such as saliva, which reacted with most Spn and NTHi strains and enhanced the clearance of these bacteria from the nasal cavity [14]. These findings indicate the inhibitory effect of PC-specific IgA in the adherence of these bacteria. However, the adherence of Spn and NTHi was not completely inhibited by TEPC-15, and pretreatment with TEPC-15 did not affect the adherence of PC-low strains, suggesting that adhesive factors other than PC such as Spn surface proteins and NTHi pili are also involved in the adherence of these bacteria [20,21].

To confirm the previously reported relationship between PC and PAF-R in bacterial adherence [10], the presence of PAF-R on Detroit 562 cells surfaces and the effects of blocking PAF-R by pretreatment with ABT-491 and PC-KLH on the adherence of Spn and NTHi were examined. FACS analysis showed the presence of PAF-R and pretreatment with ABT-491 and PC-KLH significantly reduced the adherence of PC-high Spn and NTHi. In contrast, the adherence of PC-low Spn and NTHi was not affected by pretreatment with ABT-491 and PC-KLH. Although PAF-R expression in the nasal mucosa of mice was not confirmed, similar results were obtained in the *in vivo* experiment. Cundell et al. investigated the attachment of bacterial PC to PAF-R and found that binding between PC and PAF-R enhanced the adherence of Spn and that only virulent Spn engaged PAF-R [10]. These results indicate that PAF-R and its binding to PC play an important role in the adherence of virulent strains of PC-high bacteria. Interestingly, the inhibitory activity of ABT-491 and PC-KLH on bacterial adherence was almost the same as that of TEPC-15 *in vitro* and *in vivo*, indicating that PAF-R-targeting therapy might be as effective as PC-targeting mucosal vaccine. When we look at the results of our study from a different angle, those findings indicate that PC- and PAF-R-targeting mucosal vaccine will not work for the PC-low bacteria. However, the ineffectiveness of PC-targeting vaccine on PC-low bacteria is considered an advantage of this vaccine, since PC-low Spn and NTHi are less pathogenic compared to PC-high strains and present as commensal flora in the nasopharynx of children. Moreover, we confirmed that PC expression of the other commensal flora such as streptococci and staphylococci is lower than Spn and NTHi (data not shown). These findings suggest that PC-targeting vaccine would likely not disturb the commensal bacterial flora including PC-low Spn and NTHi in the upper respiratory tract.

When the inhibitory effects of TEPC-15, ABT-491, and PC-KLH *in vitro* were compared between Spn and NTHi, the adherence of the PC-high group of Spn was reduced to the same level as that of the PC-low group, even though the adherence of the PC-high group of NTHi was still remarkably higher than that of the PC-low group despite treatment with TEPC-15, ABT-491 and PC-KLH. This might be owing to the difference in the PC expression on the bacterial surfaces. Although Spn and NTHi possess PC in the cell membrane lipoteichoic acid but also in capsular polysaccharide [22,23], PC expressed on the cell membrane of Spn is masked by the capsule. In contrast, the NTHi used in the present study lack capsular polysaccharide and thus, a greater amount of PC is exposed on the bacterial surface. In fact, the expression of PC on Spn was lower than that in NTHi in the present study. Moreover, adhesins other than PC are involved in the adherence of Spn and NTHi, and several factors other than anti-PC antibodies and PAF-R ligands contained in external secretions such as nasal secretions are associated with the inhibition of bacterial adherence, which might explain the differences in the inhibitory effects of TEPC-15, ABT-491, and PC-KLH between *in vitro* and *in vivo* experiments.

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In the present study, intracellular invasion was observed in NTHi, but not in Spn. This result is consistent with a previous study showing that invasion of Spn into epithelial cells is a limited phenomenon that occurs in a strain-specific and cell type-specific manner, and only Spn strain R6 x has been shown to invade these cells [22]. Conversely, NTHi frequently invades human epithelial cells and its invasion is known to be facilitated by the interaction between PC and PAF-R [10]. The present study demonstrated that pretreatment with TEPC-15, ABT-491, and PC-KLH significantly reduced the invasion of PC-high NTHi, but not PC-low strains, in the same manner as bacterial adherence. Thus, intracellular invasion of NTHi occurs subsequent to bacterial adhesion and interaction between PC and PAF-R is involved in the invasion as well as adherence of PC-high NTHi.

5. Conclusion

In conclusion, the present study demonstrates that the adherence of Spn and NTHi and the intracellular invasion of NTHi via interaction with PAF-R were increased in PC-high groups. Although factors other than PC and PAF-R are likely involved in the adherence and invasion of Spn and NTHi, high PC expression and the adhesive ability induced by the binding of PC to PAF-R are considered to increase the virulence of these bacteria. These findings suggest that a PC-targeting mucosal vaccine enhances the clearance of virulent PC-high Spn and NTHi by reducing the adherence of these bacteria via PC-specific IgA. However, this vaccine would likely not disturb the commensal bacterial flora in the upper respiratory tract, which comprises nonpathogenic PC-low bacteria, suggesting the safety of such a vaccine.

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