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Strain-Specific Pulmonary Defense Achieved after Repeated Airway Immunizations with Non-Typeable *Haemophilus Influenzae* in a Mouse Model

JUN KOYAMA,[1] KAMRUDDIN AHMED,[2] JIZI ZHAO,[3] MARIKO SAITO,[1] SHOZABURO ONIZUKA,[1] KEITA OMA,[1] KIWAO WATANABE,[1] HIROSHI WATANABE[1] and KAZUNORI OISHI[1,3]

[1]Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan
[2]Division of Infectious Diseases, Department of Social and Environmental Medicine, Institute of Scientific Research, Oita University, Oita, Japan
[3]Department of Special Pathogen, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Suita, Japan

KOYAMA, J., AHMED, K., ZHAO, J., SAITO, M., ONIZUKA, S., OMA, K., WATANABE, K., WATANABE, H. and OISHI, K. *Strain-Specific Pulmonary Defense Achieved after Repeated Airway Immunizations with Non-Typeable Haemophilus Influenzae in a Mouse Model.* Tohoku J. Exp. Med., 2007, **211** (1), 63-74 —— Strain-specific immune responses may play a critical role in the acute exacerbation of chronic obstructive pulmonary disease (COPD) caused by *Haemophilus influenzae* (NTHi), and the outer membrane protein P2 is one of surface antigens of NTHi, which may contribute to the strain-specific protective immunity. We examined whether repeated airway immunizations with killed-NTHi strains bearing different P2 molecules were capable of inducing protective immunity against homologous or heterologous strains in the lungs of a mouse model. Three different strains of NTHi were used in this study. Three serial intratracheal (IT) immuizations of a single strain or three different strains of NTHi led to the production of cross-reactive immunoglobulins G and A in bronchoalveolar lavage fluids. Three serial IT immunizations with a single strain enhanced the bacterial clearance of the homologous strain in the lungs, but no enhancement of bacterial clearance was found with three serial IT immunizations of heterologous strains. The enhancement in bacterial clearance, therefore, appears to be primarily strain-specific. Enhanced bacterial clearance of a hetrologous strain was also found after three serial IT immunizations of a single strain among two of the three strains employed for bacterial challenge. These findings suggest that P2 molecules and surface antigens other than P2 are involved in the development of pulmonary defense against NTHi in mice. Our data may explain, in part, why patients with COPD experience recurrent NTHi infections. ——— non-typeable *Haemophilus influenzae*; outer membrane protein P2; pulmonary defense; chronic obstructive pulmonary disease; acute exacerbation

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Correspondence: Kazunori Oishi, Department of Special Pathogen, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan.
e-mail: oishik@biken.osaka-u.ac.jp

Non-typeable *Haemophilus influenzae* (NTHi) is frequently associated with acute exacerbation of chronic obstructive pulmonary disease (COPD) (Wilson 1998; Sethi and Murphy 2001; Sethi 2004), although the role of bacterial pathogens in COPD is somewhat controversial (Hischnmann 2000; Murphy et al. 2000). Acute exacerbation occurs due to NTHi among these patients despite the presence of NTHi-specific antibodies in serum and sputum (Groeneveld et al. 1990). A recent study reported an association between a new strain of the same bacterial species and exacerbation in patients with COPD (Sethi et al. 2002). These authors also demonstrated that the development of an immunoglobulin G (IgG) response in sera from patients with an acute exacerbation of COPD was significantly higher in the case of the newly acquired strains of *H. influenzae* than preexisting strains (Sethi et al. 2004). Most of newly acquired strains were able to induce bactericidal antibodies in sera of these patients. These data suggest that strain-specific IgG found in sera plays a critical role in the recurrent exacerbation of COPD, and that the strain-specific protective immune response confers susceptibilities to infections by other strains of the same bacterial species (Sethi et al. 2002, 2004).

On the mucosal surface, secretory immunoglobulin A (IgA) plays a major role in the protective immunity (Boyaka et al. 2001). Previous studies have demonstrated immune responses to specific antigens of *Moraxella catarrhalis* or *Streptococcus pneumoniae* in airways of patients with COPD (Samukawa et al. 2000; Murphy et al. 2005). The role of strain-specific IgA in the airways, however, remains unexplained in the recurrent exacerbation of COPD.

The outer membrane protein (OMP) of NTHi contains six to eight major proteins (Sethi and Murphy 2001). P6 is a 16 kDa peptidoglycan-associated lipoprotein that is commonly found in the outer membrane of all strains of NTHi and exhibits a high degree of sequence conservation among strains (Murphy et al. 2006). In contrast, P2 is the major OMP, constituting as much as 50% of the OMP, and is an important target of the immune response to NTHi (Neary et al. 2006). The bactericidal activity of P2-specific antibodies is strictly strain-specific (Troelstra et al. 1994). Furthermore, previous studies have reported that newly acquired bactericidal antibodies from two patients with COPD after exacerbation by NTHi exhibited a recognition pattern to surface-exposed epitopes on P2 molecules and other surface proteins of NTHi (Yi et al. 1997). The authors also demonstrated that a strain-specific immune response to NTHi was directed to epitopes on the loop 5 region of the P2 molecule and was associated with bactericidal activity in animal experiments (Yi and Murphy 1997). Another study also reported the loop 6 of the P2 molecule was capable of inducing bactericidal antibodies (Neary et al. 2001). Accumulated evidence from clinical and laboratory investigations by Murphy and coworkers suggest that the P2 molecule is one of surface antigens that participate in strain-specific immune responses in the acute exacerbation of COPD by NTHi (Yi and Murphy 1997; Yi et al. 1997; Neary et al. 2001; Sethi et al. 2004). This study was, therefore, conducted to determine whether repeated airway immunizations with NTHi strains bearing different P2 molecules were capable of inducing protective immunity in lungs against homologous or heterologous strains in a mouse model.

Materials and Methods

Mice

Specific pathogen-free BALB/c mice (8-12 week old female) were purchased from Charles River Japan Laboratories (Kanagawa). The mice were maintained in barrier-protected animal facilities under specific pathogen free conditions using ventilated microisolator cages in the experimental animal facility of the Institute of Tropical Medicine, Nagasaki University. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University and conformed to National Institute of Health guidelines.

Bacterial isolates

Six strains of NTHi (H98-224, H99-115, H01-03, H04-06, H05-15, and H05-19), isolated from a patient with an acute exacerbation of COPD at different occasions, were used in this study. Each strain of NTHi was

grown overnight in brain heart infusion (BHI) broth (BBL, Becton Dickinson Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD, USA) supplemented with 5% digested horse blood at 37°C.

Immunoblot assay

The harvested bacteria were washed 2 times with sterile phosphate buffered saline (PBS). After washing, the bacteria were fixed in 75% ethanol, washed and resuspended in PBS, and stored at −80°C until used. Whole cell preparations from six strains of NTHi were heated at 95°C for 5 min then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% separating gels. Three identical patterns of OMP bands on SDS-PAGE were found among these six strains (data not shown). The pairs of identical strains were; H05-15 and H05-19, H04-03 and H04-06, and H98-224 and H99-115, respectively. We therefore employed H05-19, H04-06 and H99-115 in this study. Whole cell preparations of three strains were applied SDS-PAGE and then transferred to nitrocellulose membrane and incubated with a 1:5,000 dilution of anti-P2 rabbit sera (Yi et al. 1997). The isolated P2 protein from the NTHi strain and the anti-P2 rabbit sera were generous gifts from Dr. TF Murphy, (Buffalo, NY, USA). Horseradish peroxydase-conjugated donkey anti-rabbit IgG at 1:10,000 dilution (GE Healthcare, Bio-Sciences Corp., Piscataway, NJ, USA) was used for detection of rabbit antibodies.

Genotyping with pulsed-field gel electrophoresis (PFGE)

After digesting the genomic DNA of NTHi with *Sma*I (Takara Shuzo Co., Shiga), PFGE was performed on these three strains as described previously (Yano et al. 2000).

Polymerase chain reaction (PCR) and sequence of P2 gene

The nucleotide sequence of the PCR amplicon of *ompP2* gene was performed according to the previously published method (Hiltke et al. 2002). Briefly, bacterial genomic DNA was isolated from 400 μl of an overnight NTHi broth culture employing a Wizard genomic DNA purification kit (Promega Biotech). Using the Gene Amp PCR kit (Applied Biosystems, Tokyo) the *ompP2* gene was amplified using approximately 75 ng of bacterial DNA and primers a and b (Table 1). Reactions were carried out using a Gene Amp PCR system 9700 thermalcycler (Applied Biosystems). The reactions consisted of an initial hold for 3 min at 94°C, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 90 sec. The PCR products were purified with the ExoSAP-IT enzyme (GE Healthcare, Bio-Science Corp.) according to the instructions of the manufucture. DNA sequencing procedure was performed by primer walking method using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and primers shown in Table 1. The product was then loaded on ABI PRISM 3100 DNA Analyzer (Applied Biosystems) to determine the DNA sequence.

Intratracheal immunization with ethanol-killed bacteria

In order to develop protective immunity in the lungs against NTHi strain, 50 μl of ethanol-killed bacteria (H05-19 strain, H04-06 strain or H99-115 strain) at a concentration of 4×10^9 colony-forming units (CFU)/ml suspended in PBS was intratracheally administered to each mouse on day 0, 7 and 14 (Kurita et al. 2006). Day 0 was defined as the day of the first intratracheal (IT) immunization. Two different types of serial three IT immunizations were performed in this study. In the first type, one of these strains (H05-19 strain, H04-06 strain or H99-115 strain) was immunized on day 0, 7 and 14.

TABLE 1. Sequence of primers used in polymerase chain reaction and sequencing of ompP2 of NTHi.

Primer	Sequences
a	5′ACGCGGATCCTGGTGTTGTTTATAACAACG3′ (forward)
b	5′GGTGAAGTAAAACTTGGTC3′ (forward)
c	5′AGGCTTATTAGTCTCTCTAG3′ (forward)
d	5′ATCAGGATCCTTAGAAGTAAACGCGTAAACCTAC3′ (reverse)
e	5′CCATAGACATTAAGTATCTTCC3′ (reverse)
f	5′GCGCCTAATACTAAACCATC3′ (reverse)

In the second type, H05-19 strain was immunized on day 0, followed by the H04-06 strain on day 7 and then the H99-115 strain on day 14. Four or five animals were employed for each type of IT immunization. The procedure was performed under anesthesia induced by an intraperitoneal injection of 0.2 ml of a solution containing 3 mg of ketamine and 0.1 mg of xylazine. Heparinized blood was obtained, and the plasma separated, and bronchoalveolar lavage (BAL) was performed at the indicated times after the initial immunization as previously described (Kurita et al. 2006). Plasma and BAL fluid were stored at –80°C until used.

Determination of P6 or strain-specific antibodies

The P6-specific antibody titer in plasma or BAL fluid was determined by ELISA according to a previously published method (Kurita et al. 2006). The strain-specific antibody titer to the whole cell preparation of NTHi in plasma or BAL fluid was also determined by a previously described method, with minor modifications (Sethi et al. 2004). Because of the limitations in measuring the avidity of the strain-specific antibody to the whole cell preparation of NTHi, IgA specific and its avidity to OMP in BAL fluid were determined by ELISA employing 0.5 M sodium thiocyanate (Anttila et al. 1998). The OMPs from each NTHi strain were prepared as previously described (Kurita et al. 2006). A P6 preparation (1 μg/ml), the whole bacterial preparation (10^8 CFU/ml) or the OMP preparation (1 μg/ml) was used as the coating antigen. The isolated P6 protein was a generous gift from Dr. N. Yamanaka, Wakayama, Japan. In whole cell ELISA, 200 μl of 5% bovine serum albumin in PBS was used as the blocking solution and 50 μl of a plasma sample or BAL fluid was then added, followed by incubation at 37°C for 30 min. The plate was washed and treated with 50 μl of alkaline phosphatase-conjugated goat anti-mouse IgM, IgG, or IgA (Zymed, San Francisco, CA, USA). The optical density (OD) was measured at 405 nm. The end-point titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an OD_{450} of $\geqq$ 0.1 OD unit above the OD_{450} of negative control samples obtained from non-immunized mice.

Cell proliferation assay

In vitro antigen specific cell proliferation assays were performed using the Premix WST-1cell proliferation assay system (TAKARA, Tokyo) according to manufacture's instructions. Mononuclear cells were prepared from pulmonary lymph node (LN) of mice that had received three serial IT challenges of ethanol-killed NTHi as described above (Kurita et al. 2006). LN cells, at a concentration of 5×10^6 in 200 μl of Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (FBS), were added to the well of a 96 well cell culture plate in triplicate and were stimulated at 37°C for 72 hrs with or without antigens at concentrations of 1 or 5 μg/ml. Ovalbumin (OVA; Sigma Chemical Co., St. Louis, MO, USA) was used as a control antigen. After incubation, the assay was developed by the addition of a premix WST-1 solution (10 μg/well) to each well. OD was measured at 450 and 600 nm after 4 hrs incubation at 37°C. These data were calculated by subtracting the OD_{600} value from the OD_{450} value.

Bacterial clearance of NTHi strain in mice

Each live strain of NTHi (H05-19 strain, H04-06 strain or H99-115 strain), at a dose of a 4×10^8 CFU/mouse, was intratracheally challenged to mice which had previously received one of four different types of the serial three IT immunization of ethanol-killed NTHi strains at one week intervals, or to untreated mice. Five animals were employed in each type of IT immunization followed by bacterial challenge. Quantitative bacterial cultures of lung tissue from mice that had been euthanized with pentobarbital were performed on agar 4 hrs or 12 hrs after the bacterial challenge as previously described (Kurita et al. 2006). The lungs were removed asceptically and homogenized in 9 ml of sterile saline per gram of lung tissue prior to culturing. The detection limit of bacterial cultures of the lung tissue was 10^3 CFU/g.

Statistical analysis

Statistical analyses were performed by one-way ANOVA and multiple comparison method by Bonferroni-Dunn's test or the unpaired Student's *t*-test. Data were considered to be statistically significant, if the *p* values were less than 0.05. All data are expressed as the mean ± S.D.

Results

Immunoblot assays of whole-cell lysates and PFGE

To determine whether the P2 molecules of the three strains of NTHi (H05-19 strain, H04-06 strain or H99-115 strain) are different in molecular size, an immunoblot assay of whole cell lysates of these strains was performed using anti-P2 rabbit sera (Fig. 1A). The anti-P2 antisera

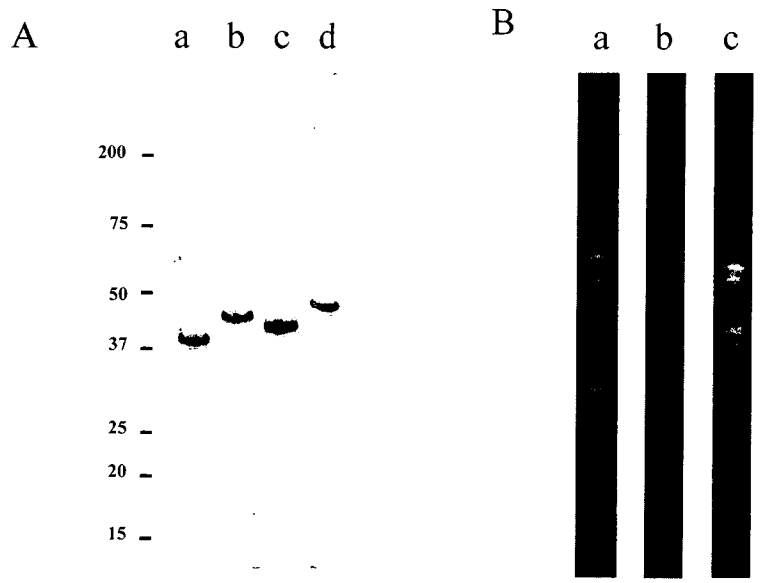

Fig. 1. Immunoblot assay of bacterial lysates of three NTHi strains and the purified P2 protein with anti-P2 sera (A) and pulse-field gel electrophoresis of *Sma*I-digested genomic DNA of three NTHi strains (B). Whole cell preparations of H05-19 strain (a), H04-06 strain (b) and H99-115 strain (c) and the purified P2 (d) were transferred to a nitrocellulose membrane and the P2 bands were visualized by mean of an anti-P2 rabbit serum. The PFGE patterns of *Sma*I-digested DNA from H05-19 strain (a), H04-06 strain (b) and H99-115 strain (c).

detected P2 molecules of these strains and the purified P2 protein, with different molecular sizes between molecular markers of 37 and 50 kDa. In addition, PFGE also demonstrated different patterns among the three strains (Fig. 1B).

OmpP2 sequence analysis

Analyses of the deduced amino acid sequences of *ompP2* of the three strains of NTHi (H05-19 strain, H04-06 strain or H99-115 strain) demonstrated sequence heterogeneity in all eight loop regions (Fig. 2). Therefore, all three strains of NTHi used in this study possess different P2 epitopes.

P6-specific cell proliferation of LN cells

In all of the three serial IT immunizations, except for the three serial IT immunizations by H05-19 strain, a concentration-dependent increase in cell proliferation was found in cultured cells isolated from pulmonary LN of mice in the presence of P6 at 1 and 5 μg/ml (Fig. 3A-D). In contrast, no cell proliferation was found in cultured cells isolated from the pulmonary LN of mice that had received any of the three types of serial IT immunizations in the presence of OVA at 1 and 5 μg/ml.

P6-specific immunoglobulins (Igs) in plasma and BAL fluid

The induction of P6-specific IgG and IgM, but not IgA, were found in plasma of mice that received any of the three types of serial IT immunizations (Table 2). In contrast, P6-specific IgG, IgM or IgA were undetectable in BAL fluids of mice that received any of the three types of serial IT immunizations.

Strain-specific Igs in plasma and BAL fluid by ELISA using the whole cell preparations

In plasma of mice that received any of the three types of serial IT immunizations, strain-specific Igs were found, except for the case of mice that received three serial IT immunizations by strain H04-06 (Table 3). Only strain H04-06-specific IgA, but not strain H05-19 or strain H99-

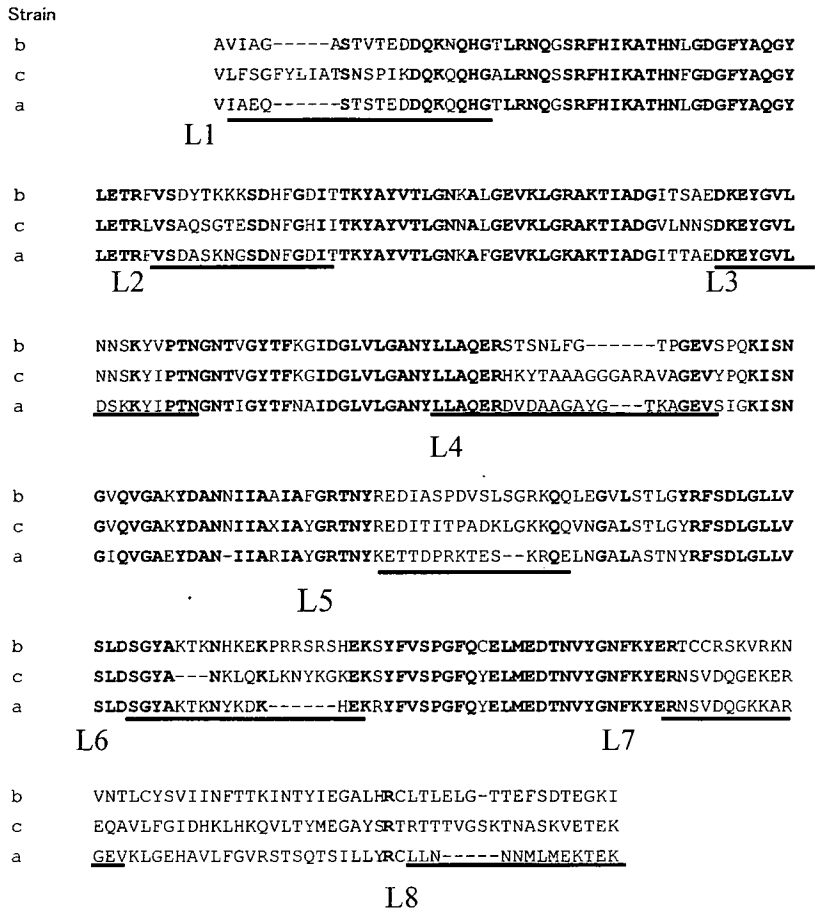

Fig. 2. Amino acid sequence of the *ompP2* regions of the three NTHi strains isolated from the same patient with COPD. Identical residues are shown in the bold. Strain a denotes H05-19 strain, strain b denotes H04-06 strain, and strain c denotes H99-115 strain, respectively. The loops 1 (L1) through loops 8 (L8) are indicated as the underlined parts.

115-specific IgA, was found in the plasma. In most types of the serial IT challenges, strain-specific IgA was detected in BAL fluids (Table 3). In case of the three serial challenges of the H04-06 strain, only strain H04-06-specific IgA was found in BAL fluids. No detectable levels of strain H05-19 or strain H99-115-specific Igs were found in BAL fluids of these mice.

Strain-specific IgA and its avidity in BAL fluid by ELISA using OMP preparations

In order to evaluate the functional strain-specific IgA, we next examined strain-specific IgA and its avidity in BAL fluids of mice (Table 4). In BAL fluids of mice that received three serial immunizations of strain H05-19 or H99-115 or three different strains, an increased IgA or its avidity specific to the OMP of strain H05-19 and H99-115 were found, compared to the OMP of strain H04-06. A similar increase in IgA and its avidity specific to OMP of strain H04-06 was shown only in BAL fluids of mice that had received three serial immunizations of strain H04-06, compared to those specific to OMP of strain H05-19 or H99-115.

Bacterial clearance in the lungs

We next examined the bacterial clearance of each NTHi strain in the lungs of mice that received three serial IT challenges. The bacterial clearance of strain H05-19 was significantly faster in cases of three serial IT challenges of strain

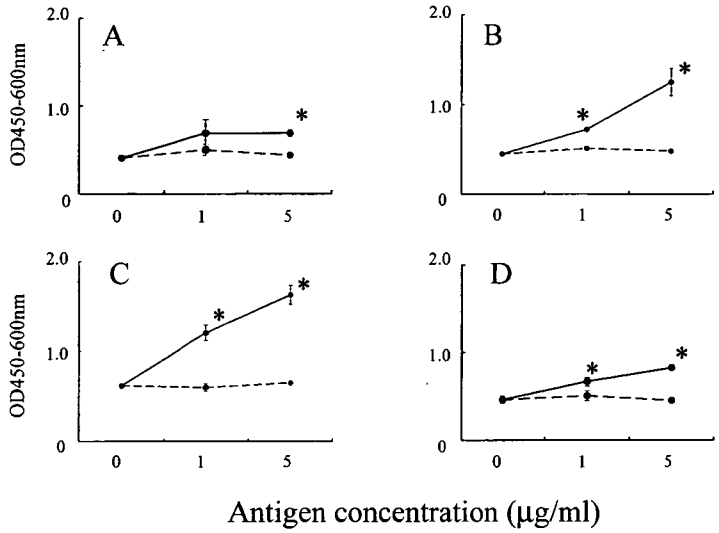

Fig. 3. In vitro cell proliferation using pulmonary lymph node cells from mice that received three serial IT challenge of killed-NTHi strain with a one-week interval in the presence of P6 (solid line) or ovalbumin (broken line). The IT immunizations include three serial immunizations of strain H05-19 (A), three serial immunizations of strain H04-06 (B), three serial immunizations of strain H99-115 (C) and three serial immunizations in the following order: strain H05-19, strain H04-06 and strain H99-115 (D). Values represent the mean ± S.D. of four animals.
*$p < 0.01$ (vs ovalbumin).

TABLE 2. Induction of P6-specific IgM and IgG in plasma in mice after three serial IT challenges of killed-NTHi strain with a one-week interval by ELISA.

Intrtracheal immunization	Ig class	Reciprocal $\log_2$ P6-specific titer in plasma
Serial three immunizations	IgM	7.0 ± 1.154
with H05-19 strain	IgG	6.5 ± 0.76
Serial three immunizations	IgM	5.5 ± 1.05
with H04-06	IgG	4.83 ± 0.75
Serial three immunizations	IgM	8.0 ± 0.89
with H99-115	IgG	6.17 ± 0.41
Serial three immunizations	IgM	7.83 ± 0.37
with three different strains	IgG	7.5 ± 0.76

Ig, immunoglobulin. Values represent the mean ± S.D. of four animals.

H05-19 or strain H99-115 than in untreated mice 12 hrs after the IT challenge (Fig. 4, upper column). No significant enhancement in the bacterial clearance of strain H05-19 was found in mice that received the three serial IT challenges of strain H04-06 or three different strains at 12 hrs post-challenge. Similarly, the bacterial clearance of strain H99-115 was significantly faster in the lungs of mice that received the three serial IT challenges of strain H05-19 or strain H99-115 than in untreated mice at 12 hrs post-challenge (Fig. 4, lower column). No significant enhancement of H99-115 strain was noted in mice that received the three serial IT challenges of strain H04-06 or the three different strains, although the bacterial densities of H99-115 strain in the lungs of mice receiving IT challenges of different strains tended to be lower than untreated mice. A

TABLE 3. Induction of strain-specific IgM, IgG and IgA in plasma and bronchoalveolar lavage (BAL) fluid of mice after three serial IT challenges of killed-NTHi strain with a one-week interval by ELISA using the whole cell preparations.

Intrtracheal immunization	Ig class	Reciprocal $\log_2$ titer in plasma			Reciprocal $\log_2$ titer in BAL fluid		
		anti H05-19	anti H04-06	anti H99-115	anti H05-19	anti H04-06	anti H99-115
Serial three immunizations	IgM	5.15 ± 0.41	6.0 ± 0	10.7 ± 0.58	N.D.	N.D.	N.D.
with H05-19 strain	IgG	10.5 ± 0.84	12.2 ± 1.47	12.0 ± 1.41	5.67 ± 0.68	5.83 ± 1.47	7.5 ± 1.29
	IgA	N.D.	7.83 ± 1.33	N.D.	7.0 ± 0.82	5.5 ± 1.22	6.83 ± 1.34
Serial three immunizations	IgM	N.D.	6.5 ± 0.58	N.D.	N.D.	N.D.	N.D.
with H04-06	IgG	N.D.	10.0 ± 0.89	7.6 ± 0.89	N.D.	N.D.	N.D.
	IgA	N.D.	N.D.	N.D.	N.D.	5.17 ± 0.75	N.D.
Serial three immunizations	IgM	N.D.	N.D.	8.0 ± 0.82	N.D.	N.D.	N.D.
with H99-115	IgG	10.5 ± 0.55	10.2 ± 1.17	10.8 ± 0.96	5.33 ± 0.58	4.25 ± 0.5	4.5 ± 0.58
	IgA	N.D.	6.0 ± 0	N.D.	4.5 ± 1.29	4.4 ± 0.55	6.8 ± 0.84
Serial three immunizations	IgM	N.D.	N.D.	9.17 ± 0.69	N.D.	N.D.	N.D.
with three different strains	IgG	12.17 ± 0.41	12.8 ± 0.41	13.0 ± 0	4.83 ± 0.41	4.67 ± 0.52	5.83 ± 0.37
	IgA	N.D.	6.33 ± 0.52	N.D.	6.0 ± 0.63	5.0 ± 0.63	4.67 ± 0.47

Ig, immunoglobulin. N.D., not detectable. Values represent the mean ± S.D. of four animals.

TABLE 4. The strain-specific IgA and its avidity in bronchoalveolar lavage (BAL) fluid of mice after three serial IT immunizations of killed-NTHi strain with a one-week interval by ELISA using the outer membrance preparations.

Intrtracheal immunization	IgA or IgA avidity	Reciprocal $\log_2$ titer in BAL fluid		
		anti H05-19	anti H04-06	anti H99-115
Serial three immunizations	IgA	10.81 ± 3.19	3.55 ± 0.95	9.36 ± 2.16
with H05-19 strain	IgA avidity	4.68 ± 0.69	2.34 ± 1.44	6.06 ± 1.59
Serial three immunizations	IgA	3.51 ± 0.20	6.10 ± 0.40	3.64 ± 0.22
with H04-06	IgA avidity	3.23 ± 0.19	5.13 ± 1.29	3.27 ± 0.20
Serial three immunizations	IgA	5.35 ± 1.76	2.85 ± 0.55	8.09 ± 1.36
with H99-115	IgA avidity	3.81 ± 0.66	1.85 ± 0.55	4.33 ± 0.25
Serial three immunizations	IgA	6.92 ± 0.52	4.64 ± 1.73	7.35 ± 0.89
with three different strains	IgA avidity	5.41 ± 0.78	3.25 ± 0.61	4.97 ± 0.43

Values represent the mean ± S.D. of four animals.

significant enhancement in bacterial clearance of strain H04-06 was found only in mice that received the three serial IT immunizations of the homologous strain, compared to untreated mice, at 4 hrs post-challenge (Fig. 4, middle column). No significant enhancement of H04-06 strain was found in mice that received the three serial IT immunizations of strain H05-19 or H99-115 or the three different strains.

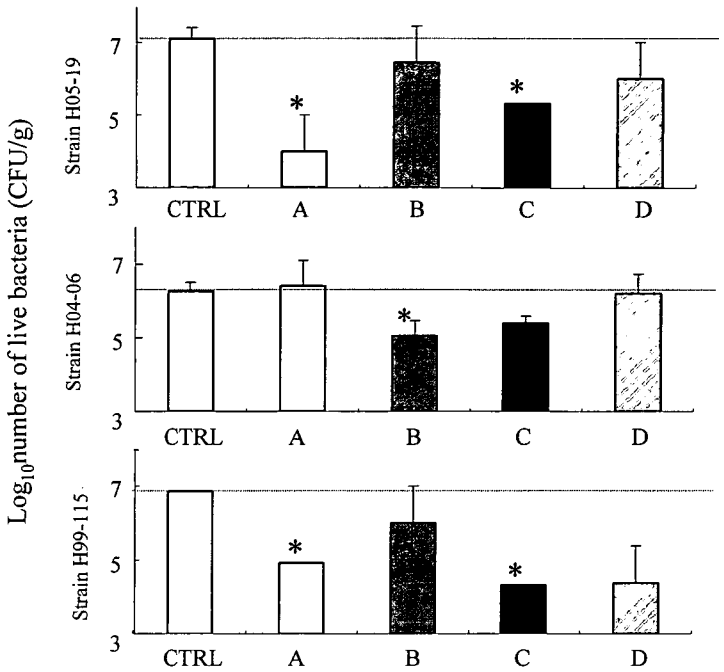

Fig. 4. The effect of three serial IT immunizations of killed-NTHi strain with a one-week intervals on the bacterial clearance of live NTHi strain H05-19, strain H04-06, and strain H99-115 are shown in the upper, middle and lower panels, respectively. The IT immunizations included three serial immunizations of strain H05-19 (A), three serial immunizations of strain H04-06 (B), three serial immunizations of strain H99-115 (C) and three serial immunizations in the following order: strain H05-19, strain H04-06 and strain H99-115 (D). The IT challenge of live strain of NTHi was done 3 weeks after the initial IT immunization with killed-NTHi. Untreated mice were used as controls (CTRL). The mice were euthanized and a quantitative bacterial culture of lung tissue was performed 12 hrs after the IT challenge for live strain H05-19 and live strain H99-115, and 4 hrs after the IT challenge for live strain H04-06. Values represent the mean ± S.D. of five animals.
*$p < 0.001$ (vs control).

Discussion

A recent study reported that the proliferative response of peripheral lymphocytes to P6 is associated with a relative protection from exacerbation by NTHi (Abe et al. 2002). We recently demonstrated a linkage between P6-specific T cell proliferation in LN cells and the induction of P6-specific IgA and IgG in BAL fluids by ELISA in mice that received repeated IT challenges of OMP of NTHi in mice (Kurita et al. 2006). Although T cell proliferation specific for P6 in LN cells was found in all mice that received the three serial IT immunizations of a single strain or three different strains of ethanol-killed NTHi in this study (Fig. 3), no P6-specific Igs including IgA, were detected in BAL fluids, but P6-specific IgG and IgM was detected in plasma. These data support the in vitro antigen presentation of P6 by dendritic cells contained in LN cells after degradation of the intratracheally challenged killed-NTHi strain. The antigen presentation in LN cells could lead to the production of Ig in plasma after the initial challenge of the OMPs of NTHi. The repeated IT immunization of OMP of NTHi, however, was required for the induction of IgG or IgA in the BAL fluids (Kurita et al. 2006). Repeated IT challenges of ethanol-killed NTHi strain failed to induce P6-specific IgG or IgA in BAL fluids in this study, because the P6 molecule, a cross-reactive epitope, is not exposed on the surface of the NTHi strain and is involved in anchoring the outer membrane (Nelson et al. 1991; Sethi and Murphy 2001). The detection of strain-specific

IgA in BAL fluids in most types of the three serial IT immunizations in the present study indicates the generation of cross-reactive IgA in the airways.

The three serial IT immunizations of a single strain significantly enhanced the bacterial clearance of the homologous strain from the lungs of mice (Fig. 4). No cross-protection was found in mice that had received three serial IT challenge of strain H04-06. These data suggest that the enhancement in bacterial clearance is primarily strain-specific. We also found that the three serial IT immunizations with strain H05-19 or H99-115 induced a cross-protective effect in the lungs of mice (Table 4). This cross-protective effect between strain H05-19 and H99-115 was associated with strain-specific IgA as well as IgG in BAL fluids (Table 3). Since there is a distinction in the amino acid sequence of loops 5 and 6 in the P2 molecule between these two strains, a cross-protective effect may be conferred by surface antigens other than P2. Possible surface antigens responsible for this cross-protective effect may include P5 adhesin and lipooligosaccharide (Sethi and Murphy 2001; Hirano et al. 2003; Novotny and Balaletz 2003). Although bacterial clearance in the lung also tended to be enhanced after an IT challenge of live H05-19 or H99-115 strain in mice that previously received the three serial IT immunizations of three different strains, the levels of enhancement were not significant in the lung of these mice (Fig. 4). Collectively, these data demonstrate that three serial IT immunizations of a single strain could lead to the production of strain-specific IgA as well as IgG, subsequently leading to an enhanced bacterial clearance of the homologous strain in the lung. The association of the enhanced bacterial clearance of strain H04-06 in the lungs of mice after three serial repeated IT challenge of the homologous strain with the presence of strain-specific IgA, but not IgG, may underscore the importance of strain-specific IgA in BAL fluids for inhibiting bacterial adherence in the airway (Taylor et al. 1990; Kurono et al. 1991).

The presence of strain-specific IgA and IgG in BAL fluid was not always associated with an enhanced bacterial clearance in lungs, because the serial IT challenges of three different strains could lead to the production of strain-specific IgA and IgG against all three strains, but none of them developed protective immunity against NTHi. These findings provide support for the view that three serial IT challenges of a single strain is sufficient to induce the production of strain-specific IgA which is capable of inhibiting the adherence of the homologous strain to the airway epithelium, while repeated IT challenges by three different strains generate strain-specific IgA which lacks such activity (Taylor et al. 1990; Kurono et al. 1991). We, therefore, examined the issue of whether strain-specific IgA and its avidity were associated with an enhanced bacterial clearance in the lungs of mice (Kauppi-Korkeila et al. 1996; Breukels et al. 2002). As we expected, the increased IgA and its avidity specific to OMP of NTHi were associated with an enhanced bacterial clearance in the lungs of mice that had received serial three IT challenges of a single strain (Table 4). No significant bacterial clearance, however, was found in the lungs of mice that had received serial three IT immunizations with different strains despite the increased IgA and its avidity in BAL fluid. Further examinations are required to elucidate this discrepancy in mice that received serial three IT immunizations by different strains.

In summary, three serial airway immunizations with a single or three different strains of NTHi stimulated the production of cross-reactive IgG and IgA in BAL fluids, but only three serial IT challenges of a single strain could induce the enhancement in bacterial clearance of the homologous strain in the lung. This enhancing effect on bacterial clearance in the lungs is, therefore, primarily induced in a strain-specific manner. In addition, enhanced bacterial clearance of a hetrologous strain was also found after three serial IT immunizations of a single strain among two of a the three strains employed for bacterial challenge. Increased strain-specific IgA and its avidity in BAL fluids was associated with an enhanced bacterial clearance in mice that had received serial IT immunizations with a homologous strain, but not in mice that had received serial IT immunizations

with heterologous strains. The data herein suggest that P2 molecules and surface antigens other than P2 are involved in the development of pulmonary defense against NTHi. Our data also suggest that a host previously infected by a NTHi continues to be susceptible to infections by other strains of NTHi, and may explain the mechanism of recurrent bacterial exacerbations of COPD. Since only three strains of NTHi with different P2 epitopes were employed in this study, the conclusions drawn are limited. Further studies will be required for a complete understanding of the strain-specific pulmonary defense against NTHi.

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肺炎球菌ワクチン

独立行政法人国立病院機構
長崎神経医療センター　統括診療部長
川上健司

大阪大学微生物病研究所感染症国際研究センター
高病原性感染症研究部門　教授
大石和徳

ワクチンの最新事情と渡航者の接種

24. 肺炎球菌ワクチン

独立行政法人国立病院機構長崎神経医療センター統括診療部長
川上 健司（かわかみ けんじ）

大阪大学微生物病研究所感染症国際研究センター高病原性感染症研究部門教授
大石 和徳（おおいし かずのり）

はじめに

肺炎球菌は細菌性肺炎の最も重要な起炎菌であり、肺炎は常に死亡の原因となることと、肺炎球菌に有効な抗菌薬の薬剤耐性が進んでいることから、ワクチンによる予防が重要となってきている。一方、乳幼児では髄膜炎という重篤な疾患の起炎菌でもあり、乳幼児への予防対策が重要視されている。

これらの疾患を予防するために、肺炎球菌ワクチンとしては、2歳以上の成人に用いる23価肺炎球菌莢膜ポリサッカライドワクチン（pneumococcal polysaccharide vaccine：PPV）と、2歳未満でも有効な多糖体―タンパク結合型ワクチン（pneumococcal conjugate vaccine：PCV）とがある。

1. 疾患

病原体である肺炎球菌は、莢膜の抗原性から90種類以上の血清型が存在する。肺炎球菌は小児の咽頭・鼻腔に常在しているが、水や土などの環境中やヒト以外の動物には通常は存在しない。したがって、感染源はこれら小児に常在する菌であり、鼻・咽頭粘膜において肺炎球菌がヒト―ヒト感染を繰り返していると推定されている。

感染症としては、咽頭・鼻腔で増殖した菌が病巣へ進み、炎症を引き起こして感染症を発症する。すなわち、咽頭・鼻腔から気道へ進めば気管支炎、肺炎となり、中耳へ進むと中耳炎、血管内に侵入すると敗血症、中枢神経を侵せば髄膜炎を引き起こす。

疾患の分類としては、侵襲性肺炎球菌感染症として敗血症、髄膜炎があり、一方、表層感染としては肺炎、気管支炎、中耳炎、副鼻腔炎、咽頭炎などがある。肺炎のうち、重症例では血管内に菌が侵入して侵襲性感染症となる。年齢で分類すると、乳幼児の疾患としては髄膜炎が生命予後や後遺症の問題から最も重要であり、高齢者に目を向けると死亡原因としての肺炎が重要である。

それぞれの疾患で、病気を引き起こす肺炎球菌の主な血清型が判明しており、ワクチンのターゲットとなる。われわれの検討[1]では、**図1**に示す通り市中肺炎の起炎菌であった肺炎球菌114株中に、PPV（Pneumovax®）に含まれている血清型の株が94株（82・5%）を占めていた。

肺炎球菌はヒトがいる全世界に存在している病原菌であるため、海外に行く場合にも国内と比較して特別な注意は必要ではない。

2. 臨床所見

肺炎球菌感染症の臨床症状は、他のインフルエンザ菌などの細菌感染症と臨床像のみでは区別はつけ難い。多くは発熱を伴い、それぞれの疾患の局所症状を呈する。肺炎であれば咳と痰、咽頭炎では咽頭痛、髄膜炎では頭痛や嘔気な

どを呈する。肺炎球菌性肺炎の中には急速に進展する劇症型も存在しており、数日で生命の危険を伴う状態に至ることがある。また、患者の免疫能が低下している場合には注意が必要であり、例えば高齢者の肺炎では、必ずしも高熱を伴わないため発見が遅れる原因となる。

細菌性肺炎の中で肺炎球菌の占める割合は最も高く、約40～60％を占めている。一方、乳幼児の髄膜炎において、肺炎球菌はインフルエンザ菌 tybe b に次いで高頻度の菌である。

肺炎球菌感染症の検査所見としては、他の細菌感染症と同様に、白血球数の増多、好中球数の増多、核の左方移動およびＣＲＰの上昇を認める。

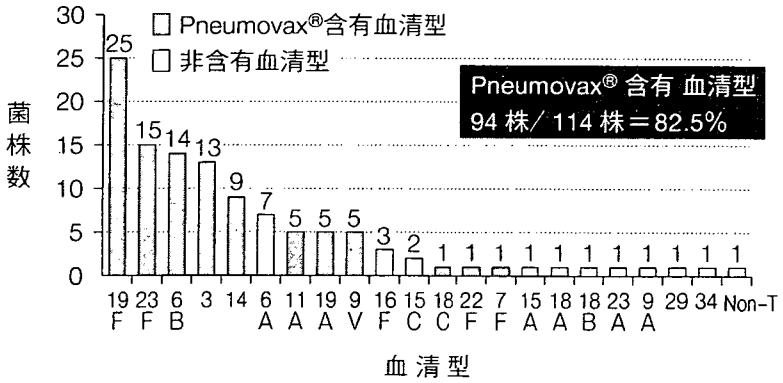

図１　市中肺炎を起こした肺炎球菌と血清型

3．診　断

肺炎球菌感染症であることを診断するためには、本来無菌である病巣から菌を検出することが最も確実である。すなわち、敗血症であれば血液培養、髄膜炎であれば髄液培養で肺炎球菌を証明すれば診断が確定する。検体のグラム染色では、好中球に貪食されたグラム陽性双球菌を認める。肺炎や気管支炎では、喀痰グラム染色や喀痰培養で肺炎球菌を検出するが、本来上気道は無菌ではなく常在菌が存在しており、起炎菌の決定には臨床症状、治療経過との総合判断が必要である。

他の起炎菌決定方法として、肺炎球菌尿中抗原テストがキット化されて実用に供されており、保険適用もあって簡易な検査法である。ただし、特に小児では咽頭に本菌が常在していることから、尿中抗原陽性であっても必ずしも本菌感染症であるとはいえない点に注意が必要である。

4．治　療

肺炎球菌感染症の治療としては、肺炎球菌に抗菌力のある抗菌薬が有用である。20世紀におけるペニシリンをはじめとする抗菌薬の開発は、本菌をはじめとする細菌感染症の治療としてきわめて有用であった。しかし、抗菌薬の使用量の増加に伴い、肺炎球菌の薬剤耐性が進んでおり、耐性菌の増加傾向は大きな問題となっている。

米国臨床検査標準委員会（ＣＬＳＩ）では、微少希釈法によるペニシリンＧに対する最小発育阻止濃度（ＭＩＣ）が、0・06μg／mℓ以下の株をペニシリン感受性肺炎球菌（ＰＳＳＰ）、0・1～1・0μg／mℓをペニシリン中間型肺炎球菌（ＰＩＳＰ）、2・0μg／mℓ以上をペニシリン耐性肺炎球菌（ＰＲＳＰ）と提唱している。成人肺炎でのＰＲＳＰの割合は25～50％を占めており、小児の髄膜炎でも45％という報告がある。

肺炎の治療については日本呼吸器病学会から２００５年に最新版の成人市中肺炎診療ガイドラインが出されており、肺炎球菌性肺炎の治療に言及している。この中で、外来治療では高用量のアモキシシリンの経口投与またはレスピラトリーキノロンの経口投与などが推奨され、入院治療ではペニシリン系注射薬の高用量投与、第４世代セフェム系注射薬、カルバペネム系注射薬などが推奨されている。

細菌性髄膜炎に関しては、２００６年に日本神経治療学会、日本神経学会および日本神経感染症学会の３学会合同による診療ガイドラインが公表されており、髄膜炎の病態治療について詳しく述べている。この中で肺炎球菌髄膜炎に対しては、髄膜炎の後遺症を減らすために、抗菌薬とともにステロイドの投与が推奨されている。

5．予　防

(1)一般的予防法

本菌感染症は咽頭に付着、増殖

した菌が病巣に進んで炎症を起こして発症する。したがって、イソジンなどによるうがいが、咽頭における増殖した菌を減少させるために有用である。

(2)ワクチン

肺炎球菌ワクチンは成人に用いられるPPVと、2歳未満にも有用なPCV-7が実用化されている。PPVは肺炎球菌の莢膜多糖体成分でできた成分ワクチンであり、23価の血清型は1、2、3、4、5、6B、7F、8、9N、9V、10A、11A、12F、14、15B、17F、18C、19A、19F、20、22F、23F、33Fである。

米国予防接種諮問委員会（ACIP）は、1997年に肺炎球菌感染症の予防が推奨される対象について示した[2]。この中で、65歳以上の高齢者に対してはPPVの有効性が証明されているとして、臨床的な利益があるAランクの推奨をしている。

わが国では1988年にPPV（Pneumovax®）が実用化されて以降、年間1～2万人が接種を受けていた。2002年以降は65歳以上の高齢者への予防接種が普及してきて年間15万人以上が接種を受けるようになり、2007年までに累計100万本を超えた。わが国における本ワクチンの接種率は欧米に比較すると未だ低く、急速に高齢化が進む現状から考えると、本ワクチン接種のさらなる普及が望まれる。わが国におけるPPVの接種対象者を**表1**に示す。

PPVは基本的に重篤な副反応が少なく、妊婦への接種は原則禁止であるが、ほかに禁忌となる対象はない。ワクチン接種に伴う副反応の多くは注射局所の疼痛、熱感、腫脹、発赤といったもので、5%以上に認められるが、治療を要するものは少なく、わが国では現在までに生命に関わるような副作用の報告はない。

現状での問題点として、PPVの再接種がわが国では認められていない点が挙げられる。米国では現在は65歳未満で一度接種した人が5年以上経過した場合には、65歳以上での再接種が認められている。2007年以後、わが国でワクチン接種後5年が経過する高齢者が毎年15万人以上発生するため、これらの人々への再接種が現実の課題となっている。わが国においても再接種の安全性を検討し、その有効性を証明することが必要な時期にきている。

なお、PPVは脾臓摘出患者の肺炎球菌感染予防に関してのみ保険適用とされ、その他PPVの接種に関する費用は自己負担となっている。地方自治体を中心として、PPVの普及のために公費負担制度が設けられており、PPVの接種率向上に寄与していると思われるが、今後は国による公費負担制度の充実が望まれる。

一方、PCVは7価のPCV-7（Prevenar®）が米国で2000年に認可され、その後実用化された。日本では2007年9月に製造販売承認申請がなされた段階である。

このワクチンは肺炎球菌の莢膜多糖体をCRM197という毒性のない変種のジフテリア毒素と結合させることにより、抗原性、免疫原性を高めて2歳以下の乳幼児であっても抗体を作ることができるように工夫されている。PCV-7は、4、6B、9V、14、18C、19F、23Fの七つの血清型の多糖体を含んでいるが、この7血清型は米国における肺炎球菌感染症のうち菌血症の86%、髄膜炎の83%、

表1　わが国におけるPPVの接種対象者

2歳以上で肺炎球菌による重篤疾患に罹患する危険性が高い次のような個人および患者

(1) 脾摘患者における肺炎球菌による感染症の発症予防（脾臓摘出後の2歳以上の場合のみ保険適用）

(2) 肺炎球菌による感染症の予防
1) 鎌状赤血球疾患，あるいはその他の原因で脾機能不全である患者
2) 心・呼吸器の慢性疾患，腎不全，肝機能障害，糖尿病，慢性髄液漏等の基礎疾患のある患者
3) 高齢者
4) 免疫抑制作用を有する治療が予定されている者で，治療開始まで少なくとも14日以上の余裕のある患者

急性中耳炎の65％をカバーしていた。米国では2001年からPCV-7接種が本格化して、そのプロトコールでは2歳までに4回の接種を行うこととなっている（表2）。ただし、2歳未満の小児への接種方法は、米国では4回であるが、欧州などでは3回でも有効であろうとしている国もあり、今後の課題となっている。

なお、PCV-7は米国では2歳未満の小児のみではなく、2～5歳の感染リスクのある小児に対する接種も推奨されている（表3）。PCV-7は髄膜炎、敗血症といった侵襲性肺炎球菌感染症の予防に寄与するのみならず、咽頭での本菌の定着をも阻害する効果が期待されており、肺炎球菌による中耳炎の減少にも寄与したとされている。一方、PCV-7の普及に伴い、七つの血清型以外の肺炎球菌感染症および肺炎球菌以外のインフルエンザ菌type bなど他の菌による感染症の増加が危惧されている。

現在、PCVはPCV-7以外に、他の7価のワクチンや9価、11価、13価などの新たなワクチンが研究開発されており、その中にはインフルエンザ菌や髄膜炎菌のタンパク成分と結合させたワクチンで、インフルエンザ菌や髄膜炎菌への感染予防効果を期待しているワクチンも開発中である。9価PCVの接種が南アフリカの乳幼児のウイルス関連肺炎の頻度を有意（31％）に減少させたという報告[3]や、ガンビアでの二重盲検試験で6～51週の乳幼児に対し9価PCVを3回接種した結果、X線で診断された肺炎がプラセボと比較して37％減少したという報告[4]があり、今後の成果が期待される。

おわりに

肺炎球菌ワクチンは、わが国ではPPVについてはようやく普及段階となっているが、さらなる普及のための公費負担問題や、再接種の問題が早期に解決されることが望まれている。またPCV-7については、わが国での実用化が欧米に大きく遅れており、早期の製造販売承認が待たれている。

表2：米国におけるPCV-7の推奨接種スケジュール

Primary Series of CATCH UP Immunization		
age at first dose (month)	primary series	booster dose
2～6	3 doses 6～8 wk apart	1 dose at 12～15 mo of age
7～11	2 doses 6～8 wk apart	1 dose at 12～15 mo of age
12～23	2 doses 6～8 wk apart	none
24～	1 dose	none
Children at HIGH RISK		
age (month)	No. previous dose	recommended
＜23	none	conjugate, 4 doses
24～59	conjugated, 4 doses	PS, 1 dose
	conjugated, 1～3 doses	PS, 1 dose
	PS, 1 dose	conjugate, 2 doses
	none	conjugate, 2 doses, PS 1 dose

PS：23価PPV.　(ACIP：MMRW 49：1, 2000)

表3　PCV-7の適応（米国CDC）

1）2歳以下の小児
2）ハイリスクの2～5歳までの小児
・ハイリスク（侵襲性感染：＞150／100,000／y）
　鎌状赤血球症，無脾症あるいは脾機能不全，HIV感染症
・おそらくハイリスク
　先天性免疫不全，慢性心疾患，慢性肺疾患，脳脊髄漏，慢性腎疾患，免疫抑制療法の必要な疾患，糖尿病
・中等度リスク（侵襲性感染：＞20／100,000／y）
　3歳未満の小児
　5歳未満の小児（自宅外ケア，Native American，African American）

●文　献●

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呼吸器感染症と粘膜免疫

——自然免疫から獲得免疫への連携

Respiratory infection and mucosal immunity

川上和義
Kazuyoshi KAWAKAMI
東北大学医学部保健学科基礎検査学講座病原検査学分野

◎気道は病原微生物などの吸入抗原にたえず曝露されているため，さまざまな免疫防御機構が発達している．デフェンシン，コレクチン(サーファクタントプロテインA，D)，補体などの非特異的液性因子およびマクロファージ，樹状細胞，好中球，NK細胞，NKT細胞，γδT細胞などの細胞性因子から構成される自然免疫，そして液性免疫および細胞性免疫によって構成される獲得免疫が重要であり，これらが気道粘膜領域において経時的・機能的に連携して病原微生物の侵入に対処している．本稿ではAIDSの重要な日和見病原真菌であるクリプトコッカスを中心に，呼吸器粘膜領域における感染防御機構について概説する．そのなかで，NKT細胞とγδT細胞はTh1-Th2サイトカインバランスを拮抗的に調節することで，クリプトコッカスに対する防御免疫応答を適正に制御しており，組織障害につながるような過剰な宿主反応を防ぐための巧妙な調節機構の存在が予想される．

Key word：真菌感染，肺，自然免疫，獲得免疫，Th1-Th2バランス

肺は呼吸を介して外界と接しており，つねに病原微生物を含めた外来異物の侵入にさらされている．下気道に侵入した5μm以上の異物は粘液によって覆われた気管や気管支粘膜によって捕捉され，肺胞にまで到達することはない．しかし，ほとんどの病原微生物を含む5μm以下の小さな異物は肺胞まで到達することが可能であり，機械的バリアーのみでは対応が困難である．気道には，機械的バリアーを越えて侵入してきた異物に対して高度に発達した免疫防御システムが存在する．これは大きく自然免疫と獲得免疫に分類することができる．

本稿では肺内における粘膜防御システムについて，とくにAIDSに合併する日和見病原真菌として重要なクリプトコッカス感染に対するこれらの免疫応答機構について著者らのデータを紹介しながら解説したい．

クリプトコッカス感染

クリプトコッカス症は，多糖体莢膜を有する酵母型真菌の*Cryptococcus neoformans*によって引き起こされる肺および全身性感染症である．莢膜は重要な病原因子であり，マクロファージからの貪食に抵抗性を示す．環境中の*C. neoformans*は莢膜を欠いており，そのために大きさが5μm以下と小さく，気道の機械的バリアーを越えて肺胞腔内まで侵入することができる．ひとたび肺胞腔まで到達すると莢膜を産生するようになり，宿主からの防御免疫応答に対し抵抗性を獲得すると考えられている．さらに，現在では*C. neoformans*は細胞内増殖真菌と認識されており，マクロファージによる殺菌からのエスケープ機構を有している[1]．そのため，自然免疫や液性免疫のみによる防御機構では感染を十分に制御できず，細胞性免疫を中心とした獲得免疫の成立が必須となる．しかし，近

年の研究では自然免疫も重要な役割を担っており，以後の獲得免疫の成立にも大きな影響を及ぼすことが明らかになりつつある．

液性自然免疫因子による感染防御

自然免疫は常時気道粘膜で機能しうる状態で存在しており，侵入した異物に対して速やかに応答することができる．気管支・肺胞粘液中には多くの液性自然免疫因子が存在するが，ここではデフェンシン，コレクチン，補体についてクリプトコッカス感染との関連について紹介する．

1. デフェンシン

デフェンシンは分子量2,000～6,000の抗菌カチオニックペプチドであり，大きくα，βデフェンシンに分けられ，それぞれ好中球，気道上皮細胞から産生分泌される．デフェンシンは膜透過性を亢進することで広く病原微生物に対して殺菌活性を示し，*C. neoformans* もαデフェンシンによって直接殺菌される[2]．

2. コレクチン

コレクチンは多糖成分認識ドメインとコラーゲン様ドメインから構成される蛋白であり，病原微生物のマンノース多糖を認識する．肺胞腔にはマンノース結合レクチンに加えてサーファクタント蛋白(surfactant protein：SP)-A，-Dが存在する．SP-Aは，マクロファージによる貪食に対してオプソニン作用を有さないものの，*C. neoformans* へ濃度依存的に結合する[3]．一方，SP-Dは莢膜を欠いた *C. neoformans* に結合し，これを凝集させることで気道上皮細胞の繊毛運動による排除を促進する[4]．

3. 補体

C. neoformans は別経路によって直接補体を活性化し，生じたC3断片が莢膜表面に蓄積する．C3やC5を欠損したマウスではクリプトコッカス感染が悪化することから，補体が本感染の防御免疫において重要な役割を担うものと考えられている．

自然免疫細胞による感染制御

肺胞腔内に肺胞マクロファージが常時存在し，外来異物の侵入を監視している．炎症時には種々の免疫細胞が集積するが，そのなかには好中球，マクロファージ，自然免疫リンパ球が含まれる．

1. 肺胞マクロファージ

肺胞マクロファージは外来異物の貪食やサイトカイン産生による免疫調節作用を通して，肺内における感染防御の第一線で重要な役割を担っている．肺胞マクロファージはオプソニン化された *C. neoformans* を効率よく貪食するとともに，各種炎症性サイトカインを産生分泌し，そして$CD4^+$T細胞に対して抗原を提示し，その増殖反応やインターフェロン(IFN)-γ産生を誘導する．

2. 好中球

通常気道内に好中球は常在しておらず，病原微生物の侵入などによって補体が活性化され，肺胞マクロファージから炎症性サイトカインやIL-8などのケモカインが産生されると速やかに血管内から肺胞腔に集積する．細胞内増殖菌であるクリプトコッカス感染では好中球の役割は限定的であると考えられている．しかし近年，好中球が貪食細胞としてだけでなく，サイトカイン産生を介して免疫調節作用を発揮するとの報告もなされている．クリプトコッカス感染でも好中球を除去することでむしろ感染が改善する[5]との報告，あるいは好中球殺菌に関連するミエロペルオキシダーゼを遺伝的に欠損したマウスでは本真菌による慢性感染が悪化するといった報告[6]がみられており，今後の詳細な解析が必要である．

3. 自然免疫リンパ球

リンパ球には，通常のT，B細胞のほかに，NK細胞，NKT細胞，γδT細胞，B1-B細胞の存在が知られている．これらの細胞群は非常に速やかに活性化され，自然免疫の時期に機能するという意味で自然免疫リンパ球とよばれる．近年の研究では自然免疫リンパ球がTh1-Th2バランスなど，獲得免疫の“質”を決定する可能性が推察されている．

① **NK細胞**……NK細胞はあらかじめ感作することなく標的細胞を傷害することができる．NK細胞がクリプトコッカス感染防御で重要な役割を担うことが以前から知られていたが，その詳細な機序は明らかではない．直接クリプトコッカスを傷害するとの報告もみられる[7]が，著者らは活性化