2.4. Purification of genomic DNA

The genomic DNA was purified from NPS. Briefly, the NPS samples were diluted 1:3 with sterilized saline and centrifuged at 12,000 rpm for 30 min at 4 °C. The pellets were collected and digested with 200 μl of lysis solution (1 M Tris pH 8.9, 4.5 v/v, nonident P-40, 4.5 v/v, Tween 20, 10 mg/ml Proteinase K) for 1 h at 60 °C. After centrifugation at 12,000 rpm for 20 min at 4 °C, the supernatants were mixed with 100 μl of 3 M sodium acetate buffer and then with 1 ml cold ethanol. Total genomic DNA was purified as precipitations. The total genomic DNA was also purified from *H. influenzae* isolates. Lysis of *H. influenzae* total DNA also used for PCR. In brief, a single colony of *H. influenzae* isolates on a chocolate agar plate was lysed in 30 μl of lysis solution for 10 min at 60 °C and for 5 min at 94 °C in the programmable thermal cycler (Gene Amp PCR System 9700, Perkin-Elmer, Norwalk, CT, USA).

2.5. Multiplex PCR

Primers specific for the *bexA* gene encoding capsular polysaccharides (H1 and H2), *p6* gene encoding a common outer membrane protein P6 (P6-S and P6-R), β-lactamase gene (TEM-S and TEM-R) and *cpsb* gene encoding type b capsular polysaccharide (typeB-S and typeB-R) were used for the multiplex PCR to identify nontypeable, type b and β-lactamase producing *H. influenzae* strains, respectively (Table 2) [7,8,14].

Each 25-μl reaction mixture contained 0.5 μl of each primers (final concentrations were 1 μM of H1 and H2, 1 μM of TEM-S and TEM-R, 1 μM of typeB-S and typeB-R, 3 μM of P6-S and P6-R), 3 μl template DNA, 12.5 μl Qiagen master mixture (Qiagen Gmbh, Germany), 2 μl of 25 mM $MgCl_2$ and 4.5 μl distilled water. The reaction mixture was subjected to amplification in the programmable thermal cycler consisting in denaturation at 94 °C for 10 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s and further extension at 72 °C for 10 min. Amplified DNA fragments were analyzed by 2% agarose gel electrophoresis.

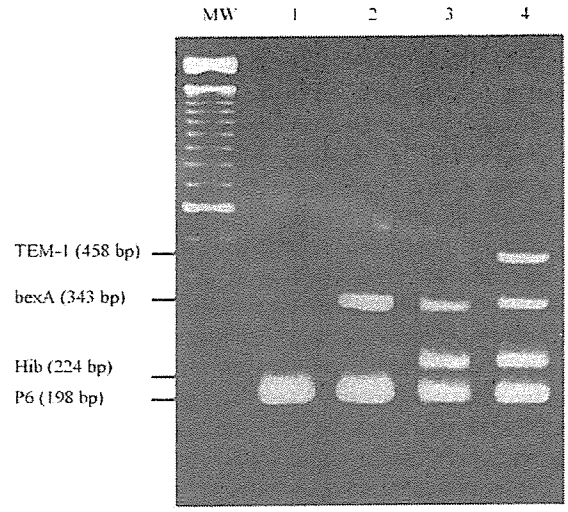

Fig. 1 Multiplex PCR of *H. influenzae*. MW, molecular weight (100 bp); Lane 1, nontypeable *H. influenzae* (ATCC49247); Lane 2, non-type b encapsulated *H. influenzae* (ATCC9327); Lane 3, type b *H. influenzae* (ATCC9334) and Lane 4, β-lactamase producing type b *H. influenzae* (clinical isolate). DNA fragments correspond as follows P6 gene (198 bp), Hib (224 bp) and *bexA* (343 bp), TEM-1 (458 bp).

3. Results

3.1. Specificity and sensitivity of multiplex PCR

The multiplex PCR were specific to *H. influenzae* strains and could identify typeable, type b and nontypeable strains including productions of β-lactamase (Fig. 1, Table 3). The negative controls of *S. aureus* and *S. pneumoniae* did not showed any amplification for the four genes (Table 3).

Table 2 Primers for the multiplex PCR

Primer name	Target	Sequence (5′–3′)	Primer length	Position	PCR product (bp)
P6-S	*P6*	ACGATGCTGCAGGCAATGGT	20	141–160	198
P6-R		CATCAGTATTACCTTCTACTAAT	23	316–338	
TEM-S	*TEM-1*	TAAGAGAATTATGCAGTGCTGCC	23	350–372	458
TEM-R		TCCATAGTTGCCTGACTCCCC	21	787–807	
HI-1	*bexA*	CGTTTGTATGATGTTGATCCAGACT	25	3552–3577	343
HI-2		TGTCCATGTCTTTCAAAATGATG	22	3873–3895	
Typeb-S	*cpsb*	AGATACCTTTGGTCGTCTGC	20	5483–5502	224
Typeb-R		CTTACGCTTCTATCTCGGTG	20	5706–5725	

Table 3 Identification of nontypeable and type b *H. influenzae*

Patient ID	Culture	Serological agglgation test	β-lactamase	Multiplex PCR							
				Bacterial lysate				Nasopharyngeal secretion			
				P6	bexA	typeb	TEM-1	P6	bexA	typeb	TEM-1
1	3+	N/T	−	+	−	−	−	+	−	−	−
2	1+	N/T	−	+	−	−	−	+	−	−	−
3	NGHi	N/A	−	N/A	N/A	N/A	−	−	−	−	−
4	3+	N/T	−	+	−	−	−	+	−	−	−
5	3+	N/T	−	+	−	−	−	+	−	−	−
6	NGHi	N/A	−	N/A	N/A	N/A	−	−	−	−	−
7	NGHi	N/A	−	N/A	N/A	N/A	−	−	−	−	−
8	1+	N/T	−	+	−	−	−	+	−	−	−
9	3+	N/T	−	+	−	−	−	+	−	−	−
10	3+	Type b	−	+	+	+	−	+	+	+	−
11	3+	N/T	−	+	−	−	−	+	−	−	−
12	3+	N/T	−	+	−	−	−	+	+	+	−
13	3+	N/T	−	+	−	−	−	+	−	−	−
14	NGHi	N/A	−	N/A	N/A	N/A	−	+	+	+	−
15	2+	Type b	−	+	+	+	−	+	+	+	−
16	NGHi	N/A	−	N/A	N/A	N/A	−	−	−	−	−
41	NGHi	N/A	−	N/A	N/A	N/A	−	−	−	−	−
42	1+	N/T	−	+	−	−	−	+	−	−	−
49	NGHi	N/A	−	N/A	N/A	N/A	−	−	−	−	−
50	1+	Type b	+	+	+	+	+	+	+	+	+
51	3+	N/T	−	+	−	−	−	+	−	−	−
52	3+	N/T	−	+	−	−	−	+	−	−	−
53	NGHi	N/A	−	N/A	N/A	N/A	−	−	−	−	−
58	3+	N/T	−	+	−	−	−	+	−	−	−
59	3+	N/T	−	+	−	−	−	+	−	−	−

NGHi, no growth of *H. influenzae*; +, positive; −, negative; N/A, not applicable; NT, nontypeable; 1+, 2+, 3+, growth in first, second and third quadrant of chocolate agar plate, respectively.

The lowest limit of the multiplex PCR to identify the genomic DNA of *H. influenzae* strain was 2×10^{-3} ng (Fig. 2).

3.2. Identification of *H. influenzae* in NPS

H. influenzae were isolated in 17 (68.0%) samples by conventional culture method. There were 3 (17.6%) serotype b strains and 14 (82.4%) non-encapsulated strains by SAT. Only one strain (4%) produced β-lactamase. All the strains identified in NPS by conventional culture method were confirmed as the similar characteristics by the multiplex PCR.

On the other hand, *H. influenzae* were identified in 18 (72.0%) out of the 25 NPS samples by the multiplex PCR. There were 5 (27.8%) type b strains possessing both *bexA* and *cpsb* genes and 13 (72.2%) non-encapsulated strains without *bexA* gene. The β-lactamase gene was identified in one sample. Two NPS samples (patient nos. 12 and 14) showed amplicons for *bexA* and *cpsb* genes while encapsulated *H. influenzae* strains were not isolated in these samples. One is the sample in which non-encapsulated strain was identified by conventional culture method and another is the sample in which *H. influenzae* were not identified by the conventional culture method (Table 3).

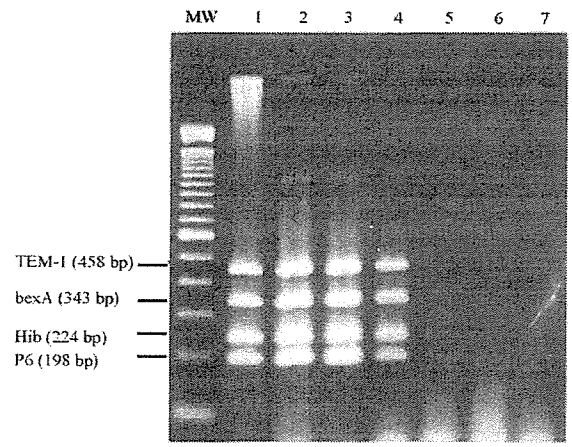

Fig. 2 Sensitivity of multiplex PCR. MW, molecular weight (100 bp); Lane 1, 50 ng; Lane 2, 5 ng; Lane 3, 500 pg; Lane 4, 50 pg; Lane 5, 5 pg; Lane 6, 500 fg and Lane 7, 50 fg.

4. Discussion

Since the type b capsular polysaccharide conjugate vaccine widely used in the United States, the incidence of invasive infectious diseases caused by type b strain in children less than 5 years old have remarkably decreased but increased relative importance of nontypeable strains and other encapsulated typeable strains [15,16]. In contrast to the United States, type b capsular polysaccharide conjugated vaccine has not licensed yet in Japan. The type b *H. influenzae* strain still remains the leading causes of meningitis in Japan [17–19]. It is worthy to identify nontypeable and encapsulated typeable strains. The SAT has been widely applied to determine the types of capsular polysaccharides of *H. influenzae*. However, the test has sometimes shown to be unreliable due to serological cross-reactions and/or lower sensitivity of sera. Laclaire *et al.* reported that two-thirds of serotype b *H. influenzae* isolates reported to CDC were incorrectly classified by SAT in 2003 but actual prevalence of Hib will be overestimated [8]. In addition to these problems, the SAT cannot be applied clinical specimens directly. It requires isolation of *H. influenzae* according to the usual laboratory cultures prior to determine the capsular types and it takes more than 48 h.

In this study, we firstly applied multiplex PCR to identify *H. influenzae* and determine the capsular characteristics of the pathogen isolated in NPS. *H. influenzae* were identified at 68–72% among NPS. About 82.4% by culture and 72.2% by the multiplex PCR of *H. influenzae* isolates were nontypeable strains. Ueyama *et al.* reported that more than 90% of strains in the nasopharynx were nontypeable [20]. About 17.6% by culture and 27.8% by PCR of *H. influenzae* were Hib strains. The surveillance of pediatric respiratory tract infectious diseases during 1980–1991 showed that only 2.6% of isolates were type b strains [21,22]. One sample (patient no. 14) in which we failed to identify *H. inlfuenzae* by conventional bacterial culture had the DNA genome of type b strain. The multiplex PCR based serotyping provided more reliable results than SAT. However, one sample (patient no. 12) in which nontypeable *H. influenzae* was isolated by bacterial culture possessed *bexA* and *cpsb* genes by the multiplex PCR. While the predominant strain in the NPS will be nontypeable, small concomitants of type b strain would be exist in the NPs. By the conventional methods alone, we cannot determine the actual prevalence of Hib that will be the minority of pathogens among the nasopharynx. Hubener *et al.* suggested that if the less common serotype represents only 5% of the total pneumococcal population, 59 colonies from each specimen would need to be serotyped to have a 95% probability of picking the second pneumococcal type [23]. The multiplex PCR allows a feasible analysis of minority strains in the nasopharyngeal *H. influenzae* population. It is necessary to apply real-time quantitative PCR for further quantitative evaluations. However, the method can easily be implied among microbiology laboratories and can assess many samples at once with rapid reliable results.

In conclusion, the possibility to use a multiplex PCR method as a qualitative assay to evaluate the true composition of possibly diverse populations of *H. influenzae* increases the usefulness as a new typeable/nontypeable technique.

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Comparative Immune Responses of Patients with Chronic Pulmonary Diseases during the 2-Year Period after Pneumococcal Vaccination▽

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Antibody responses to a 23-valent pneumococcal vaccine for *Streptococcus pneumoniae* serotypes 6B, 14, 19F, and 23F in 84 patients with chronic pulmonary diseases over a 2-year period after vaccination were examined by using a third-generation enzyme-linked immunosorbent assay. Of these patients, 28 (31%) were low responders who had developed increases of at least twofold in the levels of serotype-specific immunoglobulin G (IgG) in sera for none of the four serotypes at 1 month after vaccination. Although no specific clinical features of low responders were evident, their prevaccination levels of IgG for all serotypes were higher than those of responders. In responders, the levels of IgG specific for serotypes 14 and 23F in sera were greatly increased 1 month after vaccination and those specific for serotypes 6B and 19F were moderately increased. In contrast, no significant increases in the levels of IgG specific for serotypes 6B, 19F, and 23F in the low responders during the same period were found, but the levels of IgG specific for serotype 14 did increase. Although a rapid decline in the levels of IgG for all serotypes in responders between 1 month and 6 months after vaccination was found, the levels of IgG specific for serotypes 14 and 23F in sera remained higher than the prevaccination levels for at least 2 years after vaccination. These data suggest the need for the revaccination of responders but not low responders among patients with chronic pulmonary diseases. Revaccination as early as 3 years postvaccination is recommended for responders to increase the reduced levels of IgG in sera, especially those specific for the weak vaccine antigens.

Streptococcus pneumoniae is an important cause of pneumonia and serious invasive diseases in children and adults (4, 13, 14). The increased rate of drug-resistant pneumococci in recent years emphasizes the need for preventing pneumococcal infections by vaccination with the 23-valent pneumococcal polysaccharide vaccine (PPV) (3, 16, 19, 28).

Patients with chronic pulmonary diseases, such as chronic obstructive pulmonary diseases (COPD), are highly susceptible to pneumonia or acute exacerbation caused by *S. pneumoniae* (25). Since previous investigators reported the efficacy of PPV for preventing invasive pneumococcal diseases in patients, including those with chronic pulmonary diseases and other chronic illnesses, PPV is recommended for these patients (8, 9, 26). The nature of the effects of PPV in preventing pneumonia or acute exacerbation among patients with chronic pulmonary diseases, however, remains controversial (1, 11, 27, 30).

Antibodies to pneumococcal capsular polysaccharide (PPS) and complement provide protection against *S. pneumoniae* strains with homologous or cross-reactive capsular serotypes (18). Using a variety of methodologies, previous investigators have reported the concentrations of PPS-specific immunoglobulin G (IgG) in sera from patients with chronic pulmonary diseases, including COPD (7, 11, 22, 29). No studies, however, have examined the levels of serotype-specific IgG in sera from patients with chronic pulmonary diseases by using the third-generation enzyme-linked immunosorbent assay (ELISA) that has recently been recommended by the World Health Organization (31).

Two previous studies reported a substantial proportion of poor responders to PPV among elderly adults or patients with COPD who were receiving steroid therapy (12, 21). However, these studies failed to demonstrate the kinetics of the immune responses of this group. In addition, antibody avidity is an indicator of the strength with which an antibody binds to a complex antigen, and high-avidity antibodies are superior to low-avidity antibodies in terms of opsonophagocytic killing of *S. pneumonaie* (2, 20). No previous studies have examined the avidities of antibodies in sera from patients with chronic pulmonary diseases before and after pneumococcal vaccination.

The objective of this study, therefore, was to examine the concentrations of serotype-specific IgG and the avidity of IgG in sera from patients with chronic pulmonary diseases by using the third-generation ELISA before and after pneumococcal vaccination. We also attempt to characterize a subset of low responders among these patients and demonstrate the differ-

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TABLE 1. Comparative clinical characteristics of all subjects, responders, and low responders with chronic pulmonary diseases

Characteristic	Value for group		
	All subjects (n = 84)	Responders (n = 58)	Low responders (n = 26)
Mean age ± SD (yr)	68.1 ± 9.1	67.76 ± 8.77	69 ± 9.90
No. of males (%)	58 (69)	40 (69)	18 (69)
No. with chronic pulmonary disease (%)			
Chronic obstructive pulmonary disease	27 (32.1)	17 (29)	10 (38)
Sequelae of pulmonary tuberculosis	26 (31.0)	19 (33)	7 (27)
Bronchiectasis	12 (14.3)	6 (10)	6 (23)
Bronchial asthma	8 (9.5)	7 (12)	1 (4)
Pneumoconiosis	6 (7.1)	4 (7)	2 (8)
Interstitial pneumonia	3 (3.6)	3 (5)	0 (0)
Diffuse panbronchiolitis	2 (2.4)	2 (3)	0 (0)
No. receiving steroid therapy (%)			
Inhaled and oral steroid	10 (11.9)	9 (16)	1 (4)
Inhaled steroid alone	12 (14.3)	7 (12)	5 (18)
Oral steroid alone	6 (7.1)	5 (9)	1 (4)

ence in the kinetics of serotype-specific IgG between responders and low responders over a 2-year period after vaccination.

MATERIALS AND METHODS

Study subjects and vaccination. Eighty-four patients with chronic pulmonary diseases were enrolled in this study after providing written informed consent at 1 of 13 hospitals in the districts of Kyushu and Okinawa, Japan, between November 2001 and December 2003. The ages of the study subjects ranged from 40 to 88 years (median, 70.0 years), and 58 (69%) were male (Table 1). Of these, 28 patients (33.3%) had previously received oral steroids, inhaled steroids, or both. Each patient received a single intramuscular dose of 0.5 ml of a PPV (Pneumovax, Banyu, Japan). The dose contained 25 μg of each of 23 pneumococcal serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. None of these subjects had previously been vaccinated with a PPV. Blood samples were collected from the patients immediately before vaccination and 1 month, 6 months, 1 year, and 2 years after vaccination. Sera were separated by centrifugation, divided into small aliquots, and stored frozen at −80°C until used. All of the subjects were evaluated for serotype-specific IgG before and 1 month after vaccination.

Samples from only 40 of 84 subjects were available for all time points, before vaccination and 1 month, 6 months, 1 year, and 2 years after vaccination, because 7 subjects died during the 2-year period after vaccination and 37 of the remaining subjects lacked at least one serum sample at 6 months, 1 year, or 2 years after vaccination. The ages of these 40 subjects ranged from 40 to 80 years (mean, 67 years), and 25 (62.5%) were male. The chronic pulmonary diseases among these subjects were COPD (n = 15), sequelae of pulmonary tuberculosis (n = 15), bronchial asthma (n = 4), bronchiectasis (n = 3), and pneumoconiosis (n = 3). All studies described herein were approved by the institutional review board of each institution which is a member of the Pneumococcal Vaccine Trialist Group in Kyushu and Okinawa, and a signed consent form was obtained from each subject.

Measurement of anti-PPS IgG. Since the preabsorption of serum to both cell wall polysaccharides (CWPs) and type 22F PPS could increase the correlation between the levels of serotype-specific IgG in sera and the opsonophagocytic activities of the IgG (10), the concentrations of serotype-specific IgG were measured as previously described (31). The levels of IgG specific for the four serotypes that are the most prevalent among adult patients with pneumococcal infections in the United States and Japan were determined (15, 20). Briefly, each well of a microtiter plate (Maxisorb; Nunc, Roskilde, Denmark) was coated with 100 μl of serotype-specific PPS antigen (ATCC, Rockville, MD), and the plate was then incubated at 37°C for 5 h in a humidified chamber. The U.S. reference pneumococcal antiserum (89-SF), courtesy of Carl Frasch, was adsorbed to CWPs, but all other samples were adsorbed to CWPs (5 μg/ml) and 22F PPS (10 μg/ml) in phosphate-buffered saline–0.05% Tween 20 at room temperature for 30 min. Fifty microliters of the adsorbed sera was diluted twofold and added to the wells of a microtiter plate. The microtiter plates were incubated for 2 h at room temperature. After washing of the plates, 100 μl of diluted goat anti-human IgG-alkaline phosphatase conjugate was added to each well, and the plates were incubated for 2 h at room temperature. After washing of the plates, 100 μl of substrate solution (1-mg/ml p-nitrophenyl phosphate) was added to each well and the plates were again incubated for 2 h at room temperature. The reaction was stopped by the addition of 50 μl of 3 M NaOH to all of the wells, and the optical density at 405 nm was measured with a reference filter of 690 nm. The concentrations of serotype-specific IgG were calculated based on a comparison with the internal standard reference serum 89-SF. We defined individual subjects as responders if they developed a twofold increase in serotype-specific IgG for at least one of the four serotypes and as low responders if they developed a twofold increase in serotype-specific IgG for none of the four serotypes at 1 month postvaccination.

Measurement of the avidity of anti-PPS IgG. The avidity of serotype-specific IgG in sera was measured by using ELISA according to a previously described method (2). Twenty-eight of the 40 patients subjected to the full course of measurements of serotype-specific IgG in sera before vaccination and at 1 month, 6 months, 1 year, and 2 years were included in the avidity assay because of the limited volume of stored serum. The serum samples preadsorbed to CWPs and 22F PPS were added to the coated microtiter plates, and the plates were incubated for 2 h at room temperature. After washing of the plates, 0.5 M sodium thiocyanate was added to each well and the plates were incubated for 15 min at room temperature. After washing of the plates, diluted goat anti-human IgG-alkaline phosphatase conjugate was added to each well. After incubation for 2 h at room temperature, the substrate solution was added to the plates, followed by incubation for 2 h at room temperature. The optical density at 405 nm was measured. The avidity index was expressed as the percentage of antibodies that remained bound to the antigens after incubation with sodium thiocyanate.

Statistical analysis. The average antibody concentrations, increases (n-fold), and absolute increases are expressed as the geometric means. Differences in geometric mean concentrations (GMCs) of serotype-specific IgG over time were assessed by using the Friedman test and the Wilcoxon signed-rank test, and the differences in IgG levels between responders and low responders were assessed by using the Mann-Whitney U test for independent samples.

RESULTS

Anti-PPS IgG levels before and 1 month after vaccination. The GMCs of IgG antibodies specific for four serotypes in sera before vaccination ranged from 3.05 μg/ml for serotype 23F to 6.35 μg/ml for serotype 14 (Table 2). When the threshold of the protective levels of serotype-specific IgG against invasive pneumococcal diseases in sera is assumed to be 1 μg/ml (24), the percentages of patients who showed higher levels were 92% for serotype 6B, 99% for serotype 14, 96% for serotype 19F, and 92% for serotype 23F, much higher than those reported previously for elderly subjects (24). One month after vaccination, significant increases in the GMCs of serotype-specific IgG for all serotypes compared to those before vaccination were found for all subjects ($P < 0.01$) (Table 2). Increases in GMCs of serotype-specific IgG exceeding twofold were, however, found only for serotypes 23F and 14.

Responders and low responders to PPV. With the definition of responders and low responders in this study, the numbers of responders and low responders were 58 (69.0%) and 26 (31.0%), respectively (Table 1). No significant differences in age, sex, frequency of specific chronic pulmonary disease, and steroid use were found between the two groups. Interestingly, the prevaccination levels of serotype-specific IgG in low responders were higher than those in responders for all serotypes, although no significant differences were found between

TABLE 2. Comparison of GMCs and geometric increases (n-fold) in levels of serotype-specific IgG antibody in sera from all 84 subjects, responders, and low responders before and 1 month after vaccination[a]

Serotype	Time point	GMC of IgG (μg/ml) (95% CI) in sera from:			Geometric mean increase (n-fold) (range) in IgG in sera from:		
		All subjects (n = 84)	Responders (n = 58)	Low responders (n = 26)	All subjects (n = 84)	Responders (n = 58)	Low responders (n = 26)
6B	Pre	4.33 (3.51–5.36)	3.9 (3.01–5.04)	5.48 (3.80–7.89)			
	1 mo	6.44 (5.11–8.11)**	6.81 (5.04–9.21)**	5.68 (4.02–8.03)	1.49 (0.5–8.69)	1.75 (0.53–8.69)	1.04 (0.5–1.61)
14	Pre	6.35 (5.25–7.68)	5.82 (4.71–7.17)	7.73 (5.14–11.63)			
	1 mo	14.84 (11.51–19.14)**	18.42 (13.45–25.22)**	9.16 (6.17–13.60)*#	2.34 (0.6–46.33)	3.17 (0.83–46.33)	1.19 (0.6–1.84)
19F	Pre	5.25 (4.29–6.43)	4.74 (3.70–6.07)	6.62 (4.62–9.48)			
	1 mo	7.27 (6.04–8.75)**	7.63 (6.09–9.55)**	6.53 (4.63–9.21)	1.38 (0.35–11.41)	1.61 (0.82–11.41)	0.99 (0.35–1.82)
23F	Pre	3.05 (2.53–3.67)	2.91 (2.37–3.57)	3.37 (2.24–5.07)			
	1 mo	6.51 (5.01–8.46)**	8.39 (6.10–11.52)**	3.7 (2.45–5.58)#	2.13 (0.53–38.49)	2.88 (0.67–38.49)	1.1 (0.53–1.95)

[a] Pre, prevaccination; CI, confidence interval; **, $P < 0.01$ (for comparison with prevaccination value); *, $P < 0.05$ (for comparison with prevaccination value); #, $P < 0.05$ (for comparison with value for responders, at 1 month after vaccination).

the two groups. Significant increases in the GMCs of serotype-specific IgG for all serotypes compared to those before vaccination were found in responders 1 month after vaccination ($P < 0.01$) (Table 2). In contrast, no significant increases in IgG specific for serotypes 6B, 19F, and 23F were found at 1 month after vaccination, although a slight but significant increase in the level of IgG specific for serotype 14 compared to that before vaccination was found ($P < 0.05$) (Table 2). The GMCs for serotypes 14 and 23F were significantly higher in responders than in low responders ($P < 0.05$) (Table 2).

Influence of steroid therapy. The geometric mean increases (n-fold) in serotype-specific IgG for all serotypes 1 month after vaccination among 28 patients receiving steroid therapy and 56 patients receiving no steroid therapy were compared. The geometric mean increases (n-fold) among patients with steroid therapy and those without steroid therapy were 1.48 and 1.49 for serotype 6B, 2.28 and 2.37 for seroype 14, 1.49 and 1.33 for serotype 19F, and 2.24 and 2.08 for serotype 23F, respectively. No significant differences in increases (n-fold) in the levels of serotype-specific IgG for all serotypes between the two groups were found, which is in agreement with the results of previous studies with patients with COPD receiving steroid therapy (12, 17).

Kinetics of anti-PPS IgG during 2 years after vaccination. The GMCs of serotype-specific IgG for all serotypes decreased significantly 6 months after vaccination (P, <0.05 for serotype 6B and <0.01 for serotypes 14, 19F, and 23F) (Table 3). The GMCs of serotype-specific IgG for all serotypes declined up to 68 to 81% between 1 month and 6 months after vaccination (Table 3). The GMCs of IgG specific for types 6B and 19F declined below prevaccination levels at 6 months postvaccination and those for type 23F at 2 years postvaccination (Table

TABLE 3. GMCs and geometric increases (n-fold) in levels of serotype-specific IgG in sera from 40 patients before vaccination and 1 month, 6 months, 1 year, and 2 years after vaccination[a]

Serotype	Time point	GMC of IgG (μg/ml) (95% CI)	Geometric mean increase (n-fold) (range)	Absolute increase (μg/ml) (range)
6B	Pre	3.54 (2.6–4.81)		
	1 mo	5.03 (3.61–7.02)**	1.42 (0.5–6.13)	1.06 (−1.98–18.21)
	6 mos	3.48 (2.46–4.92)	0.98 (0.09–3.33)	
	1 yr	3.28 (2.4–4.5)	0.93 (0.25–2.67)	
	2 yrs	2.43 (1.7–3.48)	0.69 (0.11–3.29)	
14	Pre	5.47 (4.41–6.79)		
	1 mo	11.04 (8.24–14.78)**	2.02 (0.78–13.06)	1.12 (−2.75–85.13)
	6 mos	8.96 (6.64–12.08)**	1.64 (0.41–9.33)	
	1 yr	8.03 (6.12–10.54)**	1.47 (0.73–8.44)	
	2 yrs	6.92 (5.22–9.17)*	1.26 (0.27–8.14)	
19F	Pre	4.87 (3.75–6.31)		
	1 mo	6.56 (5.07–8.49)**	1.35 (0.67–11.41)	1.05 (−1.89–19.3)
	6 mos	4.6 (3.51–6.03)	0.94 (0.14–12.25)	
	1 yr	4.35 (3.46–5.48)	0.89 (0.35–4.35)	
	2 yrs	4.15 (3.19–5.41)	0.85 (0.22–5.46)	
23F	Pre	2.6 (2.03–3.32)		
	1 mo	5.54 (3.73–8.23)**	2.13 (0.67–38.49)	1.16 (−2.83–79.1)
	6 mos	3.74 (2.61–5.37)*	1.44 (0.47–27.29)	
	1 yr	3.28 (2.39–4.5)*	1.26 (0.39–18.54)	
	2 yrs	2.33 (1.61–3.36)	0.90 (0.14–12.96)	

[a] Pre, prevaccination; CI, confidence interval; *, $P < 0.05$ (for comparison with prevaccination value); **, $P < 0.01$ (for comparison with prevaccination value).

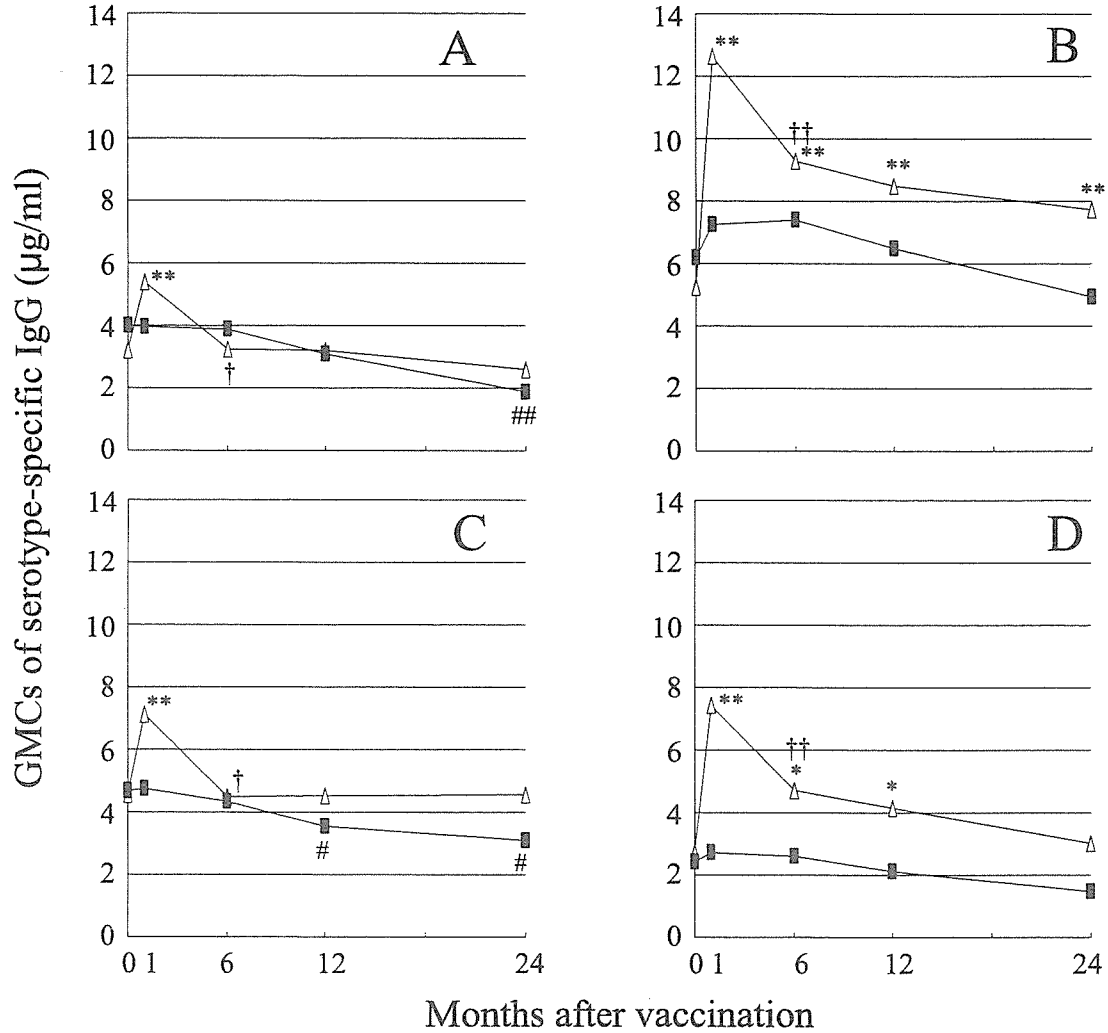

FIG. 1. Kinetics of GMCs of IgG specific for serotypes 6B (A), 14(B), 19F (C), and 23F (D) in responder (n = 27; open triangles) and low-responder (n = 13; closed squares) groups before vaccination and 1 month, 6 months, 1 year, and 2 years after vaccination as determined by ELISA. *, P of <0.05, and **, P of <0.01 (for comparison with prevaccination value); †, P of <0.05, and ††, P of <0.01 (for comparison with value for 1 month after vaccination); #, P of <0.05, and ##, P of <0.01 (for comparison with prevaccination value).

3). The GMCs of serotype 14-specific IgG 2 years postvaccination were still significantly higher than prevaccination GMCs ($P < 0.05$) (Table 3). The estimated time points after vaccination when the levels of serotype-specific IgG returned to the prevaccination levels, calculated using the logarithmic trend line, were 0.5 years for serotype 6B, 6.9 years for serotype 14, 0.6 years for serotype 19F, and 1.7 years for serotype 23F.

We next compared the kinetics of serotype-specific IgG in sera from responders (n = 27) and low responders (n = 13) during the 2-year period after vaccination (Fig. 1). The increases in type-specific IgG for all serotypes in responders 1 month after vaccination were statistically significant. While a moderate increase in IgG for serotype 6B or 19F was found, a substantial increase in IgG for serotypes 14 and 23F at the same time point was found. A rapid decline in serotype-specific IgG in sera for all four serotypes in responders within 1 year after vaccination was also found. In the case of the responders, the time intervals required for the GMCs to return to prevaccination levels were calculated to be 0.87 years for serotype 6B, 8.3 years for serotype 14, 1.1 years for serotype 19F, and 2.5 years for serotype 23F. The persistence of serotype-specific IgG above the prevaccination level was, therefore, highly varied for each serotype. In contrast, no significant increases in IgG specific for any of the serotypes in low responders at 1 month after vaccination were found. These levels remained unchanged or decreased slightly compared to the prevaccination levels for serotypes 14 and 23F between 1 month and 2 years postvaccination, while these levels decreased significantly compared to the prevaccination levels at 1 year and 2 years after vaccination for serotype 19F and at 2 years after vaccination for serotype 6B (P, <0.05 for serotype 19F; P, <0.01 for serotype 6B).

Avidity index of anti-PPS IgG. The avidity indices of serotype-specific IgG for all four serotypes in sera from all subjects, responders, and low responders before vaccination and 1 month and 2 years after vaccination are shown in Table 4.

TABLE 4. Comparison of avidity indices of serotype-specific IgG in sera from a total of 28 patients, responders, and low responders before and after vaccination[a]

Serotype	Time point	Avidity index ± SD for sera from:		
		All subjects (n = 28)	Responders (n = 20)	Low responders (n = 8)
6B	Pre	62.41 ± 19.05	63.05 ± 21.11	60.81 ± 13.64
	1 mo	61.73 ± 20.43	61.88 ± 22.25	61.35 ± 16.33
	2 yrs	55.66 ± 24.03	59.97 ± 25.03	44.88 ± 18.48#*
14	Pre	84.3 ± 14.55	86.29 ± 14.79	79.33 ± 13.53
	1 mo	82.16 ± 18.08	84.30 ± 15.90	76.78 ± 22.98
	2 yrs	83.74 ± 14.13	85.98 ± 13.44	78.16 ± 15.15
19F	Pre	73.14 ± 20.81	73.62 ± 21.26	71.96 ± 21.00
	1 mo	68.64 ± 16.82	67.61 ± 18.64	71.20 ± 11.73
	2 yrs	62.55 ± 19.46*	64.05 ± 19.82*	58.81 ± 19.28
23F	Pre	71.25 ± 16.13	73.69 ± 14.02	65.14 ± 20.24
	1 mo	74.57 ± 23.28	75.71 ± 23.94	71.72 ± 22.82
	2 yrs	69.07 ± 23.55	74.66 ± 21.84	55.11 ± 23.07

[a] Avidity indices are expressed as the percentages of antibodies that remained bound to antigens after thiocyanate treatment. Pre, prevaccination; *, $P < 0.01$ (for comparison with prevaccination value); #, $P < 0.01$ (for comparison with value for 1 month after vaccination).

Overall, no significant difference in the avidity indices for all four serotypes in all subjects between the time points before vaccination and at 1 month after vaccination was found. In addition, the avidity indices for all subjects, responders, and low responders for all four serotypes remained unchanged, except those for serotype 6B in low responders and serotype 19F in all subjects and responders, for up to 2 years after vaccination. The avidity indices were lower among low responders than among responders for all four serotypes, although the differences were statistically insignificant before vaccination and 1 month and 2 years after vaccination.

DISCUSSION

This study examined the differences in the clinical characteristics and immune responses to PPV of responders and low responders in a group of patients with chronic pulmonary diseases over a 2-year period after vaccination. Although significant increases in the levels of IgG specific for four major serotypes were found after pneumococcal vaccination, the immune responses to PPV were highly varied. Although 31% of patients with chronic pulmonary diseases were defined as low responders to PPV, no significant demographic feature was found among these subjects. Rubins et al. reported that 20% of elderly patients were found to be poor responders to PPV while none of the healthy young adults examined were poor responders, but these investigators employed the second-generation ELISA and defined a poor responder as a patient who developed a twofold increase in serotype-specific IgG for fewer than two of seven serotypes tested at both 1 and 3 months after vaccination (21). de Roux et al. also evaluated the nonresponders to PPV of each serotype who developed neither a twofold increase nor an increase of at least 1 μg/ml by using the second-generation ELISA among patients with COPD who were receiving inhaled steroids or systemic steroids (12). The frequencies of nonresponders who developed a twofold increase for fewer than two of seven serotypes were 17% and 21% among COPD patients receiving inhaled steroids and those receiving systemic steroids, respectively, in this study. The frequency of low responders to PPV in our study, therefore, is somewhat higher than those reported in these studies (12, 21). Although additional absorption to PPS 22F reduced the levels of serotype-specific IgG, the prevaccination levels of serotype-specific IgG in sera were higher than 1 μg/ml in nearly all of our patients. A tendency for increased prevaccination levels of serotype-specific IgG in the sera of low responders was also found. A recent study similarly demonstrated that elderly subjects with higher levels of serotype-specific IgG (≥5 μg/ml) in sera before vaccination tended to respond to PPV at a lower magnitude (6). The high proportion of low responses in our study may be due to the increased prevaccination levels of serotype-specific IgG in the sera of patients with chronic pulmonary diseases.

Another finding in this study is the rapid decline in the levels of serotype-specific IgG in sera 6 months after vaccination in patients with chronic pulmonary diseases. A previous study by Davis et al. reported the kinetics of levels of pneumococcal antibodies to 12 serotypes in sera from patients with COPD after vaccination with 14-valent PPV (11). Using a radioimmunoassay, the authors similarly demonstrated a gradual decline in PPS-specific antibody levels in sera over 2 years. The levels of PPS-specific IgG at 2 years postvaccination were still higher than the prevaccination levels. Sankilampi et al. also demonstrated that the concentrations of serotype-specific IgG in the elderly, as determined by the second-generation ELISA, declined to levels similar to the prevaccination levels at 3.0 years after vaccination with PPV for serotype 6B, 3.8 years for serotype 19F, 4.7 years for serotype 23F, and 7.7 years for serotype 14 in the elderly (23). A recent study reported a rapid decline of serotype 6B-specific IgG levels in sera, as determined by second-generation ELISA, at 1 year postvaccination among long-term-care residents who were 60 years of age or older (6). These data and ours indicate a gradual decline in the levels of serotype-specific IgG in sera, and these levels return to the prevaccination levels within 1 to 4 years after pneumococcal vaccination in patients with chronic pulmonary diseases or elderly patients (6, 23). In addition, the levels in sera of IgG specific for serotypes 6B, 19F, and 23F, which are weak vaccine antigens, declined faster than those of IgG specific for serotype 14 among these subjects (14). More importantly, the present study clearly demonstrates differences in the kinetics of serotype-specific IgG in sera from responders and low responders. Since low responders exhibited no significant increases in the levels of IgG specific for serotypes 6B, 19F, and 23F in sera at 1 month postvaccination, the frequency of low responders of 31% affected the kinetics of serotype-specific IgG in sera for all study subjects. Nevertheless, we found that the time point for the serotype-specific IgG to return to the prevaccination level was less than 3 years for such weak vaccine antigens, even in responders, while the time point for serotype 14 was longer than 8 years in these subjects. These data suggest that pneumococcal revaccination may be required especially for these weak vaccine antigens as early as 3 years after the initial pneumococcal vaccination for responders with chronic pulmonary diseases. Although the use of pneumococcal conjugate vac-

cines may be a possible strategy currently available for low responders, revaccination with PPV may also be effective, especially for low responders whose levels of serotype-specific IgG in sera are relatively reduced before revaccination.

The avidity indices of serotype-specfic IgG in prevaccination sera determined for four serotypes in our study were similar to data reported in a recent publication by Bogaert et al., who used serum samples collected from patients with COPD (5). A common finding in that study and ours is that the avidity index is the highest for serotype 14 and the lowest for serotype 6B. No significant increase in the avidity index of IgG specific for any of the four serotypes was found before and 1 month after vaccination with 23-valent PPV in this study. Although several previous studies demonstrated significant increases in the avidity indices among infants after immunization with a pneumococcal conjugate vaccine (2, 32), the discrepancy between the findings of these studies and ours may be due to differences in the type of pneumococcal vaccine used or differences in target subjects.

In summary, this study demonstrates differences in immune responses to PPV between responders and low responders among patients with chronic pulmonary diseases over a 2-year period after pneumococcal vaccination. Our data suggest that responders should be revaccinated at as early as 3 years postvaccination in order to increase the attenuated levels of serotype-specific IgG, especially for the weak vaccine antigens. Further studies will be required to clarify the proportion of low responders in other subsets of elderly or young adults for which PPV is recommended (9).

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

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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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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, H04-03, 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

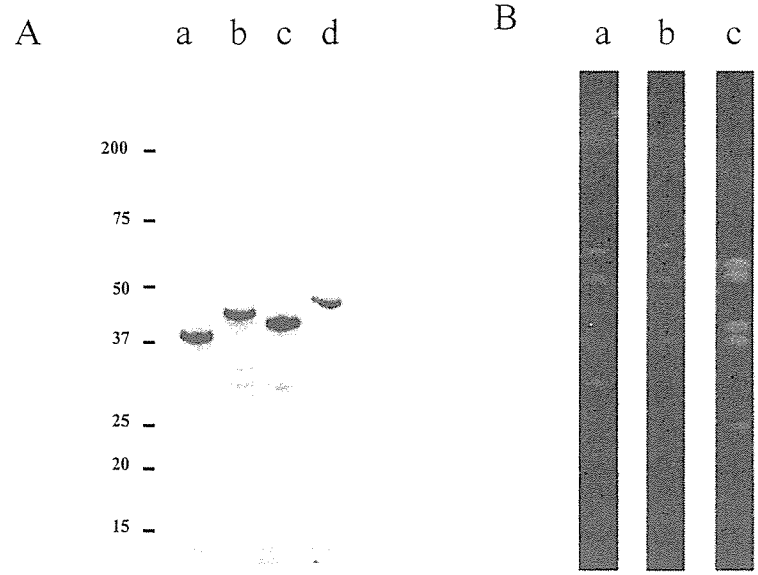

Fig. 1. Immunoblot assay of bacterial lysates of three NTHi strains and the purified P2 protein with anti-P2 sera (A) and pulse-field gel electrophoresis of *Sma*I-digested genomic DNA of three NTHi strains (B). Whole cell preparations of H05-19 strain (a), H04-06 strain (b) and H99-115 strain (c) and 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-

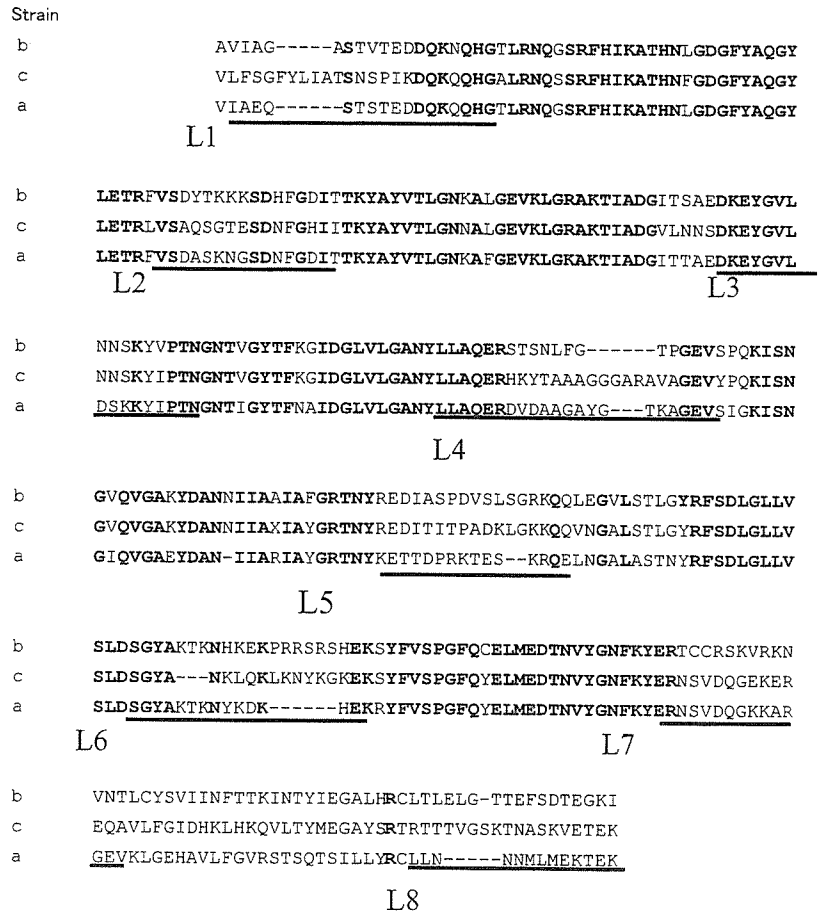

Fig. 2. Amino acid sequence of the *ompP2* regions of the three NTHi strains isolated from the same patient with COPD. Identical residues are shown in the bold. Strain a denotes H05-19 strain, strain b denotes H04-06 strain, and strain c denotes H99-115 strain, respectively. The loops 1 (L1) through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

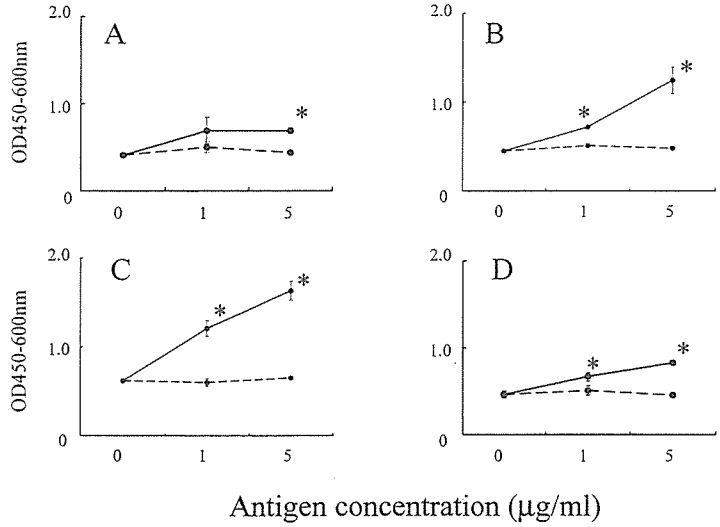

Fig. 3. In vitro cell proliferation using pulmonary lymph node cells from mice that received three serial IT challenge of killed-NTHi strain with a one-week interval in the presence of P6 (solid line) or ovalbumin (broken line). The IT immunizations include three serial immunizations of strain H05-19 (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.

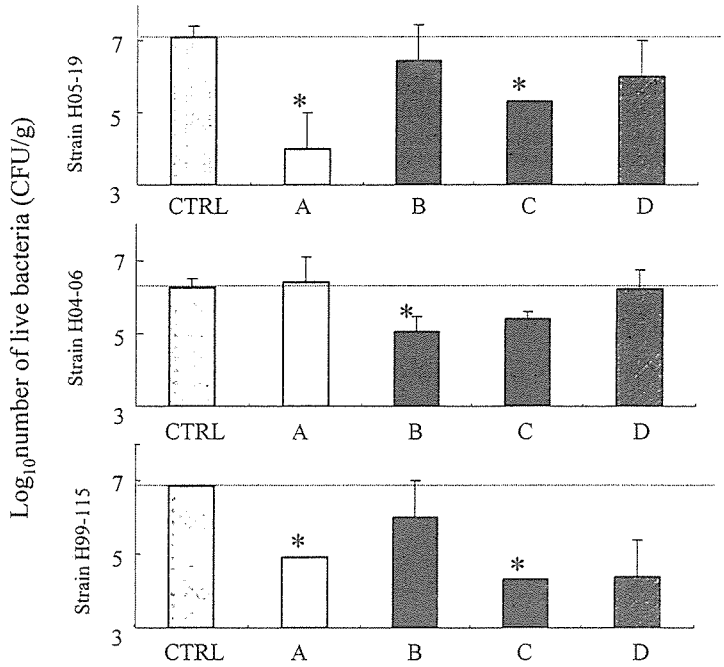

Fig. 4. The effect of three serial IT immunizations of killed-NTHi strain with a one-week intervals on the bacterial clearance of live NTHi strain H05-19, strain H04-06, and strain H99-115 are shown in the upper, middle and lower panels, respectively. The IT immunizations included three serial immunizations 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