mary viremia develops in the regional lymph nodes, and an infectious virus spreads causing a systemic illness through secondary viremia. Following systemic viremia, the mumps virus infects a number of target organs, including the central nervous system (CNS), salivary glands, glandular tissues and ovarian or testicular tissues. The most common neurological manifestation of CNS infection is aseptic meningitis. The incidence of aseptic meningitis to be hospitalized was reported to be 1–2%, and that of encephalitis was considered to be 0.02%. Although the case-fatality rate of mumps encephalitis is low, permanent sequelae, including paralysis, seizures, cranial nerve palsy, aqueductal stenosis and hydrocephalus, may occur. Acquired deafness caused by mumps is one of the leading causes of deafness in childhood, affecting approximately 5 out of 100,000 mumps patients [6,7].

In the United States, the nationwide acceptance of the measles, mumps and rubella (MMR) combined vaccine has reduced the number of mumps patients. In Japan, the MMR vaccine was introduced in 1989, but was discontinued in 1993 because of an unexpectedly high incidence of aseptic meningitis after MMR vaccination containing the Urabe strain [8,9]. Since 1993, a monovalent mumps vaccine has been used, but vaccine coverage is now estimated to be less than 30%. Annual mumps outbreaks occur with different magnitudes [10].

As for the clinical adverse reactions to the mumps vaccine, the parotid swelling observed at rate of 1–3% and aseptic meningitis occurred with the incidence of one case in 3000 recipients 2–3 weeks after vaccination [11]. It is of clinical significance to evaluate the pathophysiological relationship between aseptic meningitis and vaccination. We reported that the Hoshino vaccine strain was distinguished from wild circulating strains using the reverse transcription-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) in the HN gene using *ScaI* and *AflII* [12]. Mumps virus genome was efficiently amplified by RT-nested PCR, but this is time consuming and requires cumbersome attention to prevent carry-over contamination. Recently, we reported a rapid diagnostic method for the detection of mumps virus genome by reverse transcription loop-mediated isothermal amplification (RT-LAMP) [13]. The RT-LAMP procedure has a wide range of applications for clinical diagnostic laboratory examinations. We developed a new method for the differentiation of Hoshino vaccine strain from wild circulating strains using RT-LAMP followed by digestion with *ScaI* restriction enzyme.

2. Materials and methods

2.1. Mumps virus and clinical samples

Himeji 89/JPN.2000 (genotype B), Tokyo M-21/JPN. 2000 (genotype G), and the Hoshino vaccine seed strain were used [3]. We obtained 19 cerebro spinal fluid (CSF) samples from the patients with aseptic meningitis and 17 salivary swab samples from 16 patients with acute parotitis and 1 with orchitis after vaccination of monovalent Hoshino mumps vaccine (Kitasato Institutes, Research Center of Biologicals, Tokyo). Informed consent was obtained from a guardian of the patient. Salivary swabs were stocked in minimum essential medium (MEM) supplemented with appropriate antibiotics and 5% fetal calf serum.

2.2. RNA extraction

Total RNA was extracted from 200 μl of CSF and salivary swabs using a magnetic bead RNA purification kit (MagExtractor-viral RNA-, TOYOBO Co. Ltd., Osaka) and the RNA pellet was suspended in 25 μl of distilled water.

2.3. Mumps virus RT-LAMP

The LAMP procedure is characterized by DNA synthesis with auto-cycling strand displacements using Bst DNA polymerase (New England Biolabs, U.S.), and by a specially designed set of primers, determined by the software program for LAMP primer design (Eiken Chemical Co. Ltd., Japan), as reported by Notomi et al. [14]. The primer locations and sequence alignments are shown in Fig. 1. We synthesized six primers from genome position 7709 to 7943, as shown in Fig. 1; two outer primers (F3 and B3), two inner primers (forward inner primer, FIP and backward inner primer, BIP) and two loop primers (Loops F and B). FIP contains a complementary alignment to F1 linked with the F2 sequence, and BIP contains a complementary alignment to the B1 sequence linked with a B2 sequence. These four primers amplified the target DNA, and we synthesized two additional loop primers; F loop located between F1 and F2, and B loop located between B1 and B2. The addition of two loop primers enhanced the specificity and reactivity [14,15]. For the reaction of LAMP, a LAMP mixture was made in 25 μl of reaction mixture, containing 40 pmol of FIP and BIP, 5 pmol of F3 and B3, 20 pmol of Loop F and Loop B, 1.4 mM of each dNTP, 0.8 M betaine, 20 mM Tris–HCl, 10 mM KCl, 10 mM $(NH4)_2SO_4$, 0.1% Tween 20, 0.5 U AMV reverse transcriptase, 8 U Bst DNA polymerase and 5 μl of sample RNA. The reaction mixture was subjected to the real-time turbidimeter LA200 (TERAMECS, Japan) [13–15].

Genome RNA is first converted to cDNA with the FIP primer and the F3 primer extends the cDNA synthesis with displacement of RNA-cDNA double strand. The BIP primer anneals to the 3′ end of cDNA and extends the cDNA synthesis. The dumb-bell loop structures are synthesized, which is the key structure of further multi-branched loop products. As the LAMP reaction progressed, the reaction by-products (pyrophosphate ions) form white precipitate of magnesium pyrophosphate. Light (650 nm) emitted by light-emitting diodes passes through PCR tubes containing LAMP reaction solution and illuminates the photodiode on the opposite side. The turbidity is calculated based upon the ratio between the intensity of light received by the photodiode and the emitted

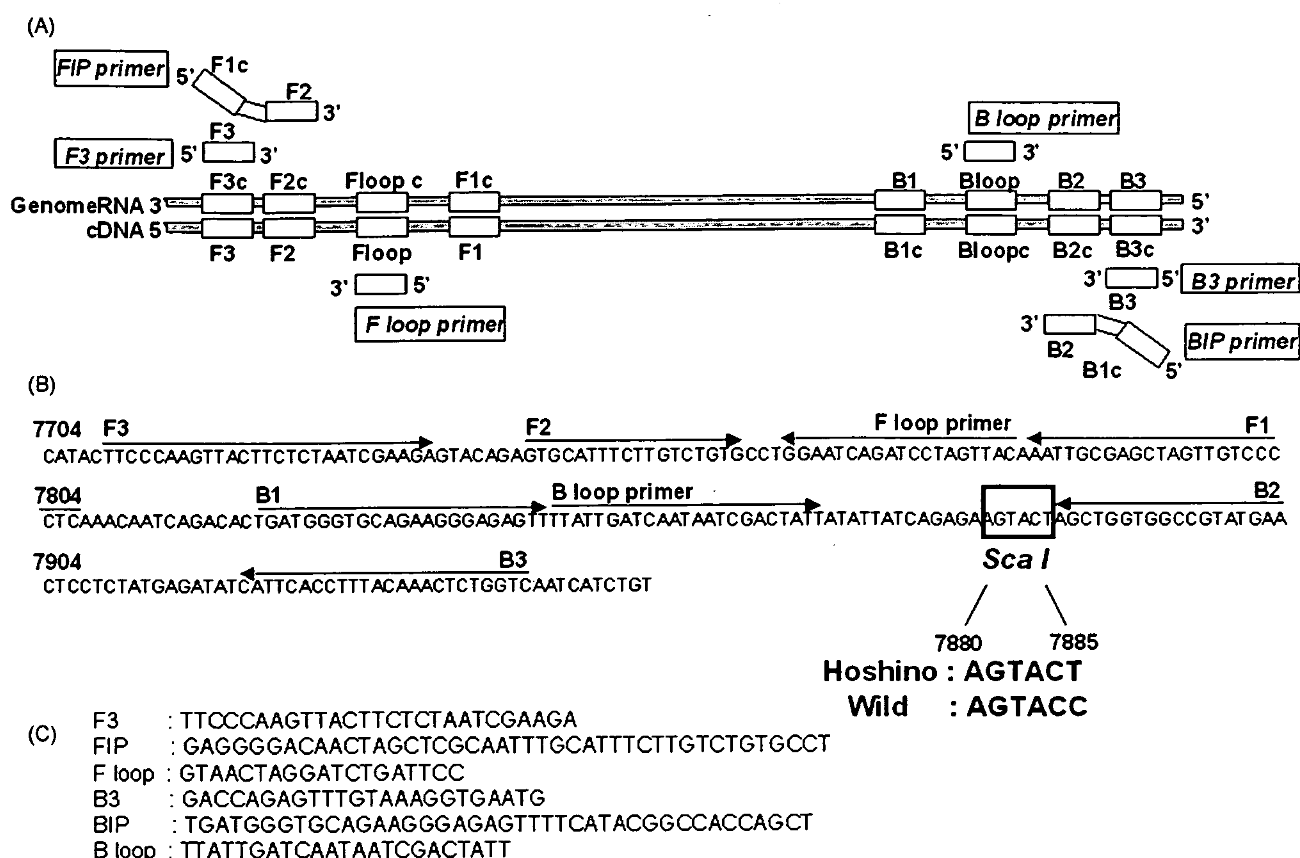

Fig. 1. Primer design of the RT-LAMP for the differentiation of the Hoshino vaccine strain from wild strains. Sequence alignment of the partial HN gene is shown from genome position 7704 to 7954. Six primers were synthesized (F3, B3, FIP, BIP, F Loop and B Loop). Arrow shows the direction of primer extension. Underlined sequence at genome position 7880–7885 is the *Sca*I site.

light intensity. Thus, the measurement of the turbidity is closely related to the DNA amplification [15].

2.4. Differentiation of the Hoshino vaccine strain from wild strains

In the HN region, the vaccine strain has a specific restriction enzyme site of *Sca*I at the gene position 7885, as is shown in Fig. 1 [12]. LAMP products were electrophoresed through 1.5% agarose gel after digestion with *Sca*I and the gel was stained with ethidium bromide.

3. Results

3.1. RT-LAMP sensitivity

To examine the sensitivity of RT-LAMP in this region, the Hoshino vaccine strain, containing 8×10^6 pfu/ml, was used. Serial dilutions of RNA by 1:10 were subjected to RT-LAMP reaction with LA200. The threshold of LAMP positivity for the spectrophotometric measurement was defined as turbidity over 0.1 [14,15], and we analyzed the correlation between the time (in seconds) to reach the threshold and infectivity of the sample. The results are shown in Fig. 2. Mumps virus genome was amplified up to 10^{-7} dilution of RNA by RT-LAMP and, thus, the detection limit was calculated as 0.8 pfu/ml. A linear correlation was obtained between the infectious titers and the time (seconds) to reach the threshold: y (infectivity 10^n pfu/ml) = −0.0043 × (seconds to reach the threshold) + 12.1.

3.2. RT-LAMP and differentiation by digestion with ScaI

A nucleotide difference is shown between the Hoshino vaccine strain and wild mumps strains in the HN region in Fig. 1. Nucleotide T at 7885 was characteristic of the Hoshino vaccine strain, while two other Japanese vaccine strains, the Miyahara and Torii, and all wild strains circulating in Japan had C at genome position 7885. Two wild genotypes B and G and the Hoshino vaccine strains were examined and the results of RT-LAMP and *Sca*I digestion are shown in Fig. 3, together with some clinical samples. RT-LAMP products showed a typical ladder pattern without digestion, but it disappeared after those of the Hoshino vaccine strain were digested with *Sca*I; whereas, those of the wild types were not digested. We examined 19 CSF samples obtained from the patients with aseptic meningitis and 17

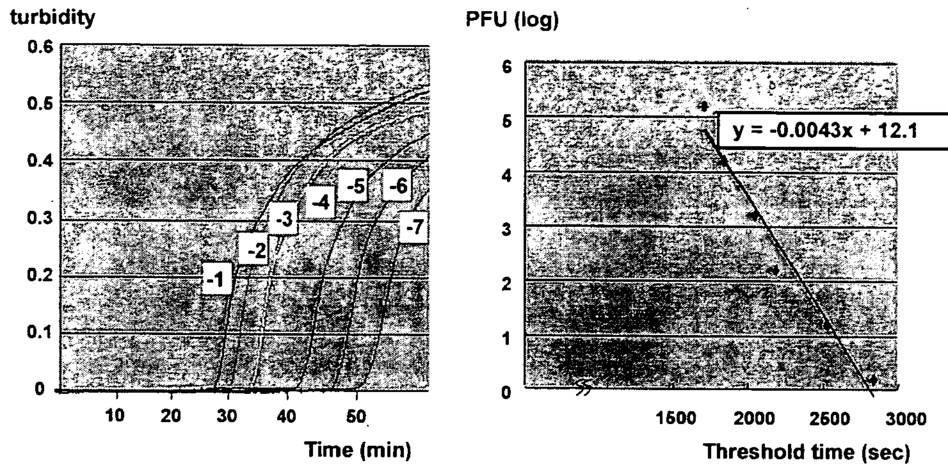

Fig. 2. Sensitivity of RT-LAMP and quantitative RT-LAMP reaction. Approximately, 8×10^6 pfu/ml of virus culture fluid (the Hoshino vaccine strain) was used. Serial 10-fold dilutions were subjected to RT-LAMP and the results of real-time RT-LAMP are shown in the left panel. RT-LAMP reaction times were monitored to reach the threshold of 0.1 and the correlation between the logarithm of virus infectivity and the RT-LAMP reaction time is shown in the right panel.

Table 1
Results of RT-LAMP and differentiation of the Hoshino vaccine strain

	n	RT-LAMP (+)		RT-LAMP (−)
		ScaI + (Hoshino)	*ScaI* − (Wild)	
Aseptic meningitis (CSF)	19	16	2	1
Acute parotitis (Salivary swab)	16	11	5	0
Orchitis (Salivary swab)	1	0	1	0

salivary swab samples obtained from 16 patients with acute parotitis and one with orchitis. The results of three samples are shown in Fig. 3. Typical ladder pattern of the LAMP products of sample Nos. 1 and 2 disappeared after digestion with *ScaI* and they were considered as the Hoshino vaccine strain and, otherwise, sample No. 3 was suspected as wild type. We summarized the results of differentiation of clinical samples in Table 1. Among 18 CSF samples positive for RT-LAMP, 16 were identified as the Hoshino vaccine strain. Among 16 salivary swab samples positive for LAMP, 11 were identified as the Hoshino vaccine strain. One case with orchitis and the five remaining samples were diagnosed as wild type infection.

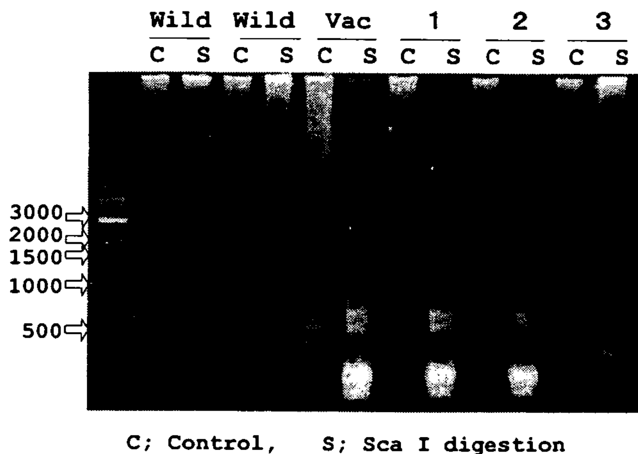

Fig. 3. The results of electrophoresis of RT-LAMP products. Two wild genotypes B and G, and the Hoshino strain were used. RT-LAMP products were electrophoresed without digestion for the control (C) and after digestion with *ScaI* (S) in the paired lanes. Three clinical samples are shown.

4. Discussion

Mumps virus has neurotropic characteristics by nature, and the most common neurological manifestation of CNS infection is aseptic meningitis [1]. In mumps infection, pleocytosis in the CSF is observed in approximately 60%, even without any neurological manifestations, and the incidence of symptomatic aseptic meningitis occurs in 1–2% [1,7]. Nagai et al. [11] compared the incidence of aseptic meningitis in natural infection and vaccine recipients. The incidence of aseptic meningitis was 13/1051 (1.24%) in patients with symptomatic natural mumps infection confirmed by virus isolation and/or detection of the mumps virus genome, and 10/21,465 (0.05%) of vaccine recipients of the Miyahara, Torii and Hoshino vaccine strains developed in Japan. Additionally, acute parotitis was reported in 551/21,465 (3.0%). The MMR vaccine is approved in most countries but, in Japan, mumps monovalent vaccine has been used as a voluntary vaccine. Consequently, we have experienced annual outbreaks of mumps estimated at more than one million cases every year because of the low acceptance of the monovalent mumps

vaccine, less than 30% [10]. Some parents have wanted to administer a mumps vaccine when a regional outbreak was noted. It is important to investigate the clinical adverse reactions after immunization for the safety issues. We reported the methods using RT-PCR and RFLP to differentiate the Hoshino vaccine strain from wild strains in CSF samples of aseptic meningitis after mumps vaccination [12]. However, it takes several hours to obtain the results.

LAMP was developed to amplify the target DNA without any temperature shifts of denaturing, annealing and extension. LAMP has been used to detect many kinds of virus genomes, mainly for DNA virus or bacterial genome [16–20]. We reported RT-LAMP to detect mumps virus genome [13] and compared the sensitivity of RT-LAMP, RT-nested PCR and virus isolation. The sensitivity of RT-LAMP for the mumps virus was equal to or slightly higher than that of RT-nested-PCR, and RT-LAMP is a simple and time-saving procedure, allowing the results to be obtained within 1 h after extraction of the viral genome. It amplified the HN gene from genome position 7949 to 8175 and LAMP products were purified and sequenced [13]. From the results of our molecular epidemiology, nucleotide T at position 8110 was characteristics of the Hoshino vaccine strain and the wild strain has C at position 8110 [3,5,12]. For the genome discrimination, LAMP products should be sequenced. Therefore, the RT-LAMP target region in the previous report [13] was changed to amplify the region from 7709 to 7943, a specific *ScaI* sequence of the Hoshino strain. After digestion with *ScaI*, we could differentiate the Hoshino vaccine strain from wild strains. Recently, circulating mumps virus genotypes were divided into 12 genotypes from A to L based upon the sequence diversity of the SH gene [21]. In Japan, we previously reported that current circulating mumps virus strains were divided into four different genotypes, B, G, K and L [3]. It would be difficult to design RT-LAMP primers to amplify the different genotypes efficiently in the SH region. Then, the RT-LAMP primer was targeted into the HN region. Although the sequence data of the HN gene was limited, none of the current circulating wild types in Japan had a *ScaI* restriction site specific for the Hoshino vaccine strain as yet [2,3,22] but genotype A (Enders, Jeryl Lynn, Rubini and SBL-1) and Edinburg 4/3 had an AGTACT sequence. We should continue molecular epidemiological surveillance to watch the importation of foreign strains or mutation of Japanese circulating strains. Mumps Hoshino vaccine strain was not detected in two patients with aseptic meningitis, and in five patients with acute parotitis. We supposed that they had been infected with the circulating wild type immediately before vaccination, and did not relate to vaccine-adverse events.

We obtained a linear correlation between the genome quantity and the reaction time when the spectrophotometric values reached to a threshold of 0.1 and calculated the virus load in a sample by monitoring the spectrophotometric turbidity value. We detected $10^{1.89}$ pfu/ml in samples of aseptic meningitis caused by Hoshino vaccine strain. In samples of aseptic meningitis caused by wild strains, we detected $10^{3.86}$ pfu/ml (data not shown). However, the number of patients was limited, and we should further investigate a larger number of cases to confirm the difference in virus load in natural infection and vaccine-associated events.

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References

[1] Carbone KM, Wolinsky JS. Mumps virus. In: Knipe DM, Howley PM, editors. Fields Virology. 4th ed. Philadelphia: Lippincott, Williams & Wilkins; 2001. p. 1381–400.

[2] Tanabayashi K, Takeuchi K, Okazaki K, Hishiyama M, Yamada A. Expression of mumps virus glycoproteins in mammalian cells from cloned cDNAs: both F and HN proteins are required for cell fusion. Virology 1992;187:801–4.

[3] Inou Y, Nakayama T, Yoshida N, Uejima H, Yuri K, Kamada M, et al. Molecular epidemiology of mumps virus in Japan and proposal of two new genotypes. J Med Virol 2004;73:97–104.

[4] Pugh RN, Akinosi B, Pooransingh S, Kumar J, Grant S, Livesley E, et al. An outbreak of mumps in the metropolitan area of Walsall, UK. Int J Infect Dis 2002;6(4):283–7.

[5] Takahashi M, Nakayama T, Kashiwagi Y, Takami T, Sonoda S, Yamanaka T, et al. Single genotype of measles virus is dominant whereas several genotypes of mumps virus are co-circulating. J Med Virol 1996;62:278–85.

[6] WHO. Mumps virus vaccines. Wkly Epidemiol Rec 2001;76(45): 345–56.

[7] Plotkin SA. Mumps vaccine. In: Plotkin, Orenstein, editors. Vaccines. 4th ed. Philadelphia: Sauders; 2004. p. 441–69.

[8] Kimura M, Kuno-Sakai H, Yamazaki S. Adverse events associated with MMR vaccines in Japan. Acta Paediatr Jpn 1996;38:205–11.

[9] Ueda K, Miyazaki C, Hidaka Y, Okada K, Kusuhara K, Kadoya R. Aseptic meningitis caused by measles-mumps-rubella vaccine in Japan. Lancet 1995;346:701–2.

[10] Infectious Disease Surveillance Center, National Institute of Infectious Diseases. Mumps, Japan, 1993–2002. IASR 2003;24:1–2.

[11] Nagai T, Okafuji T, Miyazaki C, Ito Y, Kamada M, Kumagai T, et al. A comparative study of the incidence of aseptic meningitis in symptomatic natural mumps patients and monovalent mumps vaccine recipients in Japan. Vaccine, in press.

[12] Kashiwagi Y, Kawashima H, Takekuma K, Hoshika A, Mori T, Nakayama T. Detection of mumps virus genome directly from clinical samples and a simple method for genetic differentiation of the Hoshino vaccine strain from wild strains of mumps virus. J Med Virol 1997;52:195–9.

[13] Okafuji T, Yoshida N, Fujino M, Motegi Y, Ihara T, Ota Y, et al. Rapid diagnostic method for detection of mumps virus genome by loop-mediated isothermal amplification. J Clin Microbiol 2005;43:1625–31.

[14] Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 2000;28:e63.

[15] Mori Y, Kitao M, Tomita N, Notomi T. Real-time turbidimetry of LAMP reaction for quantifying template DNA. J Biochem Biophys Methods 2004;59:145–57.

[16] Ihira M, Yoshikawa T, Enomoto Y, Akimoto S, Ohashi M, Suga S, et al. Rapid diagnosis of human herpes virus 6 infection by a novel DNA amplification method, loop-mediated isothermal amplification. J Clin Microbiol 2004;42:140–5.

[17] Iwamoto T, Sonobe T, Hayashi K. Loop-mediated isothermal amplification for direct detection of Mycobacterium tuberculosis complex, M. avium, and M. intracellular in sputum samples. J Clin Microbiol 2003;41:2616–22.
[18] Kuboki N, Inoue N, Sakurai T, Di Cello F, Grab DJ, Suzuki H, et al. Loop-mediated isothermal amplification for detection of African trypanosomes. J Clin Microbiol 2003;41:5517–24.
[19] Maruyama F, Kenzaka T, Yamaguchi N, Tani K, Nasu M. Detection of bacteria carrying the stx2 gene by in situ loop-mediated isothermal amplification. Appl Environ Microbiol 2003;69:5023–8.
[20] Parida M, Posadas G, Inoue S, Hasebe F, Morita K. Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. J Clin Microbiol 2004;42:257–63.
[21] Jin L, Rima B, Brown D, Orvell C, Tecle T, Afzal M, et al. Proposal for genetic characterization of wild-type mumps strains: preliminary standardisation of the nomenclature. Arch Virol 2005;150(9):1903–9.
[22] Kashiwagi Y, Takami T, Mori T, Nakayama T. Sequence analysis of F, SH, and HN genes among mumps virus strains in Japan. Arch Virol 1999;144:593–9.

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Development of a new neutralization test for measles virus

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Abstract

Sero-epidemiological studies are required to identify populations susceptible to measles. The hemagglutination inhibition (HI) test is no longer sensitive enough to confirm immunity to measles, and at present the particle agglutination (PA) test and enzyme-linked immunosorbent assay (EIA) are employed. The most reliable method is the neutralization test (NT), but it is time-consuming and requires experience. To simplify the NT, a recombinant measles AIK-C virus expressing green fluorescence protein (GFP-MVAIK) was constructed and used as a challenge virus. Plaques and cytopathic effects were visualized under ultraviolet light and detected easily, and measuring the intensity of the fluorescence enabled a reduction in the time-consuming steps. Neutralizing antibody titers of a complete inhibition neutralization test were equivalent to those of a 90% plaque reduction neutralization test. Comparison of four methods, HI, PA, EIA and the complete inhibition neutralization test, showed that only the results of EIA correlated well with those of the complete inhibition neutralization test, but sera with borderline levels by EIA were sometimes negative by the complete inhibition neutralization assay.
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Keywords: Recombinant measles virus; Neutralization test (NT); Hemagglutination inhibition (HI); Particle agglutination (PA); Enzyme-linked immunosorbent assay (EIA)

1. Introduction

Measles virus is a *morbillivirus* of the family *Paramyxoviridae*. Acute infection with measles virus starts in the upper respiratory tract and the virus spreads by secondary viremia to other organs and tissues, causing a variety of clinical symptoms. The virus infects immuno-competent cells, lymphocytes, and macrophages, and causes a transient immuno-suppressive state. Although measles infection is debilitating, the majority of patients recover completely without any serious complications (Griffin and Bellini, 2001; Strebel et al., 2004). Attenuated live measles vaccines have been developed and the number of cases of measles has decreased with the widespread acceptance of vaccinations in developed countries. Nevertheless, 0.45 million people are estimated to have died of measles or measles-related complications in 2004 (WHO, 2006). The Expanded Program on Immunization (EPI) has targeted as its highest priority the elimination of measles, but cannot attain the goal of controlling the disease because of the unexpectedly high infectivity of the virus, relatively poor immunogenicity of measles vaccines in infants under 9 months of age, and problems with the distribution of vaccines. The EPI recommends measles vaccination at 9 months of age in developing countries (Whittle et al., 1988).

Sero-epidemiological studies are needed to determine how many individuals are susceptible for the elimination of measles. The hemagglutination inhibition (HI) test is not sufficiently sensitive, and the particle agglutination (PA) test or enzyme-linked immunosorbent assay (EIA) is now employed (Neumann et al., 1985; Miyamura et al., 1997). The PA assay is a simple and sensitive method that does not require specialized equipment. Gelatin particles are coated with purified viral antigens and agglutinated by measles-specific antibodies. Titers of ≥1:16 are considered positive, but neutralizing antibodies are not detected among PA-positive sera with low titers (Miyamura et al., 1997). The most reliable test for confirming susceptibility is the neutralization

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test (NT), which is considered the "gold" standard, but is complicated and requires skill and experience (Neumann et al., 1985; Lee et al., 1999). The most time-consuming and critical part of the test is the final observation of cytopathic effects (CPE) under a microscope or the counting of plaques.

Current global efforts regarding vaccination are aimed at accelerating measles control. A sensitive and simple way to measure accurately levels of protective antibodies is needed urgently. A recombinant measles virus expressing green fluorescence protein (GFP-MVAIK) was developed in this study. The virus grew well in both Vero and B95a cells. Green fluorescence protein (GFP) was expressed simultaneously with measles antigens, and the growth of the virus was detected by fluorescent intensity. Using this virus as a challenge virus in the neutralization test could automate the final step in the test systems.

2. Materials and methods

2.1. Construction of recombinant cDNA of measles virus

A recombinant measles virus was rescued from full-length cDNA by reverse genetics (Schneider et al., 1997; Takeda et al., 2000; Nakayama et al., 2001; Kumada et al., 2004). An *Asc* I site was introduced between the phosphoprotein (P) and membrane (M) protein region, adding the GGCGCG sequence at position 3432 of the AIK-C genome (Mori et al., 1993). The cloned GFP genome was inserted at the Asc I site and the recombinant plasmid was co-transfected with N, P, and L expression helper plasmids under the control of T7 RNA polymerase into B95a cells. Infectious virus (GFP-MVAIK) was recovered after two passages in B95a cells. In this study, GFP-MVAIK was used instead of the Edmonston strain, a parental strain of the AIK-C vaccine used in the conventional neutralization test.

2.2. Serum samples

Sera were obtained every year from students in the first and fourth grades of a private primary school in Tokyo for a health assessment. One hundred and thirty-two pupils enter the school each year. The purpose of the study was explained to the parents and written informed consent was obtained from all participants. Each subject's history of measles immunization and past history of natural infection were confirmed through immunization records. The sera were stored at −20 °C.

2.3. Serological tests

The HI antibody titers of test sera against measles virus were examined. Serum samples were treated with 25% kaolin in 1/15 M phosphatebuffered saline to remove nonspecific factors. The supernatant was mixed with 10% green monkey red blood cells to remove nonspecific factors for agglutination and at this final stage serum samples were diluted 1:8. Serum samples were diluted serially two-fold and four units of hemagglutinin antigen was added. After 0.5% green monkey red blood cells were added, the reciprocal of the dilution which completely inhibited hemagglutination was taken as the HI antibody titer. Sera with a HI titer of ≥1:8 were considered HI-positive.

In the PA test, gelatin particles were coated with purified measles virus antigen (Serodia®-Measles, Fuji Rebio, Tokyo, Japan). Sera were diluted serially two-fold starting at a dilution of 1:8, and each diluted serum was mixed with an equal volume of gelatin particles to detect agglutination, according to the recommendation of the manufacturer. The PA antibody titers were expressed as the reciprocal of the serum dilution which induced agglutination. Sera with a PA titer of ≥1:16 were considered positive.

Purified viral particle antigens were coated for EIA on a 96-well plate and 100 μl of serum diluted 1:200 was added to each well (Measles IgG (II)-EIA "Seiken", Denka Seiken, Tokyo, Japan). After incubation with the serum sample for 60 min, anti-human IgG antibody conjugated with peroxidase was added and stained, as recommended in the instruction manual. The EIA antibody titer was expressed as the ratio of absorbance of the serum sample to that of the weak positive control reference serum supplied by the manufacturer. Sera with an EIA titer of ≥1 were considered EIA-positive.

Sera were treated for the neutralization test at 56 °C for 30 min to inactivate the complement. Fifty microliters of diluted serum was mixed with an equal volume of challenge virus containing approximately 50 PFU for the plaque reduction NT. After neutralization at 37 °C for 90 min in a 5% CO_2 incubator, the mixture was placed on a monolayer of Vero cells at 37 °C for 60 min in a 24-well plate, and overlaid with MEM containing 0.5% agarose. The plate was kept at 37 °C for 7 days in 5% CO_2. Neutralization titers were determined by calculating the serum dilution that reduced plaque numbers by 50 or 90%. Sera with a neutralization titer of ≥1:4 were considered NT-positive.

In the complete inhibition NT, sera were diluted serially two-fold, starting at an initial dilution of 1:4. Twenty-five microliter of each dilution was then mixed with an equal volume of the challenge virus approximately 100 TCID 50 and the mixture was kept at 37 °C for 90 min in 5% CO_2. The mixture was placed subsequently on a monolayer of B95a cells in a 96-well plate for complete inhibition of the cytopathic effects. The plates were kept at 37 °C for 7 days in 5% CO_2 and the neutralization titers were taken as the reciprocal of the dilution that induced complete inhibition of the appearance of CPE in B95a cells in a 96-well plate.

In order to calculate the titers automatically, the plates were processed to detect fluorescence intensity (Fluoro-Units: FU) at an emission wavelength of 528 nm and excitation wavelength of 485 nm using a fluorescence reader, FLx800 (BIO-TEK Instruments Inc., Vermont, USA).

3. Results

3.1. Expression of GFP and measles antigens

The recombinant AIK-C virus expressing GFP (GFP-MVAIK) grew well in both B95a and Vero cells. GFP was visualized in the cells infected with GFP-MVAIK with a fluorescence microscope. Measles antigens were stained by polyclonal

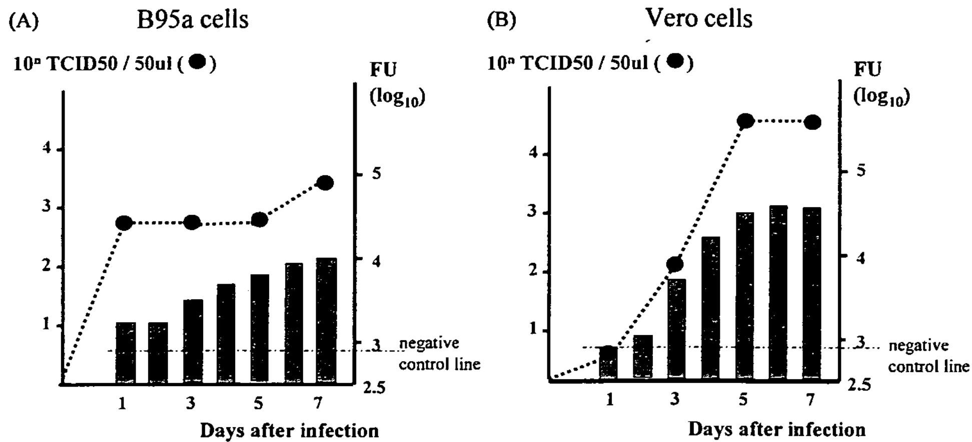
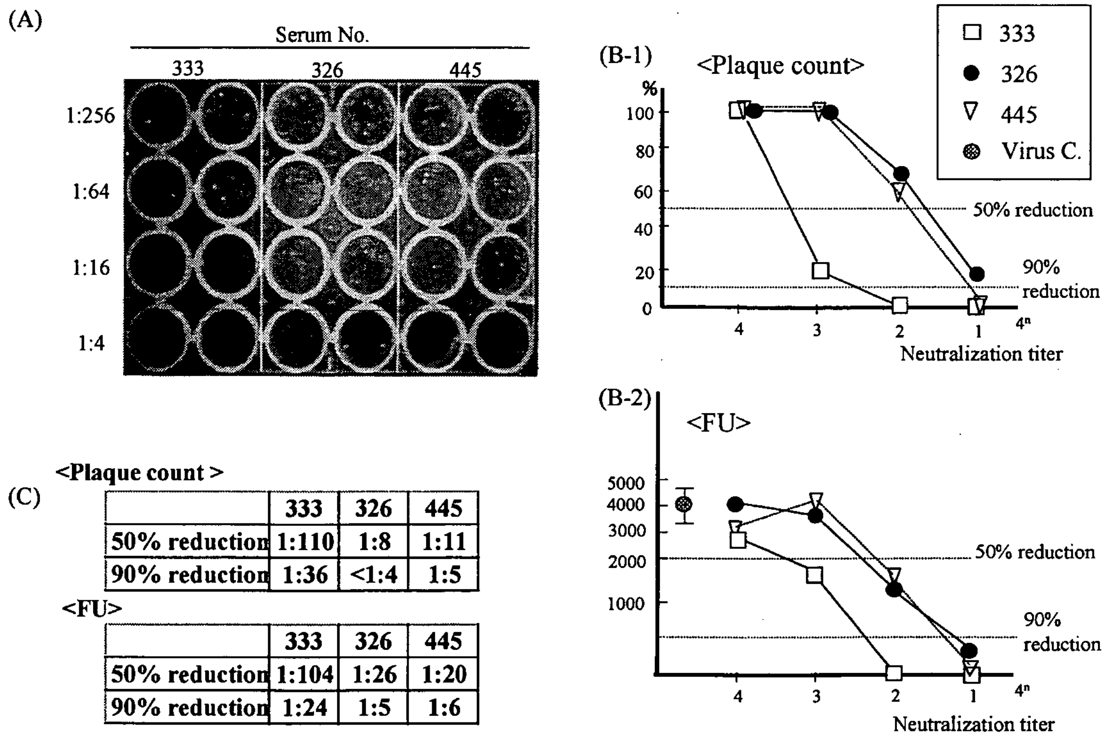


Fig. 1. Viral growth and the expression of GFP in B95a cells and Vero cells infected with GFP-MVAIK. B95a (A) or Vero cells (B) were infected with GFP-MVAIK at a moi of 0.01. Culture fluid was obtained on days 1, 3, 5, and 7 of infection, and the infectivity was examined in B95a cells. The infectivity is shown as 10^n TCID50/50 μl (closed circles). The dotted column shows the intensity in FU. The value in the control well was approximately 800 FU ($10^{2.9}$ FU) as a negative control.

antibodies against measles virus at the same locations GFP was observed under the microscope (data not shown). The correlation between the intensity of GFP and growth of the virus is shown in Fig. 1. GFP-MVAIK grew better in B95a cells than in Vero cells up to day 3 of the infection, but on day 5 or 7, growth was greater in the Vero cells. The kinetics of the GFP expression were similar to the viral growth both in B95a and in Vero cells.

3.2. Comparison of the neutralization titers assayed by conventional and new methods

The results of the conventional plaque NT are shown in Fig. 2. GFP-MVAIK was used as a challenge virus and neutralization titers of several sera were examined. The results for three samples (Nos. 326, 333, and 445) are shown. The assay plate was examined under ultraviolet light, and plaques were visualized as white spots. After the plaques were enumerated in virus control wells and at each dilution, a reduction curve was drawn, from which the theoretical titer of a 50% reduction in the plaque count was calculated (Fig. 2, B-1). The titers of sera Nos. 333, 326, and 445 were calculated as 1:110, 1:8, and 1:11, respectively. Based on a 90% reduction in the plaque count, the results were 1:36, negative, and 1:5, respectively (Fig. 2C). The intensity of the fluorescence in each well was also examined. The mean value for cell control wells was subtracted from the value for virus control wells, and in this experiment, the difference in intensity



<Plaque count>

	333	326	445
50% reduction	1:110	1:8	1:11
90% reduction	1:36	<1:4	1:5

<FU>

	333	326	445
50% reduction	1:104	1:26	1:20
90% reduction	1:24	1:5	1:6

Fig. 2. The plaque reduction NT using Vero cells and GFP-MVAIK. Three sera were used. (A) Plaques expressing GFP were visualized using ultraviolet light. (B-1) Reduction in terms of the number of plaques. (B-2) Reduction in terms of Fluoro-Units (FU). (C) Neutralizing antibody titers based on 50 and 90% reductions.

was 3897 ± 544 Fluoro-Units. From this difference, 50% and 90% reduction levels were calculated to be approximately 1950 and 400 FU, respectively. A reduction line was drawn based on the results for serial dilutions and the neutralization titer showing 1950 FU (50% reduction) and 400 FU (90% reduction) was taken as the value at the point where the lines were crossed (Fig. 2B-2). Neutralization titers were 1:104, 1:26, and 1:20 based on a 50% reduction in FU, and 1:24, 1:5, and 1:6 for a 90% reduction in FU (Fig. 2C).

In another NT for measles virus, neutralization titers were calculated as 100% inhibition of CPE in B95a cells. The results are shown in Fig. 3. CPE was visualized as a patchy region. Neutralization titers were expressed as the reciprocal of the serum dilutions that completely prevented the appearance of CPE. The mean values for the cell control wells were 658, 674, and 680 FU. CPE was not observed at a 1:8 dilution of Serum No. 445 in duplicate wells, and a 1:16 dilution of serum did not neutralize GFP-MVAIK, the mean value being 1528 FU. Thus, the neutralization titers for Nos. 445, 333, and 326 were 1:8, 1:32, and 1:4, respectively. The antibody titers determined by the complete inhibition of CPE were similar to those obtained based on a 90% reduction in plaque numbers or in FU of GFP expression. The NT for complete inhibition of CPE is simple and the neutralization titers are calculated automatically by a fluorescence microplate reader.

In most laboratories, the wild-type Edmonston strain is used for NT. The AIK-C vaccine strain was attenuated from the wild-type Edmonston strain (genotype A). The difference in neutralization titers using the Edmonston and GFP-MVAIK strains as the challenge virus was compared in 43 sera. There was no significant difference in titers in 38 sera. Five sera had four-fold lower titers by the NT using Edmonston than that using GFP-MVAIK. The correlation coefficient of neutralization titers against these two viruses was 0.936 ($p < 0.001$).

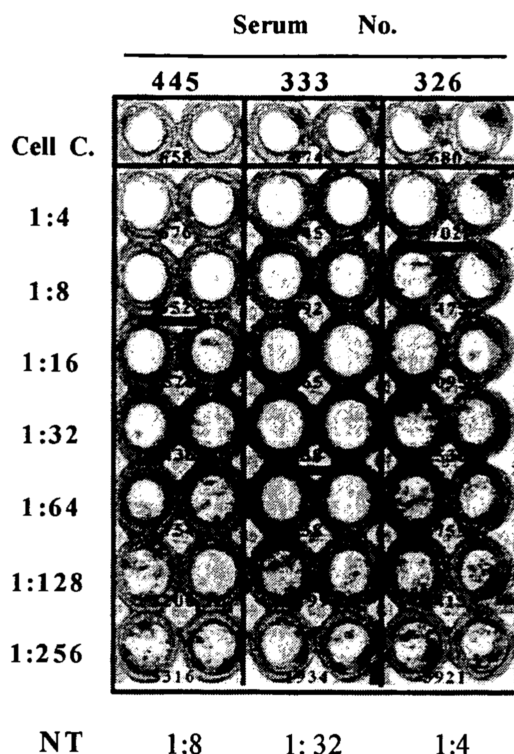


Fig. 3. The complete inhibition NT using B95a cells and GFP-MVAIK. CPE were visualized through GFP expression. Numbers between two wells demonstrate the mean value in Fluoro-Units (FU) for duplicated wells. Neutralizing antibody titers for Nos. 445, 333, and 326 were determined as 1:8, 1:32, and 1:4 based on the complete inhibition of CPE.

3.3. Comparison of HI, PA, EIA, and NT tests

Two hundred and thirty-seven serum samples were obtained from pupils in the first and fourth grades in 2002. About 98% of the students had been immunized against measles. With these

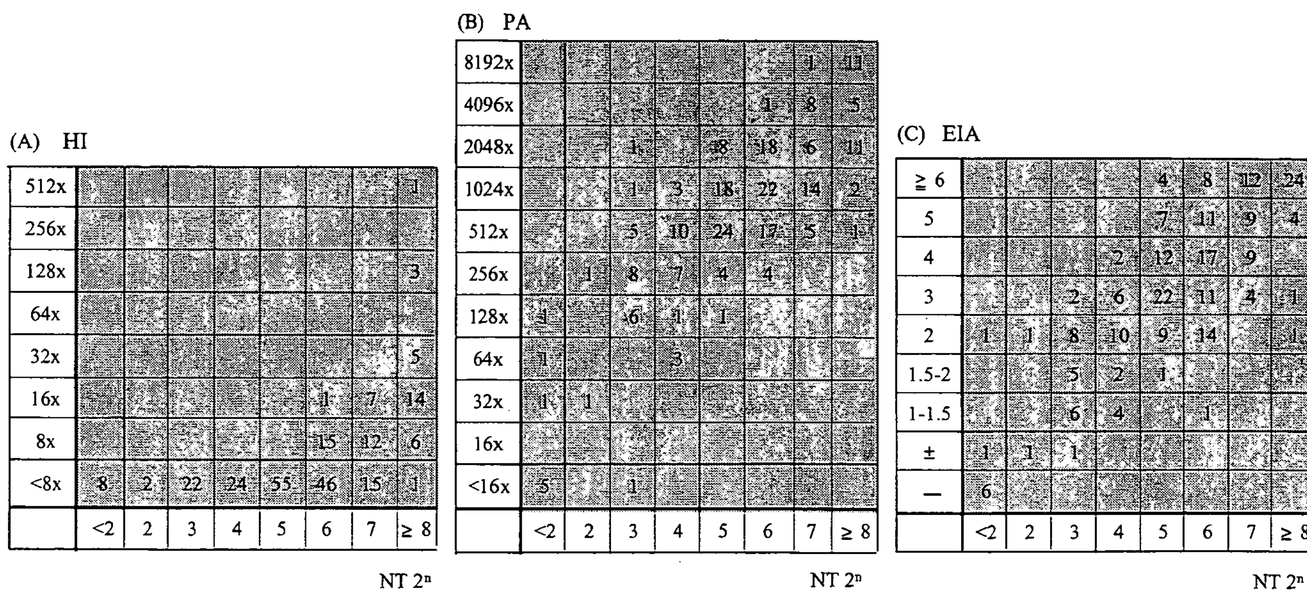


Fig. 4. Comparison of antibody titers assayed by different methods. (A) Comparison of antibody titers between NT and HI. (B) Comparison of antibody titers between NT and PA. (C) Comparison of antibody titers between NT and EIA.

Table 1
Distribution of neutralizing antibody titers of sera with PA antibodies less than or equal to 1:128

PA titers	Neutralization titers (2^n)					
	<2	2	3	4	5	6
≤16 (n=20)	19		1			
32 (n=2)	1	1				
64 (n=15)	7	3	1	3		1
128 (n=32)	6	8	11	4	3	
Total	33	12	13	7	3	1

sera, serological tests were carried out using HI, PA, the EIA, and the NT. The results are shown in Fig. 4. The correlation between HI and the NT is shown in Fig. 4(A). Only 64 samples were HI-positive (27.0%), whereas 229 samples were NT-positive (96.6%). The correlation between PA and the NT is shown in Fig. 4(B). The PA test was started at a dilution of 1:16 and sera with a PA titer of ≥1:16 were considered positive. All 216 sera with PA titers equal to or higher than 1:256 were NT-positive but some sera with PA titers less than 1:256 were NT-negative. For a more thorough analysis, 48 sera with PA titers of less than 1:256 obtained from junior and senior high school students in 2002 were added. The results for the 69 sera are shown in Table 1. Six (18.8%) of 32 sera with a PA titer of 1:128 were NT-negative, and 7 (46.7%) of 15 sera with a PA titer of 1:64 were NT-negative. The correlation between the EIA and NT is shown in Fig. 4(C). A good correlation was observed between EIA and NT, and most EIA-positive sera were NT-positive. Three sera with borderline levels of EIA (±) showed a low neutralization titer (1:4 and 1:8) and one of the three was NT-negative.

4. Discussion

In Japan, the cumulative measles vaccine coverage was 80% by 90 months of age, but the actual coverage during the most susceptible period, around 1 year of age, was less than 60% (Nakayama et al., 2003). Regional pediatric associations have launched a campaign promoting measles vaccination. At present, vaccine coverage has increased to 90% by 90 months of age. The two-dose strategy was introduced in 2006.

Most measles patients are diagnosed clinically and WHO has recommended the detection of measles-specific IgM antibodies in acute phase serum samples for the diagnosis of measles (WHO, 2003). It is important to carry out serological studies that can measure levels of protective antibody accurately to determine how many people are susceptible to measles, especially for promoting vaccination. Most studies employed the HI test which was believed to be sensitive enough to detect immunity. In the present study, 165 (95.4%) of 173 HI-negative sera were NT-positive, thus the HI test is not suitable for epidemiological studies. The PA test is highly sensitive and useful for epidemiological studies. The cut-off titer was thought to be 1:16, but some sera with a PA titer of ≤1:64 were NT-negative and all sera with a PA titer of ≥1:128 were NT-positive (Miyamura et al., 1997). In this study, 6 (18.8%) of 32 sera at 1:128 were NT-negative. At ≥1:256 in PA, all sera were NT-positive. In contrast, EIA was related more closely to the NT. The EIA test has been used in sero-epidemiological studies (Ratnam et al., 1995; Lee et al., 1999; van den Hof et al., 2003). But sera with borderline levels in the EIA were thought to be negative or have low neutralization titers. PA or EIA antibodies do not always correlate with NT activity. The antibodies detected in the NT are considered mainly anti-H and -F antibodies, which are the most important factors for preventing a measles infection. In PA or EIA, purified viral particle antigens are used which contain some components other than H and F antigens. To determine which populations are susceptible to measles, the neutralizing antibody titer should be checked, especially in the sera with a PA titer of ≤1:128 or borderline levels in the EIA. Recently, de Swart et al. (2005) reported that measles virus neutralizing antibodies were directed mainly against the H protein. Bouche et al. (1998) reported that EIA titers against an H protein antigen prepared from crude membrane preparations of H protein-transfected BHK-21 cells correlated more closely with results of the NT and HI than those of whole-virus-based EIA.

Two different forms of the NT were used in this study, a conventional plaque reduction assay using Vero cells and a complete inhibition assay using B95a cells. The complete inhibition assay is a simpler procedure than the plaque reduction assay. Ward et al. (1999) reported that the 50% plaque reduction assay was the "gold standard" and correlated well with the complete inhibition assay when neutralization titers were moderate or high, but there were discrepancies in sera with low levels of neutralizing antibodies. NT-negative sera by the complete inhibition assay showed various titers in the 50% plaque reduction assay. In the present study, results obtained by the complete inhibition assay were equivalent to a 90% reduction in plaques by the conventional assay. The complete inhibition assay is more reliable when considering levels of protection from measles in vivo, especially in the controllable stage of infection. However, the current plaque reduction NT is not suitable for large-scale serological studies because it is cumbersome and requires experience. The new method described in this study overcomes these problems by measuring the intensity of fluorescence automatically and makes it possible to test with many samples.

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References

Bouche, F., Ammerlaan, W., Berthet, F., Houard, S., Schneider, F., Muller, C.P., 1998. Immunosorbent assay based on recombinant hemagglutinin protein produced in a high-efficiency mammalian expression system for surveillance of measles immunity. J. Clin. Microbiol. 36, 721–726.

de Swart, R.L., Yuksel, S., Osterhaus, A.D., 2005. Relative contributions of measles virus hemagglutinin- and fusion protein-specific serum antibodies to virus neutralization. J. Virol. 79, 11547–11551.

Griffin, D.E., Bellini, W.J., 2001. Measles virus. In: Knipe, D.M., Howley, P.M. (Eds.), Fields virology, fourth ed. Williams & Wilkins, Lippincott, Philadelphia, pp. 1267–1312.

Kumada, A., Komase, K., Nakayama, T., 2004. Recombinant measles AIK-C strain expressing current wild-type hemagglutinin protein. Vaccine 22, 309–316.

Lee, M.S., Cohen, B., Hand, J., Nokes, D.J., 1999. A simplified and standardized neutralization enzyme immunoassay for the quantification of measles neutralizing antibody. J. Virol. Methods 78, 209–217.

Miyamura, K., Sato, T.A., Sakae, K., Kato, N., Ogino, T., Yashima, T., Sasagawa, A., Chikahira, M., Itagaki, A., Katsuki, K., Matsunaga, Y., Utagawa, E., Takeda, N., Inouye, S., Yamazaki, S., 1997. Comparison of gelatin particle agglutination and hemagglutination inhibition tests for measles seroepidemiology studies. Arch. Virol. 142, 1963–1970.

Mori, T., Sasaki, K., Hashimoto, H., Makino, S., 1993. Molecular cloning and complete nucleotide sequence of genomic RNA of the AIK-C strain of attenuated measles virus. Virus Genes 7, 67–81.

Nakayama, T., Zhou, J., Fujino, M., 2003. Current status of measles in Japan. J. Infect. Chemother. 9, 1–7.

Nakayama, T., Komase, K., Uzuka, R., Hoshi, A., Okafuji, T., 2001. Leucine at position 278 of the AIK-C measles virus vaccine strain fusion protein is responsible for reduced syncytium formation. J. Gen. Virol. 82, 2143–2150.

Neumann, P.W., Weber, J.M., Jessamine, A.G., O'Shaughnessy, M.V., 1985. Comparison of measles antihemolysin test, enzyme-linked immunosorbent assay, and hemagglutination inhibition test with neutralization test for determination of immune status. J. Clin. Microbiol. 22, 296–298.

Ratnam, S., Gadag, V., West, R., Burris, J., Oates, E., Stead, F., Bouilianne, N., 1995. Comparison of commercial enzyme immunoassay kits with plaque reduction test for detection of measles virus antibody. J. Clin. Microbiol. 33, 811–815.

Schneider, H., Spielhofer, P., Kaelin, K., Dotsch, C., Radecke, F., Sutter, G., Billeter, M.A., 1997. Rescue of measles virus using a replication-deficient vaccinia-T7 vector. J. Virol. Methods 64, 57–64.

Strebel, P.M., Papania, M.J., Halsey, N.A., 2004. Measles vaccine. In: Plotkin, S.A., Orenstein, W.A. (Eds.), Vaccine, fourth ed. Saunders, Philadelphia, pp. 389–440.

Takeda, M., Takeuchi, K., Miyajima, N., Kobune, F., Nagata, N., Suzuki, Y., Tashiro, M., 2000. Recovery of pathogenic measles virus from cloned cDNA. J. Virol. 74, 6643–6647.

van den Hof, S., van Gageldonk-Lafeber, A.B., van Binnendijk, R.S., van Gageldonk, P.G., Berbers, G.A., 2003. Comparison of measles virus-specific antibody titers as measured by enzyme-linked immunosorbent assay and virus neutralization assay. Vaccine 21, 4210–4214.

Ward, B.J., Aouchiche, S., Martel, N., Bertley, F.M.N., Bautista-Lopez, N., Serhir, B., Ratna, m.S., 1999. Measurement of measles virus-specific neutralization of the syncytium inhibition assay in comparison with the plaque reduction neutralization test. Diagn. Microbial. Infect. Dis. 33, 147–152.

Whittle, H.C., Mann, G., Eccles, M., O'Neill, K., Jupp, L., Hanlon, P., Hanlon, L., Marsh, V., 1988. Effects of dose and strain of vaccine on success of measles vaccination of infants aged 4–5 months. Lancet 30, 963–966.

WHO, 2003. WHO-recommended surveillance standard of measles. WHO-recommended standards for surveillance of vaccine-preventable diseases, 13–17.

WHO, 2006. Progress in reducing global measles deaths: 1999-2004. Wkly. Epidemiol. Rec. 81, 90–94.

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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).

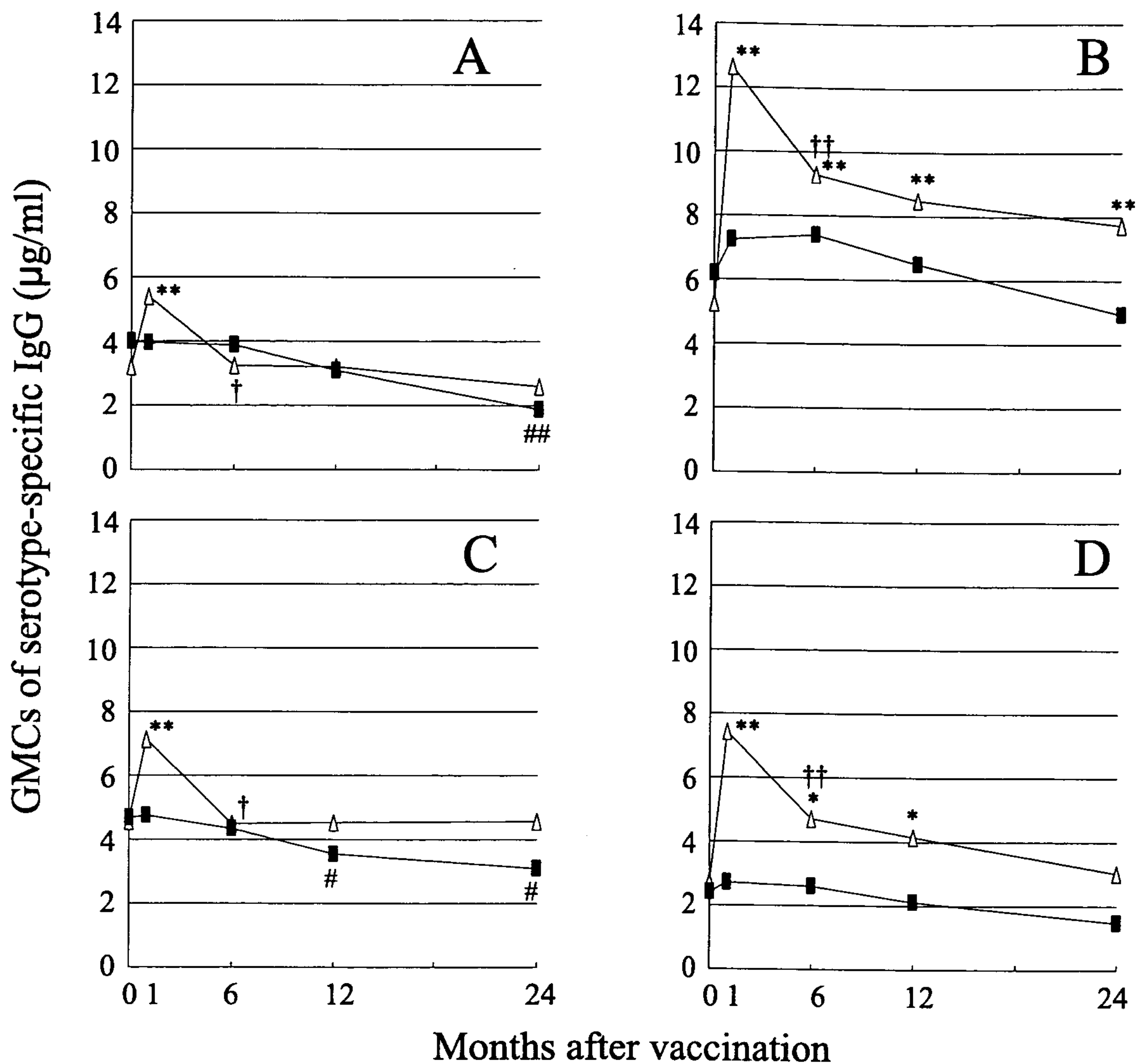

FIG. 1. Kinetics of GMCs of IgG specific for serotypes 6B (A), 14(B), 19F (C), and 23F (D) in responder (n = 27; open triangles) and low-responder (n = 13; closed squares) groups before vaccination and 1 month, 6 months, 1 year, and 2 years after vaccination as determined by ELISA. *, P of <0.05, and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

1. **Alfageme, I., R. Vazquez, N. Reyes, J. Munos, A. Fernandez, M. Hernandez, M. Merino, J. Perez, and J. Lima.** 2006. Clinical efficacy of anti-pneumococcal vaccination in patients with COPD. Thorax **61:**189–195.
2. **Anttila, M., J. Eskola, H. Ahman, and H. Kayhty.** 1998. Avidity of IgG for *Streptococcus pneumoniae* type 6B and 23F polysaccharides in infants primed with pneumococcal conjugates and boosted with polysaccharide or conjugate vaccines. J. Infect. Dis. **177:**1614–1621.
3. **Baquero, F.** 1995. Pneumococcal resistance to β-lactam antibiotics: a global geographic overview. Microb. Drug Resist. **1:**115–120.
4. **Bogaert, D., R. de Groot, and P. W. M. Hermans.** 2004. *Streptococcus pneumoniae* colonization: the key to pneumococcal disease. Lancet Infect. Dis. **4:**144–154.
5. **Bogaert, D., P. van der Valk, R. Ramdin, M. Sluijter, E. Monninkhof, R. Hendrix, R. de Goot, and P. W. M. Hermans.** 2004. Host-pathogen interaction during pneumococcal infection in patients with chronic obstructive pulmonary disease. Infect. Immun. **72:**818–823.
6. **Brandao, A. P., T. C. de Oliveira, M. C. de Cunto Brandileone, J. E. Goncalves, T. I. Yara, and V. Simonsen.** 2004. Persistence of antibody response to pneumococcal capsular polysaccharides in vaccinated long term-care residents in Brazil. Vaccine **23:**762–768.
7. **Bruyn, G. A., P. S. Hiemstra, A. Matze-van der Lans, and R. van Furth.** 1990. Pneumococcal anticapsular antibodies in patients with chronic cardiovascular and obstructive lung disease in The Netherlands. J. Infect. Dis. **162:**1192–1194.
8. **Butler, J. C., E. D. Shapiro, and G. M. Carlone.** 1999. Pneumococcal vaccines: history, current status, and future directions. Am. J. Med. **107(1A):**69S–76S.
9. **Centers for Disease Control and Prevention.** 1997. Prevention of pneumococcal disease: recommendations of the advisory committee on immunization practices (ACIP). Morb. Mortal. Wkly. Rep. **46:**1–24.
10. **Concepcion, N. F., and C. E. Frasch.** 2001. Pneumococcal type 22F polysaccharide absorption improves the specificity of a pneumococcal-polysaccharide enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. **8:**266–272.
11. **Davis, L. A., C. A. P. Aranda, G. Schiffman, and C. L. Cristianson.** 1987. Pneumococcal infection and immunologic response to pneumococcal vaccine in chronic obstructive pulmonary disease. Chest **92:**204–212.
12. **de Roux, A., N. Schmidt, M. Rose, S. Zielen, M. Pletz, and H. Lode.** 2004. Immunogenity of the pneumococcal polysaccharide vaccine in COPD patients. The effect of systemic steroids. Respir. Med. **98:**1187–1194.
13. **Fedson, D. S., and J. A. G. Scott.** 1999. The burden of pneumococcal disease among adults in developed and developing countries: what is and is not known. Vaccine **17:**S11–S18.
14. **Fedson, D. S., D. M. Musher, and J. Eskola.** 1998. Pneumococcal vaccine, p. 553–607. *In* S. Plotkin and W. A. Orenstein (ed.), Vaccines, 3rd ed. W. B. Saunders Co., Philadelphia, PA.
15. **Feikin, D. R., K. P. Klugman, R. R. Facklam, E. R. Zell, A. Schuchat, and C. G. Whitney for the Active Bacterial Core Surveillance/Emerging Infections Program Network.** 2005. Increased prevalence of pediatric pneumococcal serotypes in elderly adults. Clin. Infect. Dis. **41:**481–487.
16. **Inoue, M., N. Y. Lee, S. W. Hong, K. Lee, and D. Felmingham.** 2004. PROTEKT 1999–2000: a multicenter study of the antibiotic susceptibility of respiratory tract pathogens in Hong Kong, Japan and South Korea. Int. J. Antimicrob. Agents **23:**44–51.
17. **Lahood, N., S. S. Emerson, P. Kumar, and R. U. Sorensen.** 1993. Antibody levels and response to pneumococcal vaccine in steroid-dependent asthma. Ann. Allergy **70:**289–294.
18. **Musher, D. M., A. J. Chapman, A. Goree, S. Jonsson, D. Briles, and R. E. Baughn.** 1986. Natural and vaccine-related immunity to *Streptococcus pneumoniae*. J. Infect. Dis. **154:**245–256.
19. **Oishi, K., H. Yoshimine, H. Watanabe, K. Watanabe, S. Tanimura, K. Kawakami, A. Iwagaki, H. Nagai, H. Goto, S. Kudoh, T. Kuriyama, Y. Fukuchi, T. Matsushima, K. Shimada, K. Matsumoto, and T. Nagatake.** 2006. Drug-resistant genes and serotypes of pneumococcal strains of community-acquired pneumonia among adults in Japan. Respirology **11:**429–436.
20. **Romero-Steiner, S., D. M. Musher, M. S. Cetron, L. B. Paris, J. E. Groover, A. E. Fiore, B. D. Olikaytis, and G. M. Carlone.** 1999. Reduction in functional antibody activity against *Streptococcus pneumoniae* in vaccinated elderly individuals highly correlates with decreased IgG antibody avidity. Clin. Infect. Dis. **29:**281–288.
21. **Rubins, J. B., A. K. G. Puri, J. Loch, D. Charboneau, R. MacDonald, N. Opstad, and E. N. Janoff.** 1998. Magnitude, duration, quality, and function of pneumococcal vaccine responses in elderly adults. J. Infect. Dis. **178:**431–440.
22. **Saito, W., H. Nagai, J. Suzuki, K. Masuda, A. Tamura, N. Nagayama, S. Akagawa, Y. Kawabe, K. Machida, A. Kurashima, H. Yotsumoto, T. Saito, T. Arai, and N. Okabe.** 2005. Capsular polysaccharide antibodies after pneumococcal polysaccharide vaccination in patients with chronic respiratory disease. Nihon Kokyuki Gakkai Zasshi **43:**277–282.
23. **Sankilampi, U., P. O. Honkanen, A. Bloigu, and M. Leinonen.** 1997. Persistence of antibodies to pneumococcal capsular polysaccharide vaccine in the elderly. J. Infect. Dis. **176:**1100–1104.
24. **Sankilampi, U., P. O. Honkanen, A. Bloigu, E. Herva, and M. Leinonen.** 1996. Antibody response to pneumococcal capsular polysaccharide vaccine in the elderly. J. Infect. Dis. **173:**387–393.
25. **Sethi, S.** 2004. New developments in the pathogenesis of acute exacerbation of chronic obstructive pulmonary disease. Curr. Opin. Infect. Dis. **17:**113–119.
26. **Shapiro, E. D., A. T. Berg, R. Austrian, D. Schroeder, V. Parcells, A. Margolis, R. K. Adair, and J. D. Clemens.** 1991. The protective efficacy of polyvalent pneumococcal polysaccharide vaccine. N. Engl. J. Med. **325:**1453–1460.
27. **Simberkoff, M. S., A. P. Cross, M. Al-Ibrahim, A. L. Baltch, P. J. Geiseler, J. Nadler, A. S. Richmond, R. P. Smith, G. Schiffman, and D. S. Shepard.** 1986. Efficacy of pneumococcal vaccine in high-risk patients. Results of a Veterans Administration cooperative study. N. Engl. J. Med. **315:**1318–1327.
28. **Song, J. H., N. Y. Lee, S. Ichiyama, R. Yoshida, Y. Hirakata, F. Wang, A. Chongthaleong, N. Aswapokee, C. Chiu, M. K. Lalitha, K. Thomas, J. Perera, T. T. Yee, F. Jamal, U. C. Warsa, B. X. Vinh, M. R. Jacobs, P. C. Appelbaum, C. H. Pai, and the ANSORP Study Group.** 1999. Spread of drug-resistant *Streptococcus pneumoniae* in Asian countries: Asian Network

for Surveillance of Resistant Pathogens (ANSORP) study. Clin. Infect. Dis. **28**:1206–1211.

29. **Wernette, C. M., C. E. Frasch, D. Madore, G. Carlone, D. Goldblatt, B. Plikaytis, W. Benjamin, S. A. Quataert, S. Hildreth, D. J. Sikkema, H. Kayhty, I. Jonsdottir, and M. H. Nahm.** 2003. Enzyme-linked immunosorbent assay for quantitation of human antibodies to pneumococcal polysaccharides. Clin. Diagn. Lab. Immunol. **10**:514–519.
30. **Williams, J. H., Jr., and K. M. Moser.** 1986. Pneumococcal vaccine and patients with chronic lung diseases. Ann. Intern. Med. **104**:106–109.
31. **World Health Organization Pneumococcal Serology Reference Laboratories.** 9 June 2004, accession date. Training manual for enzyme linked immunosorbent assay for quantitation of *Streptococcus pneumoniae* serotype specific IgG (Pn PS ELISA). World Health Organization, Geneva, Switzerland. http://www.vaccine.uab.edu/ELISA%20Protocol.pdf.
32. **Wuorimaa, T., R. Dagan, M. Vakevainen, F. Bailleux, R. Haikala, M. Yaich, J. Eskola, and H. Kayhty.** 2001. Avidity and subclasses of IgG after immunization of infants with an 11-valent pneumococcal conjugate vaccine with or without aluminum adjuvant. J. Infect. Dis. **184**:1211–1215.

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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