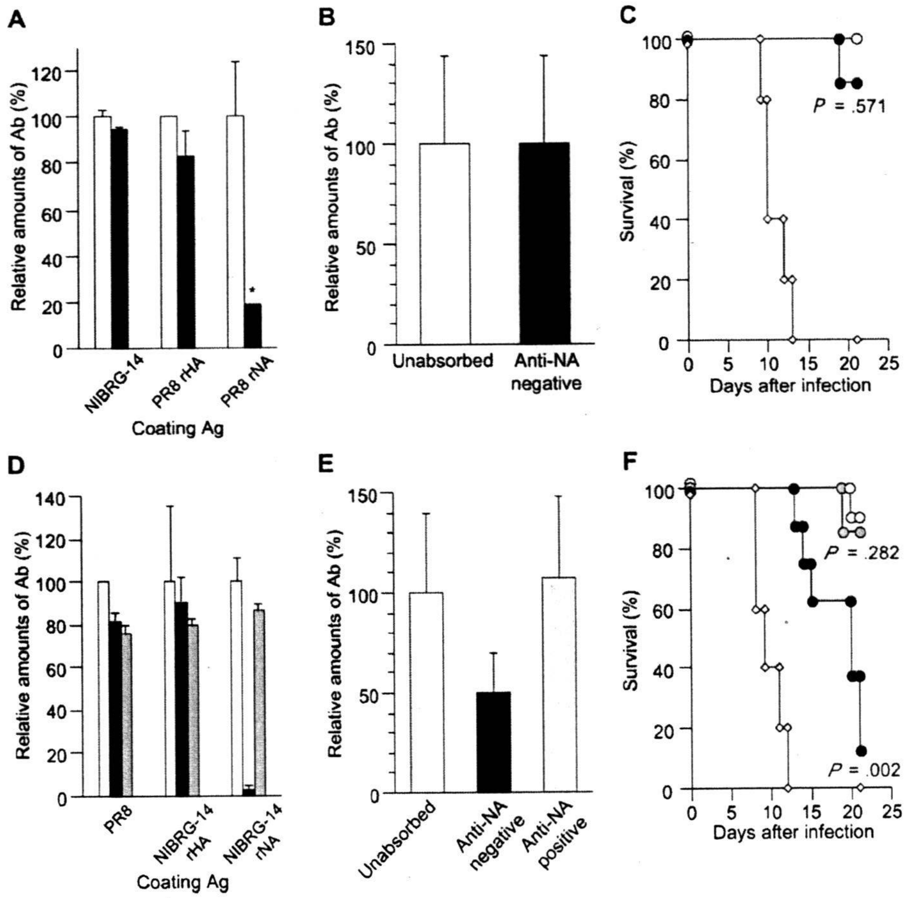


Figure 5. Protective role of neuraminidase (NA)–binding antibodies (Abs) against homologous virus challenge. Anti-NA Abs were absorbed from the serum of mice that were vaccinated with PR8 *(A–C)* or NIBRG-14 *(D–F)*, and the absorption efficiency was estimated by enzyme-linked immunosorbent assay, as shown in figure 3 *(A, D)*. For NIBRG-14, the serum from recombinant NA (rNA)–primed mice was added into NA Ab–depleted serum to restore the levels of anti-NA Abs to the levels in the unabsorbed control serum *(gray bars)*. Data are the mean ± standard deviation ($n = 3$). *Ab titers are below the detection limit. Amounts of neutralizing Abs were estimated using MN assays *(B, E)*, and the protective efficacy of each serum type in vivo *(C, F)* was estimated as shown in figure 3. Gray circles denote mice that were administered the serum mixture (anti–rNA Ab-depleted serum and anti-rNA Ab-containing serum). Data are representative of 2 independent experiments. Ag, antigen; rHA, recombinant hemagglutinin. *Black bars*, absorbed serum samples; *white bars*, unabsorbed control samples.

tenth of the MN Ab titers in the unabsorbed control serum (figure 3*B* and 3*E*). This result shows that the remaining Abs specific for the native form of HA are below the minimal threshold (one-fourth of unabsorbed serum) for the protection of infected mice (figure 1*B* and 1*C*). To compare the protective efficacy of immune serum with or without anti-HA Abs, CB17-SCID mice were intravenously administered each type of serum and then intranasally challenged with 5 LD_{50} of PR8 or a highly pathogenic H5N1 virus. Mice that were administered serum without anti-HA Abs were more susceptible to lethal infection with PR8 virus than were those that were administered unabsorbed serum (figure 3*C*) ($P = .009$, by the generalized Wilcoxon test); this finding confirms that anti-HA Abs play a pivotal role in virus neutralization in vivo. Similarly, the absorption of anti-HA Abs from the serum samples of mice that were administered NIBRG-14 vaccine significantly reduced the protective efficacy against a lethal challenge with the highly pathogenic A/Vietnam/Jp1203/2004 virus (figure 3*F*) ($P = .001$). Thus, these results support the possibility that HA-binding Abs with low HI and MN activity in anti–NIBRG-14 serum contribute to in vivo protection against a homologous virus challenge.

Association of complement with enhancement of the inhibitory effect of virus attachment by anti–NIBRG-14 serum. Feng et al. [16] have reported that low HI and MN activity of several H1-specific monoclonal Abs can be enhanced by the addition of the complement factor C1q, implying that the in vivo protective efficacy of anti–NIBRG-14 serum with compromised HI and MN activity may be improved by the addition of this complement factor to the serum. To investigate this hypothesis, we monitored the inhibitory effect of anti–NIBRG-14 serum against virus attachment to MDCK cells in the presence of C1q. Preincubation of inactivated NIBRG-14 virus with anti–NIBRG-14 serum in the presence of naive mouse serum or pu-

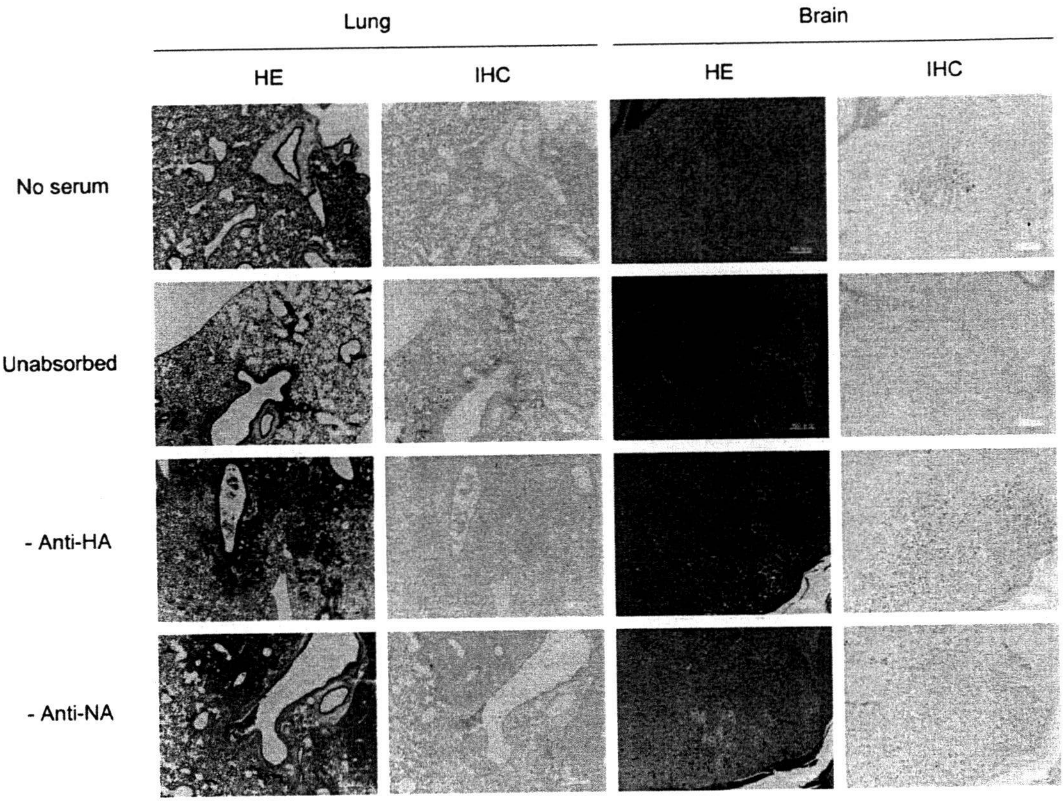


Figure 6. Histopathologic findings in mice infected with a highly pathogenic H5N1 virus. CB17–severe combined immunodeficient (SCID) mice were reconstituted with unabsorbed immune serum (unabsorbed serum) or with serum absorbed by a recombinant hemagglutinin (rHA) or an recombinant neuraminidase (rNA) column. Naive SCID mice that were not reconstituted with serum were used as control mice. At 8–10 days after infection, the lungs and brain of each mouse were collected, fixed with formalin, and analyzed by hematoxylin-eosin staining or antinucleoprotein immunohistochemical analysis (original magnification, ×50).

rified C1q partially enhanced the inhibition of virus attachment by the serum (figure 4). These data suggest that the inhibitory effect of anti–NIBRG-14 serum against virus attachment may depend on the presence of complement factors.

Contribution of NA-binding Abs to protection. The protective efficacy of anti-NA Abs was examined by an adoptive transfer of the absorbed serum. The amount of anti-NA Abs in the absorbed serum from PR8-vaccinated mice could not be detected, even by use of the highly sensitive ELISA. Therefore, the amount of NA-binding Abs remaining in this serum (<18.8% of the amount noted in control serum) could not be precisely determined (figure 5*A*). Both MN assay and the serum transfer experiments revealed that the neutralization ability of the absorbed serum was not significantly different from that of the unabsorbed control serum (figure 5*B* [$P = .500$] and 5*C* [$P = .571$]). In contrast, the absorption of anti-NA Abs from the immune serum of NIBRG-14–vaccinated mice significantly reduced the protective efficacy of the serum, and the mice that were administered this absorbed serum died more rapidly than did those that were administered the unabsorbed control serum (figure 5*F*) ($P = .002$). However, the MN assay did not detect a significant difference between the neutralization ability of the absorbed and unabsorbed serum (figure 5*E*) ($P = .056$).

To confirm whether the reduction in the protective efficacy of the serum is solely the result of the absorption of anti-NA Abs, we added the immune serum of rNA-immunized mice to the absorbed serum to restore the level of anti-NA Abs. This addition of immune serum from rNA-immunized mice restored the amount of anti-NA Abs in the absorbed serum to the level of that in the unabsorbed control serum (figure 5*D*). Of note, the adoptive transfer of serum supplemented with anti-NA Abs prolonged the survival of infected mice to the length of survival of control mice (figure 5*F*) ($P = .282$). This finding confirmed that the anti-NA Abs contained in the serum of NIBRG-14–vaccinated mice were responsible for providing immune protection. Thus, these data indicate that, even in the presence of anti-HA Abs, anti-NA Abs elicited by NIBRG-14 vaccines can afford protection against a lethal infection with homologous H5N1 virus.

Virus spread and pathologic findings in mice reconstituted with either HA or NA Ab–absorbed serum. Mice that were administered immune serum lacking either anti-HA or anti-NA Abs were subjected to histopathologic analysis. In naive mice, an abundance of viral nucleoprotein antigens was detected in both the lungs and brain, and interstitial infiltration of inflammatory cells was also observed (figure 6). In contrast, viral nucleoprotein antigen in the lungs of mice reconstituted with unabsorbed control serum was scarcely present in the lungs and was absent in the brain 10 days after infection. Absorption of either HA or NA Abs was associated with a similar phenotype, and viral antigens were abundantly detected in both the lungs and the brain. Thus, absorption of either type of Ab results in a distinct pathologic difference from SCID mice reconstituted with unabsorbed serum; however, the pathologic characteristics were comparable among mice given either HA Ab– or NA Ab–deficient serum.

DISCUSSION

The data presented in this study suggest that NIBRG-14 vaccines possess sufficient immunogenicity and induce comparable amounts of anti-HA Abs. Although the anti-HA Abs have weak HI and MN activity, they contributed to the neutralization of homologous H5N1 virus, partially with the help of complement factors in vivo. Of note, the higher level of anti-NA Abs elicited by NIBRG-14 vaccines also participated in protection, even in the presence of anti-HA Abs. These results provide new insights into the protective immunity elicited by currently stockpiled H5N1 vaccines and indicate that methods other than the conventional HI assay are required for the estimation of vaccine efficacy.

Induction of anti-HA Abs with low HI activity has been observed in mouse and ferret models of H5N1 and H2N2 avian influenza virus infection, suggesting that this phenomenon is not limited to a particular host species or to a subtype of avian influenza virus [7, 8, 17]. When whole influenza virus that bore a Ser223→Asn223 substitution in the HA of Vietnam 2004 strains was used as detection antigen, the sensitivity of the HI assay improved [7, 17]; this finding supports the insensitive detection of anti-HA Abs by conventional HI assay rather than the poor induction of anti-HA Abs (figures 1 and 2). Ser223 does not appear to be included in the antigen recognition sites of several mAbs against the HA of Vietnam 2004 strain, and the Ser223→Asn223 substitution may alter the conformation or receptor specificity of the H5 protein, which eventually increases HI sensitivity [7, 18]. Moreover, the sensitivity of the HI assay for antiavian H2 Abs was improved by using only the isolated H2 HA antigen instead of the whole virus [17]. These results suggest that the unique antigenic structure of HA or the interaction between HA and other viral antigens on the surface of avian influenza virus may significantly affect the sensitivity of the HI assay.

The addition of complement to the anti–NIBRG-14 serum partially restored the neutralization activity of the serum, similar to that of mAbs against H1 protein [16]. The large complex formed by the complement and the HA-bound Abs may cause steric interference between HA and sialic acid, as has been postulated elsewhere [19]. To our knowledge, steric interference on virus attachment has been reported only in monoclonal Abs against HA of PR8 virus. In the present study, we found that it can also occur in polyclonal Abs against H5N1 virus. Additional studies are required to clarify whether the phenomenon can be observed for all influenza viruses and whether the balance of HA and NA activities is involved in this process.

It has been shown that large amounts of anti-NA Abs contribute to providing protection to the host [11, 20]. However, when NA is conjugated with the HA antigen in the form of a whole virion, the anti-NA Ab response is inhibited by intravirionic antigenic competition [12]. Thus, the level of anti-NA Abs elicited by inactivated PR8 and NC20 vaccines may be insufficient for providing immune protection to the host (figure 5). In this context, the significant contribution of anti-NA Abs elicited by the NIBRG-14 vaccine to provision of immune protection is remarkable, and it may partially explain the protection that is independent of HI and MN Ab titers. The anti-NA Ab titer elicited by the NIBRG-14 vaccine reached a level that was approximately one-sixth of the anti-HA Ab titer (figure 2*A*), which is close to the ratio of HA to NA proteins noted on the surface of virion (5:1). This finding suggests that intravirionic competition is reduced in NIBRG-14 vaccine.

At present, we do not know the exact mechanisms underlying the enhanced induction of anti-NA Abs by NIBRG-14 vaccine. Given the labile nature of N1 protein, the differential induction of anti-NA Ab between NIBRG-14 and H1N1 vaccines may reflect that N1 protein from NIBRG-14 is more stable and immunogenic than N1 proteins from PR8 and NC20 after the process of vaccine preparation. In addition, the extent to which HA and NA antigens are cross-linked by formalin treatment may be weak on the surface of NIBRG-14, leading to less intravirionic competition.

Recent clinical studies have used clade 1 vaccines from A/Vietnam/04 strains, but most avian H5N1 viruses prevalent in the past year belong to clade 2. Thus, rgA/Indonesia/5/2005 (clade 2.1) and rgA/Anhui/1/2005 (clade 2.3) were selected as vaccine seed viruses in Japan, but the H5 proteins of both viruses possess the Ser223 residue, indicating that the HI and MN activity of the anti-HA Abs elicited by each vaccine may be compromised. Recently, it has been shown that anti-HA Abs elicited by rHA of the rgA/Indonesia/5/2005 strain showed a level of homologous HI activity that was 4.6-fold higher than that of Vietnam 2004 strain [21]. Thus, the interference of HI activity in clade 2 strains may be modest. We are currently characterizing the anti-HA and anti-NA Abs elicited by clade 2 vaccines, to clarify whether the data obtained for the NIBRG-14 vaccines can be generalized to other clades of H5N1 vaccines.

Acknowledgments

We thank Le Mai Thi Quynh at the National Institute of Hygiene and Epidemiology, Vietnam, for supplying the A/Vietnam/1194/2004 virus and

Dr. John Wood at the National Institute for Biological Standards and Controls for providing the NIBRG-14 virus. We also thank Genta Kitahara, Yoshiyuki Ushiyama, Hiroto Satake, Hiroko Kusachi, and Eri Watanabe for technical assistance and Dr. Shin-ichi Tamura for critical reading of the manuscript.

References

1. Horimoto T, Kawaoka Y. Strategies for developing vaccines against H5N1 influenza A viruses. Trends Mol Med **2006**; 12:506–14.
2. Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N Engl J Med **2006**; 354:1343–51.
3. Bresson JL, Perronne C, Launay O, et al. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. Lancet **2006**; 367:1657–64.
4. Lin J, Zhang J, Dong X, et al. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. Lancet **2006**; 368:991–7.
5. Leroux-Roels I, Borkowski A, Vanwolleghem T, et al. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. Lancet **2007**; 370:580–9.
6. Bernstein DI, Edwards KM, Dekker CL, et al. Effects of adjuvants on the safety and immunogenicity of an avian influenza H5N1 vaccine in adults. J Infect Dis **2008**; 197:667–75.
7. Hoffmann E, Lipatov AS, Webby RJ, Govorkova EA, Webster RG. Role of specific hemagglutinin amino acids in the immunogenicity and protection of H5N1 influenza virus vaccines. Proc Natl Acad Sci USA **2005**; 102:12915–20.
8. Ninomiya A, Imai M, Tashiro M, Odagiri T. Inactivated influenza H5N1 whole-virus vaccine with aluminum adjuvant induces homologous and heterologous protective immunities against lethal challenge with highly pathogenic H5N1 avian influenza viruses in a mouse model. Vaccine **2007**; 25:3554–60.
9. Couch RB, Kasel JA. Immunity to influenza in man. Annu Rev Microbiol **1983**; 37:529–49.
10. Lu X, Edwards LE, Desheva JA, et al. Cross-protective immunity in mice induced by live-attenuated or inactivated vaccines against highly pathogenic influenza A (H5N1) viruses. Vaccine **2006**; 24:6588–93.
11. Johansson BE, Bucher DJ, Kilbourne ED. Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection. J Virol **1989**; 63:1239–46.
12. Johansson BE, Moran TM, Kilbourne ED. Antigen-presenting B cells and helper T cells cooperatively mediate intravirionic antigenic competition between influenza A virus surface glycoproteins. Proc Natl Acad Sci USA **1987**; 84:6869–73.
13. Kilbourne ED, Laver WG, Schulman JL, Webster RG. Antiviral activity of antiserum specific for an influenza virus neuraminidase. J Virol **1968**; 2:281–8.
14. Bungener L, Geeraedts F, Ter Veer W, Medema J, Wilschut J, Huckriede A. Alum boosts TH2-type antibody responses to whole-inactivated virus influenza vaccine in mice but does not confer superior protection. Vaccine **2008**; 26:2350–9.
15. Baker NJ, Gandhi SS. Effect of Ca^{++} on the stability of influenza virus neuraminidase. Arch Virol **1976**; 52:7–18.
16. Feng JQ, Mozdzanowska K, Gerhard W. Complement component C1q enhances the biological activity of influenza virus hemagglutinin-specific antibodies depending on their fine antigen specificity and heavy-chain isotype. J Virol **2002**; 76:1369–78.
17. Lu BL, Webster RG, Hinshaw VS. Failure to detect hemagglutination-inhibiting antibodies with intact avian influenza virions. Infect Immun **1982**; 38:530–5.
18. Kaverin NV, Rudneva IA, Govorkova EA, et al. Epitope mapping of the hemagglutinin molecule of a highly pathogenic H5N1 influenza virus by using monoclonal antibodies. J Virol **2007**; 81:12911–7.
19. Mozdzanowska K, Feng J, Eid M, Zharikova D, Gerhard W. Enhancement of neutralizing activity of influenza virus-specific antibodies by serum components. Virology **2006**; 352:418–26.
20. Sandbulte MR, Jimenez GS, Boon AC, Smith LR, Treanor JJ, Webby RJ. Cross-reactive neuraminidase antibodies afford partial protection against H5N1 in mice and are present in unexposed humans. PLoS Med **2007**; 4:265–71.
21. Bright RA, Carter DM, Crevar CJ, et al. Cross-clade protective immune responses to influenza viruses with H5N1 HA and NA elicited by an influenza virus-like particle. PLoS ONE **2008**; 3:1–14.

Vaccine 27 (2009) 6276–6279

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

PolyI:polyC$_{12}$U adjuvant-combined intranasal vaccine protects mice against highly pathogenic H5N1 influenza virus variants

Takeshi Ichinohe[a,c], Akira Ainai[a,b], Masato Tashiro[b], Tetsutaro Sata[a], Hideki Hasegawa[a,b,*]

[a] Department of Pathology, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo, Japan
[b] Influenza Virus Research Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo, Japan
[c] Department of Immunobiology, Yale University School of Medicine, 300 Cedar Street, TAC S640, New Haven, CT, USA

ARTICLE INFO

Article history:
Received 17 November 2008
Received in revised form 22 April 2009
Accepted 25 April 2009

Keywords:
Influenza virus
H5N1
Adjuvant
Intranasal vaccine
Heterosubtypic immunity

ABSTRACT

The highly pathogenic avian H5N1 influenza virus has the potential to incite a global pandemic. Therefore, there is an urgent need to develop effective vaccines against these viruses. Because it is difficult to predict which strain of influenza will cause a pandemic, it is advantageous to develop vaccines that will confer cross-protective immunity against variants of the influenza virus. Recently, we reported that the Toll-like receptor 3 agonist, polyI:polyC$_{12}$U (Ampligen®), has been proven to be safe in a Phase III human trial, and is an effective mucosal adjuvant for intranasal H5N1 influenza vaccination. Intranasal administration of an Ampligen® adjuvanted pre-pandemic H5N1 vaccine (NIBRG14), which was derived from the A/Vietnam/1194/2004 strain, resulted in the secretion of vaccine-specific IgA and IgG in nasal mucosa and serum, respectively, and protected mice against homologous A/Vietnam/1194/2004 and heterologous A/Hong Kong/483/97 and A/Indonesia/6/2005 viral challenge.

1. Introduction

There are presently a number of pre-pandemic H5N1 vaccines in existence, which were derived from currently circulating strains of the virus. However, the continual mutation of H5N1 renders them of limited use. Therefore, it is of crucial importance to develop an influenza vaccine that confers cross-protective immunity not only against the homologous influenza virus but also against the variants that arise from mutation of the virus.

Inactivated vaccines against the influenza virus have been administered parenterally to induce viral-specific serum IgGs that are highly protective against homologous virus infection. However, they are much less effective against heterologous virus infection [1]. By contrast, a number of studies have shown that the mucosal immunity acquired through natural infection, which is mainly mediated by the secreted form of IgA (sIgA) in the respiratory tract, is more effective and cross-protective against heterologous virus infections than the systemic immunity induced by parenteral vaccination [1–3]. It is believed that sIgA is more cross-protective against heterologous influenza compared with IgG due to its divalency (higher avidity) and location [1]. In this regard, induction of virus-specific sIgA in the respiratory tract has a great advantage in conferring protection against an unpredictable pandemic of highly pathogenic avian influenza viruses.

We previously demonstrated that the synthetic double-stranded RNA (dsRNA) poly(I:C) is a promising and effective intranasal adjuvant for influenza virus vaccine. Poly(I:C) interacts with Toll-like receptor 3 (TLR3), which plays a key role in the innate immune system and activates immune cell responses. Intranasal administration with split influenza vaccine in combination with poly(I:C) increased both the mucosal and systemic humoral immune response, resulting in complete protection against homologous and heterologous influenza viruses in mice [4]. Although poly(I:C) is a potent mucosal adjuvant that induces type I interferons (IFNs) and has the potential to bridge the gap between innate and adaptive immunity [5], it has been associated with serious adverse events during clinical trials [6].

PolyI:polyC$_{12}$U (Ampligen®), a dsRNA compound that is similar to poly(I:C), degrades easily *in vivo* due to the existence of mismatched residues in the nucleotide. It has a good safety profile based on clinical trials, including a recently conducted double-blind, placebo-controlled Phase III clinical trial [7]. To date, more than 75,000 doses of Ampligen have been administered to humans, at an average dose of 400 mg, and it has been generally well tolerated. We examined the cross-protective effect of intranasal vaccine given in combination with Ampligen in mice. We demonstrated that co-administration of the vaccine with Ampligen elicited cross-protective immunity against heterologous A/Hong Kong/483/97 and A/Indonesia/6/2005 viruses.

* Corresponding author at: Influenza Virus Research Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo, Japan. Tel.: +81 42 561 0771; fax: +81 42 561 6572.
E-mail address: hasegawa@nih.go.jp (H. Hasegawa).

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.04.074

2. Materials and methods

The strains of H5N1 viruses used in this study were A/Hong Kong/483/97, A/Vietnam/1194/2004, and A/Indonesia/6/2005 [8]. The A/Hong Kong/483/97 virus was prepared in Madin–Darby canine kidney (MDCK) cells without any special step for mouse adaptation. The A/Vietnam/1194/2004 and A/Indonesia/6/2005 viruses were propagated in 10-day-old embryonated chicken eggs for 2 days at 37 °C. The formalin-inactivated whole virus vaccine (NIBRG14) was prepared from a recombinant avirulent avian virus that contains modified hemagglutinin (HA) and neuraminidase from the highly pathogenic avian influenza strain A/Vietnam/1194/2004 and other viral proteins from the influenza strain A/PuertoRico/8/34 (A/PR8, H1N1) [9]. The trivalent-inactivated influenza vaccine (split-product HA vaccines) prepared for the 2005–2006 season, including A/NewCaledonia/20/99 (H1N1), A/NewYork/55/2004 (H3N2), and B/Shanghai/361/2002, was purchased from Kitasato Institute (Saitama, Japan). PolyI:polyC_{12}U (Ampligen®) was kindly provided by Hemispherx Biopharma (Philadelphia, PA).

BALB/c mice were anaesthetized with diethyl ether and immunized 2 or 3 times, either intranasally or subcutaneously, with 1 μg of NIBRG14 [10] or trivalent split-product virus vaccines [11] with or without adjuvant at 3-week intervals. Each mouse was anaesthetized and infected by intranasal administration of 4 μl of PBS containing virus suspension with 1000 PFU of H5N1 virus into each nostril (2 μl/nostril) at 2 weeks after final vaccination. The immune response elicited after vaccination was examined 2 weeks after the final vaccination using a number of immunological assays (ELISA, hemagglutination inhibition (HI), and virus neutralization (VN) assays) [10,11]. The protective efficacy of the vaccines was examined by assessing viral titer in the nasal wash and monitoring survival rate of mice after the challenge. All animal experiments were performed in accordance with the Guides for Animal Experiments Performed at the National Institute of Infectious Diseases (NIID) and were approved by the Animal Care and Use Committee of NIID. Infection with H5N1 virus was performed under Biosafety Level 3 containment and was approved by NIID.

3. Results and discussion

3.1. Antibody responses in mice immunized intranasally or subcutaneously with NIBRG14 vaccine and Ampligen

To determine the efficacy of Ampligen as a mucosal adjuvant for H5N1 vaccines, the antibody response to NIBRG14 was examined. Mice were immunized twice by intranasal or subcutaneous administration of NIBRG14, with or without Ampligen, and their antibody response was measured by ELISA. In nasal washes, higher levels of anti-NIBRG14 IgA Ab were observed in animals immunized intranasally with 1 μg of NIBRG14 and 10 μg of Ampligen (Fig. 1A). A small IgA response was elicited by intranasal administration of NIBRG14 without adjuvant, and no IgA response was evident in any of the mice which received a subcutaneous vaccination of NIBRG14 with or without Ampligen. Neutralizing activity against homologous A/Vietnam/1194/2004 virus was detected in the sera from mice immunized either intranasally or subcutaneously, with or without adjuvant. However, no neutralizing activity against heterologous A/Hong Kong/483/97 or A/Indonesia/6/2005 viruses was detected in the sera from any immunized group, suggesting that serum IgG antibodies are insufficient to neutralize heterologous virus and IgA antibodies at the mucosal surface might be more important than serum IgG antibodies for the protection against heterologous viruses. However, nor was neutralizing activity detected in the nasal wash from any group against both homologous and heterologous viruses. We suspect that, due to the dilution by PBS when the nasal wash samples were collected, the concentration of vaccine-specific IgA in our samples was much lower than the physiological concentration in the nasal mucosa, and therefore neutralizing activity in the nasal wash may not have been detectable.

3.2. Intranasal vaccination with NIBRG14 and Ampligen protects mice against highly pathogenic avian influenza virus infection

We next examined the protective effect of intranasal vaccination with NIBRG14 in combination with Ampligen against homologous and heterologous H5N1 viruses. Mice were immunized, either intranasally or subcutaneously, with 1 μg of NIBRG14 and 10 μg of Ampligen, and then challenged by infection with homologous A/Vietnam/1194/2004, heterologous A/Hong Kong/483/97 or heterologous A/Indonesia/6/2005 viruses. All of the mice immunized intranasally with combined vaccine and Ampligen completely cleared the viruses in their nasal cavity (Table 1). By contrast, significantly higher levels of virus in nasal wash samples were detected in mice immunized subcutaneously with vaccine and Ampligen. All of the mice in both groups survived following homologous A/Vietnam/1194/2004 viral challenge (Table 1). In the heterologous viral challenge experiment, the virus titer in the nasal wash of the intranasal vaccination group was significantly lower than that of the subcutaneous vaccination group following infection with the A/Hong Kong/483/97 or A/Indonesia/6/2005 virus. Consequently, though intranasally immunized mice survived lethal infection with A/Hong Kong/483/97 or A/Indonesia/6/2005 viruses, the 100% of A/Hong Kong/483/97 and 60% of A/Indonesia/6/2005 infected mice

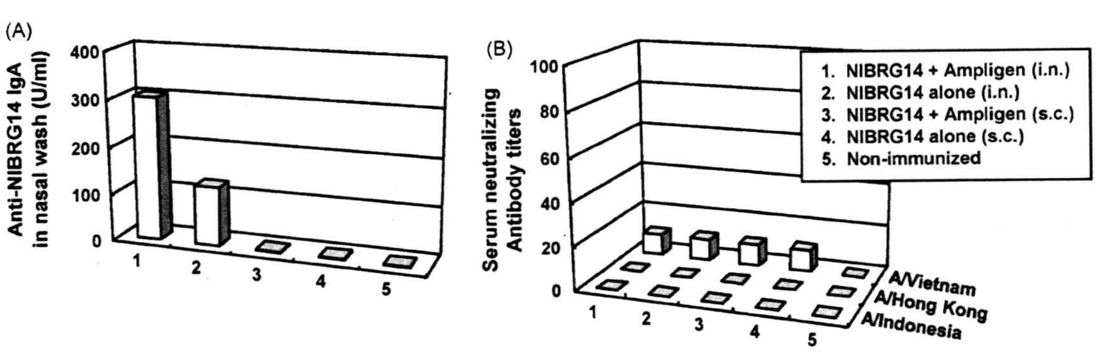


Fig. 1. Anti-NIBRG14-specific IgA and IgG responses in BALB/c mice immunized twice intranasally or subcutaneously with vaccine alone, or in combination with Ampligen. Nasal washes and serum samples were collected 14 days after the final immunization. Antibody titers were measured by ELISA (A). The serum collected at 2 weeks after the booster was analyzed for the presence of neutralizing antibodies against homologous or heterologous influenza virus (B). Inhibition of the virus was assessed by the additional reduction in infectivity beyond the background of naive mice. Sample was run in duplicate, and data are presented per group, where the ability to inhibit 100% of infection at the indicated dilution is shown.

Table 1
H5N1 virus titers in nasal washes and survival rates after challenge with homologous and heterologous viruses.

Vaccination (route)	Challenge virus					
	A/Vietnam/1194/2004		A/Hong Kong/483/97		A/Indonesia/6/2005	
	Virus titer (pfu/ml)	Survival rate (%)	Virus titer (pfu/ml)	Survival rate (%)	Virus titer (pfu/ml)	Survival rate (%)
NIBRG14 + Ampligen (i.n.)	0	100	9 ± 2	80	63 ± 24	100
NIBRG14 + Ampligen (s.c.)	112 ± 72	100	10415 ± 4649	0	1393 ± 534	40
–	1110 ± 494	0	2765 ± 2065	0	27600 ± 3355	20

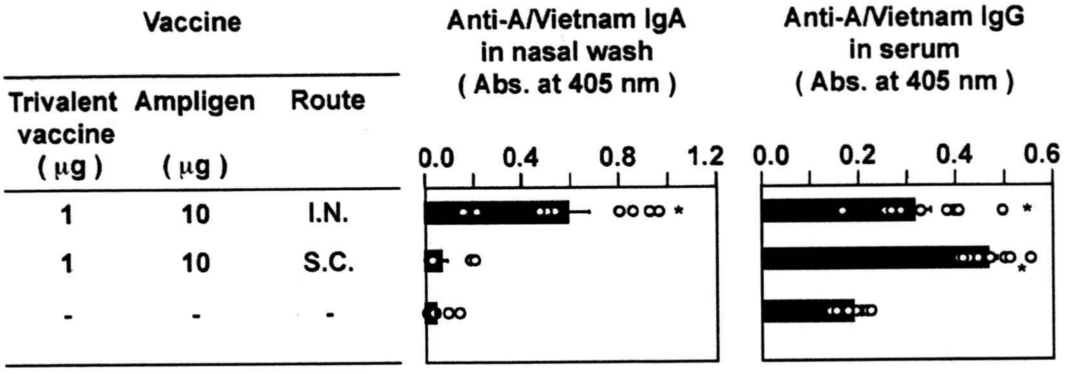


Fig. 2. Cross-reactive IgA and IgG antibodies to A/Vietnam/1194/04. The mice were initially immunized with 1 μg of trivalent-inactivated vaccines with Ampligen through intranasal or subcutaneous route. Immunization was repeated at 3 and 5 weeks after the initial immunization. The nasal washes and serum samples were collected 2 weeks after the final immunization. The concentrations of IgA and IgG antibodies titer to A/Vietnam/1194/04 were measured by ELISA. Bars represent the means ± S.E. of 1:5 diluted samples (nasal washes) or 1:200 diluted samples (sera) and open circles indicate individual animals.

immunized subcutaneously with vaccine and Ampligen succumbed to death (Table 1). These data indicate that intranasal vaccination with combined H5N1 vaccine and Ampligen is more effective than subcutaneous vaccination in protection against homologous and heterologous H5N1 influenza virus challenge [10].

3.3. Induction of cross-reactive antibodies to H5N1 virus by intranasal vaccination with seasonal influenza vaccine and Ampligen

We next characterized the cross-reactive antibody response to A/Vietnam/1194/2004 (H5N1) virus in mice immunized, either intranasally or subcutaneously, with 1 μg of seasonal influenza vaccine and 10 μg of Ampligen. Compared to that in the subcutaneously immunized mice, the concentration of IgA antibodies against A/Vietnam/1194/2004 in nasal wash samples was significantly increased in mice inoculated intranasally with the trivalent vaccine and Ampligen (Fig. 2). The concentration of IgG antibodies against A/Vietnam/1194/2004 in serum was also significantly increased in mice inoculated either intranasally or subcutaneously with the trivalent vaccine and Ampligen combination (Fig. 2). HI titers with regard to heterologous A/Vietnam/1194/2004, A/Hong Kong/483/97, and A/Indonesia/6/2005 virus were also examined *in vitro* using serum and nasal samples from the same group of mice. However, these samples did not show any appreciable cross-neutralizing activity against the H5N1 virus strains. The inability to detect any neutralizing activity in the nasal wash samples was, again, likely due to the dilution of antibodies by PBS when the nasal wash samples were collected.

3.4. Cross-protection against different H5N1 influenza virus strains by intranasal inoculation with seasonal influenza vaccine and Ampligen

We next examined whether the combination of the seasonal influenza vaccine and Ampligen could confer cross-protection against heterologous H5N1 influenza viruses, including the A/Vietnam/1194/2004, A/Hong Kong/483/97 and A/Indonesia/6/2005 strains (Table 2). We immunized mice, either intranasally or subcutaneously, with 1 μg of seasonal influenza vaccine and 10 μg of Ampligen, then monitored viral titer and survival of mice after intranasal challenge with a lethal dose of A/Vietnam/1194/2004, A/Hong Kong/483/97 or A/Indonesia/6/2005 virus. Mice that had been inoculated intranasally or subcutaneously with a combination of trivalent virus and Ampligen showed a significant reduction in A/Vietnam/1194/2004 virus titer, compared with non-inoculated mice. Furthermore, 50% of the intranasally inoculated mice survived, whereas all of the subcutaneously inoculated mice had succumbed to death by 14 days post-infection with A/Vietnam/1194/2004 virus (Table 2). In challenges with 1000 PFU of A/Hong Kong/483/97 virus, mice that had been inoculated with both the trivalent vaccine and Ampligen showed a 25% reduction in virus titer, compared with non-inoculated mice

Table 2
Cross-protective effect of inoculation with seasonal influenza vaccine and Ampligen against H5N1 influenza viruses.

Vaccination (route)	Challenge virus					
	A/Vietnam/1194/2004		A/Hong Kong/483/97		A/Indonesia/6/2005	
	Virus titer (pfu/ml)	Survival rate (%)	Virus titer (pfu/ml)	Survival rate (%)	Virus titer (pfu/ml)	Survival rate (%)
Trivalent vaccine + Ampligen (i.n.)	435 ± 231	50	103 ± 94	100	20500 ± 4843	100
Trivalent vaccine + Ampligen (s.c.)	726 ± 281	0	138 ± 54	70	12400 ± 1198	20
–	4505 ± 1113	0	484 ± 195	60	45200 ± 5492	20

(Table 2). At 14 days after challenge with A/Hong Kong/483/97, all of the intranasally inoculated mice were still alive, whereas 30% of the subcutaneously inoculated mice ($n = 10$) and 40% of the non-inoculated mice ($n = 10$) had died. Finally, in challenges with A/Indonesia/6/2005 virus, mice that had been inoculated intranasally or subcutaneously with the trivalent vaccine and Ampligen combination showed a significant reduction in virus titer compared with non-inoculated mice. At 14 days after challenge with A/Indonesia/6/2005 virus, all of the intranasally inoculated mice were still alive, whereas 80% of the subcutaneously inoculated mice had died (Table 2). Taken together, these results indicate that intranasal inoculation with the trivalent vaccine combined with Ampligen is more effective against infection with heterologous H5N1 influenza virus than subcutaneous vaccination.

4. Concluding remarks

To develop an effective influenza vaccine, it is beneficial to mimic the process of natural infection that bridges the innate and adaptive immune systems [12]. In the present study, we showed that polyI:polyC_{12}U (Ampligen®) has mucosal adjuvant activity when co-administered intranasally with formalin-inactivated H5N1 influenza whole-virion vaccine or the trivalent-inactivated influenza vaccine licensed in Japan for the 2005–2006 season. It increased both the mucosal and systemic humoral responses, and protected mice against homologous and heterologous highly pathogenic H5N1 avian influenza viruses [10,11]. Because TLR3, the receptor that is activated in response to Ampligen [13], is localized to the endosomal compartment in cells, concomitant administration of Ampligen and liposomes may be more effective than Ampligen alone. In fact, chitin microparticles, as a carrier for poly(I:C), enhanced antibody responses and provided protection against lethal H5N1 influenza virus challenge when administered in conjunction with poly(I:C) [14]. We have also observed that co-administration of H5N1 vaccine with Ampligen as a mucosal adjuvant elicited high levels of vaccine-specific IgA titer in saliva and IgG titer in the serum in Cynomolgus macaques (Ichinohe et al. unpublished data). Plans to test the efficacy of the adjuvant-combined intranasal influenza vaccine in human clinical trials will be underway in Japan as early as 2010. Finally, it would be of great benefit to develop biocompatible materials that will enhance the adhesion and uptake of vaccines in the nasal cavity and the respiratory tract. This could significantly enhance the efficacy of inoculation in humans, since the relative extent of the nasal cavity differs from that in mice, and most of the vaccine is ingested when it is intranasally administered to humans.

References

[1] Tamura S, Tanimoto T, Kurata T. Mechanisms of broad cross-protection provided by influenza virus infection and their application to vaccines. Jpn J Infect Dis 2005;58(August (4)):195–207.

[2] Asahi Y, Yoshikawa T, Watanabe I, Iwasaki T, Hasegawa H, Sato Y, et al. Protection against influenza virus infection in polymeric Ig receptor knockout mice immunized intranasally with adjuvant-combined vaccines. J Immunol 2002;168(March (6)):2930–8.

[3] Hasegawa H, Ichinohe T, Tamura S, Kurata T. Development of a mucosal vaccine for influenza viruses: preparation for a potential influenza pandemic. Expert Rev Vaccines 2007;6(April (2)):193–201.

[4] Ichinohe T, Watanabe I, Ito S, Fujii H, Moriyama M, Tamura S, et al. Synthetic double-stranded RNA poly (I:C) combined with mucosal vaccine protects against influenza virus infection. J Virol 2005;79(March (5)):2910–9.

[5] Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004;5(October (10)):987–95.

[6] Robinson RA, DeVita VT, Levy HB, Baron S, Hubbard SP, Levine AS. A phase I-II trial of multiple-dose polyriboinosic-polyribocytidylic acid in patients with leukemia or solid tumors. J Natl Cancer Inst 1976;57(September (3)):599–602.

[7] Suhadolnik RJ, Reichenbach NL, Hitzges P, Adelson ME, Peterson DL, Cheney P, et al. Changes in the 2-5A synthetase/RNase L antiviral pathway in a controlled clinical trial with poly (I)-poly (C12U) in chronic fatigue syndrome. In Vivo (Athens, Greece) 1994;8(July–August (4)):599–604.

[8] Gao P, Watanabe S, Ito T, Goto H, Wells K, McGregor M, et al. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J Virol 1999;73(April (4)):3184–9.

[9] Nicolson C, Major D, Wood JM, Robertson JS. Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system. Vaccine 2005;23(April (22)):2943–52.

[10] Ichinohe T, Kawaguchi A, Tamura S, Takahashi H, Sawa H, Ninomiya A, et al. Intranasal immunization with H5N1 vaccine plus Poly I:Poly C12U, a Toll-like receptor agonist, protects mice against homologous and heterologous virus challenge. Microbes Infect/Institut Pasteur 2007;9(Septembre (11)):1333–40.

[11] Ichinohe T, Tamura S, Kawaguchi A, Ninomiya A, Imai M, Itamura S, et al. Cross-protection against H5N1 influenza virus infection is afforded by intranasal inoculation with seasonal trivalent inactivated influenza vaccine. J Infect Dis 2007;196(November (9)):1313–20.

[12] Ichinohe T, Iwasaki A, Hasegawa H. Innate sensors of influenza virus: clues to developing better intranasal vaccines. Expert Rev Vaccines 2008;7(November (9)):1435–45.

[13] Gowen BB, Wong MH, Jung KH, Sanders AB, Mitchell WM, Alexopoulou L, et al. TLR3 is essential for the induction of protective immunity against Punta Toro Virus infection by the double-stranded RNA (dsRNA), poly (I:C12U), but not poly (I:C) differential recognition of synthetic dsRNA molecules. J Immunol 2007;178(April (8)):5200–8.

[14] Asahi-Ozaki Y, Itamura S, Ichinohe T, Strong P, Tamura S, Takahashi H, et al. Intranasal administration of adjuvant-combined recombinant influenza virus HA vaccine protects mice from the lethal H5N1 virus infection. Microbes Infect/Institut Pasteur 2006;8(October (12–13)):2706–14.

SPECIAL REPORT

Pandemic flu: from the front lines

As the novel H1N1 pandemic flu virus infects people worldwide, researchers in some of the affected countries describe in their own words the scientific and public-health challenges they face.

MEXICO

POPULATION 110 MILLION

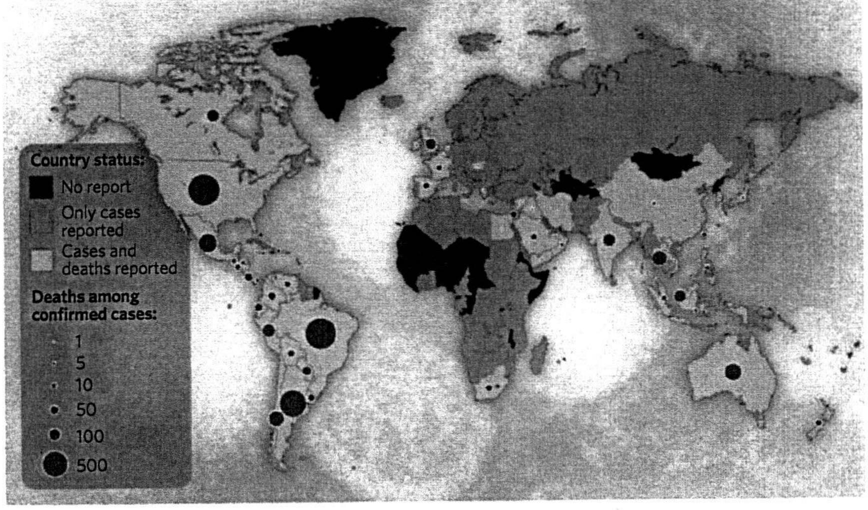
Data suggest that Mexico has seen two waves of infection — the first, which peaked in late April, affected the Mexico City area, and the second, broader wave spanned June through August in southern states, including Chiapas, Yucatan and Quintana Roo. To prepare for a potentially larger wave this winter, Mexico is raising public awareness, standardizing timely diagnosis and treatment and reinforcing equipment and management protocols in intensive-care units throughout the country.

To improve surveillance, Mexico has accelerated the upgrading of its public-health laboratory network. The national reference laboratory and 28 states will soon have real-time PCR for running diagnostic tests. This builds on a restructuring of Mexico's national surveillance and reporting systems, which started in 2007.

As Mexico's strategic reserve of antivirals would cover only 1% of the population for community cases and up to 80,000 hospitalized cases, the nation is implementing a central logistics and delivery system to assure their efficient allocation. The country also expects to have 20 million doses of the H1N1 vaccine available by December. As even this would cover only a fraction of the population, the government will prioritize health-care workers, then individuals at risk of severe disease, such as pregnant women and people with chronic underlying illnesses.

Stefano Bertozzi, executive director at the Center for Evaluation Research and Surveys at the National Institute of Public Health in Cuernavaca

REPORTED FATALITIES FROM PANDEMIC INFLUENZA



SOURCE: ECDC

AUSTRALIA

POPULATION 21 MILLION

The timing of the epidemic has differed across the country, which has meant that we needed different public-health measures and messages in individual states. The pandemic virus seems to be outcompeting the seasonal flu viruses. The great majority of flu cases around the country are now pandemic H1N1.

One interesting question is whether this pandemic virus will completely replace any of the seasonal flu strains. If it doesn't, that's going to complicate the production of future seasonal flu vaccines, as we will need a vaccine against four strains instead of the current three. The Australian government has ordered 21 million doses of dedicated pandemic virus vaccine, so if we need two doses per person, that covers half the population. There has been a lot of discussion about who should get it first, and when.

We are seeing similar patterns of disease severity to those reported worldwide, with most cases being mild. But there have been a significant number of cases with severe disease, not just in the at-risk groups, but also in healthy people. Our indigenous population is being hit harder, and we are seeing disproportionate numbers hospitalized with severe disease.

An important message for other countries that have intensive-care facilities is to expect significant pressure on them. There is a need for mechanical ventilators, and we have seen heavy use of scarce extracorporeal membrane oxygenation units.

Anne Kelso, director of the World Health Organization Collaborating Centre for Reference and Research on Influenza in Melbourne

JAPAN

POPULATION 128 MILLION

Japan stopped counting cases on 25 July and launched a new cluster surveillance system that is directly in the hands of the health ministry. Our Infectious Disease Surveillance Center no longer has any disease data feed, making it difficult to analyse epidemiological trends or disease burden. But we have received hundreds of reports through routine sentinel-based surveillance of clusters of disease from many regions and big cities, so there is extensive spread.

The demand on public-health services to report and investigate all cluster cases is overwhelming public-health staff and leading to a breakdown in the normal public-health diagnostic service in local laboratories.

With the rising numbers of cases we are seeing a corresponding increase in deaths. As elsewhere, it is younger people who are affected with more severe disease requiring hospitalization, but the overall hospitalization rate is no greater than that of human seasonal influenza. Japan has an ageing population with large numbers of people older than 65, many with at-risk underlying health conditions, but so far pandemic H1N1 seems to be largely sparing the elderly.

The country's pandemic plan was based almost entirely on a severe pandemic of H5N1 avian influenza, which limited medical consultations to just a few hospitals.

The government seems to be relaxed with the low level of epidemic by the less virulent virus since May, and seems to have yet to draw any lessons from the pandemic. As a result, local and regional authorities have now independently started to prepare for the coming flu season.

Masato Tashiro, director of the Department of Viral Diseases and Vaccine Control at the National Institute of Infectious Diseases in Tokyo

Q&A: STEPHEN MINGER
Stem-cell scientist to head GE-Geron drug screening collaboration.
www.nature.com/news

KING'S COLLEGE, LONDON

ARGENTINA
POPULATION 40 MILLION

The current epidemiological situation is a generalized spread of the virus throughout the country, although with a marked downward trend in the number of reports of the levels of influenza-like illness. The epidemic started in mid-May in Buenos Aires, and three weeks later spread to the city's larger metropolitan area. Activity peaked on 25 July, with influenza A representing 80% of the circulating respiratory viruses; 65% were H1N1-pandemic confirmed. Very few isolates were H3 and H1 seasonal.

Health systems in Argentina were overloaded because of government advice to people to consult a physician on first signs of flu symptoms such as fever or cough.

The major challenge at the lab level was in diagnosing the first cases produced by a new, unknown virus. Later, the challenge was for lab capacity to meet demand. Information transmitted to the public was not always clear enough, and the mass media had a negative role, including providing contradictory information and producing fear.

Vilma Savy, head of the respiratory virus service at the National Institute for Infectious Diseases in Buenos Aires

VIETNAM
POPULATION 85 MILLION

The first cases in Vietnam were at the end of May, a bit later than in many other parts of Asia, probably because Vietnam does not have a major international airport hub. We are now seeing an increase in disease and a small number of severe cases. Vietnam was a hotspot for H5N1 avian influenza in 2003 and 2004, and the pandemic preparation that resulted from both this and SARS has made a massive difference to the current situation.

Prior to avian flu, few hospital staff had community-acquired pneumonia on their radar; attention was concentrated on malaria, dengue fever and tuberculosis. Now clinicians have a much greater awareness of the need to look out for clusters of respiratory illnesses. There has also been greater interaction and collaboration between clinical and other researchers, and between centres across the country.

Access to vaccines and drugs remains an important issue. There is a global shortage of vaccines, and the rich countries have bought up all the first stocks. This is a really urgent issue; if we can get this right now, then many of the past issues around sharing of samples, data and general openness on emerging infectious diseases will be helped, maybe resolved. If we get it wrong, we will be back to square one. If ever there was a time for the rich world to reach out and ensure equity of access to drugs and vaccines, it is now.

Jeremy Farrar, Vietnam director for the Wellcome Trust Major Overseas Programme, director of Oxford University's Clinical Research Unit in Ho Chi Minh City, and coordinator of the South East Asia Infectious Disease Clinical Research Network

UNITED STATES
POPULATION 301 MILLION

US health-care systems have been stretched and have no surge capacity. The system cannot handle this pandemic, even it if remains moderate in severity. The same applies to many of the supplies we get. Ask anybody who has tried to order an N95 respirator recently; there aren't any. We recently surveyed a group of world-class pharmacists to identify the essential drugs needed daily to keep patients from dying. They came up with a list of more than 30 — all generics, and most made offshore, mainly in Asia, and China and India in particular. Nobody is thinking what might happen to US or global supply chains when pandemic flu hits these countries, where the primary workforce are the young, who are most affected by the virus.

The United States has a federal programme for vaccine procurement but it is administered at the state level, and the two do not always mesh up. It is still not clear how this vaccine is going to be rolled out, or whether it will be here in time.

I worry most that, given current existing public concerns about vaccines, in the autumn we might see mounting public responses and concerns about pandemic-vaccine safety, and people refusing to be vaccinated. Expect the unexpected over the next six months.

Michael Osterholm, director of the University of Minnesota's Center for Infectious Disease Research and Policy in Minneapolis

INDIA
POPULATION 1.1 BILLION

The virus is now transmitting in city clusters. Large numbers of people are turning up at designated testing facilities, swamping an already stretched surveillance system, so there is little room for monitoring mutations and reassortment. This should be done. One way would be to bring in academic labs outside of the government testing system, but sharing of clinical materials and trust is low.

Deaths have sparked a fair amount of concern and panic. Poor communication of risks by the government and the public-health system is largely to blame.

Even if this pandemic remains moderate, the impact in India is likely to be severe, owing to its high population density, low awareness of the pandemic and the propensity of the virus to infect the young (50% of Indians are under 25 years of age). Moreover, there is a high load of other infectious diseases as well as chronic conditions, groups that are at higher risks of severe forms of pandemic H1N1 disease. The health-care infrastructure is poor.

Despite this bleak outlook, India has strengths for tackling the virus, including that the government has pandemic plans in hand, and that we have a vibrant generic-pharmaceutical industry as well as a decent capacity for manufacturing vaccines. There is little clarity, however, as to India's vaccine plans, and the regulatory process is archaic, so it is not even clear whether pandemic vaccine could be rapidly approved for use in the country. The government says it has enough Tamiflu for 3 million people.

Shahid Jameel, head of the virology group at the International Centre for Genetic Engineering and Biotechnology in New Delhi

SUB-SAHARAN AFRICA
POPULATION 800 MILLION

H1N1 has not yet been reported in Nigeria, or any of the other sub-Saharan African countries with which we collaborate — Niger, Burkina Faso or the Central African Republic, although the Democratic Republic of Congo has one confirmed case. But surveillance is still very poor, and the virus may well often escape detection. International media attention to the pandemic is probably more than it deserves from an African public health point of view. Any diversion of resources from other important programmes needs to be carefully evaluated for long-term cost-benefit and sustainability.

Systems for lab surveillance and reporting of respiratory illnesses have improved since H5N1, which has hit nine sub-Saharan African countries since it first spread to the continent in 2006. With international support Nigeria, for example, has set up a central national laboratory for human influenza surveillance in Abuja, as well as several decentralized satellite labs.

There is no culture of testing for respiratory viruses, however, and the effort that went into H5N1 control is losing steam. The H5N1 virus was perceived as a major threat to the poultry industry, whereas the disease burden of pandemic flu seems low. Don't expect much mobilization for a virus where most cases are mild. ■

Claude P. Muller, head of the Institute of Immunology at the WHO Collaborative Center for Reference and Research on Measles Infections in Luxembourg

Interviews by Declan Butler
See www.nature.com/swineflu for more on pandemic flu.

J Infect Chemother (2009) 15:367–373
DOI 10.1007/s10156-009-0724-6

ORIGINAL ARTICLE

Muneki Hotomi · Dewan S. Billal · Akihisa Togawa
Yorihiko Ikeda · Shin Takei · Masamitsu Kono
Masahi Ogami · Kimiko Ubukata · Rinya Sugita
Keiji Fujihara · Noboru Yamanaka

Distribution of fibronectin-binding protein genes (*prtF1* and *prtF2*) and streptococcal pyrogenic exotoxin genes (*spe*) among *Streptococcus pyogenes* in Japan

Received: January 19, 2009 / Accepted: August 6, 2009

Abstract Two hundred and seventy-two strains of *Streptococcus pyogenes* isolated from patients with invasive and noninvasive infections in Japan were evaluated for the prevalence of fibronectin-binding protein genes (*prtF1* and *prtF2*). The possible associations of the genes with streptococcal pyrogenic exotoxin genes, macrolide resistance genes, and *emm* types were also evaluated. Overall, about 50% of *S. pyogenes* isolates carried fibronectin-binding protein genes. The prevalence of the *prtF1* gene was significantly higher among isolates from noninvasive infections (71.4%) than among isolates from invasive infections (30.8%; $P = 0.0037$). Strains possessing both the *prtF1* and *prtF2* genes were more likely to be isolates from noninvasive infections than isolates from invasive infections (50.6% vs 15.4%; $P = 0.019$). *S. pyogenes* isolates with streptococcus pyrogenic exotoxin genes (*speA* and *speZ*) were more common among isolates without fibronectin-binding protein genes. The *speC* gene was more frequently identified among isolates with fibronectin-binding protein genes ($P = 0.05$). Strains belonging to *emm*75 or *emm*12 types more frequently harbored macrolide resistance genes than other *emm* types ($P = 0.0094$ and $P = 0.043$, respectively). Strains carrying more than one repeat at the RD2 region of the *prtF1* gene and the FBRD region of the *prtF2* gene were more prevalent among strains with macrolide resistance genes than among strains negative for macrolide resistance genes. These genes (i.e., the *prtF1*, *prtF2*, and *spe* genes) may enable host-bacteria interaction, and internalization in the host cell, but may not enable infection complications such as invasive diseases.

Key words *Streptococcus pyogenes* · *prtF1* gene · *prtF2* gene · Macrolide resistance · *spe* gene · *emm* type

M. Hotomi · D.S. Billal · A. Togawa · Y. Ikeda · S. Takei · M. Kono · M. Ogami · K. Fujihara · N. Yamanaka (✉)
Department of Otolaryngology–Head and Neck Surgery, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8509, Japan
Tel. +81-73-441-0651; Fax +81-73-446-3846
e-mail: ynobi@wakayama-med.ac.jp

K. Ubukata
Laboratory of Molecular Epidemiology for Infectious Agents, Graduate School of Infection Control Sciences, Kitasato University, Tokyo, Japan

R. Sugita
Sugita ENT Clinic, Chiba, Japan

Introduction

Streptococcus pyogenes is a major etiological agent causing various infectious diseases; both noninvasive diseases, such as acute otitis media (AOM) and pharyngo-tonsillitis, and invasive diseases, such as toxic shock syndrome, bacteremia, purulent arthritis, rheumatic fever, and necrotizing fasciitis.[1] Although *S. pyogenes* has long been considered an extracellular pathogen that adheres to human mucosal epithelium, some isolates possess invasive capacity for cultured human epithelial cells.[2] Penicillins were reported to fail to eradicate *S. pyogenes* in up to 30% of patients with pharyngo-tonsillitis. The intracellular localization of *S. pyogenes* is a good explanation for antimicrobial treatment failure in *S. pyogenes* infections.[3–5]

Surface proteins have been considered as virulence factors of *S. pyogenes*, and more than a dozen surface proteins may be involved in the adherence and intracellular invasion of this pathogen.[2,6,7] Fibronectin-binding proteins encoded by the *prtF1* and *prtF2* genes have been shown to be important adhesins for the binding to the extracellular matrix of respiratory epithelial cells that results in promoting the entry of *S. pyogenes* into the cells.[8] Although the *prtF1* gene was shown to be prevalent among *S. pyogenes* strains persisting among asymptomatic carriers, a recent study showed no association between the *prtF1* gene and the source of isolates such as those obtained from invasive disease and those obtained from the throat swabs of asymptomatic carriers.[9,10] In Japan, about 77.3 % of *S. pyogenes* isolates from the throat swabs of patients with pharyngitis were reported to harbor the *prtF1* gene.[11] The prevalence of the *prtF2* gene was also not different among strains

Table 1. Primers used in this study for gene profiles

Gene	Primer sequence, 5′ → 3′		Annealing temperature	PCR product	Reference
	Forward primer	Reverse primer			
Streptococcus pyrogenic exotoxin genes					
speA	TAA GAA CCA AGA GAT GG	ATT CTT GAG CAG TTA CC	44°C	248	31
speB	AAG AAG CAA AAG ATA GC	TGG TAG AAG TTA CGT CC	44°C	955	31
speC	GAT TTC TAC TTA TTT CAC C	AAA TAT CTG ATC TAG TCC C	44°C	584	31
speF	TAC TTG GAT CAA GAC G	GTA ATT AAT GGT GTA GCC	44°C	782	31
speG	AGA AAC TTA TTT GCC C	TAG TAG CAA GGA AAA GG	44°C	155	31
speH	AGA TTG GAT ATC ACA GG	CTA TTC TCT CGT TAT TGG	44°C	416	31
speJ	ATC TTT CAT GGG TAC G	TTT CAT GTT TAT TGC C	44°C	536	31
speZ	TAA CTC CTG AAA AGA GGC T	CAT TGG TTC TTC TTG ATA AG	44°C	391	31
ssa	GTG TAG AAT TGA GGT AAT TG	TAA TAT AGC CTG TCT CGT AC	44°C	706	31
Fibronectin-binding protein genes					
prtF1	TTT TCA GGA AAT ATG GTT GAG ACA	TCG CCG TTT CAC TGA AAC CAC TCA	60°C	125	36
prtF2	GAA GAA AAG CTT CCA GAC GAG CAA GG	GGA ATC TCA GAG TTA CTT TCT GGT TCC	62°C	250	17
Streptococcus invasive locus genes					
silC	ATA TCT CCA CCA ATC ACT TTA AGT A	ACT ATA AAG ATA AGA TAC TCA ACA GT	55°C	189	17
silD	GAT GAA GTT CGT CAA GCT GACT	TCG GCT ATA GCG ATA CGT TTA ATC	55°C	148	17

isolated from asymptomatic carriers and strains isolated from patients with pharyngitis.

Streptococcus pyrogenic exotoxins, a family of highly mitogenic proteins, encoded by *spe* genes, are secreted by the vast majority of *S. pyogenes*, have very potent activities as superantigens, and play key roles in disease manifestation.[12] However, there is no study regarding the prevalence of *prtF1* and *prtF2* genes and the distribution of *spe* genes in Japan.

In this study, we evaluated the possible association between specific patterns of fibronectin-binding protein genes and the source of infections in noninvasive and invasive diseases in a Japanese population. We further examined the relationship among fibronectin-binding protein genes, streptococcus pyrogenic exotoxin genes, and the streptococcal invasive locus gene (*sil* gene). The relationship among fibronectin-binding protein genes, *emm* types, and macrolide resistance genes was also investigated.

Materials and methods

Strains

We evaluated 259 *S. pyogenes* isolates from individual patients with noninvasive diseases (tonsillitis, 168 isolates; rhinosinusitis, 51 isolates; acute otitis media (AOM), 38 isolates; conjunctivitis, 2 isolates) and 13 *S. pyogenes* isolates from individual patients with invasive diseases (septicemia, 5 isolates; purulent arthritis, 4 isolates; meningitis, 2 isolates; necrotizing fasciitis, 1 isolate; peritoneal abscess, 1 isolate). All strains were isolated between 2002 and 2004 in different regions of Japan. The patients were 128 males and 144 females, ranging in age from 1 to 72 years (mean ± SD, 21.3 ± 16.8 years). *S. pyogenes* was identified on the basis of β-hemolysis, Gram's stain, latex agglutination with a commercially available latex reagent (BioMérieux SA, Marcy l'Etoile, France), the PYR test (Nippon Becton Dickinson, Tokyo, Japan), the rapid ID 32 Strepto test (BioMérieux), and bacitracin (Oxoid, Cambridge, UK) susceptibility. All strains were stored at −80°C in Todd-Hewitt medium (Difco Laboratories, Detroit, MI, USA) until the study.

Polymerase chain reaction (PCR)

Total genomic DNA from *S. pyogenes* isolates was prepared by lysis and ethanol precipitation methods. In brief, *S. pyogenes* isolates were lysed by using lysis solution containing 1 M Tris pH 8.9, 4.5% nondent-P-40, 4.5% Tween-20, and 10 mg/ml proteinase K, and genomic DNA was precipitated by ethanol methods. The target gene was amplified in a DNA thermal cycler (Gene Amp PCR System 9700: Parkin Elmer, Norwalk, CT, USA). The primers, the annealing temperatures, and the PCR product size for the *prtF1* gene,[13,14] the *prtF2* gene,[13,15] and the *spe* genes[12] are listed in Table 1. Macrolide resistance genes (*mefA*, *ermB*, and *ermTR*) of *S. pyogenes* were determined by the PCR

Table 2. Prevalence of fibronectin-binding protein genes according to the source of the isolates

Source	Numbers of isolates	*prtF1* gene: Frequencies of RD2 repeats					Total		*prtF2* gene: Frequencies of FBRD repeats			Total		*prtF1* and *prtF2* genes: *prtF1* gene, Frequencies of RD2 repeats					*prtF2* gene: Frequencies of FBRD repeats			Total		Absent	
		1	2	3	4	6			1	2	3			1	2	3	4	6	1	2	3				
Invasive	13	2				0	2	15.4%	1	1	1	3	23.1%	2	0	0	0	0	2	0	0	2	15.4%	6	46.2%
Noninvasive	259	11	10	10	23	0	54	20.8%	4	23	8	35	13.5%	45	24	15	47	0	26	105	0	131	50.6%	39	15.2%
Total	272						56	20.6%				38	13.9%									133	49.0%	45	16.5%

methods described by Weber et al.[16] PCR detection of the streptococcal invasive locus (*sil*) gene was performed according to the method described by Bidet et al.[17] The products were analyzed by gel electrophoresis in a 2% (wt/vol) agarose gel. All the PCR experiments were conducted in duplicate.

Sequencing

The number of RD2 repeats of the *prtF1* gene and FBRD repeats of the *prtF2* gene was confirmed by the direct sequencing of several representative PCR amplified products using an Applied Bio-systems sequencing kit and ABI Prism 310 Genetic Analyzer (Applied Bio-systems, Carlsbad, CA, USA).

The *emm* typing of *S. pyogenes* was performed by DNA sequencing according to the recommendations of the Division of Bacterial and Mycotic Diseases, the Centers for Disease Control and Prevention (CDC) and the *emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm).

Statistical analysis

Comparisons between two groups were analyzed by Fisher's exact test. A P value of <0.05 was considered statistically significant. Calculations were performed using the statistical software package Prism 4 (GraphPad Software, Inc., Ja Lolla, CA, USA).

Results

Prevalence of fibronectin-binding protein genes according to sources of the isolates

Among the 13 isolates from invasive infections, *prtF1*, *prtF2*, and both genes were identified in 2 (15.4%), 3 (23.1%), and 2 (15.4%) isolates, respectively (Table 2). On the other hand, among the 259 isolates from the noninvasive infections, *prtF1*, *prtF2*, and both genes were identified in 54 (20.8%), 35 (13.5%), and 131 (50.5%) isolates, respectively. The *prtF1* and *prtF2* genes were more prevalent among isolates from noninvasive infections than among isolates from invasive infections (84.8% vs 53.8%; $P = 0.013$). The prevalence of the *prtF1* gene among isolates from noninvasive infections was greater than that among isolates from invasive infections (71.3% vs 30.8%; $P = 0.0037$), while there was no difference in the prevalence of the *prtF2* gene according to the source of the isolates 64.0% vs 38.5%; $P = 0.0788$). The prevalence of strains possessing both genes was significantly higher among isolates from noninvasive disease than among isolates from invasive diseases (50.5% vs 15.4%; $P = 0.019$).

The 129 (69.7%) of 185 isolates with the *prtF1* gene from noninvasive infections carried more than one repeat in the RD2 domain, while no isolates with the *prtF1* gene from

Table 3. Relationship of fibronectin-binding protein genes with streptococcal pyrogenic exotoxin genes and streptococcal invasive locus genes

		Numbers (%) of isolates with							
		Streptococcal pyrogenic exotoxin genes						Streptococcal invasive locus	
		speA	*speB*	*speC*	*speG*	*speH*	*speZ*	*silC*	*silD*
prtF1 gene									
Positive	189	21 (11.1%)	189 (100%)	143 (75.7%)	3 (1.6%)	1 (0.5%)	9 (4.8%)	26 (13.8%)	26 (13.8%)
Negative	83	25 (30.1%)	83 (100%)	43 (51.8%)	8 (9.6%)	2 (2.4%)	20 (24.0%)	9 (10.8%)	9 (10.8%)
prtF2 gene									
Positive	171	19 (11.1%)	171 (100%)	120 (70.2%)	5 (2.9%)	1 (0.6%)	8 (4.7%)	20 (11.7%)	20 (11.7%)
Negative	101	27 (26.7%)	101 (100%)	66 (65.3%)	6 (5.9%)	2 (2.0%)	21 (20.8%)	15 (14.9%)	15 (14.9%)
Total	272	46 (16.9%)	272 (100%)	186 (68.4%)	11 (4.0%)	3 (1.1%)	29 (10.7%)	35 (12.9%)	35 (12.9%)

invasive infections carried more than one repeat in the RD2 domain ($P = 0.0095$). In contrast, no significant differences were found in the numbers of repeats in the FBDR domain of the *prtF2* gene carried by isolates from invasive or non-invasive infections ($P = 1.00$).

Relationship of fibronectin-binding protein genes with streptococcal pyrogenic exotoxin genes and streptococcal invasive locus genes

Out of 189 *S. pyogenes* isolates with the *prtF1* gene, 21 (11.1%), 189 (100%), 143 (75.7%), 3 (1.6%), 1 (0.5%), and 9 (4.8%) isolates were positive for the *speA*, *speB*, *speC*, *speG*, *speH*, and *speZ* genes, respectively, compared with 25 (30.1%), 83 (100%), 43 (51.8%), 8 (9.6%), 2 (2.4%), and 20 (24.0%) isolates, respectively, in the 83 *prtF1*-negative isolates (Table 3). The differences in the prevalence of the *speA*, *speG*, and *speZ* genes between *the prtF1*-positive and *prtF1*-negative strains were statistically significant ($P = 0.0003$, $P = 0.0041$, and $P = 0.0001$, respectively). In contrast, the *speC* gene was predominant among the *prtF1*-positive strains (75.6%) compared with the *prtF1*-negative isolates ($P = 0.0002$).

Of 171 *S. pyogenes* isolates with the *prtF2* gene, 19 (11.1%), 171 (100%), 120 (70.2%), 5 (2.9%), 1 (0.6%), and 8 (4.7%) isolates were positive for the *speA*, *speB*, *speC*, *speG*, *speH*, and *speZ* genes, respectively, compared with 27 (26.7%), 101 (100%), 66 (65.3%), 6 (5.9%), 2 (2.0%), and 21 (20.8%) isolates, respectively, in 101 *prtF2*-negative isolates (Table 3). The prevalence of the *speA* and *speZ* genes among the *prtF2*-positive isolates was higher than that among the *prtF2*-negative isolates ($P = 0.013$ and $P = 0.0001$, respectively).

The overall prevalences of the *speA*, *speB*, *speC*, *speG*, *speH* and *speZ* genes were 16.9%, 100%, 68.4%, 4.0%, 1.1%, and 10.7%, respectively. The *speF*, *speJ*, and *ssa* genes were not identified.

The prevalences of the *silC* and *silD* genes among *prtF1*- and *prtF2*-positive isolates were similar to those among *prtF1*- and *prtF2*-negative isolates.

Relationship between fibronectin-binding protein genes and *emm* types

The 272 *S. pyogenes* isolates belonged to 32 *emm* types. The predominant *emm* types were *emm*12 (56 isolates; 20.6%), *emm*1 (39 isolates; 14.3%), *emm*75 (31 isolates; 11.4%), *emm*28 (22 isolates; 8.1%), *emm*4 (16 isolates; 5.9%), *emm*11 (15 isolates, 5.5%), *emm*89 (15 isolates; 5.5%), and *emm*58 (10 isolates; 3.7%). These eight *emm* types accounted for 75.0% of the total *S. pyogenes* isolates (Table 4).

Isolates belonging to *emm*58 (80.0%), *emm*75 (64.5%), and *emm*4 (50.0%) types possessed the *prtF1* gene alone. In contrast, only isolates belonging to *emm*89 (13.3%) possessed the *prtF2* gene. Isolates belonging to *emm*11 (86.7%), *emm*89 (86.7%), *emm*28 (81.8%), *emm*12 (80.4%), and *emm*4 (43.8%) types possessed both the *prtF1* and *prtF2* genes. About 79.5% of the *emm*1 type isolates did not possess either the *prtF1* gene or the *prtF2* gene (Table 4).

Relationship of macrolide resistance genes with fibronectin-binding protein genes and *emm* types

A total of 47 (17.3%) strains had either the *mefA*, *ermB*, or *ermTR* genes. Thirty-one of these strains (66.0%) had the *mefA gene*, 6 (12.8%) had the *ermB gene*, and 10 (21.2%) had the *ermTR* gene. The strains having macrolide resistance genes had a higher prevalence of the *prtF1* gene ($P = 0.0356$), while there was no association between the macrolide resistance genes and the *prtF2* gene ($P = 0.2492$). Strains belonging to the *emm*75 and *emm*12 types harbored macrolide resistance genes at a greater prevalence than other *emm* types ($P = 0.0094$ and $P = 0.043$, respectively). Strains that carried more than one repeat at the RD2 domain of *prtF1* and more than one repeat at the FBRD domain of *prtF2* were more prevalent among isolates with macrolide resistance genes than among isolates without macrolide resistance genes ($P = 0.0031$ and $P = 0.05$, respectively) (Table 5).

Table 4. Relationship between fibronectin-binding protein genes and *emm* types

	prtF1	*prtF2*	Both	None	Total
Source of isolates					
Invasive	2	3	2	6	13
Noninvasive	54	35	131	39	259
emm type					
1	2	2	4	31	39
2			2	1	3
3		8	1		9
4	8	1	7		16
6	1		4	1	6
9			2		2
11	1	1	13		15
12	1	8	45	2	56
18		1			1
22	4	1	2		7
28	2	2	18		22
44			1		1
48	3		1		4
49			1		1
53		1			1
54				1	1
58	8	1		1	10
63	1				1
68			3		3
73	1		4		5
75	20	1	3	7	31
77		1	1		2
80	1	1			2
87			1		1
89		2	13		15
94		5	1		6
102			1		1
103			1		1
104	1				1
112			1		1
113			1		1
st815			1		1
st3211		1		1	2
stL62		1	1		2
st1731	1				1
1759	1				1
Total	56	38	133	45	272

Table 5. Macrolide resistance gene and its relationship with FBP and *emm* type among GAS isolates

Macrolide resistance	*emm* types										Total
	1	4	9	11	12	28	58	75	77	113	
mefA											
prtF1						2		1			3
prtF2								9			9
Both	1		1	2	10						14
None	4							1			5
ermB											
prtF1							1				1
prtF2						1					1
Both	1				1	2					4
None											0
ermTR											
prtF1							2				2
prtF2		1									1
Both					4				1	1	6
None							1				1

FBP, fibronectin-binding protein; GAS, group A streptococcus

Discussion

Surface proteins such as fibronectin-binding proteins represent important virulence factors for *S. pyogenes*.[18] Binding to extracellular matrix proteins supports the persistent colonization of *S. pyogenes* on mucosal surfaces. The *prtF1* gene has been identified in a high percentage (77.3%) of strains from patients with pharyngitis.[12] In the present study, the proportion of isolates carrying the *prtF1* gene was higher among strains from noninvasive infections than among strains from invasive infections, although the prevalence of isolates with the *prtF1* gene among strains from invasive infections varied between 50% and 65% in other studies.[3,6,8,10,19-21] In contrast, the carriage rate of the *prtF2* gene was comparable to the proportion generally reported in other studies.[10,22-24] Isolates carrying the *prtF1* and *prtF2* genes simultaneously were more prevalent among strains from noninvasive infections than among strains from invasive infections.[10,22-24] Genomic analysis of the *prtF1* and *prtF2* genes has shown that both genes are inherited either within a potential pathogenicity island in the serotype M12 or at another locus on the genome of serotypes M3 and M18 as a potential consequence of genome-scale recombination events.[25-27] In the present study, the isolates with the *prtF1* gene from noninvasive infections had more RD2 and FBRD repeats than the isolates from invasive infections. However, the repeat numbers in the *prtF1* gene were unrelated to the ability to bind fibronectin. Repeat variations, such as the repeat variations in M-protein of *emm* type 6, may alter *S. pyogenes* to enable it to escape the immune system during binding to epithelial cells.[28,29] Of interest, in our study, *S. pyogenes* isolates harboring *speA* and *speZ* were more frequent among strains without the *prtF1* and *prtF2* genes, in contrast to findings for *speC*. Streptococcal pyrogenic exotoxins cause potent inflammatory responses and tissue damage. The *speA* and *smeZ* genes are considered to be correlated with the development of severe complications of diseases.[13] In contrast to the distribution of the *speA* and *speZ genes*, we found no difference regarding the distribution of the *sil* gene among *prtF1*- and *prtF2*-positive isolates. These finding suggest that the *prtF1* and *prtF2* genes may contribute mainly to the binding of *S. pyogenes* to the extracellular matrix of the host cell rather than contributing to disease complications. Although a previous study supported the idea that internalization has a potential role in the early stages of invasive diseases, recent studies have demonstrated that invasive isolates were internalized less efficiently than strains derived from patients with skin or throat infections.[30-32] However, the biological significance of the internalization of *S. pyogenes* is not clear. The strains bearing the *prtF1* and/or the *prtF2* gene might be better colonizers of the human host.

In order to determine whether particular virulent clones were circulating within Japanese populations, we further evaluated the presence of macrolide resistance genes and *emm* types in relation to the *prtF1* and *prtF2* genes. Traditionally, *S. pyogenes* has been classified on the basis of serotype diversity of the M-protein. Because of the methodological limitation of serotyping, DNA sequence-based methods for *emm* gene (*emm* typing) have been applied for characterizing *S. pyogenes*. The use of *emm* typing has allowed the recognition of several previously unknown types in different geographic areas, demonstrating the usefulness of this procedure for detecting genetic diversity among *S. pyogenes* isolates. The association between *spe* genes and *emm* type in *S. pyogenes* has also been reported.[24] In the present study, the predominant *emm* types were *emm*12, *emm*1, *emm*75, *emm*28, *emm*4, *emm*11, *emm*89, and *emm*58.[33] There was a peculiar association of *erm* genes with *prtF1* and *prtF2* genes.[8,10,34,35] The *emm*75 strains were more likely to possess *prtF1*, whereas strains belonging to other *emm* types were more likely to possess the *prtF1* and *prtF2* genes.[10,11] Macrolide resistance was reported to be detected at a higher incidence in noninvasive infections than in invasive infections. Most of the isolates with *erm* genes in the present study possessed either the *prtF1* or the *prtF2* genes. Among the isolates with the *mefA* gene, we found strains with the *prtF2* gene at a prevalence of 29.0% and both the *prtF1* and *prtF2* genes at a prevalence of 45.2%, comparable with results in previous studies regarding a nonconsistent association between the *prtF1* and *mefA* genes.[8,10] Facinelli et al.[8] observed a strong association of *erm* genes with the *prtF1* gene, while there was a less consistent association of *erm* genes with *mefA*. A recent study reported that *prtF1*-negative macrolide-susceptible or *mef*A-carrying isolates, which were poorly equipped to enter cells, produced more biofilm than macrolide-resistant *S. pyogenes*.[10,20,36]

The current observations about fibronectin-binding protein genes, streptococcus pyrogenic exotoxin genes, and macrolide resistance genes associated with *emm* genes indicates a clonal spread of particular clones across populations,[12] suggesting an increased possibility that noninvasive strains will be involved in the acquisition of antibiotic-resistance determinants by interspecies recombination. Not only virulence traits but also antibiotic resistance, especially to macrolides, can enhance bacterial fitness and can be responsible for treatment failures.

Conflict of interest None to declare.

References

1. Cunningham MW. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev 2000;13:470–511.
2. Bisno AL, Brito MO, Collins CM. Molecular basis of group A streptococcal virulence. Lancet Infect Dis 2003;3:191–200.
3. Hanski E, Caparon M. Protein F, a fibronectin-binding protein, is an adhesin of the group A streptococcus *Streptococcus pyogenes*. Proc Natl Acad Sci USA 1992;89:6172–6.
4. Markowitz M, Gerber MA, Kaplan L. Treatment of streptococcal pharyngotonsillitis: reports of penicillin's demise are premature. J Pediatr 1993;123:79–85.
5. Pichichero ME. The rising incidence of penicillin treatment failures in group A streptococcal tonsillopharyngitis: an emerging role for the cephalosporins? Pediatr Infect Dis J 1991;10: S50–5.
6. Courtney HS, Hasty DL, Dale JB. Molecular mechanisms of adhesion, colonization, and invasion of group A streptococci. Ann Med 2002;34:77–87.
7. Joh D, Wann E, Kreikemeyer B, Speziale P, Hook M. Role of fibronectin binding MSCRAMMs in bacterial adherence and entry into mammalian cells. Matrix Biol 1999;18:211–23.
8. Facinelli B, Spinaci C, Magi G, Giovanetti E, Varaldo PE. Association between erythromycin resistance and ability to enter human respiratory cells in group A streptococci. Lancet 2001;358:30–3.
9. Neeman R, Keller N, Barzilai A, Korenman Z, Sela S. Prevalence of internalization-associated gene, *prtF1*, among persisting group A streptococcus strains isolated from asymptomatic carriers. Lancet 1998;352:1974–7.
10. Baldassarri L, Creti R, Imperi M, Recchia S, Pataracchia M, Orefici G. Detection of genes encoding internalization-associated proteins in *Streptococcus pyogenes* isolates from patients with invasive diseases and asymptomatic carriers. J Clin Microbiol 2007;45: 1284–7.
11. Ma X, Kikuta H, Ishiguro N, Yoshida M, Ebihara T, Murai T, et al. Association of the *prtF1* gene (encoding fibronectin-binding protein F1) and the *sic* gene (encoding the streptococcal inhibitor of complement) with *emm* types of group A streptococci isolated from Japanese children with pharyngitis. J Clin Microbiol 2002;40: 3835–7.
12. Schmitz FJ, Beyer A, Charpentier E, Normark BH, Schade M, Fluit AC, et al. Toxin-gene profile heterogeneity among endemic invasive European group A streptococcal isolates. J Infect Dis 2003;188:1578–86.
13. Vlaminckx BJM, Mascini EM, Schellekens J, Schouls LM, Paauw A, Fluit AC, et al. Site-specific manifestations of invasive group A streptococcal diseases: type distribution and corresponding patterns of virulence determinants. J Clin Microbiol 2003;41:4941–9.
14. Terao Y, Kawabata S, Nakata M, Nakagawa I, Hamada S. Molecular characterization of a novel fibronectin-binding protein of *Streptococcus pyogenes* strains isolated from toxic shock-like syndrome patients. J Biol Chem 2002;277:47428–35.
15. Jaffe J, Natanson-Yaron S, Caparon MG, Hanski E. Protein F2, a novel fibronectin-binding protein from *Streptococcus pyogenes*, possesses two binding domains. Mol Microbiol 1996;21:373–84.
16. Weber P, Filipecki J, Bingen E, Fitoussi F, Goldfarb G, Chauvin JP, et al. Genetic and phenotypic characterization of macrolide resistance in group A streptococci isolated from adults with pharyngo-tonsillitis in France. J Antimicrob Chemother 2001;48: 291–94.
17. Bidet P, Courroux C, Salgueiro C, Carol A, Mariani-Kurkdjian P, Bonacorsi S, Bingen E. Molecular epidemiology of the *sil* streptococcal invasive locus in group A streptococci causing invasive infections in French children. J Clin Microbiol 2007;45:2002–4.
18. Molinari G, Talay SR, Valentin-Weigand P, Rohde M, Chhatwal GS. The fibronectin-binding protein of *Streptococcus pyogenes*, SfbI, is involved in the internalization of group A streptococci by epithelial cells. Infect Immun 1997;65:1357–63.
19. Delvecchio A, Currie BJ, McArthur JD, Walker MJ, Sriprakash KS. *Streptococcus pyogenes* prtFII, but not sfbI, sfbII, or fbp54, is represented more frequently among invasive-disease isolates of tropical Australia. Epidemiol Infect 2002;128:391–96.
20. Musumeci R, Lo Bue C, Milazzo I, Nicoletti G, Serra A, Speciale A, Blandino G. Internalization-associated proteins among *Streptococcus pyogenes* isolated from asymptomatic carriers and children with pharyngitis. Clin Infect Dis 2003;37:173–9.
21. Kreikemeyer B, Beckert S, Braun-Kiewnick A, Podbielski A. Group A streptococcal *rofA*-type global regulators exhibit a strain-specific genomic presence and regulation pattern. Microbiology 2002;148:1501–11.
22. Goodfellow AM, Hibble M, Talay SR, Kreikemeyer B, Currie BJ, Sriprakash KS, Chhatwal GS. Distribution and antigenicity of fibronectin-binding proteins (*SfbI* and *SfbII*) of *Streptococcus pyogenes* clinical isolates from the Northern Territory, Australia. J Clin Microbiol 2000:38:389–92.
23. Kreikemeyer B, Oehmcke S, Nakata M, Hoffrogge R, Podbielski A. *Streptococcus pyogenes* fibronectin-binding protein F2: expression profile, binding characteristics, and impact on eukaryotic cell interactions. J Biol Chem 2004;279:15850–9.
24. Bessen DE, Kalia A. Genomic localization of a T serotype locus to a recombinatorial zone encoding extracellular matrix-binding proteins in *Streptococcus pyogenes*. Infect Immun 2002;70: 1159–67.

25. Nakagawa I, Kurokawa K, Yamashita A, Nakata M, Tomiyasu Y, Okahashi N, et al. Genome sequence of an M3 strain of *Streptococcus pyogenes* reveals a large-scale genomic rearrangement in invasive strains and new insights into phage evolution. Genome Res 2003;13:1041–55.
26. Smoot JC, Barbian KD, Van Gompel JJ, Smoot LM, Chaussee MS, Sylva GL, et al. Genome sequence and comparative microarray analysis of serotype M18 group A streptococcus strains associated with acute rheumatic fever outbreaks. Proc Natl Acad Sci USA 2002;99:4668–73.
27. Jones KF, Hollingshead SK, Scott JR, Fischetti VA. Spontaneous M6 protein size mutants of group A streptococci display variation in antigenic and opsonogenic epitopes. Proc Natl Acad Sci USA 1988;85:8271-5.
28. Spinaci C, Magi G, Zampaloni C, Vitali LA, Paoletti C, Catania MR, et al. Genetic diversity of cell-invasive erythromycin-resistant and -susceptible group A streptococci determined by analysis of the RD2 region of the *prtF1* gene. J Clin Microbiol 2004;42: 639–44.
29. Tanaka D, Gyobu Y, Kodama H, Isobe J, Hosorogi S, Hiramoto Y, et al. *emm* Typing of group A streptococcus clinical isolates: identification of dominant types for throat and skin isolates. Microbiol Immunol 2002;46:419–23.
30. Jadoun J, Ozeri V, Burstein E, Skutelsky E, Hanski E, Sela S. Protein F1 is required for efficient entry of *Streptococcus pyogenes* into epithelial cells. J Infect Dis 1998;178:147–58.
31. Molinari G, Chhatwal GS. Invasion and survival of *Streptococcus pyogenes* in eukaryotic cells correlates with the source of the clinical isolates. J Infect Dis 1998;77:1600–7.
32. Brandt CM, Allerberger F, Spellerberger B, Holland R, Lutticken R, Haase G. Characterization of consecutive *Streptococcus pyogenes* isolates from patients with pharyngitis and bacteriological treatment failure: special reference to *prt*F1 and sic/drs. J Infect Dis 2001;183:670–4.
33. Billal DS, Hotomi M, Yamauchi K, Fujihara K, Tamura S, Kuki K, et al. Macrolide-resistant genes of *Streptococcus pyogenes* isolated from the upper respiratory tract by polymerase chain reaction. J Infect Chemother 2004;10:115–20.
34. Hotomi M, Billal DS, Shimada J, Yamauchi K, Fujihara K, Yamanaka N; The Surveillance Subcommittee of the Japan Society for Infectious Diseases in Otolaryngology. Current status of antimicrobial susceptibility of clinical isolates of *Streptococcus pyogenes* in Japan: report of a countrywide surveillance study. J Infect Chemother 2005;11:48–51.
35. LaPenta D, Rubens C, Chi E, Cleary PP. Group A streptococci efficiently invade human respiratory epithelial cells. Proc Natl Acad Sci USA 1994;91:12115–9.
36. Baldassari L, Creti R, Recchia S, Imperi M, Facinelli B, Giovanetti E, et al. Therapeutic failures of antibiotics used to treat macrolide-susceptible *Streptococcus pyogenes* infections may be due to biofilm formation. J Clin Microbiol 2006;44:2721–7.

Vaccine 27 (2009) 3181–3188

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Intranasal immunization with a mixture of PspA and a Toll-like receptor agonist induces specific antibodies and enhances bacterial clearance in the airways of mice

Keita Oma[a,c], Jizi Zhao[a], Hirokazu Ezoe[a], Yukihiro Akeda[a], Shohei Koyama[d], Ken J. Ishii[b], Kosuke Kataoka[e], Kazunori Oishi[a,*]

[a] *Laboratory for Clinical Research on Infectious Diseases, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan*
[b] *Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan*
[c] *The Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8523, Japan*
[d] *Respiratory Oncology and Molecular Medicine, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Miyagi, Japan*
[e] *Department of Preventive Dentistry, Institute of Health Bioscience, The University of Tokushima, Tokushima 770-8504, Japan*

ARTICLE INFO

Article history:
Received 29 September 2008
Received in revised form 16 March 2009
Accepted 19 March 2009
Available online 8 April 2009

Keywords:
PspA
TLR agonist
Mucosal adjuvant
Nasal immunization
Pneumococcal pneumonia
Pneumococcal colonization

ABSTRACT

To develop an effective nasal vaccine for *Streptococcus pneumoniae*, the effects of a panel of Toll-like receptor (TLR) agonists in combination with pneumococcal surface protein A (PspA) on induction of PspA-specific antibodies and bacterial clearance were compared in mice. Mice were nasally immunized with 10 μg of TLR agonist (TLR 2–4 and 9) and 2.5 μg of PspA once per week for 3 weeks. Significantly increased levels of PspA-specific immunoglobulin G (IgG) and IgA in the airways and PspA-specific IgG in plasma were found in mice administered PspA plus each TLR agonist, compared with mice administered PspA alone. In a sub-lethal pneumonia model using a serotype 3 pneumococcal strain, bacterial density in the lungs of mice was significantly reduced in mice administered PspA plus each TLR agonist, compared with mice administered either PspA alone or phosphate-buffered saline alone 3 h after bacterial challenge. Similarly, enhanced bacterial clearance was found in the nasopharynx of mice administered PspA plus each TLR agonist 1 day after infection with a serotype 19F strain. Our data suggest that PspA-specific antibody induced by nasal immunization with PspA plus TLR agonist is capable of reducing the bacterial load in both the nasopharynx and lungs after challenge with pneumococci with different serotypes. Despite the skewed Th1/Th2 immune responses, the effects of nasal immunization with PspA plus each TLR agonist on bacterial clearances from the lungs 3 h after infection and from nasopharynx 1 day after infection in mice were equivalent.

1. Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is a leading human pathogen causing diseases ranging from otitis media to pneumonia, bacteremia, and meningitis in children and adults. Although pneumococcal conjugate vaccine provides protective immunity against pneumonia as well as invasive disease in infants [1,2], polysaccharide-based vaccines are not ideal because they must include multiple polysaccharide serotypes and do not protect against strains with non-vaccine serotypes [3]. Previous investigators have examined several pneumococcal proteins as potential vaccine candidates with promising results [4–7]. One of these candidates, pneumococcal surface protein A (PspA) is a choline-binding protein tethered to the cell surface through its C-terminal choline-binding repeat region [4]. PspA is present on all pneumococcal strains, and anti-PspA antibody enhances bacterial clearance and induces cross-protection against infection from strains with different serotypes [8]. According to the mapping studies of the major cross-protective epitopes that reside in the ~100 amino acids of the α-helical region, PspAs have been divided into seven clades that constitute three families [9]. PspAs of families 1 and 2 are expressed by >98% of strains. Anti-PspA antibodies overcome the anti-complementary effect of PspA, allowing for increased complement activation and C3 deposition on PspA-bearing bacteria [10,11].

Nasal immunization is the most effective way to induce both mucosal secretory-IgA responses and systemic IgG responses [12]. An appropriate mucosal adjuvant is required to elicit an antigen-specific immune response in both mucosal and systemic compartments [13]. The Toll-like receptor (TLR) family is the best-studied family of pattern recognition receptors, and it recognizes

* Corresponding author. Tel.: +81 6 6879 4253; fax: +81 6 6879 4255.
E-mail address: oishik@biken.osaka-u.ac.jp (K. Oishi).

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.03.055

a broad spectrum of pathogen-associated molecular patterns from different classes of microbes [14]. TLR ligands may stimulate dendritic cells (DC), thereby acting as an effective adjuvant to allow a DC-targeted protein to induce protective CD4 T cell responses at mucosal surfaces [13,14]. The balance of Th1/Th2 immune responses appears to be dependent on each TLR ligand [15]. Th1 immune responses augment IgG2a production, while Th2 immune responses enhance IgG1 and IgE production by B cells [16–18]. The pattern of IgG subclass response may affect the bacterial clearance afforded by such humoral immunity during infections. Two recent studies employing a PspA DNA vaccine [19] and a nasal lactococcal vaccine producing PspA [20] have suggested that the induction of a balanced IgG1/IgG2a response to PspA correlates with an increased protection against pneumococcal infections. Therefore, in this study, we examined the relationship between the Th1- or Th2-associated IgG isotype response and the enhanced bacterial clearance of *S. pneumoniae* from the airways after intranasal immunization using a mixture of PspA plus each TLR 2–4 or 9 agonist in mice.

2. Material and method

2.1. Mice

Female C57BL/6 mice (6–8-week-old) were purchased from Charles River Japan, Kanagawa, Japan. Mice were transferred to microisolators and maintained in horizontal laminar flow cabinets. They were provided sterile food and water in a specific pathogen-free facility. All mice used in these experiments were free of bacterial and viral pathogens.

2.2. Bacterial strains

S. pneumoniae WU2 strain with serotype 3, expressing PspA belongs to family 1, clade 2 and is virulent in mice [21]. *S. pneumoniae* EF3030 strain with serotype 19F is a clinical isolate, expressing PspA belongs to family 1, clade 1, and is relatively avirulent in mice [22]. These strains were kindly provided by Dr. D.E. Briles, University of Alabama at Birmingham.

2.3. Recombinant PspA

PspA used for nasal immunization in this study was recombinant PspA/Rx1 (pUAB055) [5]. The recombinant plasmid pUAB055 containing the 0.9 kb *pspA* gene fragment inserted between the *pelB* leader sequence and the His-tag site in vector pET20b (a gift from Dr. S.K. Hollingshead, University of Alabama at Birmingham) was transformed into *E. coli* strain BL21 (DE3) for protein production. Rx1/PspA is of PspA family 1 (clade 2), which is the same family as both the WU2 strain and EF3030 strains. Induction with isopropylthio-β-D-galactopyranoside (Sigma, St. Louis, MO) resulted in production of 6× His-tagged recombinant PspA. The recombinant PspAs were purified by chromatography chelating-sepharose 4B pre-loaded with Ni^{+} (GE Healthcare, Buckinghamshire, England) according to the manufacturer's instruction. The fraction containing PspA was loaded onto a gel filtration superdex-75 5/30 GL column (GE Healthcare) to further purify the PspA. Contaminated endotoxin was removed from the PspA preparation by using EndoTrap^{R} (Profos AG, Rosenberg, Germany). The purified PspA preparation was analyzed for the presence of endotoxin using a chromogenic *Limulus* lysate endopoint assay, QCL-1000^{R} (Cambrex, Walkersville, MD), and it contained 1.30 ng of LPS per 1 μg of PspA. To remove LPS extensively from the PspA preparations, we used another LPS removal column, ProteoSpin^{R} (Norgen, Thorold, Canada) and prepared the PspA with a lower concentration of LPS (0.048 ng of LPS per 1 μg of PspA).

2.4. Adjuvant

Pam3CSK4 is a synthetic tripalmitoylated lipopeptide that mimics bacterial peptides [23], and is recognized by the TLR2/TLR1 heterodimer. Poly(I:C) is a synthetic analog of double-stranded RNA, a TLR3 agonist [24]. Pam3CSK4, Poly(I:C), and Ultra Pure *Escherichia coli* K12 LPS, a TLR4 agonist, were purchased from InvivoGen (San Diego, CA). CpG DNA ODN1826 (TLR9 ligand, 5′-TCCATGACGTTCCTGACGTT-3′) was purchased from Hokkaido System Science (Sapporo, Japan) [25]. Each of these adjuvants was used in a dose of 10 μg for nasal immunization, because these TLR agonists demonstrated potent adjuvant effects at this dose in mouse experiments [24–26].

2.5. Nasal immunization

Mice were immunized three times at weekly intervals intranasally with 12 μl of phosphate-buffered saline (PBS) containing 10 μg of each TLR agonist and 2.5 μg of PspA, 2.5 μg of PspA alone or 12 μl of PBS alone on day 0, days 7 and days 14. On days 21, mice were euthanized to obtain plasma, bronchoalveolar lavage fluid (BALF) and nasal wash (NW). A dose of 2.5 μg of PspA was employed for nasal immunization in this study, as nasal immunization with this dose of PspA plus 10 μg of each TLR agonist induces PspA-specific antibodies in the airways. A dose of PspA alone for nasal immunization, therefore, contained 3.25 ng of LPS. After removing the mandible, the nasal cavity was gently flushed from the posterior opening of the nose with 1 ml of PBS [27]. The NW flushing out from the anterior openings of the nose was collected. BALF was obtained by irrigation with 1 ml of PBS using of a blunted needle inserted into the trachea after tracheotomy [28].

2.6. PspA-specific antibody assays

PspA-specific antibody titers of IgG, IgG1, IgG2a or IgA in plasma, BALF and NW were determined by ELISA as previously described [28]. The coefficient variation (CV) of the levels of PspA-specific IgG, IgG1, IgG2a or IgA was also determined.

PspA was used as the coating antigen (1 μg/ml). 100 μl of sample was added to each well, followed by incubation at 37 °C for 30 min. The plate was washed, and then reacted with 100 μl of alkaline phosphatase-conjugated goat anti-mouse IgA, IgG, IgG1 or IgG2a (Zymed, San Francisco, CA). The OD at 405 nm was then measured. The end-point titers were expressed as the reciprocal Log_2 of the last dilution giving an OD_{405} of 0.1 OD unit above the OD_{405} of negative control samples obtained from non-immunized mice.

2.7. Pneumonia model

To determine the effects of nasal immunization with PspA plus each TLR agonist, *S. pneumoniae* WU2 strain at a dose of 2.0×10^6 cfu suspended in 30 μl of sterile saline was intranasally administered to both immunized and untreated mice 2 weeks after the last immunization. The 2-week interval between the last immunization and the bacterial challenge was kept to avoid the influence of each TLR agonist on pulmonary defense, as some TLRs are involved in the innate immune response to *S. pneumoniae* [29–31]. The lungs were removed aseptically from mice that had been euthanized with pentobarbital at 3 h, 6 h and 12 h post-bacterial challenge. The lung tissue was homogenized in 9 ml of sterile saline per gram of lung tissue prior to culturing and quantitative bacterial cultures of lung tissue were performed on horse blood agar. The detection limit of bacterial culture of the lung tissue was 10^3 cfu/g. The survival rate after intranasal challenge with 2.0×10^6 cfu of the WU2 strain was 100%.

2.8. Nasal carriage model

S. pneumoniae EF3030 strain at a dose of 3×10^5 cfu in suspended 30 μl of sterile saline was similarly intranasally administered to both immunized and untreated mice 2 weeks after the last immunization. One or 6 days after bacterial challenge, NW was obtained as described above, and a quantitative bacterial culture of the NW was performed.

2.9. Statistics

Statistical analyses were performed using one-way ANOVA and multiple comparison methods by Fisher's LSD. Data were considered to be statistically significant if the *P*-values were less than 0.05. All data were expressed as mean ± S.D.

3. Results

3.1. PspA-specific IgG and IgG isotypes in plasma

Nasal administration of PspA plus Pam3CSK4, Poly(I:C), LPS or CpG1826 significantly increased the levels of PspA-specific IgG in the plasma, compared with administration of PspA alone ($P<0.05$, Fig. 1A). No differences were found in the levels of PspA-specific IgG among mice nasally administered PspA plus each TLR agonist. The CV of the levels of PspA-specific IgG by PspA plus each TLR agonist was much smaller than that induced by PspA alone.

Since the preparation of PspA after removal of LPS with Endotrap contained LPS (3.25 ng per 2.5 μg of PspA), PspA-specific IgG might be elicited by the adjuvant effect of the residual LPS. We then compared the levels of PspA-specific IgG in between the plasma of mice nasally administered 2.5 μg of PspA preparations containing either 3.25 ng of LPS or 0.12 ng of LPS. No significant differences were found in the levels of PspA-specific IgG in plasma of mice after nasal immunization with two different PspA preparations (data not shown). These data suggest the residual LPS did not contribute to the induction of PspA-specific IgG in plasma as an adjuvant, and PspA itself could induce PspA-specific IgG in plasma.

To assess whether each TLR agonist induces either a Th1- or a Th2-associated IgG isotype response, plasma samples were analyzed for PspA-specific IgG1 and IgG2a isotypes (Fig. 1B). Nasal administration of PspA plus Pam3CSK4, Poly(I:C) or LPS significantly increased the levels of PspA-specific IgG1 in plasma, while PspA-specific IgG1 increased to a lesser extent in plasma of mice nasally administered PspA plus CpG1826. The IgG1 levels differed significantly between mice administered PspA plus either Pam3CSK4, Poly(I:C) or LPS and mice administered PspA plus CpG1826 ($P<0.05$, Fig. 1B). Furthermore, PspA-specific IgG1 levels were significantly higher in mice administered PspA plus either Pam3CSK4, Poly(I:C), LPS or CpG1826 than in mice administered PspA alone ($P<0.05$). In contrast, mice nasally administered PspA plus either Poly(I:C) or CpG1826 demonstrated significant increases in the levels of PspA-specific IgG2a in plasma, compared with mice administered PspA plus either Pam3CSK4, LPS or PspA alone ($P<0.01$). The CV of the levels of PspA-specific IgG1 in plasma of mice nasally administered PspA plus each TLR agonist was much smaller than that of mice nasally administered PspA alone. In contrast, the CV of the levels of PspA-specific IgG2a induced by either PspA plus each TLR agonist, except for Poly(I:C), or PspA alone was large in plasma.

3.2. PspA-specific IgG and IgA in BALF and NW

Although the levels of PspA-specific IgG were negligible in the BALF and NW of mice given PspA alone, the levels of PspA-specific IgG were significantly greater in the BALF (Fig. 2A) and NW (Fig. 3A) of mice nasally administered PspA plus either Pam3CSK4, Poly(I:C), LPS or CpG1826 than in mice nasally administered PspA alone ($P<0.05$). A PspA-specific IgG1 response was found in the BALF of mice administered PspA plus either Pam 3CSK4, Poly(I:C), LPS or CpG1826 (Fig. 2C). In contrast, significant increases of PspA-specific IgG2a were also found in the BALF of mice administered PspA plus either Poly(I:C) or PspA plus CpG1826, compared with mice administered PspA plus either Pam3CSK4 or LPS or PspA alone ($P<0.05$, Fig. 2C). However, PspA-specific IgG2a was rarely detected in the BALF of mice administered PspA plus either Pam3CSK4 or

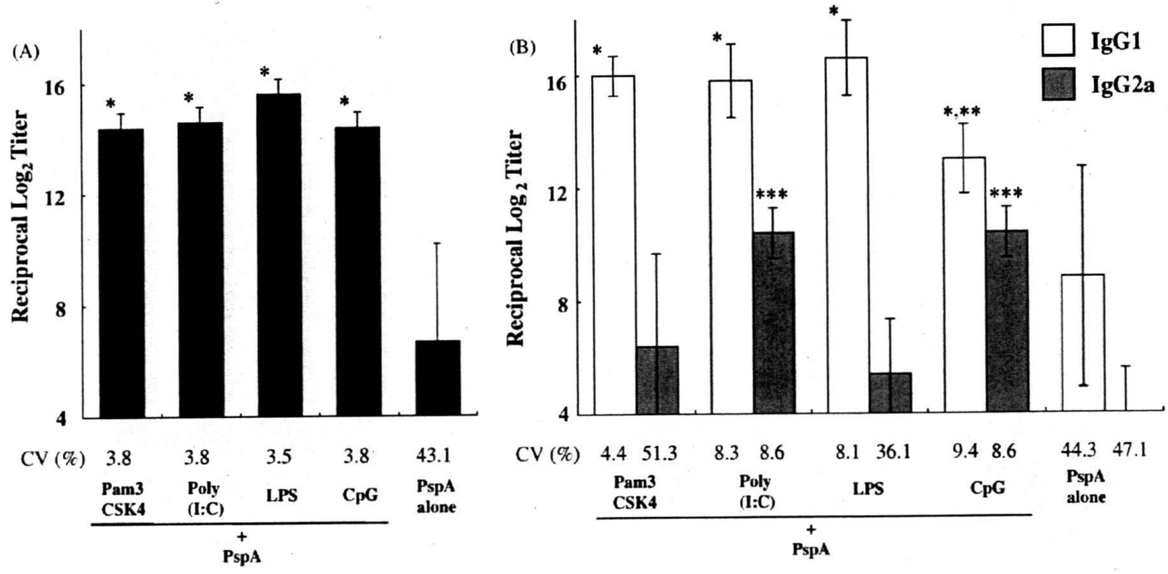


Fig. 1. Induction of PspA-specific IgG (closed bar) (A), PspA-specific IgG1 (open bar) and IgG2a (gray bar) (B) in plasma by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times weekly intervals with 10 μg of TLR agonist and 2.5 μg of PspA. One week after the final immunization, mice were euthanized to obtain plasma, and PspA-specific antibody titers were determined using ELISA. The results are expressed as means ± S.D. for six mice per group. CV, coefficient of variation; LPS, *E. coli* K12 LPS; CpG, CpG DNA ODN1826. *$P<0.05$, when compared with mice nasally administered PspA alone; **$P<0.05$, when compared with mice nasally administered PspA plus either Pam3CSK4, Poly(I:C) or LPS; ***$P<0.05$, when compared with mice nasally administered PspA plus either Pam3CSK4, LPS or PspA alone.