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

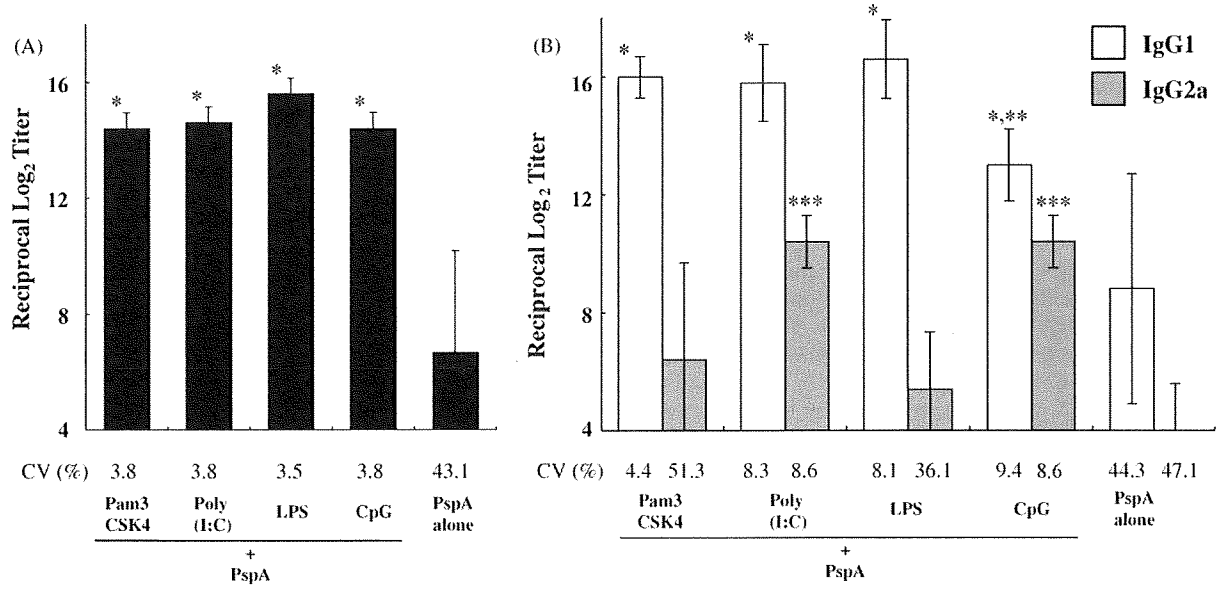


Fig. 1. Induction of PspA-specific IgG (closed bar) (A), PspA-specific IgG1 (open bar) and IgG2a (gray bar) (B) in plasma by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times weekly intervals with 10 μg of TLR agonist and 2.5 μg of PspA. 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.

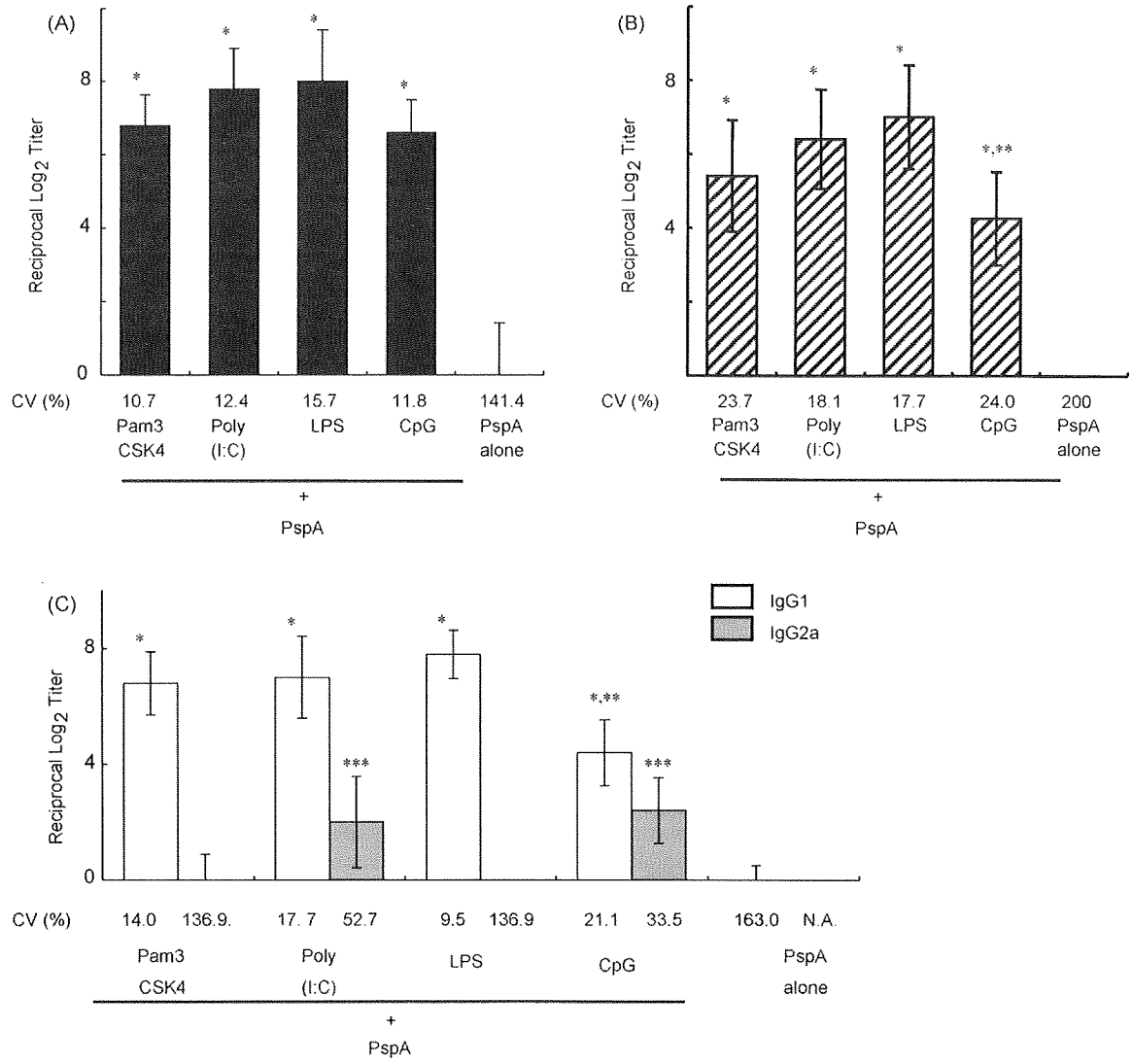


Fig. 2. Induction of PspA-specific IgG (closed bar) (A), IgA (hatched bar) (B) and PspA-specific IgG1 (open bar) and IgG2a (gray bar) (C) in bronchoalveolar lavage fluid (BALF) by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times weekly with 10 μg of TLR agonist and 2.5 μg of PspA. One week after the final immunization, mice were euthanized to obtain BALF and NW, 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; N.A., not available; 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 nasally administered PspA plus either Pam3CSK4, LPS or PspA alone.

LPS or PspA alone. Mice nasally administered PspA plus Pam3CSK4, Poly(I:C), LPS or CpG1826 demonstrated significant increases in the levels of PspA-specific IgA in the BALF (Fig. 2B) and NW (Fig. 3B), compared with mice nasally administered PspA alone ($P < 0.05$). The levels of PspA-specific IgA were significantly lower in the BALF of mice administered PspA plus CpG1826 than in mice administered PspA plus either Poly(I:C) or LPS ($P < 0.05$). The CV of the levels of PspA-specific IgG or IgA induced in the BALF by PspA plus each TLR agonist was similarly much smaller than that induced by PspA alone. A similar tendency of the CV was found in NW.

3.3. Bacterial clearance from the lungs

At 3 h post-nasal challenge with a sub-lethal dose of serotype 3 WU2 strain, the bacterial density (mean ± S.D. for Log_{10} cfu/g) in the lungs reached to 6.0 ± 0.4 and 6.0 ± 0.3 in mice nasally administered PspA alone and PBS alone, respectively (Fig. 4A). No significant difference was found between these two groups. In contrast, significant decreases were found in bacterial density in the lungs of mice nasally administered PspA plus Pam3CSK4, Poly(I:C), LPS or CpG1826, compared with mice nasally administered either PspA alone or PBS alone ($P < 0.05$). No significant differences were found in the bacterial density among mice nasally administered PspA plus each TLR agonist. At 6 h post-nasal challenge with the same dose of WU2 strain, the bacterial density (mean ± S.D. for Log_{10} cfu/g) in the lungs remained unchanged at 6.3 ± 0.4 for mice administered PBS alone (Fig. 4B). In contrast, significant decreases were found in the bacterial density in the lungs of mice nasally administered either PspA plus each TLR agonist or PspA alone, compared with mice administered PBS alone ($P < 0.05$). No significant difference was found in the bacterial density among mice nasally administered either PspA plus each TLR agonist or PspA alone. At 12 h post-nasal challenge, the bacterial density (mean ± S.D. for Log_{10} cfu/g) in the lung declined to 4.7 ± 0.7 in mice administered PBS alone (Fig. 4C). In contrast, bacteria were not detected in the lungs of mice nasally administered either PspA plus each TLR agonist or PspA alone. No bacteria were detected in the blood of any mice examined at 3 h, 6 h and 12 h post-nasal challenge.

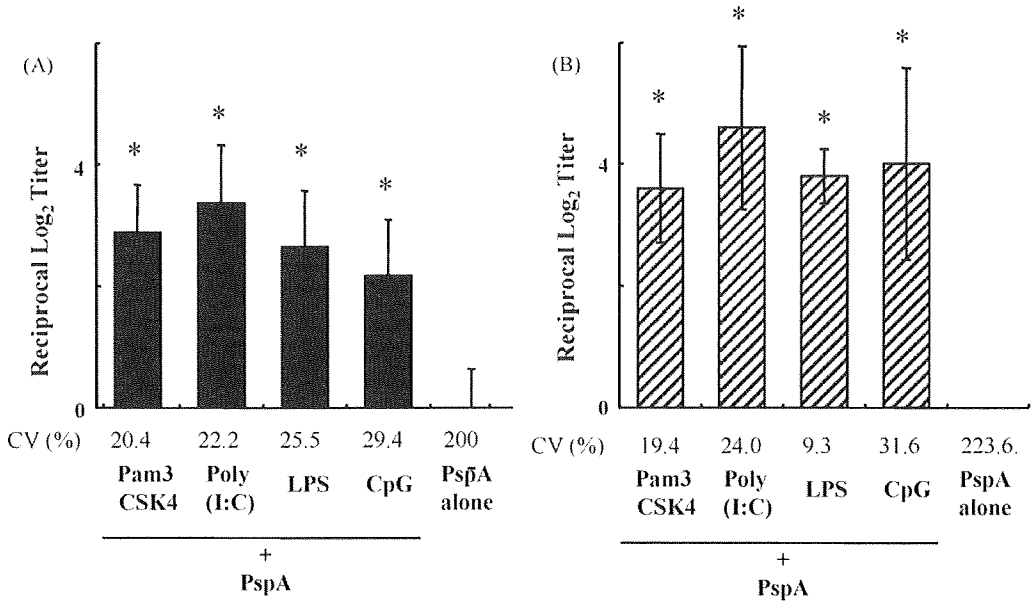


Fig. 3. Induction of PspA-specific IgG (closed bar) (A) and IgA (hatched bar) (B) in nasal wash (NW) by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times at weekly intervals with 10 μg of TLR agonist and 2.5 μg of PspA. One week after the final immunization, mice were euthanized to obtain BALF and NW, 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.

3.4. Bacterial clearance from the nasopharynx

One day after nasal challenge with 3×10^5 cfu of serotype 19F EF3030 strain, the bacterial density (mean ± S.D. for Log_{10} cfu/ml) in NW reached to 5.21 ± 0.26 and 5.08 ± 0.11 in mice administered both PspA alone and PBS alone, respectively (Fig. 5A). No significant difference was found between these two groups. In contrast, significant decreases were found in the bacterial density of mice nasally administered PspA plus either Pam3CSK4, Poly(I:C), LPS or CpG1826, compared with mice nasally administered PspA alone ($P < 0.05$). No significant differences were found in the bacterial density among mice nasally administered PspA plus each TLR agonist. Six days after challenge with 3×10^5 cfu of the EF3030 strain, the bacterial density (mean ± S.D. for Log_{10} cfu/ml) in NW declined to 4.78 ± 0.29 and 4.69 ± 0.29 for mice administered both PspA and PBS alone, respectively (Fig. 5B). No significant difference was found

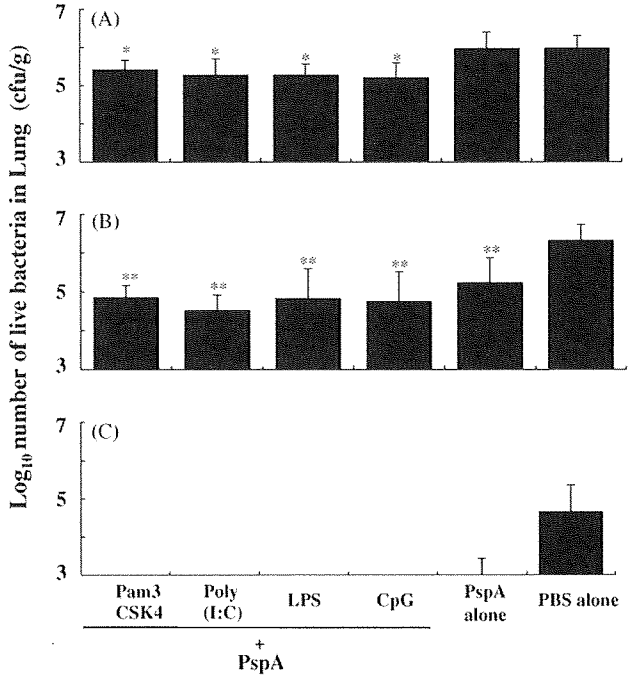


Fig. 4. The effect of intranasal immunization by PspA plus each TLR agonist on the bacterial densities in the lung tissue at 3 h (A), 6 h (B) and 12 h (C) post-challenge with *S. pneumoniae* WU2 strain. A dose of 2×10^6 cfu/mouse was nasally administered to mice previously immunized with either PspA plus each TLR agonist, PspA alone or PBS alone. Mice were euthanized to obtain the lung tissues from infected mice at indicated time-points after bacterial challenge, and quantitative bacterial cultures of lung tissue were performed. Values represent the Log_{10} cfu/g (mean ± S.D.) for six mice per group. CV, coefficient of variation; N.A., not available; LPS, *E. coli* K12 LPS; CpG, CpG DNA ODN1826. $^{*}P < 0.05$, when compared with mice nasally administered either PspA alone or PBS alone; $^{**}P < 0.05$, when compared with mice nasally administered PBS alone.

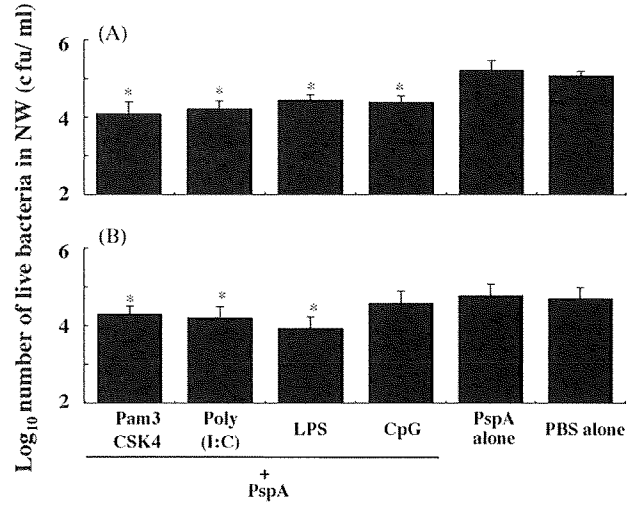


Fig. 5. The effect of intranasal immunization by PspA plus each TLR agonist on the bacterial densities in the nasopharynx 1 day (A) and 6 days (B) after challenge with *S. pneumoniae* EF3030 strain. A dose of 3×10^5 cfu/mouse was nasally administered to mice previously immunized with either PspA plus each TLR agonist, PspA alone or PBS alone. Mice were euthanized to obtain the nasal wash (NW) from infected mice at indicated time-points after bacterial challenge, and a quantitative bacterial culture of NW was performed. Values represent the Log_{10} cfu/ml (mean ± S.D.) of for six mice per group. LPS, *E. coli* K12 LPS; CpG, CpG DNA ODN1826. $^{*}P < 0.05$, when compared with mice nasally administered either PspA alone or PBS alone.

between these two groups. Similarly, significant reductions were detected in the bacterial density of mice nasally administered either PspA plus Pam3CSK4, Poly(I:C) or LPS, but not CpG1826, compared with mice nasally administered either PspA or PBS alone ($P < 0.05$). No significant differences were detected in bacterial density among mice nasally administered either Pam3CSK4, Poly(I:C) or LPS.

4. Discussion

In the present study, nasal immunization with PspA plus each TLR agonist, such as either Pam3CSK4, Poly(I:C), LPS or CpG1826, induced PspA-specific IgA and IgG in the airways as well as PspA-specific IgG in systemic circulation of mice. In contrast, nasal administration of PspA alone induced PspA-specific IgG in plasma, but neither PspA-specific IgA nor IgG in the airways. Therefore, we confirmed that each TLR agonist was an effective nasal adjuvant for the PspA antigen.

The concentrations of PspA-specific IgG in both BALF and plasma and PspA-specific IgA in both BALF and NW increased similarly in mice administered PspA plus each TLR agonist. Furthermore, Pam3CSK4 and LPS induced Th2-associated IgG isotype responses, while Poly(I:C) and CpG 1826 induced Th1- and Th2-associated IgG isotype responses. Previous studies also reported that both CpG motifs and Poly(I:C) induced a Th1 response: our data are consistent with these reports [26,32]. Moreover, the previous reports on the Th2 immune response induced by agonists of either TLR2 (Pam3Cys) or TLR4 (*lpxL1 mutant* LPS), are consistent with the results we obtained using either Pam3CSK4 or LPS [26,33].

It is of interest to determine whether the PspA-specific antibody induced in the airway by nasal immunization of PspA plus agonist of TLR has a protective role against pneumococcal infection. Arulanandam et al. demonstrated that intranasal immunization with PspA plus interleukin-12 (IL-12) induced the concentrations of PspA-specific IgG1, IgG2a and IgA in both plasma and BALF of mice, compared with administration of PspA alone [34]. Because IL-12 activates Th1 and NK cells to induce IFN-γ, the production of both Th1- and Th2-associated IgG isotypes specific for PspA were found in this study. Furthermore, the authors found that immune sera raised by PspA plus IL-12 augmented opsonophagocytic activity against *S. pneumoniae*. This response was primarily attributable to IgG2a and, to a lesser extent, IgA, although this assay evaluated antibody-mediated opsonophagocytic activity without complement. Because PspA-specific antibodies overcome the anti-complementary effects of PspA [11], in the presence of a complement, they likely mediate the efficient opsonophagocytic killing of *S. pneumoniae*.

In our sub-lethal pneumonia model using a serotype 3 WU2 strain, the significant reduction in bacterial density in the lungs of mice nasally administered PspA plus each TLR agonist at 3 h, but neither at 6 h nor at 12 h, post-infection, was associated with induction of PspA-specific IgA and IgG in the airways. No reduction of bacterial density in the lungs of mice nasally administered PspA alone at 3 h post-infection may also be explained by a negligible level of PspA-specific IgG2a and a low level of PspA-specific IgG1 in the plasma of these mice. By contrast, no differences were found in the bacterial density in the lungs of mice nasally administered PspA plus each TLR agonist nor in mice administered PspA alone at 6 h and 12 h post-nasal challenge. These findings may be explained by the extravasation of PspA-specific IgG into the alveolar space of mice given PspA alone during the progression of lung inflammation at 6 h or 12 h post-nasal challenge [35], as a relatively low, but detectable level of PspA-specific IgG was measured in the plasma of these mice after nasal immunization. A previous study demonstrated that the induction of PspA IgG1, followed by IgG2b, but not IgG2a, by oral immunization with PspA plus cholera toxin could provide protective immunity in mice [36]. Although the opsonophagocytic activity of PspA IgG1 has not been evaluated, PspA-specific IgG1 primarily induced in plasma of mice nasally administered PspA alone should transfer from plasma to the alveolar space and act as an opsonic antibody at 6 h and 12 h post-infection. Because an influx of neutrophils occurs in the lungs within several hours after bacterial challenge in mice [37], PspA-specific IgG is likely to enhance complement fixation on the surface of bacterium [11]. Thus, opsonophagocytic killing is enhanced by accumulation of neutrophils in the lung parenchyma.

The effect of PspA plus each TLR agonist to reduce bacterial density in the nasopharynx of mice continued for 6 days after pneumococcal challenge, except for PspA plus CpG1826, in a nasopharyngeal colonization model using a serotype 19F EF3030 strain. Similar levels of PspA-specific IgG and IgA in the NW of mice nasally administered PspA plus each TLR agonist cannot explain the lack of bacterial reduction found only in mice nasally administered PspA plus CpG1826 at 6 days post-challenge. Since we previously reported the discrepancy between the level of serotype-specific IgG and opsonophagocytic functions in certain host conditions [38], the functional assays of PspA-specific IgG or IgA induced by PspA plus each TLR agonist may explain a lack of bacterial reduction found only in mice nasally administered PspA plus CpG1826 at 6 days post-infection. Further studies on the time-course of the levels of PspA-specific IgG and IgA after infection also are required.

Our data suggest that the PspA-specific antibody induced in the airway by nasal immunization with PspA plus each TLR agonist reduced the density of bacterial colonization in the upper airways of mice. A previous study also reported that intranasal immunization with PspA plus cholera toxin B subunit (CTB) induced a salivary IgA response to PspA and decreased nasopharyngeal carriage in mice [39]. However, reduction in the nasaopharyngeal carriage was greater following nasal immunization with PsaA, which is an adhesin of pneumococci, than after immunization with PspA plus CTB [5]. Another study also reported that nasal immunization with PspC, which is a paralog of PspA that is also termed CbpA, plus CTB also reduced nasopharyngeal carriage in CBA/N mice at 7 days post-bacterial challenge [40]. In an infant rat model, PspC was shown to act as a cell surface adhesin and to play a major role in nasopharyngeal colonization [41]. PspA, therefore, may also play some role in bacterial adherence in the nasopharynx of mice, although opsonophagocytic killing of *S. pneumoniae* by PspA-specific antibodies cannot be ruled out.

The complement-fixing ability of the IgG2a isotype on the bacterial surfaces is higher than other IgG isotypes [42], and PspA-specific antibodies may mediate the complement-dependent opsonophagocytic killing of *S. pneumoniae*. Therefore, Th1-associated immune responses to PspA are expected to be more efficacious for preventing pneumococcal infections, as previously reported [19,20]. However, the effects on bacterial clearance by nasal immunization with PspA plus Poly(I:C) or CpG1826, which showed a balanced IgG1/IgG2a immune response to PspA, were comparable to those by nasal immunization with PspA plus either Pam3CSK4 or LPS, which showed a predominant induction of PspA-specific IgG1 in the present study. Although the function of PspA-specific IgA remains unknown, it may play a role in bacterial clearance of the airways as PspA-specific IgG play an important role [5,20,21].

Since bacterial products, such as Pam3CSK4 and LPS, are highly toxic to humans, non-toxic TLR4 agonist, such as monophosphoryl lipid A (MPL) or *lpxL1* mutant LPS, may have clinical use as a mucosal adjuvant [13,43]. PolyI:PolyC_{12}U (Ampligen^{R}), which exhibits greatly reduced toxicity and is being used in humans, can act as a mucosal adjuvant similar to Poly(I:C) for the influenza virus [44,45]. A previous study also reported that nasal administration of CpG 1826 did not induce any local or systemic tissue damage or inflammation in mice [46]. Therefore, CpG ODN may be used as a

safe mucosal adjuvant in humans. Because the antibacterial effects of nasal immunization with PspA plus a TLR agonist were evident in the present study, the combination of a safe TLR agonist and PspA has potential clinical application as a nasal pneumococcal vaccine.

The mucosal immune system in respiratory and alimentary tracts regulates immune responses to pathogenic and commensal bacteria, and quiescently maintains the mucosal surface [47]. This review suggests the presence of a multivalent mucosa-associated regulatory system of unique mononuclear cells in the upper airways, including NALT DCs which can induce antigen-specific immune responses, although the phenotype of NALT DC has not been determined. It is conceivable that soluble TLR agonists administered with PspA may have distinct mode of distribution within the mucosa. In particular, efficiency of cellular up-take by, and the resultant activation of, the antigen presenting cells including the DCs for soluble TLR agonists may be quite different from 'endogenous' TLR agonists existing as a compartment of commensal microbes, normally restricted on mucosal surface niche. This distinct delivery mode for antigens may explain, in part, why PspA-specific antibodies were induced in the airway by nasally administered PspA plus each TLR agonist, but not by PspA alone in this study.

Pivotal but complex roles of innate immune receptors in the induction of adaptive immune responses (immunogenicity) have only recently been revealed. In fact, some innate immune receptors such as RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) have also been shown to be involved in the immunogenicity of vaccines. For example, Poly(I:C), dsRNA ligand for both TLR3 and melanoma-associated gene 5 (MDA5), works as an adjuvant mainly via MDA5, and to lesser extent, TLR3 [48]. On the other hand, although influenza A virus stimulates both TLR7 and RIG-I for innate immune activation, only the TLR7–MyD88 pathway was required for the protective adaptive immune response in mice [49]. Moreover, NLRs that sense microbial and self-derived danger particle (or crystal) molecules in the cytosol [50]. Aluminum hydroxide (alum), which is a widely used adjuvant in human vaccines, stimulates the signaling of NLR pathways for a humoral adaptive immune response [51]. Alum-mediated adjuvant activity, however, remains to be controversial [52]. Taken together, activations of TLR, RLR, or NLR on antigen presenting cells including DCs by microbial stimuli seem to have non-redundant roles in inducing the following adaptive immune responses to co-administered antigens. Presumably, Pam3CSK4 and LPS trigger activation of TLR 2 and 4 on NALT DCs, respectively. Similarly, Poly(I:C) triggers activation of both MDA5 in cytoplasm and TLR3 in endosome, and CpG 1826 activates TLR9 in endosome of NALT DCs. Therefore, nasal administration of each TLR agonist, in combination with PspA, works as potent mucosal adjuvants for induction of PspA-specific antibodies in the airways.

In conclusion, the induction of PspA-specific IgA and IgG was associated with enhanced bacterial clearance of pneumococcal strains with different serotypes from the nasopharynx and lungs of mice nasally administered PspA plus each TLR agonist. Despite the difference in the Th1- and Th2-associated IgG isotype responses among TLR agonists, bacterial clearances from the lungs at 3 h post-infection in a pneumonia model, and from nasopharynx in a colonization model at 1 day post-infection, were equivalent in mice after nasal immunization with PspA plus each TLR agonist.

Acknowledgments

We are grateful to Dr. D.E. Briles and Dr. S.K. Hollingshead from the University of Alabama at Birmingham, for providing the pneumococcal strain and the recombinant plasmid, pUAB055, for this study. This work was supported by Grants-in-Aid from the Ministry of Health, Labor and Welfare of Japan on "Mechanisms, epidemiology, prevention and control of acute respiratory infection".

References

[1] Klugman KP, Madhi SA, Huebner RE, Kohberger R, Mbelle N, Pierec N, et al. A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. N Eng J Med 2003;349:1341–8.
[2] Cutts FT, Zaman SMA, Enwere G, Jaffar SJ, Levine OS, Okoko JB, et al. Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in the Gambia: randomised, double-blind, placebo-controlled trial. Lancet 2005;365:1139–46.
[3] Shapiro ED, Berg AT, Austrian R, Shroeder D, Parcells V, Margolis A, et al. The protective efficacy of polyvalent pneumococcal polysaccharide vaccine. N Eng J Med 1991;325:1453–60.
[4] Briles DE, Tart RC, Swiatlo E, Dillard JP, Smith P, Benton KA, et al. Pneumococcal diversity: considerations for new vaccine strategies with emphasis on pneumococcal surface protein A (PspA). Clin Microbiol Rev 1998;11:645–57.
[5] Briles DE, Ades E, Paton JC, Sampson JS, Carlone GM, Huebner RC, et al. Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. Infect Immun 2000;68:796–800.
[6] Ogunniyi AD, Folland RL, Briles DE, Hollingshead SK, Paton JC. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. Infect Immun 2000;68:3028–33.
[7] Briles DE, Hollingshead SK, King J, Swift A, Braum PA, Park MK, et al. Immunization of human with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. J Infect Dis 2000;182:1694–701.
[8] Nabors GS, Braun PA, Herrmann DJ, Heise ML, Pyle DJ, Gravenstein S, et al. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) varient stimulates broadly cross-reactive antibodies to heterologous PspA molecules. Vaccine 2000;18:1743–54.
[9] Hollingshead SK, Becker R, Briles DE. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. Infect Immun 2000;68:5889–900.
[10] Tu A-H, Fulgham RL, McCrory MA, Briles DE, Szalai J. Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. Infect Immun 1999;67:4720–4.
[11] Ren B, Szalai AJ, Hollingshead SK, Briles DE. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. Infect Immun 2004;72:114–22.
[12] Holmgren J, Czerkinsky. Mucosal immunity and vaccine. Nat Med 2005;11:S45–53.
[13] Freytag LC, Clements JD. Mucosal adjuvants. Vaccine 2005;23:1804–13.
[14] Kaisho T, Akira S. Toll-like receptors as adjuvant receptors. Biochim Biophys Acta 2002;1589:1–13.
[15] Pulendran B. Modulating vaccine responses with dendritic cells and toll-like receptors. Immunol Rev 2004;199:227–50.
[16] Snapper CM, Paul WE. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 1987;236:944–7.
[17] Stevens TL, Bossie A, Sanders VM, Fernandez-Botran R, Coffman RL, Mosmann TR, et al. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature 1988;334:255–8.
[18] Finkelman FD, Holmes J, Katona IM, Urban Jr JF, Beckmann MP, Paul LS, et al. Lymphokine control of in vivo immunoglobulin isotype selection. Ann Rev Immunol 1990;8:303–33.
[19] Ferreira DM, Darrieux M, Oliveira MLS, Leite LCC, Miyaji EN. Optimized immune response elicited by a DNA vaccine expressing pneumococcal surface protein A is characterized by a balanced immunoglobulin G1 (IgG1)/IgG2a ration and proinflammatory cytokine production. Clin Vaccine Immunol 2008;15:499–505.
[20] Hanniffy SB, Carter AT, Hitchin E, Wells JM. Mucosal delivery of a pneumococcal vaccine using *Lactococcus lactis* affords protection against respiratory infection. J Infect Dis 2007;195:185–93.
[21] Briles DE, Hollingshead SK, Paton JC, Ades EW, Novak L, van Ginkel FW, et al. Immunization with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with *Streptococcus pneumoniae*. J Infect Dis 2003;188:229–48.
[22] Briles DE, Novak L, Hotomi M, van Ginkel FW, King J. Nasal colonization with *Streptococcus pneumoniae* includes subpopulations of surfaces and invasive pneumococci. Infect Immun 2005;73:6945–51.
[23] Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. Proc Natl Acad Sci 2000;97:13766–71.
[24] Ichinohe T, Watanabe I, Ito S, Fujii H, Moriyama M, Tamura S, et al. Synthetic double-strand RNA Poly(I:C) combined with mucosal vaccine protects against influenza virus infection. J Virol 2005;79:2910–9.
[25] Abe N, Kodama S, Hirano T, Eto M, Suzuki M. Nasal vaccination with CpG oligodeoxynucleotide induces protective immunity against nontypeable *Haemophilus influenzae* in the nasopharynx. Laryngoscope 2006;116:407–12.
[26] Fransen F, Boog CJ, van Putten JP, van der Ley P. Agonists of toll-like receptors 3,4,7 and 9 are candidates for use as adjuvants in an outer membrane vaccine against *Neisseria meningitidis* serogroup B. Infect Immun 2007;75:5939–46.
[27] Kataoka K, McGhee JR, Kobayashi R, Fujihashi K, Shizukuishi S, Fujihashi K. Nasal Flt3 ligand cDNA elicits $CD11c^+CD8^+$ dendritic cells fro enhanced mucosal immunity. J Immunol 2004;172:3612–9.

[28] Kurita S, Koyama J, Onizuka S, Motomura K, Watanabe H, Watanabe K, et al. Dynamics of dendritic cell migration and the subsequent induction of protective immunity in the lung after repeated airway challenges by nontypeable *Haemophilus influenzae* outer membrane protein. Vaccine 2006;24:5896–903.
[29] Yoshimura A, Lien E, Ingalls RR, Toumanen E, Dziarski R, Golenbock D. Recognition of gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol 1999;163:1–5.
[30] Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipstich M, Thompson CM, et al. Recognition of pneumolysin by Toll-like receptors 4 confers resistance to pneumococcal infection. Proc Natl Acad Sci 2003;100:1966–71.
[31] Albiger B, Dahlberg S, Sandgren A, Wartha F, Beiter K, Katsuragi H, et al. Toll-like receptor 9 acts at an early stage in host defense against pneumococcal infection. Cell Microbiol 2007;9:633–44.
[32] Lin L, Gerth AJ, Peng SL. CpG DNA redirects class-switching towards "Th1-like" Ig isotype production via TLR 9 and Myd88. Eur J Immunol 2004;34:1483–7.
[33] Redecke V, Hacker H, Datta SK, Fermin A, Pitha PM, Broide DH, et al. Activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. J Immunol 2004;172:2739–43.
[34] Arulanandam BP, Lynch JM, Briles DE, Hollingshead S, Metzger DW. Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against *Streptococcus pneumoniae* infection. Infect Immun 1997;69:6718–24.
[35] Sato S, Ouellet N, Pelletier I, Shimard M, Rancourt A, Bergeron MG. Role of galectin-3 as an adhesion molecule for neutrophil extravasation during *Streptococcal pneumonia*. J Immunol 2002;168:1813–22.
[36] Yamamoto M, McDaniel LS, Kawabata K, Briles D, Jackson RJ, McGhee JR, et al. Oral immunization with PspA elicits protective humoral immunity. Infect Immun 1997:640–4.
[37] Satoh S, Oishi K, Iwagaki A, Senba M, Akaike T, Akiyama M, et al. Dexamethason impairs pulmonary defense against *Pseudomonas aeruginosa* through suppressing iNOS gene expression and peroxynitrite production in mice. Clin Exp Immunol 2002;126:266–73.
[38] Chen M, Ssali F, Mulungi M, Awio P, Yoshimine H, Kuroki R, et al. Induction of opsonophagocytic killing activity with pneumococcal conjugate vaccine in human immunodeficiency virus-infected Ugandan adults. Vaccine 2008;26:4962–8.
[39] Wu H-Y, Nham MH, Guo Y, Russel MW, Briles DE. Intranasal immunization of mice with PspA (Pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*. J Infect Dis 1997;175:839–46.
[40] Balachandran P, Brooks-Water A, Virolainen-Julkunen A, Hollingshead S, Briles D. Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of *Streptococcus pneumoniae*. Infect Immun 2002;70:2526–34.
[41] Rosenow C, Ryan P, Weiser JN, Jhonson S, Fontan P, Ortqvist A, et al. Contribution of novel cholin-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. Mol Microbiol 1997;25:819–29.
[42] Oishi K, Koles NL, Guelde G, Pollack M. Antibacterial and protective properties of monoclonal antibodies reactive with *Escherichia coli* O111:B4 lipopolysaccharide: relation to antibody isotype and complement-fixing activity. J Infect Dis 1992;165:34–45.
[43] van der Ley P, Steeghts L, Mamstra HJ, ten Hove J, Zomer B, van Alphen L. Modification of lipid A biosynthesis in *Neisseria meningitides lpxL* mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. Infect Immun 2001;69:5981–90.
[44] Thompson KA, Strayer DR, Salvato PD, Thompson CE, Klimas N, Molavi A, et al. Results of a double-blind placebo-controlled study of the double-stranded RNA drug PolyI:PolyC_{12}U in the treatment of HIV infection. Eur J Clin Microbiol Infect Dis 1996;15:580–7.
[45] Ichinohe T, Tamura S, Kawaguchi A, Ninomiya A, Imai M, Itamura S, et al. Cross-protection against H5N1 influenza virus infections is afforded by intranasal inoculation with seasonal trivalent inactivated influenza vaccine. J Infect Dis 2007;196:1313–20.
[46] Kodama S, Abe N, Hirano T, Suzuki M. Safety and efficacy of nasal application of CpG oligodeoxynucleotide as a mucosal adjuvant. Laryngoscope 2006;116:331–5.
[47] Kunisawa J, Nochi T, Kiyono H. Immunological commensalities and distinctions between airway and digestive immunity. Trends Immunol 2008;29:505–13.
[48] Kumar H, Koyama S, Ishii KJ, Kawai T, Akira S. Cutting edge: cooperation of IPS-1- and TRIF-dependent pathways in poly IC-enhanced antibody production and cytotoxic T cell responses. J Immunol 2008;180:683–7.
[49] Koyama S, Ishii K, Kumar H, Tanimoto T, Coban C, Uematsu S, et al. Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. J Immunol 2007;179:4711–20.
[50] Mariathosan S, Monack DM. Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. Nat Immunol Rev 2007;7:31–40.
[51] Eisenbarth SC, Colegio OR, O'Connor Jr W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature 2008;453:1122–6.
[52] Franchi L, Nunez G. The Nlrp3 inflammasome is critical for aluminum hydroxide-mediated IL-1 β secretion but dispensable for adjuvant activity. Eur J Immunol 2008;38:2085–9.

●原 著

肺炎球菌ワクチン再接種承認の必要性に関するアンケート調査研究

大石 和徳[1] 川上 和義[2] 永井 英明[3] 砂川 慶介[4] 渡辺 彰[5]

要旨：本邦における肺炎球菌ワクチンの再接種の実施状況と副反応の実態を明らかにすることを目的として，日本呼吸器学会，日本感染症学会の役員989名を対象に，アンケート調査を実施した．有効回答を得た385名のうち，本ワクチンの接種経験のある290名（75.3%）を調査対象者とした．この290名中，46名（15.9%）に再接種実施経験があり，252名（86.9%）は再接種の必要性を認識していた．また，その144名（49.7%）は再接種が禁忌であるが故に，患者が初回接種を控える経験をしていた．再接種の実施経験者46名のうち4名が再接種による副反応を経験していたが，いずれも軽症であった．今回の調査結果から，調査対象者の大半は再接種の必要性を認識しており，その一部は患者側の要望に応じて再接種を実施している実態が明らかになり，再接種が禁忌であることが本ワクチン接種率向上の障壁となっていることも示唆された．

キーワード：肺炎球菌ワクチン，再接種，アンケート調査
Pneumococcal polysaccharide vaccine, Revaccination, Questionnaire

緒 言

肺炎球菌は最も重要な呼吸器病原性菌であり，その菌表層は莢膜ポリサッカライドに覆われている．この莢膜ポリサッカライド（CPS）には少なくとも91種類の莢膜血清型が存在する．このCPSはT細胞非依存性抗原であり，生体内では主に血清型特異IgG2産生を誘導し，この特異抗体による補体依存性のオプソニン活性は本菌に対する感染防御の中心的役割を担っている[1]．

肺炎球菌ワクチンは23価のCPSを含有する多価ワクチンであり，その成人における敗血症や髄膜炎などの侵襲性肺炎球菌感染症（Invasive pneumococcal diseases：IPD）に対する予防効果から[1][2]，我が国では1988年に薬事承認された．この際に，米国予防接種諮問委員会（Advisory Committee on Immunization Practice；ACIP）が1982年に成人に対して14価肺炎球菌ワクチンを一回のみの接種を推奨したこともあり[3]，再接種・追加接種をしてはならない旨が添付文書に記載された．しかしながら，その後の海外における23価肺炎球菌ワクチンの再接種に関する調査においては再接種に伴う重篤な副反応は認められず，再接種のリスクは禁忌に該当しないとされている[1)~6)]．また，1997年には米国ACIPは65歳未満で肺炎球菌ワクチンを接種し，その後5年が経過した場合には再接種を推奨している[7]．我が国では，2006年10月以降，肺炎球菌ワクチンは従来の製造方法が変更されたニューモバックスNP[8]として臨床の現場で使用されているが，その再接種は依然禁忌のままである．近年，国内の高齢者において肺炎球菌ワクチン接種が普及するにつれて，臨床現場では肺炎球菌ワクチンの再接種承認を求める声が高まっている．

本研究では，我が国における肺炎球菌ワクチン再接種の実施状況と副反応の実態を明らかにすることを目的として，日本呼吸器学会理事，代議員および日本感染症学会の理事，評議員を対象にアンケート調査を実施したので報告する．

対象と方法

1）実態調査の内容と方法

我々は，日本呼吸器学会と日本感染症学会の協力のもとに，日本呼吸器学会の理事，代議員，および日本感染症学会の理事，評議員の総数989名を対象に，平成20年12月から平成21年1月にかけて，匿名回答による郵送アンケート調査を実施した．調査内容としては，1)肺炎球菌ワクチン再接種実施の状況，2）再接種対象の基礎疾患，3）再接種実施の動機，4）再接種による副反応の有無，5）再接種の必要性，6）再接種禁忌の与える初回接種への影響などであった．再接種実施の動機につい

〒565-0871 大阪府吹田市山田丘3—1
[1]大阪大学微生物病研究所感染症国際研究センター
[2]東北大学医学系研究科保健学専攻基礎検査医科学講座
[3]国立病院機構東京病院呼吸器科
[4]北里大学北里生命科学研究所・感染症学
[5]東北大学加齢医学研究所抗感染症薬開発研究部門
（受付日平成21年5月27日）

Table 1　Questionnaires for pneumococcal vaccination and revaccination

Questions		no. of subjects (%)
Pneumococcal vaccination at the affiliated institution	no. of total subjects; 385 no. of subjects answered yes (%) no. of subjects answered no (%) or with no answer	 290 (75.3) 95 (24.7)
Negative influence of the contraindication on revaccination for the first vaccination	no. of total subjects; 290 no. of subjects answered yes (%) no. of subjects answered no or with no answer (%)	 144 (49.7) 146 (50.3)
Pneumococcal revaccination	no. of total subjects; 290 no. of subjects answered yes (%) no. of subjects answered no or with no answer (%)	 46 (15.9) 244 (84.1)
Necessity for approval of revaccination	no. of total subjects; 290 no. of subjects answered yes (%) no. of subjects answered no or with no answer (%)	 252 (86.9) 38 (13.1)

ては，医師の推奨か，患者もしくは家族の希望かのいずれかを質問した．平成21年1月末までに回収されたアンケート調査票を解析した．

成　績

1．肺炎球菌ワクチン再接種の実態調査

1）アンケート対象者

送付された989通のアンケート調査用紙のうち401通(40.5%)が回収され，そのうち解析可能な調査用紙は385通（有効回答率は96.0%）であった．回答者の性別は男性が95.3%，平均年齢は53.1歳（SD：8.5）であった．回答者の所属医療機関は，大学附属病院38.2%，国公立病院28.3%，私立病院14.5%，診療所・クリニック10.9%の順で，診療科別では呼吸器内科54.3%，一般内科13.8%，感染症内科8.8%の順であった．回答者の76.7%が内科医であった．

2）肺炎球菌ワクチン接種状況

有効回答を得た385名のうち290名（75.3%）は医療施設で本ワクチンの接種を実施しており，この290名を調査対象者とした（Table 1）．接種理由は医師の推奨が52%であり，患者本人の希望は48%であった．肺炎球菌ワクチン接種を実施した患者の基礎疾患は，慢性閉塞性肺疾患（COPD），その他の慢性肺疾患，脾摘出・脾機能不全患者，慢性心不全，糖尿病の順であった．接種を推奨している年齢については75歳以上39.3%，65歳以上38.7%，80歳以上22.1%の順であった．また，調査対象者290名のうち，144名（49.7%）から再接種が禁忌となっていることが，初回接種を控える原因となったとする回答を得た（Table 1）．さらに，これらの回答者が上記の理由から初回接種を控えたとする患者数は平均19名（SD 26.7例）であり，報告総数は2,344例であった．

3）肺炎球菌ワクチン再接種の状況と副反応

調査対象者290名のうち，46名（15.9%）の医師が再接種を実施した経験ありと回答した（Table 1）．この46名のうち11名の医師から，再接種を受けた患者49症例の臨床像が報告され，他の35名の医師からの報告は得られなかった．11名の医師の再接種患者数の内訳は，1名の医師がそれぞれ15例，8例，7例，5例，4例，3例の患者に，2名の医師がそれぞれ2例の患者に，3名の医師がそれぞれ1例の患者に再接種を実施していた．再接種を受けた患者の平均年齢は74.4歳（SD：10.3）であり，その基礎疾患の内訳は，COPDを含む慢性肺疾患が19例（38.8%），慢性心不全7例（14.3%）などであった（Table 2）．初回接種から再接種までの期間は，平均63カ月（SD：11.8）であった．再接種実施の動機は，43例（87.8%）は本人もしくは家族の希望によるものであり，医師の推奨によるものは5例(10.2%)であった．また，290名の調査対象者のうち，252名（86.9%）が再接種は必要との認識を示したのに対し，回答なし，もしくは必要なしと回答したのは38名（13.1%）に過ぎなかった．

再接種を実施した調査対象者46名のうち，4名から再接種に伴う副反応の報告があった．その内訳は注射部位の局所的腫脹が2例，発疹，筋肉痛，倦怠感は各1例であった．1例では10cm以上の腫脹も経験されていたが，アナフィラキシーなど重篤な副反応の報告はなかった．

Table 2 49 reported cases with pneumococcal revaccination during last two years by the questionnaire

Demographic features	
Male sex (%)	27 (55.1)
Age, mean years (SD)	74.4 (10.3)
Duration between primary and revaccination, mean months (SD)	63 (11.8)
Underlying diseases; no. of cases (%)	
Chronic lung diseases	19 (38.8)
COPD	13 (26.5)
Other chronic lung diseases	6 (12.2)
Chronic heart disease	7 (14.3)
Diabetes mellitus	1 (2.0)
Others	19 (38.3)
Reason for revaccination; no. of cases (%)	
Request by the patient	43 (87.8)
Request by the family	1 (2.0)
Recommendation by the doctor	5 (10.2)

SD; standard deviation, COPD; chronic obstructive pulmonary disease

考　察

我が国の成人におけるIPDの実態はこれまで明らかでなかったが，最近になってChibaらはその病院ネットワークからの報告において，小児IPD症例より成人IPD症例が多く，その致命率は22%と高い実態を明らかにした[8]．IPDの発生頻度は高齢化につれて顕著に増加することから[9]，高齢化時代を迎えた我が国においてIPDは高齢者の生命を脅かす感染症の一つと言える．一方，米国の疫学調査からその頻度がIPDの約10倍多いと推定される菌血症を伴わない肺炎に対する肺炎球菌ワクチン接種の予防効果には異論があるところである[2)10)11)]．しかしながら，最近では本ワクチン接種後の成人肺炎の重症度や死亡リスクが低下するとした報告もみられる[12)13)]．このような背景から，我が国の高齢者のIPD予防および肺炎重症化予防対策の一環として本ワクチン接種が薦められる．また，高齢者においては肺炎球菌ワクチン接種による血中特異抗体濃度および感染防御効果が5年以上は維持されないことから[2)14)15)]，初回接種後の高齢者ではその後の追加接種が必要と考えられる．

今回のアンケート調査では，肺炎球菌ワクチン接種を実施している290名の調査対象者のうち，再接種実施経験者は15.9%である一方で，その86.9%は再接種の必要性を認識していた．また，初回接種の理由については患者本人の希望が約半数であるのに対し，再接種の理由はその89.8%が本人や家族の希望によっていた．これらの調査結果は，本ワクチンの再接種が禁忌であることを知りつつも，再接種の安全性と必要性を認識し，患者やその家族による再接種の希望に応じる医師の実態を浮き彫りにしている．また，肺炎球菌ワクチン接種経験者の約半数が再接種禁忌であるが故に，その初回接種を控える経験をしており，結果的に我が国における本ワクチン普及の障壁となっている可能性が示唆された．

一方，再接種経験のある46名の調査対象者のうち4名は再接種に伴う副反応の経験があったが，いずれも軽症であり，重篤な副反応は発生していなかった．最近，高山らも12例の高齢者を対象とした再接種例のうち，1例のみに接種部位の発赤，腫脹，疼痛などの軽症の副反応を認めたとしている[16]．一方，海外ではJacksonらが過去に肺炎球菌ワクチン接種歴のない901名，少なくとも5年前に肺炎球菌ワクチン接種歴のある513名の50歳から74歳までの成人を対象として，接種前の血清中特異IgG濃度とワクチン接種後の副反応について検討している[3]．彼らは，接種2日以内の接種部位の大きな局所反応（10.2cm以上）の頻度は，初回接種群（3%）より再接種群（11%）において有意に多く，また接種前の血中特異抗体濃度と接種部位の局所反応の頻度が相関することを報告している．この接種部位の局所反応については，局所の免疫複合体形成に基づくアルサス型反応が原因と考えられている[3)17)]．また，これらの再接種に伴う局所反応は平均3日で軽快したとされる．

第二世代のELISA法を用いた高齢者に対する再接種による免疫原性の検討では，いずれも接種前の血清中特異IgG濃度（6～7種類の血清型）は再接種後に有意に増加することが報告されている[5)6)]．しかしながら，Torlingらは再接種4週後の特異抗体IgG濃度のピーク（7.47μg/ml）は初回接種4週後のピーク（19.06μg/ml）と比較して有意に低値であったとしている[5]．このよう

に，T細胞非依存性抗原の特性から，肺炎球菌ワクチンの再接種により，初回接種時と同等程度の特異抗体産生誘導は認められるが，そのブースター効果は期待できない．

本論文はアンケートによる再接種の実態調査結果であるため，その科学的証拠としては限界がある．しかしながら，本アンケート調査研究から，1）調査対象者の大半は再接種の必要性を認識し，その一部は患者側の再接種の要望に応じていること，2）調査対象者の約半数は再接種が禁忌であるが故に，その初回接種を控える経験をしていること，3）再接種の実施経験者46名からの報告では，再接種に伴う重篤な副反応は認められていないことが明らかになった．今回の調査結果と国外における本ワクチンの再接種の安全性と免疫原性の成績から，我が国における高齢者に対しても再接種が早期に承認されるべきである．

謝辞：稿を終えるにあたり，本アンケート調査にご協力いただきました日本呼吸器学会理事，代議員および日本感染症学会理事，評議員各位に深甚の謝意を表します．本調査研究は厚生労働科学研究費補助金（医薬品・医療機器等レギュラトリーサイエンス総合研究事業)「ワクチンの有効性向上のためのエビデンスお呼び方策に関する研究」(班長：神谷齊）により，同研究班の平成20年度事業として実施した．

引用文献

1) Jackson LA, Neuzil KM. Pneumococcal polysaccharide vaccine. In : Plotkin S, Orenstein W, Offit P, ed. Vaccine. 5 th ed. Saunders, 2008 ; 569—604.
2) Shapiro ED, Berg AT, Austrian R, et al. The protective efficacy of polyvalent pneumococcal polysaccharide vaccine. N Eng J Med 1991 ; 325 : 1453—1460.
3) Pneumococcal polysaccharide vaccine. Recommendation of the immunization Practices Advisory Committee. Ann Intern Med 1982 ; 96 : 203—205.
4) Jackson LA, Benson P, Sneller V-P, et al. Safety of revaccination with pneumococcal polysaccharide vaccine. JAMA 1999 ; 281 : 243—248.
5) Torling J, Hedlund J, Konradsen HB, et al. Revaccination with the 23-valent pneumococcal polysaccharide vaccine in middle-aged ad elderly persons previously treated for pneumonia. Vaccine 2003 ; 22 : 96—103.
6) Lackner TE, Hamilton RG, Hill JJ, et al. Pneumococcal polysaccharide revaccination : immunoglobulin G seroconversion, persistence, and safety in frail, chronically ill old subjects. J Am Geriatr Soc 2003 ; 51 : 240—245.
7) Center for Disease Control. Prevention of pneumococcal diseases : Recommendations of Advisory Committee on Immunization Practice. MMRW 1997 ; 46 : 1—23.
8) Chiba N, Mozozumi M, Sunaoshi K, et al. Serotype and antibiotic resistance of isolates from patients with invasive pneumococcal disease in Japan. Epidemiol Infect 2009.
9) Lexau CA, Lynfield R, Danila R, et al. Changing epidemiology of invasive pneumococcal disease among older adults in the era of pediatric pneumococcal conjugate vaccine. JAMA 2005 ; 294 : 2043—2051.
10) Ortqvist A, Hedlund J, Burman L, et al. Randomised trial of 23-valent pneumococcal capsular polysaccharide vaccine in prevention of pneumonia in middle-aged and elderly people. Lancet 1998 ; 351 : 399—403.
11) Jackson LA, Neuzil KM, Yu O, et al. Effectiveness of pneumococcal vaccine in older adults. N Eng J Med 2003 ; 348 : 1747—1755.
12) Fisman DN, Abrutyn E, Spaude KA, et al. Prior pneumococcal vaccination is associated with reduced death, complications, and length of stay among hospitalized adults with community-acquired pneumonia. Clin Infect Dis 2006 ; 42 : 1093—1101.
13) Johnstone J, Marrie TJ, Majumdar SR, et al. Effect of pneumococcal vaccination in hospitalized adults with community-acquired pneumonia. Arch Intern Med 2007 ; 167 : 1938—1943.
14) Sankilampi U, Honkanen PO, Bloigu A, et al. Antibody response to pneumococcal capsular polysaccharide vaccine in the elderly. J Infect Dis 1996 ; 173 : 387—393.
15) Chen M, Hisatomi Y, Furumoto A, et al. Comparative immune response of patients with chronic pulmonary diseases during the 2 year period after pneumococcal vaccination. Clin Vaccine Immunol 2007 ; 14 : 139—145.
16) 高山直秀，菅沼明彦，柳澤如樹．当院ワクチン外来における肺炎球菌ワクチン接種状況．厚生労働科学研究費補助金岡部班・神谷班合同班会議（新予防接種研究班）平成20年度報告書．2009 ; 197—198.
17) Sankilampi U, Honkanen PO, Pyhala R, et al. Associations of prevaccination antibody levels with adverse reactions to pneumococcal and influenza vaccines administered simultaneously in the elderly. Vaccine 1997 ; 10 : 1133—1137.

Abstract

A questionnaire study on the necessity of approval for revaccination of the pneumococcal polysaccharide vaccine

Kazunori Oishi[1], Kazuyoshi Kawakami[2], Hideaki Nagai[3], Keisuke Sunakawa[4] and Akira Watanabe[5]

[1]International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University

[2]Department of Medical Microbiology, Mycology and Immunology, Tohoku University Graduate School of Medicine

[3]Department of Respirology, National Hospital Organization Tokyo Hospital

[4]Graduate School of Infection Control Sciences, Kitasato University, Laboratory of Infectious Diseases

[5]Research Division for Development of Anti-Infective Agents, Institute of Development, Aging and Cancer, Tohoku University

To clarify the current situation of revaccination with pneumococcal polysaccharide vaccine (PPV) and the adverse effects caused by revaccination with PPV in the elderly in Japan, a questionnaire study was carried out among the 989 members of the directors and councillors of the Japanese Respirology Society and the Japanese Association for Infectious Diseases. Of 385 evaluable respondents, 290 who had had experience giving PPV immunization were regarded as the study subjects. Of whom 46 subjects (15.9%) had had experience of PPV revaccination. However, 252 subjects (86.9%) recognized that PPV revaccination is necessary. In addition, of the 290 subjects, 114 subjects (49.7%) had experienced a patient refusing the first vaccination with PPV because of contraindications for PPV revaccination. Of 46 subjects with experience of PPV vaccination, 4 subjects found adverse effects in the recipients of PPV revaccination. The adverse effects found were not serious. The present study demonstrated that most of the study subjects recognized the necessity of PPV revaccination, and in part, those subjects implementing PPV revaccination were responding to requests by patients or their family. It was also suggested that the contraindication for PPV revaccination could prevent the increase of the coverage rate of PPV.

Virology 394 (2009) 259–265

Contents lists available at ScienceDirect

Virology

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

Nuclear location of minor capsid protein L2 is required for expression of a reporter plasmid packaged in HPV51 pseudovirions

Kazunari Kondo [a,b], Yoshiyuki Ishii [a], Seiichiro Mori [a], Shiho Shimabukuro [a], Hiroyuki Yoshikawa [b], Tadahito Kanda [a,*]

[a] Center for Pathogen Genomics, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[b] Department of Obstetrics and Gynecology, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba-shi, Ibaraki 305-8575, Japan

ARTICLE INFO

Article history:
Received 21 May 2009
Returned to author for revision
17 June 2009
Accepted 26 August 2009
Available online 19 September 2009

Keywords:
HPV51
L2 function
Pseudovirion

ABSTRACT

The deduced amino acid (*aa*) sequence of L2 of the newly sequenced HPV51 strain, isolated by Matsukura and Sugase (Ma-strain), was markedly different from that of the prototype HPV51 isolated by Nuovo et al. (Nu-strain) (GenBank M62877) in two regions: *aa* 95–99 (region I) and *aa* 179–186 (region II). The two regions of Ma-strain were homologous to those of the other mucosal HPVs. The *aa* sequences of the N-terminal and C-terminal regions of Ma-L2 and Nu-L2 were identical and contained the nuclear localizing signal (NLS). When expressed in HEK293 cells, Ma-strain L2 (Ma-L2) was located in the nucleus but Nu-strain L2 (Nu-L2), in the cytoplasm. The chimeric L2s having both Nu-L2 regions I and II were located in the cytoplasm, and those having one of them were located both in the nucleus and cytoplasm, suggesting that Nu-L2 regions I and II inhibit the NLS function. For a better understanding of a role of L2 in infection, pseudovirion (PV) preparations were produced with a reporter, Ma-strain L1, and various L2s (Ma-L2, Nu-L2, or the chimeric L2s). These PV preparations contained structurally similar particles composed of L1 and L2 and the packaged reporter plasmid at a similar level. The reporter expression was not induced in HEK293 cells after inoculation with PVs containing the L2s that are incapable of localizing in the nucleus when expressed alone. Among PVs containing L2s capable of localizing in the nucleus, the reporter expression was induced only by PVs containing Ma-L2 region I. Thus, the results indicate that the expression of the reporter in the HPV51 PV requires the nuclear localizing ability of L2 and another unknown function associated with region I.

Introduction

Human papillomavirus (HPV) is a small nonenveloped virus with circular double-stranded DNA genome (8000 base-pairs) packaged in an icosahedral capsid composed of two capsid proteins L1 (major) and L2 (minor) (Howley and Lowy, 2001). Various HPVs, which have been identified in proliferative lesions including cervical cancer, are classified into more than 100 genotypes based on the homology of nucleotide sequence of L1 gene (Howley and Lowy, 2001). HPV infects the epithelium and propagates in the differentiating keratinocytes or establishes a latent state in the epithelial basal cells (Howley and Lowy, 2001). There are no cell cultures supporting efficient HPV propagation.

Infectious HPV pseudovirions have been developed (Unckell et al., 1997; Stauffer et al., 1998; Buck et al., 2004) and are used as a surrogate virus to study biological functions and immunogenicity of the capsid proteins (Sapp and Selinka, 2005; Buck et al., 2005). The mRNAs transcribed from the codon-modified L1 and L2 genes escape from the mechanism inducing the rapid degradation of the authentic L1- and L2-mRNAs in the undifferentiated cells (Sokolowski et al., 1998; Collier et al., 2002). L1 and L2 expressed in cultured cells from the codon-modified genes self-assemble to form L1/L2 virus-like particles (L1/L2-VLPs) in the nucleus. When L1 and L2 are expressed in the SV40 T-antigen positive cells transfected with a reporter plasmid carrying SV40-ori, the replicating reporter plasmid is packaged into the L1/L2-VLP to produce a pseudovirion (PV) (Buck et al., 2004). Because the efficiency of the packaging is not high, a semi-purified PV preparation contains complete PVs and empty L1/L2-VLPs. The reporter is expressed at a readily detectable level in the cells inoculated with the PV preparation.

According to the registered nucleotide sequence of HPV51 DNA (GenBank M62877), isolated from cervical condyloma by Nuovo et al. (1988) and sequenced by Lungu et al. (1991), the deduced amino acid (*aa*) of the HPV51 L2 is markedly different from those of the other mucosal HPVs in two regions, *aa* 95–99 (region I) and *aa* 179–186 (region II) (Fig. 1A). This led us to newly sequence the L2 gene of another HPV51 isolated from a Japanese woman with CIN (Matsukura and Sugase, 1995). In this study, we found that the two regions of the new isolate were different from those of the prototype but homologous to those of the other mucosal HPVs. We prepared and

* Corresponding author.
E-mail address: kanda@nih.go.jp (T. Kanda).

0042-6822/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2009.08.034

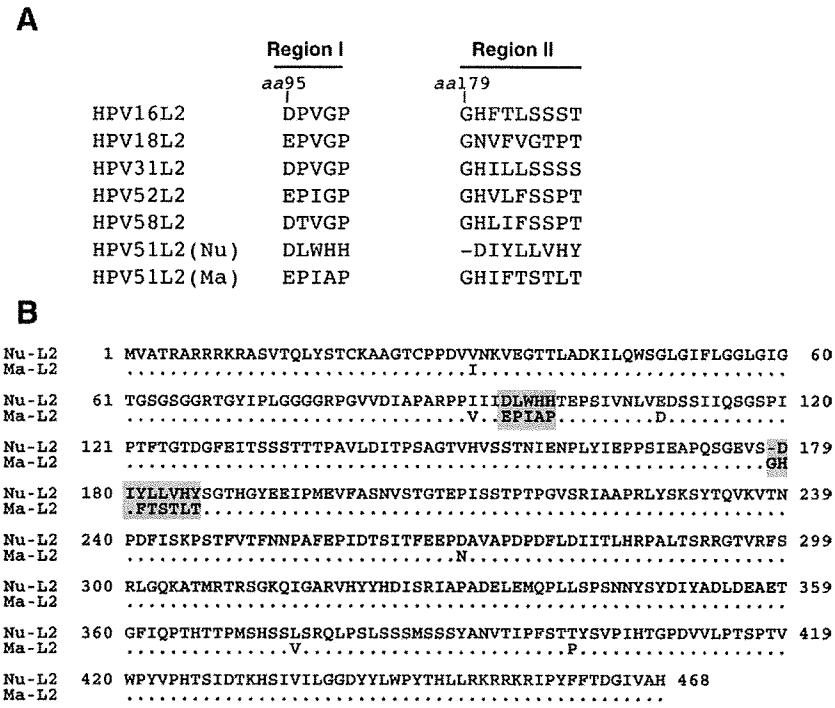


Fig. 1. Amino acid sequence of L2. (A) Amino acid sequences of L2 *aa* 95–99 (region I) and *aa* 179–186 (region II) of prevalent mucosal HPV L2s. HPV51 isolated by Nuovo et al. (1988) (Nu-strain) is indicated as HPV51-Nu and HPV51 isolated by Matsukura and Sugase (1995) (Ma-strain) is indicated as HPV51-Ma. (B) Total amino acid sequences of L2 of the two HPV51 strains. The upper line (Nu-L2) shows amino acid sequence of L2 of HPV51 Nu-strain (deduced from the nucleotides sequences of GenBank M62877). The lower line (Ma-L2) shows that of HPV51 Ma-strain.

characterized PVs by using L2s of the two strains. We found that the reporter expression from HPV51 PVs appears to require two L2 functions: a capacity to localize in the nucleus when expressed alone and unknown function associated with region I.

Results

Sequencing of HPV51 L1 and L2 genes

We sequenced the L1 and L2 genes of HPV51 isolated by Matsukura and Sugase (Ma-strain) (Matsukura and Sugase, 1995) (GenBank accession numbers for L1 and L2 genes of Ma-strain are GQ487711 and GQ487712, respectively) and compared the deduced aa sequences with those of the registered HPV51 (Nuovo et al., 1988) (named Nu-strain in this study). The *aa* sequence of the Ma-strain L2 (Ma-L2) was different from that of Nu-strain L2 (Nu-L2) at 6 *aa* positions and in regions I and II (Fig. 1). The *aa* sequences of the two regions of Ma-L2 were homologous to those of the other mucosal HPVs (Fig. 1A).

The *aa* sequence of Ma-strain L1 (Ma-L1) was different at only four positions (L at *aa* 52, G at *aa* 264, S at *aa* 265, and T at *aa* 272) from those of Nu-strain L1 (Nu-L1).

Subcellular localization of the L2s

We produced codon-modified genes encoding Ma-L1, Ma-L2, and Nu-L2, using appropriate synthetic oligonucleotides. Then, six DNA fragments encoding chimeric L2s, from Ch1-L2 to Ch6-L2 (Fig. 2A), were produced by PCR-based DNA synthesis.

Fig. 2B shows immunofluorescence staining of the L2s transiently expressed alone in HEK293FT cells. HEK293 cells were transfected with the expression plasmid for L2s and fixed with paraformaldehyde 1 day later. The cells were incubated with the cross-reactive anti-L2 serum, which had been produced by immunizing mice with HPV16 L2 peptide (*aa* 11–200), and incubated with Alexa Fluor 488-conjugated anti-mouse IgG rabbit serum. L2 was detected by fluorescence microscopy. Ma-L2 and Ch3-L2 were located almost exclusively in the nucleus. Nu-L2, Ch1-L2, and Ch6-L2 were located in the cytoplasm. Ch2-L2 was located mostly in the nucleus with fluorescence somewhat weaker than that of Ma-L2, along with some cells showing fluorescence in both the nucleus and the cytoplasm. Ch4-L2 and Ch5-L2 were located in the nucleus, with some cells showing cytoplasmic fluorescence as well.

When Nu-L2 was co-expressed with Ma-L1, which has a strong nuclear localizing signal (Zhou et al., 1991), the localization of Nu-L2 was shifted to the nucleus (data not presented), suggesting that Nu-L2 made a complex with Ma-L1 and moved to the nucleus with the help of Ma-L1.

Nuclear localizing signal of HPV51 L2

Ma-L2 and Nu-L2 had two nuclear localizing signals (NSLs) at their both ends. The DNA fragments of MA-L2 corresponding regions of *aa* 5–11 (encoding RARRRDR), *aa* 95–99 (region I, encoding VIIEPIAP-TEPSIVNLVD), *aa* 179–187 (region II, encoding GHIFTSTLT), and *aa* 452–457 (encoding RKRRKR) were fused to the N-terminus of enhanced green fluorescent protein (EGFP). The resultant fusion genes were transiently expressed in HEK293FT cells and localization of EGFP was examined by fluorescent microscopy (Fig. 3). The plain EGFP was located in both of the cytoplasm and the nucleus. The EGFP with the N-terminal region or the C-terminal region was clearly accumulated in the nucleus. The localization of EGFP with region I was similar to that of plain EGFP. The EGFP with region II was located in the nucleus slightly more than in the cytoplasm in some cells, but the nuclear accumulation in those cells was much less than the EGFP with the N- or C-terminal NLS. Because the *aa* sequences of Nu-L2 N-terminal and C-terminal regions are identical with those of Ma-L2, the NSLs of Nu-L2 have the same NSL sequences. Thus, the data indicate that both terminal regions evidently contain NSLs like the L2s of HPV6b and HPV16 (Sun et al., 1995; Darshan et al., 2004), suggesting that Nu-L2 regions I and II inhibit the NLS function of Nu-L2.

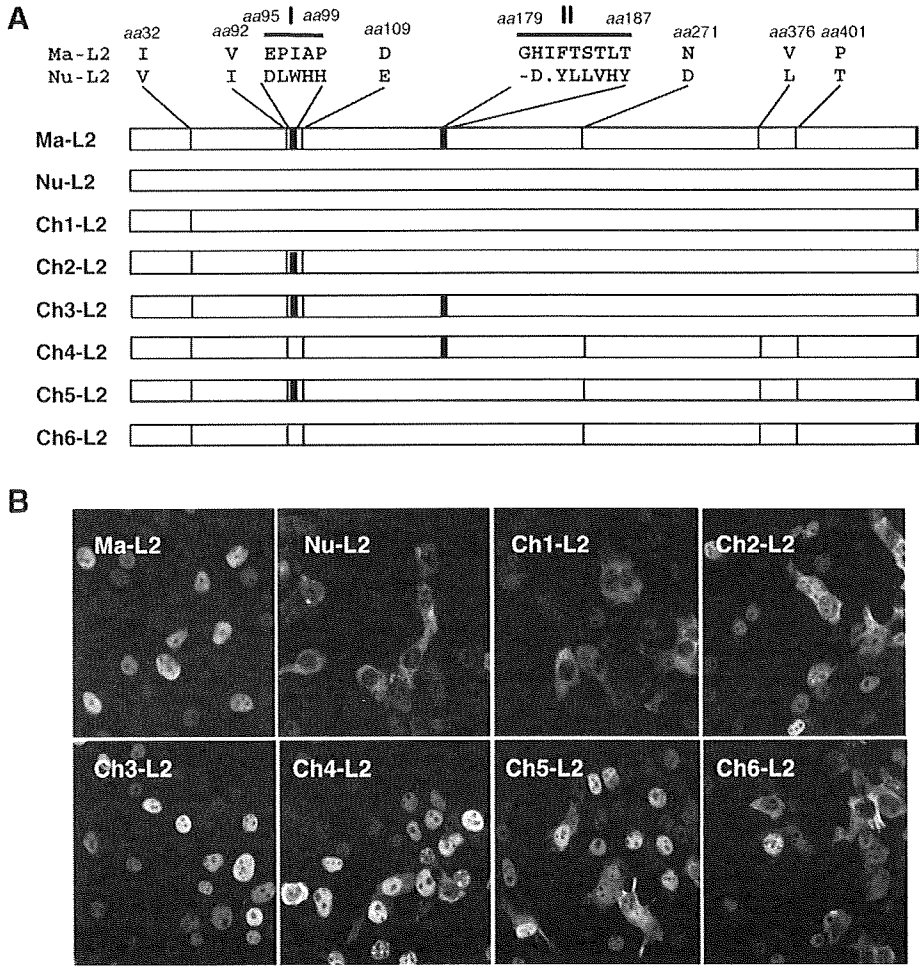


Fig. 2. Subcellular localization of the L2s. (A) Schematic representation of the L2s used in this study. (B) Subcellular localization of the L2s. 293FT cells were transfected with the expression plasmid for L2. One day later, the cells were fixed and L2 was detected with anti-L2 mouse antiserum and Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) (Invitrogen). The cells were coated with ProLong Gold anti-fade reagent (Invitrogen) and examined with a confocal microscope (FluoView1000, OLYMPUS, Tokyo, Japan).

Preparation and characterization of HPV51 pseudovirions

Since L2 is believed to play a critical role in the early process of infection (Pereira et al., 2009), we prepared and characterized HPV51 PVs containing either Ma-L2 or Nu-L2. The PVs were produced by using the codon-modified genes. HEK293FT cells were transfected with the L1- and L2-expression plasmids together with pYSEAP, a reporter plasmid expressing secretary alkaline phosphatase (SEAP). Sixty hours later the nuclear extract was centrifuged on a density-gradient as previously described (Kondo et al., 2007). A fraction containing the majority of the PVs was used as a PV preparation. The PV preparation produced with Ma-L2, Nu-L2, Ch1-L2, Ch2-L2, Ch3-L2, Ch4-L2, Ch5-L2, or Ch6-L2 was named as PV51MaL2, PV51NuL2, PV51Ch1L2, PV51Ch2L2, PV51Ch3L2, PV51Ch4L2, PV51Ch5L2, or PV51Ch6L2, respectively. For comparison, the nuclear extract from HEK293 cells transfected with the L1-expression plasmid and pYSEAP, without L2-expression plasmid, was similarly processed to produce PV51L2(−).

Transmission electron microscopy showed that PV51MaL2 and PV51NuL2 were composed of homogeneous spherical particles with a diameter of approximately 55 nm (Fig. 4A).

SDS-gel electrophoresis showed that all the PV preparations, except for PV51L2(−), contained both L1 and L2 (Fig. 4B). The

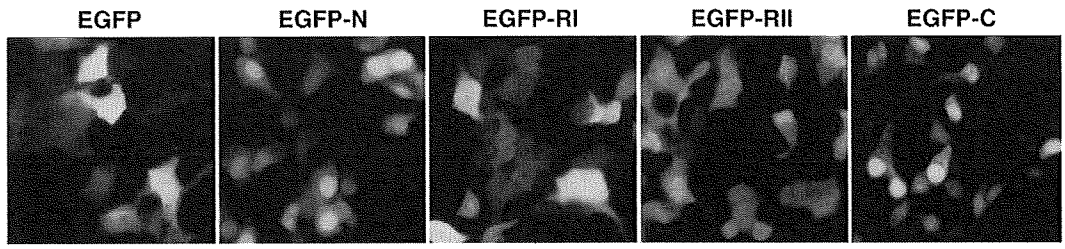
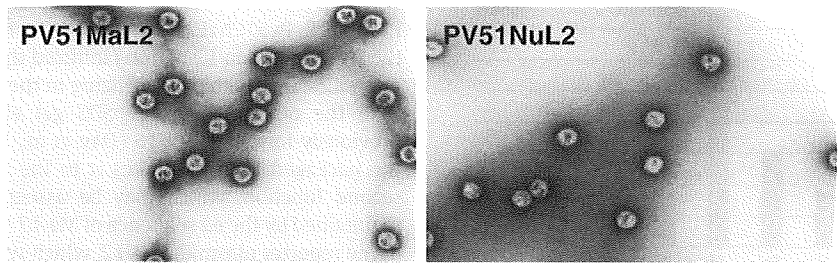
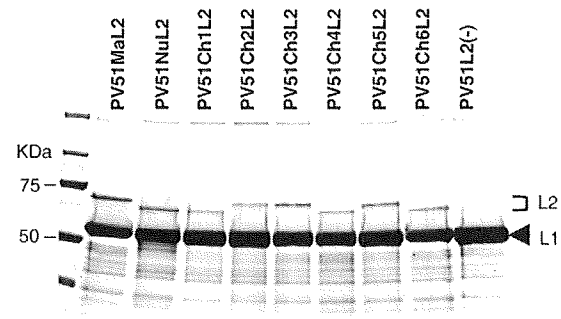


Fig. 3. Subcellular localization of the enhanced green fluorescent protein (EGFP) fused with nuclear localizing signal of Ma-L2. 293FT cells were transfected with the expression plasmid for EGFP or EGFP fused with N-terminal region (*aa* 5–11) (EGFP-N), region I (*aa* 92–109) (EGFP-RI), region II (*aa* 179–187) (EGFP-RII), and C-terminal region (*aa* 452–457) (EGFP-C). One day later, the cells were fixed and examined with a confocal microscope (FluoView1000, OLYMPUS, Tokyo, Japan).

A



B



C

	DNase resistant SEAP DNA (ng/1 μgL1protein)
PV51MaL2	0.6
PV51NuL2	0.9
PV51Ch1L2	1.4
PV51Ch2L2	0.8
PV51Ch3L2	0.7
PV51Ch4L2	0.9
PV51Ch5L2	1.5
PV51Ch6L2	1.2
PV51L2(-)	1.5

Fig. 4. Characterization of HPV51 pseudovirion preparations. (A) Electron micrograph of PV51MaL2 and PV51NuL2. The pseudovirion preparation was allowed to settle on carbon-coated copper grids and negatively stained with 4% uranylacetate. The grids were examined in a HITACHI model H-7600 transmission electron microscope and photographed at an instrumental magnification of ×200,000. Bar = 50 nm. (B) SDS–gel electrophoresis of the pseudovirion preparations. The pseudovirion preparation was mixed with an equal volume of SDS sample buffer and boiled. Then the sample was electrophoresed on 10% SDS–polyacrylamide gel. Protein was stained with SYPRO Ruby Protein Gel Stain (Life Technologies Corp.) and detected with a Typhoon 9410 image analyzer (GE Healthcare Life Sciences). (C) Level of the reporter plasmid packaged in the pseudovirion. The mixture of the pseudovirion preparation and DNase-I was incubated at 37 °C for 1 hour. DNase-resistant DNA was extracted and quantified by real-time PCR with primers complementary to the reporter plasmid, pYSEAP.

aliquots of the preparation were electrophoresed on an SDS–polyacrylamide gel and stained with SYPRO Ruby Protein Gel Stain (Life Technologies Corp.). Protein in the gel was detected with a Typhoon 9410 image analyzer (GE Healthcare Life Sciences). The L1-contents in the PV preparations, which were estimated by comparison of L1-bands with BSA-bands of known amount (from 0.01 to 0.1 μg per lane) on an SDS–gel (data not shown), ranged from 0.4 to 0.8 μg/μl. The PV preparation containing approximately 1 μg of L1 was electrophoresed and the proteins in the gel were stained similarly. PV51MaL2 contained slightly more L2 than the other PVs. Nu-L2, Ch2-L2, Ch3-L2, and Ch5-L2 migrated faster than the other L2s, indicating that region I was associated with the faster migration.

The PV preparations were found to contain the packaged reporter plasmid at a similar level (Fig. 4C). The PV preparations containing 2 to 4 μg of L1 were digested with DNase-I extensively and the level of the DNase-resistant pYSEAP was measured by a real-time PCR method. The numbers of reporter copies and the particles were calculated from the DNA and L1 contents, respectively. The PV preparations were estimated to contain approximately 0.3 to 1 genome copies per 100 particles; that is, the complete PVs constituted approximately 0.3% to 1% of the particles in a preparation. It is noteworthy that PV51L2(−) contained a similar level of the packaged reporter, indicating that the presence of L2 did not affect the packaging efficiency.

Expression of the reporter in HEK293FT cells inoculated with HPV51 pseudovirions

Fig. 5 shows the levels of reporter expression in HEK293FT cells inoculated with the PVs. The 293FT cells (10^4 cells) were inoculated with the PV preparation containing approximately 5×10^4 genome copies and incubated at 37 °C for 3 days. Then, the SEAP activity of the culture medium was measured. The cells inoculated with PV51L2(−)

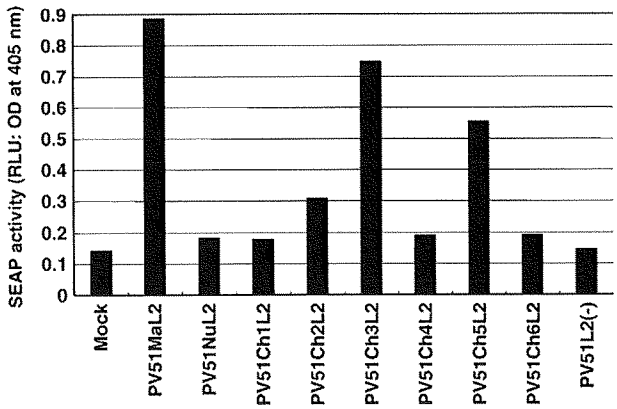


Fig. 5. Expression of secretary alkaline phosphatase (SEAP) in 293FT cells inoculated with the pseudovirion preparations. HEK293 cells inoculated with the pseudovirion preparation were incubated for 3 days and SEAP activity of the culture medium was measured by the colorimetric SEAP assay. Mock: SEAP activity of the culture medium of the cells not inoculated with the pseudovirion. RLU: relative light unit.

did not express the reporter, indicating that L2 is required for the reporter expression. The cells inoculated with PV51MaL2, PV51Ch3L2, and PV51Ch5L2 expressed the reporter efficiently and those with PV51Ch2L2 expressed less efficiently. The cells inoculated with PV51NuL2, PV51Ch1L2, PV51Ch4L2, and PV51Ch6L2 did not express the reporter. Evidently, L2s in PV51NuL2, PV51Ch1L2, PV51Ch4L2, and PV51Ch6L2 lacked the function required for the reporter expression.

Discussion

This study showed that the prototype HPV51 (Nuovo et al., 1988) is likely defective because the PV containing its L2 lacks infectivity (Fig. 5). Regions I and II of the prototype L2 were markedly different from those of the new isolate (Matsukura and Sugase, 1995), which were homologous to those of the other mucosal HPVs (Figs. 1A and B). The prototype may have been cloned from defective DNA that was integrated in the chromosome or maintained as an episome in the cervical condyloma.

Ma-L2 expressed alone in HEK293FT cells was located exclusively in the nucleus (Fig. 2B), perhaps by using its NLSs at the both ends as previously shown for L2s of HPV6b and 16 (Sun et al., 1995; Darshan et al., 2004). By contrast, Nu-L2 expressed in HEK293FT cells was located exclusively in the cytoplasm (Fig. 2B), despite the presence of NLS. Regions I and II of Nu-L2 probably affect the conformation of Nu-L2 and the resultant change may inhibit the activity of NSLs. The NLS function of chimeric L2s, having either region I or region II of Nu-L2 (Ch2-L2, Ch4-L2, and Ch5-L2), may have been partially reduced and that of chimeric L2s, having both regions (Ch1-L2 and Ch6-L2), markedly reduced.

Nu-L2 and three chimeric L2s, Ch1-L2, Ch4-L2, and Ch6-L2, migrated faster than the other L2s on the SDS–gel electrophoresis (Fig. 4B). Because only these L2s contain region I of Nu-L2, the faster migration is probably associated with the conformational modification induced by Nu-L2 region I.

Nu-L2 was translocated to the nucleus in the cells expressing Ma-L1, suggesting that Nu-L2 binds to L1 and makes a complex, as described previously for HPV11 L1 and L2 (Finnen et al., 2003), and the complex moves to the nucleus by using the NLS of L1. The L1-mediated translocation seems to make L2s with the reduced NSL activities possible to assemble into the particles in the nucleus.

PV51MaL2 and PV51NuL2 contained spherical particles (Fig. 4A) composed of L1 and L2 (Fig. 4B). PV51MaL2, PV51NuL2, PV51Ch1L2, PV51Ch2L2, PV51Ch3L2, PV51Ch4L2, PV51Ch5L2, and PV51Ch6L2 contained L1 and L2 at a comparable ratio and the DNase-resistant (very likely packaged) reporter plasmid at similar levels (Fig. 4C). However, the reporter was expressed in HEK293FT cells inoculated with PV51MaL2, PV51Ch2L2, PV51Ch3L2, and PV51Ch5L2 but not with Pv51NuL2, Pv51Ch1L2, or Pv51Ch4L2, and Pv51Ch6L2 (Fig. 5). Thus, the PVs having Ma-L2 region I induced the expression of the reporter. These L2s are capable of localizing in the nucleus and migrate slower than the other L2s in the SDS–gel electrophoresis. As reported previously for L2 of HPV16 (Day et al., 2004), probably L2 binds to the packaged DNA and takes it to the nucleus. Then, an unknown second function, which may be associated with L2 conformation maintained by the *aa* sequence of Ma-L2 region I, helps the expression of the reporter plasmid. Ch4-L2, which was located in the nucleus but migrated faster, probably lacks the second function.

The reporter plasmid was packaged into PV51L2(−) similarly, indicating that L2 is not involved in the packaging (Fig. 4C) as reported by Buck et al. (2005). The particles containing the reporter plasmid were approximately 0.3% to 1% of the particles in the PV preparations. The extremely low packaging efficiency suggests that the packaging occurs by chance. For the survival of HPV51 in humans, HPV genomic DNA must have an unidentified mechanism, such as a packaging signal, to induce efficient packaging.

In summary, this study clearly showed that although HPV51 L2 is not involved in the packaging of the reporter into the L1/L2-VLP in the process of pseudovirion production, the expression of the packaged reporter requires the functions of L2: its nuclear localizing capacity and unknown function associated with *aa* 95–99.

Materials and methods

Cell

HEK293FT cells, a cell line expressing a high level of SV40 T-antigen, was purchased from Life Technologies Corp. (Carlsbad, CA, USA). The cells were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids (Life Technologies Corp.), 1% GlutaMax-I (Life Technologies Corp.), and Geneticin (500 μg/ml; Life Technologies Corp.).

Expression plasmids

The L1 and L2 genes of the cloned Ma-strain HPV51 DNA (a gift from Dr. Toshihiko Matsukura) (Matsukura and Sugase, 1995) were sequenced with an ABI 3130xl sequencer (Life Technologies Corp.). The *aa* sequences of L1 and L2 of Matsukura strain were deduced from the nucleotide sequences and those of Nuovo strain were deduced from the nucleotide sequences in the database (GenBank M62877). The nucleotide sequence of the codon-modified gene encoding the L1 or the L2 was designed with the following codons, which are used most frequently in human mRNAs; Ala: GCC; Cys: TGC; Asp: GAC; Glu; GAG; Phe: TTC; Gly: GGC; His: CAC; Ile: ATC; Lys: AAG; Leu: CTG; Asn: AAC; Pro: CCC; Gln: CAG; Arg; AGG; Ser: AGC; Thr: ACC; Val: GTG; Trp: TGG; Tyr: TAC. The codon-modified genes were produced by PCR with sense and antisense synthetic oligonucleotides that had an annealing region (approximately 20 nucleotides) at its 3′ end. HPV16L1 gene inserted between *Not*I and *Hin*dIII sites in p16L1h (gifts from J. T. Schiller), a plasmid expressing the codon-modified HPV16 L1, was replaced with the codon-modified L2 genes encoding Ma-L1, Ma-L2, and Nu-L2 to produce pMa-L1, pMa-L2, and pNu-L2, respectively.

Three regions of the codon-modified Nu-L2 gene, nt1-96, nt1-327, and nt1-561 (A at the first ATG of Nu-L2 gene was numbered as nt1) were replaced with corresponding regions of codon-modified Ma-L2 genes by using PCR to produce Ch1-L2, Ch2-L2, and Ch3-L2 genes, respectively. The regions of the codon-modified Ma-L2 gene, nucleotide (*nt*) 285 to 296 and *nt* 536 to 560 were replaced with corresponding regions of codon-modified Nu-L2 gene by using PCR to produce Ch4-L2 and Ch5-L2 genes, respectively. The region of Ch4-L2

gene, nt1 to nt456, was ligated with the region of Ch5-L2 gene, nt457 to nt1410, at the *PmlI* site to produce Ch6-L2 gene. These genes were inserted similarly to p16L1h to produce pCh1-L2, pCh2-L2, pCh3-L2, pCh4-L2, pCh5-L2, and pCh6-L2, respectively.

Expression plasmids for EGFP fused with L2 NSLs were produced by insertion of the annealed synthetic oligonucleotides encoding MVAT plus none, *aa* 5–11, *aa* 92–109, *aa* 179–187, and *aa* 452–457 of Ma-L2 at the multicloning site of EGFP gene (pCMS-EGFP; Clontech Inc. Mountain View, CA). The resultant fusion EGFP contained additional *aa* sequences at its N-terminus.

The plasmid expressing secretary alkaline phosphatase from SV40 early promoter, pYSEAP, was a gift from J. T. Schiller.

The nucleotide sequences of the all the plasmids constructed in this study were verified by sequencing.

Fluorescence microscopy

HEK293FT cells in an 8-well chamber plate (Nunc, Rochester, NY) were transfected with 0.2 μg of the expression plasmid for L2 by using Optifect transfection reagent (Invitrogen). One day later, the cells were fixed with 4% paraformaldehyde in PBS and incubated with 0.5% Triton X-100 in PBS. The cells were reacted with anti-L2 serum obtained by immunizing mice with HPV16L2 peptide (the region of *aa* 11–200), which cross-reacts with L2s of various mucosal HPVs, followed by Alexa Fluor 488 goat anti-mouse IgG (H + L) (Invitrogen). The cells were coated with ProLong Gold anti-fade reagent (Invitrogen) and examined with a confocal microscope (FluoView1000, OLYMPUS, Tokyo, Japan).

HEK293FT cells in an 8-well chamber plate (Nunc, Rochester, NY) were transfected with 0.2 μg of the expression plasmid for EGFP fused with Ma-L2 NSL by using Optifect transfection reagent (Invitrogen). One day later, the cells were fixed with 4% paraformaldehyde in PBS, coated with ProLong Gold anti-fade reagent (Invitrogen), and imaged in a BZ-8000 fluorescence microscope (Keyence, OSAKA, Japan).

Production of pseudovirions

HEK293FT cells (Life Technologies Corp.), which had been seeded in a 10-cm culture dish ($1X10^7$ cells) at 16 hours before the transfection, were transfected with a mixture of an L1-plasmid (13.5 μg), an L2-plasmid (3 μg), and pYSEAP (13.5 μg) by using Fugene HD (Rosch Diagnostics GmbH, Mannheim, Germany). Sixty hours later, the cells were harvested with trypsin. The cells were suspended in 0.5 ml of lysis buffer (PBS containing 1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.35% Brij58 [Sigma-Aldrich Inc., St. Louis, MO], 0.1% Benzonase [Sigma-Aldrich Inc.], 0.1% Plasmid Safe ATP dependent-DNase [EPICENTRE Corp. Madison, WI]) and incubated for 30 hours at 37 °C with slow rotation. The lysate was cooled on ice for 5 minutes, mixed with 5 M NaCl solutions to adjust the concentration of NaCl to 0.85 M, and further kept on ice for 10 minutes. Then, the lysate was centrifuged at 5000*g* for 10 minutes at 4 °C. The supernatant was layered on an Optiprep gradient (from top to bottom, 27%, 33%, and39% in PBS containing 1 mM $CaCl_2$, 10 mM $MgCl_2$, and 0.8 M NaCl) and centrifuged at 50,000 rpm for 3.5 hours at 16 °C in an SW55Ti rotor (Beckman Coulter Inc. Fullerton, CA). Fractions (300 μl each) were obtained by puncturing the bottom. Aliquots (5 μl per fraction) were analyzed by SDS-gel electrophoresis. The fraction that contained L1 most abundantly was used as a pseudovirion (PV) preparation.

Electron microscopy

The particles in a PV preparation were allowed to settle on carbon-coated copper grids and stained with 4% uranylacetate. The grids were examined in a HITACHI model H-7600 transmission electron microscope and photographed at an instrumental magnification of ×200,000.

Quantification of L1 protein

A PV preparation (0.1 μl) or standard protein solution consisting of the known amount (~0.1–1 μg/μl) of bovine serum albumin (BSA) was mixed with an equal volume of SDS sample buffer and boiled. These samples were electrophoresed on 10% SDS–polyacrylamide gel. Protein was stained with SYPRO Ruby Protein Gel Stain (Life Technologies Corp.) and detected with a Typhoon 9410 image analyzer (GE Healthcare Life Sciences). The amount of L1 protein in the PV preparation was estimated from comparing the images obtained with a Typhoon 9410 imaging analyzer and Image Quant TL software (GE Healthcare, Chalfont St. Giles, UK).

Quantification of DNase-resistant reporter DNA

Five microliters of a PV preparation was mixed with 195 μl of PBS containing $MgCl_2$ (10 mM) and DNase-I (70 U). After incubating at 37 °C for 1 hour, DNA was extracted by using QIAamp DNA Blood kit (Qiagen GmbH, Hilden, Germany). pYSEAP DNA in the sample was quantified by real-time PCR using forward primer (AGAACCCG-GACTTCTGGAAC) and reverse primer (GGCAGCTGTCACCGTAGACA).

Quantification of SEAP activity

A PV preparation was inoculated to HEK293FT cells (2×10^4), which had been seeded 1 day prior to the inoculation in 96-well flat-bottom tissue culture-treated plates (Corning Costar Corp., New York, NY). The cells were incubated for 3 days and SEAP activity of the culture medium was measured by the colorimetric SEAP assay (NCI home page: http://home.ccr.cancer.gov/lco/colorimetricseap.htm).

Acknowledgments

We thank Dr. Kunito Yoshiike for critical reading of the manuscript. This work was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare for the Third-Term Comprehensive 10-year Strategy for Cancer Control.

References

Buck, C.B., Pastrana, D.V., Lowy, D.R., Schiller, J.T., 2004. Efficient intracellular assembly of papillomaviral vectors. J. Virol. 78, 751–757.

Buck, C.B., Pastrana, D.V., Lowy, D.R., Schiller, J.T., 2005. Generation of HPV pseudovirions using transfection and their use in neutralization assays. Methods Mol. Med. 119, 445–462.

Collier, B., Öberg, D., Zhao, X., Schwartz, S., 2002. Specific inactivation of inhibitory sequences in the 5′ end of the human papillomavirus type 16 L1 open reading frame results in production of high levels of L1 protein in human epithelial cells. J. Virol. 76, 2739–2752.

Darshan, M.S., Lucchi, J., Harding, E., Moroianu, J., 2004. The L2 minor capsid protein of human papillomavirus type 16 interacts with a network of nuclear import receptors. J. Virol. 78, 12179–12188.

Day, P.M., Baker, C.C., Lowy, D.R., Schiller, J.T., 2004. Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. Proc. Natl. Acad. Sci. U. S. A. 101, 14252–14257.

Finnen, R.L., Erickson, K.D., Chen, X.S., Garcea, R., 2003. Interaction between papillomavirus L1 and L2 capsid proteins. J.Virol. 77, 4818–4826.

Howley, P.M., Lowy, D.R., 2001. Papillomaviruses and their replication, In: Lnipe, D.M., Howley, P.M. (Eds.), 4th ed. Fields Virology, Vol. 2. Lippincott, Williams and Wilkins, Philadelphia, pp. 2197–2229.

Kondo, K., Ishii, Y., Ochi, H., Matsumoto, T., Yoshikawa, H., Kanda, T., 2007. Neutralization of HPV16, 18, 31, and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region. Virology 358, 266–272.

Lungu, O., Nuovo, G.J., Crum, C.P., Silverstein, S.J., 1991. Biologic properties and nucleotides sequence analysis of human papillomavirus type 51. J. Virol. 65, 4216–4225.

Matsukura, T., Sugase, M., 1995. Identification of genital human papillomavirus in cervical biopsy specimens: segregation of specific virus types in specific clinicopathologic legions. Int. J. Cancer 61, 13–22.

Nuovo, G.J., Crum, C.P., De Villiers, E.M., Levine, R.U., Silverstein, S.J., 1988. Isolation of a novel human papillomavirus (type 51) from a cervical condyloma. J. Virol 62, 1452–1455.

Pereira, R., Hitzeroth, I.I., Rybicki, E.P., 2009. Insights into the role and function of L2, the minor capsid protein of papillomaviruses. Arch. Virol. 154, 187–197.
Sapp, M., Selinka, H.C., 2005. Pseudovirions as specific tools for investigation of virus interactions with cells. Methods Mol. Biol. 292, 197–212.
Sokolowski, M., Tan, W., Jellne, M., Schwartz, S., 1998. mRNA instability elements in the human papillomavirus type 16 L2 coding region. J. Virol. 72, 1504–1515.
Stauffer, Y., Raj, K., Masternak, K., Beard, P., 1998. Infectious human papillomavirus type18 pseudovirions. J. Mol. Biol. 283, 529–536.
Sun, X.Y., Frazer, I., Müller, M., Gissmann, L., Zhou, J., 1995. Sequences required for the nuclear targeting and accumulation of human papillomavirus type 6B L2 protein. Virology 213, 321–327.
Unckell, F., Streeck, R.E., Sapp, M., 1997. Generation and neutralization of pseudovirions of human papillomavirus type33. J. Virol. 71, 2934–2939.
Zhou, J., Doorbar, J., Sun, X.Y., Crawford, L.V., McLean, C.S., Frazer, I.H., 1991. Identification of the nuclear localization signal of human papillomavirus type 16 L1 protein. Virology 185, 625–632.

Biochemical and Biophysical Research Communications 387 (2009) 525–530

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Identification of nucleolin as a protein that binds to human papillomavirus type 16 DNA

Hidetaka Sato [a,b], Rika Kusumoto-Matsuo [a], Yoshiyuki Ishii [a], Seiichiro Mori [a], Tomomi Nakahara [a], Fumiko Shinkai-Ouchi [c], Kei Kawana [b], Tomoyuki Fujii [b], Yuji Taketani [b], Tadahito Kanda [a], Iwao Kukimoto [a,*]

[a] Pathogen Genomics Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[b] Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[c] Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

ARTICLE INFO

Article history:
Received 25 June 2009
Available online 16 July 2009

Keywords:
Human papillomavirus
Nucleolin
Host factor
Genome maintenance

ABSTRACT

Transcription, replication, and segregation of human papillomaviruses (HPVs) are regulated by various host factors, but our understanding of host proteins that bind to the HPV genome is limited. Here we report the results of a search of cellular proteins that can associate with specific genomic regions of HPV type 16 (HPV16). We found that human nucleolin, an abundant nucleolar protein, was preferentially captured *in vitro* by an HPV16 genomic fragment from nucleotide positions (nt) 531–780. Electrophoretic mobility shift assays with a bacterially expressed nucleolin revealed that nucleolin bound to an HPV16 genomic region between nt 604 and 614 in a sequence-dependent manner. Chromatin immunoprecipitation analysis showed that both exogenous and endogenous nucleolin bound to a plasmid containing the HPV16 genomic region in HeLa cells. Furthermore, nucleolin associated with the HPV16 genome stably maintained in HPV16-infected W12 cells, suggesting that the nucleolin binding may be involved in the dynamics of the HPV genome in cells.

Introduction

Human papillomaviruses (HPVs), which are recognized as the causative agents of cervical cancer, have circular double-stranded DNA genomes with sizes close to 8 kbp [1]. HPV infects basal cells in the epidermis and its genome is maintained as episomes, whereas the viral genome amplification occurs in upper differentiating epithelium [2]. Due to the limited coding capacity of its small genome, HPV relies heavily on the function of host cell proteins for viral transcription, replication and segregation [3,4]. The transcription of the HPV genome is driven by two major promoters: in HPV type 16 (HPV16) genome, the early promoter P_{97} directs early gene transcription, while the late promoter P_{670} induces capsid expression. After initial unwinding of the replication origin by the coordinated action of the HPV E1 and E2 proteins, the HPV DNA replication progresses with the use of cellular replication proteins. For viral genome maintenance, the HPV genome is passively segregated by being tethered to host chromosomes, then passed into nuclei of daughter cells. Because of a lack of cell culture systems for efficient HPV propagation, molecular mechanisms of these processes are not fully understood.

Nucleolin is an abundant, ubiquitously expressed protein that is found in the nucleolus, the nucleoplasm, and on the cell surface, and is involved in regulation of ribosomal DNA (rDNA) transcription and the maturation of pre-ribosomal RNA [5]. In addition, nucleolin exerts several nuclear functions related to the transcription of several genes by the RNA polymerase II [6–8], genotoxic stress response [9], and chromosome congression in mitosis [10].

To gain new insights into how the dynamics of the HPV genome are regulated by host factors, we searched for cellular proteins that can bind to the two promoter regions of HPV16 using an unbiased proteomic approach. We describe the identification and characterization of nucleolin as an HPV16 genome-binding protein that may play a role in regulation of the HPV life cycle.

Materials and methods

Isolation of HPV16 genome-binding protein and its identification by peptide mass fingerprinting. Three HPV16 DNA fragments, I (nt 7791–120, 234 bp), II (nt 131–360, 230 bp), and III (nt 531–780, 250 bp), were generated by PCR using following primers: I forward, 5′biotin—TAC ATG AAC TGT GTA AAG GTT AGT CA-3′; I reverse, 5′-TGT GGG TCC TGA AAC ATT GCA GTT CTC TTT-3′; II forward, 5′biotin—AGA AAG TTA CCA CAG TTA TGC ACA GA-3′; II reverse, 5′-GTT CCA TAC AAA CTA TAA CAA TAA TGT CTA-3′; III forward, 5′biotin—CAA GAA CAC GTA GAG AAA CCC AGC TG-3′; III reverse, 5′-GTG TGT

* Corresponding author. Address: Pathogen Genomics Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-murayama, Tokyo 208-0011, Japan. Fax: +81 42 567 5632.
E-mail address: ikuki@nih.go.jp (I. Kukimoto).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.07.055

GCT TTG TAC GCA CAA CCG-3′. The biotinylated PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega) and coupled to Dynabeads M-280 streptavidin (Dynal, Norway) in a buffer consisting of 10 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, and 1 M NaCl. The HeLa nuclear extract was prepared by Dignam's procedure and incubated with the HPV16 DNA-coupled magnetic beads at 4 °C overnight. The beads were then washed three times in a wash buffer (10 mM Hepes, pH 7.9, 200 mM NaCl, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, and 10% glycerol). The bound proteins were released from the beads in SDS-sample buffer by boiling for 5 min and fractionated in a 4–20% gradient SDS–polyacrylamide gel (Daiichi Pure Chemicals, Japan), followed by silver staining. The 95-kDa protein band bound to fragment III was excised from the gel and subjected to in-gel trypsin digestion. The resultant peptide mixtures were analyzed by MALDI-QIT-TOF MS (AXIMA-QIT, Shimazu Biotech, Japan). Mascot software (Matrix Science) was used for protein identification.

Electrophoretic mobility shift assay (EMSA). The full-length cDNA of human nucleolin was amplified by RT-PCR from mRNA of HeLa cells. The resultant cDNA sequence completely matched the nucleolin sequence in the GenBank (NM 005381). To generate an expression plasmid for glutathione *S*-transferase (GST) fused nucleolin, the cDNA fragment encoding nucleolin amino acid (aa) from 289 to 710 was amplified by PCR with the full-length nucleolin cDNA as a template, and cloned into pGEX-2TK (GE Healthcare). GST–nucleolin and GST were expressed in *Escherichia coli* and purified using a GSTrap HT column and an AKTAprime (GE Healthcare). The EMSA was performed as described previously [11]. The DNA/protein complex was separated on a 5% polyacrylamide gel and visualized by autoradiography on X-ray films.

Chromatin immunoprecipitation (ChIP) assay. An expression plasmid for N-terminally FLAG-tagged nucleolin (FLAG-nucleolin) was constructed by cloning the full-length cDNA of nucleolin into p3xFLAG-CMV10 (Sigma). The ChIP assay was performed as described previously [11] with some modifications. Briefly, HeLa cells were transfected with pGL3-P_{670} or pGL3-Basic (Promega) together with the FLAG-nucleolin expression plasmid or p3xFLAG-CMV10 using FuGENE6 (Roche). At 48 h after the transfection, the cells were fixed with 1% formaldehyde at 37 °C for 5 min, lysed and sonicated using a Bioruptor (Cosmobio, Japan). The sonicated extract was immunoprecipitated with specific antibodies that had been coupled to Dynabeads M-280 sheep anti-mouse IgG (Dynal). Antibodies used were anti-FLAG M2 (Sigma), anti-nucleolin (MS-3, Santa Cruz, or 4E2, Abcam), or control mouse IgG (Santa Cruz). After washing the beads, the immunoprecipitated DNA/protein complexes were eluted, and reverse cross-linked. DNA was purified and subjected to PCR for HPV16 DNA (from nt 501 to 670), 18S rDNA, or pGL3-Basic. PCR primers were as follows: HPV16 forward, 5′-CCG GTC GAT GTA TGT CTT GTT GCA GAT CAT-3′; HPV16 reverse, 5′-CAT CCT CCT CCT CTG AGC TGT CAT TTA ATT-3′; 18S rDNA forward, 5′-GCC TGG ATA CCG CAG CTA GGA ATA ATG G-3′; 18S rDNA reverse, 5′-TTG ATT AAT GAA AAC ATT CTT GGC AAA TG-3′; pGL3-Basic forward, 5′-AGA CCC ACG CTC ACC GGC TCC AGA-3′; pGL3-Basic reverse, 5′-ACG AGC GTG ACA CCA CGA TGC CTG T-3′. The amounts of the immunoprecipitated DNA were quantified by real-time PCR analysis using a LightCycler 480 (Roche) with the LightCycler 480 SYBR Green I Master reagent (Roche). W12 cells were cultured in an undifferentiated state as described [12], and the ChIP assay was performed without plasmid transfection.

Generation of nucleolin-knockdown cells and Western blotting. The stable nucleolin-knockdown cell line derived from HeLa cells was established by transfection of an expression plasmid for small hairpin RNA (shRNA) against nucleolin and subsequent selection of cells in the presence of 10 μg/ml puromycin. The shRNA-expression plasmid was constructed by cloning the shRNA target sequence for nucleolin (5′-GGA AGA CGG TGA AAT TGA T-3′) [13] into pBAsi-hU6 (Takara, Japan). For Western blot analysis, cell extracts were prepared by boiling cells in SDS-sample buffer. Protein samples were separated on a 12% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany), and probed with a specified primary antibody and a peroxidase-conjugated secondary antibody. Antibodies used were anti-nucleolin (MS-3, Santa Cruz, or 3G4B20, Active Motif), anti-PCNA (PC10, Santa Cruz), and anti-FLAG M2. Specific proteins were visualized using an ECL Western blot detection system (GE Healthcare).

Results

Identification of nucleolin as a protein binding to an HPV16 genomic fragment

Two regions in the HPV16 genome were chosen as targets to screen for cellular proteins that might potentially regulate transcription and/or replication of HPV (I and III in Fig. 1A). Fragment I (nt 7791–120) contains the regulatory sequence for the HPV16 early promoter P_{97} and the replication origin, whereas fragment III (nt 531–780) includes the late promoter P_{670}. To find specific binding proteins for these fragments, fragment II (nt 131–360) was used as a control for comparison, because no particular binding proteins were assigned to this region. These DNA fragments were generated by PCR so as to have 5′-biotin-labeled ends, coupled to streptavidin-conjugated magnetic beads and then incubated in a nuclear extract prepared from HeLa cells. After washing the beads, bound proteins were released and separated by SDS–PAGE, followed by silver staining. Among many protein bands detected, fragment III selectively bound to a 95-kDa protein (Fig. 1B), while no specific proteins were detected for fragment I. To identify the 95-kDa protein, the band was excised from the gel, digested with trypsin, then subjected to peptide mass fingerprinting. The list of observed mass fingerprints significantly fitted the predicted mass fingerprint of human nucleolin (p value = 0.0094). Western blot analysis with an anti-nucleolin antibody verified an enrichment of nucleolin in the bound fraction of fragment III (Fig. 1C).

In vitro binding of nucleolin to the HPV16 DNA

An electrophoretic mobility shift assay (EMSA) revealed that a recombinant nucleolin bound to a site in the HPV16 genome from nt 591 to 620. Human nucleolin from amino acid 289 to 710, which contains four RNA-binding domains and a C-terminal glycine/arginine-rich region (Fig. 2A), was expressed as a fusion protein with GST (GST–nucleolin) in bacteria and affinity purified (Fig. 2B). This truncated nucleolin has been shown to exhibit DNA-binding activity [6]. GST–nucleolin was examined in EMSA for its capability to form a complex with overlapping DNA probes having the HPV16 sequences from nt 531 to 660. Among the HPV16 probes tested, probe f generated a shifted band with GST–nucleolin (Fig. 2C).

Nucleolin has been previously reported to bind to a different location in the HPV18 genome *in vitro*, and the binding site has been assigned to the sequence 5′-TTGCTTGCATAA-3′ (nt 7642–7653 in the HPV18 genome) [14]. Similarity between the HPV18 sequence and probe f was explored, and the same sequence motif, 5′-TTGCXXXCAXA-3′, was found in the two sequences (Fig. 2D, upper panel). To test whether this sequence motif was recognized by nucleolin, base substitutions that have been shown to abolish the nucleolin binding to the HPV18 site were introduced into probe f. The mutations completely disrupted the GST–nucleolin binding to the probe (Fig. 2D), demonstrating that nucleolin bound to this motif in probe f in a sequence-dependent manner.