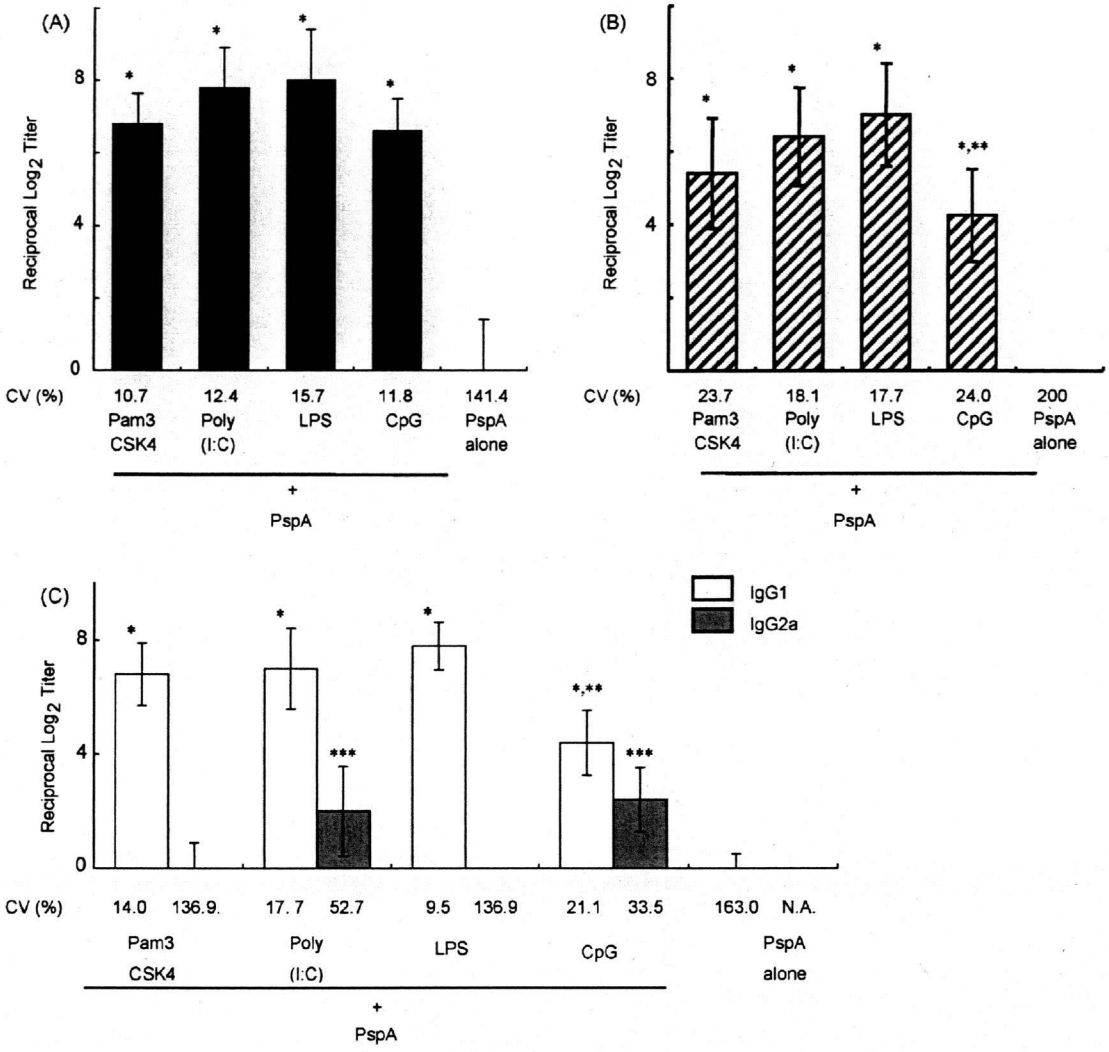


**Fig. 2.** Induction of PspA-specific IgG (closed bar) (A), IgA (hatched bar) (B) and PspA-specific IgG1 (open bar) and IgG2a (gray bar) (C) in bronchoalveolar lavage fluid (BALF) by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times 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.

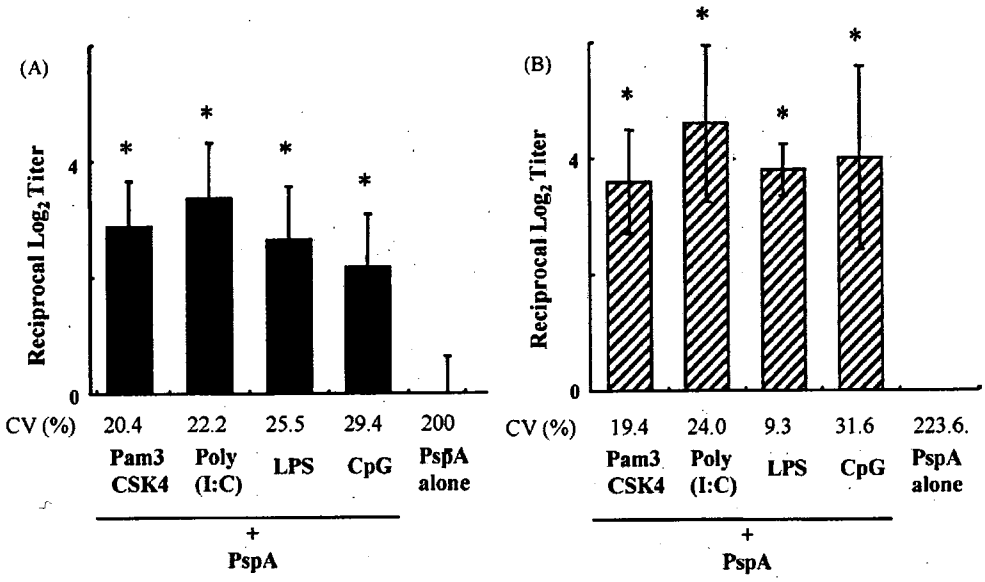


**Fig. 3.** Induction of PspA-specific IgG (closed bar) (A) and IgA (hatched bar) (B) in nasal wash (NW) by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times at weekly intervals with 10 μg of TLR agonist and 2.5 μg of PspA. One week 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 $3 \times 10^5$ 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 $3 \times 10^5$ 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

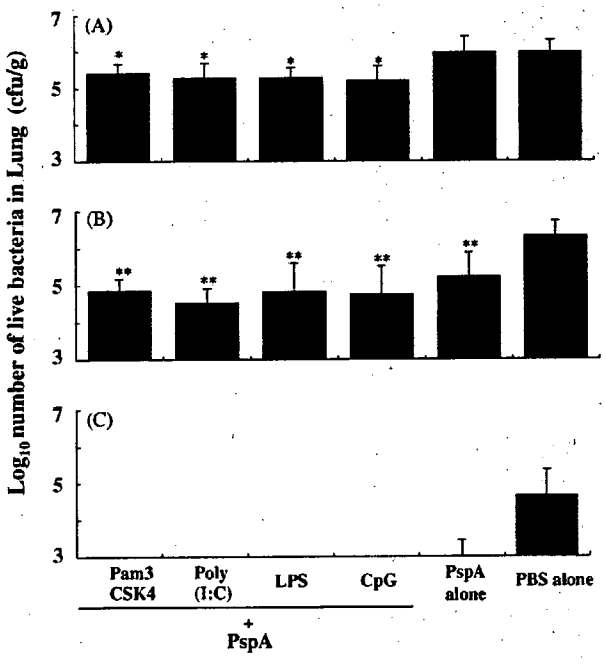


**Fig. 4.** The effect of intranasal immunization by PspA plus each TLR agonist on the bacterial densities in the lung tissue at 3 h (A), 6 h (B) and 12 h (C) post-challenge with *S. pneumoniae* WU2 strain. A dose of $2 \times 10^6$ cfu/mouse was nasally administered to mice previously immunized with 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.

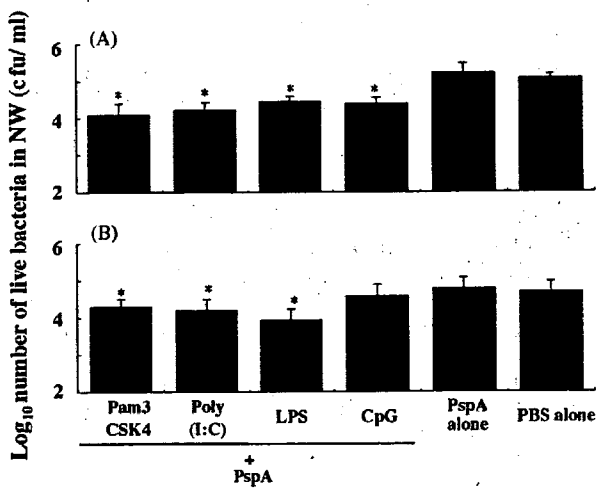


**Fig. 5.** The effect of intranasal immunization by PspA plus each TLR agonist on the bacterial densities in the nasopharynx 1 day (A) and 6 days (B) after challenge with *S. pneumoniae* EF3030 strain. A dose of $3 \times 10^5$ cfu/mouse was nasally administered to mice previously immunized with either 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]. Polyl:Poly$C_{12}$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.

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# Development of mucosal adjuvants for intranasal vaccine for H5N1 influenza viruses

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**Abstract:** An increasing number of infections of highly pathogenic avian influenza virus (H5N1) in humans has been reported in South-East Asia and other areas of the world. High mortality (>60%) of this viral infection and its pathosis of systemic infection are features of this new human disease. Moreover, there is great concern that this avian H5N1 virus could cause a pandemic of new influenza in humans, once it acquires the ability for human to human transmission. To prevent such highly contagious infectious diseases as influenza, it is essential to prepare effective vaccines. Especially in the case of new influenza virus, we cannot predict the strain which will cause the pandemic. In such a situation, a vaccine that induces cross-protective immunity against variant viruses is extremely important. However currently used parenteral seasonal influenza vaccine is strain-specific, and is less effective against variant viruses. In order to overcome the weakness of current vaccines we need to learn from the immune responses induced by natural infection with influenza viruses. In the case of mucosally acquired acute respiratory infection such as influenza, mucosal immunity induced by natural infection plays important role in protection against the infection, as mucosal secretory IgA antibody plays an important role in cross-protection. In this review we describe the advantages and development of mucosal vaccine against highly pathogenic H5N1 influenza viruses.

**Keywords:** influenza virus, mucosal immunity, secretory IgA antibody, adjuvant

## Influenza virus and its infection signal

Influenza is a contagious acute respiratory disease of birds and mammals caused by infection of the upper respiratory tract by viruses of the family Orthomyxoviridae. Types A and type B infect humans and cause respiratory symptoms and also encephalopathy in infants. Recently it has been reported that infection by highly pathogenic influenza viruses (HPIV) and the avian influenza virus (H5N1) in humans can be fatal. In cases where infection sites were not restricted to the respiratory system, it spread systemically including the gastrointestinal (GI) tract.[1] Although most human H5N1 infections have been caused by the direct transmission of virus from infected poultry, there is fear that a pandemic could result if subsequent transmission of H5N1 virus occurred between infected humans.[2] Therefore, there is an urgent and important public health need to develop effective vaccines against this highly pathogenic strain of avian influenza virus.

Annual epidemics of influenza are caused when the antigenic properties of the viral surface proteins hemagglutinin (HA) and neuraminidase (NA) are altered. HA is involved in binding of the virus to sialic acids on the surface of susceptible cells.[3] NA cleaves terminal sialic acid residues from carbohydrate moieties on the surfaces of infected cells, promoting the release of progeny viruses from infected cells. It has been shown that both HA and NA are among the most protective of the various viral proteins against influenza when immunized with plasmid DNAs encoding HA and NA.[4]

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Influenza virus has single-stranded RNA as its genome, and this single-stranded RNA is recognized as an infection signal by host cells through Toll-like receptor 7 (TLR-7).[5] In the course of viral replication, double-stranded RNA is produced, which is recognized by TLR-3 as an infection signal. Thus, influenza virus is recognized by host immune cells at the very early stage of infection by the host through pathogen signals, and these receptors and the host immune system initiate the mucosal and systemic immune system against present and future viral infection. By verifying a series of events occurring at the infection site, we use our increased understanding of the immune response to develop and apply strategies to combat influenza viral infection.

## Innate immunity and adjuvant effect

Innate immunity is a set of nonspecific mechanisms that constitute the body's naturally occurring immune response to infection by microbes at any site. In influenza virus infection, the upper respiratory mucosal surface is the effector site of the innate immune system. The mechanical barrier of the mucosal epithelium, surface mucus, secretion of antimicrobial peptides such as defensins, secretion of type I and II interferons (IFNs), natural killer cells, and complement factor all play important roles in innate immunity at the respiratory mucosa (Figure 1). Among these, the IFN response is required to signal viral infection. During influenza virus infection, genomic single-stranded RNA, and double-stranded RNA produced during viral replication, have been implicated as the molecular signals of infection that trigger IFN production.

The innate immune system senses viral infection by recognizing a variety of viral components, including double-stranded (ds) RNA, and triggering antiviral responses. The cytoplasmic helicase protein retinoic-acid-inducible protein I (RIG-I, also known as Ddx58) and melanoma differentiation-associated gene 5 (MDA5, also known as Ifih1 or Helicard) have been implicated in recognition of viral dsRNA. Viral dsRNA binds to RIG-I and MDA5 in the cytoplasm, which leads to activation of IFN regulatory factors.[6] In vitro studies suggest that RIG-I and MDA5 recognize both RNA viruses, and polyinosine-polycytidylic acid (poly(I:C)), a synthetic dsRNA analog. RIG-I is essential for the production of IFNs in response to RNA viruses, including paramyxovirus, influenza virus, and Japanese encephalitis virus, whereas MDA5 is critical for the detection of picornavirus.[6] The recognition of viral infection by the innate immune system

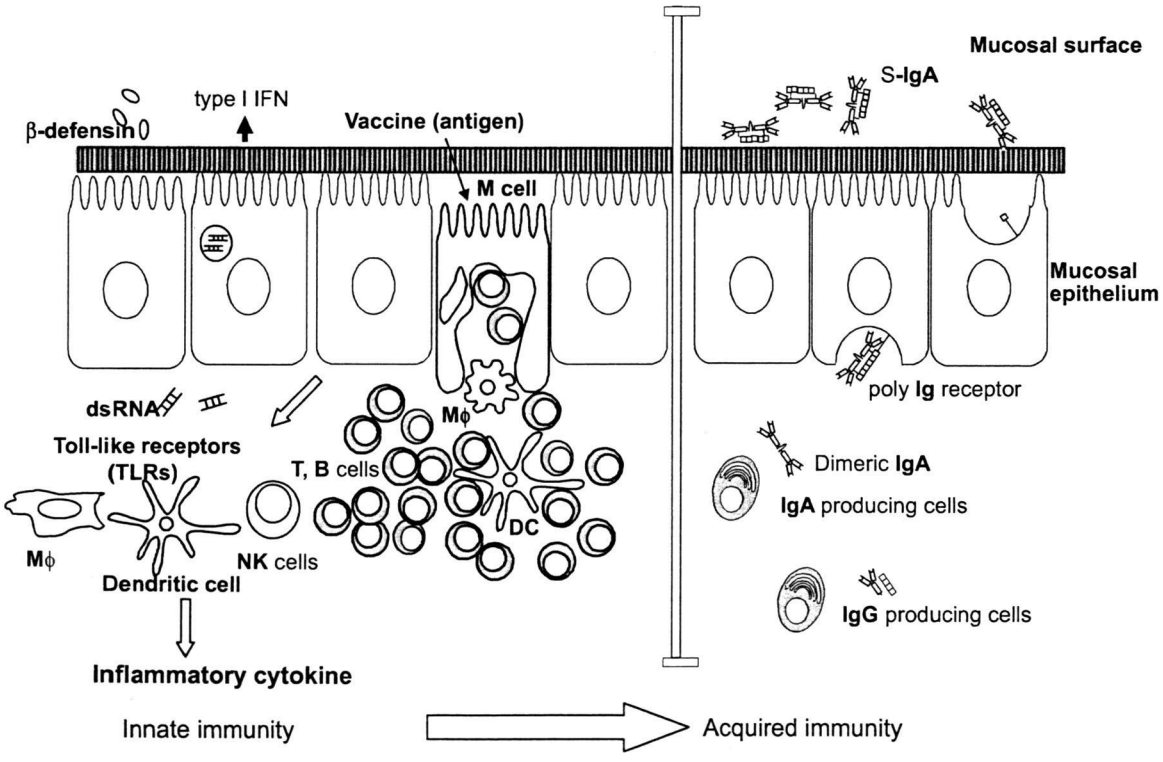


**Figure 1** Defence mechanism at mucosal site, innate and adaptive immunity.

bridges the transition between innate and adaptive immune responses. This is a particularly important facet of innate immunity involved in mucosal immune responses. We can take advantage of the mucosal innate immune response to enhance vaccine efficacy, which we will discuss later in this review.

Among the several innate immune receptors, the Toll-like receptor family plays a central role in the recognition of viral nucleic acid. This recognition leads to the induction of type I IFN. We previously demonstrated that the synthetic double-stranded RNA (dsRNA) poly(I:C), a TLR-3 agonist, has mucosal adjuvant activity when co-administered intranasally with an influenza HA vaccine, and increases both the mucosal and systemic humoral response, resulting in complete protection against challenge by homologous avirulent (H1N1) and highly pathogenic (H5N1) influenza viruses in mice.[7,8] Sloat and Cui[9] also reported that mice immunized intranasally with recombinant anthrax protective antigen adjuvanted with poly(I:C) developed strong systemic and mucosal anti-anthrax antigen responses with lethal toxin neutralization activity. Thus, the signals conducted by innate immune receptors work as adjuvants which act as a bridge between innate immunity and acquired immunity.

## Mucosal vaccine

Seasonal influenza vaccines are prepared based on the prediction of the expected strain of epidemic of the next season. These are parenterally injected vaccines which does not prevent the infection itself, which reduce the severity and complications after the infection. Parenteral vaccines can induce the neutralizing IgG antibody in the serum but they cannot induce the secretory IgA antibody which acts on the mucosal surface. Secretory IgA antibodies on the mucosal membrane surface are highly effective for preventing infection because they react on the surface of the mucosal membrane before the pathogens attach to the epithelial cell surface, which is the first target of influenza viral infection. Moreover, serum IgG antibodies are less effective against drifted viral strains because they act more specifically than secretory IgA antibodies. Secretory IgA antibodies have cross-protective effects against variant strains of the influenza virus. The exact mechanism of the cross-reactive effects of IgA is still unknown, but this phenomenon is a great advantage in preventing infection. In fact, natural influenza virus infection was shown to be superior to vaccination with inactivated virus in inducing cross-protection against infection by mutated viruses within a particular subtype of the A-type virus in humans.[10–12] Another reason why the mucosal immune system is adept at preventing infection is that the effector sites are not restricted to the originally sensitized mucosa. IgA-specific antibody forming cell (AFC) precursors migrate from mucosal sites throughout the entire body via site-specific homing pathways. This system is referred to as the common mucosal immune system.[13–17] Because of the advantages of induction of mucosal immunity for preventing influenza, several strategies have been used to attempt to development a mucosal vaccine. For effective induction of secretory IgA by inactivated vaccine, mucosal co-administration of vaccine with adjuvant is necessary. As a mucosal adjuvant, a bacterial toxin such as cholera toxin (CT) or *Escherichia coli* heat-labile toxin (LT) have been used experimentally.[18,19] Although LT is an effective adjuvant for the production of mucosal IgA, it has adverse clinical side effects, such as facial paralysis (Bell's palsy).[20] New, clinically safe and effective adjuvants are necessary for the administration of intranasal influenza vaccines to humans. The most promising candidate for mucosal adjuvant is PolyI:Poly$C_{12}$U (Ampligen®), which is synthetic dsRNA and has a good safety profile based on clinical trials, including a recent double-blind, placebo-controlled Phase III clinical trial for chronic fatigue syndrome (CFS).[21–23] To date, >75,000 doses of Ampligen® have been administered to humans, at an average dose of 400 mg, and it has been generally well tolerated. Recently, it was shown that PolyI:Poly$C_{12}$U was as effective as poly(I:C) in inducing maturation of human monocyte-derived dendritic cells in vitro.[24] So PolyI:Poly$C_{12}$U (Ampligen®) was examined as an adjuvant for mucosal influenza H5N1 vaccine administered intranasally in mice together with synthetic dsRNAs (poly(I:C) and Ampligen®) as powerful TLR-3 agonists.

## Highly pathogenic avian influenza virus H5N1

The first outbreak of the highly pathogenic avian influenza virus H5N1 was reported in humans and birds in Hong Kong in 1997, during which six out of 18 infected people died.[25] Subsequently, re-emergence of the H5N1 virus associated with a high fatality rate (greater than 60%) has been reported in southern China, Vietnam, Thailand, Cambodia, Indonesia, Turkey, and Iraq. From January 2003 to September 2008, 387 laboratory-confirmed human cases of H5N1 were reported to the World Health Organization (WHO). Although most human H5N1

infections have been caused by the direct transmission of virus from infected poultry, there is fear that a pandemic could result if subsequent transmission of H5N1 virus occurred between infected humans.[2] Because the ability to be transmitted from human to human represents the final barrier to a new pandemic of H5N1, there is an urgent and important public health need to develop effective vaccines in preparation for such a pandemic. However developing a vaccine against the H5N1 virus poses a number of problems. A highly contained facility is required, and the virus grows very poorly in embryonated eggs because it kills chickens. Attenuation of the vaccine strain is necessary to eliminate these problems. Currently licensed human vaccines are strain-specific and do not protect against heterotypic influenza viruses. This is problematic, because influenza A (H5N1) continues to evolve into antigenically distinct clades. The question remains of how an effective vaccine can be prepared for an impending pandemic of a new influenza, which might be caused by a highly pathogenic strain of avian influenza virus. Influenza virus A (H5N1) is not the only strain that could cause a new pandemic in humans.

H5 vaccine candidates must be continually updated to match the antigenicity of circulating viruses because of the differences in HA antigenicity among 1997, 2003, and 2004 H5 viruses.[26] In addition, it is difficult to predict which strain of virus (H5 or other avian-associated HA) will be responsible for a pandemic. In such circumstances, the ideal approach is to prepare a vaccine that confers strong cross-protective immunity against variants of a particular virus strain. Mucosal immunity induced through natural infection by influenza virus has potent cross-protective activity, compared with subcutaneous vaccination-induced systemic immunity. Cross-protective activity is correlated with mucosal secretory IgA, which is not induced after subcutaneous vaccination.[27] In order to induce cross-protective mucosal immunity through influenza vaccination, we have examined the effect of intranasal administration of an inactivated viral vaccine with various adjuvants, and found that mucosal IgA plays an important role in cross-protection against variant influenza A and B virus infection.[7,28–30] Nicholson and colleagues reported that the H5N1 vaccine is poorly antigenic in humans, and requires adjuvant to elicit a detectable antibody response.[31] Several groups looking at avian influenza H5N1 vaccines have reported that intranasal administration of a formalin-inactivated whole virus vaccine with or without mutant *E. coli* LT adjuvant (R192G), or an adenoviral vector-based influenza vaccine, protected mice from lethal challenge by a heterologous H5N1 virus.[32–34]

## Development of adjuvant-combined inactivated nasal vaccines

Subcutaneous injection of inactivated vaccines would be an effective strategy in an epidemic caused by a homologous virus, as it induces specific serum IgG, but would be less effective in an epidemic caused by a heterologous virus. On the other hand, live attenuated vaccines effectively protect against heterologous virus infection by inducing secretory IgA, IgG, and cytotoxic lymphocyte (CTL) responses. However, because their safety has been proven only in healthy people between the age of 5 and 49, their use is approved only for this group of people in the US. Intranasal administration of inactivated vaccines represents a potential solution to overcoming these problems.

In clinical trials, inactivated whole virus particles and split-product vaccines have been shown to be effective in preventing live virus infection when administered intranasally.[35–39] Moreover, intranasal administration of an inactivated whole virion vaccine induced a broad spectrum of heterosubtypic immunity in mice, which was not observed using an ether-split vaccine.[33] The stronger immunogenicity of the inactivated whole virion vaccine was likely due to the stimulation of innate immunity by genomic single-stranded RNA, via TL-R7.[5,40]

Intranasal administration of an inactivated ether-split vaccine and the synthetic dsRNA poly(I:C) conferred effective cross-protection in the upper respiratory tract (RT) against viral variants (drift viruses) of influenza A, or B-type viruses.[7] Because most viruses produce dsRNA during replication,[41] synthetic dsRNA likely acts as a molecular mimic of viral infection. The mammalian TLR-3 receptor recognizes dsRNA, and activates the NF-κB[42] pathway, resulting in activation of type I IFN, which in turn enhances the primary antibody response to subcutaneous immunization of soluble materials.[43] This adjuvant activity of type I FN appears to play an important role in bridging the gap between innate and adaptive immunity.[43]

In mice, intranasal administration of an ether-split vaccine from PR8 (influenza strain H1N1) and poly(I:C) adjuvant induced a strong anti-HA IgA and IgG response in nasal washes and serum, respectively, while vaccination without poly(I:C) induced very little response. In addition, administration of either an A/Beijing (H1N1) or A/Yamagata (H1N1) vaccine and poly(I:C) conferred complete protection against PR8 virus challenge in a mouse model of nasal infection,

suggesting that intranasal vaccination with poly(I:C) adjuvant confers cross-protection against variant viruses. Although the systemic antigen-specific T-cell responses were induced by intranasal vaccination with poly(I:C) adjuvant, T-cell responses against heterologous influenza viruses were weak. Moreover, TLR3, which is a receptor for dsRNA in nasal-associated lymphoid tissue (NALT), was upregulated at the level of mRNA expression upon intranasal administration of a split vaccine and poly(I:C).[7] Recently, a clinically safe dsRNA, PolyI:PolyC$_{12}$U (Ampligen®), was investigated as a dsRNA adjuvant for intranasal avian flu vaccines.

To evaluate the adjuvant effect of Ampligen®, the protective effect of intranasal administration of vaccine and Ampligen® adjuvant against homologous (A/Vietnam) and heterologous (A/Hong Kong and A/Indonesia) H5N1 influenza virus challenge was examined[44] (Figure 2). Two groups of mice were immunized either intranasally or subcutaneously with 1 μg of vaccine from Vietnam strain and 10 μg of Ampligen®, then challenged by intranasal administration of 1000 PFU of H5N1 influenza virus at 2 weeks after the final immunization. A third group of control mice was immunized intranasally with 10 μg of Ampligen® alone. In response to homologous viral challenge, all the mice immunized intranasally with vaccine and Ampligen® completely cleared viruses in their nasal cavity. By contrast, significantly higher levels of virus in nasal wash were detected in mice immunized subcutaneously with vaccine and Ampligen®. All mice of both groups survived after homologous A/Vietnam/1194/2004 viral challenge. In the heterologous virus challenge group, virus titers in nasal wash of intranasal vaccination group were significantly lower than in the subcutaneous vaccination group after A/Hong Kong or A/Indonesia viral challenge. Consequently, although intranasally immunized mice survived a potentially lethal infection with A/Hong Kong or A/Indonesia viruses, most influenza-challenged mice died (Figure 2). These results clearly indicated that intranasal administration of H5N1 vaccine and Ampligen® adjuvant is more effective than subcutaneous vaccination against homologous and heterologous H5N1 influenza virus challenge.

BALB/c mice were immunized three times intranasally or subcutaneously with trivalent inactivated influenza vaccine licensed in Japan for the 2005–2006 season.[45] The vaccine included A/New Caledonia/20/99 (H1N1), A/New York/55/2004 (H3N2), and B/Shanghai/361/2002 viral strains and was administered with PolyI:PolyC$_{12}$U (Ampligen®) as an adjuvant. The immunized mice were challenged with A/Hong Kong, A/Vietnam, or A/Indonesia H5N1 influenza viruses 2 weeks after the final immunization. Mice immunized intranasally manifested cross-reactivity of mucosal IgA and serum IgG with H5N1 virus as well as a reduced H5N1 viral titer in nasal wash, and their survival was higher after H5N1 virus challenge compared with nonimmunized animals. Subcutaneous immunization did not induce a cross-reactive IgA response and did not afford protection against H5N1 viral infection. These results suggest that intranasal immunization with annual influenza vaccine may overcome the problem of a limited supply of H5N1 virus vaccine by providing cross-protective mucosal immunity in humans against H5N1 viruses with pandemic potential.

## Cross-protection by other vaccines

Parenteral inactivated vaccines, including split-product, subunit vaccines and whole virion vaccines, induce mainly serum IgG antibodies that are weakly cross-protective against drift viruses within a subtype. These IgG antibodies would be effective against an epidemic of homologous virus, but would rarely be effective against an epidemic caused by a heterologous virus. Thus, an inactivated parenteral vaccine can effectively protect against an epidemic caused by a homologous virus, but would be relatively ineffective against an epidemic caused by a heterologous virus.

On the other hand, a cold-adapted, live-attenuated virus vaccine licensed in Russia and in the USA[46–48] appeared to mimic the natural course of infection, and provided cross-protective immunity against different subtypes of viruses by inducing secretory (S)-IgA antibodies, serum IgG antibodies, and a CTL response.[49,50] The advantage of live viral vaccines is that they induce not only mucosal IgA and serum IgG antibody responses, but also CTL responses, and confer cross-protection against different subtypes of influenza virus. Current cold-adapted (ca) live-attenuated influenza virus vaccines are growth-restricted to the upper RT. Using reverse genetics, a live attenuated vaccine was generated that encodes a modified form of H5 HA and wild-type-type N1 neuraminidase from influenza A virus strain H5N1, with the remaining gene segments derived from the cold-adapted (CA) influenza A vaccine donor strain. This vaccine was immunogenic in mice.[51] Four weeks after receiving a single intranasally administered dose of CA vaccine, mice were fully protected from lethal challenge with homologous and antigenically distinct, heterologous wt strain H5N1 viruses from different genetic sublineages.[51] Because live attenuated vaccine can induce immune responses equivalent to those induced by natural infection, a live vaccine that has no side

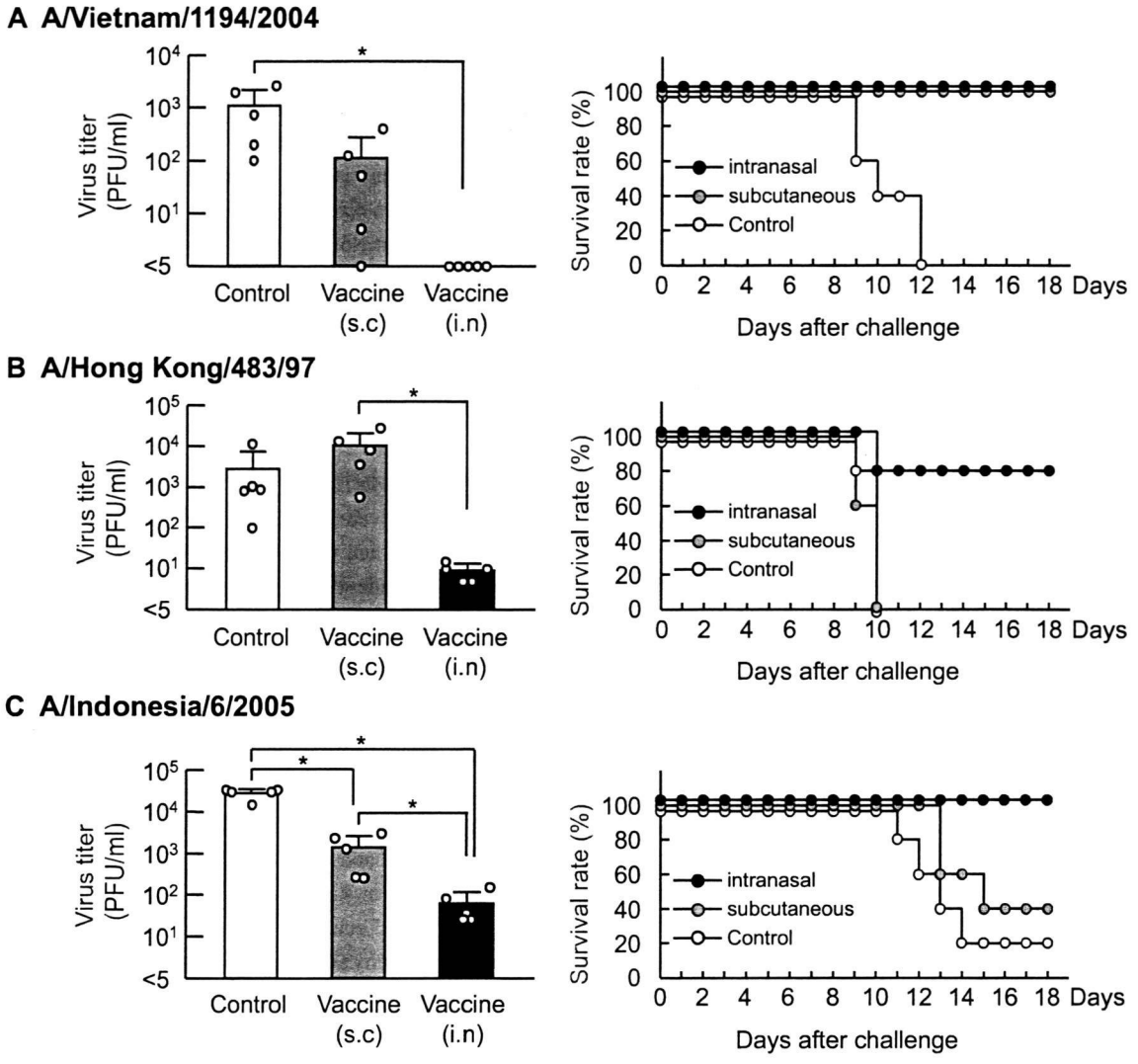


**Figure 2** H5N1 virus titers in nasal washes and survival rates after lethal challenge with homologous A/Vietnam, heterologous A/Hong Kong, or heterologous A/Indonesia viruses. Mice were immunized intranasally (solid bar) or subcutaneously (gray bar) with vaccine and Ampligen®, then challenged by intranasal administration of 1000 PFU of A/Vietnam (**A**), A/Hong Kong (**B**), or A/Indonesia (**C**) virus 14 days after the final immunization. Nasal washes were collected three days post infection (d.p.i), and virus titers were measured by plaque assay. Each bar represents the mean ± SD of five mice and open circles indicate individual animals. For statistical analysis, virus titers were compared to those from control mice (open bar) that received intranasal administration of 10 μg of Ampligen® alone. Survival rates were monitored for 18 days. Copyright © 2007. Reproduced with permission from Ichinohe T, Kawaguchi A, Tamura S, et al. Intranasal immunization with H5N1 vaccine plus Poly I:Poly C12U, a Toll-like receptor agonist, protects mice against homologous and heterologous virus challenge. *Microbes Infect.* 2007; 9:1333–1340.
**Note:** *$p < 0.05$.

effects would be good candidate of pandemic vaccine, if it could be produced.

## Conclusion

Now that a pandemic of new influenza virus seems possible, and because it will be difficult to know when a pandemic will occur or which strain of virus will be the cause, it is in our best interests to develop broadly effective and safe vaccines against the influenza virus. For the development of a broadly effective vaccine, induction of mucosal immunity is an inevitable requirement, as mucosal secretory IgA plays an important role in cross-protection. Vaccines designed to induce mucosal immunity are necessary to combat a new influenza pandemic. As stated above, one of the requirements for inducing mucosal immunity is administration of the vaccine at mucosal sites, such as the nasal mucosa. For this reason, intranasal administration of inactivated vaccine plus adjuvant, or live attenuated vaccines, are promising candidates for

inducing cross-protective immunity against variant influenza viruses. However, for safety reasons, the ideal vaccine for induction of cross-protective mucosal immunity may be an inactivated vaccine. Recently, several candidate adjuvants that are effective in mucosal vaccine administration have emerged, including dsRNA (Ampligen®),[7,8] CMPs, SMPs,[30] and mutant CT.[52] These mucosal adjuvants represent promising approaches to the development of safe and effective vaccines for a potential influenza pandemic.

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SHORT REPORT

# Neonatal pertussis presenting as acute bronchiolitis: direct detection of the *Bordetella pertussis* genome using loop-mediated isothermal amplification

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**Abstract** We report a 28-day-old female infant with pertussis presenting as severe acute bronchiolitis with cyanosis. On admission, the patient's symptoms were similar to those of acute bronchiolitis. However, occasional apneic episodes with cyanosis and peripheral lymphocytosis suggested neonatal pertussis and prompted us to examine the presence of *Bordetella pertussis* using loop-mediated isothermal amplification (LAMP) based on the insertion sequence IS481. LAMP of the nasopharyngeal and intratracheal aspirates was positive for *B. pertussis* and a diagnosis of neonatal pertussis was made. As the clinical features of pertussis in neonates and early infancy are not characteristic, LAMP is a useful tool for rapid diagnosis of *B. pertussis* infection.

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**Keywords** *B. pertussis* · Bronchiolitis · LAMP · Neonatal pertussis

**Abbreviations**
LAMP Loop-mediated isothermal amplification

## Case report

A 28-day-old female infant, who was born at 39 weeks gestation weighing 2,928 g, was referred to us because of cough, wheezing, and cyanosis. The infant had begun to have manifestations of mild viral upper respiratory tract infection at the age of 21 days. The respiratory symptoms worsened abruptly and cyanosis was observed on the day before admission. The child's mother and 6-year-old sister, who had both received DPT vaccination, had complained of mild coughs two weeks before the onset of the patient's illness, but had not been treated with any antibiotics.

On admission to our hospital, the patient was dyspneic without pyrexia, and oxygen saturation decreased to 88% during a fit of coughing. The heart rate was 160/min and the respiratory rate was 45–60/min. Routine hematological tests showed a white blood cell count of 28,600/μl with a lymphocyte count of 16,331/μl, and C-reactive protein was 0.04 mg/dl. Chest X-ray showed mild pulmonary emphysema. The nasopharyngeal fluid was negative on immunoassays for respiratory syncytial virus, influenza A and B, and adenovirus. The patient's symptoms on admission strongly suggested bronchiolitis. In addition, the apneic episodes with cyanosis and lymphocytosis suggested

neonatal pertussis. Initially, the patient was treated with piperacillin and hydrocortisone. However, her condition deteriorated and she developed severe apnea and bradycardia during the night after admission. Chest X-ray indicated the presence of atelectasis in the upper lobe of the right lung. Following increasing respiratory distress over the next two days, intubation was required, and she was ventilated at high pressure. Clarithromycin was started on day three after admission. Despite undergoing mechanical ventilation, the patient had frequent episodes of severe apnea with bradycardia and required manual ventilation for recovery. Nasopharyngeal and intratracheal aspirates obtained on day five were positive for *Bordetella pertussis* using a loop-mediated isothermal amplification (LAMP) assay based on the insertion sequence IS481 target (Fig. 1). PCR analyses of the nasopharyngeal and intratracheal aspirates were also positive for *B. pertussis* (data not shown). Viral isolation and bacterial cultures from intratracheal aspirates were negative. The patient made favourable progress from the fifth day of intubation and ventilation was stopped on the eighth day. Piperacillin and clarithromycin were continued for eight days and three weeks, respectively. LAMP remained positive 30 days after initiation of treatment, when the infant had no clinical manifestations of pertussis, and was finally negative for the presence of the *B. pertussis* genome on the 33rd day. LAMP was negative for *B. pertussis* in the patient's parents and sister.

## Discussion

Young infants with *B. pertussis* infection present with atypical symptoms, including apnea, cyanosis, and wheezing, and the laboratory findings, like leukocytosis and lymphocytosis, are not always specific. Therefore, such cases are often treated as acute bronchitis or bronchiolitis. The initial manifestations found in our patient strongly suggested that she had contracted acute bronchiolitis. Manifestations such as bronchiolitis are not rare in early infantile pertussis, and Sotomayor et al. reported that a diagnosis of bronchiolitis or pneumonia was made in 14 of 46 infantile pertussis cases in the early stages [4]. Although pertussis can be diagnosed from culture and serological studies, culture requires 7–10 days to isolate *B. pertussis* and serology is frequently negative in young infants with pertussis, like our patient. Confirmation of the diagnosis by serology requires acute- and convalescent-phase sera, delaying the diagnosis of *B. pertussis* infection.

PCR is more sensitive than culture-based methods and is not readily affected by prior antibiotic therapy. Its main disadvantage is that PCR assays have limited availability, and false positives can occasionally occur [3]. In our case, *B. pertussis* was detected using both LAMP and PCR. LAMP has been developed as a novel method to amplify DNA, and it has been reported to be a rapid method for the diagnosis of *B. pertussis* infection that requires only about 60 min and is less expensive than PCR [2]. The sensitivity and specificity of LAMP for *B. pertussis* are 83 and 95%, respectively [2]. The LAMP assay promises to become a useful tool for the rapid diagnosis of pertussis in clinical laboratories without requiring specific equipment, such as a thermal cycler and electrophoresis system. As cross-reactivity with *B. bronchiseptica* in IS481-based PCR has been reported, the possibility that a LAMP assay based on an IS481 target might also cross-react with *B. bronchiseptica* cannot be excluded completely [2]. However, with few exceptions, *B. bronchiseptica* is not pathogenic in humans, and our patient had certainly contracted *B. pertussis* infection.

Of particular interest is the persistence of a positive LAMP reaction long after commencing treatment. Bonacorsi et al. reported a very-low-birth-weight neonate in whom treatment with josamycin was unsuccessful and PCR for *B. pertussis* was positive for 78 days after the initial treatment [1]. The reason for the persistence of positive DNA results remains unclear, but the high sensitivity of LAMP and the difficulty of eliminating pathogens in infants with compromised immunity may contribute to long-term persistence of the *B. pertussis* genome. Further studies of culture- and genome-based methods for the detection of *B. pertussis* are required to clarify the optimal duration of treatment for neonatal *B. pertussis* infection.

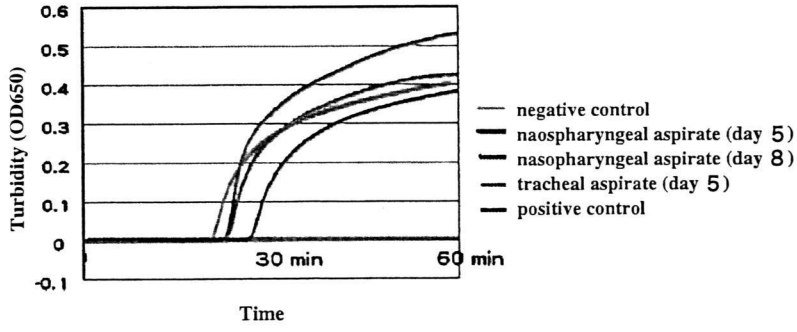


**Fig. 1** The results of LAMP for detecting the *Bordetella pertussis* genome. LAMP of nasopharyngeal (on days 5 and 8) and tracheal (on day 5) aspirates showed a positive reaction within 30 min

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PEDIATRIC RESEARCH

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# The Effects of Disodium Cromoglycate on Enhanced Adherence of *Haemophilus influenzae* to A549 Cells Infected With Respiratory Syncytial Virus

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**ABSTRACT:** Nontypeable *Haemophilus influenzae* (NTHi) secondary infection often complicates respiratory syncytial virus (RSV) infections. Previous studies have revealed that RSV infections enhance NTHi adherence to airway epithelial cells. In this study, we investigated the effects of disodium cromoglycate (DSCG) and corticosteroids, which are frequently used for the treatment of wheezing often related to RSV infections, on the adherence of NTHi to RSV-infected A549 cells. DSCG inhibited enhanced adherence of NTHi to RSV-infected A549 cells, whereas dexamethasone (Dex) and fluticasone propionate (Fp) did not. DSCG suppressed the expression of ICAM-1, which is one of the NTHi receptors. Furthermore, DSCG exhibited an inhibitory effect on RSV infections. It is suggested that DSCG exerts an anti-RSV effect, and consequently attenuates the expression of NTHi receptors. ***(Pediatr Res* 66: 168–173, 2009)**

Respiratory syncytial virus (RSV) is one of the major pathogens of upper and lower respiratory tract infections in children. RSV infection at a younger age often involves the lower respiratory tract and is frequently associated with expiratory wheezing, which is referred to as bronchiolitis or wheezy bronchitis, asthma, and pneumonia (1). It is known that RSV infections can be complicated by bacterial superinfections (2–5). Nontypeable *Haemophilus influenzae* (NTHi) is one of the most common bacteria involved in mixed RSV-bacterial bronchopulmonary infections in pediatric patients (2,4). It has long been recognized that a preceding local respiratory viral infection seems to play an important role in the pathogenesis of infections by bacteria, including NTHi. The mechanisms underlying bacterial superinfections include virus-induced local destruction of the epithelium, which compromises the host's physiologic barrier, and virus-induced modulation of the immune response (6). In addition, enhanced bacterial adherence to virus-infected cells is considered an important factor increasing the risk of bacterial superinfections (7).

Recent studies demonstrated that some respiratory viruses including RSV lead to both expression of viral glycoproteins and up-regulation of cellular molecules including ICAM-1 (CD54), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), and platelet activating factor receptor (PAFr) on the host-cell membrane. Both could serve as bacterial receptors and promote bacterial adhesion to the cells (8–11). Strategies for preventing interaction between RSV and bacteria may reduce the incidence of secondary bacterial complications of RSV infection.

Disodium cromoglycate (DSCG) and corticosteroids, which are recognized as inhalation drugs for the management of bronchial asthma, are also used for the treatment of acute infantile wheezing and exacerbation of asthma that are often related to RSV infections. The effects of these medicines against RSV infections and secondary bacterial complications are not clear. In this study, we investigated the effects of DSCG and corticosteroids on the *in vitro* interaction between RSV and NTHi.

## MATERIALS AND METHODS

***Epithelial cell culture.*** A549 human pneumocyte type II carcinoma cells (RIKEN Cell Bank, Tsukuba, Japan: RCB0098) were used for the RSV infection experiments. A549 cells were grown at 37°C in 5% $CO_2$ in DMEM (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO). HEp-2 cells (human laryngeal epithelial carcinoma, RIKEN Cell Bank: RCB1889) were used for RSV growth and plaque assays. HEp-2 cells were grown at 37°C in 5% $CO_2$ in Minimum Essential Medium Eagle (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated FBS.[1]

***Virus.*** Human RSV serotype A (A2 strain) (provided by Dr. Tsutsumi, Graduate School of Medicine, Sapporo Medical University, Sapporo, Japan) was grown in HEp-2 cells. Supernatant fluids were clarified and titrated for infectivity testing by plaque assay as described previously (12). The viral growth medium comprised Minimum Essential Medium Eagle with 1% heat-inactivated FBS.

***Bacteria.*** NTHi attachment assay was performed with NTHi strain 03H113, 05H11, and 06H18, clinical isolates obtained from the pediatric patients' airways. All the other assays were performed with NTHi strain 03H113. Strain 03H113, 05H11, and 06H118 express high-molecular weight 1 and 2 (HMW1/HMW2) adhesins and P5 fimbriae. Strain 03H113 also expresses Hap and lacks Hia and pilli (Hif A), 05H11 expresses Hap and Hia and lacks Hif A, and 06H18 lacks Hap, Hia, and Hif A. The gene expression of these adhesins was examined by PCR. NTHi were grown on chocolate agar plates at 37°C in 5% $CO_2$ overnight. One or two colonies were propagated in brain-heart infusion (BHI) broth (Becton Dickinson, MD) supplemented with nicotinamide adenine dinucleotide and haemin (both at 10 mg/L) at 35°C overnight. A portion of this culture was inoculated as a preculture into a fresh sample of BHI broth, at the final concentration of 5%. The new culture was then incubated for 3 h at 35°C. Before assays, bacteria were washed three

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**Abbreviations: Dex,** dexamethasone; **DSCG,** disodium cromoglycate; **FBS,** fetal bovine serum; **Fp,** fluticasone propionate; **MOI,** multiplicity of infection; **NTHi,** nontypeable *Haemophilus influenzae*; **RSV,** respiratory syncytial virus

times with PBS (Nikken, Kyoto, Japan) and then diluted with tissue culture medium to $1 \times 10^6$ CFU/mL. Bacterial numbers were determined by OD at 600 nm and confirmed by plating dilutions on agar plates.

***Viral infection.*** A549 cells were grown as confluent monolayers and then incubated with RSV at a multiplicity of infection (MOI) of 1–7.5 virus/cell for 1 h at 37°C in 5% $CO_2$. The cells were then washed with PBS, followed by replacement of the medium and incubation for 48 h at 37°C in 5% $CO_2$.

***NTHi attachment assay.*** The NTHi attachment assay was performed using a modified method described previously (13). The A549 cells were grown as confluent monolayers in 24-well tissue culture plates (IWAKI, ATG, Chiba, Japan). The cells were then inoculated with RSV and incubated for 48 h at 37°C in 5% $CO_2$. The monolayers were then washed twice, and 1 mL of DMEM-containing NTHi was inoculated into each well (MOI = 1). After incubation for 1 h at 37°C in 5% $CO_2$, the monolayers were washed gently three times with PBS to remove loosely adherent bacteria. The A549 cells were then detached using 0.05% trypsin-EDTA (GIBCO), and serial dilutions were plated on chocolate agar for the quantitative colony counts. For each assay, triplicate wells for each condition were examined, and the numbers of adherent bacteria were normalized as to the numbers of epithelial cells.

***Drugs.*** DSCG is clinically used as a solvent inhalation for prophylactic and acute treatment for asthma and infantile wheezing. The concentration of the clinically used solvent of DSCG is 10 mg/dL (20 mM). The experiments on DSCG were examined below the concentration of 20 mM, which is thought as clinically relevant, and no visible cytotoxicity was observed morphologically, though the concentration at pulmonary alveolous is unknown. DSCG [Intal; disodium 5,5′-(2-hydroxytrimethlenedioxy) bis 4-oxo-4*H*-1-benzopyran-2-carboxylate] was kindly provided by Astellas Pharmaceutical Co., Ltd., Tokyo, Japan. Dexamethasone crystalline (Dex) and fluticasone propionate (Fp) were examined at the concentrations below $10^{-6}$M, which have been reported to exhibit anti-inflammatory effect *in vitro*. Dex and Fp were from Sigma Chemical Co.

***Cell surface receptor expression.*** It has been reported that ICAM-1 expressed by airway epithelial cells is one of the major NTHi receptors (8,14), and RSV infection up-regulates ICAM-1 expression by A549 cells (8). Previous studies have revealed that Dex- or Fp-attenuated cytokine-induced ICAM-1 expression in human airway epithelial cells *in vitro* (15,16) and ICAM-1 expression in the bronchial epithelium was inhibited after treatment with inhaled DSCG in patients with bronchial asthma *in vivo* (17).

Therefore, first we determined the effects of DSCG, Dex, and Fp on the cytokine-induced ICAM-1 expression in A549 cells. As reported previously (16), A549 cells were incubated with IL-4 (Sigma Chemical Co., 20 ng/mL) plus TNF-$\alpha$ (Sigma Chemical Co., 20 ng/mL) for 24 h to induce ICAM-1 expression. ICAM-1 expression in A549 cells was assayed by fluorescence-activated cell sorting (FACS). A549 cells were grown as confluent monolayers in 6-well tissue culture plates and stimulated with cytokines or inoculated with RSV, and then incubated with or without each drug. At 24- to 48-h intervals, A549 cells were detached from the plates using Cell Dissociation Solution (Sigma Chemical Co.) and washed with PBS, and $10^6$ cells were resuspended in 100 $\mu$L of FACS buffer (1% FBS and 0.1% sodium azide in PBS). Cells were incubated with 20 $\mu$L of phycoerythrin (PE)-conjugated mouse anti-human ICAM-1 (CD54) MAb (IgG1; Becton Dickinson Biosciences, Cockeysville, MD) or an isotype-matched control antibody (IgG1; Becton Dickinson Biosciences) for 30 min at 4°C. Cells were then washed extensively and fixed with Cell FIX (Becton Dickinson Biosciences) and analyzed on a flow cytometer (FACS Calibur, Becton Dickinson Biosciences). The mean fluorescence intensity of cells was estimated after subtracting the background produced by the isotype control Ab.

***Inhibition of bacterial adhesion.*** The ability of NTHi to adhere to RSV-infected A549 cells was assessed by blocking ICAM-1 on the cell surface. A549 cells at 48 h after RSV-infection (MOI = 2.5) were incubated with different concentrations (5–50 $\mu$g/mL) of purified mouse anti-human ICAM-1 MAb (Calbiochem, Darmstadt, Germany; clone 8.4A6; $IgG_1$, Alexis Biochemicals, Lausen, Switzerland; clone RR1/1; $IgG_1$) or the isotype control (IgG1; BD Biosciences, San Jose, CA) for 1 h at 37°C in 5% $CO_2$ and then washed with PBS, followed by NTHi adhesion assay.

***Effects of drugs on RSV infection.*** A549 cells were grown as confluent monolayers in 6-well tissue culture plates and then inoculated with RSV at MOI of 2.5. After viral adsorption, A549 cells were incubated with or without each drug. Furthermore, to determine the inhibitory effect of DSCG on RSV infection, treatment with DSCG was evaluated using two other protocols involving treatment of DSCG at different points in viral infection as described previously (18). For treatment of cells before viral adsorption, cells were cultured for 24 h at 37°C in 5% $CO_2$ in the presence or absence of DSCG. For treatment of cells during viral adsorption, viral solutions were first preincubated for 30 min at room temperature with the indicated concentrations of DSCG, and then inoculated with the virus-DSCG mixtures. To determine cell-associated viral contents, the cells were washed with PBS extensively and replaced with new medium at 48 h after viral inoculation, after which the cells were harvested with cell scrapers (IWAKI), homogenized by secure vortexing for 1 min and spun down, and then the supernatants were stored at −80°C. Cell-associated viral contents were quantitated by plaque assay using HEp-2 cells as described previously (12).

***Viral syncytium assay.*** Monolayer cultures of A549 cells in 6-well culture plates were infected with RSV at MOI of 2.5 at 37°C in 5% $CO_2$. After a 1-h adsorption period, the monolayers were washed with PBS and then overlaid with fresh medium with indicated concentrations of drugs. At 48-h post infection, the cell monolayers were examined microscopically for syncytium formation.

***Statistical analysis.*** Each NTHi adherent assay and RSV plaque assay was performed in triplicate of wells, and the results are expressed as the means ± SD. Between-group comparisons were tested using Mann-Whitney's *U* test. $p < 0.05$ was considered significant.

This study has been approved by the Institutional Review Board of Chiba University.

## RESULTS

***NTHi adherence to RSV-infected/noninfected A549 cells.*** The number of any NTHi strain of 03H113, 05H11, or 06H18 attached to RSV-exposed A549 cells was significantly higher than for noninfected A549 cells ($p < 0.05$; Fig. 1*A*). Because the attachment assay of these three strains exhibited similar results, the additional analyses were performed using the strain 03H113. The adhesion of NTHi (strain 03H113) to A549 cells at 48 h after inoculation with RSV at MOI of 2.5 and 7.5 increased by 12.7- and 16.9-fold, respectively (Fig. 1*B*).

***Effects of drugs on NTHi adherence to RSV-infected A549 cells.*** To determine the effects of DSCG, Dex, and Fp on the RSV-induced increase in NTHi adherence to A549 cells, A549 cells inoculated with RSV at MOI of 2.5 were incubated with medium containing the indicated concentra-

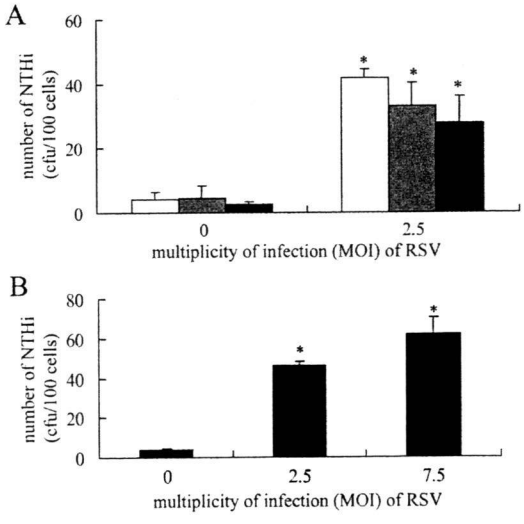


**Figure 1.** Adhesion of NTHi to A549 cells increased after infection with RSV. A549 cells were incubated with NTHi at 48 h after RSV inoculation. The numbers of adherent bacteria were normalized as to those of epithelial cells. *Panel A* shows that antecedent infection with RSV resulted in a statistically significant increase in adhesion of NTHi strain 03H113 (*white bar*), 05H11 (*gray bar*), or 06H18 (*black bar*) compared with noninfected cells (*$p < 0.05$). *Panel B* shows that adherence of NTHi (strain 03H113) increased with the MOI of RSV. Data are expressed as the means ± SD for three samples. Between-group comparisons were performed using Mann-Whitney's *U* test. *$p < 0.05$ *versus* noninfected control.

tions of the drugs for 48 h at 37°C in 5% $CO_2$. DSCG reduced the number of NTHi attached to RSV-infected A549 cells, significantly ($p < 0.05$; Fig. 2*A*). At the concentration of 20 mM, DSCG reduced NTHi adherence to 30% of that in the vehicle control. Meanwhile, Dex and Fp did not reduce NTHi adherence to RSV-infected cells significantly (Fig. 2*B*).

***Effects of drugs on ICAM-1 expression by A549 cells.*** The increase of ICAM-1 expression caused by A549 cells at 48 h after RSV infection was investigated by FACS analysis. The increase in ICAM-1 expression was RSV dose dependent (Fig. 3*A*).

To determine the contribution of ICAM-1 to NTHi adherence to RSV-infected cells, we performed an inhibition study on ICAM-1. Blocking of ICAM-1 by preincubating RSV-infected A549 cells with anti-ICAM-1 MAb reduced the number of adherent bacteria. Anti-ICAM-1 MAb (25 μg/mL) inhibited NTHi adhesion by 49% compared with the isotype control ($p < 0.05$; Fig. 3*B*).

DSCG, Dex, and Fp reduced the cytokine-induced and RSV infection-induced ICAM-1 expression. The reducing effects of each drug were dependent on the concentration of the drug, and the maximum reducing concentrations of each drug are shown in Figure 3*C* and *D*. DSCG, Dex, and Fp reduced the cytokine-induced ICAM-1 expression significantly compared with the control, whereas no significant differences between the drugs were observed (Fig. 3*C*). Meanwhile, a reducing effect on RSV infection-induced ICAM-1 expression by

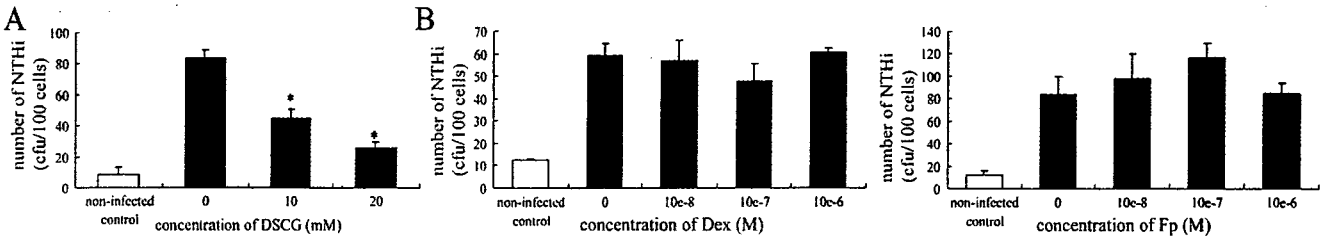


**Figure 2.** Effects of drugs on NTHi adherence to A549 cells infected with RSV (MOI = 2.5, 48 h) are shown by *black bars*. The results for RSV noninfected controls are shown by *white bars* (*A*, *B*). Between-group comparisons were performed using Mann-Whitney's *U* test. Incubation with DSCG for 48 h after RSV adsorption reduced the number of NTHi attached to RSV infected-A549 cells compared to that without DSCG significantly (*$p < 0.05$; *A*). Dex and Fp did not reduce NTHi adherence to RSV-infected A549 cells significantly (*B*). Data are expressed as the means ± SD for three samples.

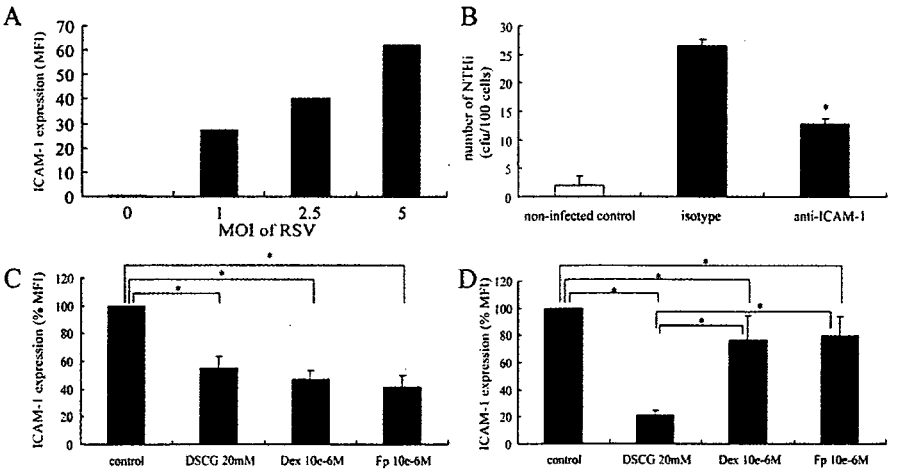


**Figure 3.** *A*, ICAM-1 expression on A549 cells post-RSV infection (48 h) was analyzed by FACS. ICAM-1 expression on A549 cells infected with RSV increased with the MOI of RSV. ICAM-1 expression is expressed as the mean fluorescence intensity (MFI) of cells. These experiments were repeated more than twice with similar results. *B*, Adhesion of NTHi to A549 cells infected with RSV was blocked by anti-ICAM-1 MAb or isotype control. The data shown are for representative experiments with the anti-ICAM-1 MAb (clone 8.4A6) or isotype control (25 μg/mL). The result for the RSV noninfected control is shown by a *white bar*. Preincubation of A549 cells with anti-ICAM-1 MAb significantly reduced the adhesion of NTHi compared with preincubation with the isotype control Ab (*$p < 0.05$). Data are expressed as the means ± SD for three samples. Between-group comparisons were performed using Mann-Whitney's *U*-test. *C*, Cytokine-induced ICAM-1 expression on A549 cells. A549 cells were stimulated with IL-4 plus TNF-α (20 ng/mL) and then incubated with or without the indicated drugs for 24 h. The data shown are for the maximum reducing concentration of each drug. ICAM-1 expression was reduced significantly by DSCG, Dex, or Fp compared with the control without a drug (*$p < 0.05$), whereas no significant differences between the drugs were observed. ICAM-1 expression is expressed as the mean fluorescence intensity of cells. Data are expressed as the means ± SD for three samples. Between-group comparisons were performed using Mann-Whitney's *U*-test. *D*, ICAM-1 expression by A549 cells at 48 h after RSV infection (MOI = 2.5). A549 cells were incubated with or without drugs. ICAM-1 expression was reduced significantly by DSCG, Dex, or Fp compared with the control without a drug (*$p < 0.05$). RSV infection-induced ICAM-1 expression on A549 cells incubated with DSCG (21.1 ± 3.6%) was significantly weak compared with those incubated with Dex or Fp. (*$p < 0.05$). ICAM-1 expression is expressed as the relative MFI of cells. Data are expressed as the means ± SD for three samples. Between-group comparisons were performed using Mann-Whitney's *U*-test.

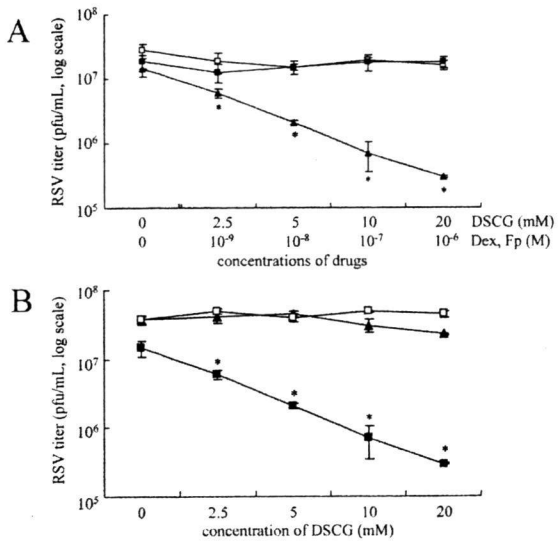


**Figure 4.** *A*, Effect of DSCG (*closed triangles*), Dex (*open squares*), or Fp (*closed squares*) on RSV infectivity for A549 cells. A549 cells were incubated with DSCG, Dex, or Fp at each concentration for 48 h after viral adsorption (MOI = 2.5). Only DSCG reduced the RSV titer significantly ($*p < 0.05$). The viral titer was determined by plaque assay and is shown as a log scale. Data are expressed as the means ± SD for three samples. Between-group comparisons were performed using Mann-Whitney's *U* test. *B*, Inhibitory effect of DSCG on RSV infection was examined by treating A549 cells with DSCG at different stages of viral infection. For treatment of cells before adsorption (*closed triangles*), the cells were cultured for 24 h at 37°C in 5% $CO_2$ in the presence or absence of DSCG. For treatment of cells during viral adsorption (*open squares*), viral solutions were first preincubated for 30 min at room temperature with the indicated concentrations of DSCG. The cells were then inoculated with the virus-DSCG mixtures. For treatment of cells after viral adsorption (*closed squares*), the cells were first inoculated with the virus, and then treated with or without the indicated concentrations of DSCG. The viral titer was determined by plaque assay and is shown as a log scale. Only the treatment after viral adsorption significantly suppressed the viral titer in a dose-dependent manner. Between-group comparisons were performed using Mann-Whitney's *U* test. $*p < 0.05$, *vs* control, 0 mM DSCG. Data are expressed as the means ± SD for three samples.

DSCG was significantly stronger than Dex and Fp ($p < 0.05$; Fig. 3*D*).

***Effects of drugs against RSV infection.*** To determine whether DSCG, Dex, and Fp have inhibitory ability as to RSV infection, titration of RSV treated with each drug after viral adsorption was performed by plaque assay. The RSV titer decreased significantly with increasing concentration of DSCG ($p < 0.05$; Fig. 4A). Dex and Fp did not inhibit RSV infection significantly (Fig. 4B).

To determine the inhibitory effect of DSCG on RSV infection, we treated A549 cells with DSCG at different points of viral infection. Only the treatment after viral adsorption significantly suppressed the viral titer in a dose-dependent manner ($p < 0.05$; Fig. 4*C*).

A characteristic of RSV infection *in vitro* is that infected cells fuse with adjacent infected or uninfected cells to form giant syncytia (Fig. 5*A*). When DSCG was added to infected cells, inhibition of RSV-induced syncytium formation was observed (Fig. 5*B*). Meanwhile, Dex and Fp did not cause inhibition of syncytium formation (Fig. 5*C*).

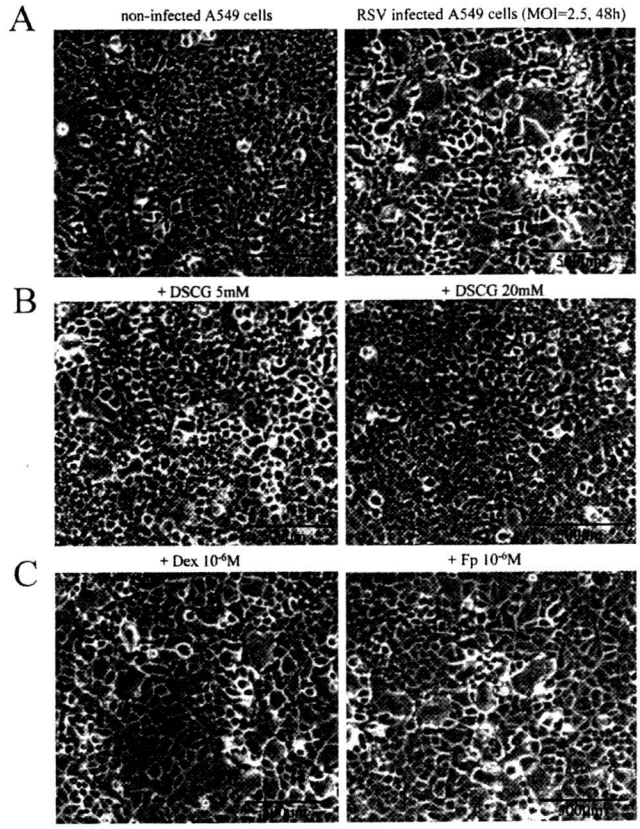


**Figure 5.** Effect of DSCG, Dex, or Fp on the morphologic changes of A549 cells infected with RSV. The monolayers of A549 cells were examined microscopically at ×100 magnification. Syncytium formations appeared on A549 cells infected with RSV (MOI = 2.5) for 48 h (*A*). Inhibition of RSV-induced sycytium formation was observed with increasing concentrations of DSCG (*B*). RSV-induced syncytium formation was not inhibited by Dex or Fp (*C*).

## DISCUSSION

It is known that RSV infections are often associated with secondary bacterial infections and that bacterial superinfections increase the severity of RSV infections. Preventing secondary bacterial infections could be a key for the management of lower respiratory infections with RSV.

In this study, we showed that RSV infection enhanced NTHi adherence to A549 cells, as demonstrated by previous studies (8,13). The effects of DSCG, Dex, and Fp, which are often used as therapies for wheezing and bronchial asthma, on NTHi adherence to RSV-infected A549 cells were investigated at clinically relevant concentrations. Only DSCG, *i.e.* not Dex or Fp, reduced the number of adherent NTHi.

It has been reported that ICAM-1 acts as a major receptor for NTHi on RSV-infected A549 cells (8). In our experiments, RSV infection up-regulated ICAM-1 expression on A549 cells, and NTHi adherence to RSV-infected cells was inhibited by blocking of ICAM-1. These results indicate that ICAM-1 contributes to the enhanced adherence of NTHi to RSV-infected A549 cells.

DSCG, Dex, and Fp have been reported to attenuate ICAM-1 *in vivo* or *in vitro* (15–17). In this study, DSCG attenuated ICAM-1 expression induced by RSV infection more strongly than corticosteroids. It is suggested that the