Table 2. Titers of IgA and IgG antibodies cross-reactive with A/Vietnam/1194/2004 (H5N1), and virus titer after challenge, in immunized mice.

Vaccine			End point–dilution titer to A/Vietnam/1194/2004, mean ± SE[a]		Hemagglutination inhibition[b]		A/Vietnam virus titer in nasal wash, pfu/mL[c]
Trivalent vaccine, μg	Ampligen, μg	Route	IgA in nasal wash	IgG in serum	Virus	Antibody titer in serum	
1	10	in	36 ± 28	1200 ± 400	A/Vietnam (H5N1)	<10	<5
					A/HongKong (H5N1)	<10	
					A/Indonesia (H5N1)	<10	
1	...	in	ND	ND	A/Vietnam (H5N1)	<10	120 ± 40
					A/HongKong (H5N1)	<10	
					A/Indonesia (H5N1)	<10	
...	10	in	ND	ND	A/Vietnam (H5N1)	<10	93 ± 44
					A/HongKong (H5N1)	<10	
					A/Indonesia (H5N1)	<10	
1	10	sc	ND	1600	A/Vietnam (H5N1)	<10	152 ± 24
					A/HongKong (H5N1)	<10	
					A/Indonesia (H5N1)	<10	
1	...	sc	ND	4800 ± 1600	A/Vietnam (H5N1)	<10	548 ± 370
					A/HongKong (H5N1)	<10	
					A/Indonesia (H5N1)	<10	
...	10	sc	ND	ND	A/Vietnam (H5N1)	<10	1248 ± 455
					A/HongKong (H5N1)	<10	
					A/Indonesia (H5N1)	<10	
...	...	...	ND	ND	A/Vietnam (H5N1)	<10	1576 ± 665
					A/HongKong (H5N1)	<10	
					A/Indonesia (H5N1)	<10	

NOTE. Mice were immunized as described in the Note to table 1. At 2 weeks after the final immunization, the animals were challenged by intranasal (in) administration of 1000 pfu of A/Vietnam/1194/04 virus. At 3 days after challenge, nasal-wash and serum samples were collected, and the titers of IgA and IgG antibodies to A/Vietnam/1194/04 were measured by ELISA.

[a] Data are for 5 mice. These titers were achieved by diluting the samples until optical-density values reached background levels.

[b] Against homologous viruses in serum, measured at 2 weeks after the final immunization and expressed as reciprocals of the highest dilution that completely inhibits hemagglutination of 4 hemagglutinin units of virus.

[c] Data are for 5 mice; measurement was by a plaque assay using with Mardin-Darby canine kidney cells. $^{*}P < .05$, vs. the nonimmunized group.

that in noninoculated mice, the concentration of IgG antibodies to HA in serum was significantly increased in mice inoculated intranasally with both the trivalent vaccine and Ampligen and in mice inoculated subcutaneously with the trivalent vaccine with or without Ampligen (table 1). A serum IgG response was not induced either in mice inoculated intranasally with vaccine without Ampligen or in mice inoculated, via either route, with Ampligen alone.

HI titers with regard to the A/NewCaledonia (H1N1), A/NewYork (H3N2), and B/Shanghai virus strains were examined in vitro, by use of serum samples from the same group of mice in which antibodies were examined. The serum samples from mice inoculated subcutaneously with vaccine with or without Ampligen showed high HI activity, with titers of 160–320, whereas serum samples from mice inoculated intranasally with Ampligen without the trivalent vaccine showed little response. No HI titer was detected in serum samples from noninoculated mice, from those inoculated intranasally with vaccine without Ampligen, or from those inoculated, via either route, with Ampligen alone.

Induction of H5N1-reactive antibody by intranasal inoculation with both seasonal influenza vaccine and Ampligen. We next characterized the cross-reactive antibody response to A/Vietnam/1194/04 (H5N1) in the various groups of inoculated mice. Compared with that in the noninoculated mice, the concentration of IgA antibodies to A/Vietnam/1194/04 in nasal-wash samples was significantly increased in mice inoculated intranasally with both the trivalent vaccine and Ampligen (table 2). The concentration of IgG antibodies to A/Vietnam/1194/04 in serum was also significantly increased in mice inoculated intranasally with both the trivalent vaccine and Ampligen and in mice inoculated subcutaneously with the trivalent vaccine with or without Ampligen (table 2).

HI titers with regard to heterologous A/Vietnam/1194/2004 (H5N1), A/HongKong (H5N1), and A/Indonesia/6/2005 (H5N1) virus were examined in vitro using serum samples from

the same group of mice in which antibodies were examined. However, these antibodies did not show any appreciable cross-neutralizing activity against H5N1 virus strains (table 2). We did not detect HI activity against A/NewCaledonia (H1N1), A/NewYork (H3N2), B/Shanghai, A/Vietnam/1194/2004 (H5N1), A/HongKong (H5N1), and A/Indonesia/6/2005 (H5N1) virus strains in the nasal-wash samples from inoculated mice (data not shown). The lack of such activity was likely due to the dilution of antibodies intrinsic to collection of nasal-wash samples.

We also examined the cross-protective effect that Ampligen combined with the trivalent vaccine has in mice challenged with 1000 pfu of A/Vietnam/1194/04 (H5N1) virus. In noninoculated mice, the virus titer was $10^{3.2}$ pfu/mL in nasal-wash samples obtained 3 days after challenge. Compared with those from noninoculated mice, nasal-wash samples from mice inoculated intranasally with either the trivalent vaccine alone or Ampligen alone and from mice inoculated subcutaneously with both the trivalent vaccine and Ampligen showed a (nonsignificant) 1-log-unit reduction in virus titer; in contrast, nasal-wash samples from mice inoculated intranasally with both the trivalent vaccine and Ampligen did not contain detectable virus (table 2). The nasal-wash and serum samples from the same groups of mice were tested for the ability to inhibit hemagglutination induced by the A/Vietnam/1194/04, A/HongKong/483/97, or A/Indonesia/6/05 viruses; no HI activity was detected in the nasal-wash or serum samples from the different inoculated groups (data not shown).

Cross-protection against different H5N1 influenza virus strains by intranasal inoculation with seasonal influenza vaccine. We next examined whether the combination of the trivalent vaccine and Ampligen conferred cross-protection against heterologous H5N1 influenza viruses, including the A/Vietnam/1194/04, A/HongKong/483/97, and A/Indonesia/6/05 strains (figure 1). In challenges with 1000 pfu of A/Vietnam/1194/04 virus, mice that had been inoculated with both the trivalent virus and Ampligen showed a significant reduction in virus titer, compared with noninoculated mice (figure 1*A*). Furthermore, at 14 days after challenge with A/Vietnam/1194/04 virus, 50% of the intranasally inoculated mice ($n = 10$) were still alive, whereas all of the subcutaneously inoculated mice ($n = 10$) and all of the noninoculated mice ($n = 10$) were dead. The subcutaneously inoculated mice showed the typical ruffled fur and neurological symptoms, such as tremor and spinning, evidenced by moribund mice; in contrast, the mice not challenged with A/Vietnam/1194/04 virus showed no ruffled fur and appeared to be healthy. In challenges with 1000 pfu of A/HongKong/483/97 virus, mice that had been inoculated with both the trivalent vaccine and Ampligen showed a 25% reduction in virus titer, compared with noninoculated mice (figure 1*B*). At 14 days after challenge with A/HongKong/483/97, all of the intranasally inoculated mice were still alive, whereas 30% of the subcutaneously inoculated mice ($n = 10$) and 40% of the noninoculated mice ($n = 10$) had died. Finally, in challenges with 1000 pfu of A/Indonesia/6/05 virus, mice that had been inoculated with both the trivalent vaccine and Ampligen showed a significant reduction in virus titer, compared with noninoculated mice (figure 1*C*). At 14 days after challenge with A/Indonesia/6/05 virus, all of the intranasally inoculated mice were still alive, whereas 80% of the subcutaneously inoculated mice ($n = 5$) and 80% of the noninoculated mice ($n = 5$) had died. Taken together, these results indicate that intranasal inoculation with both the trivalent vaccine and Ampligen was more effective against challenge with heterologous H5N1 influenza virus than was subcutaneous inoculation.

T cell responses induced by intranasal inoculation with both the trivalent vaccine and Ampligen. We next examined whether intranasal inoculation with the trivalent vaccine with or without Ampligen induced a T cell response against either homologous virus strains or heterologous H5N1 viruses. At 10 days after the final inoculation, T cells were isolated from the spleens of vaccinated mice and were cultured with irradiated antigen-presenting cells, in the absence or presence of the trivalent-vaccine components (at concentrations of 0.1 μg/mL and 1.0 μg/mL). T cells from mice inoculated with both the trivalent vaccine and Ampligen were proliferated and produced IFN-γ in a manner dependent on the concentration of the trivalent-vaccine antigens (figure 2*A*); however, these antigens had no effect on the proliferation of and IFN-γ production by T cells isolated either from mice inoculated with the trivalent vaccine alone (figure 2*B*) or from noninoculated mice (figure 2*C*), and mice intranasally inoculated with both the trivalent vaccine and Ampligen did not show a cross-reactive T cell response to A/Vietnam/1194/04 (H5N1) whole-virus antigen (figure 2*A*). These results suggest that intranasal inoculation with both the trivalent vaccine and Ampligen induces a systemic T cell response to homologous vaccine strains but not to heterologous H5N1 virus strains.

DISCUSSION

We have evaluated both the immunogenicity of the trivalent inactivated influenza vaccine (HA vaccine) prepared for the 2005–2006 season and its cross-protective efficacy against different H5N1 influenza viruses when it is administered either alone or with Ampligen. Compared with noninoculated mice, those inoculated, intranasally or subcutaneously, with both the trivalent vaccine and Ampligen manifested a lower virus titer in nasal-wash samples at 3 days after challenge with H5N1 influenza virus. However, whereas intranasal inoculation effectively improved the survival rate of mice infected with antigenically distinct H5N1 virus strains, subcutaneous inoculation did not. We speculate that this cross-protection may be me-

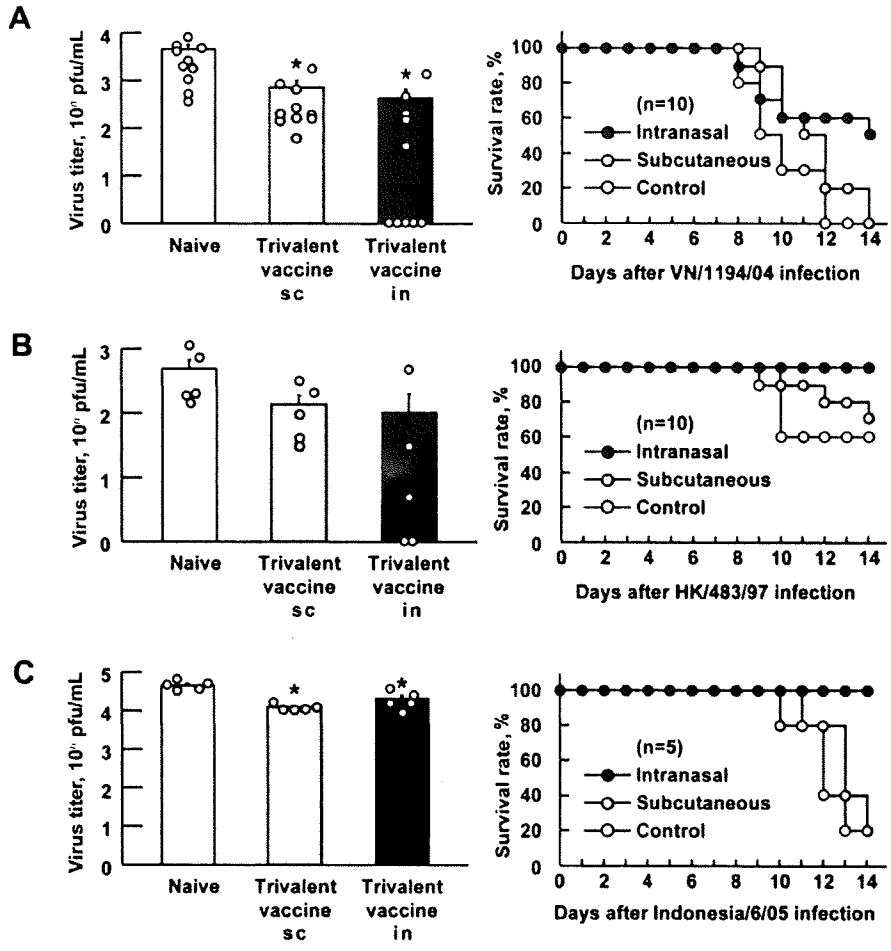

Figure 1. Cross-protective effect of inoculation with trivalent inactivated influenza vaccine and Ampligen against highly pathogenic H5N1 influenza viruses. Mice were inoculated, intranasally (in) or subcutaneously (sc), with trivalent inactivated vaccine and Ampligen, as described in table 1. At 14 days after the final inoculation, the mice were challenged by in administration of 1000 pfu of A/Vietnam/1194/04 virus *(A)*, A/HongKong/483/97 virus *(B)*, or A/Indonesia/6/05 virus *(C)*. At 3 days after challenge, nasal-wash samples were collected, and the titer of each virus was determined *(left panels)*; data are means ± SEs 5 mice/group. White circles indicate values for individual mice. Survival rates of the mice in each group ($n = 5$ or $n = 10$) also were monitored, for 14 days after challenge with H5N1 *(right panels)*. *$P < .05$ vs. noninoculated (Naive) mice.

diated by the mucosal immune response, probably via secretory IgA antibodies to viral proteins. Elsewhere, we have shown that polymeric immunoglobulin receptor–knockout mice do not secrete IgA and show less cross-protective efficacy against variant influenza virus infection, when challenged with either influenza A virus or influenza B virus [26, 27]. However, anti-HA or anti-NA antibodies that normally act as neutralizing antibodies do not seem to contribute to such cross-protection, because none of the nasal-wash and serum samples collected from inoculated mice in the present study exhibited cross-neutralizing activity in vitro (table 2); these antibodies neutralized the HI activity of the viruses whose subtypes are the same as those of the respective vaccine strains (table 1). These findings may be interpreted as suggesting that IgA antibodies specific for viral internal proteins are important for heterosubtypic protection, because some secretory IgA antibodies may neutralize virus infectivity during transcytosis in the infected epithelial cells [28–30].

In the present study, intranasal inoculation with both the trivalent vaccine and Ampligen induced a systemic T cell response to homologous virus strains but not to the heterologous H5N1 virus strain. These results further support the notion that the heterologous protection achieved by such inoculation is largely attributable to cross-reactive secretory IgA. The cross-

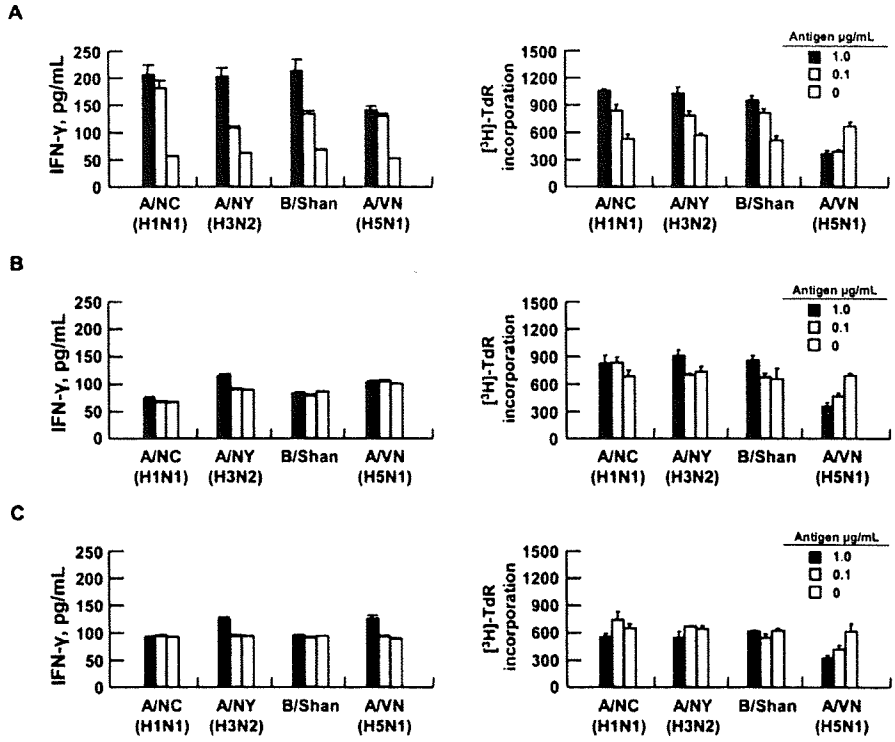

Figure 2. Interferon (IFN)–γ *(left panels)* and [³H]thymidine incorporation *(right panels)* by T cells from mice inoculated intranasally (in) with the trivalent vaccine and Ampligen *(A)*, mice inoculated with the trivalent vaccine alone *(B)*, and noninoculated mice *(C)*, as described in table 1. Splenic T cells were isolated from the mice 10 days after the final inoculation and were cultured with irradiated antigen-presenting cells in the absence or presence of A/NewCaledonia (A/NC), A/NewYork (A/NY), B/Shanghai (B/Shan), or A/Vietnam/1194/04 (A/VN) antigens, at concentrations of 0.1 μg/mL and μg/mL. After 4 days of culture, the concentration of IFN-γ in culture supernatants was measured by ELISA; the minimum detectable dose of mouse IFN-γ is <1 pg/mL. Data are means ± SDs for 2 independent experiments, each performed with T cells from 5 mice/group.

protective immunity seems to correlate with virus replication in noninoculated mice. Although noninoculated and intranasally inoculated mice were challenged with the same titer of the A/Vietnam (H5N1) virus, the virus titer in the noninoculated mice was different in every experiment, a finding that explains the observed difference in cross-protective effects (table 2 and figure 1).

Our other recent studies have presented a strong case for the use of double-stranded RNA (dsRNA) as an adjuvant for intranasal vaccines [8, 9]. dsRNA is a powerful Toll-like receptor –3 agonist in the induction of innate immune responses; particularly significant is its apparent ability, as adjuvant, to broaden the range of viral mutant strains against which the wild-type vaccine provides full or partial protection, a property that will become increasingly important as new strains of influenza virus appear with ever increasing frequency and exhibit the potential to evolve into a global pandemic. The limitation to this approach has been that traditional dsRNAs are experimental agents, which have not been approved for human use and/or have exhibited significant toxicity (i.e., in the case of polyI:polyC) in human clinical trials. The one exception is the dsRNA product, Ampligen (polyI:polyC$_{12}$U). Ampligen has been studied in >700 patients with a cumulative systemic drug exposure of 76,000 doses; Ampligen has completed pivotal phase II/III trials for chronic fatigue syndrome and has an excellent safety record in humans.

In summary, intranasal inoculation with both the trivalent inactivated influenza vaccine and Ampligen induced cross-protective mucosal immunity to heterologous H5N1 influenza virus in mice. Intranasal inoculation with both the annual influenza vaccine and Ampligen may thus represent a strategy that, in humans, can generate protective mucosal immunity against newly emerging and highly pathogenic avian influenza viruses.

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Original article

Intranasal immunization with H5N1 vaccine plus Poly I:Poly $C_{12}U$, a Toll-like receptor agonist, protects mice against homologous and heterologous virus challenge

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Abstract

The avian H5N1 influenza virus has the potential to cause a new pandemic. Since it is difficult to predict which strain of influenza will cause a pandemic, it is advantageous to produce vaccines that confer cross-protective immunity. Mucosal vaccine administration was reported to induce cross-protective immunity by inducing secretion of IgA at the mucosal surface. Adjuvants can also enhance the development of fully protective mucosal immunity. Here we show that a new mucosal adjuvant, polyI:poly$C_{12}U$ (Ampligen®), a Toll-like receptor 3 agonist proven to be safe in a Phase III human trial, is an effective adjuvant for H5N1 influenza vaccination. Intranasal administration of a candidate influenza vaccine with Ampligen resulted in secretion of IgA, and protected mice that were subsequently challenged with homologous A/Vietnam/1194/2004 and heterologous A/HK/483/97 and A/Indonesia/6/2005 virus.

Keywords: Influenza virus; H5N1 subtype; Ampligen; Adjuvant; Immunoglobulin A

1. Introduction

The first outbreak of the highly pathogenic avian influenza virus H5N1 was reported in humans and birds in Hong Kong in 1997, during which 6 out of 18 infected people died [1]. Subsequently, re-emergence of H5N1 highly pathogenic influenza viruses 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 June 2007, 310 laboratory-confirmed H5N1-infected human cases were reported to the World Health Organization. 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.

H5 Vaccine candidates must be continually updated to match the antigenicity of circulating viruses because of the difference in HA antigenicity among 1997, 2003, and 2004 H5 viruses [3]. In addition, it is difficult to predict which strain of virus (H5 or other avian-associated hemagglutinin) will be responsible for a pandemic. In such circumstances, the ideal

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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 to subcutaneous vaccination-induced systemic immunity. Cross-protective activity is correlated with mucosal secretory IgA, which is not induced following subcutaneous vaccination [4]. 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 [5–8]. Nicholson et al. reported that the H5N1 vaccine is poorly antigenic in humans, and requires adjuvant to elicit a detectable antibody response [9]. Several groups looking at avian influenza H5N1 vaccines have reported that intranasal administration of a formalin-inactivated whole virus vaccine with or without mutant *Escherichia coli* heat-labile toxin (LT) adjuvant (R192G), or an adenoviral vector-based influenza vaccine, protected mice from lethal challenge by a heterologous H5N1 virus [10–12]. Although LT is an effective adjuvant for the production of mucosal IgA, it has adverse clinical side effects, such as the facial paralysis of Bell's palsy [13]. New, clinically safe and effective adjuvants are necessary for the administration of intranasal influenza vaccines to humans.

We previously demonstrated that the synthetic double-stranded RNA (dsRNA) poly(I:C), a Toll-like receptor 3 (TLR-3) agonist, has mucosal adjuvant activity when co-administered intranasally with an influenza hemagglutinin (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 [7,14]. Sloat et al. [15] 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. However, while TLR agonists, including poly(I:C), are potent mucosal adjuvants that induce type I interferons (IFNs), and have the potential to bridge the gap between innate and adaptive immunity [16], they have been associated with serious adverse events during clinical trials. Poly(I:C) induced a number of side effects in human, including renal failure and hypersensitivity reactions in some patients in a previous clinical trial using as high as 75 mg of poly(I:C)/m^2 on day 0 and then daily from day 7 to a maximum of 35 days [17].

PolyI:PolyC_{12}U (Ampligen®) is similar to dsRNA and has a good safety profile based on clinical trials, including a recently conducted double-blind placebo-controlled Phase III clinical trial [18]. 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:PolyC_{12}U was as effective as poly(I:C) in inducing the maturation of human monocyte derived dendritic cells in vitro [19].

In this study, we examined the protective effect of intranasal administration in mice of an H5N1 vaccine together with synthetic dsRNAs (PolyI:C and Ampligen) as powerful TLR-3 agonists. The H5N1 vaccine used was NIBRG14, which was derived from a highly pathogenic influenza virus (A/Vietnam/1194/04) isolated from a patient with H5N1 influenza, and was prepared by the UK National Institute for Biological Standards and Control (NIBSC) [20]. We demonstrate that co-administration of the vaccine with either poly(I:C) or Ampligen as mucosal adjuvants elicited protective immunity not only against homologous virus (A/Vietnam/1194/04), but also heterologous viruses, including past (A/HK/483/97) and recent (A/Indonesia/6/05) clinical highly pathogenic H5N1 influenza virus isolates.

2. Materials and methods

2.1. Mice

Six- to eight-week-old female BALB/c mice were purchased from Japan SLC. Mice were kept under specific-pathogen-free conditions approved by the Institution Animal Care and Use Committee of National Institute of Infectious Diseases.

2.2. Viruses

The strains of H5N1 viruses used in this study were A/Hong Kong/483/97 (A/HK/483/97), A/Vietnam/1194/2004 (A/Vietnam/1194/04), A/Indonesia/6/2005 (A/Indonesia/6/05) [21]. The HK/483/97 virus, isolated from patient with fatal influenza A H5N1 disease in the Government Virus Unit, the Queen Mary Hospital, Hong Kong, was prepared in Mardin–Darby canine kidney (MDCK) cells without any special step for mouse adaptation. The A/Vietnam/1194/04 virus and A/Indonesia/6/05 from patients with H5N1 disease were propagated in 10-day-old embryonated chicken eggs for 2 days at 37 °C. These viruses were stored at −80 °C and viral titers were quantified by plaque assay using MDCK cells.

2.3. Preparation of vaccine and adjuvants

The vaccine used in these studies was a formalin-inactivated whole virus vaccine, NIBRG14, derived from a recombinant avirulent avian virus containing modified HA and neuraminidase (NA) from the highly pathogenic avian influenza strain A/Vietnam/1194/2004 virus, and other viral proteins from influenza strain A/PR/8/34 (H1N1) [20]. Modified HA lacks the multibasic amino acids at the cleavage site. The HA vaccine (split-product virus vaccine) from A/PR/8/34 (A/PR8; H1N1) was used in the examination of RNA expression experiments. Synthetic poly(I:C) dsRNA was kindly provided by Toray Industries Inc. (Kamakura, Kanagawa, Japan). Poly I:Poly C_{12}U (Ampligen®) was kindly provided by Hemispherx Biopharma (Philadelphia, PA).

2.4. Immunization and virus challenge

Five mice for each experimental group were anaesthetized with diethyl ether and immunized intranasally with 1 μg of total protein amount of vaccine, with or without adjuvant. Four weeks later, they were re-immunized in the same manner. According to a modification of the procedure of Yetter and coworkers [22], each mouse was anesthetized and infected by intranasal administration of 4 μl of PBS containing virus suspension with 1000 PFU of H5N1 virus into each nostril (2 μl/nostril). As 2 μl of the virus suspension remained in the local nasal area and could not enter the lung tissue, the initial viral infection was limited to the nasal area. H5N1 virus infection experiments were carried out in Biosafety Level 3 containment facilities, approved by the Guides for Animal Experiments Performed at National Institute of Infectious Diseases.

2.5. Measurement of the virus titer and antibody titers

Serum and nasal wash fluid were collected for measurement of the virus titer and antibodies against vaccine from mice that were sacrificed under anesthesia with chloroform. The levels of IgA and IgG Abs against vaccine determined by enzyme-linked immunosorbent assay (ELISA) as described previously [5]. Standards for vaccine-reactive IgA and IgG antibody titration were prepared from the nasal wash or serum of survived mice after H5N1 virus challenge, and expressed as the same arbitrary units (160-unit). The antibody titers of unknown specimens were determined from the standard regression curve constructed by twofold serial dilution of the 160-unit standard for each assay.

Virus neutralization tests by antisera were performed as described previously [23].

The virus titer was measured as follows. One milliliter of nasal wash was harvested from each mice, and aliquots of 200 μl of serial 10-fold dilutions of the nasal wash fluid were inoculated into MDCK cells in six-well plates. After 1 h incubation, each well was overlaid with 2 ml of agar medium according to the method of Tobita and coworkers [24]. The number of plaques in each well was counted at 2 days after inoculation. All of the experiments were repeated independently at least three times. The data are presented as means ± standard error (S.E.).

2.6. RNA isolation, cDNA synthesis, and real-time PCR

The mRNA levels of IRF-3, IRF-7, RIG-I, MDA5, Fas, and TRAIL in nasal-associated lymphoid tissues (NALTs) in vaccinated or influenza virus-infected mice was measured by real-time quantitative PCR after reverse transcription. Mice were inoculated with influenza virus or intranasally administered influenza HA vaccine (A/PR8) with or without poly (I:C). The NALTs were collected sequentially up to 72 h after administration. The NALTs from three mice in each group was pooled and total RNA was extracted using an SV-Total RNA Isolation kit (Promega, Madison, WI) and cDNA were synthesized using Omniscript Reverse Transcriptase (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Real-time quantitative PCR was performed using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) with a QuantiTect Probe PCR kit (Qiagen), TaqMan probes (Applied Biosystems), and primers (Sigma Genosys, Ishikari, Japan) (Table 1) designed with Primer Express (Applied Biosystems). The system uses two dye layers to detect the presence of target and control sequences. The FAM dye layer yields results for quantification of the target mRNA. The detection of RIG-I and MDA5 mRNAs was performed with TaqMan Gene Expression Assays (Applied Biosystems). PCR was carried out in a volume of 20 μl; initial denaturation at 50 °C for 2 min and 95 °C for 15 min, was followed by 45 cycles of 94 °C for 15 s and 60 °C for 1 min. Relative mRNA abundance was calculated using the comparative delta-Ct method [25].

2.7. Statistics

Comparisons between experimental groups were made with the t-test for paired observations, and $P < 0.05$ was considered significant.

3. Results

3.1. Intranasal immunization of H5N1 vaccine with poly(I:C) protects against highly pathogenic avian influenza virus infection

To determine the efficacy of poly(I:C) as a mucosal adjuvant for H5N1 vaccines, the antibody response to NIBRG14, a whole virus vaccine derived from virus strain A/Vietnam/1194/04, was examined in mice immunized intranasally with

Table 1
Primers for quantitative PCR (probes labeled 5′ FAM, 3′ TAMRA)

Target	Sequences	
IRF3	Forward:	TGA CAC CAA TGG CAA AAG CA
	Reverse:	CCC AAG ATC AGG CCA TCA A
	Probe:	CCT CAC TCC CAG GAA AAC CTA CCG AAG TTA
IRF7	Forward:	CAG CCT TGG GTT CCT GGA T
	Reverse:	CCC ACC ACT GCC TGT AGC A
	Probe:	CCA TCA TGT ACA AGG GCC GCA C
Fas	Forward:	GCG ATT CTC CTG GCT GTG A
	Reverse:	GGC TCA AGG GTT CCA TGT TC
	Probe:	CAC TGT GTT CGC TGC GCC T
TRAIL	Forward:	CCT TAG GCC AGA AGA TTG AAT CC
	Reverse:	CCT AAA GAG CAC GTG GTT GAG A
	Probe:	TCC TCT CGG AAA GGG CAT TCA T
β-actin	Forward:	CAC CGA TCC ACA CAG AGT ACT TG
	Reverse:	CAG TGC TGT CTG GTG GTA CCA
	Probe:	CAG TAA TCT CCT TCT GCA TCC TGT CAG CAA

mRNA expression levels of RIG-I and MDA5 were measured using the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA).

0.1–1 μg of NIBRG14 and various amounts (0 to 10 μg) of poly(I:C). Mice were immunized with vaccine and poly(I:C) twice, 6 and 2 weeks before infection. Mice immunized with vaccine alone (no adjuvant), or adjuvant alone had a small or an undetectable Ab response (Fig. 1). Animals immunized with 1 μg of vaccine and 10 μg of poly(I:C) had the highest concentration of anti-NIBRG14-specific IgA and IgG Abs in their nasal wash and serum, respectively. Next, we examined the protective effect of intranasal administration of NIBRG14 and poly(I:C) against homologous (A/Vietnam/1194/04) H5N1 influenza virus infection. The mean virus titer in the nasal washes of control mice was $10^{2.3}$ PFU/ml 3 days post inoculation (d.p.i.) with 1000 PFU, and none of the control mice survived more than 14 d.p.i. Intranasal immunization with vaccine alone had a partial protective effect, when comparing nasal wash virus titers to that of controls, and 80% of the mice in this group survived. However, intranasal immunization with H5N1 vaccine and poly(I:C) adjuvant resulted in a marked protective effect against viral infection (Fig. 1). Thus, intranasal administration of H5N1 vaccine with poly(I:C) adjuvant protected mice against homologous H5N1 influenza virus infection.

3.2. Antibody responses against H5N1 influenza virus in BALB/c mice immunized intranasally or subcutaneously with NIBRG14 vaccine and Ampligen as an adjuvant

We next examined the efficacy of Ampligen (PolyI:Poly$C_{12}U$) as a mucosal adjuvant for H5N1 vaccines. Mice were immunized twice by intranasal or subcutaneous administration of A/Vietnam/1194/04 whole virus vaccine (NIBRG14) with or without Ampligen, and their antibody response was examined by ELISA. In nasal washes, the highest concentration of anti-NIBRG14 IgA Ab was observed in animals immunized intranasally with 1 μg of NIBRG14 and 10 μg of Ampligen (Fig. 2). A small IgA response was elicited by intranasal administration of NIBRG14 without adjuvant, and no IgA response was evident in mice that received intranasal administration of Ampligen alone (control group), or in any of the mice who received a subcutaneous vaccination (Fig. 2). The neutralization antibody titer to A/Vietnam/1194, A/HK/483/97, and A/Indonesia/6/05 viruses was examined *in vitro* using sera from the same group of mice. The neutralizing activity to homologous virus (A/Vietnam/1194/04) was detected in the sera from immunized mice intranasally or subcutaneously with or without adjuvant. However, no neutralizing activity to heterologous viruses (A/HK/483/97, A/Indonesia/6/05) was detected in the sera from all immunized mice group.

3.3. Intranasal administration with H5N1 vaccine with Ampligen protected mice from variant H5N1 influenza virus challenge

We next examined the protective effect of intranasal administration of vaccine and Ampligen adjuvant against homologous (A/Vietnam/1194/04) and heterologous (A/HK/483/97 and A/Indonesia/6/05) H5N1 influenza virus challenge (Fig. 3). Two groups of mice were immunized either intranasally or subcutaneously with 1 μg of NIBRG14 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, the mean nasal wash virus titer of control mice was $10^{3.04}$ PFU/ml 3 d.p.i., and none of the mice survived more than 12 d.p.i. (Fig. 3A). In mice immunized subcutaneously with vaccine

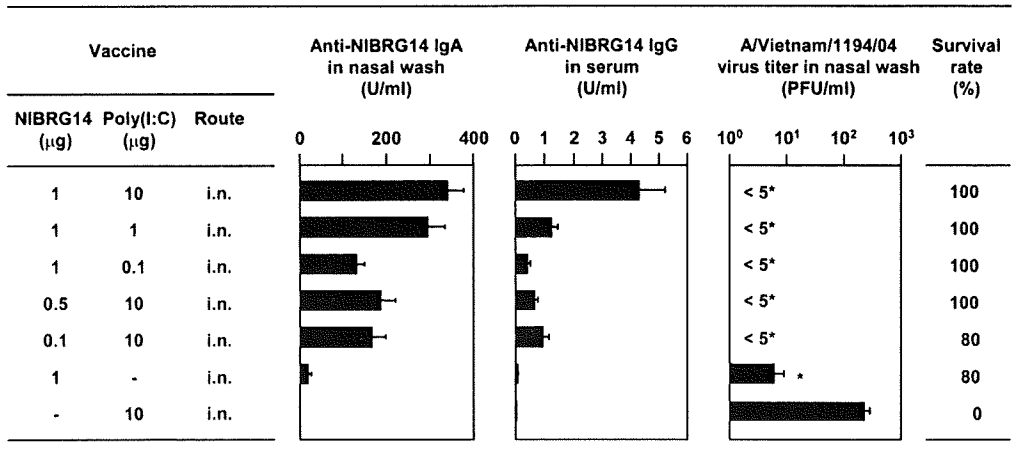

Fig. 1. Anti-NIBRG14-specific IgA and IgG antibody titers, A/Vietnam/1194/04 virus titers, and survival rate of mice after lethal challenge with A/Vietnam/1194/04 virus. Anti-NIBRG14-specific IgA and IgG responses in BALB/c mice immunized twice intranasally with the indicated doses of vaccine (NIBRG14) and/or poly(I:C) adjuvant. Nasal washes and serum samples were collected 14 days after the second immunization. Antibody titers of five mice from in each group were measured by ELISA. The same groups of mice were then infected intranasally with 1000 PFU of A/Vietnam/1194/04 virus 14 days after the second immunization. Nasal washes were collected 3 d.p.i., and virus titers were measured by plaque assay. Each column represents the mean ± standard error (S.E.) of five animals. For statistical analysis, virus titers were compared to control mice that received intranasal administration of 10 μg of poly(I:C) without vaccine. Survival rates were monitored for 16 d.p.i. $^*P < 0.05$.

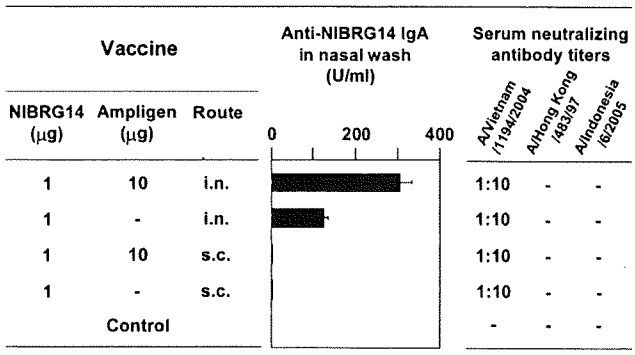

Fig. 2. Anti-NIBRG14-specific IgA and IgG responses in BALB/c mice immunized twice intranasally or subcutaneously with vaccine alone, or in combination with Ampligen®. Nasal washes and serum samples were collected 14 days after the final immunization. Antibody titers were measured by ELISA; data represent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

and Ampligen, there was a 1 log reduction in nasal wash viral titer compared to control mice, and all mice survived up to 18 d.p.i. (Fig. 3A). In contrast, none of the mice immunized intranasally with vaccine and Ampligen had virus titers in their nasal washes, and 100% of the mice survived up to18 d.p.i. (Fig. 3A). In the heterologous, A/HK/483/97 virus challenge group, the mean nasal wash virus titer of control mice was $10^{3.44}$ PFU/ml 3 d.p.i., and none of the mice survived more than 10 d.p.i. (Fig. 3B). In mice immunized subcutaneously with vaccine and Ampligen, there was no evidence of a protective effect based on nasal viral titers, and none of the mice survived more than 10 d.p.i. (Fig. 3B). However, in mice immunized intranasally with vaccine and Ampligen, there was a significant reduction in virus titer in the nasal washes compared to the subcutaneous vaccination group, and 80% of mice survived up to 18 days following virus challenge (Fig. 3B). In the group challenged with heterologous A/Indonesia/6/05 virus, the mean nasal wash virus titer of the control group was $10^{4.44}$ PFU/ml 3 d.p.i., and 20% of the mice survived up to 18 d.p.i. (Fig. 3C) In addition, 1000 PFU of A/Indonesia/6/05 was not 100% lethal. In mice immunized subcutaneously with vaccine and Ampligen, there was a significant reduction in nasal wash virus titer compared to control mice, and 40% of the mice survived up to 18 d.p.i. (Fig. 3C). In mice immunized intranasally with vaccine and Ampligen, there was a significant reduction in virus titers compared to the subcutaneous vaccination and control groups, and 100% of the mice survived up to 18 d.p.i. (Fig. 3C). 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.

3.4. Expression levels of IRF-3, IRF-7, RIG-I, MDA5, Fas, and TRAIL mRNA in the NALT after intranasal administration of vaccine with dsRNA

Lastly, we examined mRNA expression levels of IRF-3, IRF-7, RIG-I, MDA5, Fas, and TRAIL to define the mechanism of adjuvant activity when dsRNA was administered intranasally with influenza vaccine (Table 1). The expression of IRF-7 in the NALT was up-regulated in mice administered vaccine and poly(I:C) without infection, or mice challenged with A/PR8 influenza virus, while that of IRF-3 was unchanged under either condition (Fig. 4A and B). The mRNA level of RIG-1, a cytoplasmic dsRNA receptor, but not MDA5 was rapidly induced in mice administered vaccine and poly(I:C) without infection, or mice challenged with A/PR8 influenza virus, compared to the group receiving vaccine alone (Fig. 4C). The mRNA levels of Fas and TRAIL were unchanged among the three groups (Fig. 4E and F). These results suggested that up-regulation of IRF-7 and RIG-I, which are key molecules in the recognition of viral dsRNA and the induction of type-I IFNs in the early stages of viral replication, mediate the adjuvant effect of dsRNA, and enhance the immune response in the nasal mucosa.

4. Discussion

In the current study, we evaluated the immunogenicity and cross-protective effect of the NIBRG14 H5N1 influenza vaccine co-administered intranasally with poly(I:C) or Ampligen adjuvant. It has been suggested that the most effective immunization strategies for protection against influenza virus infection should involve the induction of a mucosal immune response at the nasal mucosal epithelium, which is the initial site of virus infection [5,26]. Single subcutaneous immunization with an inactivated H5 influenza vaccine with incomplete Freund's adjuvant or aluminum hydroxide adjuvant conferred protection against heterologous H5N1 influenza virus challenge in a lethal mouse model [27]. In the current study, we demonstrated that intranasal immunization with adjuvant-combined vaccine, but not subcutaneous vaccination, induced an A/Vietnam/1194/04-specific IgA response in the nasal mucosa (Figs. 1 and 2), and conferred a broad spectrum of cross-protective immunity against heterologous A/HK/483/97(H5N1) and A/Indonesia/6/05(H5N1) viruses (Fig. 3). In intranasal vaccination group, A/Vietnam/1194/04 HA-reactive IgA and IgG were detected in the nasal wash and serum, respectively. However, the neutralizing activity of the serum against homologous A/Vietnam/1194/04 virus but not heterologous A/Hong Kong/483/97 and A/Indonesia/6/05 was observed exclusively (Fig. 2). No neutralizing activity was detected in the nasal wash in any group in the present study against both homologous and heterologous viruses. The concentration of anti-A/Vietnam/1194/04 HA IgA in the nasal wash was much lower than that of anti-A/Vietnam/1194/04 HA IgG in the serum, because the nasal wash was diluted with PBS for collection from the nasal mucosa. It seems that the concentration of anti-A/Vietnam/1194/04 HA IgA is

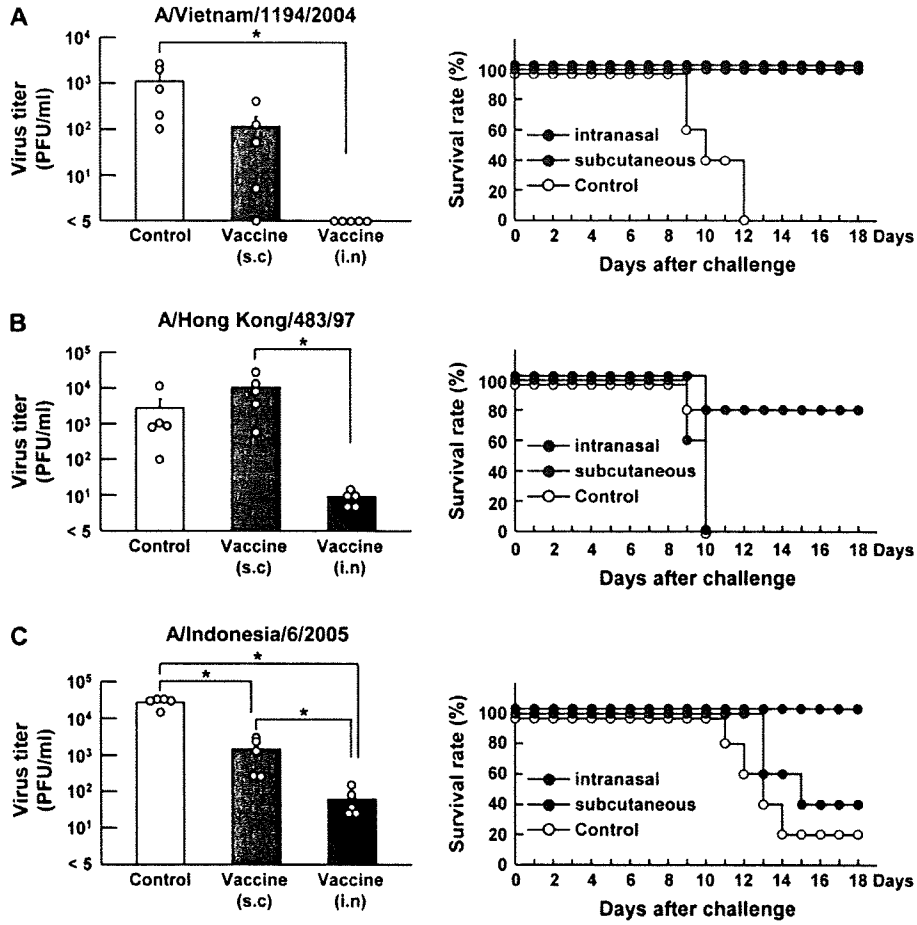

Fig. 3. H5N1 virus titers in nasal washes and survival rates after lethal challenge with homologous A/Vietnam/1194/04, heterologous A/HK/483/97, or heterologous A/Indonesia/6/05 viruses. Mice were immunized intranasally (black column) or subcutaneously (gray column) with vaccine and Ampligen, then challenged by intranasal administration of 1000 PFU of A/Vietnam/1194/04 (A), A/HK/483/97 (B), or A/Indonesia/6/05 (C) virus 14 days after the final immunization. Nasal washes were collected 3 d.p.i., and virus titers were measured by plaque assay. Each column represents the mean ± S.E. of five mice and open circles indicate individual animals. For statistical analysis, virus titers were compared to those from control mice (open column) that received intranasal administration of 10 μg of Ampligen alone. Survival rates were monitored for 18 days. $^*P < 0.05$.

much lower than the physiological concentration in the nasal mucosa, and therefore neutralizing activity in the nasal wash may not be detected. In this experiment, we used the same PFU value of each strain of H5N1 viruses to compare the virus titer in the nasal wash and survival rate after the challenge between subcutaneously vaccinated and intranasally vaccinated group within the same challenging strain. Although A/Indonesia/6/05 was not 100% lethal at this challenging dose, intranasal administration of the vaccine improved the survival rate from 20% to 100%, while subcutaneous vaccination modified the survival rate from 20% to 40%. These results indicate that intranasal vaccination is more effective than subcutaneous vaccination in protecting against heterologous H5N1 influenza virus infection.

Cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT) have been used as adjuvants to enhance mucosal immune responses [28]. Although CT and LT are effective adjuvants, they have several undesirable side-effects in humans, including dysfunction of the VIIth cranial nerve [13]. Therefore, the development of other, safer adjuvants is required. Previously, we demonstrated that poly(I:C) has mucosal adjuvant activity in combination with a split-product influenza vaccine (A/PR8, H1N1), and does not have any adverse effects on the central nervous system [7]. Although poly(I:C) is a potent mucosal adjuvant, that induces type I IFNs and could potentially bridge the gap between innate and adaptive immunity [16], its poor safety record in clinical trials imposes a significant regulatory barrier [17].

In this study, we also explored the potential mechanism underlying the adjuvant effect of dsRNA. Intranasal administration of poly(I:C) and the split-product vaccine, or A/PR8 virus, but not vaccine alone (no adjuvant), induced mRNA expression of IRF-7 and RIG-I in NALT. Previously, we showed that TLR-3, which recognizes dsRNA, was upregulated, and IFN-α/β was induced, as were Th1- and Th2-related cytokines, after intranasal administration of poly(I:C) and the split-product HA vaccine [7]. dsRNA is recognized not only by TLR-3 and RNA helicase, RIG-I and MDA5, and induces activation of NF-κB and production of type-I IFNs [29,30]. Thus, it appears that the mucosal adjuvant effect of dsRNA results in

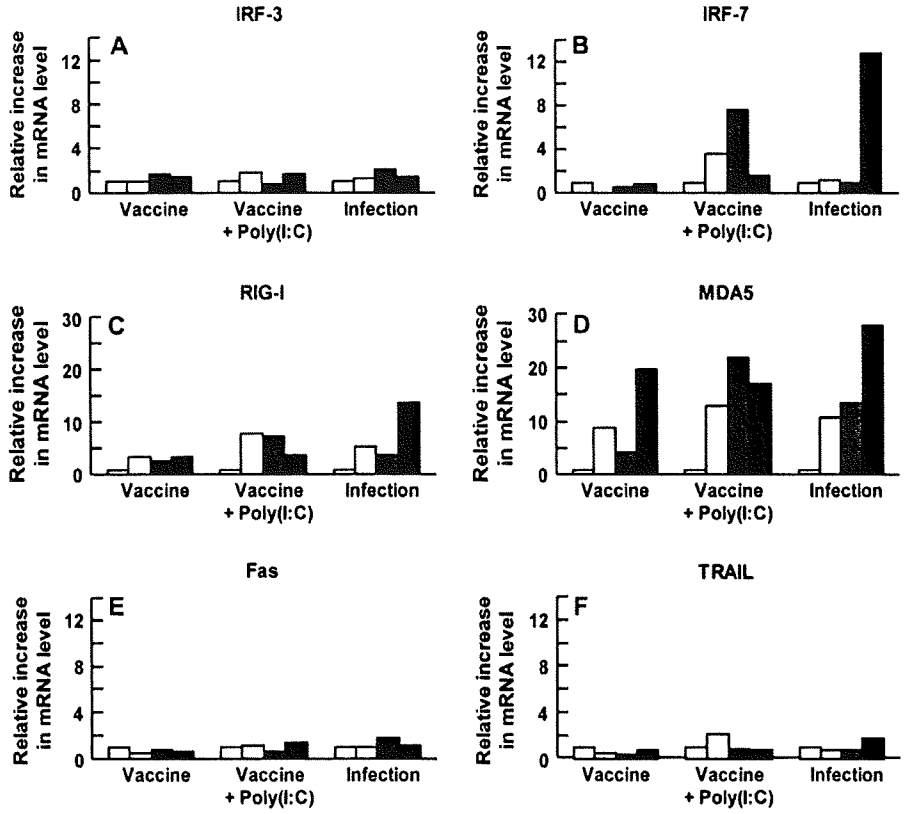

Fig. 4. Expression of mRNAs of IRF-3 (A), IRF-7 (B), RIG-I (C), MDA5 (D), Fas (E), and TRAIL (F) in NALT. Total RNA was extracted from NALT at 0 h (open column), 6 h (light gray column), 24 h (dark gray column), and 72 h (closed column) after intranasal administration of HA vaccine (A/PR8) alone, HA vaccine plus Poly (I:C), or infected with 1000 PFU of A/PR8 viruses. Real-time quantitative RT-PCR was used to determine mRNA expression levels of pooled total RNAs extracted from NALT of three mice.

marked antibody responses, together with enhanced expression of TLR-3, RIG-I, IRF-7, type-I IFNs, and Th1- and Th2-related cytokines. It also confers a cross-protective effect against lethal challenge with heterologous H5N1 influenza viruses. The expression of Fas and TRAIL did not change upon virus infection or intranasal administration of vaccine with dsRNA adjuvants.

In summary, intranasal administration of Ampligen combined with H5N1 vaccine derived from a highly pathogenic influenza virus clinical isolate induced cross-protective mucosal immunity against heterologous H5N1 influenza virus infection. Intranasal vaccination co-administered with the TLR-3 agonist, Ampligen, appears to offer an effective strategy against an influenza pandemic, regardless of the strain of H5N1. The potential shortage of vaccine during a pandemic might also be addressed by preparing a stock of this vaccine in advance using the United States Food and Drug Administration "animal rule" as a global model for regulatory approval by various governmental regulatory agencies.

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Development of a mucosal vaccine for influenza viruses: preparation for a potential influenza pandemic

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Highly pathogenic avian H5N1 influenza A virus has caused influenza outbreaks in poultry and migratory birds in Southeast Asia, Africa and Europe, and there is concern that it could cause a new pandemic. This fear of an emerging pandemic of a new influenza strain underscores the urgency of preparing effective vaccines to meet the pandemic. One way to mitigate current concerns is to develop an influenza vaccine that is fully functional against drift influenza viruses. In our current situation, in which we cannot predict which strain will cause a pandemic, cross-protective immunity using potential and novel mucosal vaccines plays a particularly important role in preventing the spread of highly pathogenic influenza virus.

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Highly pathogenic avian influenza viruses

Highly pathogenic avian H5N1 influenza A virus has caused influenza outbreaks in poultry and migratory birds in Asia, Europe and Africa. Subsequently, human H5N1 disease with high fatality rates has been reported in southern China [1], Vietnam [2], Thailand [3], Cambodia [4], Indonesia, Turkey, Azerbaijan, Egypt, Iraq and Nigeria [101]. The viruses associated with these fatal human outbreaks have the potential to cause a new pandemic. To date, most of the human cases were due to infection from birds and very few involved transmission of the virus from human to human. However, there was a cluster of cases of deadly H5N1 influenza that occurred in 2006, in which the WHO deemed that human-to-human transmission was the most probable cause of viral spread [5,6]. The ability to be transmitted from human to human represents the final barrier to a new pandemic of this deadly avian strain of H5N1 influenza virus, and the increasing fear of emergence of such a new strain compels us to prepare effective vaccines should such a pandemic arise. The effort to develop effective vaccines against highly pathogenic avian influenza H5N1 viruses is an urgent and global public-health priority [7]. However, there are a number of problems in developing vaccines against highly pathogenic avian influenza H5N1 viruses. A highly contained facility is required as the virus grows very poorly in embryonated eggs since it kills the host (chicken) quickly. Attenuation of the vaccine strain is necessary to limit these problems. Currently licensed human vaccines are strain specific and do not protect against heterotypic influenza viruses. This is problematic, as influenza A (H5N1) continues to evolve into antigenically distinct clades. Therefore, the question remains, how can we prepare an effective vaccine for an impending pandemic of a new influenza strain that might be caused by a highly pathogenic strain of avian influenza virus? Moreover, influenza virus A (H5N1) is not the only strain that could cause a new pandemic in humans. Once a virus acquires the ability to infect via human-to-human transmission, a serious pandemic can ensue. In this situation, safe and effective vaccines are likely

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to be the only way to protect the public. As resistance of influenza virus A (H5N1) to antiviral drugs has already been reported [8], effective human vaccines against this and other potentially deadly influenza viruses are urgently needed.

Influenza

Influenza is a contagious acute respiratory disease caused by infection of the upper respiratory tract by influenza virus. Recently, it was shown that infection by highly pathogenic influenza viruses is not restricted to respiratory systems and can expand to the gastrointestinal tract [9]. However, the respiratory mucosa is the primary target tissue of viral infection. Annual epidemics of influenza occur when the antigenic properties of the viral surface protein hemagglutinin (HA) and neuraminidase (NA) are altered. HA is one of the major viral surface glycoproteins and is involved in the binding of the virus to sialic acids on the surface of susceptible cells [10]. NA is also a viral surface glycoprotein that cleaves terminal sialic acid residues from carbohydrate moieties on the surfaces of infected cells, promoting the release of progeny viruses. The mechanism of altered antigenicity is twofold: antigenic shift, caused by genetic rearrangement between human and animal viruses after double infection of host cells, which can cause a pandemic; and antigenic drift, caused by small changes in the HA and NA proteins on the virus surface, which can cause influenza epidemics. The emergence of variant virus strains by these two mechanisms is the cause of influenza epidemics.

Among the eight genes encoded by influenza virus (*HA, NA, M, NP, NS, PA, PB1* and *PB2*), immunity against HA and NA plays a central role in protection against influenza. This has been supported by studies demonstrating failure to confer protective immunity via the passive transfer of conserved M1- or NP-specific antibodies, and studies showing the effectiveness of a DNA vaccine encoding NA or HA [11,12]. It has been shown that both surface glycoprotein HA- and NA-encoding DNAs are more protective against influenza compared with other DNAs expressing various viral proteins [11]. Thus, in developing an effective influenza vaccine for use during a pandemic, HA and NA would be the target antigens.

Innate immunity

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 the case of 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 (NK) cells and complement factor, all play important roles in innate immunity at the respiratory mucosa. Among these, the IFN response is required to signal viral infection. During influenza virus infection, genomic single-stranded RNA, and double-stranded (ds)RNA produced during viral replication, have been implicated as the molecular signals of infection that trigger IFN production. For example, production of vast amounts of IFN-α in response to wild-type influenza virus requires endosomal recognition of influenza virus genomic RNA and signaling through Toll-like receptor (TLR)7 and MyD88 [13]. dsRNAs produced during viral replications are recognized by TLR3 expressed in human alveolar and bronchial epithelial cells [14]. Thus, type I IFNs, such as IFN-α and -β, and interleukins (ILs), such as IL-6 or -12, play important roles in the response to influenza virus infection.

The innate immune system senses viral infection by recognizing a variety of viral components, including dsRNA and triggering antiviral responses. The cytoplasmic helicase proteins 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 the recognition of viral dsRNA. Viral dsRNA binds to RIG-I and MDA5 in the cytoplasm, which leads to the activation of IFN regulatory factors [15]. *In vitro* studies suggest that RIG-I and MDA5 recognize both RNA viruses and polyinosine–polycytidylic acid (poly[I:C]), a synthetic dsRNA analogue. 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 [15]. The recognition of viral infection by the innate immune system bridges the transition between innate and adaptive immune responses. This is a particularly important facet of innate immunity when considering 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.

Other innate immune systems, including human β-defensin and mannan-binding lectin, block viral fusion by creating a protective barricade of immobilized surface protein [16]. Accumulation of NK cells in local lymphoid tissue in response to inoculation of chitin microparticles (CMPs) in the nasal mucosa also protects mice from lethal H5N1 influenza virus infection [ICHINOHE T ET AL., UNPUBLISHED DATA].

Mucosal immunity

The mucosal immune system plays an important role in the prevention of influenza virus infection in the upper respiratory system. Secretory immunoglobulin (sIg)A and IgG antibodies and cytotoxic T lymphocytes (CTLs) ($CD8^+$) are involved in recovery from influenza following viral infection in naive mice. One of the reasons 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 precursors migrate from mucosal sites throughout the entire body through site-specific homing pathways. This system is referred to as the common mucosal immune system [17-21]. The other benefit of the mucosal immune system is the presence of cross-reactive sIgA, which plays a fundamental role in mucosal immunity (FIGURE 1). In fact, natural influenza virus infection is 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 [22-24].

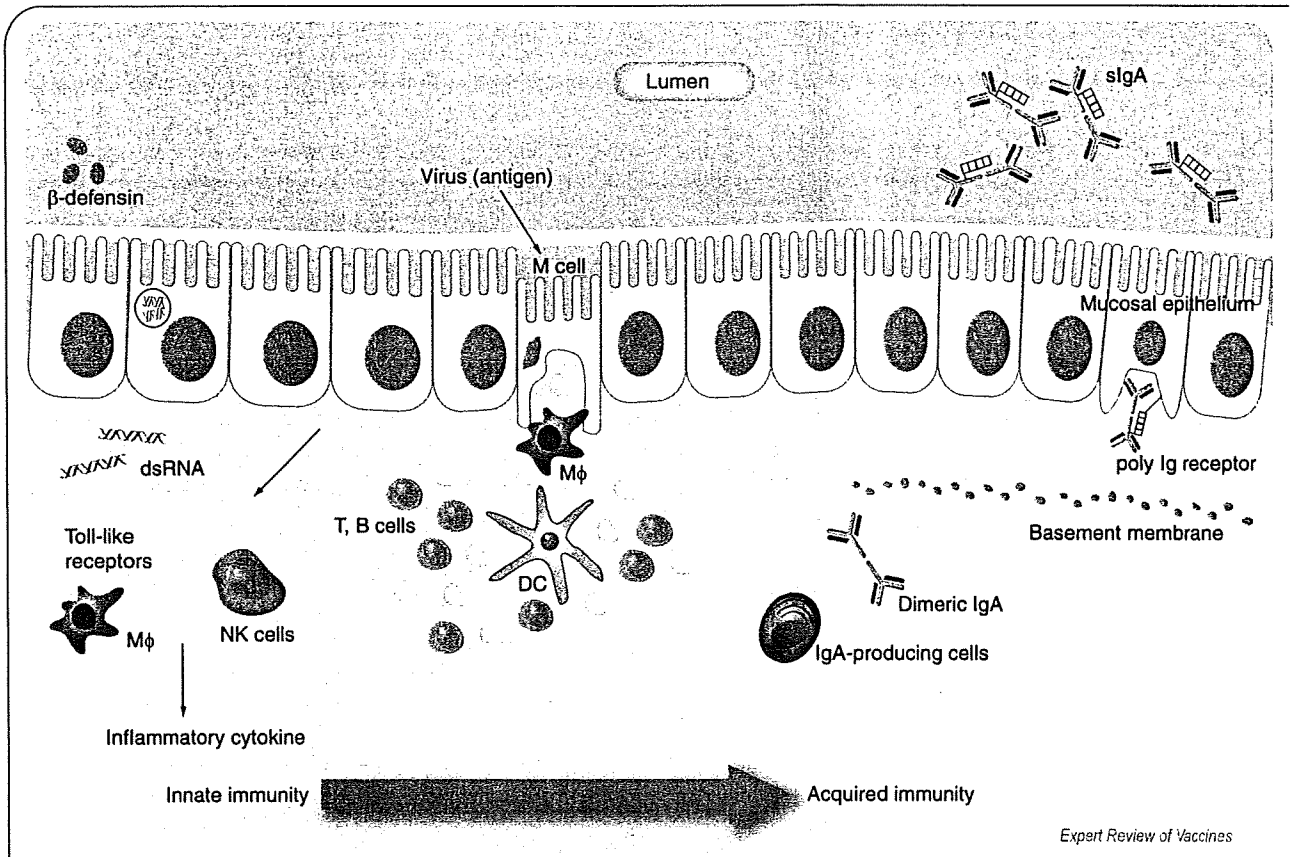

Figure 1. Defense mechanism on the mucosal surface.
DC: Dendritic cell; ds: Double stranded; Ig: Immunoglobulin; Mϕ: Macrophage; NK: Natural killer; sIgA: Secretory immunoglobulin A.

During the course of natural infection by influenza virus, the mucosal membrane becomes protected against viral infection. People who recover from natural infection with a particular strain of influenza virus are protected against subsequent influenza viral infection. When reinfected with the drifted influenza viruses they also showed reduced shedding of virus compared with people who were not previously infected [25]. Following initial viral infection, protection of the mucosal membrane is almost certainly mediated by mucosal immunity. Mucosal immune responses are induced at mucosal-associated lymphoid tissues. Subsequent infection then induces a secondary IgA antibody response in the local lymphoid tissue. This $CD4^+$ T cell-dependent induction of IgA response to influenza infection can be generated in the absence of B-cell signaling through the major histocompatibility complex (MHC) class II, establishing that the process is driven by nonantigen-specific bystander help [26].

Safe and effective vaccines that induce cross-protective mucosal immunity are likely to be the single most important public health tool for decreasing morbidity and mortality during a pandemic of influenza. The importance of cross-protective immunity against subtypic and heterosubtypic viruses in preventing a new pandemic of influenza will now be discussed.

Vaccines

Cross-protection by currently available 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 (TABLE 1). These IgG antibodies would be effective against an epidemic of homologous virus but would rarely be effective against an epidemic caused by a heterologus 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 the USA [27–29] appeared to mimic the natural course of infection and provided cross-protective immunity against different subtypes of viruses by inducing sIgA antibodies, serum IgG antibodies and a CTL response [25,30]. However, in the USA, use of a live, attenuated virus vaccine is approved only for people between the ages of 5 and 49 years, meaning the major high-risk groups, infants and the elderly, are excluded.

Cross-protection by new vaccines

Parenteral adjuvant-combined vaccines

Virosomal subunit vaccines, containing HA and NA incorporated into unilameller liposomes composed of phosphatidylcholine

(Berna Biotech, Bern, Switzerland), are licensed and have been developed as parenteral vaccines for eliciting strong antibody responses [31,32]. Subunit vaccines emulsified with Microfluidized Emulsion 59 (MF59, FLUAD, Chiron, CA, USA) have also been developed and elicit strong antibody responses [33]. MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine showed higher seroconversion rates than A/Hong Kong/156/97, A/Hong Kong/213/03, A/Thailand/16/04 and A/Vietnam/1203/04, compared with nonadjuvanted vaccines [34,35]. Aluminium hydroxide and aluminium phosphate are effective adjuvants for inactivated whole-virion vaccines [36].

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 protect against heterologous virus infection effectively by inducing sIgA, IgG and CTL responses (TABLE 1). However, the safety was proved only in healthy people between the age of 5 and 49 years. As a result, they are only approved for use in healthy people aged 5–49 years in the USA. Intranasal administration of inactivated vaccines represents a potential solution to overcome these problems.

In clinical trials, inactivated whole-virus particles and split-product vaccines are effective in preventing live virus infection when administered intranasally [37–41]. 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 [42]. The stronger immunogenicity of the inactivated whole-virion vaccine was probably due to the stimulation of innate immunity by genomic single stranded RNA, via TLR-7 [43,44].

Intranasal administration of an inactivated ether-split vaccine and the synthetic dsRNA poly(I:C) conferred effective cross-protection in the upper respiratory tract against viral variants (drift viruses) of influenza A- or B-type viruses [45]. Most viruses produce dsRNA during replication [46], thus, synthetic dsRNA probably acts as a molecular mimic of viral infection. The mammalian TLR3 recognizes dsRNA and activates the nuclear factor-κB pathway [47], resulting in the activation of type I IFNs that, in turn, enhances the primary antibody response to subcutaneous immunization of soluble materials [48]. This adjuvant activity of IFN-I appears to play an important role in bridging the gap between innate and adaptive immunity [48].

In mice, intranasal administration of an ether-split vaccine from PR8 (influenza virus strain H1N1) and poly(I:C) adjuvant induced strong anti-HA IgA and IgG responses in nasal washes and serum, respectively, whereas 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. 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) [45]. Recently, a clinically safe, commercially available dsRNA, poly(I:C_{12}U) (Ampligen®), was investigated as a dsRNA adjuvant for intranasal avian influenza vaccines [HASEGAWA, UNPUBLISHED DATA].

In another attempt to develop mucosal vaccines against influenza, an immune regulatory cell, NK T cells (NKT), were used. A nasal vaccine using NKT cell-specific glycolipid ligand, α-galactosylceramide, as an adjuvant can induce cross-protection against variant influenza viruses, including highly pathogenic H5-type virus derived from human avian influenza patients [HASEGAWA, UNPUBLISHED DATA].

Intranasal immunization of mice with an inactivated vaccine together with cholera toxin B subunits (CTB) containing a trace amount of the holotoxin (CTB*) induced not only sIgA and strong cross-protection in the upper respiratory tract against infection by variant viruses of the same subtype, but also serum IgG and weak cross-protection in the lower respiratory tract against variant virus infection [49–52]. These findings were consistent with those of previous reports [53–55]. Although CTB* is an effective adjuvant for producing sIgA, it also has some side effects, such as nasal discharge. The use of *Escherichia coli* heat-labile enterotoxin (LT), which is structurally and functionally similar to cholera toxin (CT), as an adjuvant with nasal influenza vaccines may also be clinically unsafe, as intranasal administration of influenza vaccine with LT has been linked to several cases of Bell's palsy (facial paralysis) [56]. Attempts to reduce the toxic side effects of adjuvant therapy have involved introduction of mutations into CTBs [57] or use of physiological adjuvants, such as the complement component C3d [58].

Chitin is a natural polysaccharide of *N*-acetyl-D-glucosamine, and CMPs 1–20 μm in diameter represent another candidate immune-enhancing adjuvant, as they can be derived from safe, nonmicrobial sources, such as shrimp, crab and lobster. Chitin is nonallergenic, biodegradable and biocompatible. Chitin-derived products are now widely used in the medical, veterinary, cosmetic, health supplement and environmental industries [59]. Chitin is also a major component of fungal spores and induces a T-helper (Th)1 response. The innate immune system of the lung is well adapted to the clearance of airborne spores, largely through phagocytosis by macrophages. This process involves the secretion of IL-12 and -18 from macrophages, which enhances the Th1 immune responses [60]. Intranasal application of CMPs results in the elevation of Th1 cytokines, including IL-12, IFN-γ and TNF-α [60,61], and stimulation of NALT by CMPs serves as a bridge between the innate and adaptive immune systems [60]. Chitosan, a partially deacetylated form of chitin, has also been used as a vaccine adjuvant owing to its mucoadhesive properties and has been shown to enhance antibody responses to mucosally delivered vaccine antigens [62].

Surfclam microparticles (SMPs), comprising of 98.9% calcium micropowder from fired natural surf clams, range in size from 1 to 10 μm in diameter and represent a good candidate

Table 1. Characteristics of current and developing or new vaccines.

Vaccine	Immune responses*			Cross-protective immunity*	Ref.
	IgA	*IgG*	*CTL*		
Current vaccines					
1. Parenteral inactivated vaccine					
Split-product and subunit vaccine	-	++	-	-	[73–76]
2. Live virus vaccine					
Cold-adapted, live virus vaccine	+	+	+	++	[27–29]
Developing or new vaccines					
1. Inactivated vaccine					
i) Adjuvant-combined parenteral vaccine	-	++	-	+	[31–33,36]
ii) Nonparenteral vaccine					
- Nasal vaccine	++	+	-	++	[37–42,77]
- Adjuvant-combined nasal vaccine	++	+	-	++	[45,50,52,59,63,71,78,79]
- Epidermal vaccine	+	++	-	++	[64,65,80]
iii) DNA vaccine	-	+	++	++	[11,12,67]
2. Live virus vaccine					
i) Recombinant live virus vaccine	+	+	+	++	[81–85]
ii) Virus-vectored vaccine	+	+	-	+	[86]

*+: Exist; ++: Strong; -: Absent.
CTL: Cytotoxic T lymphocyte; Ig: Immunoglobulin.

vaccine carrier owing to their size and the surface features of the microparticles. The adjuvant effect of SMPs and the induction of cross-protective immune responses against homologous and heterologous influenza variants by intranasal administration of an inactivated influenza HA vaccine together with SMPs has been demonstrated [63].

Epidermal vaccines

Epidermal powder immunization (EPI) using special delivery systems (PowderJect ND5.2™) can deliver a powdered trivalent influenza vaccine efficiently to the epidermis in humans [64,65]. EPI vaccination of trivalent influenza vaccine induced seroconversion to all strains. Using an inactivated virus vaccine, EPI facilitated antigen recognition and uptake by Langerhans' cells and dendritic cells in the dermis, eliciting both high serum IgG levels and a low mucosal IgA antibody response. Although the EPI system elicits a good immune response, reduction of antigen dosage would be required for practical application.

DNA vaccines

The injection of plasmid DNA encoding influenza A virus (H1N1) nuclear protein (NP) into BALB/c mice resulted in the generation of NP-specific CTLs and protection against challenge infection by the H3N2 strain of virus [66]. Thus, cross-protection against different subtypes of influenza can be elicited by the injection of NP-expressing DNA (TABLE 1). However, protection conferred by NP-expressing DNA is not as effective as that obtained by HA or NA-expressing DNA. In order to enhance the efficacy of DNA vaccines, expression of a fusion of antigen and C3d was evaluated. A DNA vaccine encoding the fusion protein HA–C3d was more effective in inducing cross-reactive antibodies than nonfused forms of HA and protected mice from lethal challenge by influenza viruses of different subtypes [67].

Live virus vaccines

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 (TABLE 1). Current cold-adapted live, attenuated influenza virus vaccines are growth-restricted to the upper respiratory tract. Using reverse genetics, a live, attenuated vaccine that encodes a modified form of HA and wild-type NA from influenza A virus strain H5N1, with the remaining gene segments derived from the ca influenza A vaccine donor strain was generated. This vaccine was immunogenic in mice [68]. After 4 weeks following a single intranasally administered dose of ca vaccine, mice were fully protected from lethal challenge with homologous and antigenically distinct, heterologous wild-type strain H5N1 viruses from different genetic sublineages [68]. Live, attenuated vaccine can induce equivalent immune responses compared with

natural infection, so if we can produce a live vaccine without side effects, it would be a good candidate as a pandemic vaccine.

Viral vector-based vaccines

Development of viral vector-based vaccines encoding H5N1 influenza HA is one of the newest strategies for preparing for a new pandemic. This is an egg-independent strategy. Since avian influenza is highly lethal in chickens, use of embryonated eggs, which are essential for the production of influenza vaccines, would be problematic in a pandemic. A replication-incompetent, human adenoviral vector-based H5 influenza vaccine (Had-H5HA) induced both humoral and cell-mediated immune responses against avian H5N1 influenza virus isolated from people [69]. Immunization of mice with Had-H5HA conferred effective protection against H5N1-related disease and death and primary viral replication in response to antigenically distinct strains of H5N1 influenza virus [69]. Adenovirus vector-based vaccines may prove to be useful in epidemics involving certain strains of influenza virus for which growth of vaccines in embryonated eggs would be problematic. Adenovirus vector-based influenza vaccine could mimic the natural infection of influenza virus. However, pre-existing antiadenovirus immunity prevents the vaccine virus from infection. Moreover, there is still concern about the safety of adenoviral vector for human use after the fulminant hepatitis accident [70].

Perspectives on developing a new pandemic vaccine

In the current climate of fear of a new influenza virus pandemic and in light of the fact that 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 interest 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 sIgA plays an important role in cross-protection. Vaccines designed to induce mucosal immunity are necessary for meeting a pandemic of new influenza. As stated above, one of the requirements of inducing mucosal immunity is the administration of vaccine at mucosal sites, such as the nasal mucosa. For this reason, intranasal administration of inactivated vaccine plus adjuvants, live, attenuated vaccines or recombinant live vector-based 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 one. Recently, several candidate adjuvants that are safe and effective in mucosal administration have emerged, including dsRNA (Ampligen) [45,71], CMPs, SMPs [63] and mutant CT [72]. These mucosal adjuvants represent promising approaches to the development of safe and effective vaccines for a potential influenza pandemic.

Expert commentary

Current subcutaneous influenza vaccines are designed to limit disease severity, but not to prevent the disease itself. Moreover, a vaccine against a specific strain can only be developed after the pandemic has begun. This system only works when the vaccine strain is the same as the circulating strain. We now need a vaccine that provides protection from viral infection, which means the prevention of infection at the local site. The mucosal immune system plays an important role in the prevention of mucosally infective pathogens, such as influenza virus; therefore, a mucosal vaccine is highly desirable. The main problem being faced at this time, with the fear of the emergence of a new influenza strain and its pandemic, would probably be resolved with usable mucosal vaccines.

Moreover, mucosal vaccines can provide preventive immunity on the surface of the mucosal epithelium, which gives cross-protective immunity against variant strains of the virus.

The application of adjuvants is the main issue for the preparation of mucosal vaccines in humans, because a mucosal adjuvant is essential for an effective immune responses on the mucosal surface. Recently, we have developed several candidates of human-applicable adjuvants including synthetic dsRNA. When combined with a mucosal vaccine against influenza, the risk of the pandemic could be reduced.

Five-year view

In our opinion, inactivated intranasal influenza vaccine provides the most promising preventive tool for influenza virus infection. The influenza virus changes its antigenicity every year and one cannot predict the strain of the new emerging influenza virus in humans. Cross-protective immunity can limit infection and spreading of variant viruses. The advantage of mucosal immunity in the prevention of influenza is already clear; however, until recently, there were no

Key issues

- Highly pathogenic avian H5N1 influenza A virus has caused influenza outbreaks in poultry and migratory birds in Southeast Asia, Africa and Europe, and there is concern that it could cause a new pandemic.
- We cannot predict which strain will cause a pandemic.
- Currently licensed human vaccines are subtype specific and do not protect against heterotypic influenza viruses.
- The mucosal immune system provides cross-reactive secretory immunoglobulin A, which plays a fundamental role in mucosal immunity.
- Adjuvant-combined intranasal vaccine can provide safe and effective mucosal immunity against variant influenza virus infection.

human-applicable adjuvants for mucosal vaccines. Knowledge of the innate immune system provides us with a better understanding to fill the gap between innate immunity and adaptive mucosal immunity. We are now at the beginning of the application of inactivated mucosal influenza vaccines for human use. However, once the safety and effectiveness of mucosal vaccines are proved, they will prove invaluable. We aim to finish clinical trials of adjuvant-combined intranasal influenza vaccine, both seasonal and pandemic, within the next 5 years.

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