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

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ABSTRACT

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

1. Introduction

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

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

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

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

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2. Materials and methods

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

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

3. Results and discussion

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

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

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

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

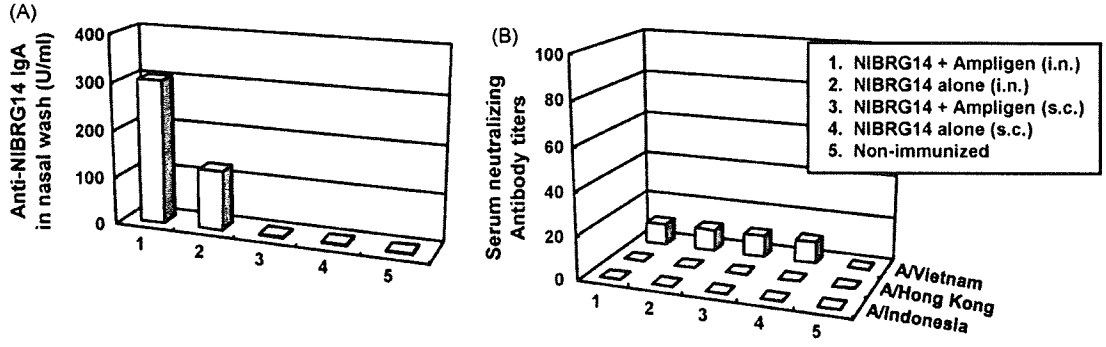

Fig. 1. Anti-NIBRG14-specific IgA and IgG responses in BALB/c mice immunized twice intranasally or subcutaneously with vaccine alone, or in combination with Ampligen. Nasal washes and serum samples were collected 14 days after the final immunization. Antibody titers were measured by ELISA (A). The serum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

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

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

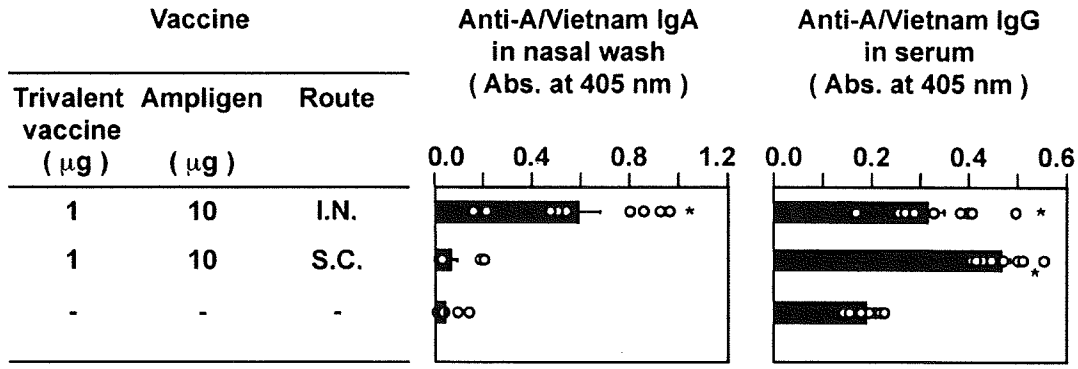

Fig. 2. Cross-reactive IgA and IgG antibodies to A/Vietnam/1194/04. The mice were initially immunized with 1 μg of trivalent-inactivated vaccines with Ampligen through intranasal or subcutaneous route. Immunization was repeated at 3 and 5 weeks after the initial immunization. The nasal washes and serum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

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

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

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

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

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

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

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

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

4. Concluding remarks

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

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Induction of Cross-Protective Immunity Against Influenza A Virus H5N1 by an Intranasal Vaccine With Extracts of Mushroom Mycelia

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The identification of a safe and effective adjuvant that is able to enhance mucosal immune responses is necessary for the development of an efficient inactivated intranasal influenza vaccine. The present study demonstrated the effectiveness of extracts of mycelia derived from edible mushrooms as adjuvants for intranasal influenza vaccine. The adjuvant effect of extracts of mycelia was examined by intranasal co-administration of the extracts and inactivated A/PR8 (H1N1) influenza virus hemagglutinin (HA) vaccine in BALB/c mice. The inactivated vaccine in combination with mycelial extracts induced a high anti-A/PR8 HA-specific IgA and IgG response in nasal washings and serum, respectively. Virus-specific cytotoxic T-lymphocyte responses were also induced by administration of the vaccine with extract of mycelia, resulting in protection against lethal lung infection with influenza virus A/PR8. In addition, intranasal administration of NIBRG14 vaccine derived from the influenza A/Vietnam/1194/2004 (H5N1) virus strain administered in conjunction with mycelial extracts from *Phellinus linteus* conferred cross-protection against heterologous influenza A/Indonesia/6/2005 virus challenge in the nasal infection model. In addition, mycelial extracts induced proinflammatory cytokines and CD40 expression in bone marrow-derived dendritic cells. These results suggest that mycelial extract-adjuvanted vaccines can confer cross-protection against variant H5N1 influenza viruses. The use of extracts of mycelia derived from edible mushrooms is proposed as a new safe and effective mucosal adjuvant for use for nasal vaccination against influenza virus infection. ***J. Med. Virol. 82:128–137, 2010.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: avian influenza; adjuvant; immunoglobulin A; heterosubtypic immunity

INTRODUCTION

When developing a vaccine, both prophylactic effectiveness and safety must be considered. The mucosal immune system of the respiratory tract, which is a primary site of influenza infection, is usually the first immunological barrier against influenza virus infection. The influenza virus is able to cause annual epidemics of influenza by altering the antigenic properties of its surface hemagglutinin (HA), the antigenic glycoprotein that is responsible for binding of the virus to sialic acids

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on the surface of susceptible cells. Inactivated vaccines against the influenza virus are administered parenterally to induce the production of anti-HA IgG antibodies that are highly protective against homologous virus infection, but less effective against heterologous virus infection [Ichinohe et al., 2008]. In contrast, a number of studies have shown that the mucosal immunity acquired through natural infection, which is mainly due to the secreted form of IgA in the respiratory tract, is more effective and provides greater cross-protection against virus infections than systemic immunity induced by parenteral vaccination in humans and mice [Asahi et al., 2002]. In this regard, induction of secreted IgA in the respiratory tract has a stronger potential to confer protection against unpredictable epidemics of influenza.

In the effort to develop effective intranasal vaccines, cholera toxin and *Escherichia coli* heat-labile toxin have been used as adjuvants to enhance the mucosal immune response [Tamura et al., 2005]. Although these toxins effectively provoke mucosal immune responses, they elicit adverse clinical side effects, such as nasal discharge and the facial paralysis of Bell's palsy [Mutsch et al., 2004]. Therefore, other adjuvants that are both effective and safe for human use have been developed for clinical application with intranasal influenza vaccine [Coulter et al., 2003; Hasegawa et al., 2005; Ichinohe et al., 2005, 2006, 2007a,b; Asahi-Ozaki et al., 2006].

It has been reported that extracts derived from certain mushrooms can elicit an innate immune response, resulting in activation of NF-κB, and strongly stimulate cellular and humoral immunity [Kim et al., 2003; Kuo et al., 2006]. These mushroom extracts induce phenotypic and functional maturation of dendritic cells, tumoricidal activity in macrophages, and augmentation of natural killer cell activity [Sorimachi et al., 2001; Kodama et al., 2005; Kim et al., 2006]. It has also been shown that oral administration of mushroom extracts has an anti-inflammatory effect [Bernardshaw et al., 2006] and decreases IgE levels through modulation of the Th1/Th2 balance [Inagaki et al., 2005; Lim et al., 2005]. In an experimental peritonitis model, mice that were treated orally with edible mushroom (*Agaricus blazei*) extracts prior to bacterial challenge showed significantly lower levels of septicemia and improved survival rates [Bernardshaw et al., 2006]. Extracts from these mushrooms also have been used in immunotherapy to prevent tumor growth and metastasis [Ukawa et al., 2000; Sanzen et al., 2001]. These findings prompted an investigation into whether the administration of intranasal influenza vaccine in combination with mushroom extracts would induce a protective immune response against a lethal and heterologous virus challenge. To accomplish this, the effectiveness of 12 mycelial extracts as an immune-enhancing adjuvant was assessed by comparison with the effects of the adjuvant, poly(I:C). The results of the present study demonstrate for the first time that intranasal administration of inactivated influenza virus vaccine in combination with mycelial extracts as a mucosal adjuvant induces cross-protective immune responses against homologous and heterologous variant influenza viruses, including highly pathogenic influenza A H5N1 virus isolates.

MATERIALS AND METHODS

Mice

Six- to 8-week-old female BALB/c mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). MyD88-deficient mice were kindly provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan) [Adachi et al., 1998]. Mice were kept under specific pathogen-free conditions approved by the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases.

Vaccines and Influenza Viruses

HA vaccine (split-product virus vaccine) was prepared at the Kitasato Institute (Saitama, Japan) from members of the family Orthomyxoviridae, genus *Influenzavirus A, B*, species *Influenzavirus A*, including influenza A/PuertoRico/8/34 (A/PR8; H1N1). The virus was grown in allantoic cavities of 10- to 11-day-old fertile chicken eggs, purified and disintegrated with ethyl ether. The vaccines contained all proteins from the virus particle; however, the major component of the vaccine was HA (about 30% of the total protein). The A/PR8 virus used for the challenge experiments was adapted for use in mice by subculturing 148 times in ferret, 596 times in mouse, and 73 times in 10-day-old fertile chicken eggs.

The strains of influenza A virus H5N1 used in this study were A/Vietnam/1194/2004 and A/Indonesia/6/2005 [Gao et al., 1999]. The influenza A/Vietnam/1194/2004 virus and influenza A/Indonesia/6/2005 virus obtained 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. The H5N1 vaccine used in these studies was NIBRG14, a formalin-inactivated whole virus vaccine derived from a recombinant avirulent avian virus containing modified HA and neuraminidase from the highly pathogenic avian influenza A/Vietnam/1194/2004 virus and other viral proteins from influenza A/PR/8/34 (H1N1) [Nicolson et al., 2005]. Modified HA lacks the multibasic amino acids at the cleavage site.

Preparation of Adjuvants

The mycelia extracts of *Phellinus linteus*, *Cordyceps militaris*, *Lyophyllum decastes*, *Macrolepiota gracilenta*, *Naematoloma sublateritium*, *A. blazei*, *Grifola frondosa*, *Ganoderma lucidum*, *Hericium erinaceum*, *Inonotus obliquus*, *Lentinula edodes*, and *Pleurotus nebrodensis* were kindly provided by Intelligence Biological Institute Co., Ltd (Nirasaki, Yamanashi, Japan). The extracts of mycelia were prepared as described previously [Inagaki et al., 2005]. Synthetic double-stranded RNA poly(I:C) was kindly provided by Toray

Industries, Inc. (Kamakura, Kanagawa, Japan). Lipopolysaccharide and Zymosan A from *Saccharomyces cerevisiae* were purchased from Sigma (St. Louis, MO).

Immunization and Infection

Five mice from each experimental group were anesthetized with diethyl ether and primarily immunized by dropping 1 μg of vaccine per mouse with various adjuvants into both nostril. Four weeks later, they were re-immunized in the same manner with the same adjuvant. For A/PR8 virus infection, two different infection protocols were used. Under the first protocol, each mouse was anesthetized and infected by intranasal application of 20 μl of virus suspension (1,000 PFU in PBS; 40 LD_{50}). This procedure induced total respiratory tract infection, which resulted in virus shedding from the nose and lungs, and led to death from viral pneumonia about 7 days later. Under the second protocol, anesthetized mice were infected by dropping 2 μl of virus suspension (1,000 PFU in PBS) into each nostril. The nasal-restricted volume (4 μl) of virus suspension induced nasally localized infection, which was not lethal. The nasal and lung wash virus titers were used as indices of protection in the upper and lower respiratory tracts of immunized mice, respectively. For infection with influenza A H5N1 virus, each mouse was anesthetized and 4 μl of PBS containing virus suspension with 1,000 PFU of H5N1 was administered intranasally (2 μl/nostril). The virus suspension remained in the local nasal area and could not enter the lung tissue, and the initial viral infection was limited to the nasal area, leading to death about 8 days later. H5N1 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.

Measurement of Virus Titer and Antibody Titer

Serum, nasal washings, and bronchoalveolar washings were collected for measurement of virus titer and antibody titer from mice euthanized under anesthesia with chloroform. To collect nasal washings, a hypodermic needle was inserted into the posterior opening of the nasopharynx and 1 ml of PBS containing 0.1% bovine serum albumin was injected three times (1 ml total). Bronchoalveolar washings were collected by washing the trachea and lungs twice by injection of 1 ml PBS containing 0.1% BSA (2 ml total). The levels of IgA and IgG antibodies versus HA molecules purified from the A/PR8 viruses or NIBRG14 vaccine were determined by ELISA as described previously [Ichinohe et al., 2005, 2007a]. Briefly, ELISA was performed sequentially from the solid phase (EIA plates; Costar, Cambridge, MA) with a ladder of reagents as follows: first, HA molecules purified from influenza A/PR8 virus or NIBRG14; second, nasal washings, bronchoalveolar washings, or serum; third, either goat anti-mouse IgA antibody (α-chain specific; Amersham Biosciences, Piscataway, NJ) or goat anti-mouse IgG antibody (γ-chain-specific; Amersham Biosciences) conjugated with biotin; fourth, streptavidin conjugated with alkaline phosphatase (Life Technologies, Rockville, MD); and fifth, *p*-nitrophenylphosphate. The amount of chromogen produced was determined by measuring the absorbance at 405 nm using an ELISA reader. A twofold serial dilution of either purified A/PR8 HA-specific IgA (320 ng/ml) or A/PR8 HA-specific monoclonal IgG (160 ng/ml) was used as a standard, as described previously [Asahi et al., 2002]. The binding kinetics of the standard A/PR8 HA-specific monoclonal IgG was comparable with A/PR8 HA-specific IgG obtained from immunized mice. The A/PR8 HA-specific antibody concentration of each sample was determined from standard regression curves constructed for each assay with a programmed SJeia Autoreader (Model ER-8000; Sanko Junyaku, Tokyo, Japan). Standards for NIBRG14-reactive IgA and IgG antibody titration were prepared from the nasal washings or serum of survived mice after H5N1 virus challenge, and expressed using the same arbitrary units (160-unit). The NIBRG14-reactive antibody titer of each sample was determined from the standard regression curve constructed by twofold serial dilution of the 160-unit standard for each assay.

Before the hemagglutination inhibition tests, receptor-destroying enzyme (RDE II; Denka Seiken Co., Ltd, Tokyo, Japan) was added to the RBC-treated sera at 37°C overnight to inactivate non-specific hemagglutination inhibitors, followed by incubation at 56°C for 1 hr to inactivate RDE. Briefly, hemagglutination inhibition tests were performed by mixing 25 μl aliquots of serial twofold dilutions of the treated serum samples with four HA units of virus in microtiter plates and incubating them at room temperature for 30 min. Then, 50 μl of 0.5% chicken RBCs were added to each well and incubated at room temperature for 30–40 min. The hemagglutination inhibition titer was expressed as the reciprocal of the highest serum dilution that completely inhibited hemagglutination of four HA units of the virus.

The virus titer was measured as follows: 200 μl aliquots of serial 10-fold dilutions of the nasal washings were inoculated into MDCK cells in six-well plates. After incubation for 1 hr, each well was overlaid with 2 ml of agar medium. The number of plaques in each well was counted 2 days after inoculation. All experiments were repeated independently at least three times, and the data are presented as means ± SD.

Antigen-Specific T-Cell Response

Antigen-specific T-cell responses were measured as described previously [Ichinohe et al., 2005]. Spleens were harvested from mice 1 week after booster vaccination. After preparation of a single-cell suspension, T-cells were purified by depletion of $CD11b^+$ (Mac-1), $CD45R^+$ (B220), $DX5^+$, and $Ter\text{-}119^+$ cells using a magnetic cell sorter (MACS: Militenyi Biotec, Bergisch, Germany). To prepare antigen-presenting cells, splenocytes from normal BALB/c mice were depleted of

CD90 (Thy1.2)$^+$ cells by MACS and irradiated at 2,000 cGy.

T-cells were purified from the spleen (1×10^5 cells/well) and cultured with irradiated antigen-presenting cells (5×10^5 cells/well) in the presence or absence of A/PR8 vaccine (0.1, 1, or 10 μg/ml). After 4 days of culture, the cytokine concentration in the culture supernatant was measured by ELISA using a Mouse interferon-γ Immunoassay Kit (Biosource International, Camarillo, CA) according to the manufacturer's instructions.

Bone Marrow-Derived Dendritic Cell Preparation and Mycelial Extract Sensitivity Analysis

Bone marrow cells were isolated from the femurs and tibiae of wild-type or MyD88-deficient mice and bone marrow-derived dendritic cells were prepared as described [Inaba et al., 1992]. Lipopolysaccharide (1 μg/ml), Zymosan (2 μg/ml), *P. linteus* (5 μg/ml), *M. gracilenta* (5 μg/ml), *L. edodes* (5 μg/ml), or *G. frondosa* (5 μg/ml) was added on day 5 after cultivation with granulocyte–macrophage colony stimulating factor (Wako, Tokyo, Japan). On day 6, culture supernatants were collected for tumor necrosis factor (TNF)-α titration. Concentrations of TNF-α were determined by ELISA using a Mouse TNF-α Immunoassay Kit (Biosource International) according to the manufacturer's instructions.

Statistical Analysis

Comparisons between experimental groups were performed by Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Local and Systemic Antibody Responses in BALB/c Mice Immunized Intranasally With the Hemagglutinin Vaccine and Mycelia Extracts as Adjuvants

The immune-enhancing effects of 12 varieties of mycelia extracts as mucosal adjuvants for intranasal influenza vaccine were investigated in BALB/c mice (Fig. 1). The mice were immunized twice intranasally with 1 μg of HA vaccine in combination with various adjuvants and the activities of the mycelial extracts were compared. Mice treated with mycelia extracts from *P. linteus*, *M. gracilenta*, *G. frondosa*, and *L. edodes*-adjuvanted vaccines developed sufficient levels of both HA-specific IgAs in the nasal washings and IgGs in the serum (Fig. 1). No specific antibodies were detected in the nasal washings or serum from control mice immunized with non-adjuvanted vaccine (Fig. 1).

Intranasal Immunization With the Hemagglutinin Vaccine Combined With Mycelia Extracts Protects Against Lethal Influenza Virus Lung Infection in Mice

Next, the protective effects of intranasal immunization with HA vaccine combined with mycelia extracts against lethal influenza virus lung infection were examined (Fig. 2). Mice immunized with *P. linteus*-adjuvanted vaccine showed equivalent amounts of IgG in the lung washings and serum when compared with those immunized with poly(I:C)-adjuvanted vaccine, and the viral titer of the lung washings was remarkably decreased compared with that of the control group (Fig. 2A). *M. gracilenta*- or *L. edodes*-adjuvanted

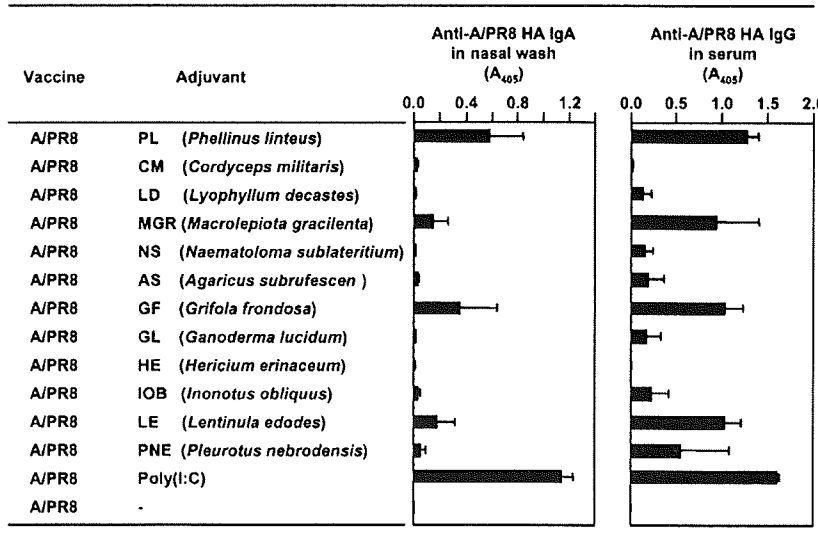

Fig. 1. Anti-A/PR8 hemagglutinin-specific IgA and IgG responses in BALB/c mice immunized intranasally with hemagglutinin vaccine alone, or in combination with various mycelia extracts or poly(I:C). Nasal washings and serum samples were collected 14 days after the final immunization. Antibody titers were measured by ELISA. Data represent the means ± SE of three mice per group.

vaccines conferred a partial but significant reduction in lung wash virus titers (Fig. 2A), while the virus titer in the lung washings of mice immunized with *G. frondosa*-adjuvanted vaccine was not altered compared to that of the control mice (Fig. 2A). The hemagglutination inhibition titer from each group corresponded with the IgG titer in lung washings and serum (Fig. 2A).

To examine the protective effects of these mycelia extract-adjuvanted vaccines against lethal influenza virus challenge, mice were inoculated with a lethal dose (40 LD_{50}) of influenza A/PR8 virus (Fig. 2B). Mice that had been immunized previously with *P. linteus*- or poly(I:C)-adjuvanted vaccine exhibited no remarkable change in body weight 14 days after virus challenge. In mice that had been immunized with vaccine adjuvanted with mycelia extracts from *M. gracilenta*, *G. frondosa*, or *L. edode*, body weights decreased gradually until day 6 after virus challenge, and then recovered from days 7 to 14 (Fig. 2B). Control mice that had been immunized with non-adjuvanted vaccine suffered from marked loss of body weight. The survival rate of mice immunized with *P. linteus*-adjuvanted vaccine was 100% at 14 days after virus challenge, suggesting that *P. linteus*-adjuvanted vaccine protected the mice against lethal lung infection as effectively as vaccine containing the poly(I:C) adjuvant (Fig. 2B). Meanwhile, the survival rates of mice immunized with vaccines adjuvanted with mycelial extracts from *M. gracilenta*, *G. frondosa*, or *L. edode* ranged from 40% to 60% at day 14 after lethal lung infection (Fig. 2B). All mice immunized with non-adjuvanted vaccine were deceased by 7 days after challenge.

Intranasal Administration of *Phellinus linteus*-Adjuvanted H5N1 Vaccine Protects Mice From Highly Pathogenic H5N1 Influenza Virus Variant Challenge

Next, the efficacy of *P. linteus*-adjuvanted vaccine against homologous (A/Vietnam/1194/2004) and heterologous (A/Indonesia/6/2005) H5N1 influenza virus challenge was examined in BALB/c mice (Fig. 3). The mice were immunized twice intranasally with 1 μg of formalin-inactivated whole H5N1 virus vaccine (NIBRG14) alone, or in combination with 500 μg of *P. linteus* extract. At 2 weeks after the final immunization, the mice were challenged by intranasal administration of 1,000 PFU of H5N1 influenza viruses.

The concentrations of anti-NIBRG14 IgA and IgG antibodies in nasal washings and serum, respectively, were much higher in animals immunized intranasally with *P. linteus*-adjuvanted NIBRG14 vaccine than in mice immunized with the vaccine alone or in non-immunized mice (Fig. 3A). In response to homologous viral challenge (A/Vietnam/1194/2004), the mice immunized with *P. linteus*-adjuvanted vaccine showed a significant reduction in virus titer compared with control mice (Fig. 3A). The mice vaccinated with *P. linteus*-adjuvanted vaccine survived longer than 14 days post-infection, while the mice immunized with

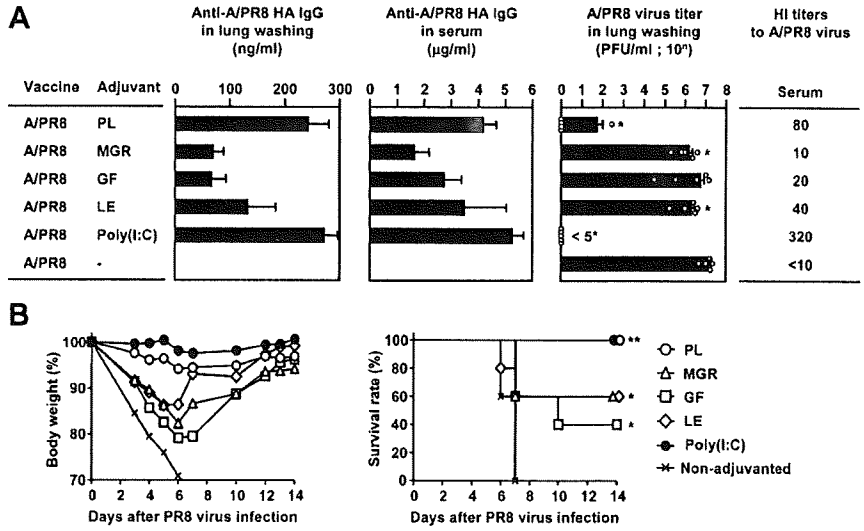

Fig. 2. **A**: Anti-A/PR8 hemagglutinin-specific IgG antibodies in lung washings and serum, and A/PR8 virus titer in lung washings. The mice were immunized twice intranasally with 1 μg of hemagglutinin vaccine alone, or in combination with extracts of mycelia from *P. linteus* (PL), *Macrolepiota gracilenta* (MGR), *Grifola frondosa* (GF), *L. edodes* (LE), or poly(I:C). Two weeks after the final immunization, the immunized mice were challenged by administration of 1,000 PFU (40 LD_{50}) of A/PR8 influenza viruses into the lung, and samples were collected 3 days after the challenge. The concentrations of IgG antibodies and virus titers from five mice from each group were measured by ELISA and plaque assay using MDCK cells, respectively. Each column represents the mean ± SE of five mice per group and open circles indicate individual animals. The virus titers were statistically compared to those of non-adjuvanted mice ($^*P < 0.05$). The hemagglutination inhibition (HI) titers against homologous A/PR8 influenza virus in the serum were measured at 2 weeks after the final immunization. The data are presented per group, and expressed as reciprocals of the highest dilution that completely inhibits hemagglutination of four HA units of the virus. **B**: Body weight and survival curves of the immunized mice after lethal A/PR8 virus challenge. Each point represents the ratio relative to the initial body weight (average) of five mice for each day after challenge (left panel). The survival rates were monitored for 14 days (right panel). $^{**}P < 0.01$ versus control mice, log-rank test.

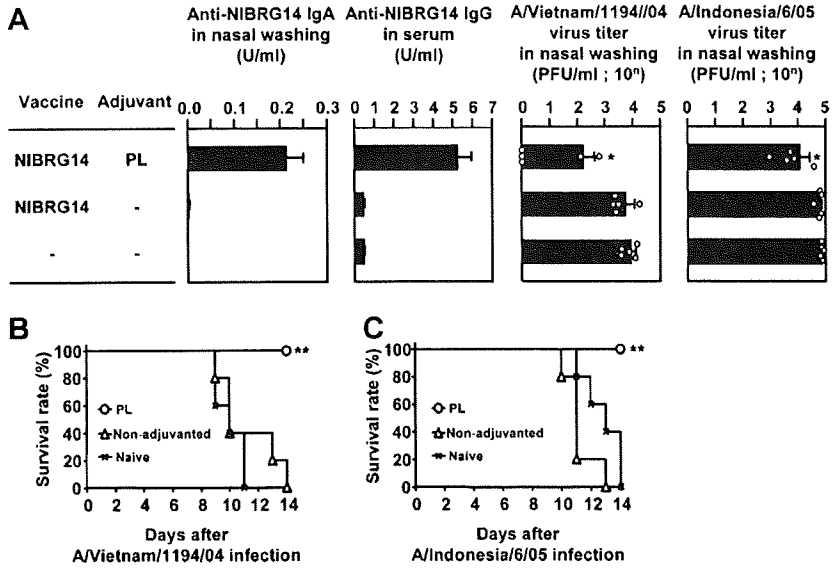

Fig. 3. Anti-NIBRG14-specific IgA and IgG responses, and H5N1 virus titers in nasal washings and survival rates after lethal challenge with homologous influenza A/Vietnam/1194/04 and heterologous influenza A/Indonesia/6/05 viruses. **A**: Anti-NIBRG14-specific IgA and IgG responses and H5N1 virus titer in the nasal washings. The mice were immunized twice intranasally with vaccine alone, or in combination with mycelia extracts of *Phellinus linteus* (PL), then challenged by intranasal administration of 1,000 PFU of influenza A/Vietnam/1194/04 or influenza A/Indonesia/6/05 virus 14 days after the final immunization. Nasal washings and serum samples were collected 3 days after the challenge. The concentrations of IgA and IgG antibodies and virus titers from five mice from each group were measured by ELISA and plaque assay using MDCK cells, respectively. Each column represents the mean values ± SE of five mice per group, and open circles indicate individual animals. The virus titers were statistically compared to those of non-immunized mice ($^*P < 0.05$). (**B**,**C**) The survival curves of mice immunized according to the same schedule as in Figure 2A after lethal influenza A/Vietnam/1194/04 (B) or influenza A/Indonesia/6/05 (C) virus challenge is depicted. The survival rates were monitored for 14 days. $^{**}P < 0.01$ versus control mice, log-rank test.

non-adjuvanted vaccine or non-immunized mice succumbed to disease by days 14 and 11, respectively (Fig. 3B). In the group challenged with heterologous A/Indonesia/6/2005 virus, mice immunized with *P. linteus*-adjuvanted vaccine showed a significant reduction in virus titer compared to the control mice (Fig. 3A) and survived longer than 14 days post-infection (Fig. 3C), while none of the mice immunized with non-adjuvanted vaccine or non-immunized mice survived more than 14 days post-infection (Fig. 3C). None of the surviving mice exhibited any clinical signs of infection, such as ruffled hair or emaciation, following the virus challenge. These results clearly indicate that intranasal administration of H5N1 vaccine in combination with *P. linteus* extract protects mice against highly pathogenic homologous and heterologous influenza A virus H5N1 infection.

Intranasal Immunization With the Hemagglutinin Vaccine With Mycelia Extract Induces a Weak Systemic T-Cell Response

To examine whether intranasal administration of influenza vaccine induces a T-cell response, levels of interferon-γ were measured in supernatants of T-cells from spleen and cervical lymph nodes of immunized mice in co-culture with antigen-presenting cells and PR8 vaccine (Fig. 4). Briefly, T-cells isolated from the spleen or cervical lymph nodes of mice 7 days after the final immunization were cultured with irradiated antigen-presenting cells in the presence or absence of A/PR8 vaccine at 0.1, 1.0 or 10 μg/ml. Low but significant levels of interferon-γ were detected in the splenic T-cells of mice immunized with *P. linteus*-adjuvanted vaccine (Fig. 4A). However, there was no significant effect on interferon-γ production in splenic T-cells of mice immunized by A/PR8 vaccine adjuvanted with *M. gracilenta*, *G. frondosa*, or *L. edode* mycelial extracts. Similarly, *P. linteus*-adjuvanted vaccine induced little T-cell response in cervical lymph nodes (Fig. 4B). These results suggest that immunization with a combination of vaccine and mycelia extract induces a relatively weak T-cell response.

MyD88 Participates in Mycelia Extract Stimulated Production of Proinflammatory Cytokines in Bone Marrow-Derived Dendritic Cells

Crude mycelia extract contains proteoglycans, hemicellulase, and β-glucans [Ukawa et al., 2000] and activates innate immune responses via CD14/TLR4 or dectin-1 (a β-glucan-specific C-type lectin receptor)-dependent pathways [Saijo et al., 2007; Taylor et al., 2007]. Because MyD88 is a general adaptor/regulator molecule for the Toll/IL-1R family of receptors [Medzhitov et al., 1998], cytokine production was measured in wild-type and MyD88-deficient bone marrow-derived dendritic cells following stimulation with mycelia extract. Bone marrow-derived dendritic

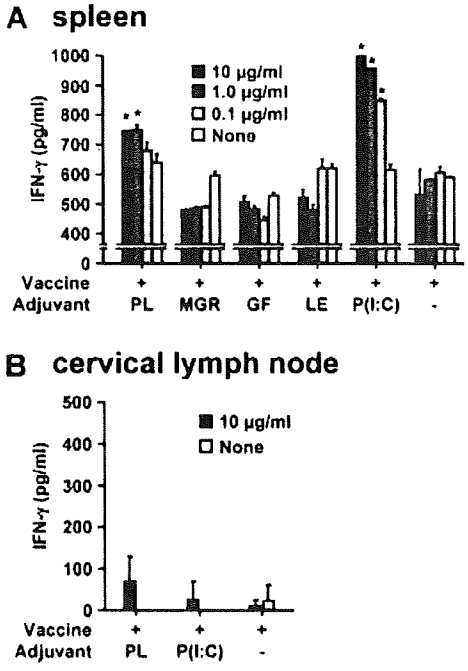

Fig. 4. In vitro responses of influenza A/PR8 virus-specific T-cells derived from mice immunized intranasally with hemagglutinin vaccine alone, or in combination with various mycelial extracts or poly(I:C). Spleens (**A**) and cervical lymph nodes (**B**) were isolated 1 week after the final immunization and re-stimulated with T-cell-depleted splenocytes that had been pulsed with the indicated concentration of A/PR8 hemagglutinin vaccine. Production of interferon-γ in the culture supernatant was measured by ELISA at 4 days after the antigen re-stimulation. These results are presented as the means of two independent experiments. $^*P < 0.05$ versus non-stimulated sample.

cells from wild-type or MyD88-deficient mice were stimulated with lipopolysaccharide, Zymosan, or mycelia extracts in vitro for 24 hr, and their ability to secrete TNF-α was examined. After stimulation with Zymosan, *P. linteus*, *M. gracilenta*, *G. frondosa*, or *L. edode* extracts, TNF-α production was partially but significantly reduced in MyD88-deficient dendritic cells in comparison to wild-type dendritic cells, and was drastically reduced after lipopolysaccharide stimulation in MyD88-deficient dendritic cells as compared to wild-type (Fig. 5). All mycelia extracts strongly induced TNF-α and IL-6 as much as stimulation with lipopolysaccharide or Zymosan in bone marrow-derived dendritic cells, but not IL-12 or p70. In addition, while lipopolysaccharide and Zymosan strongly enhanced CD40 expression in dendritic cells, treatment with mycelial extracts from *P. linteus*, *M. gracilenta*, *G. frondosa*, or *L. edode* only modestly enhanced CD40 expression in dendritic cells. These results indicate that proinflammatory cytokine production in bone marrow-derived dendritic cells in response to stimulation by mycelial extracts is partly dependent on MyD88 and that the adjuvant activity of mycelia extracts may be achieved through activation of dendritic cells.

DISCUSSION

The results of the present study clearly demonstrate that extracts of mycelia from edible mushrooms, especially *P. linteus*, are an effective mucosal adjuvant when administered intranasally with influenza vaccine. Nasal immunization induced not only an increase in mucosal secretory IgA, but also a high titer of anti-HA IgG in the serum. This immune reaction resulted

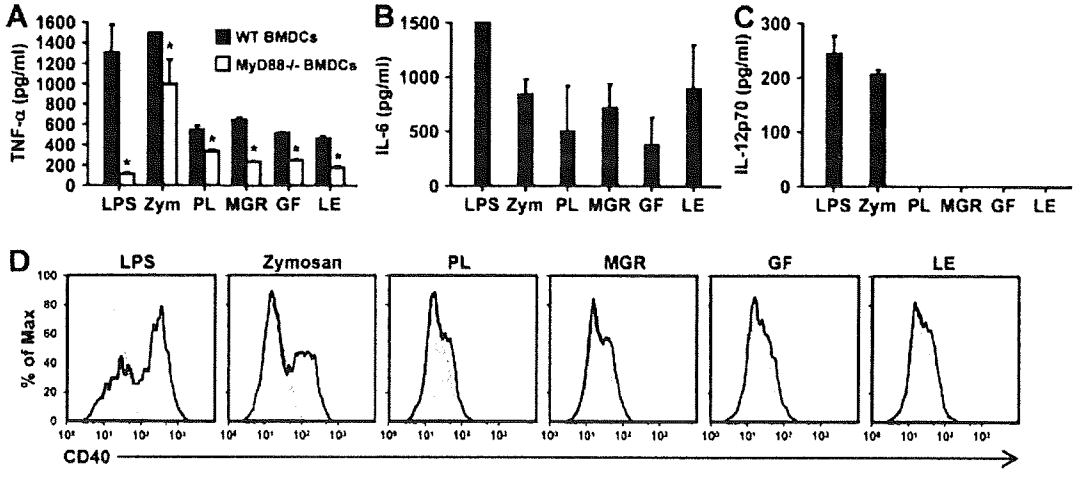

Fig. 5. Tumor necrosis factor (TNF)-α (**A**), IL-6 (**B**), and IL-12p70 production from bone marrow-derived dendritic cells (BMDCs). BMDCs (5×10^5 cells/well) from wild-type (filled columns) or MyD88-deficient mice (open columns) were stimulated with lipopolysaccharide (LPS), Zymosan (Zym.), *Phellinus linteus* (PL), *Macrolepiota gracilenta* (MGR), *Grifola frondosa* (GF), or *Lentinula edodes* (LE) for 24 hr as described in Materials and Methods Section. The culture supernatants were collected and the concentration of TNF-α (A), IL-6 (B) and IL-12p70 (**C**) were measured by ELISA. Data represent the means ± SD of duplicate samples. $^*P < 0.05$, wild-type versus MyD88-deficient dendritic cells. The expression of the co-stimulatory molecule CD40 was measured by flow cytometry after 24 h of stimulation (**D**). The gray histograms show the expressions on the unstimulated cells, and the bold lines show the expression of CD40.

in cross-protective immune responses against both homologous and heterologous influenza variants, including highly pathogenic H5N1 influenza virus isolates. Administration of the formalin-inactivated whole H5N1 vaccine (NIBRG14) or the PR8 HA vaccine combined with *P. linteus* extract following a two-dose immunization protocol was able to confer protection against infection with lethal influenza A/Vietnam/1194/2004 (H5N1) virus, influenza A/Indonesia/6/2005 (H5N1) virus, and lethal lung infection (40 LD_{50}) by PR8 influenza virus, respectively. These results indicate that *P. linteus* extract is an effective mucosal adjuvant when administered intranasally with influenza vaccine. The *P. linteus*-adjuvanted vaccines induced poor T-cell responses, indicating that cross-protection may be mediated primarily by the mucosal immune response, probably via the activity of secretory IgA antibodies against viral proteins. It has been shown that polymeric immunoglobulin receptor-knockout mice do not secrete IgA and exhibit less cross-protective efficacy against variant influenza virus infection [Asahi et al., 2002]. Although neutralizing activity against heterologous A/Vietnam/1194/2004 (H5N1) virus was not detected in the nasal washings, this was likely due to dilution with PBS when the nasal washings were collected. The concentration of vaccine-specific IgA in the nasal wash samples was much lower than the physiological concentration in the nasal mucosa, and therefore neutralizing activity in the nasal washings may not have been detectable.

Intranasal vaccination is advantageous for protection against influenza virus infection due to the induction of secretory IgA at the mucosal surface, which elicits a more effective cross-protective immunity compared to serum IgG. In fact, the cross-protective effects of *P. linteus*-adjuvanted vaccine were observed even against virulent heterologous H5N1 variants. Antigen-specific T-cell responses were weak in mice that had been immunized intranasally with vaccine and mycelia extracts from *P. linteus*, indicating that homologous and heterologous protection is primarily accomplished by secretory IgA at the mucosal surface.

Although mycelia extracts did not activate dendritic cells to the same extent as lipopolysaccharide or Zymosan, they induced high levels of cytokines such as TNF-α and IL-6 as much as stimulation with lipopolysaccharide or Zymosan. It has been shown that dectin-1 mediates the phagocytosis of β-glucan-bearing ligands, including yeast-derived particles such as Zymosan [Herre et al., 2004]. The phagocytosis of pathogens is a critical host defence mechanism, not only for clearance of the invading microorganism, but also for the generation of antigenic fragments for presentation to $CD4^+$ T-cells to induce a subsequent adaptive immune response [Dzionek et al., 2001]. Toll-like receptors 3, 7, 8, and 9 are localized to intracellular compartments and specialize in recognition of viral nucleic acids in the endosome [Iwasaki and Medzhitov, 2004]. In this regard, concomitant administration of mycelial extracts and a toll-like receptor agonist, such as synthetic double-stranded RNA poly(I:C), synthetic imidazoquinoline compounds, or oligodeoxynucleotides containing cytosine–guanine motifs may be more effective than either mycelial extracts or toll-like receptor agonists alone, by reason of accumulation of vaccine with a toll-like receptor agonist into endosomal compartments that express toll-like receptors 3, 7, 8, and 9. In fact, complexing toll-like receptor 3 or 9 agonists to cationic liposomes markedly potentiated their ability to activate immune responses [Zaks et al., 2006]. These synergistic effects may contribute to the enhancement of mucosal adjuvant effects, leading to complete protection against viral challenge.

A major objective of intranasal influenza vaccine development is the design of an adjuvant that can provide effective mucosal immune activity and at the same time is stable and safe for clinical application in humans. Although poly(I:C) is a potent mucosal adjuvant, it has been associated with some adverse events during clinical trials of intravenous administration. Poly(I:C) induced a number of side effects in humans, including renal failure and hypersensitivity, in a previous clinical trial using dosages as high as 75 mg of poly(I:C)/m^2 at day 0 followed by daily administration from day 7 to a maximum of 35 days [Robinson et al., 1976]. Although the crude mycelia extracts used in the present study are not as effective as poly(I:C) as an adjuvant, identification of the active ingredients has the potential to produce an adjuvant as effective as poly(I:C). The mycelial extracts are derived from edible mushrooms and are separated by boiling, indicating that the active ingredients in the adjuvant are thermotolerant, whereas poly(I:C) loses adjuvant activity after boiling at 95°C for 5 min [Ichinohe et al., 2005]. It has also been shown that oral administration of mushroom extracts decreases IgE levels by modulation of Th1/Th2 balance. Inagaki et al. [2005] reported that oral administration of *P. linteus* significantly inhibited the IgE-dependent mouse triphasic cutaneous reaction, and Lim et al. [2005] demonstrated that *P. linteus* given orally decreased IgE concentration in serum and murine mesenteric lymph node lymphocytes, and increased concanavalin A-induced interferon-γ secretion in mesenteric lymphocytes. These characteristics offer great advantages for clinical application.

For the clinical application, we need to evaluate the effective dose of vaccine in human trials. The effectiveness of intranasal vaccine with injection dose (15 μg) in humans could be roughly assessed by the ability of ~0.1 μg vaccine to induce an immune response to provide protection against infection in BALB/c mice immunized intranasally according to a two-dose regimen [Tamura et al., 2005]. These data suggest that the vaccine dose in BALB/c mice in the current study (1 μg of vaccine per mouse) corresponded to ~10 times the dose in subcutaneous injection in human. Identification of the active ingredients in the crude mycelial extracts could reduce the doses. Further investigation is necessary to identify the active ingredients to produce more potent mucosal adjuvant.

In summary, intranasal immunization with influenza vaccine and extract of mycelia from *P. linteus*-induced cross-protective mucosal immunity against heterologous H5N1 influenza viruses in mice. Intranasal immunization with influenza vaccine containing *P. linteus* extract may thus represent a strategy to generate protective mucosal immunity in humans against newly emerging and highly pathogenic avian influenza viruses. Because prophylactic agents, including vaccines, must ensure sufficient safety for clinical use, further studies are required to determine whether such a nasal vaccine would be effective for use in humans.

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Zymosan Enhances the Mucosal Adjuvant Activity of Poly(I:C) in a Nasal Influenza Vaccine

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The synthetic double-stranded RNA polyriboinocinic polyribocytidylic acid [poly(I:C)] is a potent mucosal adjuvant in mice immunized intranasally with an inactivated influenza vaccine. In an attempt, to increase the effectiveness of a nasal poly(I:C)-combined vaccine, the effect of zymosan, a cell wall extract from *Saccharomyces cervisiae* was investigated, on the adjuvant activity of poly(I:C) in BALB/c mice. The addition of zymosan (10 μg) as an adjuvant in mice which were immunized intranasally with a poly(I:C) (1–5 μg)-combined vaccine (1 μg) enhanced the ability of the mice to mount an effective immune response to a lethal dose of influenza virus, and resulted in a synergistic increase in secretory IgA and serum IgG antibody levels. To define the mechanism by which zymosan enhanced the adjuvant activity of poly(I:C), bone marrow-derived dendritic cells (BM-DCs) were cultured in the presence of poly(I:C) and/or zymosan. There was a synergistic increase in cytokine production (TNF-α, IL-6, IL-10, and IFN-β) in BM-DCs, together with an increase in the expression of co-stimulatory molecules (CD86 and CD40) in response to co-treatment with poly(I:C) and zymosan. This synergistic effect on cytokine production was mimicked by co-treatment with poly(I:C) and a Toll-like receptor 2 (TLR2) ligand, which represented one of the components of zymosan. The results of the current study suggest that one of the mechanisms by which zymosan enhances the adjuvant activity of poly(I:C) is through increased cytokine production by DCs involving the synergistic activation of poly(I:C)-induced TLR3- and zymosan-induced TLR2-mediated signaling pathways. ***J. Med. Virol. 82:476–484, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: influenza virus; intranasal vaccine; mucosal adjuvant; poly(I:C); zymosan

INTRODUCTION

Influenza is highly a contagious acute respiratory disease caused by infection with the influenza virus, which attacks the host's respiratory tract mucosa [Lamb and Krug, 2001; Wright and Webster, 2001]. The influenza virus can alter the antigenic properties of the viral surface hemagglutinin (HA) protein, which results in annual epidemics of influenza. To control these epidemics, the traditional approach is parenteral administration of an inactivated vaccine. Parenteral vaccines induce primarily HA-specific serum IgG antibodies, which are highly protective against homologous virus infection, but less effective against heterologous virus infection. By comparison, natural virus infection or intranasal administration of an adjuvant-combined vaccine induces secretory HA-specific IgA antibodies in the respiratory tract, which are protective not only against homologous virus infection but also against heterologous virus infection [Couch and Kasel, 1983; Liew et al., 1984; Underdown and Schiff, 1986; Murphy and Clements, 1989]. Thus, mucosal influenza vaccines are superior to parenteral vaccines in providing cross-protection against virus infection [Tamura et al., 1992a,b], and several investigators have reported attempts to develop a mucosal vaccine by intranasal administration of an inactivated vaccine together with various mucosal adjuvants [Hasegawa et al., 2005; Ichinohe et al., 2006; Kamijuku et al., 2008].

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It was demonstrated that poly(I:C), a synthetic double-stranded RNA, is a potent adjuvant when administered with a nasal influenza vaccine [Ichinohe et al., 2005]. Poly(I:C) binds to TLR3, one of the pattern recognition receptors (PRRs) that recognizes highly conserved microbial structures on host dendritic cells (DCs), macrophages and leukocytes. TLR3 ligands can activate the innate immune system through the up-regulation of cytokine production or the expression of co-stimulatory molecules by DCs, macrophages and leukocytes [Iwasaki and Medzhitov, 2004; Kawai and Akira, 2006], which is in turn essential for enhancing the acquired immune response.

TLR-independent PRRs, including NOD-like receptors, RIG-I like receptors, and C-type lectin receptors, have also been identified as activators of the innate immune response [O'Neill, 2008]. C-type lectins promote phagocytosis of nonopsonized microbes and induce the production of cytokines in DCs, macrophages and leukocytes [Robinson et al., 2006]. Dectin-1, a C-type lectin receptor, recognizes β-glucans, which are carbohydrate polymers found mainly on the cell walls of fungi [Brown, 2006], and plays a crucial role in protection against some fungal infections [Saijo et al., 2007; Taylor et al., 2007]. Dectin-1 signaling is mediated by spleen tyrosine kinase (Syk), and promotes NF-κB activation [Rogers et al., 2005; Gross et al., 2006], resulting in the production of interleukin 10 (IL-10) and IL-2 [Rogers et al., 2005]. Co-activation of Dectin-1/Syk and TLR2/MyD88 signaling pathways results in a synergistic increase in the production of IL-12 and tumor necrosis factor α (TNF-α) [Gantner et al., 2003]. This synergy is mediated by zymosan, a cell wall extract from *Saccharomyces cervisiae* that is composed of β-glucan (55%), mannan, protein, lipid, chitin [Di Carlo and Fiore, 1958], and an unknown TLR2 ligand [Gantner et al., 2003; Slack et al., 2007]. These results suggest that the combined effect of several ligands for different PRRs might be more effective as an adjuvant than a single ligand for one PRR.

In the current study, the effect of zymosan on the adjuvant activity of poly(I:C) in mice immunized intranasally with an inactivated influenza virus was investigated. Zymosan together with poly(I:C) enhanced the ability of immunized mice to mount a protective response to infection with a lethal dose of virus, and caused a synergistic increase in secretory IgA and serum IgG levels. Zymosan plus poly(I:C) also enhanced synergistically cytokine production by BM-DCs in culture, with an accompanying increase of the expression of co-stimulatory molecules. A TLR2 ligand, which represented one component of zymosan, was involved specifically in the synergistic enhancement of cytokine production in DCs by combined zymosan/poly(I:C) treatment. These results suggest that one of the mechanisms by which zymosan enhances the adjuvant activity of poly(I:C) is through a synergistic increase in cytokine production by DCs, involving the co-activation of poly(I:C)-stimulated TLR3-mediated signaling pathways and zymosan-stimulated TLR2-mediated signaling pathways.

MATERIALS AND METHODS

Mice

Six- to 8-week-old female BALB/c mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). All animal experiments were carried out in accordance with the Guide for Animal Experiments Performed at the National Institute of Infectious Diseases (NIID), and were approved by the Animal Care and Use Committee of NIID.

Influenza Viruses and HA Vaccines

Influenza virus strain A/Puerto Rico/8/34 (A/PR8; H1N1) was grown in the allantoic cavities of 10- to 11-day-old fertile chicken eggs. The HA vaccine (split-product virus vaccine), in which the HA was the major component of the vaccine (approximately 30% of total protein), was prepared from purified influenza virus, according to the method of Davenport et al. [1964] at the Kitasato Institute (Saitama, Japan).

Vaccination and Virus Challenge

Poly(I:C) was kindly provided by Toray Industries, Inc. (Kamakura, Kanagawa, Japan). Zymosan A from *S. cervisiae* was purchased from Sigma–Aldrich (St. Louis, MO). Mice were anesthetized with diethyl ether and immunized by dropping 5 μl of PBS(−) containing 1 μg of A/PR8 HA vaccine together with poly(I:C) (1, 5, or 10 μg) and/or zymosan (1, 10, 50, or 100 μg) into each nostril. Three weeks later, mice were re-immunized in the same manner. Two weeks after the second immunization, mice were anesthetized and subjected to virus challenge with a small volume or large volume dose of A/PR8 virus. For the small volume dose, mice were infected by dropping 2 μl of A/PR8 virus suspension [1,000 plaque-forming units (PFUs) in PBS] into each nostril, according to the modified procedure of Yetter et al. [1980]. This procedure caused an infection that was confined largely to the upper respiratory tract, but was not lethal (influenza model). For the large volume dose, mice were infected with a lethal dose of A/PR8 virus (1,000 PFU; $40LD_{50}$ in 20 μl), which resulted in death from pneumonia approximately 7 days after infection (viral pneumonia model). Three days after virus challenge, serum specimens were collected from the heart with a syringe. Nasal wash and bronchoalveolar wash specimens were obtained from immunized mice by washing the nasal cavity of the isolated upper head and the isolated lungs with 1 and 2 ml, respectively, of PBS(−) containing 0.1% bovine serum albumin and antibiotics [0.1% BSA PBS(−)] [Asahi et al., 2002]. Survival of the vaccinated mice was followed for 2 weeks after viral challenge.

Measurement of Virus Titer

Virus titer in nasal or bronchoalveolar wash specimens was measured according to the method of

Tobita et al. [1975]. Briefly, 200 μl aliquots of serial 10-fold dilutions of the nasal wash were inoculated into Madin-Darby canine kidney (MDCK) cells in a six-well plate. After allowing the plates to incubate for 1 hr, each well was overlaid with 2 ml of agar medium. The number of plaques was counted following crystal violet staining 2 days after inoculation.

ELISA and Measurement of Neutralizing and Hemagglutinin Inhibitory Antibody Titers

The levels of HA-specific IgA and IgG antibodies were determined by enzyme-linked immunosorbent assay (ELISA) using purified HA from A/PR8 virus, as described previously [Tamura et al., 1992a; Asahi et al., 2002]. Aliquots of nasal wash, bronchoalveolar wash or serum specimens were added to the wells of an EIA plate (Costar, Cambridge, MA) coated with purified HA. HA-specific antibodies were detected using biotin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or IgA (Kirkegaard & Perry Laboratories, Gaithersburg, MD) antibodies and alkaline phosphatase-conjugated streptavidin (Zymed, South San Francisco, CA). The detection reaction was initiated by the addition of *p*-nitrophenylphosphate in 10 mM diethanolamine (pH 9.8) containing 0.5 mM $MgCl_2$. Absorbance of 405 nm was measured using a Microplate Reader Model 680 (BIO-RAD, Hercules, CA). Purified HA-specific monoclonal IgG or polyclonal IgA were used as standards.

Virus neutralization was carried out as described previously [Ichinohe et al., 2005]. Briefly, A/PR8 virus was mixed with an equal volume of serial twofold dilutions (from 1:32) of antisera. The mixtures were incubated for 1 hr at 37°C and then plated in duplicate on MDCK cells, as described for the plaque assay. Neutralization titer was defined as the reciprocal of the end-point dilution that reduced by more than 50% the number of plaques relative to that of sera from naïve mice.

The hemagglutination inhibition (HI) assay was performed according to the method of Sever [1962].

Preparation of BM-DCs

Conventional BM-DCs were generated as described by Lutz et al. [1999]. Briefly, the femur and tibia of mice aged 6–12 weeks were removed and the bone marrow cells were collected in PRMI1640 medium supplemented with 10% Fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), HEPES (25 mM), and 2-ME (50 μM). Bone marrow cells were seeded in RPMI1640 medium containing 10 ng/ml of recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF; Wako, Tokyo, Japan). Fresh medium containing GM-CSF was added 3 days after plating, and then one half of the culture supernatant was exchanged with fresh medium on days 6, 8, and 10. BM-DCs collected on day 10 or 11 were used for the experiments.

BM-DC Stimulation In Vitro and Measurement of Cytokine Production

BM-DCs (5×10^5 cells per well) were plated on non-treated 24-well plates in the presence of zymosan, poly(I:C), or both. Cells were cultured for 24 hr, after which culture supernatants were collected and assayed for the presence of cytokines. Cytokine concentration was measured by using ELISA kits for TNF-α, IL-12p70, IL-6, IL-10 (eBioscience, San Diego, CA) and IFN-β (PBL Biomedical Laboratories, New Brunswick, NJ), according to the manufacturer's instructions. The TLR2 ligand Pam_3CSK_4 (InvivoGen, San Diego, CA), β-glucan from *S. cervisiae* (Dectin-1 ligand, Sigma–Aldrich), and CTB* [cholera toxin B subunit (Sigma–Aldrich) supplemented with 0.2% cholera toxin (Sigma–Aldrich)] were used as cell stimuli. For all stimulation assays, 1 μg/ml of lipopolysaccharide (LPS; Sigma–Aldrich) was used as a control. Cells were collected in PBS containing 0.5% FCS, 5 mM EDTA, and 0.1% sodium azide (EFP buffer) for analysis by flow cytometry.

Flow-Cytometry

To examine BM-DC maturation, cells were washed in EFP buffer, and then stained with FITC-conjugated anti-CD86 or anti-CD40 antibody, plus PE-conjugated anti-CD11c antibody in the presence of anti-FcγRII/III antibody. Propidium iodide (PI; Sigma–Aldrich) was added to each sample before analysis. The mean fluorescence intensity (MFI) derived from CD86 and CD40 within the population of PI-negative/CD11c-positive cells was estimated. The anti-CD11c antibody (clone N418) was purchased from eBioscience. Anti-CD86 (clone GL1), anti-CD40 (clone 3/23), anti-FcγRII/III (clone 2.4G2), and isotype control antibodies were from BD Bioscience (San Jose, CA). Data was acquired using a FACSCalibur system (BD Bioscience) and analyzed using CellQuest (BD Bioscience) or FlowJo software (Treestar, Ashland, OR).

Statistical Analysis

Differences between paired groups were determined using the Student's *t*-test. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Antibody Response and Protection Against Viral Infection in Mice Immunized by Intranasal Administration of an HA Vaccine Together With Poly(I:C) and/or Zymosan

The effect of zymosan on the adjuvant activity of poly(I:C) in a nasal influenza vaccine was examined in BALB/c mice. Mice were immunized twice intranasally with 1 μg of an A/PR8 HA vaccine together with poly(I:C) or zymosan alone, or a combination of poly(I:C) and zymosan. Two weeks after the second immunization, the mice were infected with a small or large volume dose of A/PR8 virus. Three days after virus challenge, serum

and nasal wash specimens were assayed for A/PR8 HA vaccine-specific antibody responses, and nasal and bronchoalveolar wash specimens were assayed for virus titer.

The antibody response of mice immunized in the presence of poly(I:C) or zymosan alone, or a combination of poly(I:C) and zymosan was determined. Figure 1 shows A/PR8 HA-specific IgA levels in nasal wash specimens, and A/PR8 HA-specific IgG levels, neutralizing antibody and HI antibody titers in serum from immunized mice. Immunization in the presence of poly(I:C) (5 or 10 μg) alone induced IgA and IgG antibodies, as well as neutralizing antibody and HI antibody responses, and the response to 5 μg of poly(I:C) was weaker than to 10 μg of poly(I:C). In the presence of zymosan (1, 10, 50, or 100 μg) alone, there were relatively low levels of IgA and IgG antibodies, and weak neutralizing antibody and HI antibody responses, which increased with increasing doses of zymosan. The antibody response to 100 μg of zymosan was similar to that of 5 μg of poly(I:C). The presence of both poly(I:C) (1 or 5 μg) and zymosan (10 μg) induced a synergistic increase in IgA and IgG levels, and neutralizing antibody responses, and the effect increased with increasing doses of poly(I:C). These results demonstrated that the combination of poly(I:C) and zymosan as an adjuvant induces a synergistic increase in nasal wash IgA and serum IgG levels, as well as serum neutralizing responses, which are functional indicators of protection against infection.

The ability of immunized mice to mount a protective response to viral infection was investigated next. Figure 2 shows viral titers in the nasal and bronchoalveolar wash specimens from immunized mice. Immunization in the presence of 5 or 10 μg of poly(I:C) alone induced partial or complete protection, respectively, in the nasal cavity (Fig. 2A), and weak or strong protection, respectively in the lungs, although protection in the lung was partial (Fig. 2B). In the presence of 10 or 100 μg of zymosan alone, there was partial or complete protection, respectively in the nasal cavity (Fig. 2A) and weak or strong protection, respectively, in the lung, despite the partial protective effect (Fig. 2B). The combination of poly(I:C) (1 or 5 μg) and 10 μg of zymosan induced complete protection in both the nasal cavity and lung (Fig. 2A,B). The ability of immunized mice to mount a protective response to virus infection correlated well with their antibody responses, which increased with increasing doses of poly(I:C) and/or zymosan (Fig. 1). When changes in body-weight and the survival rates of immunized mice after challenge with a lethal dose of virus were examined (Fig. 3A,B), mice which were immunized with vaccine in the presence of 5 μg of poly(I:C) alone had a survival rate of 80% 14 days after challenge and a slight loss of body weight, whereas mice which were immunized in the presence of 10 μg of zymosan alone had a survival rate of 20% and exhibited a significant loss in body weight. All mice which were immunized in the presence of 1 or 5 μg of poly(I:C) plus 10 μg of zymosan survived more than 14 days with no loss in body weight. These results demonstrated that the combination of poly(I:C) and zymosan as an adjuvant enhances the ability of immunized mice to mount a protective antibody response to virus infection,

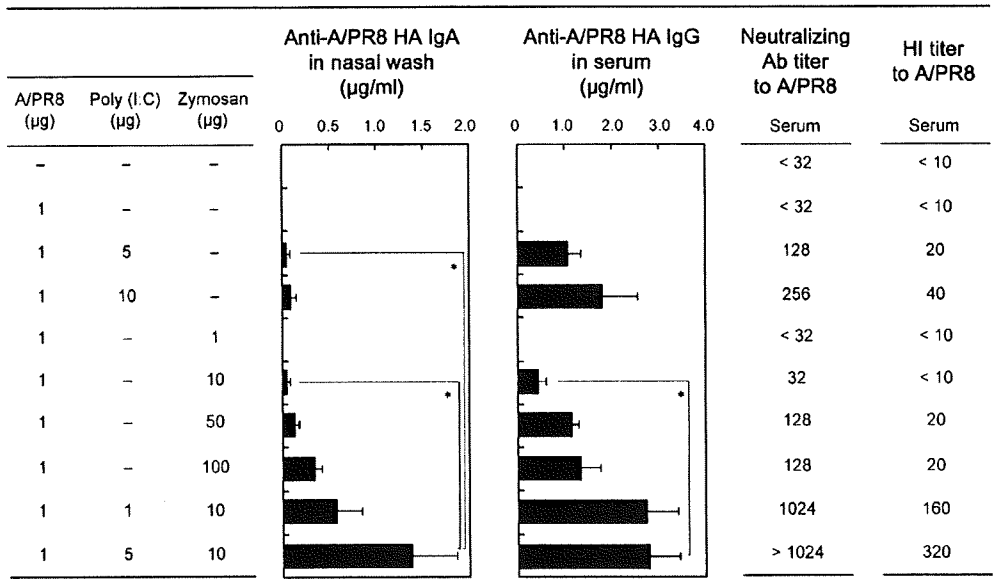

Fig. 1. A/PR8 HA-specific Ab responses in mice immunized intranasally with an A/PR8 HA vaccine in the presence of poly(I:C) and/or zymosan. BALB/c mice were immunized intranasally with an A/PR8 HA vaccine (1 μg) in the presence of poly(I:C) (1, 5, or 10 μg) or zymosan (1, 10, 50, or 100 μg) alone, or with both, on days 0 and 21. Two weeks after the second immunization, mice were subjected to challenge by intranasal infection with a small volume (1,000 PFU, 2 μl into each nostril) or a large volume (1,000 PFU, 20 μl into a nostril) of A/PR8 virus. Nasal wash, lung wash and serum specimens were collected 3 days after virus challenge. A/PR8 HA-specific antibodies, neutralizing antibodies and HI titers were measured in nasal wash and serum specimens. Bars represent means ± standard error (SE). $^*P < 0.05$.

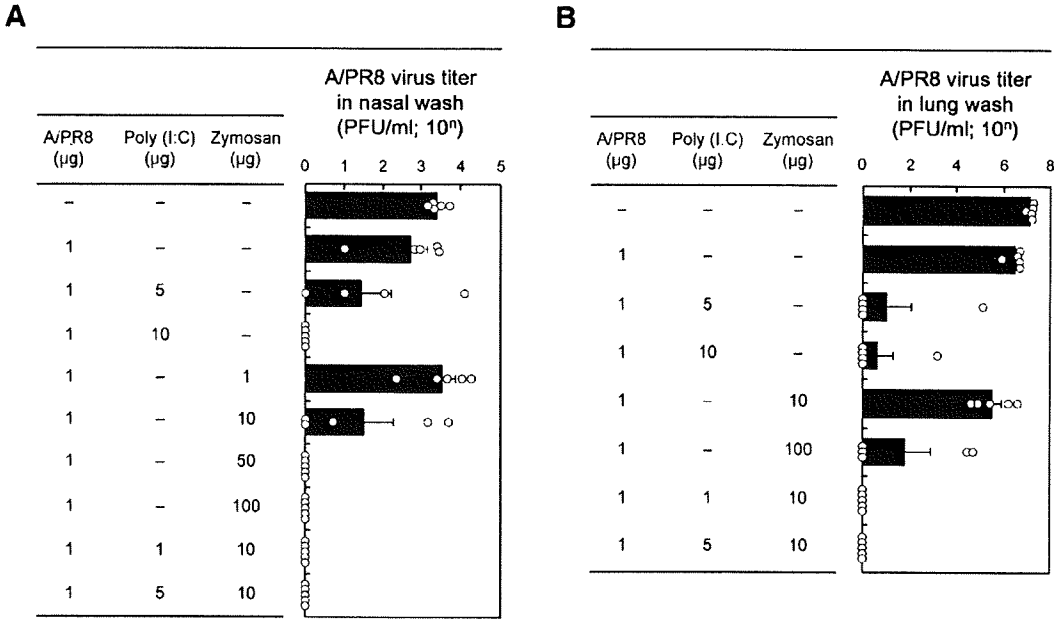

Fig. 2. Protection against influenza virus infection in mice immunized intranasally with an A/PR8 HA vaccine in the presence of poly(I:C) and/or zymosan. Mice were immunized as described for Figure 1, and then infected intranasally with a small volume of A/PR8 virus (1,000 PFU) (**A**), which induces non-lethal influenza, or a large volume (**B**), which induces lethal pneumonia. Nasal (A) or bronchoalveolar (B) wash specimens were obtained 3 days after infection. As an index of protection against infection, viral titers in the specimens were determined by plaque assay. Bars represent means ± SE. White circles indicate values for individual mice. $^*P < 0.05$.

paralleling the induction of synergistic antibody responses to the vaccine.

Synergistic Enhancement of Cytokine Production in Cultured BM-DCs by Co-Treatment With Poly(I:C) and Zymosan

The effect of co-stimulation with poly(I:C) and zymosan on the production of cytokines in conventional BM-DCs in culture was investigated. The levels of various cytokines in the supernatants of cultured BM-DCs (5×10^5 cells/ml) in the presence of various stimuli were measured by ELISA. Figure 4A shows the levels of TNF-α, IL-6, and IL-10 produced by BM-DCs in response to poly(I:C) and/or zymosan. In the presence of 10 μg of poly(I:C) or 1 μg of zymosan alone, cells produced 0.35 and 0.47 ng/ml of TNF-α, respectively, whereas co-treatment resulted in a synergistic increase in the production of TNF-α (1.67 ng/ml). The production of IL-6 and IL-10 was also increased in a synergistic manner by co-treatment with 1 μg of zymosan plus 10 μg of poly(I:C), to levels that were three- and twofold higher than in the presence of 10 μg of poly(I:C) alone, respectively. A synergistic increase in TNF-α and IL-6 was also observed when cells were co-treated with 1 μg of poly(I:C) and 1 μg of zymosan. Similar results were obtained in the presence of A/PR8 HA-vaccine (Fig. 4B). The highest levels of TNF-α, IL-6 and IL-10 were achieved when cells were treated with 10 μg of poly(I:C) and 1 μg of zymosan. As a negative control, there was no induction of cytokine production in response to HA vaccine alone. As shown in Figure 4C, IFN-β production in the presence of poly(I:C) plus zymosan was 5- and 12-fold higher than in the presence of 10 μg of poly(I:C) or 1 μg of zymosan alone, respectively (Fig. 4C). These results suggested that the production of type-1 interferon (IFN-β) and pro-inflammatory cytokines (TNF-α, IL-6, or IL-10) by BM-DCs is enhanced synergistically by co-treatment with poly(I:C) and zymosan. Treatment of cells with CTB* failed to invoke an increase in cytokine production under the current experimental conditions, even though both CTB* and poly(I:C) can function as potent mucosal adjuvants and induce protective immune responses against influenza in BALB/c mice [Tamura et al., 1994; Ichinohe et al., 2005].

The expression of CD86 and CD40 on the surface of BM-DCs following 24 hr treatment with poly(I:C) and/or zymosan in the absence of the HA vaccine was examined by flow cytometry (Fig. 4D). The expression of CD86 was up-regulated by (in increasing order of the magnitude of their effect) zymosan alone, poly(I:C) alone and poly(I:C) plus zymosan. Similarly, the expression of CD40 was also up-regulated, in increasing order of their effect, by zymosan alone, poly(I:C) alone and poly(I:C) plus zymosan. Thus, although either poly(I:C) or zymosan was able to promote the maturation of BM-DCs, the combination of poly(I:C) and zymosan appeared to promote maturation more rapidly than either poly(I:C) or zymosan alone. These results suggest that the combination of poly(I:C) and zymosan activates