Development of mucosal adjuvants for intranasal vaccine for H5N1 influenza viruses

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

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

Influenza virus and its infection signal

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

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

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

Innate immunity and adjuvant effect

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

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

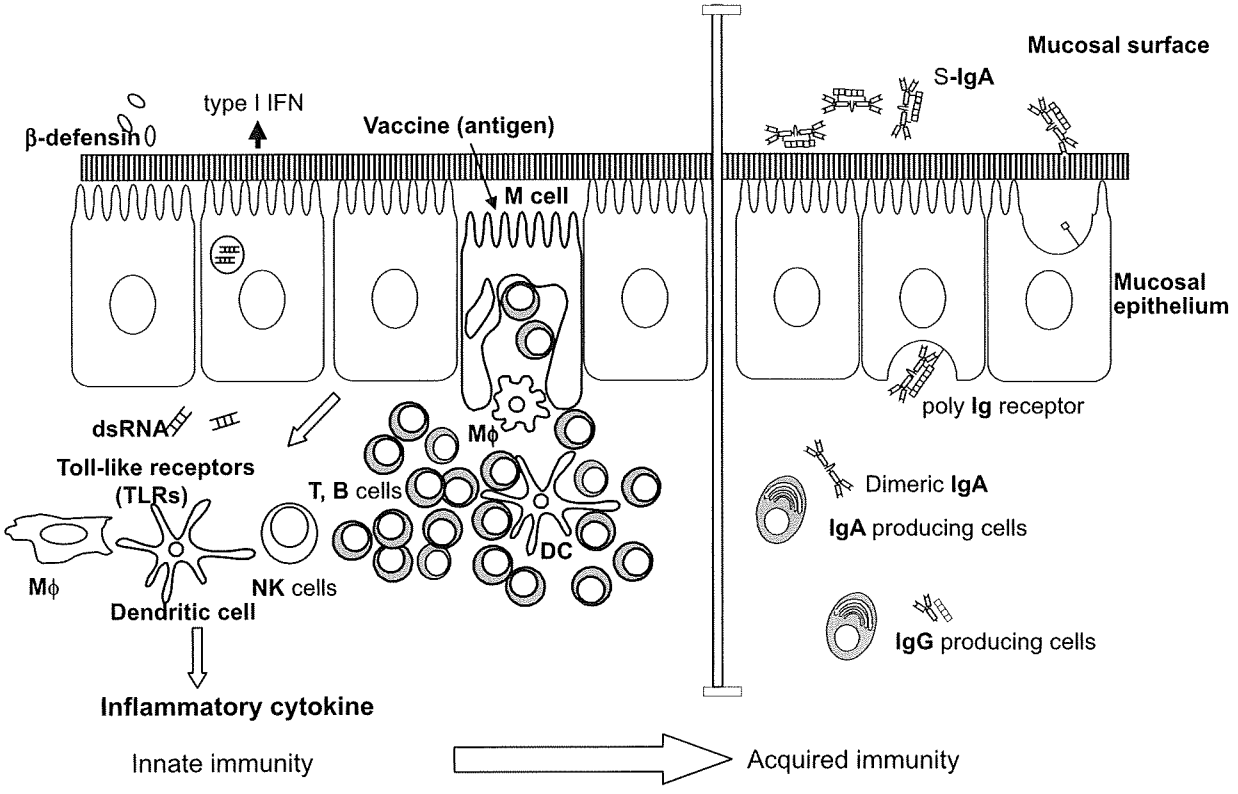

Figure 1 Defence mechanism at mucosal site, innate and adaptive immunity.

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

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

Mucosal vaccine

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

Highly pathogenic avian influenza virus H5N1

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

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

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

Development of adjuvant-combined inactivated nasal vaccines

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

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

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

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

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

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

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

Cross-protection by other vaccines

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

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

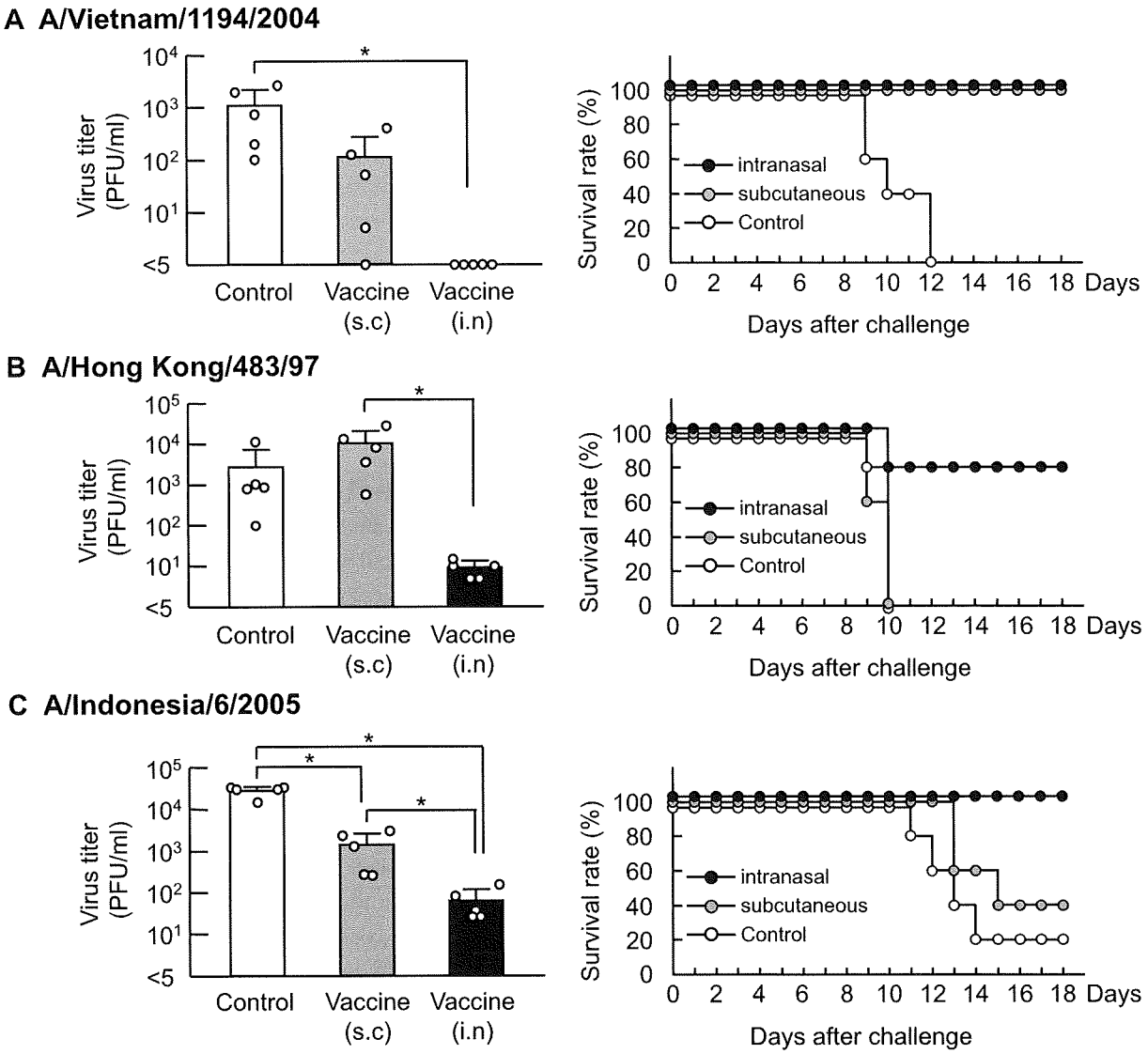

Figure 2 H5N1 virus titers in nasal washes and survival rates after lethal challenge with homologous A/Vietnam, heterologous A/Hong Kong, or heterologous A/Indonesia viruses. Mice were immunized intranasally (solid bar) or subcutaneously (gray bar) with vaccine and Ampligen®, then challenged by intranasal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

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

Conclusion

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

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

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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,

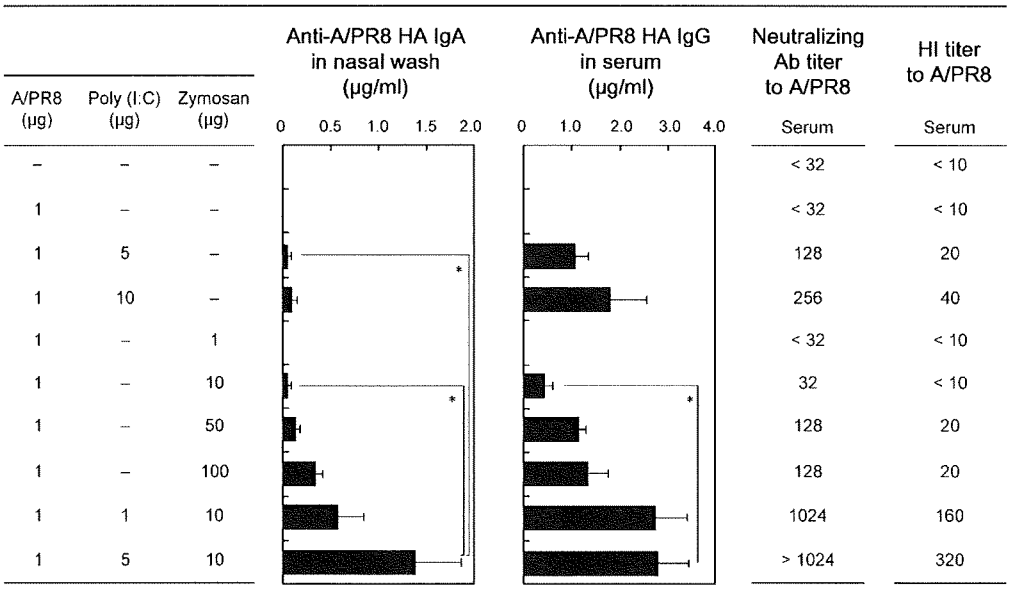

Fig. 1. A/PR8 HA-specific Ab responses in mice immunized intranasally with an A/PR8 HA vaccine in the presence of poly(I:C) and/or zymosan. BALB/c mice were immunized intranasally with an A/PR8 HA vaccine (1 μg) in the presence of poly(I:C) (1, 5, or 10 μg) or zymosan (1, 10, 50, or 100 μg) alone, or 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). *$P < 0.05$.

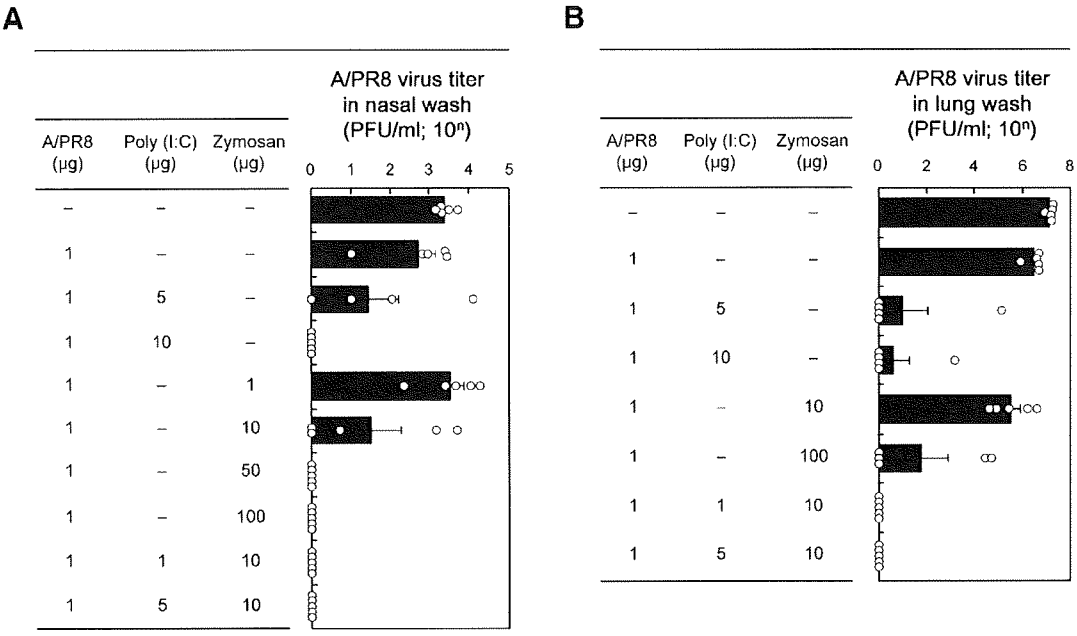

Fig. 2. Protection against influenza virus infection in mice immunized intranasally with an A/PR8 HA vaccine in the presence of poly(I:C) and/or zymosan. Mice were immunized as described for Figure 1, and then infected intranasally with a small volume of A/PR8 virus (1,000 PFU) (**A**), which induces 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

BM-DCs more rapidly than either agent alone, and synergistically enhances cytokine production.

Requirement of TLR2 Ligand for Enhanced Cytokine Production by BM-DCs in Response to Poly(I:C) and Zymosan

Lastly, the mechanism was examined by which zymosan enhanced the production of cytokines in combination with poly(I:C). Zymosan is composed of 55% β-glucan, which is a ligand of Dectin-1, and an unknown ligand of TLR2 [Di Carlo and Fiore, 1958; Gantner et al., 2003; Slack et al., 2007]. To determine which of these components of zymosan contributed to the synergistic effect on cytokine production in BM-DCs, cells were treated with Pam_3CSK_4 (0.1 or 1 μg), which is a TLR2-specific ligand, or β-glucan (0.1, 1, or 10 μg), either alone or in the presence of 5 μg poly(I:C). As shown in Figure 5, TNF-α production in the presence of 1 μg of Pam_3CSK_4 and 5 μg of poly(I:C) was 100- and 3-fold higher than in the presence of Pam_3CSK_4 and poly(I:C) alone, respectively. Similarly, the production of IL-6, IL-12p70 and IL-10 in response to 1 μg of Pam_3CSK_4 was increased significantly by co-treatment with 5 μg of poly(I:C). By comparison, when BM-DCs were cultured in the presence of β-glucan, there were no significant differences in cytokine production in the presence or absence of poly(I:C). Furthermore, there were no significant differences in the expression of CD86 and CD40 in cells cultured with β-glucan in the presence or absence of poly(I:C) (data not shown). These results suggest that the ability of zymosan to enhance the production of cytokines in BM-DCs in the presence of poly(I:C) is due to the TLR2 ligand component of zymosan.

DISCUSSION

The development of an effective mucosal influenza vaccine is very important if nasal vaccines are to be deployed instead of parenteral vaccines. The development of an effective inactivated mucosal vaccine requires that a suitable mucosal adjuvant be used with the vaccine. Previously, it was shown that the synthetic double-stranded RNA poly(I:C) is an effective adjuvant when administered with an inactivated nasal influenza vaccine [Ichinohe et al., 2005]. In the current study, in an attempt to enhance further the effectiveness of the nasal influenza vaccine, the effect of zymosan was investigated on the adjuvant activity of poly(I:C) in mice immunized intranasally with an inactivated influenza vaccine (1 μg). Zymosan (10 μg) plus poly(I:C) (1–5 μg) increased synergistically the levels of nasal IgA and serum IgG antibodies in immunized mice (Fig. 1). This synergistic increase in antibody production resulted in an enhanced ability of the immunized mice to mount a protective response to influenza virus challenge and protected mice from viral pneumonia (Fig. 2), with a corresponding increase in survival rate without body weight loss (Fig. 3). These results suggest that zymosan is an effective adjuvant for enhancing the effectiveness of the poly(I:C)-combined nasal influenza vaccine.

The mechanism by which zymosan enhanced the adjuvant activity of poly(I:C) was investigated using an in vitro BM-DC culture system. Zymosan is a cell wall extract from *S. cervisiae* that contains 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]. Zymosan has been used for over 50 years as a model microorganism to investigate phagocytosis and the inflammatory response both in vivo and in vitro [Di Carlo and Fiore, 1958]. β-glucan, one of components of zymosan, is recognized by Dectin-1, a C-type lectin that mediates phagocytosis of microbial agents by DCs or macrophages [Herre et al., 2004; Brown, 2006; Robinson et al., 2006]. In preliminary experiments, it was confirmed that zymosan facilitates the uptake of FITC-labeled HA vaccines prepared from A/Yamagata virus (H1N1) by BM-DCs (unpublished

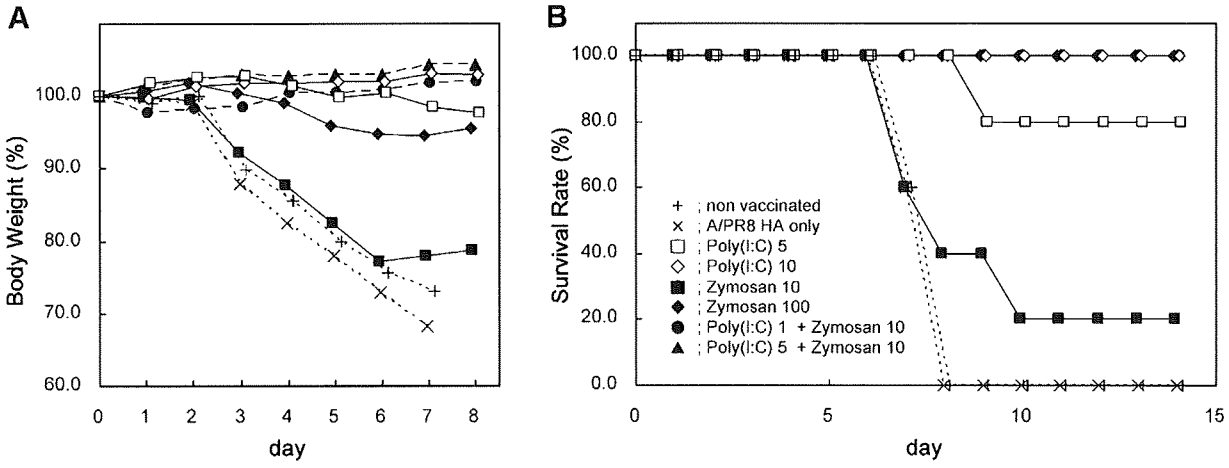

Fig. 3. Survival and body weight of mice immunized and infected with influenza virus. Mice were immunized as described for Figure 1 and then infected with a large volume of A/PR8 virus. Changes in body weight (**A**) and survival rate (**B**) were monitored for 8 and 14 days after infection, respectively. Body weight is represented as relative to the initial mean body weight of five mice.

data). Thus, β-glucan appears to be involved in the uptake of HA vaccine by BM-DCs in the presence of zymosan.

When microbial pathogens or structures, such as zymosan, are phagocytosed, surface TLRs, including TLR4 and TLR2, are recruited to the phagosome [Blander and Medzhitov, 2006]. TLR-mediated intracellular signaling pathways are divided into two main categories, MyD88-dependent and TRIF-dependent signaling pathways, depending on the type of adaptor molecule that is engaged [Takeda and Akira, 2005]. Simultaneous or sequential stimulation of MyD88-dependent and TRIF-dependent signaling pathways by their respective ligands induces a synergistic increase in the production of TNF-α, IL-6, and IFN-β; although tolerance is induced by agonists that act through the same pathway [Bagchi et al., 2007]. A similar result was noted in the current study, in that poly(I:C), a ligand of TLR3/TRIF, when combined with Pam_3CSK_4 which is a TLR2 ligand and activator of TLR2/MyD88 signaling, synergistically enhanced the production of various cytokines (Fig. 5). On the other hand, poly(I:C) in combination with β-glucan, also a constituent of zymosan and an activator of Dectin-1/Syk signaling, had no such effect on cytokine production (Fig. 5). These results suggest that simultaneous stimulation of MyD88-dependent and TRIF-dependent signaling pathways by their respective ligands induces a synergistic increase in cytokine production.

The mechanism by which an inactivated influenza vaccine in the presence of poly(I:C) and zymosan is recognized by DCs and induces an elevated antibody response likely involves many steps. The vaccine is taken up by DCs by phagocytosis and provides a potential source of peptides that can bind to major histocompatibility complex (MHC) class II molecules on the surface of the DC [Blander and Medzhitov, 2006]. Antigenic recognition of the complex of peptide/MHC class II on the surface of DCs by $CD4^+$ T cells induces antibody production. The β-glucan component of zymosan, which is recognized by Dectin-1, might facilitate the phagocytosis of zymosan [Robinson et al., 2006] together with the vaccine, whereas poly(I:C) can be endocytosed by DCs and bind to intracellular TLR3, perhaps within the endosome [Iwasaki and Medzhitov, 2004; Kawai and Akira, 2006]. Activation of two different signaling pathways, TLR3/TRIF by poly(I:C) and TLR2/MyD88 by zymosan, results in the synergistic increase in cytokine production by DCs. Alternatively, the synergistic effect of the two adjuvants might be accomplished within the same endosomal compartment, as poly(I:C) could be phagocytosed simultaneously with zymosan, and phagosomes could fuse with endosomes containing TLR3 during the process of phagosome maturation. In the current study, the levels of cytokines involved in the activation of the innate immune response (IFN-β, IL-6, and TNF-α) as well as the acquired immune response (IL-6, IL-10, and IL-12) were increased synergistically in the presence of poly(I:C) and zymosan (Figs. 4 and 5). These results suggest a possible mechanism by which zymosan enhances the activity of poly(I:C) as an adjuvant of the influenza HA vaccine: (1) stimulation

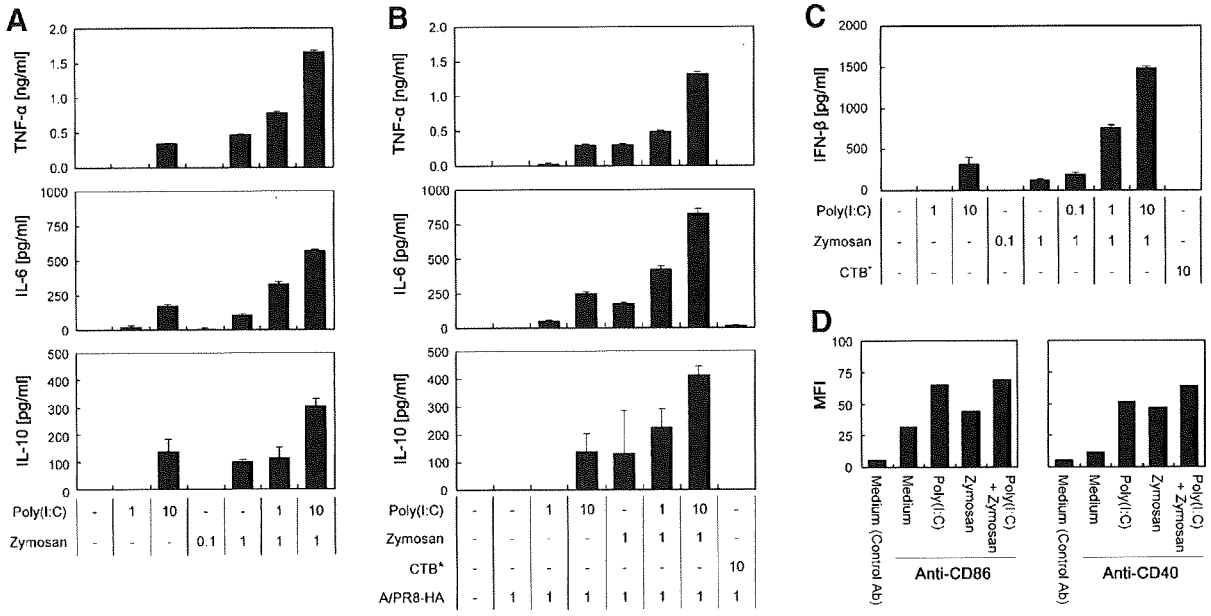

Fig. 4. Cytokine production by BM-DCs and maturation of BM-DCs in the presence of poly(I:C) and/or zymosan. BM-DCs (5×10^5 cells/well) were cultured for 24 hr in the presence of poly(I:C) (0.1, 1, or 10 μg) or zymosan (0.1 or 1 μg) alone, or with both, in the absence (A and C) or presence (B) of an A/PR8 HA vaccine (1 μg). The production of TNF-α, IL-12p70, and IL-10 (A and B) was measured by sandwich ELISA. IFN-β (C) was also quantified by ELISA. ELISAs were performed in triplicate for each sample. Each data is representative of two independent experiments, and represent the means ± standard deviation (SD). (D) BM-DCs (5×10^5 cells/well) were cultured with poly(I:C) (10 μg) or zymosan (1 μg) alone, or with both, and then the expression of the co-stimulatory molecules CD86 and CD40 was analyzed flow cytometry. Data represents the mean fluorescence intensity (MFI) for each molecule within the population of CD11c-positive BM-DCs.

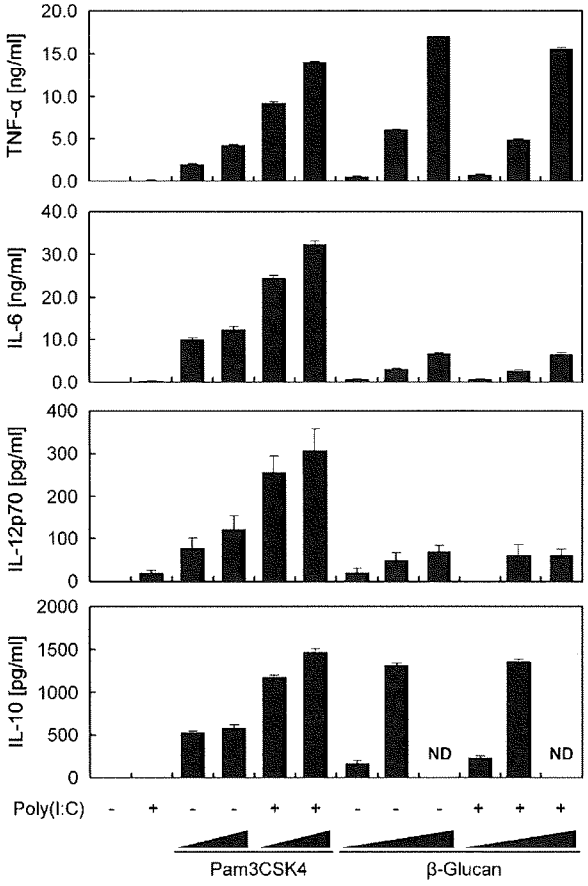

Fig. 5. Cytokine production by BM-DCs in the presence of poly(I:C) and either Pam3CSK4 or β-glucan. BM-DCs (5×10^5 cells/well) were cultured for 24 hr in the presence of poly(I:C) (5 μg), Pam_3CSK_4 (0.1 or 1 μg), or β-glucan (0.1, 1, or 10 μg) alone, or the indicated combinations of the three molecules. The production of TNF-α, IL-6, IL-12p70, and IL-10 in culture supernatants was determined by ELISA. Data represent the means ± SD of two or three independent experiments. ND; not done.

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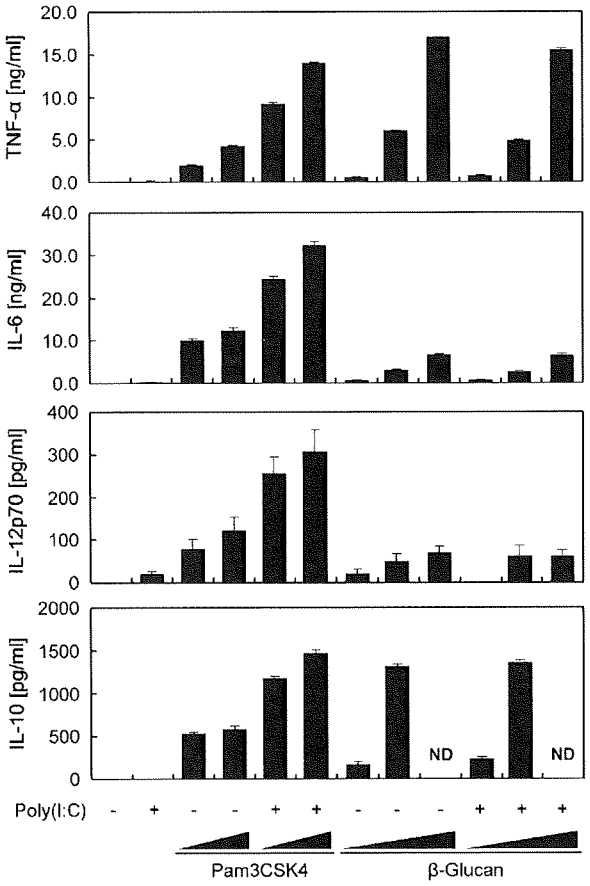

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Induction of Cross-Protective Immunity Against Influenza A Virus H5N1 by an Intranasal Vaccine With Extracts of Mushroom Mycelia

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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; hetero-subtypic 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