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

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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-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

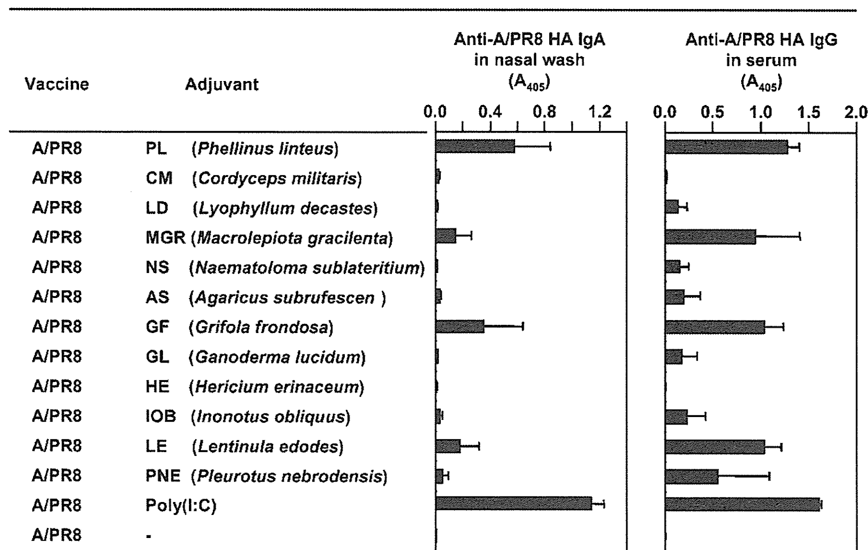

Fig. 1. Anti-A/PR8 hemagglutinin-specific IgA and IgG responses in BALB/c mice immunized intranasally with hemagglutinin vaccine alone, or in combination with various mycelia extracts or poly(I:C). Nasal washings and serum samples were collected 14 days after the final immunization. Antibody titers were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

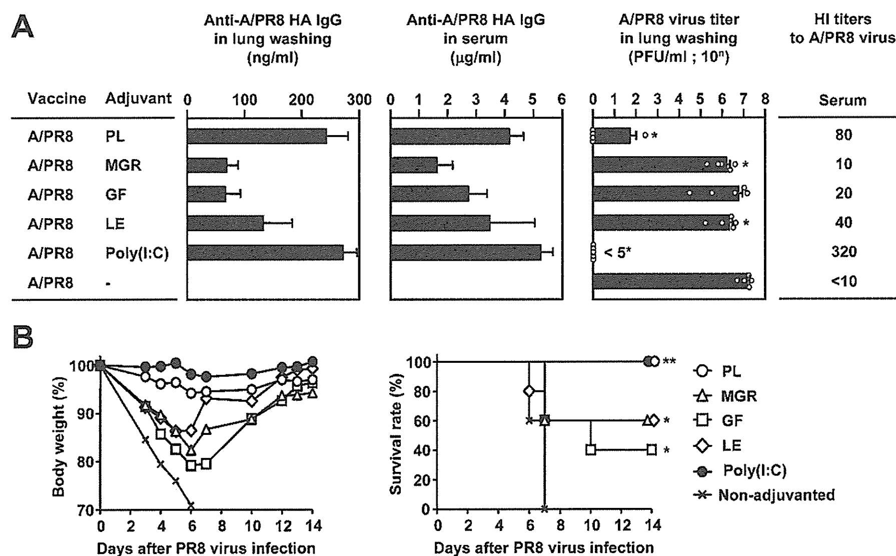

Fig. 2. **A**: Anti-A/PR8 hemagglutinin-specific IgG antibodies in lung washings and serum, and A/PR8 virus titer in lung washings. The mice were immunized twice intranasally with 1 µg of hemagglutinin vaccine alone, or in combination with extracts of mycelia from *P. linteus* (PL), *Macrolepiota gracilenta* (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.

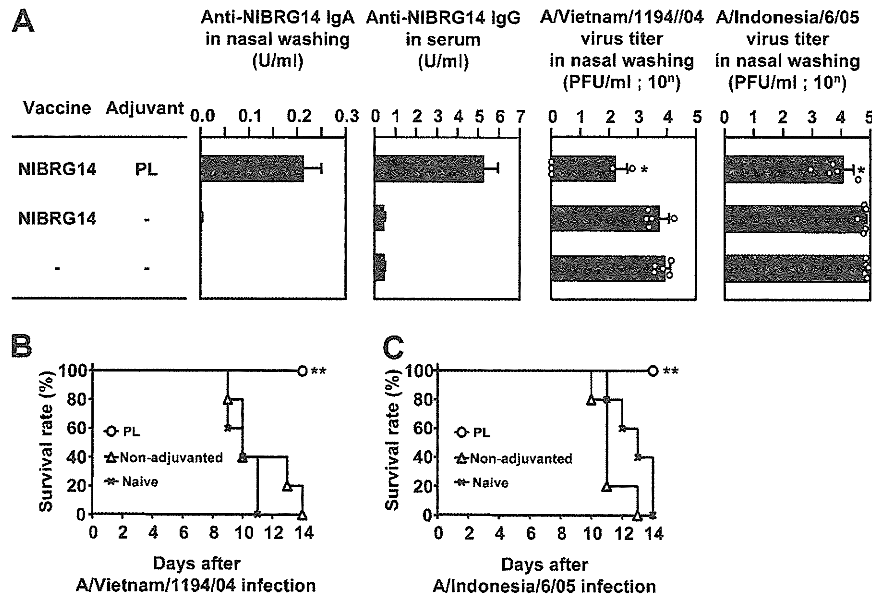

Fig. 3. Anti-NIBRG14-specific IgA and IgG responses, and H5N1 virus titers in nasal washings and survival rates after lethal challenge with homologous influenza A/Vietnam/1194/04 and heterologous influenza A/Indonesia/6/05 viruses. **A**: Anti-NIBRG14-specific IgA and IgG responses and H5N1 virus titer 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

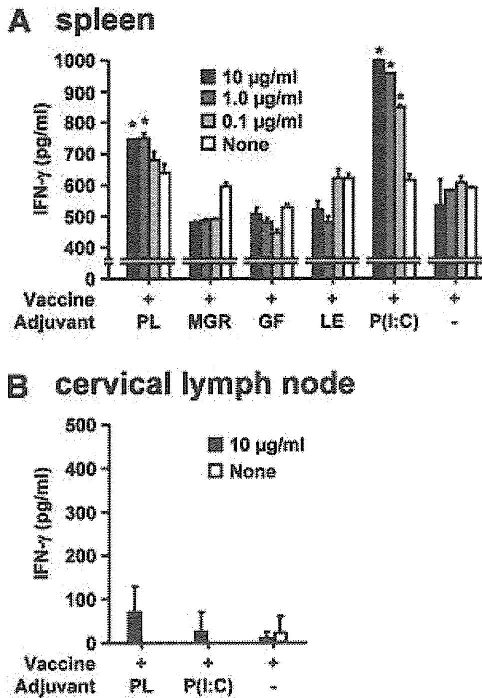

Fig. 4. In vitro responses of influenza A/PR8 virus-specific T-cells derived from mice immunized intranasally with hemagglutinin vaccine alone, or in combination with various mycelial extracts or poly(I:C). Spleens (**A**) and cervical lymph nodes (**B**) were isolated 1 week after the final immunization 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

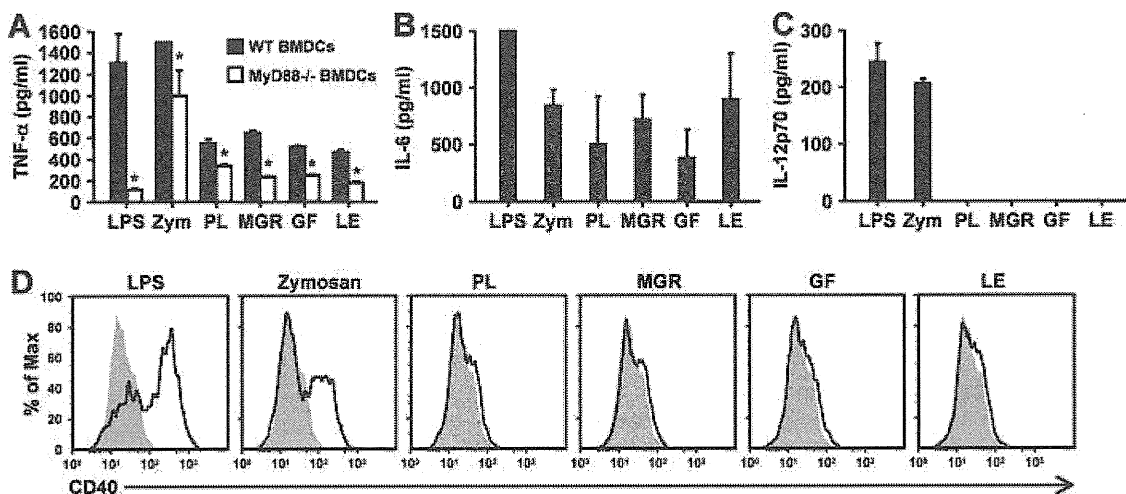

Fig. 5. Tumor necrosis factor (TNF)-α (**A**), IL-6 (**B**), and IL-12p70 production from bone marrow-derived dendritic cells (BMDCs). BMDCs (5×10^5 cells/well) from wild-type (filled columns) or MyD88-deficient mice (open columns) were stimulated with lipopolysaccharide (LPS), Zymosan (Zym.), *Phellinus 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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REFERENCES

Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity 9:143–150.

Asahi Y, Yoshikawa T, Watanabe I, Iwasaki T, Hasegawa H, Sato Y, Shimada S, Nanno M, Matsuoka Y, Ohwaki M, Iwakura Y, Suzuki Y, Aizawa C, Sata T, Kurata T, Tamura S. 2002. Protection against influenza virus infection in polymeric Ig receptor knockout mice immunized intranasally with adjuvant-combined vaccines. J Immunol 168:2930–2938.

Asahi-Ozaki Y, Itamura S, Ichinohe T, Strong P, Tamura S, Takahashi H, Sawa H, Moriyama M, Tashiro M, Sata T, Kurata T, Hasegawa H. 2006. Intranasal administration of adjuvant-combined recombinant influenza virus HA vaccine protects mice from the lethal H5N1 virus infection. Microbes Infect 8:2706–2714.

Bernardshaw S, Hetland G, Grinde B, Johnson E. 2006. An extract of the mushroom Agaricus blazei Murill protects against lethal septicemia in a mouse model of fecal peritonitis. Shock 25:420–425.

Coulter A, Harris R, Davis R, Drane D, Cox J, Ryan D, Sutton P, Rockman S, Pearse M. 2003. Intranasal vaccination with ISCOMATRIX adjuvanted influenza vaccine. Vaccine 21:946–949.

Dzionek A, Sohma Y, Nagafune J, Cella M, Colonna M, Facchetti F, Gunther G, Johnston I, Lanzavecchia A, Nagasaka T, Okada T, Vermi W, Winkels G, Yamamoto T, Zysk M, Yamaguchi Y, Schmitz J. 2001. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. J Exp Med 194:1823–1834.

Gao P, Watanabe S, Ito T, Goto H, Wells K, McGregor M, Cooley AJ, Kawaoka Y. 1999. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J Virol 73:3184–3189.

Hasegawa H, Ichinohe T, Strong P, Watanabe I, Ito S, Tamura S, Takahashi H, Sawa H, Chiba J, Kurata T, Sata T. 2005. Protection against influenza virus infection by intranasal administration of hemagglutinin vaccine with chitin microparticles as an adjuvant. J Med Virol 75:130–136.

Herre J, Marshall AS, Caron E, Edwards AD, Williams DL, Schweighoffer E, Tybulewicz V, Reis e Sousa C, Gordon S, Brown GD. 2004. Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. Blood 104:4038–4045.

Ichinohe T, Watanabe I, Ito S, Fujii H, Moriyama M, Tamura S, Takahashi H, Sawa H, Chiba J, Kurata T, Sata T, Hasegawa H. 2005. Synthetic double-stranded RNA Poly(I:C) combined with mucosal vaccine protects against influenza virus infection. J Virol 79:2910–2919.

Ichinohe T, Watanabe I, Tao E, Ito S, Kawaguchi A, Tamura S, Takahashi H, Sawa H, Moriyama M, Chiba J, Komase K, Suzuki Y, Kurata T, Sata T, Hasegawa H. 2006. Protection against influenza virus infection by intranasal vaccine with surf clam microparticles (SMP) as an adjuvant. J Med Virol 78:954–963.

Ichinohe T, Kawaguchi A, Tamura S, Takahashi H, Sawa H, Ninomiya A, Imai M, Itamura S, Odagiri T, Tashiro M, Chiba J, Sata T, Kurata T, Hasegawa H. 2007a. Intranasal immunization with H5N1 vaccine plus Poly I:Poly C(12)U, a Toll-like receptor agonist, protects mice against homologous and heterologous virus challenge. Microbes Infect (Institut Pasteur) 9:1333–1340.

Ichinohe T, Tamura S, Kawaguchi A, Ninomiya A, Imai M, Itamura S, Odagiri T, Tashiro M, Takahashi H, Sawa H, Mitchell WM, Strayer DR, Carter WA, Chiba J, Kurata T, Sata T, Hasegawa H. 2007b. Cross-protection against H5N1 influenza virus infection is afforded by intranasal inoculation with seasonal trivalent inactivated influenza vaccine. J Infect Dis 196:1313–1320.

Ichinohe T, Iwasaki A, Hasegawa H. 2008. Innate sensors of influenza virus: Clues to developing better intranasal vaccines. Expert Rev Vaccines 7:1435–1445.

Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 176:1693–1702.

Inagaki N, Shibata T, Itoh T, Suzuki T, Tanaka H, Nakamura T, Akiyama Y, Kawagishi H, Nagai H. 2005. Inhibition of IgE-dependent mouse triphasic cutaneous reaction by a boiling water fraction separated from mycelium of Phellinus linteus. Evid Based Complement Altern Med 2:369–374.

Iwasaki A, Medzhitov R. 2004. Toll-like receptor control of the adaptive immune responses. Nat Immunol 5:987–995.

Kim GY, Park SK, Lee MK, Lee SH, Oh YH, Kwak JY, Yoon S, Lee JD, Park YM. 2003. Proteoglycan isolated from Phellinus linteus activates murine B lymphocytes via protein kinase C and protein tyrosine kinase. Int Immunopharmacol 3:1281–1292.

Kim GY, Ko WS, Lee JY, Lee JO, Ryu CH, Choi BT, Park YM, Jeong YK, Lee KJ, Choi KS, Heo MS, Choi YH. 2006. Water extract of Cordyceps militaris enhances maturation of murine bone marrow-derived dendritic cells in vitro. Biol Pharm Bull 29:354–360.

Kodama N, Asakawa A, Inui A, Masuda Y, Nanba H. 2005. Enhancement of cytotoxicity of NK cells by D-Fraction, a polysaccharide from Grifola frondosa. Oncol Rep 13:497–502.

Kuo MC, Weng CY, Ha CL, Wu MJ. 2006. Ganoderma lucidum mycelia enhance innate immunity by activating NF-kappaB. J Ethnopharmacol 103:217–222.

Lim BO, Jeon TI, Hwang SG, Moon JH, Park DK. 2005. Phellinus linteus grown on germinated brown rice suppresses IgE production by the modulation of Th1/Th2 balance in murine mesenteric lymph node lymphocytes. Biotechnol Lett 27:613–617.

Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, Janeway CA, Jr. 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol cell 2:253–258.

Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, Spyr C, Steffen R. 2004. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 350:896–903.

Nicolson C, Major D, Wood JM, Robertson JS. 2005. Generation of influenza vaccine viruses on Vero cells by reverse genetics: An H5N1 candidate vaccine strain produced under a quality system. Vaccine 23:2943–2952.

Robinson RA, DeVita VT, Levy HB, Baron S, Hubbard SP, Levine AS. 1976. A phase I-II trial of multiple-dose polyriboinosic-polyribocytidylic acid in patieonts with leukemia or solid tumors. J Natl Cancer Inst 57:599–602.

Saijo S, Fujikado N, Furuta T, Chung SH, Kotaki H, Seki K, Sudo K, Akira S, Adachi Y, Ohno N, Kinjo T, Nakamura K, Kawakami K, Iwakura Y. 2007. Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. Nat Immunol 8:39–46.

Sanzen I, Imanishi N, Takamatsu N, Konosu S, Mantani N, Terasawa K, Tazawa K, Odaira Y, Watanabe M, Takeyama M, Ochiai H. 2001. Nitric oxide-mediated antitumor activity induced by the extract from Grifola frondosa (Maitake mushroom) in a macrophage cell line, RAW264.7. J Exp Clin Cancer Res 20:591–597.

Sorimachi K, Akimoto K, Ikehara Y, Inafuku K, Okubo A, Yamazaki S. 2001. Secretion of TNF-alpha, IL-8 and nitric oxide by macrophages activated with Agaricus blazei Murill fractions in vitro. Cell Struct Funct 26:103–108.

Tamura S, Tanimoto T, Kurata T. 2005. Mechanisms of broad cross-protection provided by influenza virus infection and their application to vaccines. Jpn J Infect Dis 58:195–207.

Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H, Haynes K, Steele C, Botto M, Gordon S, Brown GD. 2007. Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nat Immunol 8:31–38.

Ukawa Y, Ito H, Hisamatsu M. 2000. Antitumor effects of (1 → 3)-beta-D-glucan and (1 → 6)-beta-D-glucan purified from newly cultivated mushroom, Hatakeshimeji (Lyophyllum decastes Sing.). J Biosci Bioeng 90:98–104.

Zaks K, Jordan M, Guth A, Sellins K, Kedl R, Izzo A, Bosio C, Dow S. 2006. Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes. J Immunol 176:7335–7345.

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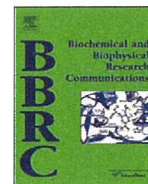

A CTL-based liposomal vaccine capable of inducing protection against heterosubtypic influenza viruses in HLA-A*0201 transgenic mice

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ABSTRACT

The current vaccination strategy against influenza is to induce the production of antibodies directed against surface antigens of viruses. However, the frequent changes in the surface antigens of influenza viruses allow the viruses to avoid antibody-mediated immunity. On the other hand, it is known that cytotoxic T-lymphocyte (CTL) populations directed against internal antigens of influenza A virus are broadly cross-reactive to influenza virus subtypes. In the present study, liposomal conjugates with CTL epitope peptides derived from highly conserved internal antigens of influenza viruses were evaluated for their ability to protect against infection with influenza viruses. Liposomal conjugates with peptide M1 58-66, an HLA-A*0201-binding CTL epitope present within the amino-acid sequence of the M1 coding region, successfully induced antigen-specific $CD8^+$ T-cells and CTLs in HLA-A*0201-transgenic mice. Moreover, after nasal infection with either the H1N1 or H3N2 virus, viral replication in the lung was significantly inhibited in the immunized mice. These protective activities lasted at least 6 months after the immunization. Thus, these results suggest that liposome-coupled CTL epitope peptides derived from highly conserved internal antigens of influenza viruses might be applicable to the development of vaccines that induce protection against infection with heterosubtypic influenza viruses.

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Introduction

A novel swine-origin influenza A (H1N1) virus (S-OIV) emerged in Mexico in March 2009 and continues to spread globally via human-to-human contact. Although S-OIV belongs to the same hemagglutinin (HA) serotype as seasonal human influenza A (H1N1) viruses, vaccination with seasonal influenza vaccine does not result in cross-reactive antibodies [1], possibly because the protein sequence divergence of the S-OIV H1 from human seasonal influenza H1 is wide: around 20–24% [2]. The current vaccination strategy is to elicit neutralizing antibody responses against HA, a surface glycoprotein of influenza viruses. Although antibody-mediated immunity to the surfaces of viruses reduces the probability of infection and morbidity, an antibody against one influenza virus type or subtype can provide limited or no protection against another. Moreover, an antibody to one antigenic variant of an influenza virus might not protect against a new antigenic variant of the same type or subtype, as a consequence of the frequent development of antigenic variants through antigenic drift. Therefore, although vaccines designed to induce antibodies against HA provide reasonable protection against the homologous viruses, it is feared that the vaccines currently being produced may have HA sequences so different from those of any pandemic strain that the vaccines would have little or no efficacy, due to the high rate of viral diversification [3].

Upon natural infection, it is known that the host responds by inducing humoral and cellular immunity against the pathogen. Humoral immune responses are represented by the production of antibodies that bind to the surfaces of bacteria and viruses, whereas cellular immune responses mediate immunity to intracellular pathogens. Since Effros et al. [4] reported in 1977 that influenza A virus-specific cytotoxic T-lymphocytes (CTLs) are broadly cross-reactive for cells of the same MHC class I type infected with serologically distinct H1N1 and H3N2 viruses, it has been known that cytotoxic T-cells specific to internal proteins show high cross-reactivity between strains and between subtypes, reflecting more than 90% conservation of the internal proteins [5]. Lee et al. [6] recently reported that memory T-cells established by seasonal human influenza A infection cross-react with H5N1 in healthy individuals who have not been exposed to H5N1 viruses. $CD8^+$ T-cells of the participants recognized multiple synthesized

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influenza peptides, including peptides from the H5N1 strain. Thus, vaccine formulas that include heterosubtypic T-cell-mediated immunity might confer broad protection against avian and human influenza A viruses.

In general, extracellular antigens are presented via MHC class II molecules to $CD4^+$ T-cells, whereas intracellular antigens are presented via MHC class I molecules to $CD8^+$ T-cells. To induce influenza-specific CTLs, vaccine antigens must be loaded onto the class I MHC processing pathway in the antigen-presenting cells (APCs) via cross-presentation. In the cross-presentation, exogenous proteins cross over to the endogenous pathway to gain access to MHC class I molecules [7]. However, the currently approved alum adjuvant, which was first described by Glenny and Pope [8] in 1926, is known to be effective only for the induction of humoral immunity, not for the induction of cell-mediated immunity [9]. Consequently, the development of a novel vaccine adjuvant is essential for the production of the $CD8^+$ T-cell vaccine.

In our previous study [10], we produced surface-coupled liposomal antigens that were presented by APCs to $CD8^+$ T-cells via MHC class I molecules. Therefore, this antigen preparation was expected to be applicable to the development of viral vaccines to induce virus-specific CTLs that effectively eliminate virus-infected cells [11]. Since the liposomal conjugates induced CTLs efficiently when CTL epitope peptides were coupled to the surfaces of liposomes [10], the liposomal conjugates were expected to be applicable to the development of peptide vaccines. We recently demonstrated that liposome-coupled peptide $NP_{366-374}$, derived from nucleoprotein (NP) of influenza H3N2 viruses, induced antigen-specific CTLs and successfully suppressed influenza H3N2 viral replication in the lung in C57BL/6 mice [12]. In the present study, we evaluated a possible application of liposome-coupled peptides to the development of an influenza vaccine using HLA-A∗0201-restricted CTL epitope peptides and HLA-A∗0201-transgenic mice.

Materials and methods

Mice. Mice express a transgenic HLA-A∗0201 monochain, designated as HHD, in which human b2-microglobulin (b2m) is covalently linked to a chimeric heavy chain composed of HLA-A∗0201 (a1 and a2 domains) and H-$2D^b$ (a3, transmembrane, and cytoplasmic domains) [13]. Eight- to twelve-week-old mice were used for all experiments. The mice were housed in appropriate animal care facilities at Saitama Medical University, Saitama, Japan. Experiments in the present study were approved (No. 214) by the Animal Research Committee of Saitama Medical School and the mice were handled according to international guidelines for experiments with animals.

Reagent. Synthetic CpG ODN (5002: TCCATGACGTTCTTGATGTT) was purchased from Hokkaido System Science (Sapporo, Japan) and was phosphorothioate-protected to avoid nuclease-dependent degradation.

Synthetic peptides. The HLA-A∗0201-restricted, influenza virus matrix protein-derived peptide M1 58-66 (GILGFVFTL) [14] and two newly determined peptides—NS1 128-136 (IILKANFSV), which corresponds to residues 128–136 of the nonstructural protein 1 and PB1 410-418 (GMFNMLSTV), which corresponds to residues 410–418 of the polymerase PB1 protein—were synthesized by Operon Biotechnologies (Tokyo, Japan).

Coupling of peptides to liposomes. Liposomal conjugates with peptides derived from internal antigens of influenza virus were prepared essentially in the same way as described previously [10] using disuccinimidyl suberate (DSS). Briefly, a mixture of 10 ml of anhydrous chloroform solution containing 0.136 mM DOPE and 24 ml of TEA was added in drops to 26.6 ml of anhydrous chloroform solution containing 0.681 mM DSS and stirred for 5 h at 40 °C. The solvent was evaporated under reduced pressure, and 18 ml of a 2:1 mixture of ethyl acetate and tetrahydrofuran was added to dissolve the residue. To the solution was added 36 ml of 100 mM sodium phosphate (pH 5.5) and 90 ml of saturated NaCl aqueous solution. The mixture was, shaken for 1 min and allowed to separate. To remove undesirable materials, the upper layer was washed with the same buffer. After evaporation of the solvent, 3 ml of acetone was added to dissolve the residue. Then, 100 ml of ice-cold acetone was added in drops and kept on ice for 30 min to precipitate. Crystals were collected and dissolved in 5 ml of chloroform. After evaporation, 34.4 mg of DOPE-DSS was obtained, then 0.18 mM DOPC, 0.03 mM DOPE-DSS, 0.21 mM cholesterol, and 0.06 mM DOPG were dissolved in 10 ml of chloroform/methanol. The solvent was removed under reduced pressure and 5.8 ml of phosphate buffer (pH 7.2) was added to make a 4.8% lipid suspension. The vesicle dispersion was extruded through a 0.2 mm polycarbonate filter to adjust the liposome size. A 2 ml suspension of DSS-introduced liposome and 0.5 ml of 10 mg/ml peptide solution were mixed and stirred for 3 days at 4 °C. The liposome-coupled and -uncoupled peptides were separated using CL-4B column chromatography. The resulting PBS solution of peptide-liposome conjugates, containing approximately 0.7 mg peptides/ml, was sterile filtered and kept at 4 °C until use.

Cell line. Madin-Darby canine kidney (MDCK) cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS).

Influenza viruses. Two influenza A virus strains—H1N1 (A/PR/8/34) and H3N2 (A/Aichi/2/68)—were propagated in 10-day-old embryonated hen's eggs at 35 °C for 3 days. Egg allantoic fluid containing either virus was then harvested and stocked at −80 °C. Virus titers were determined by calculating the 50% tissue culture infectious dose ($TCID_{50}$) using MDCK cells [15]. The value of 50% mouse lethal dose (LD_{50}) of the H1N1 (A/PR/8/34) virus was determined in mice that were inoculated intranasally (i.n.) under anesthesia. The A/PR/8/34 (H1N1) virus was purchased from ATCC, and the H3N2 (A/Aichi/2/68) virus was kindly provided by Dr. Hiroshi Kida of Hokkaido University, Japan.

Immunization. Mice were subcutaneously (s.c.) immunized with 100 μl solution of the peptide-liposome conjugates in the presence of 5 μg CpG.

Intracellular cytokine staining. Intracellular cytokine staining (ICS) was performed as described previously [16]. Briefly, after one week following immunization, 2×10^6 spleen cells of immunized mice were incubated with 10 mM of the peptide M1 58-66 for 5 h at 37 °C in the presence of brefeldin A (GolgiPlug™, BD Biosciences, San Jose, CA). After Fc receptors were blocked with the rat anti-mouse CD16/CD32 mAb (Fc Block™, BD Biosciences), cells were stained with FITC-conjugated rat anti-mouse CD8α mAb (BD Biosciences) for 30 min at 4 °C. The cells were then fixed, permeabilized, and stained with phycoerythrin (PE)-conjugated rat anti-mouse interferon-gamma (IFN-γ) mAb (BD Biosciences). After the cells were washed, flow cytometric analyses were performed.

Table 1
Amino acid sequences of CTL epitopes derived from internal antigens of influenza viruses.

Virus strain	M1 58-66	NS1 128-136	PB1 410-418
H1N1 (A/PR/8/34)	GILGFVFTL	IILKANFSV	GMFNMLSTV
H3N2 (A/Aichi/2/68)	GILGFVFTL	VMLKANFSV	GMFNMLSTV
H1N1 (A/New York/4290/2009)	GILGFVFTL	IVLKANFSV	GMFNMLSTV
H5N1 (A/Hong Kong/483/97)	GILGFVFTL	IILKANFSV	GMFNMLSTV

Disagreements are underlined.

In vivo CTL assay. In vivo CTL assay was carried out as described previously by Suvas et al. [17]. Briefly, spleen cells from naive HHD mice were equally split into two populations. One population was pulsed with 10 mM of peptide M1 58-66 and labeled with a high concentration (2.5 μM) of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR). The other popula-

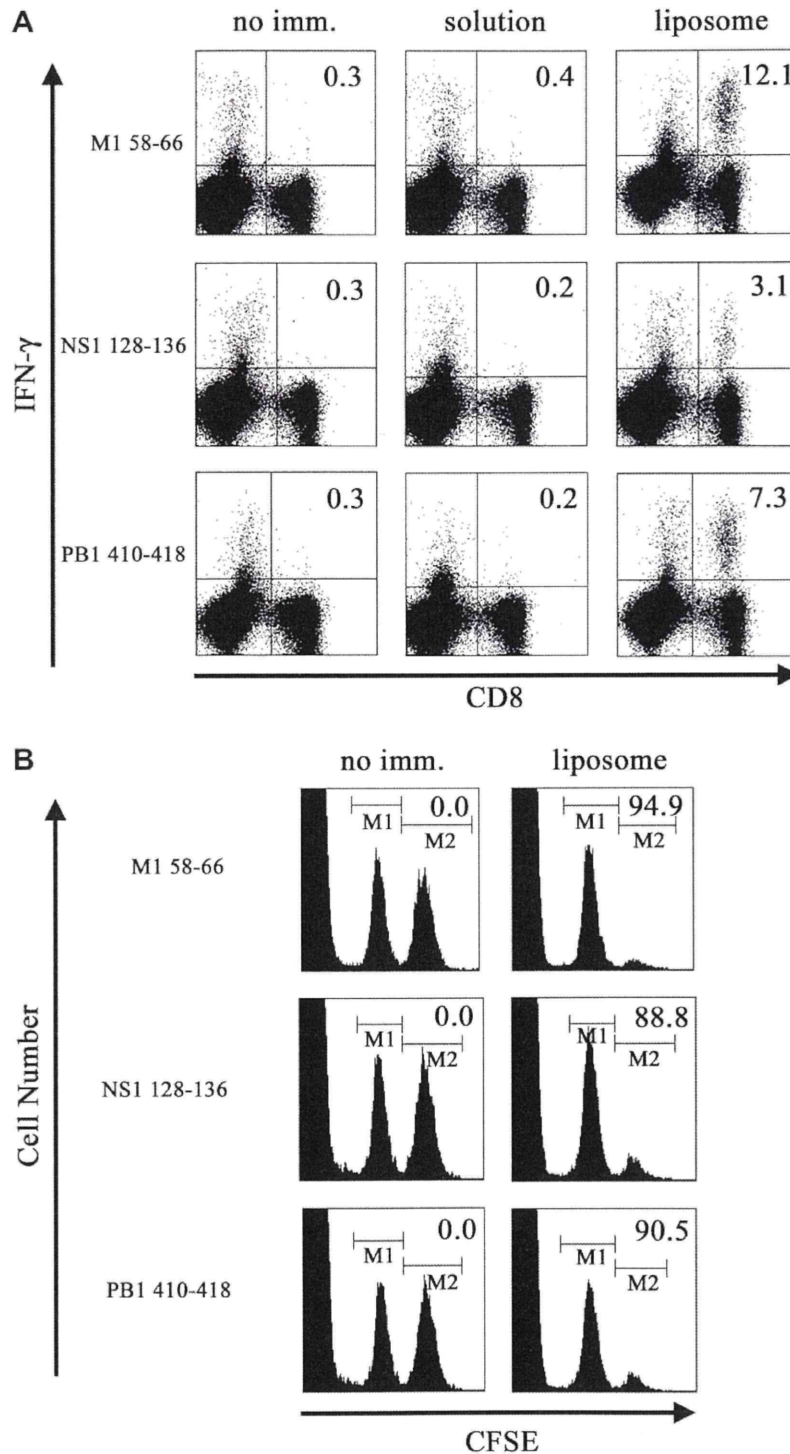

Fig. 1. Induction of antigen-specific CD8⁺ T-cell and CTL responses. Mice received immunization with the liposome-coupled peptides M1 58-66, NS1 128-136, and PB1 410-418 (liposome) or liquefied peptides in the same amounts as liposome conjugates (solution) in the presence of CpG. Control mice received no immunization (no imm.). One week after the immunization, ICS (A) and *in vivo* CTL assay (B) were performed as described in Materials and methods. (A), ICS of antigen-specific CD8⁺ T-cells among spleen cells of A2Tg mice. Cells were stained for their surface expression of CD8 (*x*-axis) and their intracellular expression of IFN-γ (*y*-axis). The numbers shown indicate the percentage of CD8⁺ cells that were positive for intracellular IFN-γ. (B), induction of CTLs by liposome-coupled peptides. The numbers shown indicate the percentage of total cells killed. The data shown are representative of three independent experiments.

tion was unpulsed and labeled with a lower concentration (0.25 μM) of CFSE. An equal number (1×10^7) of cells from each population was mixed together and adoptively transferred intravenously (i.v.) into mice that had been immunized once with a liposomal peptide two weeks earlier. Twelve hours later, spleen cells were prepared and analyzed by flow cytometry. To calculate specific lysis, the following formula was used: % specific lysis = (1 − {(number of $CFSE^{low}$ cells in normal mice)/(number of $CFSE^{high}$ cells in normal mice)}/{(number of $CFSE^{low}$ cells in immunized mice)/(number of $CFSE^{high}$ cells in immunized mice)}) × 100.

Viral challenge experiment. Mice were anesthetized by an intraperitoneal injection of ketamine (175 mg/g weight) (Sigma-Aldrich) and xylazine (3.5 mg/g weight) (Bayer Holding Ltd., Tokyo, Japan), and were challenged intranasally (i.n.) with either $5 \times LD_{50}$ (1×10^4 $TCID_{50}$) of H1N1 (A/PR/8/34) virus or 1×10^4 $TCID_{50}$ of H3N2 (A/Aichi/2/68) virus resuspended in 40 ml of PBS per animal. The mice were weighed daily and monitored for mortality for two weeks. For virus titration, mice were sacrificed on day 2, 4, or 6 after the virus challenge, and the virus titers in their lungs were determined by calculating $TCID_{50}$ using MDCK cells as described [15]. In brief, the lungs were homogenized in 1 ml of PBS and the homogenate was clarified by centrifugation at 2000 rpm for 10 min. The lung homogenates were then serially 10-fold diluted in 96-well U-bottomed plates, 5 wells per dilution, starting from 10^{-1} to 10^{-7} in DMEM with 5% FCS. MDCK cells in D-5 were added to all wells (2.5×10^4 cells/well) and incubated at 35 °C in 5% CO_2. One day later, the culture medium in each well was replaced by DMEM containing 2 mg/ml acetylated trypsin (Sigma-Aldrich), and the plates were incubated in a CO_2 incubator at 35 °C for 4 more days. After the addition of 50 ml of 0.5% chicken red blood cell suspension in PBS to each well, the agglutination pattern for each sample was observed and virus titers were determined by calculating $TCID_{50}$ [15]. Three to six mice were used in each experimental group. The limit of detection in this assay was 10^3 $TCID_{50}$/mouse.

Statistical analyses. Statistical analyses were performed using Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

Results

Induction of antigen-specific $CD8^+$ T-cells and CTLs by liposome-coupled CTL epitopes derived from internal proteins of influenza viruses

HLA-A*0201 (A2)-binding epitopes were predicted among the amino-acid sequences of six coding regions—M1, NP, PA, PB1, PB2, and NS—in the H1N1 influenza virus strain A/PR/8/34 (PR8) using programs available on the Internet. The predicted epitopes were then synthesized and chemically coupled to liposomes to evaluate their abilities to induce antigen-specific $CD8^+$ T-cells and CTLs by means of ICS and an *in vivo* cytotoxicity assay, respectively. Immunization of the liposome-coupled, HLA-A2-restricted epitope peptides, M1 58-66, NS1 128-136, and PB1 410-418 (Table 1), induced significant levels of antigen-specific $CD8^+$ T-cells in HLA-A2-transgenic (A2Tg) mice, as evaluated by ICS (Fig. 1A). Moreover, as shown in Fig. 1B, all the peptide-liposome conjugates in Fig. 1A induced significant *in vivo* CTL responses, indicating that all the predicted CTL epitopes were loaded onto MHC class I and were recognized by CTLs. On the other hand, solutions of the predicted peptides did not induce antigen-specific $CD8^+$ T-cells in A2Tg mice immunized even in the presence of CpG (Fig. 1A). As shown in Table 1, all three peptides were preserved well not only in H1N1 (A/PR/8/34) and H3N2 (A/Aichi/2/68) seasonal influenza viruses but also in the pandemic H1N1 2009 virus (A/New York/4290/2009) and in a highly pathogenic avian influenza virus, H5N1 (A/Hong Kong/483/97), except that NS1 128-136 partially changed in H3N2 (A/Aichi/2/68) and H1N1 (A/New York/4290/2009). Among the CTL epitopes determined in the present study, peptide M1 58-66 was already reported by Gotch et al. in 1987 [14].

Virus challenge experiment

Among the above-described HLA-A2-restricted CTL epitopes, virus challenge experiments were performed using peptide M1 58-66. A2Tg mice were immunized subcutaneously with liposome-coupled, HLA-A2-restricted peptide M1 58-66. One week after the immunization, they were infected intranasally with the influenza virus H1N1 (A/PR/8/34) or H3N2 (A/Aichi/2/68) strain. As shown in Fig. 2, viral growth in the lung was suppressed significantly in the immunized mice 2–6 days after the infection with either H1N1 (A) or H3N2 (B) viruses. Thus, immunization with liposome-coupled peptides successfully induced protection against influenza viruses regardless of the influenza virus subtypes in A2Tg mice.

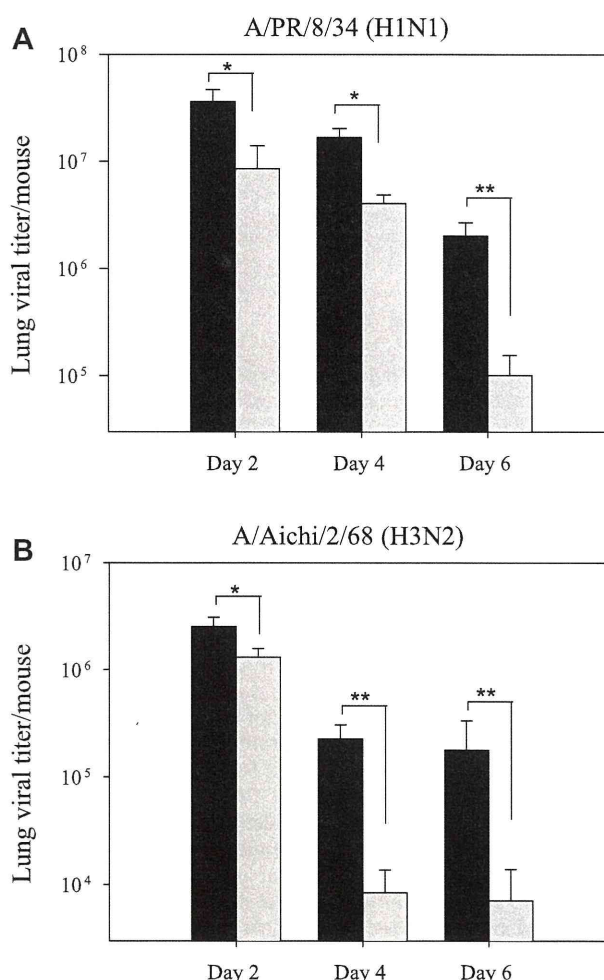

Fig. 2. Viral titers in lungs of mice following H1N1 or H3N2 virus challenge. Mice received immunization with either liposome-coupled peptide M1 58-66 liposome conjugates in the presence of CpG (gray bars) or none (black bars). One week later, mice were challenged i.n. with either the H1N1 (A) or the H3N2 virus (B). On day 2, 4, or 6 post-infection, viral titers in the lungs were determined by calculating $TCID_{50}$ using MDCK cells as described in Materials and methods. Data represent mean and SE of 5 mice per group. $^{*}p < 0.05$. $^{**}p < 0.01$.

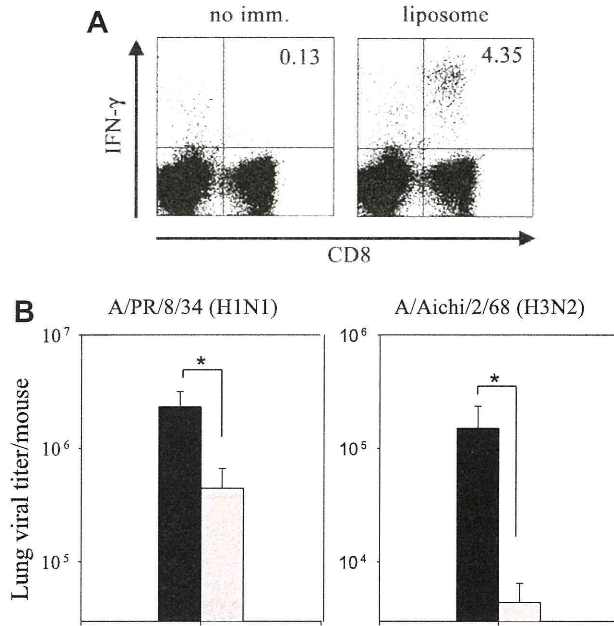

Fig. 3. Induction of long-lasting immunity by peptide-liposome conjugates. Mice were immunized with either liposome-coupled peptide M1 58-66 in the presence of CpG or none. 90 days after the immunization, ICS (A) and viral challenge experiments (B) were performed. (A), ICS of $CD8^+$ T-cells specific for peptide M1 58-66 among spleen cells of A2Tg mice immunized with liposome-coupled peptide (liposome) or none (no imm.). (B), viral replication in the lungs of mice infected with influenza viruses in mice immunized with liposome-coupled peptide (gray bars) or none (black bars). Data represent the mean and SE of 5 mice per group. $^*p < 0.05$.

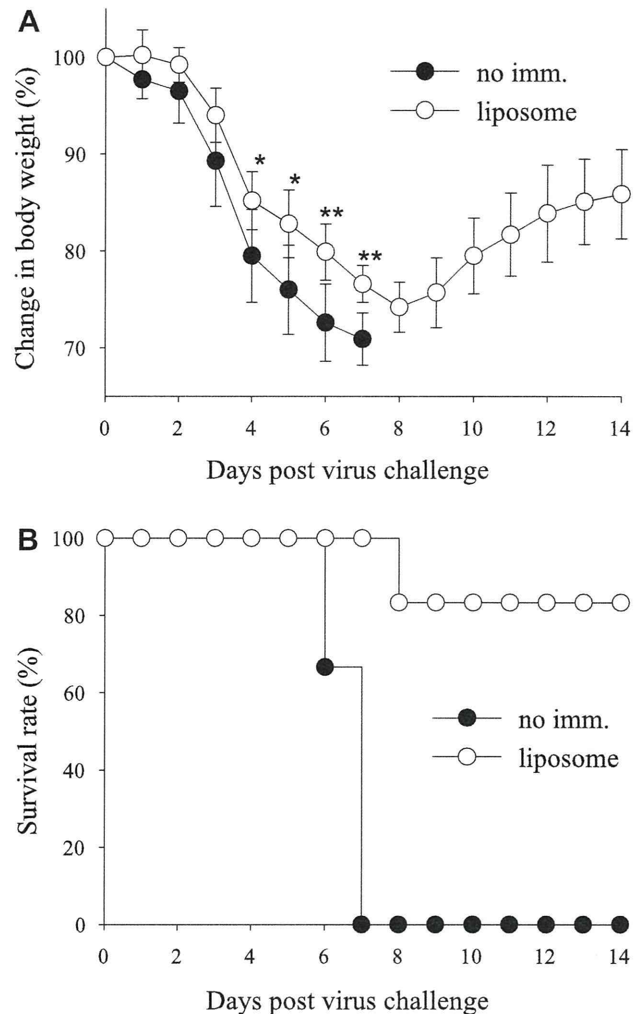

Fig. 4. Maintenance of protective ability against a lethal dose of H1N1 virus in mice immunized with liposome-coupled peptide. Mice were immunized with either liposome-coupled peptide M1 58-66 in the presence of CpG (open symbols) or none (solid symbols). Six months after the immunization, mice were challenged i.n. with $5 \times LD_{50}$ of H1N1 (A/PR/8/34) virus. The mice were weighed and monitored daily for mortality up to 14 days post-challenge. (A), changes in body weight of mice calculated as a percentage of the mean weight per group as compared with starting body weight. Data represent mean body weight and SE of 6 mice per group. $^*p < 0.05$ and $^{**}p < 0.01$ as compared with non-immunized control mice. (B), survival percentage. The mice were euthanized after they had lost 30% of their initial body weight.

Induction of memory $CD8^+$ T-cells

After confirming the induction of protective ability by liposomal peptide in the effector phase, we investigated whether or not this immunization induced memory $CD8^+$ T-cells. As shown in Fig. 3, $CD8^+$ T-cells specific to peptides M1 58-66 were detected significantly in immunized mice at 90 days after the immunization (Fig. 3A). In addition, viral growth in the lung was suppressed significantly after nasal challenge with either the H1N1 or H3N2 influenza virus (Fig. 3B). Thus, it was demonstrated that the immunization readily induced memory $CD8^+$ T-cells.

Induction of long-lasting protection against lethal doses of influenza viruses

We next investigated whether or not the long-lasting immunity demonstrated above would help protect mice against infection with lethal doses of influenza viruses. Six months after the immunization with liposome-coupled M1 58-66 peptides, mice were challenged with a lethal dose of influenza virus H1N1 PR8 strain. As shown in Fig. 4, although the immunized mice lost body weight up to 8 days after infection, the rate of loss was significantly lower than that in a non-immunized control group at 4–7 days, and the immunized mice recovered body weight thereafter (Fig. 4A). Finally, 5 out of 6 immunized mice stayed alive while all of the non-immunized control mice died within 7 days after infection with a significant loss of body weight and morbidity (Fig. 4B).

Discussion

The present study demonstrated that liposome-coupled CTL epitope peptides derived from internal antigens of influenza virus protected against infection with heterosubtypic influenza viruses. Since the early finding that influenza A virus-specific CTLs are broadly cross-reactive for cells of the same MHC class I type infected with serologically distinct H1N1 and H3N2 viruses [4], numerous investigators have reported on immunodominant and cross-reactive CTL epitopes derived from conserved internal antigens, such as NP [18,19], M1 [14,19,20], and NS1 [5] of the influenza viruses. It was hoped that CTL epitopes could be used to develop a broadly protective influenza vaccine [21]. To date, candidate $CD8^+$ T-cell vaccines have been reported to induce protection even partially [22], suggesting that $CD8^+$ T-cells certainly help protect against influenza. Therefore, the $CD8^+$ T-cell vaccine strategy against influenza was expected to support antibody-focused vaccine strategies by "reducing" [21] or "dampening" [5] the impact of the next pandemic.

The liposome-coupled CTL epitope peptides efficiently induced antigen-specific $CD8^+$ T-cells and CTLs (Fig. 1), and suppressed viral

replication in the lungs of mice infected with either H1N1 or H3N2 influenza viruses (Fig. 2). In addition, since a single immunization just one week before infection successfully reduced viral replication in the lung, this vaccination protocol would be expected to counter the rapid spread of an influenza pandemic. The efficacy of the liposome-coupled peptides in inducing CTL-based protective immunity was likely due to the characteristics of liposome-coupled antigens, which are very readily recognized by APCs [23] and which effectively induce cross-presentation via MHC class I in the APCs [10], in addition to their safety, in that they are least likely to induce allergic responses [24]. Moreover, the liposome-coupled CTL epitope peptides were capable of inducing long-lived $CD8^+$ memory T-cells without including $CD4^+$ T-cell epitope in the composition of the vaccine (Fig. 3). In fact, mice immunized with liposome-coupled M1 58-66 peptides remained protected for at least 6 months after immunization (Fig. 4).

The CTL epitopes employed in the present study are contained not only in the seasonal influenza viruses but also in the currently emerging S-OIV and in the extremely virulent avian H5N1 influenza viruses (Table 1), suggesting that this liposomal peptide vaccine might be effective for protection against infection with both seasonal and pandemic influenza viruses. In addition, the liposome employed in the present study was originally developed as an antigenic carrier that effectively induces humoral immunity (i.e., antibody production) against liposome-coupled antigens [25]. Therefore, the antigen-liposome conjugates are capable of inducing both humoral and cellular immunity against influenza viruses, by combined coupling of antibody and CTL epitopes to the surfaces of liposomes.

Conclusions

The results obtained in the present study demonstrated that liposome-coupled CTL epitope peptides derived from highly conserved internal antigens of influenza viruses might be applicable to the development of a broadly protective influenza vaccine that could confer protective immunity against both seasonal and pandemic influenza.

Acknowledgments

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References

[1] CDC, Morbidity and Mortality Weekly Report, Serum cross-reactive antibody response to a novel influenza A (H1N1) virus after vaccination with seasonal influenza vaccine, MMWR 58 (2009) 521–524.

[2] D. Gatherer, The 2009 H1N1 influenza outbreak in its historical context, J. Clin. Virol. 45 (2009) 174–178.

[3] B. Olsen, V.J. Munster, A. Wallensten, et al., Global patterns of influenza A virus in wild birds, Science 312 (2006) 384–388.

[4] R.B. Effors, P.C. Doherty, W. Gerhard, et al., Generation of both cross-reactive and virus-specific T-cell populations after immunization with serologically distinct influenza A viruses, J. Exp. Med. 145 (1977) 557–568.

[5] J.H.C.M. Kreijtz, G. Mutsert, C.A. Baalen, et al., Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus, J. Virol. 82 (2008) 5161–5166.

[6] L.Y. Lee, D.L.A. Ha, C. Simmons, et al., Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals, J. Clin. Invest. 118 (2008) 3478–3490.

[7] W.R. Heath, F.R. Carbone, Cross-presentation, dendritic cells, tolerance and immunity, Annu. Rev. Immunol. 19 (2001) 47–64.

[8] A.T. Glenny, C.G. Pope, Immunology notes. XXIII. The antigenic value of toxoid precipitated by potassium alum, J. Pathol. Bacteriol. 29 (1926) 31–40.

[9] H. Aggrebeck, J. Wantzin, I. Heon, Booster vaccination against diphtheria and tetanus in man: comparison of three different vaccine formations-III, Vaccine 14 (1996) 1265–1272.

[10] M. Taneichi, H. Ishida, K. Kajino, et al., Antigen chemically coupled to the surface of liposomes are cross-presented to $CD8^+$ T cells and induce potent antitumor immunity, J. Immunol. 177 (2006) 2324–2330.

[11] T. Uchida, M. Taneichi, Clinical application of surface-linked liposomal antigens, Mini Rev. Med. Chem. 8 (2008) 184–192.

[12] T. Nagata, T. Toyota, H. Ishigaki, et al., Peptides coupled to the surface of a kind of liposome protect infection of influenza viruses, Vaccine 25 (2007) 4914–4921.

[13] S. Pascolo, N. Bervas, J.M. Ure, et al., HLA-A2.1-restricted education and cytolytic activity of $CD8^+$ T lymphocytes from β2 microglobulin (β2m) HLA-A2.1 monochain transgenic H-$2D^b$ β2m double knockout mice, J. Exp. Med. 185 (1997) 2043–2051.

[14] F. Gotch, J. Rothbard, K. Howland, et al., Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2, Nature 326 (1987) 881–882.

[15] R. Cottey, C.A. Rowe, B.S. Bender, Influenza virus, in: Current Protocols in Immunology, John Wiley & Sons Inc., 2001, pp. 19.11.1–19.11.32.

[16] M. Matsui, O. Moriya, M.L. Belladonna, Adjuvant activities of novel cytokines, interleukine (IL)-23 and IL-27 for induction of hepatitis C virus-specific cytotoxic T lymphocytes in HLA-A*0201 transgenic mice, J. Virol. 78 (2004) 9093–9104.

[17] S. Suvas, U. Kumaraguru, C.D. Pack, et al., $CD4^+CD25^+$ T Cells regulate virus-specific primary and memory $CD8^+$ T cell responses, J. Exp. Med. 198 (2003) 889–901.

[18] S.L. Epstein, W. Kong, J.A. Miplon, et al., Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein, Vaccine 23 (2005) 5404–5410.

[19] A.R.M. Townsend, J. Rothbard, F.M. Gotch, et al., The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides, Cell 44 (1986) 959–968.

[20] E.G.M. Berkhoff, D. Wit, M.M. Geelhoed-Mieras, et al., Functional constrains of influenza A virus epitopes limit escape from cytotoxic T lymphocytes, J. Virol. 79 (2005) 11239–11246.

[21] P.C. Doherty, A. Kelso, Toward a broadly protective influenza vaccine, J. Clin. Invest. 118 (2008) 3273–3275.

[22] P.G. Thomas, R. Keating, D.J. Hulse-Post, et al., Cell-mediated protection in influenza infection, Emerg. Infect. Dis. 12 (2006) 48–54.

[23] Y. Tanaka, M. Kasai, M. Taneichi, et al., Liposomes with differential lipid components exert differential adjuvanticity in antigen-liposome conjugates via differential recognition by macrophages, Bioconjug. Chem. 15 (2004) 35–40.

[24] M. Taneichi, S. Naito, H. Kato, et al., T cell-independent regulation of IgE antibody production induced by surface-linked liposomal antigen, J. Immunol. 169 (2002) 4246–4252.

[25] Y. Nakano, M. Mori, S. Nishinohara, et al., Surface-linked Liposomal antigen induces IgE-selective unresponsiveness regardless of the lipid components of liposomes, Bioconjug. Chem. 12 (2001) 391–395.

IL-4/IL-13 antagonist DNA vaccination successfully suppresses Th2 type chronic dermatitis

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Summary

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Conflicts of interest
None declared.

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Background Atopic dermatitis (AD) is a chronic disease with a Th2-type-cytokine dominant profile. Several cytokines and related peptides have been used for the treatment of AD but they were ineffective because of their limited biological half-life. We have recently developed a highly efficient mouse dominant negative interleukin (IL)-4/IL-13 antagonist (IL-4DM), which blocks both IL-4 and IL-13 signal transductions.

Objective To examine the effects of IL-4DM *in vivo* in an AD model induced by the repeated exhibition of oxazolone (OX).

Methods Plasmid DNA was injected intraperitoneally to cause an experimental AD-like dermatitis. The effect was evaluated by ear thickness, histological findings, and mast cells counts in the inflamed skin. The plasma IgE and histamine levels were measured. Cytokine production in skin and splenocytes were also analysed.

Results Mice treated with control plasmid developed marked dermatitis with mast cells and eosinophil infiltration, and had increased plasma IgE and histamine levels with a Th2 type splenocyte cytokine profile. Treatment with mouse IL-4 DNA augmented the ear swelling and thickness with an increased dermal eosinophil count, plasma histamine level, and production of splenocyte IL-4. However, IL-4DM treatment successfully controlled the dermatitis, decreased the mast cell and eosinophil count, and suppressed plasma IgE and histamine levels. Splenocytes produced an increased level of IFN-γ.

Conclusion These data showed that the simultaneous suppression of IL-4/IL-13 signals successfully controlled Th2-type chronic dermatitis. IL-4DM DNA treatment is a potent therapy for AD and related diseases.

Interleukin (IL)-4 plays a central role in Th2-cytokine-dominant inflammatory skin diseases such as atopic dermatitis (AD).[1–3] IL-4 is responsible for the differentiation of allergen-specific Th2 cells together with its closely related cytokine IL-13 for the class switching of activated B cells to IgE-producing cells. The effects of IL-13 are similar to IL-4 on B cells, monocytes, and other cell types, but T cells appear to lack an IL-13 binding receptor component and do not respond to IL-13.[4] The structural basis for the overlapping functions of IL-4 and IL-13 is a shared receptor subunit, and IL-4Rα organizes intracellular signals in response to both cytokines.[5,6] Signal transduction is induced by heterodimerization of the IL-4Rα with a second subunit; which may vary according to the cell types. The specific inhibition of IL-4 can be achieved by antagonistic IL-4 mutants. Variants of human IL-4 that bind to the receptor subunit IL-4Rα, but not to the other subunit γ-chain (γc) or IL-13Rα1 are competitive antagonists of IL-4.[7,8] IL-13 is inhibited by similar variants, which form unproductive complexes with IL-4Rα.[5,9] The single-site human IL-4 mutant Y124D has been used as an IL-4/IL-13 inhibitor in various studies,[7–17] but this variant retains some residual agonistic activity, which could be relevant for *in vivo* applications.[7,8] In contrast, IL-4 and IL-13 double mutant R121D/Y124D lacks detectable activity and appears to be an effective antagonist for human IL-4 and IL-13.[5,18]

We have recently developed a highly efficient murine IL-4 antagonist DNA (IL-4DM), in which the amino acids glutamine 116 and tyrosine 119 were changed for aspartic acid.[19] This murine mutant DNA is analogous to the R121D/Y124D double mutant. IL-4DM binds with high affinity to the murine

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IL-4Rα without inducing signal transduction, and has no detectable activity upon the proliferation or differentiation of murine cells. An appropriate amount of IL-4DM completely inhibits responses by wild-type IL-4.[19] Like its human analogue, the IL-4DM mutant is also an antagonist of IL-13 (B. Schnarr *et al.*, unpublished data[37]). Recent experiments with monocytes from mice lacking a functional γc gene showed that IL-4DM is a complete inhibitor of IL-4 in the absence of γc as well.[20] In this study we have examined the effects of IL-4DM *in vivo*, using an AD model induced by the repeated exhibition of oxazolone (OX). The repeated application of a hapten such as OX on mice causes an initial delayed-type hypersensitivity that changes to an immediate-type response in the late phase with elevated IgE production and deviation of Th-cell responses. The skin lesions that appear in the late phase are compatible with the clinical findings as well as the cytokine profile observed in AD.[21–23] The inhibitory effect of IL-4DM on IL-4 and IL-13 on the immune response was comparable with that of knockout mice lacking either IL-4[24] or IL-4Rα. Treatment with IL-4DM prevented contact hypersensitivity responses with the increased production of interferon (IFN)-γ.

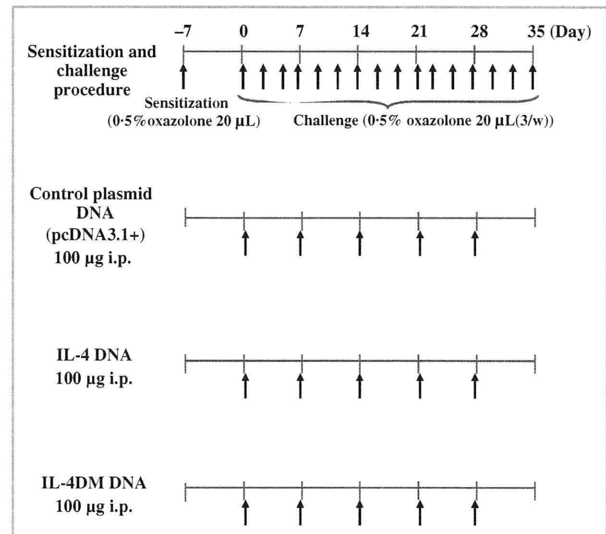

Fig 1. Schedule for induction of chronic contact hypersensitivity and administration of compounds. Mice received intraperitoneal (i.p.) injection of 100 μg of each plasmid DNA on days 0, 7, 14, 21 and 28.

Materials and methods

Animals

BALB/c male mice aged 5 weeks were purchased from Japan SLC Co. (Shizuoka, Japan) and were used at the age of 6 weeks. Age-matched wild-type BALB/c mice were used as controls. All animals were cared for according to the ethical guidelines approved by the Institutional Animal Care and Use Committee of Mie University.

Reagents

The cDNA coding region of mouse IL-4 was amplified by a polymerase chain reaction (PCR) based on the cDNA sequence of mouse IL-4. The mouse IL-4 fragment was inserted into *Bam*HI and *Eco*RI-filled in pcDNA3.1+ (Invitrogen, San Diego, CA, U.S.A.) under the TPA leader sequence, and then digested by *Bam*HI and *Sac*I. A Quickchange™ Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) was used for the mutagenesis of mouse IL-4. The oligonucleotide primers used to prepare a mouse IL-4 double mutant (IL-4DM, Q116D/Y119D) were CTAAAGAGCATCATGGATATGGATGACTCGTAGTCTAGAG and CTCTAGACTACGAGTCATCCATATCCATGATGCTCTTTAG. The IL-4 mutant fragments were ligated into pcDNA3.1+.[25] Mouse IL-4, IL-4DM plasmid DNAs were purified using the Plasmid Mega kit (Qiagen, Chatsworth, CA, U.S.A.) and diluted with sterilized physiological saline. OX was purchased from Sigma (St Louis, MO, U.S.A.) and was dissolved in acetone/olive oil (4 : 1).

Administration of DNA

Mice were treated by intraperitoneal injection of 100 μg of IL-4DM DNA on days 0, 7, 14, 21 and 28. A control plasmid (pcDNA3.1+) vector and IL-4 DNA were also injected on the same day (Fig. 1).

Sensitization and challenge procedures

As shown in Figure 1, mice were initially sensitized by pasting 20 μL of 0·5% OX solution to their left ear 7 days before the first challenge (day 7) and then 20 μL of 0·5% OX solution was repeatedly applied on the left ear three times per week from day 0 as reported previously.[23] Ear swelling was measured with thickness gauge calipers before and 30 min after OX challenge to the pinna of the ear on day 35. The ear swelling response was expressed as the difference between the values taken before and 30 min after application.

Histological analysis

Ear skin specimens obtained 6 h after the final challenge on day 35 were fixed in 10% buffered neutral formaldehyde and embedded in paraffin wax. Histological sections were of 6 μm thickness and they were stained with haematoxylin and eosin. The sections were also stained with 0·5% toluidine blue for the identification of mast cells. The cell counts were performed in six consecutive microscopic fields at × 400 magnification.

Measurement of plasma IgE and plasma histamine

Blood was collected under ether anaesthesia 6 h after the last challenge. Plasma IgE levels were determined by a sandwich enzyme-linked immunosorbent assay (ELISA). In brief, 96-well immunoplates (Corning Inc., Corning, NY, U.S.A.) were coated with 100 μL of an antimouse IgE capture antibody (2 μg mL^{-1}) (BD PharMingen, San Diego, CA, U.S.A.) overnight at 4 °C. Plasma samples of 100 μL were diluted 60-fold with PBS

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containing 10% fetal calf serum (FCS) were placed in the wells. After incubation for 1 h at room temperature, 100 μL of biotin-conjugated antimouse IgE antibody (2 μg mL^{-1} in blocking buffer) (BD PharMingen) was added to each well. The plates were incubated at room temperature for 1 h, followed by six washes, incubated with 100 μL of horseradish peroxidase avidin D (FUNAKOSHI, Tokyo, Japan) 1 : 1000 in blocking buffer, and then incubated for 30 min at room temperature. A substrate solution of 100 μL containing 1·5 mg ABTS (Sigma-Aldrich, St Louis, MO, U.S.A.) in 5 mL of a 0·1 mol L^{-1} citric acid solution was added, and kept for 30 min at room temperature in a dark place. Thereafter the reaction was terminated by adding 50 μL of 2 mol L^{-1} H_2SO_4, and the optical density of each well at 405 nm was determined by using a microplate reader. A standard curve was prepared using mouse anti-TNP IgE standard (BD PharMingen). Plasma histamine levels were analysed using the commercial sandwich ELISA kit from Immunoteck (Marseille, France) according to the manufacturer's protocol.

Purification of mRNA from mouse ears

At 6 h after the final challenge, the skin of the left ear was sampled. The specimen was homogenized and the total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction; 1 mL of homogenate was vigorously mixed with 200 μL of chloroform, and centrifuged at 12 000 **g** for 15 min at 4 °C. The aqueous phase was separated and mixed with 0·5 mL of 2-propanol (Nacalai Tesque, Kyoto, Japan) to precipitate RNA. After centrifugation, the precipitate was washed with 1 mL of 75% ethanol (Nacalai Tesque) and dried. RNA was suspended in 50 μL of RNase-free water, and the concentration was measured based on the absorbance at 260 nm, and the quality was confirmed by electrophoresis. cDNA was prepared from 10 μg of mRNA using archive kit (ABI, Foster City, CA, U.S.A.) according to the manufacturer's protocol.

Cytokine mRNA expression in skin

The transcriptional activity in the lesional skin samples was measured with a PCR. The amplification of cDNA was performed in 50 μL of a master mixture containing 0·5 μg of cDNA, 200 nmol deoxynucleotide triphosphate, 5 μL of PCR buffer, 2 U of Taq polymerase (ABI) and 2 μmol of each specific primer for the DNA of interest. The following primers were used for PCR reactions (5′–3′), mouse IFN-γ: TCAAGTGGCATAGATGTGGAAGAA and TGGCTCTGCAGGATTTTCATG; mouse IL-2: CCTGAGCAGGATGGAGAATAACA and TCCAGAACATGCCGCAGAG; mouse IL-4: CACTGACGGCACAGAGCTATTGATG and TCATGGTGCAGCTTTCGATGAATC; mouse IL-10: CTCTTACTGACTGGCATGAGGATCAGCAGG and TCTTCACCTGCTCCACTGCCTTGCTCTTAT; mouse IL-12: TCCTGCACTGCTGAAGACATC and TCTCGCCATTATAGATTCAGAGAC; mouse IL-13: AGACCAGACTCCCCTGTGCA and TGGGTCCTGTAGATGGCATTG; mouse β-actin: TGGAATCCTGTGGCATCCATGAAAC and TAAAACGCAGCTCAGTAACAGTCCG.[26] PCR was performed under the following conditions: 95 °C for 5 min, followed by 35 or 40 cycles of 95 °C for 30 s, 56 °C (IFN-γ, IL-12) or 60 °C (IL-2, IL-4, IL-10, IL-13, β-actin) for 30 s, and 72 °C for 1 min were carried out. After the final cycle, the temperature was maintained at 72 °C for 7 min. PCR amplified fragments were electrophoresed through 1·5% agarose gels in tris-acetate EDTA buffer containing ethidium bromide, and the gels were scanned under ultraviolet light. The mRNA of β-actin was used as an internal control. The signal intensity of each reverse transcriptase (RT)-PCR product was estimated using an ATTO Lane & Spot Analyzer (ATTO, Shizuoka, Japan).

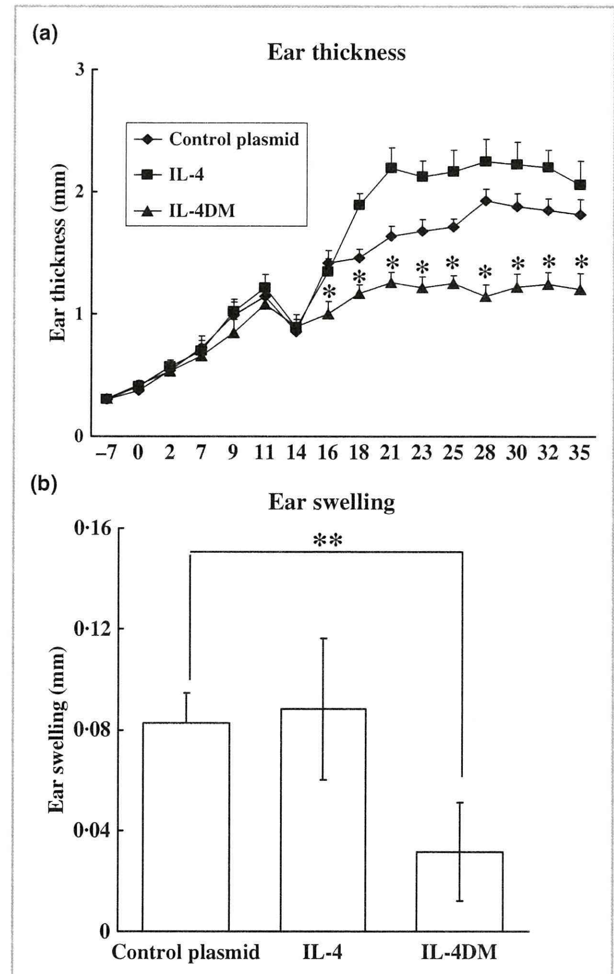

Fig 2. The effects of interleukin (IL)-4DM, IL-4, and control plasmid (pcDNA3.1) on ear swelling induced by repeated application of oxazolone (OX). (a) Ear thickness was measured before each OX challenge. Each point represents the mean ± SD of seven or eight mice. *$P < 0·05$: significantly different from the control group and IL-4 (Student's t-test). (b) Inhibition of the effecter phase of chronic hypersensitivity by IL-4DM, IL-4, and control plasmid DNA transfer. The ear swelling was measured 30 min after applying OX. The ear swelling in the IL-4DM groups was significantly suppressed compared with those in the IL-4 and control plasmid DNA groups. *Significant difference from the control by Student's t-test at $P < 0·05$.

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Cytokine production from splenocytes

A suspension of 2×10^6 splenocytes were made in a solution of 200 μL RPMI-1640 medium (Nikken Bio Medical Laboratory, Kyoto, Japan) containing 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), 50 UI penicillin, 50 μg mL^{-1} streptomycin, and 5 μg mL^{-1} soluble antimouse CD3 (BD Bioscience), and 10 μg mL^{-1} antimouse CD28 (BD Bioscience). Cells were dispensed in triplicate into 96-well flat-bottomed microplates (Sumitomo Bakelite, Tokyo, Japan). After incubation for 48 h at 37 °C in a humidified incubator (5% CO_2), culture supernatants were collected and analysed for IFN-γ (Quantikine; R&D Systems, Minneapolis, MN, U.S.A.) or IL-4 (Quantikine; R&D Systems) production with an ELISA according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using Student's t-test and Mann–Whitney U-test. Values are expressed as mean ± SEM. A 95% confidence limit was taken as significant ($P < 0.05$).

Result

Ear thickness with the treatment of IL-4DM, IL-4, or control plasmid

In the control group, the ear thickness increased from the beginning of the challenge, and increased gradually through the experiments (Fig. 2a). The agonistic IL-4 DNA treatment augmented increase of the ear thickness after day 16. In contrast, IL-4DM DNA treatment significantly suppressed increase of the ear thickness compared with that of control plasmid or IL-4DNA-treated mice.

Effects of IL-4DM on the oxazolone-induced acute-phase ear swelling

The ear swelling was also measured 30 min after OX application on day 35, and the difference between before and 30 min after application was calculated. IL-4DM DNA treatment suppressed the ear swelling significantly compared with that of the control DNA-injected group (Fig. 2b). However, IL-4DNA showed no suppressive effects.

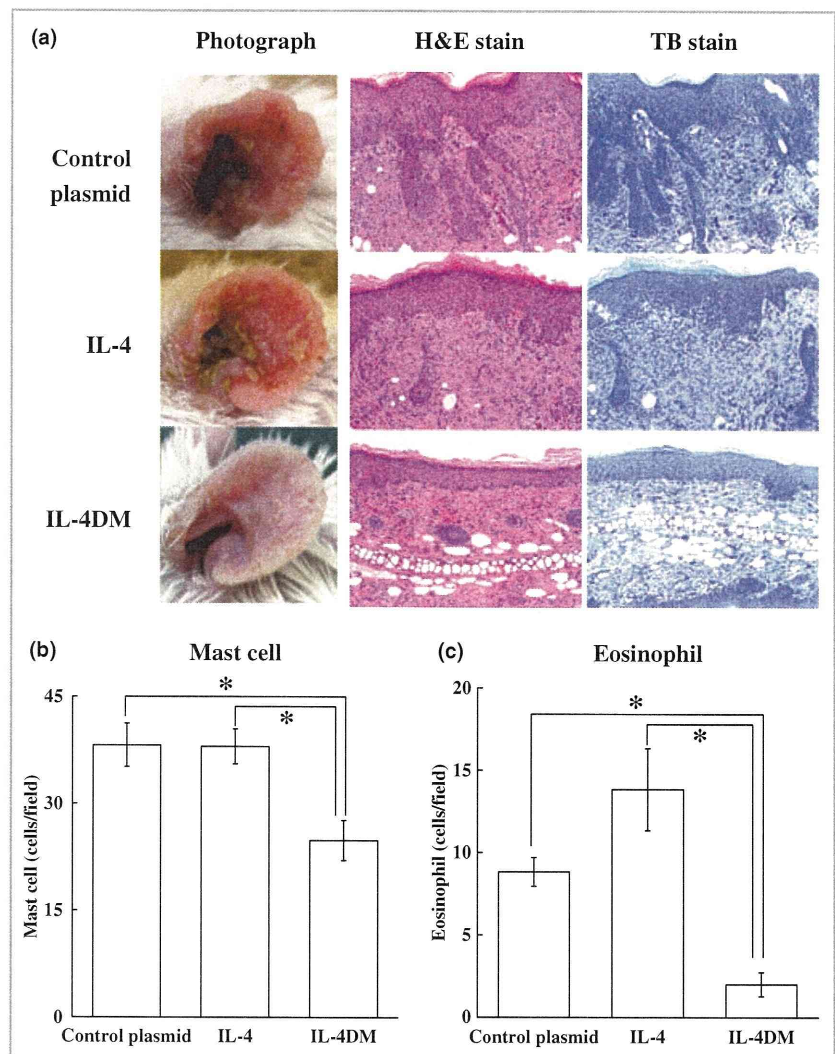

Fig 3. (a) Representative photographs and histological feature of oxazolone (OX)-treated skin lesion. OX-sensitized ear revealed hyperkeratosis, acanthosis, and parakeratosis in control and interleukin (IL)-4-treated mice. An increased number of infiltrating lymphocytes, macrophages and mast cells was observed in the skin lesions, all of which are typical histological findings observed in patients with atopic dermatitis. In contrast, acanthosis was clearly suppressed, and skin infiltration of granulocytes, eosinophils, and mast cells was decreased in the IL-4DM-treated mice as compared with control plasmid-treated mice (original magnification × 200). (b) The number of dermal mast cells was counted, and found to be decreased in the IL-4DM-treated mice. (c) The number of dermal eosinophils was also counted in 10 high power fields. The skin infiltration of eosinophils was significantly decreased in the IL-4DM-treated mice. Data are expressed as the mean ± SEM. *Significant difference by Student's t-test at $P < 0.05$.

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