Nasal Immunization with a Vaccinia Virus-based Pentavalent H5N1 Avian Influenza Vaccine Induces Cellular Immunity in the Respiratory Tract of Mice

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Abstract

A previous study showed that vaccinia virus-vectored pentavalent H5N1 influenza vaccine adjuvanted with IL-15 injected via the parenteral route induced broadly effective protective immunity. However, influenza virus is known to infect the mucosal surfaces of the respiratory tract, and nasal immunization induces effective protective immunity against respiratory tract infection. Therefore, we explored the vaccine potential of nasal immunization with the vaccinia virus-vectored multivalent pandemic influenza vaccine candidate. BALB/c mice were nasally immunized with the Wyeth strain of vaccinia virus-based pentavalent H5N1 vaccine adjuvanted with IL-15 or Wyeth virus. Four weeks after the first immunization, the mice received a booster, and antigen (Ag)-specific antibody (Ab) and cellular immune responses induced in the vaccinated mice were analyzed 3 weeks later. Nasal immunization of the pentavalent Wyeth-based H5N1 vaccine induced broadly reactive Ag-specific systemic Ab responses compared with Wyeth-vaccinated mice. The secretory IgA Ab response was induced in the lower respiratory tract, although the degree of induction was marginal. The number of IFN- γ -producing cells, and the frequencies of IFN- γ ⁺ CD4⁺, TNF- α ⁺ CD4⁺ and TNF- α ⁺ CD8⁺ T cells were significantly increased in the lungs of mice immunized with Wyeth-based H5N1 vaccine. The results of this study suggested that although the immunogenicity of Wyeth-based pentavalent H5N1 vaccine expressing IL-15 was insufficient as a mucosal vaccine, cellular immunity was preferentially induced in the respiratory mucosal compartment, which is the site of influenza virus infection, when the vaccine was delivered via the nasal mucosa.

Keywords :

avian influenza vaccine,
H5N1, vaccinia virus,
nasal immunization

Introduction

Influenza is a contagious acute respiratory disease caused by influenza virus infection. Influenza epidemics include seasonal influenza and pandemics (1). The appearance of novel and highly pathogenic influenza virus strains may lead to respiratory disorders with significant morbidity and high mortality, and may cause pandemics. The first influenza pandemic (Spanish Flu) occurred in 1918, and was caused by the influenza A H1N1 virus. Influenza A H2N2 infection caused a further pandemic in 1958 (Asian Flu). The third

pandemic, in 1968, was caused by influenza A H3N2 (Hong Kong Flu). The most recent pandemic occurred in 2009, and was caused by influenza A H1N1 of swine origin (2). Novel mutations can potentially break the xenogenic barrier and generate serious pandemics. Furthermore, some subtypes of avian influenza virus have been shown to sporadically infect both avian species and humans. These subtypes include influenza A H5N1, H7N2, H7N3, H7N7, H7N9, H9N2, and H10N7. Although the pathogenicity of these subtypes is generally low, the H5N1 and H7N9 subtypes are highly pathogenic; the development of effective vaccines against such highly pathogenic influenza strains is urgently required in preparation for possible future outbreaks (3, 4).

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An inactivated, detergent-extracted H5N1 vaccine demonstrated potency, and has been accepted as a pandemic vaccine (5). However, the immunogenicity of this vaccine may be poor and high levels of antigens (Ags) and adjuvant are required for vaccination. Therefore, it is necessary to develop a vaccine with a more efficient delivery system, and a proper adjuvant and delivery route. The efficacy of various H5N1 vaccines has been studied, and live attenuated H5N1 vaccine and live vector-delivered H5N1 vaccines were shown to be effective compared with inactivated subunit or split vaccines (6, 7). Furthermore, a vaccinia virus (Wyeth strain) expressing five Ags, i.e., hemagglutinin, H5, neuraminidase, N1, and nucleoprotein (NP), derived from the H5N1 influenza virus A/Vietnam/1203/2004, and matrix polypeptides M1 and M2, derived from the H5N1 influenza virus A/CK/Indonesia/PA/2003 with the molecular adjuvant, IL-15, was generated in a previous study (8). This pandemic influenza vaccine candidate was shown to induce effective Ag-specific antibody (Ab) responses in serum against diverse subtypes of influenza viruses, and also produced cellular immune responses and protected against H5N1 infection when injected subcutaneously (8).

Vaccination via the mucosal route induces both systemic and local mucosal immune responses, resulting in two layers of protection against infectious diseases (9). As influenza is a respiratory disease and influenza virus infects humans primarily through the mucosal route (1), induction of influenza-specific immune responses in the bronchial mucosa is a logical means of preventing influenza virus infection. Nasal immunization is known to induce mucosal immunity in the bronchial compartment (10, 11).

The present study was performed to assess the Ag-specific immune responses induced by nasal immunization with Wyeth, expressing five H5N1-derived Ags along with IL-15 (Wyeth/IL-15/5Flu), and to evaluate the vaccine potential of this influenza vaccine candidate.

Materials and methods

Mice

Female BALB/c mice were obtained from Japan SLC, Inc. (Tokyo, Japan). All animal experimental protocols were approved by the Nihon University Animal Care and Use Committee, and all animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals of Nihon University School of Dentistry (Matsudo, Japan).

Antigen and immunization

The recombinant Wyeth vaccinia viruses with five influenza genes (H5 hemagglutinin, N1 neuraminidase, NP, M1, and M2) along with IL-15 (Wyeth/IL-15/5Flu) were generated in a previous study (8). Mice were immunized nasally with 25- μ L aliquots (12.5 μ L per nostril) of phosphate-buffered saline (PBS) containing 10^7 plaque-forming units (PFU) of Wyeth/IL-15/5Flu or Wyeth ($n=6$ for each group). A second booster dose was given 4 weeks later. Plasma and bronchoalveolar lavage samples were collected 3 weeks after boosting. The trachea was cannulated with a catheter (BD Angiocath; Becton Dickinson and Company, Franklin Lakes, NJ, USA) and the lungs were washed with 0.6 mL of PBS to obtain bronchoalveolar lavage samples, as described elsewhere (12).

Detection of Ag-specific Ab responses

Influenza Ag-specific Ab titers were determined by enzyme-linked immunosorbent assay (ELISA), as described previously (13). Briefly, plates were coated with recombinant H5 hemagglutinin (0.5 μ g/mL) or the lysate of H1N1-infected MDCK cell culture supernatant inactivated by irradiation in 0.2 M sodium carbonate coating buffer (1×10^6 PFU/mL) for overnight. The plates were then washed and blocked with blocking buffer containing 20 mM Tris, 150 mM NaCl, 0.1% Tween 20, and 0.1% bovine serum albumin. After a 2-hour incubation at room temperature, 32-fold dilution of plasma or 2-fold dilution of bronchoalveolar lavage samples was added, followed by incubation for 1 hour. After washing, horseradish peroxidase (HRP)-labeled goat anti-mouse γ or α heavy chain-specific Ab (Southern Biotech, Birmingham, AL, USA) was added, followed by incubation for 1 hour. Finally, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and H_2O_2 were added for color development.

Enumeration of IFN- γ -secreting cells

Mononuclear cells (MNCs) were obtained from the spleen by gently teasing the tissue through a sterile stainless steel screen. The lung MNCs were obtained using a discontinuous density gradient of Percoll (GE Healthcare, Chicago, IL, USA) after digestion with collagenase (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). MNCs were restimulated with or without H1N1 A/PR/8/34 virus at a multiplicity of infection (MOI) of 10, *in vitro* for 1 hour. ELISpot assay for IFN- γ -secreting cells was performed using a commercial kit (Mabtech, Nacka Strand, Sweden).

according to the manufacturer's instructions. Briefly, MNCs were added into 96-well nitrocellulose plate coated with anti-IFN- γ antibody. After incubation, the detection antibody was added, and then the plate was developed with TMB substrate to detect IFN- γ -secreting cells. Complete RPMI 1640 containing 10% fetal bovine serum, 50 μ M 2-mercaptoethanol, 10 mM HEPES, 100 μ U/mL penicillin, and 100 μ g/mL streptomycin was used as blocking and culture media. The number of IFN- γ -secreting cells was counted using a stereomicroscope (SZX7) and cellSens standard 1.16 software (Olympus, Tokyo, Japan).

Intracellular cytokine staining

MNCs restimulated with H1N1 A/PR/8/34 virus as described above were further cultured for 5 hours, followed by additional culture for 10 hours with GolgiPlug containing Brefeldin A (Becton Dickinson and Company). The MNCs were stained with fluorescein isothiocyanate (FITC) - conjugated anti-CD4 or anti-CD8 monoclonal (m) Abs followed by intracellular staining with phycoerythrin (PE) - labeled anti-IFN- γ or -TNF α mAbs (Becton Dickinson and Company). Flow cytometry analysis was performed using a FACSCalibur system (Becton Dickinson and Company). Data were analyzed using FLOWJO software (Tree Star, Ashland, OR, USA).

Statistics

The data are presented as means $\pm$ standard error of the mean (SEM). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple-comparison test with BellCurve (Social Survey Research Information Co., Ltd. Tokyo, Japan) for Excel software (Microsoft Corp., Redmond, WA, USA). In all analyses, $p < 0.05$ was taken to indicate statistical significance.

Results

Nasal recombinant vaccinia vaccine expressing H5N1 influenza Ags elicits Ag-specific Ab responses

Initially, we examined whether nasal immunization with the recombinant Wyeth strain of vaccinia virus expressing Ags derived from H5N1 influenza A virus, i. e., H5 hemagglutinin, N1 neuraminidase, M1 and M2 matrix proteins, and NP (Wyeth/IL-15/5Flu) (8), could induce Ag-specific Ab responses. Mice nasally immunized with this vaccinia-based vaccine showed significant greater Ag-

specific plasma IgG Ab responses to H1N1 virus compared with Wyeth vaccinia virus-immunized mice (Fig. 1A). Further, Wyeth/IL-15/5Flu-immunized mice showed elevated anti-H5 IgG Ab responses (Fig. 1B). In contrast, H5-specific secretory (S) IgA Abs were produced at low but detectable levels in the lung (Fig. 1C). However, SIgA anti-H1N1 Abs were not detected in the lungs of Wyeth-IL-15/5Flu-immunized mice (data not shown). As expected, nasal immunization with Wyeth vector induced no SIgA Ab responses in any of the Ags examined. These results indicated that nasal immunization with Wyeth/IL-15/5Flu induces Ag-specific Ab responses.

Nasal recombinant vaccinia vaccine expressing H5N1 influenza Ags produced IFN- γ -secreting cells in systemic tissue and the lower respiratory tract

We next analyzed the number of IFN- γ -secreting cells produced in the spleen and lungs of mice nasally immunized with Wyeth/IL-15/5Flu. The results of ELISpot assay showed significantly greater numbers of IFN- γ -secreting cells among the MNCs isolated from the spleen and lungs of Wyeth/IL-15/5Flu-vaccinated mice after restimulation of the MNCs with H1N1 virus *in vitro*. Further, the numbers of IFN- γ -producing cells in the lung were higher than in the spleen. In contrast, mice vaccinated with Wyeth vector virus showed fewer IFN- γ -secreting cells in the spleen and lung compared with those vaccinated with 5Flu Ag-expressing vector virus (Fig. 2A and B). These results indicated that nasal immunization with Wyeth/IL-15/5Flu induced broadly reactive cellular immune responses against influenza virus.

Nasal recombinant vaccinia vaccine expressing H5N1 influenza Ags increased cellular immunity-related cytokine-producing cells in the lung

We next analyzed cellular immunity-related cytokine-producing cells in Wyeth/IL-15/5Flu-vaccinated mice. Splenic and lung MNCs were isolated from vaccinated mice, and restimulated with H1N1 virus *in vitro* followed by FACS analysis to determine intracellular cytokine production. Our results showed that the frequency of TNF- α ⁺ CD4⁺ cells was significantly increased in the spleen of Wyeth/IL-15/5Flu vaccinated mice (Fig. 1A). Furthermore, the frequencies of TNF- α ⁺ CD4⁺, IFN- γ ⁺ CD4⁺, and IFN- γ ⁺ CD8⁺ T cells were significantly increased in the lungs of vaccinated mice (Fig. 1B). These results indicated that the levels of

Fig. 1. Influenza-specific antibody (Ab) responses. (A) H1N1 virus-specific plasma IgG, (B) recombinant (r) H5-specific plasma IgG, and (C) rH5-specific IgA in bronchoalveolar lavage. Groups of mice were nasally immunized with 10^7 plaque-forming units (PFU) of Wyeth ($n=6$) or Wyeth/IL-15/5Flu ($n=6$, closed bar) on days 0 and 28. Plasma and lung wash were collected 3 weeks after the second immunization, and 32-fold dilution of plasma and 2-fold dilution of bronchoalveolar lavage were subjected to enzyme-linked immunosorbent assay (ELISA). The results are expressed as means $\pm$ SEM. *, $p < 0.05$ and **, $p < 0.01$ compared with Wyeth-treated group. ND, not detectable.

cellular immunity-related cytokines were increased in the lungs compared with the spleen of mice vaccinated with Wyeth/IL-15/5Flu via the nasal mucosal route.

Discussion

In this study, we evaluated the efficacy of vaccinia virus-based pentavalent H5N1 influenza vaccine as a mucosal vaccine. Nasal immunization with this Wyeth strain of vaccinia virus-vectored pentavalent vaccine elicited systemic IgG Ab responses to avian H1 and H5 subtypes, as well as cellular immune responses. A previous study demonstrated the potential of this Wyeth-vectored pandemic influenza vaccine candidate, as the pentavalent H5N1 influenza vaccine candidate induced humoral Ab responses with broad cross-reactivity against avian influenza virus subtypes when administered via the parenteral route. Thus, subcutaneous immunization with this pentavalent Wyeth-based vaccine candidate is effective against influenza. Vaccination via the parenteral route induced protective immunity only in the systemic compartment (14). However, influenza virus infection occurs mainly via the mucosal surface of the respiratory tract (1) and nasal delivery of Ags is the best way to induce protective immunity in the respiratory tract (10, 11). Therefore, we explored the potential of this Wyeth-based vaccine as a mucosal vaccine. The results of the present study indicated that intranasal delivery of the Wyeth-based H5N1 vaccine candidate induced broadly reactive humoral systemic IgG Ab re-

sponses (Fig. 1A and B). However, the induction levels of these IgG Ab responses were lower than those in mice vaccinated via the subcutaneous route at the same dose (data not shown). In this context, it has been shown that even though mucosal immunization resulted in both systemic and mucosal immune responses, parenteral delivery of Ags induced superior systemic immune responses (15). In contrast, similar levels of systemic IgG Ab responses were induced by immunization with formalin-fixed inactivated influenza virus, both nasally and subcutaneously (16).

Furthermore, nasal pentavalent Wyeth-based vaccine induced low but detectable mucosal SIgA Ab responses in the bronchial mucosa (Fig. 1C). On the other hand, previous studies have shown that nasal vaccination with inactivated influenza virus plus enterotoxin from *Escherichia coli* as a mucosal adjuvant elicited mucosal SIgA Abs in lung tissue (16). A recent study showed that nasal vaccination with HA1 derived from H5N1 subunit vaccine fused with the immunoenhancer, foldon, together with mucosal adjuvant including CpG, induced elevated levels of mucosal SIgA Ab responses in the respiratory tract (17). Another study showed that heat-inactivated H9N2 whole virus plus polyethyleneimine elicited significant SIgA Abs in the mucosal compartment when administered nasally (18). Taken together, these observations indicated that inactivated and subunit nasal vaccine candidates require mucosal adjuvant for SIgA induction, and fusion partners to enhance

Fig. 2. Numbers of IFN- γ -secreting cells in the spleen (A) and lung (B). Groups of mice were nasally immunized with 10⁷ PFU of Wyeth ($n=6$) or Wyeth/IL-15/5Flu ($n=6$, closed bar) on days 0 and 28. Mononuclear cells (MNCs) were isolated from the spleen and lungs of mice 3 weeks after the second immunization, restimulated with H1N1 A/PR/8/34 virus at a multiplicity of infection (MOI) of 10 *in vitro*, and then subjected to IFN- γ -specific ELISpot assay. The results are expressed as means $\pm$ SEM. *, $p < 0.05$.

immunogenicity (11). Therefore, we employed the cytokine IL-15, which contributes to enhancement of CD8⁺ T cell-mediated cellular immunity (13), as an immune stimulator. Furthermore, the introduction of IL-15 genes into the vector virus enhanced Ag-specific Ab production against coexpressed vaccine Ags (8, 19). A previous study showed that IL-15 plays an important role in intestinal IgA induction by B1 cells (20). However, the present study indicated that IL-15 did not enhance mucosal IgA responses following nasal immunization with IL-15-expressing Wyeth-based influenza vaccine (Fig. 1C).

The mucosal surface is protected by various host defense systems, including the mucus layer and antimicrobial peptides (21). Thus, vaccine Ags deposited directly on the mucosal surface encounter host defenses similar to microbial pathogens, resulting in low adsorption efficiency of the vaccine (22), which may lead to reduced induction of immune responses. Taken together, our results and the findings of these previous studies suggest that nasal immunization with Wyeth-vectored pentavalent vaccine elicits Ag-specific Ab responses in both systemic and mucosal compartments, although vaccine Ags delivered via the nasal route seem to have inferior immunogenicity to those delivered by parenteral injection.

Our results showed that nasal Wyeth-vectored pentavalent influenza vaccine induced cellular immunity, including IFN- γ production, via both CD4⁺ and CD8⁺ T cells, and

TNF- α production by CD4⁺ T cells in the lung. In contrast, cellular immunity-related cytokine production in the spleen was not enhanced by nasal immunization, with the exception of TNF- α production by CD4⁺ T cells (Figs. 2 and 3). These results indicated that cellular immunity was preferentially induced in the respiratory tract when vaccine was delivered via the intranasal route. In this regard, the main sites where protective immunity was induced differed depending on the route of Ag delivery, and intranasal immunization induced superior immune responses in the respiratory tract compared with oral immunization (10, 23). A recent study showed that the frequency of lung CD4⁺ IFN- γ ⁺ cells was increased by nasal immunization with H5N1 monovalent split vaccine, including HA together with cationic polymers. Although secretion of IFN- γ by CD8⁺ cells was slightly increased in this previous study, Ag-specific CD8⁺ cells showed marked expansion in the lung (24). Further, the combination of live attenuated influenza virus and nasal delivery was reported to be required for the induction of lung T cell responses (25). On the other hand, the splenic CD4⁺ and CD8⁺ T cells isolated from mice vaccinated subcutaneously with Wyeth-based pentavalent H5N1 vaccine showed increased IFN- γ production (8). Furthermore, it has been shown that T cell responses were preferentially induced in the spleen when adenovirus-based *Mycobacterium tuberculosis* vaccine was administered via the intramuscular route, whereas nasal vaccination of this

Fig. 3. Frequencies of cellular immunity-related cytokine-positive T cell subsets. Groups of mice were nasally immunized with 10^7 PFU of Wyeth/IL-15/5Flu ($n=6$) on days 0 and 28. The MNCs were isolated from the spleen (A) or lungs (B) of mice 3 weeks after the second immunization, restimulated with H1N1 A/PR/8/34 virus at a MOI of 10 *in vitro*, and then analyzed for intracellular TNF- α and IFN- γ expression in CD4⁺ and CD8⁺ T cells. The results are expressed as means $\pm$ SEM. *, $p < 0.05$ compared with MNCs without restimulation *in vitro*.

vaccine mainly induced T cell responses in the airway lumen (15). Taken together, our results and those of other studies indicated that live Wyeth virus-vectored pentavalent H5N1 vaccine induced cellular immunity in the respiratory tract when delivered via the nasal mucosal route, suggesting that nasal vaccine is appropriate for induction of influenza virus-specific cellular immune responses at the site of infection.

The results of the present study showed that nasal immunization with IL-15-expressing Wyeth-based pentavalent H5N1 vaccine induced Ag-specific systemic IgG Ab responses and marginal mucosal IgA Ab responses in the lower respiratory tract. Nasal delivery of this vaccine candidate preferentially induced cellular immunity in lung tissues rather than the spleen. These results suggest that although the immunogenicity of nasal Wyeth/IL-15/5Flu vaccine is insufficient, vaccine delivery by the nasal mucosa induced appropriate cellular immunity against influenza

virus infection in lung tissue.

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