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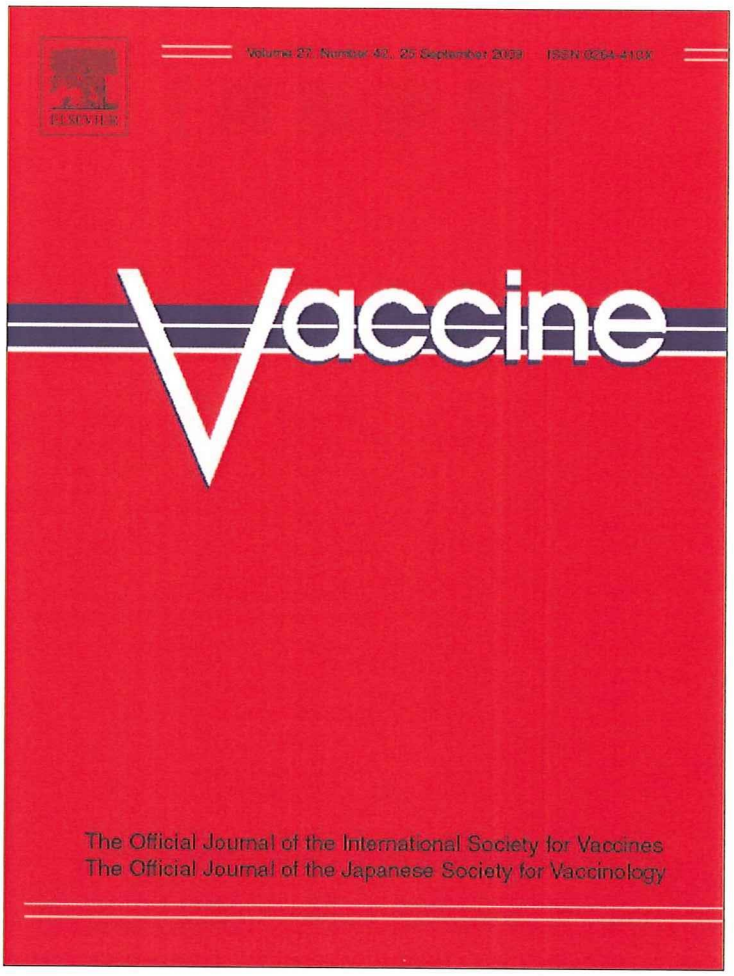

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Poly(γ-glutamic acid) nano-particles combined with mucosal influenza virus hemagglutinin vaccine protects against influenza virus infection in mice

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ABSTRACT

Adding poly(γ-glutamic acid) nano-particles (γ-PGA-NPs), a safe, natural material, to subcutaneous immunization with influenza virus hemagglutinin (HA) vaccine increases the protective immune responses against influenza virus in mice. Here, we examined whether intranasal administration of the HA vaccine with γ-PGA-NPs would induce protection from influenza virus challenge in mice. Intranasal immunization with the mixture of γ-PGA-NPs and HA vaccine from an influenza virus strain A/PR/8/34 (H1N1) or A/New Caledonia/20/99 (H1N1) enhanced protection of mice from A/PR/8/34 infection. Intranasal immunization with A/New Caledonia/20/99 HA vaccine and γ-PGA-NPs induced cell-mediated immune responses and neutralizing antibody production for both A/New Caledonia/20/99 and A/PR/8/34. These data suggest that γ-PGA-NPs may have potential for clinical applications as a mucosal adjuvant.
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1. Introduction

Influenza viruses belong to the *Orthomyxoviridae* family and are a major cause of respiratory disease in humans. Pandemics of influenza A virus are responsible for substantial mortality and morbidity, particularly in high-risk groups, which include the elderly and individuals with chronic underlying medical conditions [1]. Influenza virus infections in the elderly often lead to secondary bacterial infections that result in severe symptoms and occasionally death [2–5].

Influenza vaccines effectively prevent severe influenza virus infections; moreover, Nichol et al. [6,7] reported that influenza virus vaccines decrease the number of cases requiring hospitalization due to highly contagious respiratory diseases. In addition, in an animal study, influenza virus vaccine effectively protected the host animals from severe influenza virus–bacteria superinfectious diseases [8]. However, although natural infection can confer resistance to virus infection to a certain degree [9,10], inactivated parenteral vaccine is less protective against antigenic drift variants within a subtype of the influenza virus and does not provide protection against viruses from different subtypes [10–12].

Intranasal immunization with influenza virus hemagglutinin (HA) vaccines and adjuvant induces cross-protection against variants within a subtype and against different subtypes [11]. Furthermore, several studies revealed that the IgA for the influenza virus in the respiratory tract is more effective against virus infections than is the serum IgG antibody for the influenza virus that is induced by systemic immunization with parenteral vaccines in humans and mice [13,14]. Therefore, recent studies have aimed at finding an immunization procedure for stimulating mucosal IgA antibody production by influenza virus HA vaccine. Tamura et al. [15,16] demonstrated in mice that intranasal immunization with influenza virus HA vaccines and cholera toxin B subunit (CTB) can protect the host against drift variants within a subtype of the influenza virus and against different subtypes of influenza viruses. They also found [15,16] that intranasal immunization, but not subcutaneous or intraperitoneal immunization with the vaccine and CTB induced cross-reactive IgA antibody production in the respiratory tract. Therefore, they suggested that the cross-protection may be associated with sufficient level of secreted IgA antibody.

Most influenza virus HA vaccines are poor immunogens when delivered to the mucosa without adjuvant [8,17]. In the 1980s and 1990s, CTB and *Escherichia coli* heat-labile toxin (LT) were used as adjuvants in studies of mucosal vaccines [18,19]. However, these

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toxins cause severe diarrhea and nasal discharge; therefore, a safer and more effective adjuvant is needed for intranasal influenza virus vaccines. Recent studies have demonstrated that synthetic double-stranded RNA polyriboinosinic acid–polyribocytidylic acid (poly(I:C)) and modified pulmonary surfactant are safe and potent candidates for use as an adjuvant in mucosal influenza virus vaccines [17,20]. These materials induce dendritic cell (DC) activation, which plays an important role in mucosal adjuvant activity.

We recently demonstrated that subcutaneous immunization with influenza virus A/FM/1/47 or A/New Caledonia/20/99 HA vaccine and poly(γ-glutamic acid) nano-particles (γ-PGA-NPs) enhances protective immune responses against strain A/FM/1/47 or A/New Caledonia/20/99 infection [21]. γ-PGA is a bacterial capsular exopolymer produced by certain strains of *Bacillus natto*, which is a natural component of natto, a healthful traditional Japanese food item. γ-PGA-NPs are naturally degraded by γ-glutamyl transpeptidase, which is widely distributed throughout the body [22]. The γ-PGA-NPs are taken up by DCs and subsequently localized to lysosomal compartments. Immature DCs stimulated with γ-PGA-NPs produce TNF-α and IL-12, and up-regulate expression of CD40, CD80 (B7-1), and CD86 (B7-2), resulting in an enhanced T-cell stimulatory capacity. These maturational changes in the DCs involve the MyD88-mediated NF-κB signaling pathway [22].

In the present study, we examined whether γ-PGA-NPs could function as an effective mucosal adjuvant for influenza virus HA vaccine. Here we report that the addition of γ-PGA-NPs to the intranasal influenza virus HA vaccine in mice induced cross-protective immune responses against influenza virus infection.

2. Materials and methods

2.1. Animals, virus strains, and HA vaccine

Female BALB/c mice were obtained from Japan SLC Inc. (Hamamatsu, Japan) and Charles River Japan (Yokohama, Japan), and bred in our facility at the National Institute of Biomedical Innovation. All the mice used in this study were 4 weeks old. Influenza A virus strains A/New Caledonia/20/99 (H1N1) and A/PR/8/34 (H1N1) for the neutralization test were prepared at Kanonji Institute, the Research Foundation for Microbial Diseases of Osaka University (Kanonji Institute; Kanonji, Japan). Mouse adapted A/PR/8/34 (H1N1) strain for a virus challenge test was kindly provided from Dr. Hideki Hasegawa (National Institute of Infectious Diseases, Tokyo, Japan). Influenza virus HA vaccines (split-product virus vaccines) were prepared from the influenza virus A/New Caledonia/20/99 strain and A/PR/8/34 at Kanonji Institute.

2.2. Synthesis and preparation of γ-PGA-NPs, and preparation of HA-loaded γ-PGA-NPs

γ-PGA (number average molecular weight, Mn = 380,000) was kindly donated by Meiji Seika Co., Ltd. (Tokyo, Japan). Nano-particles composed of γ-PGA hydrophobic derivatives were prepared as previously described [21,23]. Briefly, γ-PGA was hydrophobically modified by L-phenylalanine ethylester (L-Phe) (Sigma, St. Louis, MO) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (WSC) (Wako Pure Chemical Industries, Osaka, Japan). The purified γ-PGA-graft-L-Phe was characterized by ^{1}H NMR to determine the degree of L-Phe grafting. In this study, γ-PGA-graft-L-Phe with a 53% degree of grafting was used. Nano-particles composed of γ-PGA-graft-L-Phe were prepared by a precipitation and dialysis method. First, the γ-PGA-graft-L-Phe (10 mg) was dissolved in 1 ml of dimethyl sulfoxide (DMSO), and an equal volume of saline was added, yielding a translucent solution. The solution was then dialyzed against distilled water with cellulose membrane tubing (50,000 molecular weight cut-off) to remove the organic solvents. The dialyzed solution was then freeze-dried. HA-loaded γ-PGA-NPs were prepared as described previously [21].

2.3. Vaccination, sample preparation, and measurement of anti-IgG and IgA antibodies for influenza virus antigen

BALB/c mice were anesthetized and inoculated subcutaneously (s.c.; inoculation volume was 200 μl) or intranasally (i.n.; inoculation volume was 20 μl) with PBS or PBS containing 0.5 μg influenza virus HA vaccine (HA vaccine), 100 μg HA vaccine encapsulated- or HA vaccine immobilized-PGA-NP, or a mixture of 0.5 μg HA vaccine and 10, 30, or 100 μg γ-PGA-NPs, or 10 μg poly(I:C), on days 0 and 28. Mouse sera, nasal wash fluids, and bronchoalveolar lavage (BAL) fluids were harvested 14 days after the final immunization, and analyzed for influenza virus antigen-specific IgG and IgA antibodies using procedures described previously [24]. The results are presented as the difference between the absorbances of the pre- and post-immunization samples. The reciprocal endpoint titers of influenza virus antigen-specific IgA and IgG antibodies were defined as the highest dilutions giving an A_{450} of 0.2.

2.4. Measurement of anti-influenza virus neutralization antibody titer

The neutralizing antibody titer in the mouse sera was determined by a micro-cytopathic effect (CPE) neutralizing test. For CPE neutralizing test, the sera, BAL fluids, and nasal wash fluids were subjected to twofold serial dilutions. The antibody solutions (100 μl) were mixed 1:1 (v/v) with a suspension containing 100 $TCID_{50}$ of highly purified A/New Caledonia/20/99 or A/PR/8/34 particles and incubated at 37 °C for 1 h. The virus-antibody mixture was then transferred onto Madin-Darby canine kidney (MDCK) cell monolayers in 96-well plates and incubated at 37 °C for 1 h. The cells were then washed with Minimum Essential Medium (MEM) supplemented with 0.01 M HEPES, 0.2% Bovine albumin, and 10 μg/ml of trypsin (MEM maintenance medium), 100 μl MEM maintenance medium was added to each well, and the cells were incubated at 37 °C in a 5% CO_2 incubator. The CPE status was observed 72 h after the infection. The culture medium was removed by absorption and the adherent MDCK cells were fixed with 4% formaldehyde–PBS for more than 1 h. The formaldehyde–PBS was then removed by absorption, and the fixed cells were stained with 0.1% Naphthol blue black for 30 min. After the plates were washed, 50 μl of 0.1 M NaOH was added to each well, and the A_{595} was read using a microplate reader. The neutralizing antibody titer was expressed as the reciprocal of the highest serum, BAL fluid, or nasal wash fluid dilution that yielded 50% neutralization of 100 $TCID_{50}$ of virus.

2.5. Cell proliferation assay and cytokine ELISAs

The spleen of each immunized mouse was harvested 14 days after the final vaccination. Mononuclear cells (MNCs; 2.5×10^5 cells) isolated from the organs were suspended in 200 μl of RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 5×10^{-5} M β-mercaptoethanol (complete RPMI 1640 medium), and restimulated for 4 days with various concentrations of A/New Caledonia/20/99 or A/PR/8/34 HA vaccine in 96-well flat-bottomed microtiter plates. Cell proliferation was assayed by treating the cells for the last 18 h of culture with [^{3}H] thymidine (0.5 μCi/well; Perkin-Elmer, Wellesley, MA). The incorporated radioactivity was counted using a TopCount NXT™ microplate scintillation luminescence counter (Perkin-Elmer). For cytokine assays, MNCs (1.25×10^6 cells) were added to each well of 48-well plates in 0.5 ml of complete RPMI 1640 medium and stimulated with the

indicated concentrations of the HA vaccine for 36 h at 37 °C and in 5% CO_2. The amount of interleukin (IL)-4, IL-6, and γ-interferon (IFN-γ) in each culture supernatant was determined using the OptEIA ELISA set (BD Biosciences-Pharmingen, San Diego, CA).

2.6. IFN-γ ELISPOT assay

Ninety-six-well filtration plates (Millipore, Bedford, MA) were coated with anti-mouse IFN-γ mAb (5 μg/ml, clone RA-6A2, BD Biosciences-Pharmingen) overnight. After the plates were washed four times with PBS, 200 μl of PBS supplemented with 10% FBS was added to each well, and the plates were incubated at 37 °C. MNCs (10^6 cells) from influenza virus HA vaccine- and/or adjuvant-treated mice suspended in 100 μl of complete RPMI 1640 medium were added to each well of the filtration plates, along with UV-inactivated influenza virus particles suspended in 100 μl of complete RPMI-1640 medium, and the plates were incubated for 48 h at 37 °C. The plates were then washed six times with PBS supplemented with 0.1% Tween 20 (PBS-T) and incubated with biotin-conjugated anti-mouse IFN-γ mAb (1 μg/ml, clone XMG-1.2, BD Biosciences-Pharmingen) for 2 h at room temperature. After five washes with PBS-T, horseradish peroxidase (HRP)-conjugated streptavidin (1:800; BD Biosciences-Pharmingen) was added to the wells, and the plates were incubated for 1 h at room temperature. The plates were then washed five times with PBS-T, followed by a 5-min incubation in stable TMB-H solution (Moss Inc., Pasadena, MD) to develop the reaction. The reaction was stopped by washing the plates with double-distilled water. The spots were then counted with a KS-ELISPOT reader (Carl Zeiss, Obercochen, Germany).

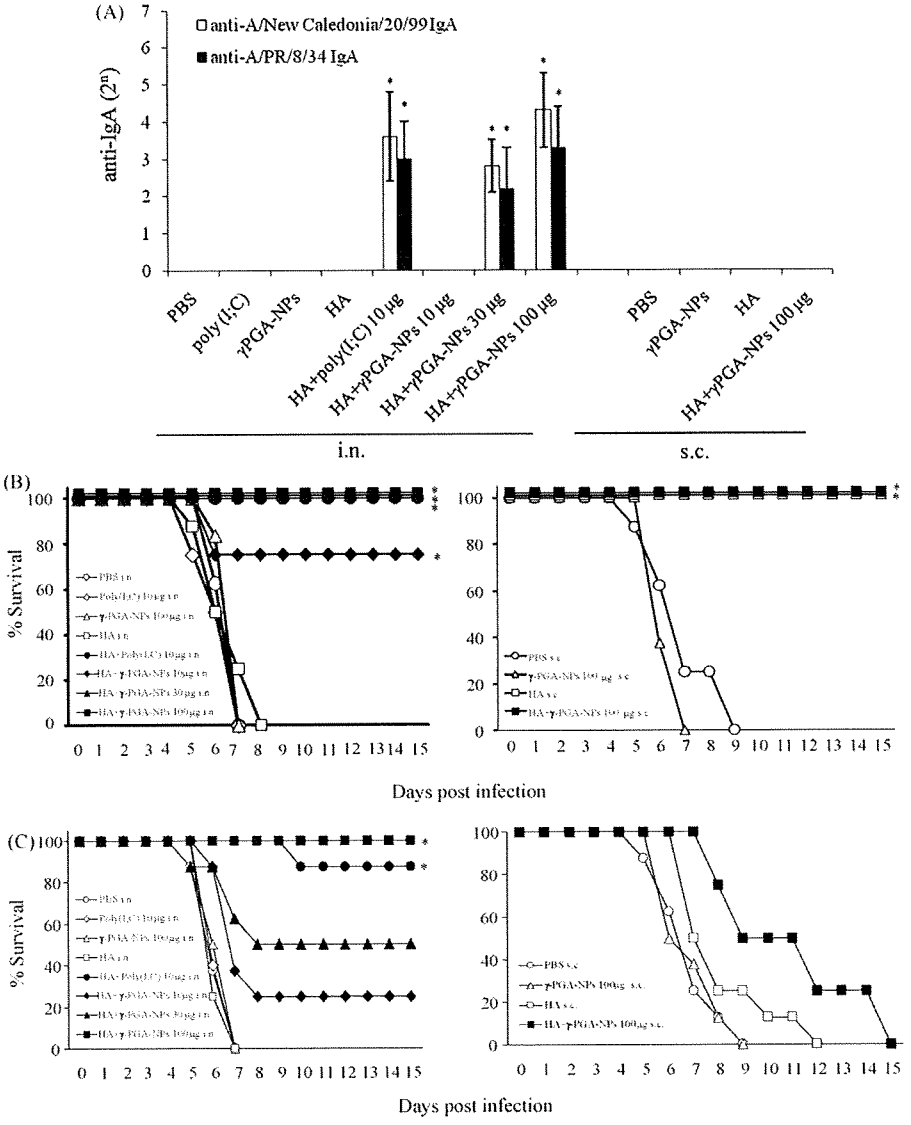

Fig. 1. Effect of adding γ-PGA-NPs to the intranasal influenza virus HA vaccine on the protective immune responses against influenza virus infections. (A) Groups of mice (5 per group) were intranasally (i.n.) or subcutaneously (s.c.) inoculated twice with PBS, 10 μg of poly(I:C), 100 μg of γ-PGA-NPs, 0.5 μg of A/New Caledonia/20/99 HA vaccine (HA), or a mixture of the HA and poly(I:C) or γ-PGA-NPs as described in Section 2. Fourteen days after the final immunization, mouse nasal wash fluid was collected and the titers of anti-IgA antibody for A/New Caledonia/20/99 and A/PR/8/34 were measured by ELISA. Bars represent means ± standard deviations of 5 mice. (B and C) Groups of 8 mice were immunized intranasally with A/PR/8/34 HA vaccine (B) or A/New Caledonia/20/99 HA vaccine (C) as described in (A). On day 42, the mice were infected with 2000 pfu of A/PR/8/34, and their mortality was assessed for the next 15 days. *$p < 0.05$ versus the group of mice immunized with HA alone.

2.7. Virus challenge

Mice were inoculated intranasally with 20 μl of PBS containing 2000 pfu (200 × LD_{50}) of the mouse-adapted A/PR/8/34 strain. The mortality of the mice was assessed daily for up to 15 days thereafter.

2.8. Statistical evaluations

Fisher's exact test was performed using Statcel2 software (OMS, Tokyo, Japan) to evaluate the differences between groups in the mortality experiments. To analyze the data in the other experiments, non-parametric Student's *t*-tests were used. *p*-Values of <0.05 were considered significant.

3. Results

3.1. Protective efficacy of intranasal immunization with A/PR/8/34 or A/New Caledonia/20/99 HA vaccine and γ-PGA-NPs against A/PR/8/34 infection in mice

We examined the efficacy of γ-PGA-NPs as a mucosal adjuvant for influenza virus HA vaccine. Mice (4 weeks old) were immunized subcutaneously or intranasally with A/New Caledonia/20/99 HA vaccine alone, a mixture of γ-PGA-NPs and the HA vaccine, or a mixture of poly(I:C) and the HA vaccine, on days 0 and 28. On day 42, nasal wash fluids were collected and the anti-influenza virus IgA antibody titers in the fluids were assessed. As shown in Fig. 1A, adding 10, 30, or 100 μg of γ-PGA-NPs, or 10 μg of poly(I:C) to the HA vaccine, but not the HA vaccine alone yielded detectable anti-IgA antibody titers for A/New Caledonia/20/99, however, subcutaneous administration with the HA vaccine and/or 100 μg of γ-PGA-NPs did not. Furthermore, the addition of γ-PGA-NPs or poly(I:C) to the HA vaccine also enhanced the anti-IgA antibody titer for a different strain, A/PR/8/34. Since the addition of poly(I:C) to the influenza virus HA vaccine enhances the protection of mice from infection with variants within a subtype and against different subtypes [20], we examined whether intranasal immunization of mice with the mixture of the HA vaccine and γ-PGA-NPs would protect them likewise. Therefore, to examine the cross-protection within a subtype, we used two different strains, A/New Caledonia/20/99 and A/PR/8/34 HA in this study. Mice were immunized intranasally with a mixture of γ-PGA-NPs and A/New Caledonia/20/99 HA or A/PR/8/34 HA vaccine, followed by infection with A/PR/8/34. Fifteen days after infection, all of the mice that had been immunized with A/PR/8/34 HA vaccine alone or A/New Caledonia/20/99 HA vaccine alone had died. All of the mice immunized intranasally with the mixture of influenza virus HA vaccine from A/PR/8/34 or A/New Caledonia/20/99 and 10 μg of poly(I:C) survived from lethal A/PR/8/34 infection (Fig. 1B and C). Immunization with mixture of A/PR/8/34 HA vaccine and more than 30 μg of γ-PGA-NPs survived all mice against A/PR/8/34 infection (Fig. 1B), although immunization with mixture of A/New Caledonia/20/99 HA vaccine and 10, 30, or 100 μg of γ-PGA-NPs survived 25%, 50%, and 100% of mice against A/PR/8/34 infection, respectively (Fig. 1C). We also examined whether subcutaneous immunization with the mixture of γ-PGA-NPs and A/PR/8/34 or A/New Caledonia/20/99 HA vaccine protected the host from a lethal infection with A/PR/8/34. Immunization with A/PR/8/34 HA vaccine, or the mixture of γ-PGA-NPs and A/PR/8/34 HA vaccine, survived all mice from the A/PR/8/34 infection (Fig. 1B), whereas no mice immunized subcutaneously with the A/New Caledonia/20/99 HA vaccine or the mixture of γ-PGA-NPs and A/New Caledonia/20/99 HA vaccine were survived (Fig. 1C). Therefore, the protection by subcutaneous vaccination was less effective for protection from the different influenza virus strain than that by intranasal vaccination. Here we used 4 weeks old mice for the experiments, therefore we examined whether the protection was also seen in older mice (7 weeks old). The intranasal immu-

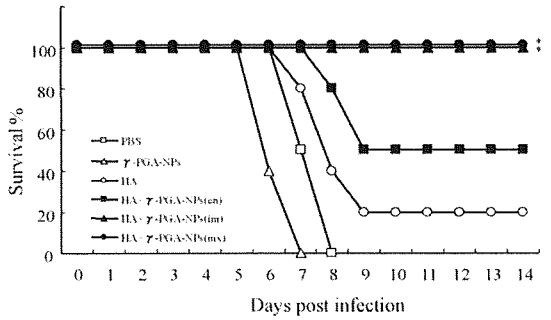

Fig. 2. Effect of different configurations of the combined HA vaccine and γ-PGA-NPs on protective immune responses against a lethal influenza virus infection. Groups of 5 mice were intranasally immunized with PBS, 100 μg of γ-PGA-NPs, 0.5 μg of A/New Caledonia/20/99 HA vaccine (HA), HA-encapsulated γ-PGA-NPs (HA + γ-PGA-NPs(en)), HA-immobilized γ-PGA-NPs (HA + γ-PGA-NPs(im)), or a mixture of HA and γ-PGA-NPs (HA + γ-PGA-NPs(mx)) on days 0 and 28. On day 42, the mice were infected with 2000 pfu of A/PR/8/34 and their mortality was assessed for the next 14 days. *$p < 0.01$ versus the group of mice immunized with HA alone and that with HA-vaccine packaged-γ-PGA-NPs.

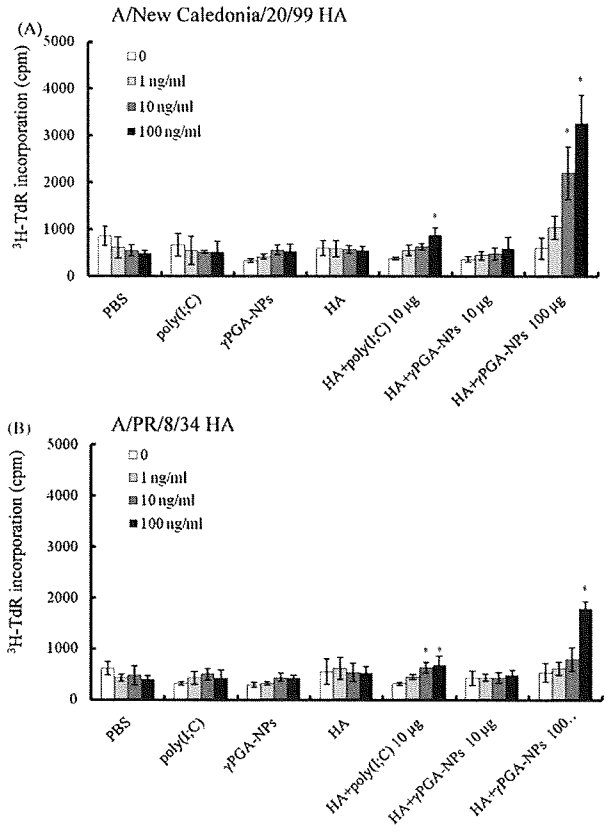

Fig. 3. Influenza virus HA vaccine-specific cell proliferation in mice. Groups of mice (5 per group) were inoculated intranasally with PBS, 10 μg of poly(I:C), 100 μg of γ-PGA-NPs, 0.5 μg of A/New Caledonia/20/99 HA vaccine (HA), a mixture of the HA and 10 μg of poly(I:C), or a mixture of the HA and 10 or 100 μg of γ-PGA-NPs on days 0 and 28. On day 42, splenic mononuclear cells (MNCs) were harvested and restimulated with the indicated concentrations of A/New Caledonia/20/99 HA (A) or A/PR/8/34 HA (B) for 4 days, and ^{3}H-TdR incorporation was assessed. The experiments using 5 mice per group were done in triplicate and repeated three times independently, and the one of the three experiments is shown here. Bars represent means ± standard deviations of 5 mice. *$p < 0.05$ versus the group of mice immunized with HA alone and that with HA-vaccine packaged-γ-PGA-NPs.

nization with a mixture of A/New Caledonia/20/99 or A/PR/8/34 HA vaccine and γ-PGA-NPs, also protected the mice from A/PR/8/34 lethal infection (data not shown).

Since vaccine antigens can be immobilized on the γ-PGA-NP surface or packaged inside it [23,25], we examined whether A/New Caledonia/20/99 HA vaccine-immobilized- or the HA-vaccine-packaged-γ-PGA-NPs, like the HA vaccine and γ-PGA-NP mixture, also protected mice from A/PR/8/34 infection. As shown in Fig. 2, intranasal immunization with HA-vaccine-packaged-γ-PGA-NPs resulted in the survival of only 50% of the mice from lethal influenza virus infection, while the mixture of the HA vaccine and γ-PGA-NPs resulted in all of the mice surviving the infection. Although all the mice receiving the HA vaccine-immobilized γ-PGA-NPs survived the infection, they all became sick with weight loss a few days after the infection; however, all of the mice recovered within 10 days after the infection (data not shown). In contrast, the mice immunized with the mixture of the HA vaccine and γ-PGA-NPs did not show any clinical symptoms or weight loss (data not shown).

3.2. *Influenza virus antigen-specific cell proliferation and cytokine secretion of the splenic mononuclear cells (MNCs) of mice immunized intranasally with a mixture of influenza virus HA vaccine and γ-PGA-NPs*

Several investigations have suggested that intranasal immunization induces not only mucosal but also systemic immunity [26,27]. Therefore, we examined whether intranasal immunization with the mixture of influenza virus HA vaccine and γ-PGA-NPs induced systemic immune responses for influenza viruses. Mice were immunized intranasally with HA vaccine from A/New Caledonia/20/99, a mixture of the HA vaccine and γ-PGA-NPs, or a mixture of the HA vaccine and poly(I:C) on days 0 and 28. On

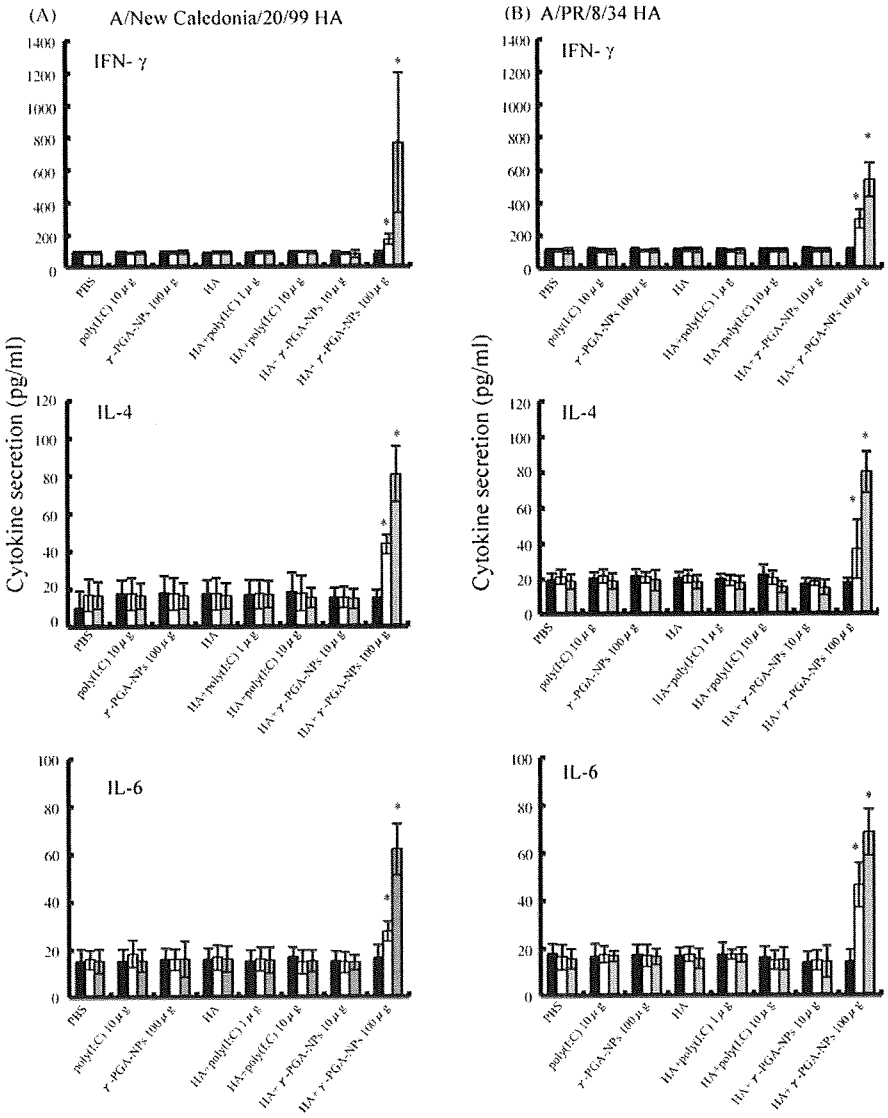

Fig. 4. Effect of adding γ-PGA-NPs to the intranasal influenza virus HA vaccine on Th1- and Th2-cytokine production. Groups of 5 mice were intranasally inoculated twice with PBS, 10 μg of poly(I:C), 100 μg of γ-PGA-NPs, 0.5 μg of A/New Caledonia/20/99 HA vaccine (HA), a mixture of the HA and 1 or 10 μg of poly(I:C), or a mixture of the HA and 10 or 100 μg of γ-PGA-NPs, and the splenic MNCs were restimulated with 100 ng/ml (white bars) or 1 μg/ml (gray bars) HA from A/New Caledonia/20/99 (A) or A/PR/8/34 (B), or without HA (black bars). After 60 h of restimulation, the culture supernatants were harvested and the amounts of IFN-γ, IL-4, and IL-6 in the supernatants were determined by cytokine ELISAs. The experiments using 5 mice per group were done in triplicate and repeated twice independently, and the one of the two experiments is shown. Bars represent means ± standard deviations of 5 mice. $^*p < 0.05$ versus the group of mice immunized with HA alone.

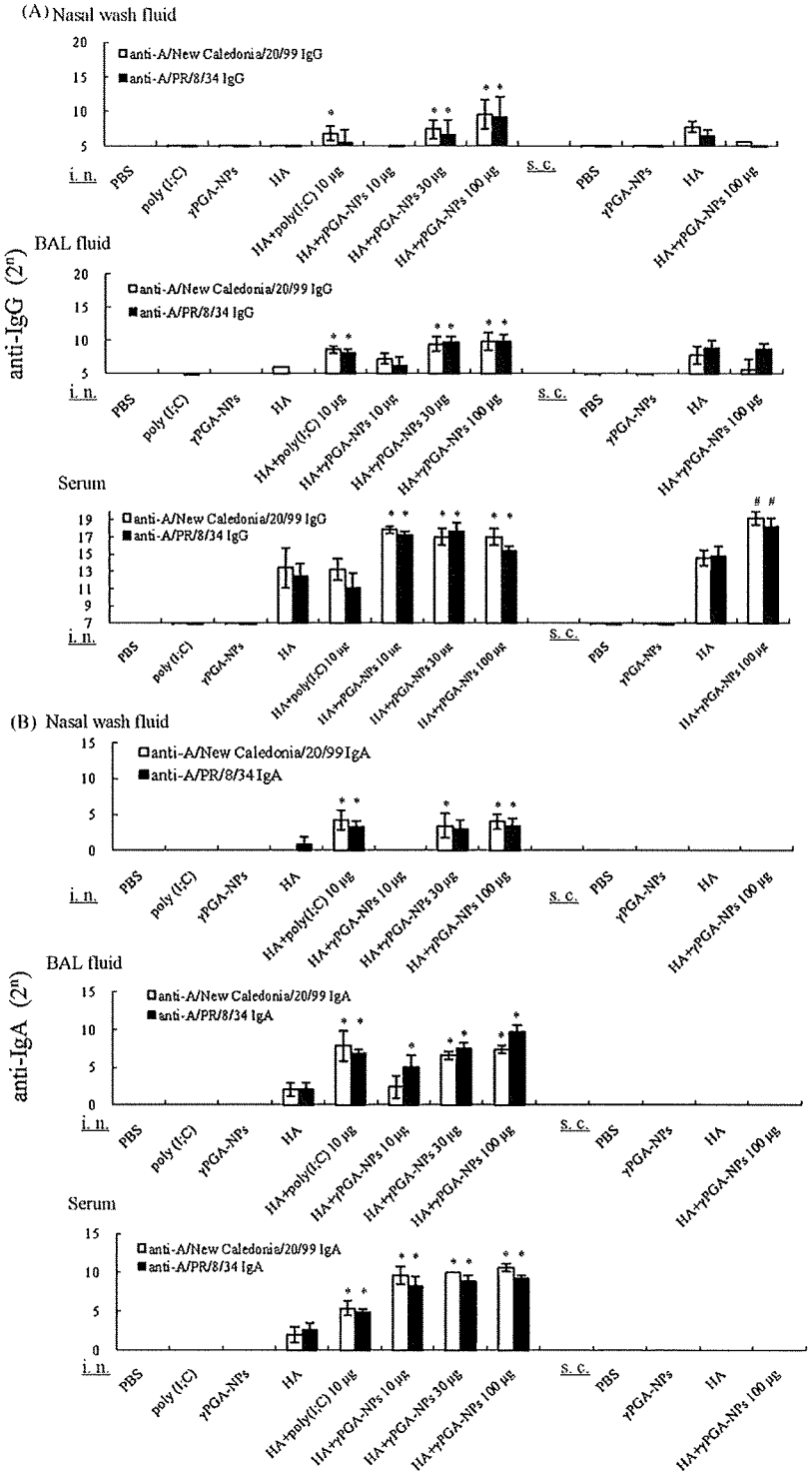

Fig. 5. Effect of adding γ-PGA-NPs to the HA vaccine on the level of anti-HA and neutralizing antibodies in the sera of immunized mice. Groups of mice (5 per group) were inoculated intranasally (i.n.) or subcutaneously (s.c.) with PBS, 10 μg of poly(I:C), 100 μg of γ-PGA-NPs, 0.5 μg of A/New Caledonia/20/99 HA vaccine (HA), a mixture of HA and 10 μg of poly(I:C), or a mixture of HA and 10 or 100 μg of γ-PGA-NPs as described in Section 2. Fourteen days after the final immunization, mouse sera, BAL fluids, and nasal wash fluids were harvested and the antibody titers for A/New Caledonia/20/99 (white bars) and A/PR/8/34 (black bars) in the sera were assessed: (A) anti-IgG antibody, and (B) anti-IgA antibody. Since non-specific IgG antibody was detected in nasal wash fluids, BAL fluids and sera, influenza virus antigen-specific IgG detectable titer was set more than 2^5, 2^5, and 2^7 in nasal wash fluids, BAL fluids and sera, respectively. Bars represent means ± standard deviations of 5 mice. $^*p < 0.05$ versus the group of mice immunized subcutaneously with HA alone. $^\#p < 0.05$ versus the group of mice immunized intranasally with HA alone.

day 42, the splenic mononuclear cells (MNCs) were harvested. The MNCs were restimulated with HA vaccine from A/New Caledonia/20/99 or A/PR/8/34 at the indicated concentrations for 4 days. As shown in Fig. 3A, the A/New Caledonia/20/99 HA vaccine-stimulated MNCs from the mice immunized with the HA vaccine with γ-PGA-NPs proliferated, but those from the PBS-, poly(I:C)-, γ-PGA-NPs-, or the HA vaccine-administered mice did not. Furthermore, the A/PR/8/34 HA vaccine-stimulated MNCs from the mice immunized with A/New Caledonia/20/99 HA vaccine and γ-PGA-NPs proliferated greater than did immunization with the HA vaccine and poly(I:C) (Fig. 3B).

We next examined the productions of type I helper T (Th1) and type II helper T (Th2) cytokines in the HA vaccine-restimulated MNCs *in vitro*. As shown in Fig. 4, intranasal immunization with the mixture of the HA vaccine and γ-PGA-NPs induced a greater secretion of IFN-γ (Th1), IL-4 (Th2), and IL-6 (Th2) than did immunization with the mixture of the HA vaccine and poly(I:C) or the HA vaccine alone.

3.3. Enhancement of influenza virus antigen-specific humoral and cellular immune responses by the administration of influenza virus HA vaccine with γ-PGA-NPs

We next determined whether influenza virus HA vaccine-specific humoral and cellular immune responses were increased by the addition of γ-PGA-NPs to the intranasal immunization

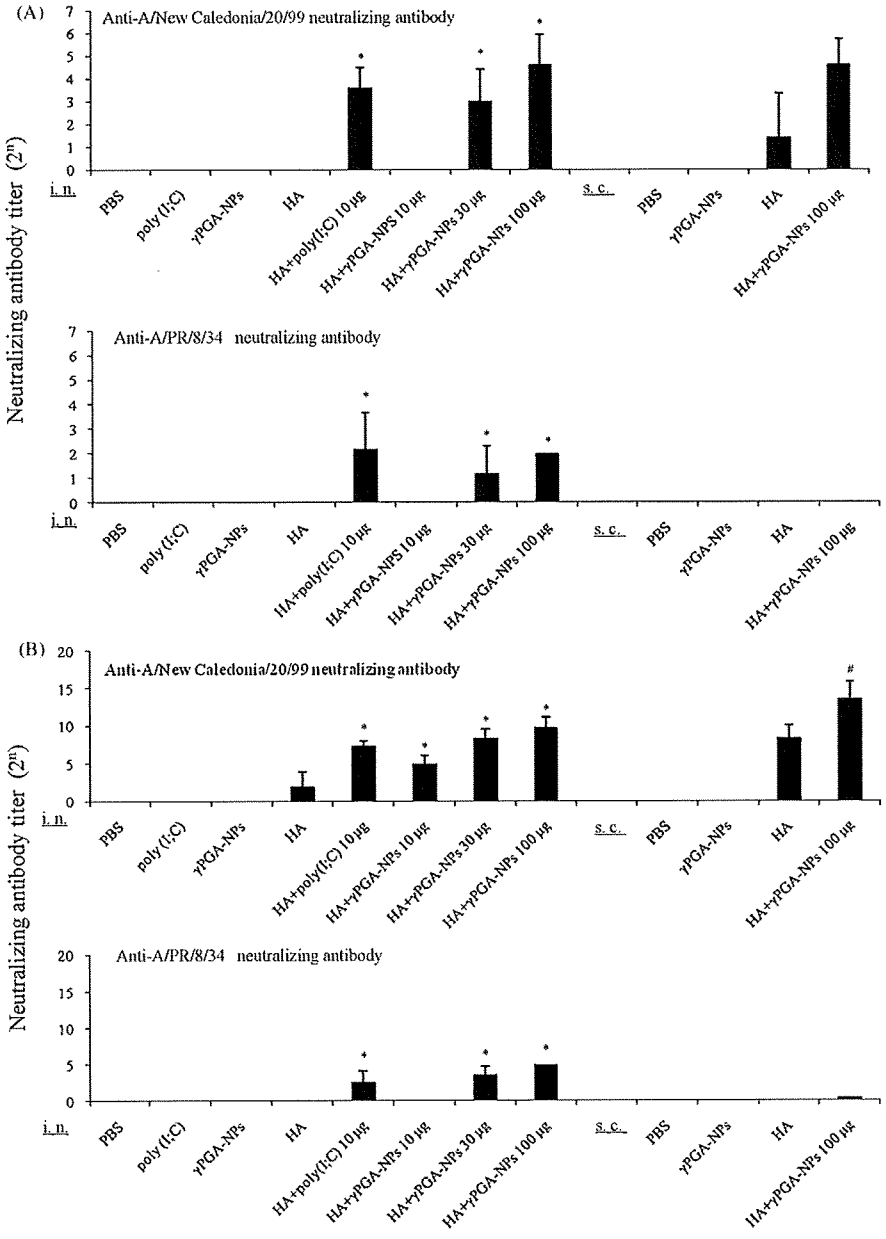

Fig. 6. Effect of adding γ-PGA-NPs to the HA vaccine on the level of anti-influenza virus neutralizing antibodies in the sera of immunized mice. Groups of mice (5 per group) were immunized and the BAL fluids and sera were harvested as described in Fig. 5. The neutralizing antibody titers in BAL fluids (A) and sera (B) were determined by a CPE neutralizing test. Bars represent means ± standard deviations of 5 mice. $^{*}p < 0.05$ versus the group of mice immunized intranasally with HA alone. $^{\#}p < 0.05$ versus the group of mice immunized subcutaneously with HA alone.

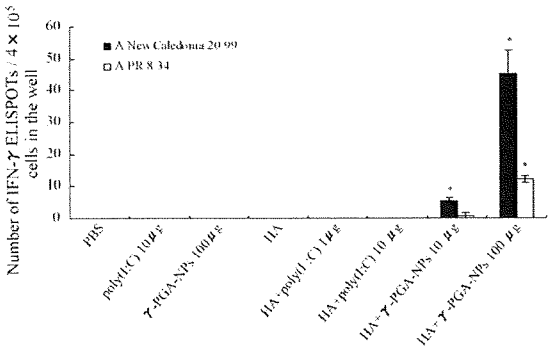

Fig. 7. Enhanced influenza virus-specific cell-mediated immune responses by the addition of γ-PGA-NPs to the intranasal influenza virus HA vaccine. Groups of mice (5 per group) were inoculated intranasally with the materials indicated in the figure on days 0 and 28. On day 42, MNCs were harvested and stimulated with 10^7 pfu/ml of A/New Caledonia/20/99 or A/PR/8/34 virus, which was inactivated by pretreatment with 5000 J/m^2 of ultraviolet radiation, in anti-IFN-γ mAb-coated 96-well filtration plates for 48 h. After stimulation, the plates were stained and the number of IFN-γ spots per 4×10^5 cells was counted as described in Section 2. Bars represent means ± standard deviations of 5 mice. *$p < 0.05$ versus the group of mice immunized intranasally with HA alone.

with influenza virus HA vaccine. Mice were immunized twice intranasally or subcutaneously, and the levels of anti IgA and IgG antibodies for A/New Caledonia/20/99 HA vaccine antigens in their sera, nasal wash fluids, and BAL fluids were assessed. As shown in Fig. 5, intranasal immunization with the mixture of A/New Caledonia/20/99 HA vaccine and γ-PGA-NPs, as well as the mixture of the HA vaccine and poly(I:C), induced higher titers of anti IgG and IgA antibodies for A/New Caledonia/20/99 and A/PR/8/34 than intranasal immunization with the HA vaccine alone. Subcutaneous immunization with A/New Caledonia/20/99 HA vaccine or the mixture of the HA vaccine and γ-PGA-NPs also induced high titers of IgG antibody, but not IgA antibody for A/New Caledonia/20/99 or A/PR/8/34.

We next tested whether a greater titer of neutralizing antibodies for A/New Caledonia/20/99 and A/PR/8/34 was induced in the sera of the mice immunized with A/New Caledonia/20/99 HA vaccine and γ-PGA-NPs. As shown in Fig. 6, intranasal immunization with the mixture of the HA vaccine and γ-PGA-NPs induced titers of neutralizing antibodies for both A/New Caledonia/20/99 and A/PR/8/34 in the BAL fluids and sera, while subcutaneous immunization with A/New Caledonia/20/99 HA vaccine or the mixture of the HA vaccine and γ-PGA-NPs induced titers of neutralizing antibodies only for A/New Caledonia/20/99 (Fig. 6). In addition, we did not detect neutralizing antibody titer in nasal wash fluids of immunized mice (data not shown). The possible reason is that the nasal wash fluids were diluted by PBS to collect from nasal mucosa.

To determine whether the mixture with γ-PGA-NPs also enhanced the influenza virus-specific cell-mediated immune response, an IFN-γ ELISPOT assay was performed. Interestingly, immunization with the mixture of A/New Caledonia/20/99 HA vaccine and 100 μg of γ-PGA-NPs resulted in a greater number of IFN-γ spots in MNCs restimulated with UV-treated A/New Caledonia/20/99 and A/PR/8/34 virus (Fig. 7).

4. Discussion

We demonstrated in the present study that the addition of γ-PGA-NPs to intranasal immunization with influenza virus HA vaccine, but not to subcutaneous immunization, enhanced the cross-protection against influenza virus infection.

CTB and LT are well known to be effective mucosal adjuvants for intranasal influenza virus HA vaccine in experimental mice [15,16,28,29]. Adding these toxins to the intranasal influenza virus HA vaccine results in secretions of cross-reactive anti-influenza virus neutralizing antibodies in the nasal mucosa and lungs sufficient to protect mice from subsequent influenza virus infection. Furthermore, subcutaneous CTB injection with the HA vaccine enhances anti-IgG secretion for the same influenza virus strain in sera [11]. However, since CTB and LT have unpleasant side effects, including nasal discharge, in humans, it is undesirable to use them for a vaccine adjuvant [20]. Recently, poly(I:C) was revealed as a new, effective, and safe mucosal adjuvant for influenza virus HA vaccine [20]. In the present study, we revealed that intranasal immunization with A/New Caledonia/20/99 HA vaccine and more than 10 μg of γ-PGA-NPs enhanced the protective immune responses against infection with A/New Caledonia/20/99. We also found that intranasal immunization with the HA vaccine and more than 30 μg of γ-PGA-NPs enhanced cross-reactive immune responses against variants within a subtype. In addition, γ-PGA is a bacterial capsular exopolymer produced by certain strains of *Bacillus natto*, which is a natural component of the Japanese food item, natto, and γ-PGA-NPs are naturally degraded in the body [22]. Furthermore, repeated injections with milligram amounts of γ-PGA-NPs in mice did not reveal any pathogenic symptoms (unpublished data). Although we still have to confirm that γ-PGA-NPs are not toxic or allergenic for humans, the nano-particles might be a safe and effective adjuvant for intranasal influenza virus HA vaccine. However, it still remains elusive whether the intranasal immunization with the HA vaccine and γ-PGA-NPs can protect from different strains.

Mucosal viral antigen vaccination with CTB or LT promotes not only mucosal, but also systemic immune responses [26,27]. This raised the possibility that mucosal immunization with the HA vaccine and γ-PGA-NPs might also promote antigen-specific systemic as well as mucosal immune responses. In the present study, we demonstrated that intranasal immunization with the HA vaccine and 100 μg of γ-PGA-NPs activated splenic lymphocytes, resulting in the induction of HA-specific cell proliferation, Th1 and Th2 cytokine secretions, T-cell-mediated immune responses, and anti-HA IgG secretion in sera. These results indicated that the intranasal immunization of the HA vaccine with γ-PGA-NPs promoted antigen-specific systemic as well as mucosal immune responses.

Quan et al. [10] reported that intranasal immunization with inactivated influenza virus and CTB induced cross-protective neutralizing antibody secretion, not only in the nasal cavity and lung, but also in sera. Furthermore, they suggested that the cross-protection by the intranasal vaccination was mainly due to the anti-influenza virus neutralizing antibody in the mucosa and sera rather than to Th1 cells and CTLs. In the present study, we revealed that intranasal immunization with the HA vaccine and γ-PGA-NPs induced the production of anti-neutralizing antibodies for different variants within a subtype in the BAL fluids and sera (Fig. 6). Furthermore, a cross-protective influenza virus antigen-specific cell-mediated immune response was detected in mice immunized with the HA vaccine and γ-PGA-NPs (Fig. 7). On the other hand, subcutaneous immunization with the HA vaccine and γ-PGA-NPs did not induced cross-reactive neutralizing antibody production (Fig. 6). Furthermore, the subcutaneous immunization with A/New Caledonia/20/99 HA vaccine did not protect any mice from A/PR/8/34 infection (Fig. 1C). Intranasal immunization with A/New Caledonia/20/99 HA vaccine and γ-PGA-NPs induced lesser cell-mediated immune response against A/PR/8/34 compared with that with A/PR/8/34 and γ-PGA-NPs (Fig. 7). These results may suggest that the cross-reactive neutralizing antibody may contribute to the cross-protection caused by intranasal immunization with the mixture of influenza HA vaccine and γ-PGA-NPs. Previous study clearly has shown that intranasal immunization with influenza HA

virus vaccine and CTB induced production of cross-reactive IgA, but lower levels of IgG [30]. In our study, the intranasal immunization, but not subcutaneous immunization, with the HA vaccine and γ-PGA-NPs induced cross-reactive IgA antibody (Fig. 5) and neutralzing antibody productions (Fig. 6), with cross-protection (Fig. 1), indicating that the cross-reactive IgA antibody produced by adding γ-PGA-NPs may play a role for induction of cross-protection. However, we do not have the data which shows direct evidence, thus, further study will be required to show the evidence.

γ-PGA-NPs are 200 nm sized nano-particles, and various molecules including vaccine antigens can be immobilized on their surface or packaged inside them [20,21]. The particles act as drug carriers, moving substances into DCs and directing the activation of DCs by enhancing the NF-κB activity. In addition, immature DCs (iDCs) stimulated with γ-PGA-NPs, produce TNF-α and IL-12 and up-regulate expression of CD40, CD80 (B7-1), and CD86 (B7-2), resulting in an enhancement of the DCs' T-cell stimulatory capacity [22]. We previously found that a simple mixture of influenza virus HA vaccine and γ-PGA-NPs, without conjugation, enhances the cell-mediated and humoral immune responses more than when HA is attached to the γ-PGA-NPs or packaged inside [21]. In addition, in the present study, mucosal immunization with the mixture of A/New Caledonia/20/99 HA vaccine and γ-PGA-NPs induced greater protection from A/PR/8/34 infection than the attached or encapsulated forms of the inoculant. It remains unknown why the simple mixture of influenza virus HA vaccine and γ-PGA-NPs induced greater protective immune responses than did the other γ-PGA-NP preparations. After the HA vaccine and γ-PGA-NPs were mixed, some of the γ-PGA-NPs bound to the vaccine antigens naturally and some unconjugated antigens and γ-PGA-NPs remained separate (data not shown). Since the activation of NF-κB in DCs acts as a powerful vaccine adjuvant [31], one possible scenario is that in the mixture, the separated HA and γ-PGA-NPs are taken up by DCs independently at the same time, and the γ-PGA-NPs themselves may induce DC activation directly, resulting in the enhancement of unconjugated influenza virus antigen-induced immunity.

In summary, intranasal immunization with a mixture of influenza virus HA vaccine and γ-PGA-NPs elicited sufficient cross-protective immune responses against influenza virus infection to promote survival in mice. Influenza virus vaccines for humans require the ability to induce effective cross-protection with minimal side effects. Therefore, our novel vaccine design might mark the beginning of a breakthrough in meeting these requirements.

Acknowledgments

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LETTERS

In vitro and *in vivo* characterization of new swine-origin H1N1 influenza viruses

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Influenza A viruses cause recurrent outbreaks at local or global scale with potentially severe consequences for human health and the global economy. Recently, a new strain of influenza A virus was detected that causes disease in and transmits among humans, probably owing to little or no pre-existing immunity to the new strain. On 11 June 2009 the World Health Organization declared that the infections caused by the new strain had reached pandemic proportion. Characterized as an influenza A virus of the H1N1 subtype, the genomic segments of the new strain were most closely related to swine viruses[1]. Most human infections with swine-origin H1N1 influenza viruses (S-OIVs) seem to be mild; however, a substantial number of hospitalized individuals do not have underlying health issues, attesting to the pathogenic potential of S-OIVs. To achieve a better assessment of the risk posed by the new virus, we characterized one of the first US S-OIV isolates, A/California/04/09 (H1N1; hereafter referred to as CA04), as well as several other S-OIV isolates, *in vitro* and *in vivo*. In mice and ferrets, CA04 and other S-OIV isolates tested replicate more efficiently than a currently circulating human H1N1 virus. In addition, CA04 replicates efficiently in non-human primates, causes more severe pathological lesions in the lungs of infected mice, ferrets and non-human primates than a currently circulating human H1N1 virus, and transmits among ferrets. In specific-pathogen-free miniature pigs, CA04 replicates without clinical symptoms. The assessment of human sera from different age groups suggests that infection with human H1N1 viruses antigenically closely related to viruses circulating in 1918 confers neutralizing antibody activity to CA04. Finally, we show that CA04 is sensitive to approved and experimental antiviral drugs, suggesting that these compounds could function as a first line of defence against the recently declared S-OIV pandemic.

Sequence analyses of recently emerged swine-origin H1N1 viruses (S-OIVs) revealed the absence of markers associated with high pathogenicity in avian and/or mammalian species, such as a multibasic haemagglutinin (HA) cleavage site[2] or lysine at position 627 of the PB2 protein[3]. To characterize the new viruses *in vitro* and *in vivo*, we amplified the following S-OIVs in Madin–Darby canine kidney (MDCK) cells: A/California/04/09 (CA04), A/Wisconsin/WSLH049/09 (WSLH049), A/Wisconsin/WSLH34939/09 (WSLH34939), A/Netherlands/603/09 (Net603) and A/Osaka/164/09 (Osaka164). WSLH34939 was isolated from a patient who required hospitalization, whereas the remaining viruses were isolated from mild cases. These viruses represent the currently recognized neuraminidase (NA) variants among S-OIVs: CA04, NA-106V, NA-248N; Osaka164, NA-106I, NA-248N; WSLH049, NA-106I, NA-248D; WSLH34939, NA-106I, NA-248D; and Net603, NA-106V, NA-248N.

In MDCK cells and primary human airway epithelial cells, CA04 grew to titres comparable to those typically obtained for contemporary human H1N1 influenza viruses (Supplementary Fig. 1). Confocal, transmission electron and scanning electron microscopy revealed virions of remarkably filamentous shape (Supplementary Fig. 2), in marked contrast to the spherical shape observed with negatively stained virions (http://www.cdc.gov/h1n1flu/images.htm). The biological significance of the morphology of CA04 remains unknown.

To evaluate the pathogenicity of S-OIV in mammalian models, we conducted studies in mice, ferrets, non-human primates and pigs. BALB/c mice intranasally infected with a high dose ($>10^4$ plaque-forming units (p.f.u.)) of CA04 (Supplementary Fig. 3) experienced weight loss and those infected with the highest dose of this virus were humanely killed, in contrast to animals infected with a recent human H1N1 virus (A/Kawasaki/UTK-4/09, KUTK-4). The 50% mouse lethal dose (MLD_{50}) was $10^{5.8}$ p.f.u. for CA04 and $>10^{6.6}$ p.f.u. for

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KUTK-4. For the additional S-OIV isolates tested, the MLD_{50} values were $>10^{6.4}$ p.f.u. for Osaka164, $>10^{6.6}$ p.f.u. for WSLH049, $10^{4.5}$ p.f.u. for WSLH34939 and $>10^{5.8}$ p.f.u. for Net603.

On day 3 after infection of mice, similar titres were detected in nasal turbinates of mice infected with 10^5 p.f.u. of S-OIVs or KUTK-4 (Supplementary Table 1); however, S-OIVs replicated more efficiently in the lungs of infected animals, which may account for the prominent bronchitis and alveolitis with viral antigen on day 3 after infection with CA04 (Supplementary Fig. 4a, b). On day 6 after infection, virus titres followed a similar trend and the lungs of CA04-infected mice showed bronchitis and alveolitis with viral antigen, although signs of regeneration were apparent (Supplementary Fig. 4c). We detected viral-antigen-positive bronchial epithelial cells, but not alveolar cells, on day 3 after infection of mice infected with KUTK-4 (Supplementary Fig. 4e). By day 6, infection in KUTK-4-inoculated mice had progressed to bronchitis and peribronchitis; however, viral antigen was rarely detected in these lesions (Supplementary Fig. 4f).

There were marked differences in the induction of pro-inflammatory cytokines in the lungs of mice infected with CA04 compared with KUTK-4 (Supplementary Fig. 5a–c). Infection with KUTK-4 resulted in limited induction of pro-inflammatory cytokines/chemokines in the lungs, in marked contrast to infection with CA04. Increased production of interleukin-10 (IL-10; Supplementary Fig. 5a) in lungs of CA04-infected mice at day 6 after infection probably reflects a host response to dampen over-exuberant pulmonary inflammation and promote tissue repair. Infection with CA04 led to strong induction of both interferon-γ (IFN-γ) and IL-4 in the lungs. The selective induction of the T_H2 cytokine IL-5 in CA04-infected, but not in KUTK-4-infected, mice on day 6 after infection is noteworthy (Supplementary Fig. 5b), but further studies are needed to understand the relevance of this finding to viral control. IL-17 has been reported to have a role in protection against lethal influenza and also in eliciting inflammatory responses[4,5]; however, the enhanced viral replication and lung pathology observed in CA04-infected mice was not linked to dysregulated IL-17 production.

Cynomolgus macaques (*Macaca fascicularis*) have been used to study highly pathogenic avian H5N1 viruses[6,7] and the 1918 pandemic virus[8]. Infection of cynomolgus macaques with CA04 (see Methods for detailed procedures) resulted in a more prominent increase in body temperature than infection with KUTK-4 (Supplementary Fig. 6). This difference might originate from the observed differences in virus titres (Table 1 and Supplementary Table 2). No remarkable difference in body weight loss was found between the two groups (data not shown). CA04 replicated efficiently in the lungs and other respiratory organs of infected animals, similar to highly pathogenic influenza viruses[6,8] (Table 1). By contrast, conventional human influenza viruses are typically limited in their replicative ability in the lungs of infected primates[6,8] (Table 1), although a seasonal H1N1 virus was isolated from one animal on day 7 after infection. Pathological examination revealed that CA04 caused more severe lung lesions than did KUTK-4 (Fig. 1 and Supplementary Fig. 7). On day 3 after infection with CA04, alveolar spaces were occupied by oedematous exudate and inflammatory infiltrates (Fig. 1a, b); severe thickening of alveolar walls was also observed (Fig. 1b). Viral-antigen-positive cells were distributed in the inflammatory lesions, and many of these cells were elongated with thin cytoplasm and hemming around the alveolar wall, indicating type I pneumocytes (Fig. 1c). In addition to type I pneumocytes, CA04 viral antigens were also detected in considerable numbers of cuboidal, cytokeratin-positive cells, hence identified as type II pneumocytes (Fig. 1d and Supplementary Fig. 8), as has been reported for highly pathogenic avian H5N1 influenza viruses[6]. Upon infection with KUTK-4, large sections of infected lungs showed thickening of the alveolar wall on day 3 after infection (Fig. 1e). Although the infiltration of inflammatory cells was prominent at the alveolar wall (Fig. 1f), viral antigens were sparse and detected in type I (but not type II) pneumocytes (Fig. 1g). By contrast, the lungs of non-infected animals show clear alveolar spaces (Fig. 1h).

On day 7 after infection, lung pathology remained more severe for CA04- than for KUTK-4-infected lungs (Supplementary Fig. 7), although regenerative changes were seen for CA04. Nonetheless, considerable numbers of antigen-positive cells were still detectable (Supplementary Fig. 7c). Collectively, these findings demonstrate that CA04 causes more severe lung lesions in non-human primates than does a contemporary human influenza virus.

Induction of pro-inflammatory cytokines/chemokines in the lungs of CA04-infected macaques was variable at day 3 after infection (Supplementary Fig. 9). However, consistent with persisting lung pathology and inflammation on day 7 after infection, the levels of MCP-1, MIP-1α, IL-6 and IL-18 were markedly higher in the lungs of two of three CA04-infected macaques.

Ferrets are widely accepted as a suitable small-animal model for influenza virus pathogenicity and transmissibility studies. Infection of ferrets with S-OIVs or KUTK-4 did not cause marked changes in body temperature or weight in any group (data not shown). Although all test viruses were detected in nasal turbinates at similar titres on day 3 after infection (Supplementary Table 3), S-OIVs replicated to higher titres in trachea and lungs.

Pathological examination detected similar levels of viral antigen in the nasal mucosa of both CA04- and KUTK-4-infected ferrets (Supplementary Fig. 10a and e). However, the lungs of CA04-infected ferrets showed more severe bronchopneumonia with prominent viral antigen expression in the peribronchial glands and a few alveolar cells (Supplementary Fig. 10b–d) on day 3 after infection. By contrast, most of the lung appeared normal after infection with

Table 1 | Virus titres in organs of infected cynomolgus macaques

Organ	A/California/04/09 (H1N1)						A/Kawasaki/UTK-4/09 (H1N1)					
	Day 3 after infection			Day 7 after infection			Day 3 after infection			Day 7 after infection		
	1	2	3	4	5	6	7	8	9	10	11	12
Nasal mucosa	4.7	3.3	–	–	–	–	–	–	–	–	–	–
Oro/nasopharynx	6.3	4.4	4.7	–	7.9	–	–	–	4.3	–	–	4.8
Tonsil	6.4	–	–	–	7.1	–	–	–	2.8	–	–	3.0
Trachea	5.9	2.0	5.6	–	–	–	2.0	4.1	–	3.7	–	5.4
Bronchus (right)	5.7	2.9	4.3	–	5.1	–	–	2.5	–	3.5	–	3.8
Bronchus (left)	5.9	–	6.1	–	5.1	–	–	–	–	3.3	–	5.1
Lung (upper right)	5.7	5.6	4.5	–	–	–	2.7	–	–	–	–	–
Lung (middle right)	5.6	6.4	6.9	–	–	–	2.3	2.6	2.5	–	–	–
Lung (lower right)	6.1	4.5	6.0	–	–	–	2.6	2.6	–	–	–	3.4
Lung (upper left)	4.7	4.3	6.4	–	–	–	–	–	–	–	–	–
Lung (middle left)	5.8	4.3	6.3	–	–	–	–	–	–	–	–	–
Lung (lower left)	6.7	4.5	6.6	–	–	–	–	–	–	–	–	2.3
Conjunctiva	3.6	–	–	–	–	–	–	–	–	–	–	–

Cynomolgus macaques were inoculated with $10^{7.4}$ p.f.u. of virus (6.7 ml) through multiple routes (see Methods). Three macaques per group were killed on days 3 and 7 after infection for virus titration. No virus was recovered from lymph nodes (chest), heart, spleen, kidneys or liver of any of the animals. A dash indicates that virus was not detected (detection limit: 2 $\log_{10}$ p.f.u. g^{-1}). Numbers 1 to 12 indicate animal identification number. Values indicate virus titre (mean $\log_{10}$ p.f.u. g^{-1}).

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