

研究成果の刊行に関する一覧表（平成23年度）

書籍

著者氏名	論文タイトル名	書籍全体の 編集者名	書 籍 名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻 号	ページ	出版年
<u>Okamoto,S.</u> , Matsuura,M., Akagi,T., Akashi,M., Tanimoto,T., Ishikawa,T., Takahashi,M., <u>Yamanishi,K.</u> , Mori,Y.,	Poly(gamma-glutamic acid)nano-particles combined with mucosal influenza virus hemagglutinin vaccine Protects against influenza Virus infection in mice	Vaccine	27(42)	5896-5905	2009
Itoh, Y., Ozaki, H.,Ishigaki, H., Sakoda, Y., Nagata,T., Soda, K., IsodaN., Miyake, T., Ishida, H., Okamoto, K., Nakayama,M., Tsuchiya, H., Torii, R., <u>Kida, H.</u> , and Ogasawara, K	Subcutaneous inoculation of a whole virusparticle vaccine preparedfrom a non-pathogenic virus library induces protective immunity against H7N7 highly pathogenic avian influenza virus in cynomolgus macaques.	Vaccine	28	780-789	2010

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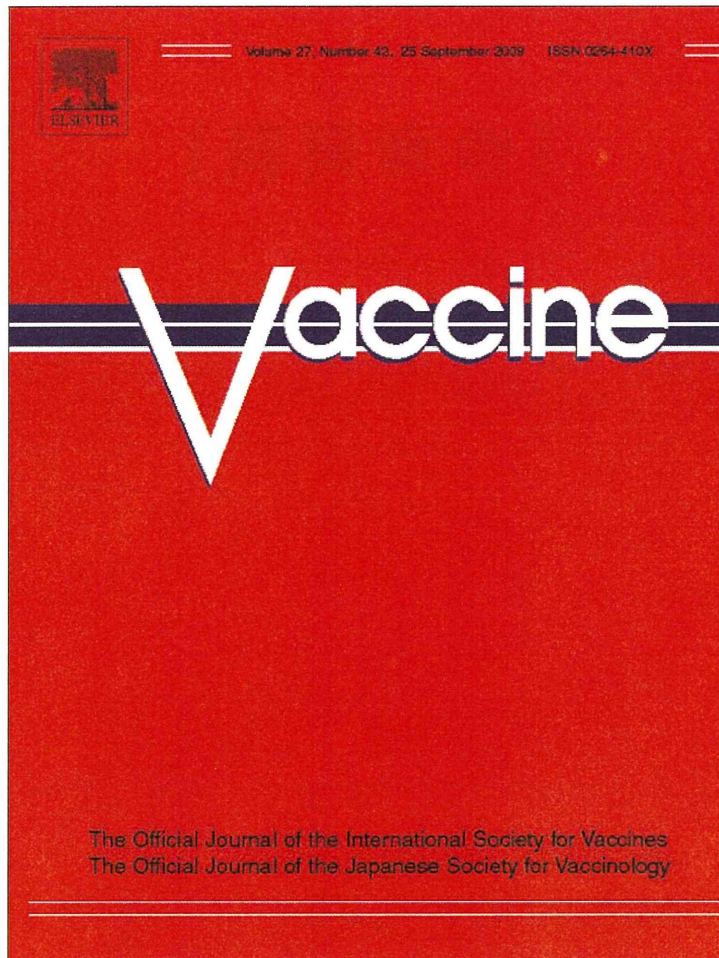
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【参考資料】

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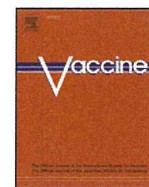
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Vaccine

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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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ARTICLE INFO

Article history:

Received 18 October 2008

Received in revised form 10 July 2009

Accepted 15 July 2009

Available online 31 July 2009

Keywords:

Influenza vaccine

Poly- γ -glutamic acid nano-particles

(γ -PGA-NPs)

Mucosal immunization

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- κ B 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, M_n = 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 ^1H 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 dis-

tilled 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 μ l) or intranasally (i.n.; inoculation volume was 20 μ l) with PBS or PBS containing 0.5 μ g influenza virus HA vaccine (HA vaccine), 100 μ g HA vaccine encapsulated- or HA vaccine immobilized-PGA-NP, or a mixture of 0.5 μ g HA vaccine and 10, 30, or 100 μ g γ -PGA-NPs, or 10 μ g 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 μ l) were mixed 1:1 (v/v) with a suspension containing 100 TCID₅₀ 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 μ g/ml of trypsin (MEM maintenance medium), 100 μ l MEM maintenance medium was added to each well, and the cells were incubated at 37 °C in a 5% CO₂ 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 μ l 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 TCID₅₀ 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 μ l 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 [^3H] thymidine (0.5 μ Ci/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% CO₂. 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 μ g/ml, clone RA-6A2, BD Biosciences-Pharmingen) overnight. After the plates were washed four times with PBS, 200 μ l of PBS supplemented with 10% FBS was added to each well, and the plates were incubated at 37 °C. MNCs (10⁶ cells) from influenza virus HA vaccine- and/or adjuvant-treated mice suspended in 100 μ l of complete RPMI 1640 medium were added to each well of the filtration plates, along with

UV-inactivated influenza virus particles suspended in 100 μ l 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 μ g/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).

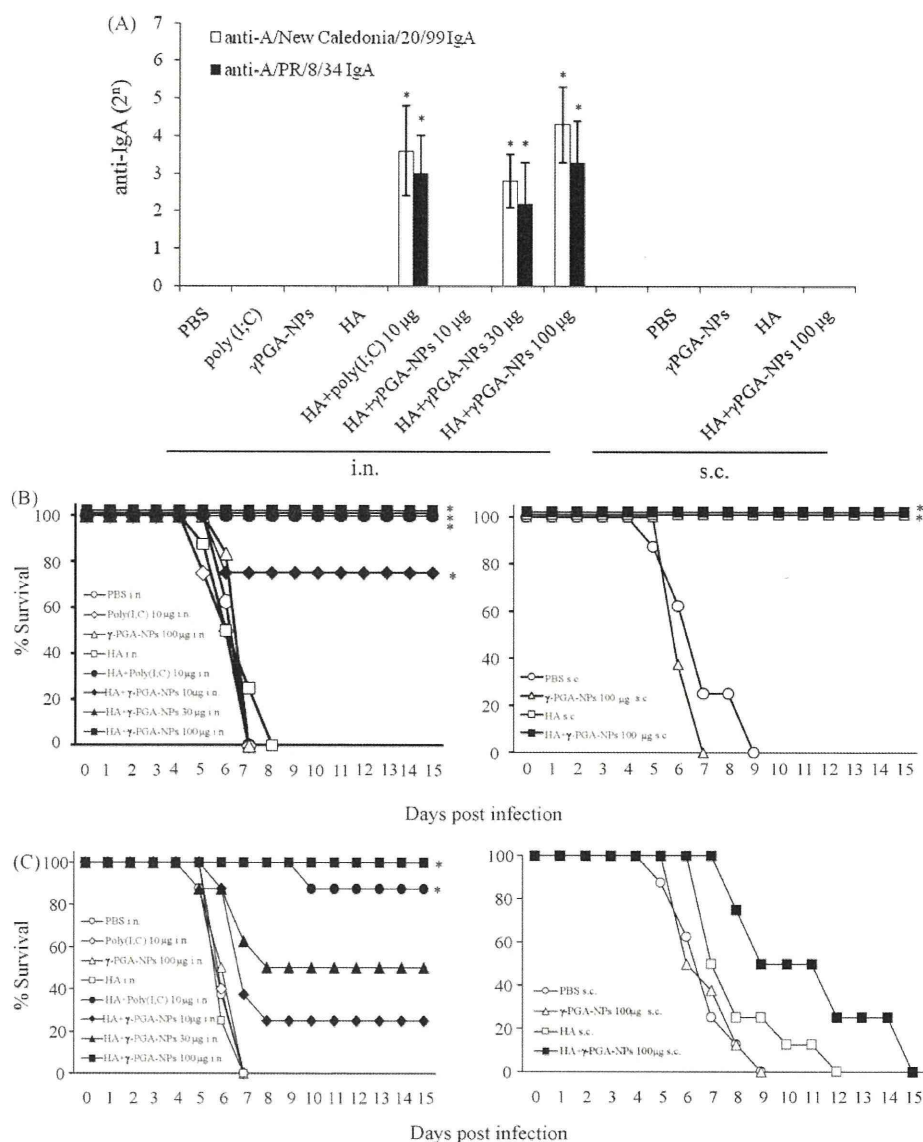


Fig. 1. Effect of adding γ -PGA-NPs to the intranasal influenza virus HA vaccine on the protective immune responses against influenza virus infections. (A) Groups of mice (5 per group) were intranasally (i.n.) or subcutaneously (s.c.) inoculated twice with PBS, 10 μ g of poly(I:C), 100 μ g of γ -PGA-NPs, 0.5 μ g 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 \pm 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 μ l of PBS containing 2000 pfu ($200 \times LD_{50}$) 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 μ g of γ -PGA-NPs, or 10 μ g 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 μ g 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 using 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

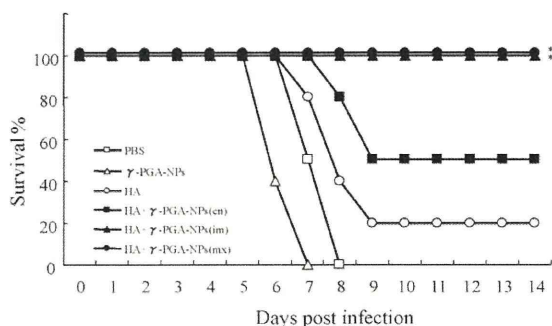


Fig. 2. Effect of different configurations of the combined HA vaccine and γ -PGA-NPs on protective immune responses against a lethal influenza virus infection. Groups of 5 mice were intranasally immunized with PBS, 100 μ g of γ -PGA-NPs, 0.5 μ g of A/New Caledonia/20/99 HA vaccine (HA), HA-encapsulated 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.

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 μ g 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 μ g 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 μ g 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-

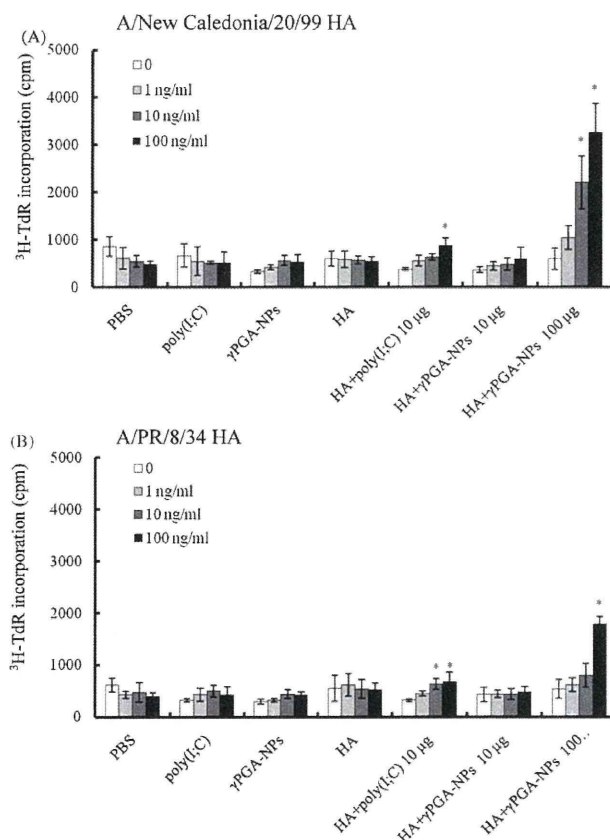


Fig. 3. Influenza virus HA vaccine-specific cell proliferation in mice. Groups of mice (5 per group) were inoculated intranasally with PBS, 10 μ g of poly(I:C), 100 μ g of γ -PGA-NPs, 0.5 μ g of A/New Caledonia/20/99 HA vaccine (HA), a mixture of the HA and 10 μ g of poly(I:C), or a mixture of the HA and 10 or 100 μ g 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 \pm 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 immu-

nized 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

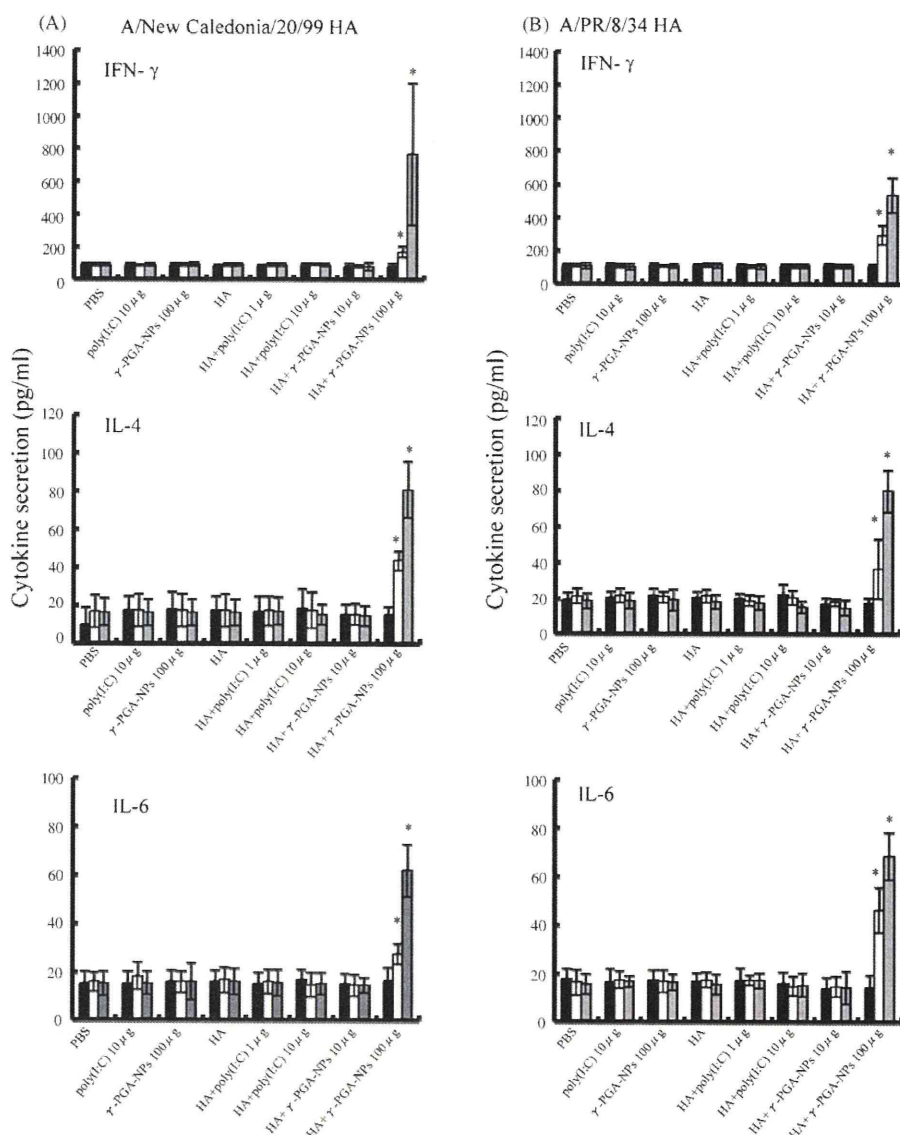


Fig. 4. Effect of adding γ -PGA-NPs to the intranasal influenza virus HA vaccine on Th1- and Th2-cytokine production. Groups of 5 mice were intranasally inoculated twice with PBS, 10 μ g of poly(I:C), 100 μ g of γ -PGA-NPs, 0.5 μ g of A/New Caledonia/20/99 HA vaccine (HA), a mixture of the HA and 1 or 10 μ g of poly(I:C), or a mixture of the HA and 10 or 100 μ g of γ -PGA-NPs, and the splenic MNCs were restimulated with 100 ng/ml (white bars) or 1 μ g/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 \pm standard deviations of 5 mice. * p < 0.05 versus the group of mice immunized with HA alone.

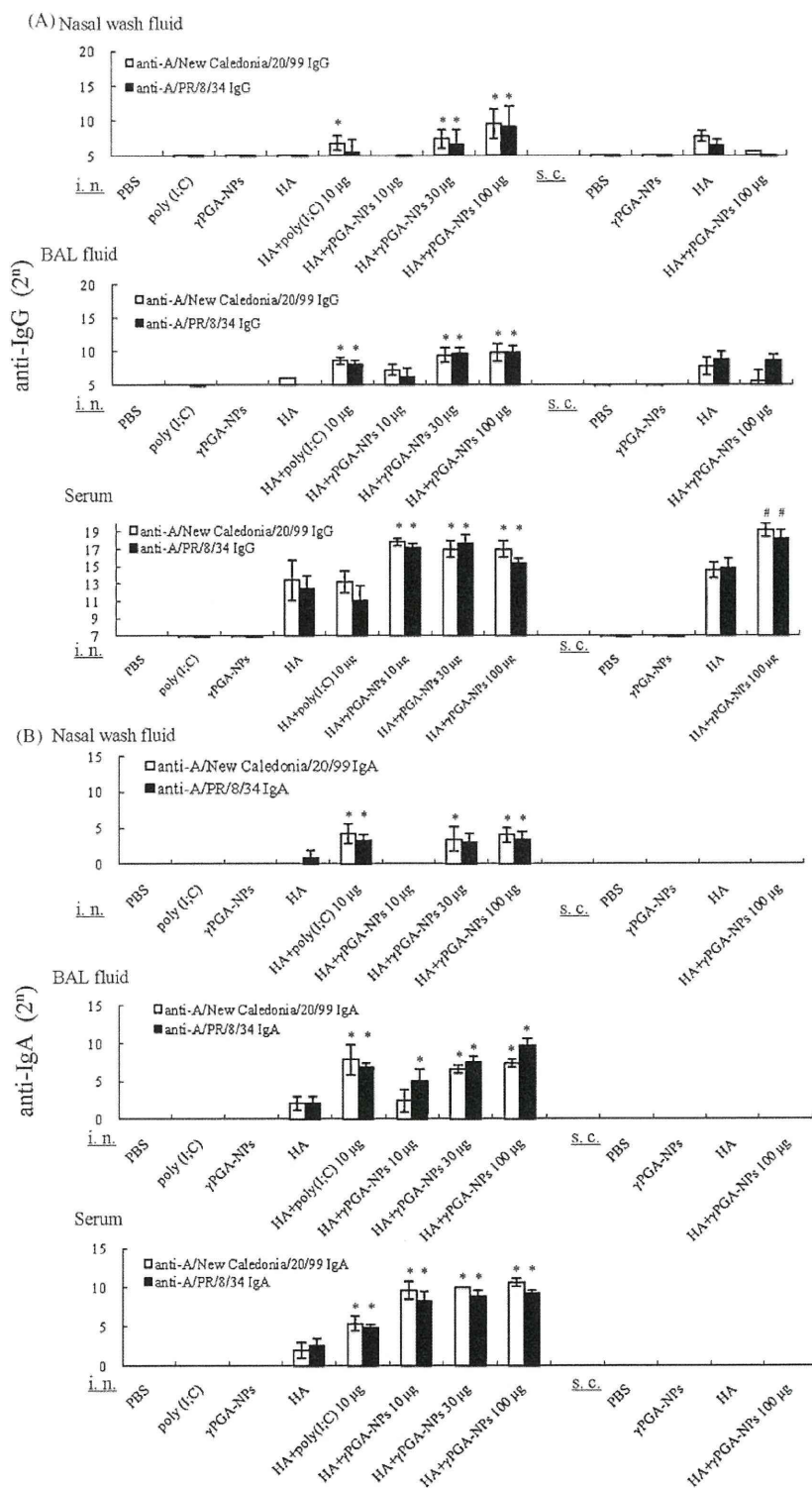


Fig. 5. Effect of adding γ -PGA-NPs to the HA vaccine on the level of anti-HA and neutralizing antibodies in the sera of immunized mice. Groups of mice (5 per group) were inoculated intranasally (i.n.) or subcutaneously (s.c.) with PBS, 10 μ g of poly(I:C), 100 μ g of γ -PGA-NPs, 0.5 μ g of A/New Caledonia/20/99 HA vaccine (HA), a mixture of HA and 10 μ g of poly(I:C), or a mixture of HA and 10 or 100 μ g 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 \pm 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.

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

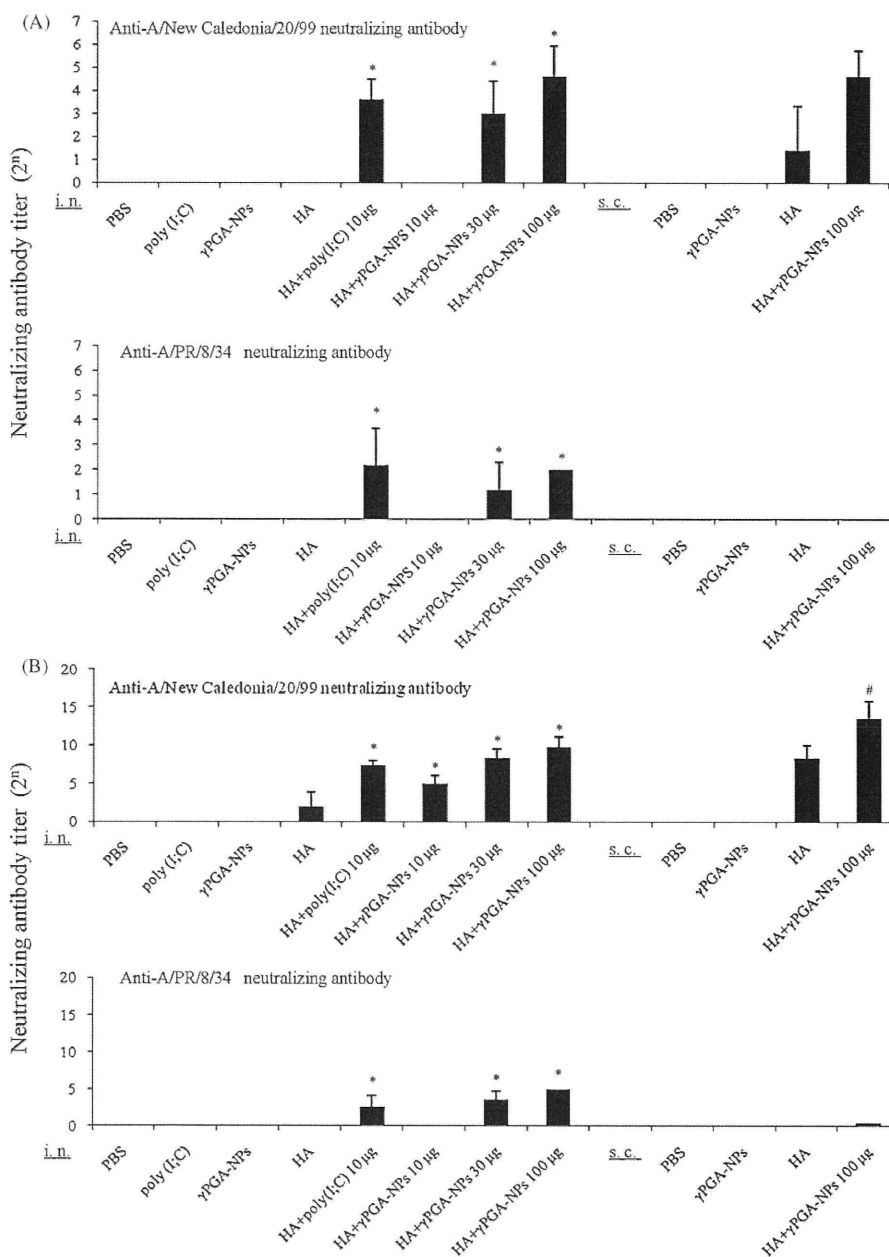


Fig. 6. Effect of adding γ -PGA-NPs to the HA vaccine on the level of anti-influenza virus neutralizing antibodies in the sera of immunized mice. Groups of mice (5 per group) were immunized and the BAL fluids and sera were harvested as described in Fig. 5. The neutralizing antibody titers in BAL fluids (A) and sera (B) were determined by a CPE neutralizing test. Bars represent means \pm 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.

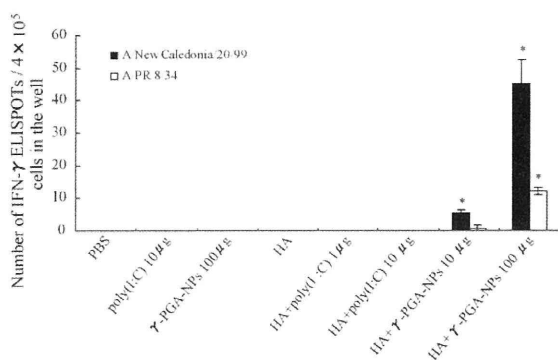


Fig. 7. Enhanced influenza virus-specific cell-mediated immune responses by the addition of γ -PGA-NPs to the intranasal influenza virus HA vaccine. Groups of mice (5 per group) were inoculated intranasally with the materials indicated in the figure on days 0 and 28. On day 42, MNCs were harvested and 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 \pm 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 $100 \mu\text{g}$ 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 $10 \mu\text{g}$ 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 $30 \mu\text{g}$ 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 $100 \mu\text{g}$ 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 induce 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 neutralizing 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- κ B 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- κ B 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

We thank Drs. Kosuke Kataoka (Tokushima University Graduate School of Oral Sciences) and Shigetada Kawabata (Osaka University Graduate School of Dentistry) for technical advice. We also thank Dr. Hironori Yoshii, Ms. Eiko Moriishi, and Ms. Yuko Ueda for technical assistance. This study was partly supported by grants from the Japanese Ministry of Health, Welfare, and Labor, the Japanese Ministry of Education, Culture, Sports, Science, and Technology, and CREST, Japan Science and Technology Agency.

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