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Onjisaponins, from the root of *Polygala tenuifolia* Willdenow, as effective adjuvants for nasal influenza and diphtheria–pertussis–tetanus vaccines

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Abstract

Active substances from hot water extracts from 267 different Chinese and Japanese medicinal herbs were screened for mucosal adjuvant activity with influenza HA vaccine in mice. The extract from the root of *Polygala tenuifolia* was found to contain potent mucosal adjuvant activity. The active substances were purified and identified as onjisaponins A, E, F, and G. When each onjisaponin (10 μg) was intranasally (i.n.) inoculated with influenza vaccine (10 μg) in mice, serum hemagglutination-inhibiting (HI) antibody titers increased 3–14 times over control mice administered vaccine alone after 4 weeks. When each onjisaponin (10 μg) was i.n. inoculated with the vaccine (10 μg) followed by i.n. vaccination of the vaccine alone after 3 weeks, serum HI antibody titers increased 27–50 fold over those mice given i.n. vaccinations without onjisaponins. These same conditions also significantly increased nasal anti-influenza virus IgA antibody titers. Two inoculations with onjisaponin F (1 μg) and influenza HA vaccine (1 μg) at 3 weeks intervals, significantly increased serum HI antibody and nasal anti-influenza virus IgA and IgG antibody titers after only 1 week over mice given HA vaccine alone after the secondary vaccination. Intranasal vaccination with onjisaponin F inhibited proliferation of mouse adapted influenza virus A/PR/8/34 in bronchoalveolar lavages of infected mice. Separate intranasal vaccinations with onjisaponins A, E, F, and G (10 μg) each and diphtheria–pertussis–tetanus (DPT) vaccine (10 μg) of mice followed by i.n. vaccination with DPT vaccine alone after 4 weeks showed significant increases in serum IgG and nasal IgA antibody titers after 2 weeks following secondary vaccination over mice vaccinated with DPT vaccine alone. All onjisaponins showed little hemolytic activity at concentrations up to 100 μg/ml. The results of this study suggest that onjisaponins may provide safe and potent adjuvants for intranasal inoculation of influenza HA and DPT vaccines. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Adjuvant; DPT vaccine; Influenza vaccine; Mucosal immunity; Onjisaponin

1. Introduction

Subcutaneous injection is the most common method for delivery of vaccines. Aerial infectious organisms such as influenza virus, *Bordetella pertussis* or *Corynebacterium diphtheriae*, are known to infect via mucous membranes of the respiratory tract. To prevent such infections at early stages, it would be advantageous to develop vaccines that could elicit a local mucosal immune response over those needing longer immune responses by subcutaneous delivery [1]. Therefore, nasal administration of vaccines for respiratory pathogens has attracted much attention as a means of delivering vaccines over subcutaneous injections due to

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localized inducement of an immune response [2]. However, studies have known that nasal administration of vaccines may not receive sufficient immunostimulation with vaccine alone. Therefore, the use of adjuvants to enhance local mucosal immune responses has been documented [3].

Cholera toxin (CT) from *Vibrio cholerae* and heat-labile enterotoxin (LT) from *Escherichia coli* are known potent mucosal immune adjuvants when co-administered with specific antigens [4–6]. Although both CT and LT are able to function as mucosal adjuvants, their extreme toxicity prevents their use in humans. To resolve this problem, the use of recombinant B subunits of these toxins (CTB* or LTB) has been studied [7–9]. Another approach has been used B subunits with trace amounts of the holotoxins (0.1–0.5%) (CTB* or LTB*) [10–12]. In these studies, the recombinant holotoxin free CTB and LTB both showed potent mucosal adjuvant activities at relatively high dose but the B subunits at low doses affected the synergic effect of the holotoxins as adjuvants for a vaccine. A third approach has been used chemically modified [13] or mutated toxin with 1/1000 less toxicity than the native toxin yet retaining its adjuvant activity [14–19]. Although the use of recombinant B subunits of CT and LT and chemically modified or mutated toxins provide potential safe and potent mucosal adjuvants, the search for other adjuvants which can be used to develop safe and effective nasal vaccines is warranted.

In this study, hot water extracts from 267 different types of Chinese and Japanese medicinal plants were screened for substances that exhibited adjuvant activity with nasal influenza vaccine. It was found that the root of *Polygala tenuifolia* contained the most potent adjuvants when combined with nasal influenza or diphtheria–pertussis–tetanus (DPT) vaccine. The substances having adjuvant activities were identified as onjisaponins A, E, F, and G, also known as triterpenoidsaponins which previously have never been shown to exhibit mucosal adjuvant activities with nasal vaccines. Using laboratory mice, the efficacy in developing vaccines that could be administered locally in the mucous membrane of respiratory tracts using onjisaponins as adjuvants with nasal influenza and DPT vaccines was explored.

2. Materials and methods

2.1. Materials

CT and the B subunit of CT, which was estimated to contain 0.01–0.1% of CT (CTB*), were purchased from Sigma Chemical Co., St. Louis, MO. Superfos Quil A, a crudely enriched saponin preparation from the bark of *Quillaja saponaria* Molina was obtained from Accurate Sciences, Westbury, NY.

2.2. Influenza vaccine formulation and immunization

Influenza hemagglutinin (HA) vaccines, a split-product vaccines, were prepared from influenza viruses A/PR/8/34 (A/PR8, H1N1) and A/Beijing/262/95 (A/Beijing, H1N1) by the method of Davenport et al. [20] in Center for Biologicals of the Kitasato Institute, Saitama, Japan. The dose of HA vaccine was expressed as the concentration of HA molecule in the HA vaccine as determined by SDS-PAGE. HA vaccine, diluted with phosphate buffered saline (PBS) (pH 7.4), was mixed with the indicated doses of onjisaponins, CTB* or CT. Female BALB/c mice (6–7 weeks old), purchased from Japan SLC, Inc., Hamamatsu, Japan, were anesthetized by an intraperitoneal injection of sodium amobarbital (0.2 ml of a saline solution of 11 mg/ml), and then inoculated intranasally by dropping 10 μl of the preparation containing vaccine (1 or 10 μg of HA per mouse) and adjuvant into each nostril gradually to prevent suffocation. In some experiments, mice were boosted with the same antigen with or without onjisaponin 3 or 4 weeks later.

2.3. DPT vaccination

Diphtheria-purified pertussis–tetanus combined (DPT) vaccine (containing purified pertussis antigen 15 μg/ml, tetanus toxoid (TT) 2 Lf/ml and diphtheria toxoid (DT) 20 Lf/ml) was prepared in the Center for Biologicals of the Kitasato Institute (Saitama, Japan). DPT vaccine was mixed with onjisaponins, CTB* or Quil A. Mice were anesthetized and inoculated intranasally by dropping 10 μl of the mixture of vaccine and adjuvant (10 μg/mouse) into each nostril. The mice were secondary inoculated with the DPT vaccine alone 4 weeks after the first vaccination.

2.4. Infection of influenza virus

Mouse adapted influenza virus A/PR8 (H1N1), which was passed 148 times in ferret, 596 times in mice, and 72 times in 10-day old fertile chicken eggs, was used for infection [21]. Mice were anesthetized and infected with A/PR8 virus by intranasally dropping 20 μl of the viral suspension containing $10^{4.1}$ 50% egg-infecting dose ($E1D_{50}$) ($20 \times LD_{50}$). This procedure induced a total respiratory tract infection that caused virus shedding from the lung and led to death from viral pneumonia 5–10 days later. The lung virus titers, which were assayed 3 days after infection, were used as indices of protection in the lower respiratory tract of immunized mice [22].

2.5. Specimens

Mice were anesthetized with ether and then bled from the heart with a syringe. Serum was separated

from the blood by centrifugation and used for antibody titration. After bleeding, mice were incised ventrally along the median line from the xiphoid process to the point of the chin. The trachea and lungs were taken out and washed twice by inflating the lung through the trachea with a total of 2 ml PBS containing 0.1% bovine serum albumin (BSA) [21,23]. The bronchoalveolar wash was used for virus titration after the removal of cellular debris by centrifugation at 2500 rpm for 20 min at 4 °C. After the lower jaw of the mouse was cut off, a syringe needle was inserted into the posterior opening of the nasopharynx and then a total of 1 ml PBS containing 0.1% BSA was injected into the opening; the outflow was collected as nasal wash. The nasal wash was centrifuged to remove cellular debris and used for antibody titration.

2.6. Virus titrations

Serial 10-fold dilutions of the bronchoalveolar washes were prepared, and each dilution was injected into five embryonated eggs. The presence of virus in each egg was determined by the hemagglutinating capacity of the allantoic fluid 2 days after infection. The virus titer of each bronchoalveolar wash, expressed as the 50% egg-infecting dose (EID_{50}), was calculated from the lowest dilutions of allantoic fluid of eggs with virus. The virus titer in each experimental group was represented by the mean $\pm$ standard deviation (S.D.) of virus titer per milliliter of each wash specimen from all mice in each group [21]. Virus titers $> 10^{0.7}$ were detected in this system and virus titers $< 10^{0.5}$ were treated as $10^{0.4}$ for calculation of mean $\pm$ S.D.

2.7. Determination of anti-influenza virus antibodies (Abs)

The titers of Abs against influenza virus HA molecules in the serum were determined by standard microtiter method of hemagglutination-inhibiting (HI) assay after removal of non-specific inhibitors [24].

The titers of IgA and IgG Abs against influenza virus in the nasal wash were measured by ELISA, as described previously [23,25]. The wells of a 96-well ELISA plate (Linbro) were coated with 100 μl of the influenza HA vaccine (5 μg/ml) in 10 mM carbonate/bicarbonate buffer, pH 9.6. The plate was incubated for 2 h at room temperature and then washed three times with 200 μl/well of PBS containing 0.05% Tween 20 and 0.1% NaN_3 (PBS-Tween). The blocking solution, containing 1% BSA and 0.1% NaN_3 in PBS, was placed in the wells (200 μl) and incubated overnight at 4 °C. After washing with PBS-Tween, each sample (100 μl) was added to a set of three wells. Because IgA and IgG Abs recognize the same antigenic epitope, the IgA and IgG Abs in each sample were separated by Protein G Sepharose in advance. Samples for ELISA were prepared as follows; Protein G Sepharose packed into Ultrafree®-MC centrifugal filter units with low binding Durapore® membrane (pore size 0.45 μm; Millipore) was equilibrated with 20 mM sodium phosphate buffer, pH 7.0 (binding buffer). The nasal wash diluted with the binding buffer was applied to the Protein G Sepharose column, and then the column was washed with the same buffer by centrifugation at 1000 rpm for 5 min. IgA Ab was obtained in the unabsorbed fraction, and then the IgG Ab was eluted from the column with 0.1 M glycine-HCl buffer, pH 2.7. The eluate was neutralized with 1 M Tris–HCl buffer, pH 9.0, immediately. The unabsorbed fraction and neutralized eluate were diluted to five times with the blocking solution and used for ELISA. The plates were incubated for 2 h at room temperature and washed with PBS-Tween. Alkaline-phosphate-coupled goat anti-mouse IgA or IgG diluted with the blocking solution (1:1000) was added to each well (100 μl). The plates were incubated overnight at room temperature and then washed with PBS-Tween. Finally, *p*-nitrophenylphosphate (1 mg/ml) in 10% diethanolamine buffer containing 0.5 mM $MgCl_2$ at pH 9.8 (150 μl) was added to each well. After the incubation at 37 °C, the absorbance of the wells was read at 405 nm in a Microplate Reader (MRX-MD, Dynex). The Ab titer of unknown specimen was expressed by the relative absorbance to that of the specimen from positive control mice given the CTB*-combined vaccine.

2.8. Determination of anti-DPT Abs

The amounts of IgA and IgG Abs against DPT vaccine in the nasal wash and serum were measured by ELISA. The wells of a 96-well ELISA plate (Nunc-Immuno™ plate PolySorp™, Nalge Nunc International Corp.) were coated with 100 μl of pertussis toxin (PT) from *B. pertussis* strain Tohama phase I (Seikagaku Corp., Tokyo) (5 U/ml), DT (the Kitasato Institute) (10 Lf/ml, 4 μg/ml as protein nitrogen) or TT (the Kitasato Institute) (2 Lf/ml, 0.8 μg/ml as protein nitrogen) in PBS (pH 7.4). The plate was incubated overnight at 4 °C and then washed with PBS containing 0.1% Tween 20 and 0.02% NaN_3 (PBS-Tween). Each sample diluted with 0.2% BSA in PBS was added to a set of two wells. The plates were incubated for 60 min at 37 °C and washed with PBS-Tween. Alkaline-phosphatase-coupled goat anti-mouse IgG or IgA (Zymed Laboratories) diluted with 0.2% BSA in PBS was added to each well. The plates were incubated for 60 min at 37 °C and washed with PBS-Tween. Finally, *o*-nitrophenylphosphate in 10% diethanolamine buffer (pH 9.8) was added to each well. After the incubation at room temperature (25 °C) for 60 min, the absorbance of the wells was measured. Anti-PT Ab titer was ex-

pressed as ELISA units (EU)/ml using the FDA reference antiserum as standard. Anti-DT and TT Ab titers were expressed as international units (IU)/ml.

2.9. Hemolytic activity

Serial 1/2 dilutions of onjisaponins in PBS (final volume, 100 μl) were made in a V bottom microtiter plate. Sheep red blood cells (40% sheep blood and 60% Alsever's solution; Japan Bio-test) were washed three times by low speed centrifugation followed by resuspension of the red cell pellet in PBS to the original volume. The red cell pellet was diluted to 2.5 × the original volume and then used in the hemolysis assay. Twenty-five microliters of the resuspended cells were added to each well in the microtiter plate and mixed by pipetting. After incubation at room temperature for 30 min, the plates were spun at 1000 rpm for 5 min in a centrifuge to sediment unhemolyzed cells. Fifty microliters of the supernatant from each well were transferred to the well of a flat bottom microtiter plate. Absorbance caused by released hemoglobin was determined at 490 nm with a TOHSO microtiter plate reader.

2.10. Preparation of screening sample

Medicinal plants (10 g) were decocted with water (100 ml) to half volume. The hot-water extract was filtered with a stainless-steel mesh. The filtrate was centrifuged and passed through a Sephadex LH-20 column to remove tannins. The unadsorbed fraction was lyophilized and then dissolved or suspended in original volume of water.

2.11. Purification of adjuvant active substances from the root of P. tenuifolia

The root of *P. tenuifolia* Willdenow was obtained from Tsumura and Co. (Tokyo, Japan). The root of *P. tenuifolia* (500 g) was decocted with 10 l of distilled water to half volume. The hot water extract was filtered through a stainless-steel mesh. Decoction of the residue was repeated, and the two filtrates were combined. The filtrate was further filtered with glass fiber filter paper, and the final filtrate was lyophilized. After the lyophilizate (133.90 g) was dissolved in water (1.79 l), the supernatant which was obtained by centrifugation, was added to four volumes of ethanol and stirred overnight at room temperature to precipitate insoluble materials. The resulting supernatant, which showed potent adjuvant activity, was evaporated to dryness and dissolved in water (1 l). The water-soluble fraction was extracted with *n*-hexane (600 ml) to remove the lipid fraction. The resulting aqueous layer was further extracted with chloroform (1 l) and subsequently with water-saturated *n*-butanol (3 l) to obtain organic layers. Each of the organic layers, which showed potent adjuvant activity, were evaporated to dryness, suspended in water (1 l) and dialyzed against water for 10 days with a dialysis membrane of 10 000 m.w. cut off. The inner potions of dialysis membrane (non-dialysable fraction) were evaporated to dryness to give a chloroform-soluble or a *n*-butanol-soluble non-dialysable active fraction. These fractions were chromatographed by high-performance liquid chromatography (HPLC) with Hydroxyapatite column (PENTAX column SH-0710M, $\phi 7.5 \times 100$ mm) using water-acetonitrile as the eluent, and the adjuvant active fractions were obtained from non-dialysable fractions both of chloroform and *n*-butanol layers. The active fractions were further chromatographed by HPLC on Phenyl column (Senshu-pak C6H5-N, $\phi 4.6 \times 250$ mm^2) using water-acetonitrile as the eluent, and purified four active substances were obtained. The active substances were identified to be onjisaponins A, E, F and G (Fig. 1) by comparison with authentic standards on thin-layer chromatography (TLC) and HPLC.

2.12. Statistical analysis

All values obtained from experiments were expressed as mean ± S.D. Data were analyzed by one-way factorial analysis of variance (ANOVA), and significance was defined as $P < 0.05$.

3. Results

3.1. Screening of adjuvant activities from hot-water extracts of medicinal plants

Adjuvant activities against intranasal vaccination of influenza HA vaccine were screened from 267 kinds of hot-water extracts of medicinal plants with the mice. The Sephadex LH-20 unadsorbed fractions of the extracts were mixed with equal volume of influenza HA vaccine, and the mixture (10 μg/mouse as vaccine) was administered intranasally to the mice. Four weeks after the administration, HI Ab titer in serum was determined. Among the medicinal plants tested the fraction from root of *P. tenuifolia* showed the most potent adjuvant activity. The active substances were purified by hydroxyapatite and Phenyl column HPLC. The active substances were identified to be onjisaponins A, E, F and G (Fig. 1) as described in the Section 2.11.

3.2. Adjuvant activity of onjisaponins against primary intranasal administration of influenza HA vaccine

Mice were inoculated with the mixture of influenza A/PR8 HA vaccine (10 μg/mouse) and each onjisaponin (10 μg/mouse) intranasally. Four weeks after the

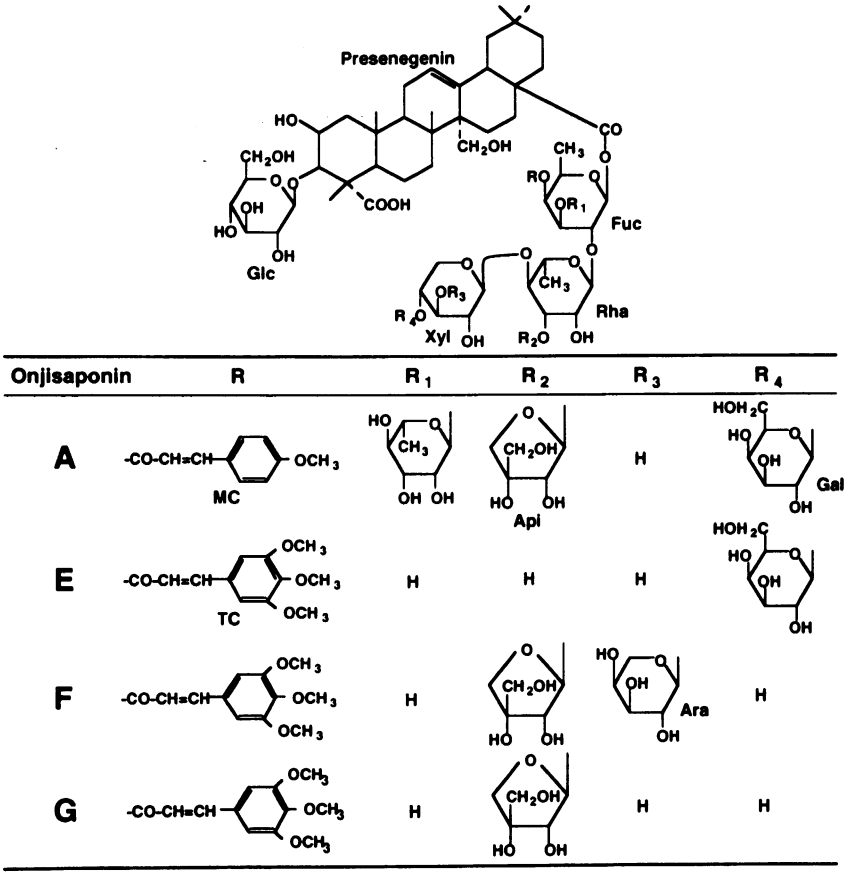

Fig. 1. Chemical structures of onjisaponins from the root of *P. tenuifolia*. MC = Monomethoxy cinnamic acid. TC = Trimethoxy cinnamic acid.

inoculation, serum samples were tested for HI Ab titers. When mice were inoculated with only HA vaccine, a low level of HI Ab titer was detected (Fig. 2). When mice were inoculated with the mixture of HA vaccine and onjisaponin A, E or F, HI Ab titers significantly increased 8–14 times in comparison with those of only HA vaccine-treated mice (Fig. 2). Onjisaponin G also significantly increased HI Ab titer 3–5 times. These results indicate that onjisaponins enhance production of serum Ab against HA of influenza vaccine. Monomethoxy cinnamic acid (MC) which is a structural moiety of onjisaponin A and trimethoxy cinnamic acid (TC) which is that of onjisaponins E, F and G (Fig. 1) did not enhance serum HI Ab titer (Fig. 2).

3.3. Adjuvant activity of onjisaponin against secondary intranasal inoculation of influenza HA vaccine

Each onjisaponin was inoculated with HA vaccine in both the primary and secondary vaccinations and compared to primary and secondary vaccinations with HA vaccine alone to compare adjuvant activities. Ab titers of mice primarily vaccinated with onjisaponins and HA vaccine followed by a secondary vaccination with HA vaccine alone were also determined. Three weeks after the first vaccination with the mixture of influenza A/PR8 HA vaccine (10 μg/mouse) and each onjisaponin (10 μg/mouse) into nostril, only A/PR8 HA vaccine (10 μg/mouse) was administered intranasally to the mice. A week after the second vaccination, serum and nasal

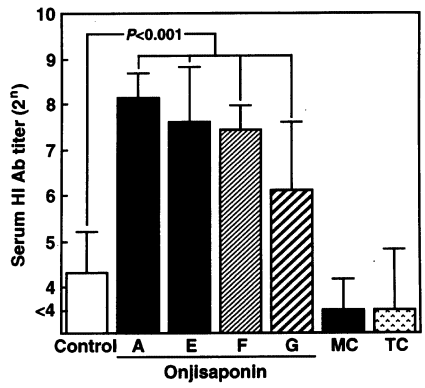

Fig. 2. Effect of onjisaponins and cinnamic acids on serum HI antibody (Ab) titer against primary intranasal inoculation of influenza HA vaccine. BALB/c mice (7 weeks old) were inoculated intranasally with the mixture of influenza A/PR/8/34 HA vaccine and sample (each 10 μg/mouse). Four weeks after vaccination, serum HI Ab titer was determined. Values represent mean ± S.D. ($n = 10$). MC = Monomethoxy cinnamic acid. TC = Trimethoxy cinnamic acid.

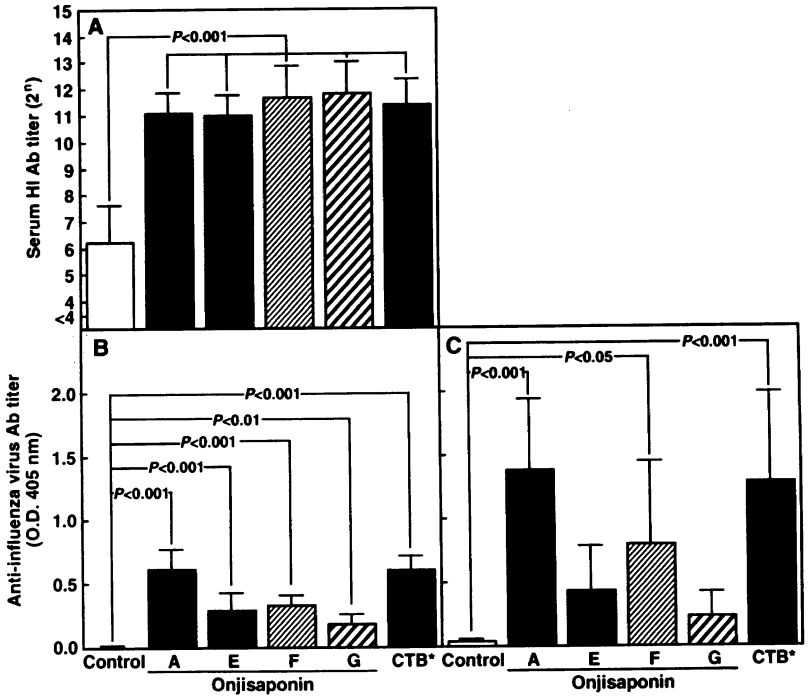

Fig. 3. Effect of onjisaponins, which were inoculated in primary vaccination, on serum HI and nasal anti-influenza virus Ab titers against secondary intranasal inoculation of influenza HA vaccine. Mice were inoculated intranasally with the mixture of influenza A/PR8 HA vaccine and adjuvant (each 10 μg/mouse). Three weeks after primary vaccination, A/PR8 HA vaccine (10 μg/mouse) was inoculated intranasally to the mice. A week after secondary vaccination, serum HI Ab titer (A), anti-influenza virus IgA (B) and IgG (C) Ab titers of nasal wash were determined as described in Section 2. Values represent mean ± S.D. ($n = 6–10$).

wash were prepared. Onjisaponins A, E, F and G enhanced serum HI Ab titers 27–50 times over HA vaccine alone, and the adjuvant activities of these onjisaponins were almost equal to that of CTB* at the same dose (Fig. 3(A)). When HA vaccine alone was primarily and secondarily immunized intranasally, a very low level of anti-HA IgA (Fig. 3(B)) and IgG Abs (Fig. 3(C)) were detected in the nasal wash. However, combination of primary immunization of the vaccine with onjisaponin A and secondary vaccination of the vaccine alone enhanced anti-HA IgA and IgG Ab titers in the nasal wash as similar to those of CTB* (Fig. 3(B) and (C)). Onjisaponin F also enhanced anti-HA IgA and IgG Ab titers in the nasal wash, whereas onjisaponins E and G enhanced only anti-HA IgA Ab titer significantly. These results indicate that onjisaponins also induce production of Ab even after secondary immunization and may have a memory effect against the HA vaccine which was together with the onjisaponins.

Mice were inoculated intranasally with the mixture of influenza A/Beijing HA vaccine (1 μg/mouse) and onjisaponin A or F (1 μg/mouse). Three weeks after the primary immunization, the mixture of vaccine (1 μg/mouse) and onjisaponin (1 μg/mouse) were re-inoculated intranasally to the mice. Onjisaponin F significantly enhanced serum HI Ab titers in comparison with influenza HA vaccine alone, but the activity was a little lower than that of the positive control, CTB*, at the same dose (Fig. 4(A)). Primary and secondary immunizations of the vaccine with onjisaponin F induced a little higher anti-influenza virus IgA and IgG Ab titers in the nasal wash compared with the control (Fig. 4(B) and (C)), but the IgA Ab titer was a little lower than that of CTB* (Fig. 4(B)); CTB* did not show any adjuvant activity in elevating anti-influenza virus IgG Ab titer (Fig. 4(C)). Onjisaponin A did not enhance nasal antiviral IgA and IgG Abs at the dose of 1 μg/mouse. These results indicate that even 1 μg of onjisaponin F induces the production of anti-influenza virus Ab in the nasal cavity and serum.

3.4. Adjuvant activity of onjisaponin F on protection of mice against influenza virus infection

The effectiveness of intranasal administration of influenza HA vaccine and onjisaponin F on influenza virus infections were tested by using mouse adapted influenza virus. Mice were inoculated intranasally twice with the mixture of A/PR8 HA vaccine (1 μg/mouse) and onjisaponin F (1 μg/mouse) at 4 weeks intervals, and infected intranasally with the A/PR8 virus 2 weeks after secondary vaccinations. Three days after the infection, pulmonary virus titers were determined as an index of

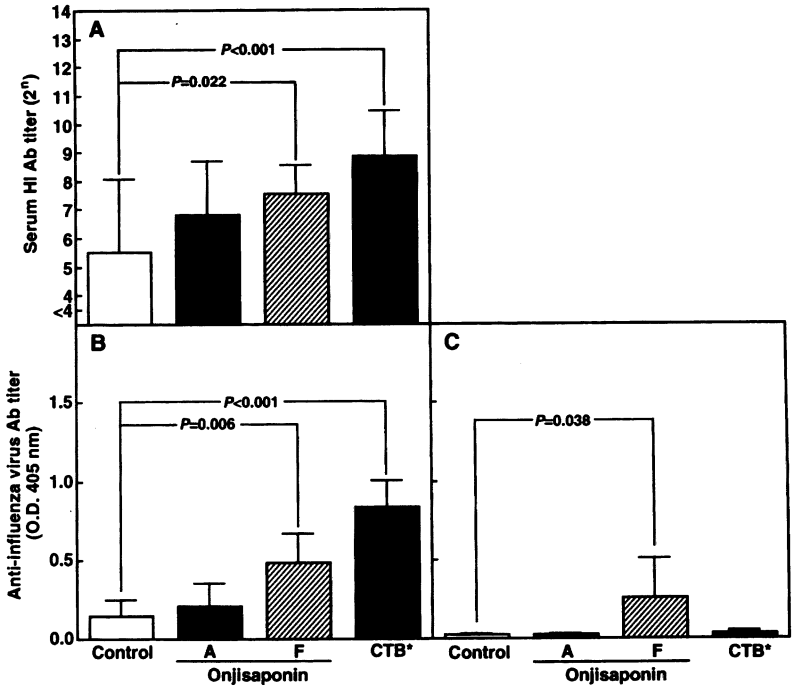

Fig. 4. Effect of onjisaponins, which were inoculated in primary and secondary vaccinations, on serum HI and nasal anti-influenza virus Ab titers against secondary intranasal inoculation of influenza HA vaccine. Mice were inoculated intranasally with the mixture of influenza A/Beijing/262/95 HA vaccine and onjisaponin A, F or CTB* (each 1 μg/mouse). Three weeks after primary vaccination, the mixture of HA vaccine and sample (each 1 μg/mouse) was inoculated intranasally to the mice. A week after secondary vaccination, serum HI Ab titer (A), anti-influenza virus IgA (B) and IgG (C) Ab titers of nasal wash were determined. Values represent mean ± S.D. ($n = 4$–10).

protection. Control mice receiving vaccine alone, failed to produce detectable protective Abs (data not shown) and no protection was observed against virus challenge (Fig. 5). However, complete protection was provided by the inoculation of vaccine (0.1 μg/mouse) with positive control, CT (0.1 μg/mouse) (Fig. 5), and none of the mice immunized with this regimen were infected. High levels of both nasal antiviral IgA Abs and serum antiviral IgG Abs were detected in the same mice (data not shown). Significant protection against infection was also observed by the inoculation of vaccine (1 μg/mouse) with onjisaponin F (1 μg/mouse) (Fig. 5), and one-third of the mice immunized with this regimen were not infected. In this experimental group of mice, high levels of both nasal antiviral IgA Abs and serum antiviral IgG Abs were also detected in the mice (data not shown). These results indicate that onjisaponin F is an effective adjuvant for intranasal administration of influenza HA vaccine to protect influenza virus infection.

3.5. Adjuvant activity of onjisaponins against intranasal administration of DPT vaccine

Mice were inoculated intranasally with the DPT vaccine and each onjisaponin as described in Section 2. When the mice were administered with DPT vaccine alone, IgG Ab titers against PT (Fig. 6(A)), DT (Fig. 6(B)) and TT (Fig. 6(C)) in the serum were very low. When mice were inoculated with DPT vaccine and onjisaponin A, serum anti-PT and anti-DT IgG Ab titers were enhanced to the same level as those of the mice which were administered with DPT vaccine and CTB* (Fig. 6(A) and (B)). The same dose of onjisa-

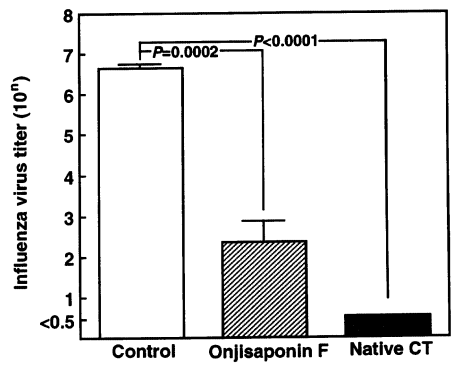

Fig. 5. Protection of influenza virus infection by intranasal administration of HA vaccine and onjisaponin F. Mice (6 weeks old) were inoculated intranasally with the mixture of A/PR8 HA vaccine and onjisaponin F (each 1 μg/mouse). Four weeks after primary vaccination, the mixture of HA vaccine and onjisaponin F (each 1 μg/mouse) was inoculated intranasally to the mice. Two weeks after secondary vaccination, mice were challenged with mouse adapted influenza virus A/PR8. Three days after challenge, bronchoalveolar washes were titrated for virus (EID_{50}). Values represent mean ± S.D. ($n = 3$).

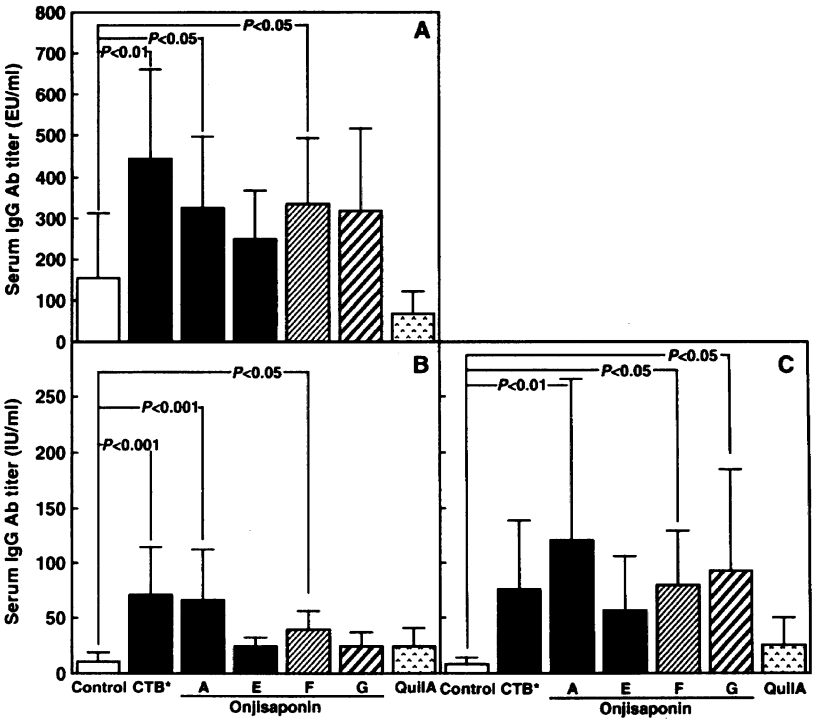

Fig. 6. Effect of onjisaponins on augmentation of serum IgG Ab titer against secondary intranasal administration of DPT vaccine. Mice (7 weeks old) were inoculated intranasally with DPT vaccine and sample (10 μg/mouse). Four weeks after primary vaccination, DPT vaccine was inoculated intranasally to the mice. Two weeks after secondary vaccination, anti-PT (A), anti-DT (B) and anti-TT (C) IgG Ab titers of the serum were determined with ELISA as described in Section 2. Values represent mean ± S.D. ($n = 5–10$).

ponin A enhanced anti-TT IgG Ab titer but CTB* and onjisaponin E showed little effects (Fig. 6(C)). Onjisaponin F also enhanced serum anti-PT, anti-DT and anti-TT IgG Ab titers, but onjisaponin G raised only anti-TT IgG Ab titer in the serum, significantly (Fig. 6). Quil A, which is a mixture of saponins prepared from the bark of *Quillaja saponaria*, did not show any adjuvant activity to enhance the serum IgG Ab titers against PT, DT and TT when Quil A was administered intranasally at same dose of onjisaponins.

When the mice were inoculated with DPT vaccine alone, IgA Ab titers against PT (Fig. 7(A)), DT (Fig. 7(B)) and TT (Fig. 7(C)) in the nasal wash were low. When the mice were inoculated with DPT vaccine and onjisaponin A or F, nasal anti-PT, anti-DT and anti-TT IgA Ab titers were enhanced to the same level as that of the mice which were administered DPT vaccine with CTB* (Fig. 7(A), (B) and (C)). Onjisaponin G also enhanced nasal anti-DT IgA and anti-TT IgA Ab titers (Fig. 7(B) and (C)). Quil A enhanced nasal anti-PT IgA Ab titer weakly, but did not show potent adjuvant activity against DT and TT. These results indicate that onjisaponins A and F show potent adjuvant activity against intranasal administration of DPT vaccine at the same level as that of CTB*, and are more effective than that of Quil A in the serum and nasal cavity.

3.6. Hemolytic activities of onjisaponins

It is generally known that saponins have hemolytic activity. Therefore hemolytic activities of the onjisaponins were measured using SRBC. The onjisaponins tested showed different hemolytic activities. Onjisaponins E and F showed little hemolytic activity at final concentrations up to 200 μg/ml (highest concentration tested) (Fig. 8). Onjisaponins A and G also showed little hemolytic activity at concentrations up to 100 μg/ml, whereas onjisaponin G showed weak hemolytic activity and onjisaponin A showed apparent hemolytic activity at a concentrations of 200 μg/ml (Fig. 8).

4. Discussion

Influenza is a highly infectious acute respiratory disease caused by specific influenza viruses that cause both world-wide pandemics and local outbreaks. The slight or radical alteration of the surface HA and sialidases are possible mechanisms by which these viruses change so rapidly [26]. An effective immune response induced by vaccinations to prevent influenza outbreaks is highly desirable. Current vaccines, which contain inactivated influenza viruses are administered subcutaneously. These vaccines have been shown to induce production

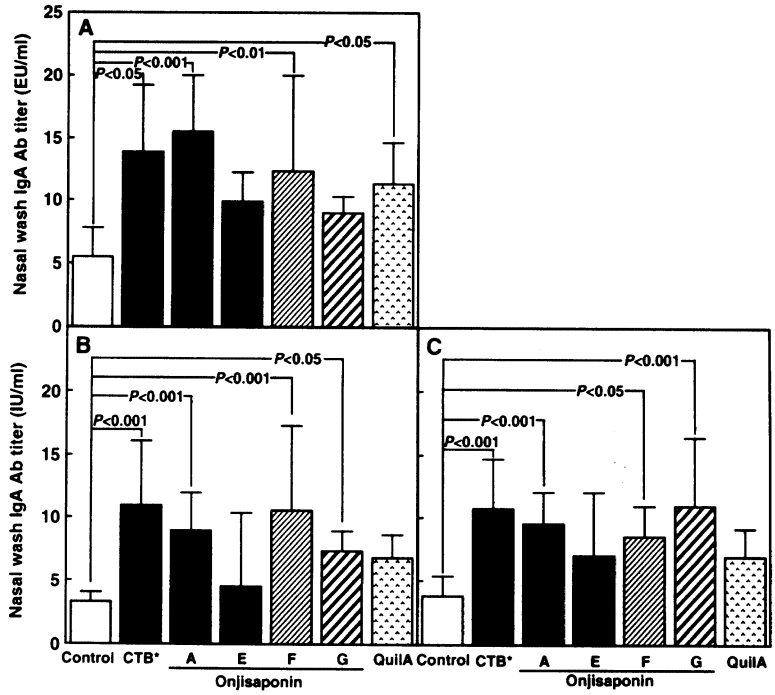

Fig. 7. Effect of onjisaponins on augmentation of nasal IgA Ab titer against secondary intranasal administration of DPT vaccine. Mice were treated as described in the legend of Fig. 6. Anti-PT (A), anti-DT (B) and anti-TT (C) IgA Ab titers of nasal wash were determined with ELISA as described in Section 2. Values represent mean ± S.D. ($n = 2$–10).

of high levels of serum antiviral IgG Abs which have a protective effect against homologous viral infections. However, these types of vaccinations are less effective against heterologous viral infections within the same viral subtypes. This explains the ineffectiveness of current influenza vaccines when a vaccine strain is different from an epidemic strain. Therefore, the advocacy for the intranasal administration of vaccines for the induction of mucosal IgA Ab and systemic IgG Ab against influenza viruses has been recognized [3] because mucosal IgA Ab can cross react with the same subtypes of virus strains. However, the administration of a vaccine by itself cannot efficiently induce the secretion of IgA Abs.

The purpose of this study was to determine whether adjuvant active substances could be isolated from certain Chinese and Japanese medicinal herbs. The investigators of this study screened the hot water extracts of 267 different Kampo (Japanese crude herbal drugs) medicinal herbs for adjuvant active substances to be tested as adjuvants with influenza vaccines. Significant adjuvant activity was found in the extracts from the root of *P. tenuifolia*. The active substances were purified from the extract by HPLC and identified as onjisaponins A, E, F, and G (Fig. 1). Using laboratory mice, each onjisaponin was administered intranasally with influenza vaccine and the results compared with mice inoculated with vaccine alone. Serum HI Ab titers were significantly increased using onjisaponins as adjuvants (Fig. 2). After primary administration of influenza vaccine with onjisaponins, re-inoculation with influenza vaccine alone enhanced both nasal anti-influenza virus IgA and serum HI Ab titers over those mice initially administered influenza vaccine itself (Fig. 3). These results indicate that onjisaponins purified from the root of *P. tenuifolia* have potent adjuvant activities when administered intranasally in combination with influenza vaccine.

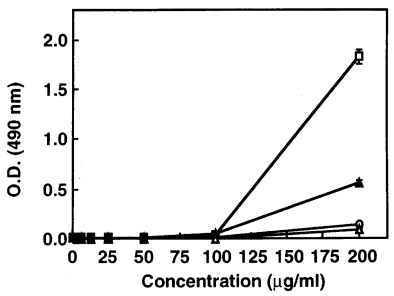

Fig. 8. Hemolytic activity of onjisaponins. Serial 1/2 dilutions of onjisaponins A (□), E (○), F (△) and G (▲) in PBS (100 μl/well) were made in a V bottom microtiter plate. Twenty-five microliters of the SRBC suspension in PBS were added to each well in the microtiter plate. After incubation at room temperature for 30 min, the plates were centrifuged at 1000 rpm for 5 min. Fifty microliters of supernatant from each well were transferred to the same well of a flat bottom microtiter plate and then absorbance was determined at 490 nm. Values represent mean ± S.D. of triplicate samples.

Influenza virus specific IgA Ab has been shown to play an essential role in protecting the host against infection by the virus in the respiratory tract [27]. To test the protective role, onjisaponin F, which showed the most potent adjuvant activity, was administered with influenza vaccine. Fig. 5 shows that when 1 μg of onjisaponin F and 1 μg of influenza HA vaccine were administered intranasally to mice, the influenza virus titers in the bronchoalveolar lavage fluid (BALF) were significantly reduced. These results indicate that onjisaponin F can induce protective immunity against intranasal infection by influenza virus. Interestingly, native CT showed more potent protective activity against proliferation of influenza virus in the bronchoalveolar cavity than onjisaponin F at the same dose. However, the extreme toxicity of CT to humans may inhibit its use and call for the use of recombinant B subunits of the holotoxin [7–9]. Therefore, the efficacy of using onjisaponin F as an adjuvant with influenza vaccine for treatment must be further evaluated in comparison with CT, recombinant CTB and/or CT derivatives.

Previous studies on the purification of onjisaponins from the root of *P. tenuifolia* have been described [28,29]. Onjisaponin B (senegin III) was reported to enhance the production of antigen-specific Abs in the serum after intraperitoneal injection [30]. However, mucosal adjuvant activities of onjisaponins A, E, F, and G have never been reported. This study is the first to show that onjisaponins A, E, F, and G have adjuvant activities by intranasal inoculation with influenza HA vaccine and DPT vaccine by inducing antigen-specific IgA Abs in nasal washes (Figs. 4 and 7). Because nasal vaccines stimulate both respiratory mucosal immune and systemic immune systems (Figs. 2, 3 and 6), the results of this study suggest that use of onjisaponins may be useful adjuvants when administered intranasally in combination with nasal vaccines. Such use may provide vaccines that may protect against both local and systemic infections by specific pathogens.

Quil A, a purified mixture of *Quillaja* saponins from the bark of *Q. saponaria* Molina, is known to have potent adjuvant activity and has been used as an adjuvant for veterinary vaccines [31]. However, Quil A has not been used in human vaccines because of its high toxicity. In addition, Quil A has been reported to have potent hemolytic activity. QS-21, a purified saponin from Quil A, has been shown to have both potent adjuvant activity [32,33] and hemolytic activity at 25 μg/ml [34]. The results of this study show that onjisaponins A, E, F, and G had no hemolytic activity at 100 μg/ml and onjisaponins E and F had no hemolytic activity at even 200 μg/ml (Fig. 8). These results suggest that onjisaponins may be safer than both Quil A and QS-21 when used as vaccine adjuvants. Further investigations on the safety, acute and chronic toxicities, and local stimulatory activities of onjisaponins are under progress.

The onjisaponins and QS-21 both contain an oleanan structure as the aglycon portion of the molecules. QS-21, however, has an aldehyde residue at position 23 of the aglycon and a hydroxyl group at position 16. Onjisaponins have a carboxyl group at position 23 and hydroxyl groups at positions 2 and 27. Previous studies indicate that the aldehyde group at position 23 of QS-21 is essential for its adjuvant activity [35]. However, the results of this study show that a carboxyl function at position 23 instead of an aldehyde group can be just as effective for inducing adjuvant activity. The carbonyls of aldehyde and carboxyl groups can form hydrogen bonds and may be involved in productive binding. Studies on how the functional groups at position 23 for both QS-21 and onjisaponins interact in inducing adjuvant activity are worth pursuing. The finding that MC and TC which are moieties of onjisaponins A, E, F, and G did not enhance serum HI Ab titers suggest that other parts and functionalities of the onjisaponins are required for biological activity. Chemical modifications of onjisaponins are currently under investigation to further elucidate the structure and function relationships of the various chemical groups and their mechanisms of action.

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