Intranasally and Orally Effective Adjuvants from Chinese and Japanese Medicinal Herbs for Nasal Influenza Vaccine

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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 \textit{Polygala tenuifolia} was found to contain potent mucosal adjuvant activity. The active substances were purified and identified as onjisaponins A, E, F and G. Two inoculations of mice 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. Onjisaponins also showed adjuvant effect for intranasal vaccination of diphtheria-pertussis-tetanus (DPT) vaccine. Traditional Japanese herbal (Kampo) formulation, Sho-seiryu-to (Xiao-Qing-Long-Tang in Chinese) showed oral adjuvant activity for nasally administered influenza vaccine. The active substance was isolated from one of the component herbs, the tuber of \textit{Pinellia ternata}, and was identified as 9S, 12S, 13S-trihydroxy-10E-octadecenoic acid (pinellic acid) by spectroscopic and synthetic methods. Oral administration of pinellic acid (1 µg) to mice given primary and secondary intranasal inoculation of influenza HA vaccine (1 µg) enhanced antiviral IgA antibody titers in nasal and bronchoalveolar washes. Onjisaponins and pinellic acid showed negligible hemolytic activity at effective dose for adjuvant activity. The results of these studies suggest that onjisaponins and pinellic acid may provide safe and potent adjuvants for intranasal inoculation of influenza HA vaccine.

INTRODUCTION

Subcutaneous injection is the most common method for delivery of vaccines. Aerial infectious organisms such as influenza virus 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. Therefore, nasal administration of vaccines for respiratory pathogens has attracted much attention as a means of delivering vaccines over subcutaneous injections due to localized inducement of an immune response. 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. Although the use of less toxic mutated bacterial 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.

Because several traditional Japanese herbal (Kampo) medicines have been used for the treatment of the “cold” syndrome where the influenza virus has been known to be one of the causative agent, their component herbs have a possibility to contain antiviral and/or immunostimulating substances. In this study, hot water extracts from 267 different...
Chinese and Japanese medicinal plants were screened for intranasally active substances that exhibited adjuvant activity with the nasal influenza vaccine. Oral administration of the Kampo formulation, Sho-seiryu-to (SST, Chinese name: Xiao-Qing-Long-Tang), has been used clinically for treatment of cold syndromes. SST has been shown to exhibit antiviral activity against the influenza A virus and the influenza B virus by stimulating production of antiviral IgA antibodies (Abs) in the upper respiratory tract of mice (Nagai and Yamada, 1998; Yamada and Nagai, 1998). Moreover, oral administration of SST enhanced nasal antiviral IgA Ab and bronchoalveolar and serum antiviral IgG Ab titers after secondary nasal inoculation of the vaccine (Nagai and Yamada 1998; Yamada and Nagai, 1998). Therefore, the present study also attempted to elucidate the active component herb in SST, and to isolate and characterize the effective compound exhibiting oral adjuvant activity for the nasally administered influenza vaccine.

MATERIALS AND METHODS
For preparation of a 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 the original volume of water. Component herbs of SST (Pinelliae Tuber, Ephedrae Herba, Schisandraceae Fructus, Cinnamomi Cortex, Paeoniae Radix, Asiasari Radix, Glycyrrhizae Radix and Zingiberis Siccatus Rhizoma) were purchased from Uchida Wakanyaku (Tokyo, Japan). Pinelliae Tuber (tuber of Pinellia ternata) was cultivated at the Guizhou Province in China in 1992. The B subunit of Escherichia coli heat-labile enterotoxin containing a trace amount of the holotoxin (LTB*) was kindly provided from the Research Center for Biologicals of the Kitasato Institute (Kitamoto-shi, Japan). The B subunit of cholera toxin containing a trace amount of the holotoxin (CTB*) was purchased from Sigma (St. Louis, MO). The influenza HA vaccine was prepared from a mouse adapted influenza virus A/PR/8/34 (A/PR8, H1N1) by the method of Davenport et al. Biotinylation of the HA vaccine was conducted using a biotin (long arm) NHS (Vector Laboratories, Burlingame, CA) according to the instructions of the manufacturer.

RESULTS
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 unabsorbed 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 Polygala 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) by comparison with authentic standards on TLC and HPLC (Nagai et al., 2001).

Adjuvant Activity of Onjisaponins against Primary or Secondary 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 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. 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. These results indicate that onjisaponins enhance production of serum Ab against HA of
influenza vaccine (Nagai et al., 2001). 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 the influenza HA vaccine alone, but the activity was a little lower than that of the positive control, CTB*, at the same dose (Fig. 2A). 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. 2B and C), but the IgA Ab titer was a little lower than that of CTB* (Fig. 2B); CTB* did not show any adjuvant activity in elevating anti-influenza virus IgG Ab titer (Fig. 2C). 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 (Nagai et al., 2001).

Adjuvant Activity of Onjisaponin F on Protection of Mice against Influenza Virus Infection

The effectiveness of intranasal administration of the influenza HA vaccine and onjisaponin F on influenza virus infections were tested by using a 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 week 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 protection. Control mice receiving vaccine alone failed to produce detectable protective Abs (data not shown) and no protection was observed against virus challenge (Fig. 3). However, complete protection was provided by the inoculation of vaccine (0.1 µg/mouse) with positive control, cholera toxin (CT) (0.1 µg/mouse) (Fig. 3), 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. 3), 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 the influenza HA vaccine to protect the influenza virus infection (Nagai et al., 2001). Onjisaponin A and F also showed 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, which is a mixture of saponins prepared from the bark of Quillaja saponaria, in the serum and nasal cavity.

Hot Water Extract of Pinelliae Tuber Enhances Ab Production for Nasal Influenza Vaccine

A traditional Japanese herbal (Kampo) medicine, SST, is composed of eight component herbs. Hot water extracts from the four component herbs (Pinelliae Tuber, Schisandrae Fructus, Asiasari Radix and Zingiberis Siccatum Rhizoma) not in Kakkon-to (Ge-Gen-Tang in Chinese), which has no anti-influenza virus activity, were each orally administered to BALB/c mice from 9 days before to 16 days after intranasal inoculation of the influenza HA vaccine, and the titers of anti-influenza virus Ab in each serum were measured. Oral administration of hot water extract from Pinelliae Tuber with intragastric gavage significantly enhanced the titer of anti-influenza virus IgG Ab, and that from Zingiberis Siccatum Rhizoma slightly increased the titer, whereas those from other herbs had no effect (Fig. 4A). In order to evaluate the adjuvant effect of the hot water extract from Pinelliae Tuber on the production of secreted anti-influenza virus IgA Ab in the respiratory tract, the hot water extract was administered to the mice, which received secondary intranasal inoculations of influenza HA vaccine. When the mice were
vaccinated by intranasal inoculation of HA vaccine together with the known adjuvant, LTB*, followed by a secondary intranasal vaccination 28 days after the first vaccination, significant increases in antiviral IgA Ab titers were observed in the bronchoalveolar washes (Fig. 4B). Oral administration of hot water extract of Pinelliae Tuber (20 mg/mouse/day) 9 days before to 16 days after the primary intranasal vaccination also significantly increased the antiviral IgA Ab titers in the bronchoalveolar washes 14 days after the secondary vaccination (Fig. 4B), suggesting that the hot water extract of Pinelliae Tuber contained adjuvant active substances for the production of anti-influenza virus IgA Abs in the respiratory tracts.

Purification and Identification of Adjuvant Active Substance from Pinelliae Tuber

In order to identify the adjuvant active substance(s) from Pinelliae Tuber, the hot water extract was fractionated, and purified by in vivo oral adjuvant activity guided purification. Purified active substance was identified as a 9, 12, 13-trihydroxy-10-octadecenoic acid by spectroscopic method. Since the absolute configurations of hydroxy groups at C-12 and -13 could not be determined by conventional dibenzoyl theory, all theoretical stereoisomers (9S, 12S, 13S; 9S, 12R, 13S; 9S, 12S, 13R; 9S, 12R, 13R; 9R, 12S, 13S; 9R, 12R, 13S; 9R, 12S, 13R; 9R, 12R, 13R) were synthesized (Sunazuka et al., 2002). By comparison of the analytical data between these synthetic trihydroxy fatty acids and the active substance, the purified active substance, the compound was identified as 9S, 12S, 13S-trihydroxy-10E-octadecenoic acid, and named pinellic acid (Fig. 5) (Sunazuka et al., 2002; Nagai et al., 2002).

Adjuvant Activity of Pinellic Acid with Intranasal Administration of Influenza Vaccine

Mice were orally administered pinellic acid (1 µg/mouse) with intragastric gavage followed by intranasal inoculation of HA vaccine (1 µg/mouse), and 3 weeks later, the same procedure repeated. The anti-influenza virus IgA and IgG Ab responses in the nasal and bronchoalveolar washes and serum in the vaccinated mice were examined 1 week after secondary vaccination. The antiviral IgA Ab responses, induced in the nasal and bronchoalveolar washes of mice given pinellic acid with the vaccine, were enhanced 5.2- and 2.5-fold, respectively, compared with control mice given vaccine and solvent alone (Fig. 6A and B). Secondary antiviral IgG Ab responses, induced in the bronchoalveolar washes and sera of mice given pinellic acid with the vaccine, were increased 3-fold compared with the controls (Fig. 6C and D). These results suggest that the adjuvant activity of orally administered pinellic acid may be specific for eliciting antigen-specific Ab production in both upper and lower respiratory tracts and serum after secondary intranasal vaccinations with influenza HA vaccine (Nagai et al., 2002).

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. 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 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 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. Present 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. Because nasal vaccines stimulate both respiratory mucosal immune and systemic immune systems, 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. Among the oral adjuvants developed for mucosal vaccines, CT is a potentially useful candidate. However, the toxicity associated with CT presents an obstacle for human use. A potentially useful alternative is to employ recombinant subunits of CT with small quantities of the holotoxin. In the present study, we show that oral administration of pinellic acid, which was isolated from the tuber of Pinellia ternata, one of component herbs of the Kampo formulation, SST, as an adjuvant for intranasal inoculation of influenza HA vaccine results in systemic humoral responses as well as production of IgA at local mucosal sites. Presently, there are no currently available oral adjuvants for mucosal vaccines for use in humans. The results of this study show that pinellic acid may prove to be a useful and safe adjuvant for mucosal vaccines.

Literature Cited
Fig. 1. Chemical structures of onjisaponins. MC = Monomethoxy cinnamic acid. TC = Trimethoxy cinnamic acid.
Fig. 2. Effect of onjisaponins on anti-influenza virus Ab titers against secondary intranasal inoculation of influenza HA vaccine. Mice were inoculated intranasally with the mixture of influenza A/Beijing/HA vaccine and onjisaponin A, F or CTB* (each 1 µg/mouse) twice. A week after secondary vaccination, serum HI (A), anti-influenza virus IgA (B) and IgG (C) Ab titers of nasal wash were determined. Values represent mean ± S.D. (n = 4-10).

Fig. 3. Protection of influenza virus infection by intranasal administration of HA vaccine and onjisaponin F. Mice were inoculated intranasally with the mixture of A/PR8 HA vaccine and onjisaponin F (each 1 µg/mouse) twice. 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. Values represent mean ± S.D. (n = 3).
Fig. 4. Effect of hot water extracts of component herbs of Sho-seiryu-to on production of antiviral Abs for nasal inoculation of influenza HA vaccine in mice. Mice were treated p.o. with hot water extract of Pinelliae Tuber (PT), Schisandraceae Fructus (SF), Asiasari Radix (AR), Zingiberis Rhizoma (ZSR) or water 21 times from 9 days before to 16 days after i.n. vaccination. (A) Anti-influenza virus IgG Ab titers in serum were determined after primary vaccination. (B) Anti-influenza virus IgA Ab titers in bronchoalveolar washes were determined after secondary vaccination. Values represent mean ± S.E. (n = 5-6).

Fig. 5. Structure of pinellic acid (9S, 12S, 13S-trihydroxy-10E-octadecenoic acid).
Fig. 6. Effect of oral administration of pinellic acid on production of antiviral Abs for secondary nasal inoculation of influenza HA vaccine in mice. Anti-influenza virus IgA Ab titers of nasal (A) and bronchoalveolar washes (B), and antiviral IgG Ab titers of bronchoalveolar washes (C) and serum (D) were determined 7 days after secondary vaccination. Values represent mean ± S.E. (n = 6-7).