Oral adjuvant activity for nasal influenza vaccines caused by combination of two trihydroxy fatty acid stereoisomers from the tuber of *Pinellia ternata*

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A R T I C L E   I N F O

Article history:
Received 25 December 2009
Received in revised form 27 February 2010
Accepted 6 March 2010

Keywords:
Influenza vaccine
Mucosal immunity
Oral adjuvant
Pinellic acid
Stereoisomer

A B S T R A C T

Pinellic acid from the tuber of *Pinellia ternata* was isolated as an effective oral adjuvant for nasal influenza vaccine, and identified 95,125,135-trihydroxy-10E-octadecenoic acid (95,125,135) by the enantioselective total synthesis [Nagai et al, Int. Immunopharmacol., 2, 1183-93 (2002); Shirahata et al., Tetrahedron, 62, 9483-96 (2006)]. However, present study showed that synthetic 95,125,135 that was nearly 100% pure was not effective as an oral adjuvant. HPLC analysis also showed that the adjuvant active pinellic acid fraction from tuber of *P. ternata* contained the 95,125,135 as the main component and at least two minor components. Therefore seven other chemically synthesized stereoisomers were tested in combination with the 95,125,135 for oral adjuvant activity. Only the 95,125,135 in combination with the 95,125,138 isomer in a weight ratio of 90.4:9.6 (pinellic acid mixture, PAM) was a potent oral adjuvant and elicited both antiviral IgA antibody (Ab) in bronchoalveolar lavage fluids and nasal washes and antiviral IgG1 Ab in mice sera. Oral administration of the PAM followed by nasal influenza vaccination and infection with A/PR/8/34 virus showed increases in survival rate (22%, control versus 78% test) in mice orally administered PAM as adjuvant. Histopathological examination of lung tissue of mice given oral PAM with vaccine followed by influenza virus infection showed attenuated infiltration of inflammatory cells with decreases in the alveolar spaces and increases in the alveolar septa. The result of this study refutes the our previous study and suggests that the combination of 95,125,135 and 95,125,138 isomers is necessary for effective oral adjuvant activity when used in conjunction with nasal influenza vaccine.

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1. Introduction

Influenza virus infection is a worldwide health concern for people with compromised immune systems, respiratory diseases, and the elderly inflicted with cardiopulmonary disease. Influenza vaccine is an useful prophylaxis for influenza virus infection [1]. Trivalent influenza vaccine consists of inactivated H1N1 and H3N2 subtypes of influenza A virus, and influenza B virus has been used as current vaccine by subcutaneous injection. Subcutaneous injection of this vaccine is known to induce production of serum antiviral IgG antibodies (Abs) that provide a protective effect against proliferation of influenza virus in lung tissue [2]. The primary site of influenza virus infection is the nasal cavity. Intranasal inoculation of influenza HA vaccine has been tried to increase its safety and to prevent the antigenic variation of influenza viruses [2]. However, to date, vaccinations in the nasal cavity have been shown to be less effective than subcutaneous vaccinations. Therefore, an effective vaccine that could be administered intranasally and stimulate the production of mucosal IgA Abs against influenza virus would be highly desirable [3]. Previous studies have shown that nasal administration of vaccines by themselves may not provide sufficient immunostimulation [4,5], and the use of adjuvants to enhance local mucosal immune responses has been documented [6].

Several traditional Japanese herbal (Kampo) medicines have been used for the treatment of “cold” syndrome [7] in which influenza virus is known to be a major causative pathogen [1]. Oral administration of the Kampo medicine, shoseiryuto (SST, Chinese name: xiao-qing-long-tang, Korean name: so-cheong-ryong-tang), has been used clinically for treatment of cold syndromes. Studies using BALB/c mice showed SST had potent antiviral activity against H1N1 and H3N2 subtypes of influenza A virus and influenza B virus as evidenced by
increases in antiviral IgA Abs in the nasal wash and bronchoalveolar lavage fluid (BALF) [8,9]. Moreover, oral administration of SST enhanced serum hemagglutination-inhibiting Ab titers after primary nasal inoculation of influenza HA vaccine in mice [8] and increased nasal antiviral IgA Ab and BALF and serum antiviral IgG Ab titers after secondary nasal inoculation of the vaccine [9]. These results suggest that SST contains components that enhance immunostimulating activity against nasally inoculated influenza viral antigens. SST is a multi-herbal formula which consists of 8 component herbs. The tuber of Pinellia ternata Breitenbach (Pinelliae Tuber) was identified to be an essential component for oral immunostimulating activity [10]. The active component was isolated and purified from the tuber of P. ternata and characterized as pinellic acid [10]. Pinellic acid was identified as 9S,12S,13S-trihydroxy-10E-octadecenoic acid by infrared spectroscopy, proton magnetic resonance, mass spectrometry, and circular dichroism by comparison with all eight chemically synthesized stereoisomers [11–13].

Although the synthetic product (9S,12S,13S-trihydroxy-10E-octadecenoic acid) was nearly 100% pure by spectroscopic analysis, and characterized as pinellic acid [10]. Pinellic acid was active component was isolated and purified from the tuber of P. ternata and characterized as pinellic acid [10]. Pinellic acid was identified as 9S,12S,13S-trihydroxy-10E-octadecenoic acid by infrared spectroscopy, proton magnetic resonance, mass spectrometry, and circular dichroism by comparison with all eight chemically synthesized stereoisomers [11–13].

All stereoisomers of pinellic acid (9S,12R,13S) was prepared from the tuber of P. ternata as previously described [10]. All stereoisomers of pinellic acid (9S,12S,13S, 9S,12R,13S, 9R,12S,13S, 9R,12S,13R, 9R,12R,13S and 9R,12R,13R-trihydroxy-10E-octadecenoic acid; Fig. 1) were synthesized as previously described [11–13]. Mouse-adapted influenza virus A/PR/8/34 (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 kindly provided from The Kitasato Institute Research Center for Biologica (Kitamoto-shi, Saitama, Japan). Influenza HA vaccines were prepared from influenza virus A/New Caledonia/20/99 (H1N1) and mouse-adapted influenza virus A/PR8 by the method of Davenport et al. [14]. Biotinylination of the HA vaccine was conducted using a biotinylation kit (sulfo-OSu) (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions.

2.2. HPLC analysis of pinellic acid

The HPLC analysis of pinellic acid was performed as previously described [15]. Pinellic acid (1 mg) was dissolved in methanol (1 ml). The solution was filtered using a DISMIC-13HP PTFE membrane filter (0.45 µm, Toyo Roshi Kaisha, Tokyo, Japan).

2.3. Animals

Six week old female BALB/c mice were purchased from Clea Japan (Tokyo, Japan). The animals were housed in plastic cages in an air-conditioned room at 23 ± 2 °C with a relative humidity of 55 ± 10% under a 12-h light–dark cycle, fed a standard laboratory diet and given water ad libitum. Animal experiments were approved by the Animal Research Committee of Kitasato University, and performed in accordance with the Guidelines for Care and Use of Laboratory Animals at Kitasato University and the Guidelines for Proper Conduct of Animal Experiments from Science Council of Japan.

2.4. Vaccination and pinellic acid administration

Seven week old female BALB/c mice at the onset of primary vaccinations were used in all experiments. Mice were orally administered at the dose of 0.1 or 1 µg/mouse (<5 or ~50 µg/kg) of pinellic acid in 0.2% MeOH/water (<0.001 endotoxin units/mouse determined with Endospace® test, Seikagaku Biobusiness, Tokyo, Japan), anesthetized by intraperitoneal injection of Nembutal® (sodium pentobarbital, Dainippon Sumitomo Pharma, Osaka, Japan) and vaccinated by a primary intranasal inoculation of 1 µl phosphate-buffered saline (PBS) containing 0.5 µg/ml of influenza vaccine in each nostril. The mice were given a secondary oral administration of pinellic acid at the same dose of the primary administration, and intranasal inoculation of the vaccine (0.5 µg/1 µl in PBS) into each nostril at 21 days post the primary vaccination. Mice were divided into control group (oral administration of 0.2% MeOH/water), 9S,12S,13S group [oral administration of 9S,12S,13S-pinellic acid (1 µg/mouse)], 9S,12S,13S+9S,12R,13S group [oral administration of the mixture of 9S,12S,13S-pinellic acid (0.904 µg/mouse) and 9S,12R,13S-pinellic acid (0.096 µg/mouse)], or 9S,12R,13S group [oral administration of 9S,12R,13S-pinellic acid (0.1 µg/mouse)] with nasal vaccination.

2.5. Specimens

At 7 days post the secondary vaccination, the mice were sacrificed using ether and then bled from the axillary artery, and the blood was allowed to clot. Serum samples were separated from the blood by centrifugation. The bled mice were incised ventrally along the median line from the xiphoid process to the point of the chin. The trachea with the lung attached were removed and washed twice by inflating the lung through the trachea with 2 ml of PBS containing 0.1% bovine serum albumin (BSA) [10,16]. The BALF was centrifuged at 1200 × g for 20 min to remove cellular debris. The lower jaw was excised from the head of the mouse. A hypodermic needle was
inserted into the posterior opening of the nasopharynx, and 2 ml of PBS containing 0.1% BSA was injected to collect the nasal wash. The nasal washes were centrifuged at 1200 × g for 20 min to remove cellular debris. Serum, BALF and nasal wash were kept at −80 °C and used for titration of anti-influenza virus Abs.

2.6. Determination of anti-influenza virus Abs

The amounts of IgA or IgG1 Abs to influenza HA vaccine in nasal wash, BALF and serum were measured by the modified fluorometric reverse (Ab-capture) ELISA [10,17]. Briefly, the wells of a 96-well ELISA plate (Immulon® 4 HBX, Thermo Fisher Scientific, Rockford, IL, USA) were coated with 100 µl of anti-mouse IgA or IgG1 mAb (BD Pharmingen, San Diego, CA, USA) (1 µg/ml) in 50 mM carbonate–bicarbonate buffer (pH 9.5) containing BSA (10 µg/ml), and incubated at 37 °C for 3 h. After the solution was removed, the blocking solution, 1% nonfat dry milk (Snow Brand Milk Products, Sapporo, Japan) in PBS, was placed in each well (300 µl) and incubated at 37 °C for 1 h. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween). Serial dilutions of nasal wash, BALF or serum with SuperBlock® blocking buffer in PBS (Thermo Fisher Scientific) (diluted to 1:10 with PBS containing 0.05% Tween 20) were added to the wells (100 µl). The wells were sealed with adhesive tape and the plates were incubated overnight at room temperature. After washing with PBS-Tween, 1 µg/ml of biotinylated influenza HA vaccine in the blocking solution was added to each well (100 µl). The plates were incubated at room temperature for 1 h with shaking on a microplate mixer. After washing the wells, streptavidin-β-galactosidase conjugate (Calbiochem, EMD Chemicals, Gibbstown, NJ, USA), diluted to 1:1000 with the blocking solution, was added and incubated at room temperature for 1 h with shaking. After the final wash, 0.1 mM 4-methylumbelliferyl-β-D-galactoside (Sigma-Aldrich, St. Louis, MO, USA) in buffer A (10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM MgCl₂ and 0.1% BSA) was added to each well (100 µl). The plate wells were sealed with tape, and then incubated at 37 °C for 2 h. The enzyme reaction was stopped by the addition of 100 µl of 0.1 M glycine–NaOH (pH 10.3), and the fluorescence of the 4-methylumbelliferone measured (ex. 355 nm, em. 460 nm) using a Fluoroskan II spectrofluorometer (Thermo Fisher Scientific). Endpoint titers of influenza HA vaccine-specific Abs were expressed as reciprocal log_{10} titers.

2.7. Infection of influenza virus

Mouse-adapted influenza virus A/PR8 was used for infection [16,17]. Mice were anesthetized by an intraperitoneal injection of Nembutal® (sodium pentobarbital), and infected with A/PR8 virus by intranasally dropping 20 µl (2 × LD₅₀) of the viral suspension into one nostril. 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 survival rates were observed for 21 days.

2.8. Lung histology

The lung was inflated with 1 ml of 3.7% paraformaldehyde and then fixed in 3.7% paraformaldehyde for 2 days. After fixation, the lung was embedded in paraffin with Tissue-Tek® Infiltration Processor (Sakura Finetek Japan, Tokyo, Japan) and 2 µm sections were cut with a Sledge Microtome IVS-410 (Sakura Finetek Japan). Sections were mounted on glass slides and stained with hematoxylin and eosin. Mucus production and goblet cell hyperplasia were examined by periodic acid–Schiff (PAS) staining.

2.9. Statistical analysis

All results were expressed as the mean ± standard error of the mean (S.E.M.). Data were analyzed by one-way factorial analysis of variance (ANOVA). Differences between two groups were analyzed by Student’s t-test. Differences among three groups or more were analyzed by Dunnett’s test. Differences in the survival rate between groups after lethal challenge with influenza virus were analyzed using the logrank test in the Kaplan–Meier method with StatView® software (SAS Institute, Cary, NC, USA). P-values < 0.05 (p < 0.05) were considered to be significant.
3. Results

3.1. HPLC analysis of pinellic acid

The pinellic acid fraction, which was previously reported to have oral adjuvant activity [10], prepared from the tuber of P. ternata was analyzed again after HPLC and the elution pattern presented two minor peaks before and after (r.t. = 31.18 min and 31.65 min respectively) the main peak (r.t. = 31.39 min) with the peak area ratios = 8.9:84:1:3.6 (Fig. 2A). The 9S,12R,13S-trihydroxy-10E-octadecenoic acid synthesized in large scale showed only a main peak (r.t. = 31.39 min) (Fig. 2B). All eight synthetic stereoisomers of pinellic acid are shown with their respective retention times (Fig. 2C). The 9R,12R,13S isomer had a nearly identical retention time (31.38 min) as the 9S,12S,13S isomer. The 9R,12S,13S and 9S,12R,13S isomers were eluted with the longest retention times (31.48 min) (Fig. 2C).

3.2. Combination effect of pinellic acid stereoisomers on adjuvant activity for intranasal administration of influenza vaccine

Previously, all eight pinellic acid stereoisomers (Fig. 1) were synthesized in small scale and tested for oral adjuvant activity by intranasal inoculation of influenza HA vaccine [12,13]. Using 9S,12S,13S-trihydroxy-10E-octadecenoic acid (synthesized in small scale) as an adjuvant, both the primary antiviral IgA Ab induced responses in the nasal washes and the secondary IgG1 Ab responses in the sera of mice inoculated with vaccine were enhanced when compared to mice given only solvent as the control adjuvant [12,13]. When the seven other stereoisomers of pinellic acid (9R,12R,13S; 9S,12S,13R; 9R,12R,13R; 9R,12S,13S; 9S,12R,13S; 9R,12S,13R; and 9R,12R,13R) were administered in similar fashion, each showed little or no adjuvant activity [12,13]. The initial finding that 9S,12S,13S-trihydroxy-10E-octadecenoic acid appeared to be the active component of Pinelliae Tuber composed in SST prompted a large stereoselective synthesis of this isomer. However, when this synthetically pure 9S,12S,13S-trihydroxy-10E-octadecenoic acid was tested for adjuvant activity by the methods described above, the antiviral IgA Ab responses in the nasal washes and BALF, and the antiviral IgG1 Ab responses in mice sera and BALF were negative (Fig. 3). These findings led us to study the effects of combining the various stereoisomers of pinellic acid for adjuvant activity using the HPLC % peak ratio (90.4:9.6) observed in the adjuvant active pinellic acid fraction prepared from Pinelliae Tuber [10]. Oral administration of the pinellic acid fraction (1 µg/mouse) showed a potent adjuvant activity for intranasal inoculation of influenza vaccine (1 µg/mouse) in previous study [10]. However, intranasal administration of pinellic acid showed only weak adjuvant activity for the nasal influenza vaccine [10]. Therefore the adjuvant activity of mixture of pinellic acid isomers at the dose of 1 µg/mouse for the nasal influenza vaccine (1 µg/mouse) through the oral route was tested in this study. The adjuvant activity of 9R,12R,13S isomer at the dose of 0.1 µg/mouse was also tested for the possibility that only 9S,12R,13R isomer is an active component containing in the mixture. Only the 9S,12S,13S isomer in combination with the 9S,12R,13S isomer in a 90.4:9.6% ratio respectively resulted in significant adjuvant activity when orally administered to BALB/c mice at 1 µg/mouse (~50 µg/kg body weight) as determined by enhancement of anti-influenza virus IgA Ab in the nasal washes and the BALF and IgG1 Ab in sera when compared to solvent alone as an adjuvant control (Fig. 3A, B and D). In addition, both the 9S,12S,13S isomer (1 µg/mouse) and the 9S,12R,13S isomer (0.1 µg/mouse) did not show any adjuvant activity when used alone as an adjuvant (Fig. 3). All six other isomers in combination with the 9S,12S,13S isomer showed little or no adjuvant activity using the above protocols (Table 1). For simplification purposes, the 9S,12S,13S isomer in combination with the 9S,12R,13S isomer in a % ratio of 90.4:9.6 is designated PAM (pinellic acid mixture).

Fig. 3. Effect of mixture of pinellic acid stereoisomers (9S,12S,13S:9S,12R,13R = 90.4:9.6) on the anti-influenza virus antibody titers. Anti-influenza virus IgA Ab titers of nasal wash (A) and BALF (B), and antiviral IgG1 Ab titers of nasal wash (C) and serum (D) were determined at 7 days post the last intranasal inoculation of influenza vaccine by ELISA. Bars represent mean ± S.E.M. (n = 6). Control: 0.2% MeOH/water. Doses of 9S,12S,13S form and mixture of stereoisomer (9S,12S,13S:9S,12R,13R = 90.4:9.6) were 1 µg/mouse (~50 µg/kg). Dose of 9S,12R,13R form was 0.1 µg/mouse (~5 µg/kg).
3.3. Protection of influenza virus infection by nasal influenza vaccine with pinellic acid stereoisomers

BALB/c mice were administered orally the PAM at a dose of 1 µg/mouse followed by intranasal inoculation of influenza vaccine A/PR8 (1 µg/mouse) followed by a secondary oral administration of the PAM (1 µg/mouse) and intranasal inoculation of the vaccine (1 µg/mouse) at 21 days post the primary administration. Mice were then infected with A/PR8 virus by dropping intranasally 20 µl of the viral suspension containing 2 × 10^6 pfu at 7 days post the secondary inoculation, and the survival rates were observed for 21 days. The mice administered solvent (control) orally and intranasal inoculated with influenza vaccine intranasally began to die at 9 days post A/PR8 virus infection and the survival rate was 22% at 21 days post virus infection (Fig. 4A). The test mice administered PAM orally and intranasally inoculated with the influenza vaccine began to die at 9 days post A/PR8 virus infection; however, the survival rates was 78% at 21 days post virus infection. Reduction in body weight of mice infected with the influenza virus was less in mice orally administered PAM and inoculated with influenza vaccine at 6–8 days post infection in comparison with the mice not administered the PAM (Fig. 4B). These results show that oral administration of the PAM adjuvant protects mice from influenza virus infection.

Influenza virus infection also induced marked infiltration of inflammatory cells, especially lymphocytes, eosinophils and neutrophils, into the lung tissue of influenza vaccine-inoculated mouse (Fig. 5A). A noticeable decrease of alveolar space and increase of alveolar septa were observed by the infiltration of inflammatory cells and fibrosis of lung tissue (Fig. 5A). These observations indicate severe bronchiolar pneumonia due to bronchial hyperplasia. Infection of influenza virus after nasal vaccination with oral administration of the PAM attenuated the infiltration of inflammatory cells with decreases in the alveolar spaces and increases in the alveolar septa as compared to the vehicle control (Fig. 5B). Also, influenza virus infection caused marked goblet cell hyperplasia and mucus hypersecretion within the bronchi in the lung tissue of influenza vaccine-inoculated mouse (Fig. 5C). The influenza virus-induced mucus secretion was significantly abated by the administration of the PAM as adjuvant in test mice when compared to a vehicle control (Fig. 5D). These results suggest that the PAM as adjuvant in conjunction with nasally administered influenza vaccine decreases the inflammation in the lung of the influenza virus-infected mice based on histochemical analysis.

4. Discussion

This study examines the combination of two pinellic acid stereoisomers (9S,12S,13S and 9S,12R,13R) that elicited oral adjuvant activity when applied to nasal influenza vaccines. Previous studies showed that pinellic acid prepared from the tuber of *P. ternata* possessed potent adjuvant activity with nasal influenza vaccine and was identified as 9S,12S,13S-trihydroxy-10E-octadecenoic acid [10]. The stereochemistry of the 9S,12S,13S-trihydroxy-10E-octadecenoic acid was confirmed by stereoselective chemical synthesis of all eight stereoisomers [11–13]. Unexpectedly, the 9S,12S,13S isomer synthesized in a large scale and near 100% purity did not show any adjuvant activity. The present study showed that the pinellic acid fraction from the tuber of *P. ternata* showed three adjacent peaks (one major and two minor peaks) as determined by HPLC. The main peak had 84% of the total peak area of the observed three peaks and its retention time corresponded to that of the 9S,12S,13S isomer. These results suggested that the pinellic acid fraction prepared from the tuber of *P. ternata* contained the 9S,12S,13S isomer as its major component with at least 2 additional minor components. Unfortunately, the amounts of the two minor components were insufficient for analysis and identification by instrumental methods. Based on HPLC retention times, the minor peak before the major peak was presumed to be the 9S,12R,13S or 9R,12S,13R isomer and the later major peak was the 9S,12R,13S or 9R,12S,13S isomer. These findings suggested that the tuber of *P. ternata* contained at least 3 pinellic acid isomers that elicited potent adjuvant activity. Because the 9S,12S,13S isomer produced in small scale synthesis showed potent adjuvant activity, it was presumed that this product contained small amounts of other isomers as byproducts. The synthesis of all eight pinellic acid stereoisomers has been previously described [11–13]. Previous studies have shown that in the synthesis of 9S,12S,13S-trihydroxy-

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**Table 1**

Adjuvant effects of mixtures of pinellic acid stereoisomers for nasal influenza vaccine.

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<thead>
<tr>
<th>Main peak of pinellic acid (90.4%)</th>
<th>Sub peak of stereoisomer (9.6%)</th>
<th>Anti-influenza virus IgA</th>
<th>Anti-influenza virus IgG1</th>
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<td>Nasal wash BALF BALF Serum</td>
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n.d. = not determined.

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**Fig. 4.** Effects of mixture of pinellic acid stereoisomer (9S,12S,13S; 9S,12R,13R = 90.4:9.6) on survival rate and body weight loss by influenza virus-induced pneumonia in mice. The time course of survival rate (A) and body weight (B) of immunized mice after lethal A/PR/8/34 virus challenge. The survival rate and body weight were monitored for 21 days after the challenge. Control; 0.2% MeOH/water. (A) Asterisk indicates statistical significant difference (p = 0.0143, Kaplan–Meier method Logrank test) from the control (n = 9). (B) Each point shows mean ± S.E.M. Asterisk indicates statistical significant difference (p < 0.05) from the control.
containing in PAM was ruled out, because 0.1 µg/mouse of 
10E-octadecenoic acid, small amounts of the 9R,12S,13S (4.76%),
9R,12R,13R (2.5%), and 9S,12R,13R (0.12%) isomers were present as
byproducts [13]. Therefore the possibility that the potent adjuvant
activity of the chemically synthesized 9S,12S,13S isomer was due to
combinational effects from other isomers was examined. In this study,
the weight ratios of the pinellic acid stereoisomers observed in the
HPLC peak elution profiles in the pinellic acid fraction from P. ternata
were used to construct a pinellic acid mixture. Since all eight
stereoisomers could potentially serve as adjuvants, all isomers were
tested in various combinations as oral adjuvants with nasally
inoculated influenza vaccine. Because the 9S,12S,13S isomer was the
main component by HPLC analysis from the pinellic acid fraction of
P. ternata, its concentration was maintained at 90.4 wt.% and all other
isomers combined with the 9S,12S,13S isomer at 9.6 wt.%. Only the
9S,12S,13S and the 9S,12S,13S isomer combination with the weight
ratio of 90.4:9.6 elicited potent oral adjuvant activity and this mixture
was designated the pinellic acid mixture (PAM) for this study. The
possibility that only 9S,12S,13S isomer is an active component
containing in PAM was ruled out, because 0.1 µg/mouse of
9S,12S,13S isomer did not show any adjuvant activity. It should be
noted that in our attempts to synthesize all pinellic acid stereoisomers,
small amounts of byproducts could not be avoided. Recently,
stereoselective synthesis of pinellic acid was reported by other groups
[18–23]. However, the stereochromal purity of the products has not
been established. Only when the absolute stereoselective synthesis of
pinellic acid isomers becomes available and all isomers obtained in
100% purity can an objective optimum ratio of the 9S,12S,13S isomer
with other isomers be established for serving as oral adjuvants for
nasally administered influenza vaccines.

When BALB/c mice were treated with SST (1 g/kg, 10 times) orally
from 7 days before to 5 days after the infection and infected with
mouse-adapted influenza virus A/PR8 by nasal-site restricted infec-
tion, SST caused increases in influenza virus hemagglutinin-specific
IgA antibody-secreting cells in nasal lymphocytes at 6 days post
infection when compared to water-treated (control) mice [24,25].

Oral administration of SST also augmented IL-2 receptor β chain+
(activated) T-cell in Peyer’s patch lymphocytes. Previous studies
revealed that SST showed potent anti-influenza virus activity through
augmentation of the antiviral IgA antibody titer in the nasal and
bronchoalveolar cavities of the mice [8]. These results suggest that
oral administration of SST shows anti-influenza virus activity in the
nasal cavity by activation of T-cell in Peyer’s patch lymphocyte and
stimulation of production of anti-influenza virus IgA antibody in nasal
lymphocyte. It is known that a common mucosal immune system
(CMIS) is present [26] and Peyer’s patch cells are one of the inductive
sites of CMIS in gut-associated lymphoid tissue (GALT) in inducing the
production of IgA Ab [27]. Migration studies using Lewis rats showed
that lymphocytes from Peyer’s patch migrate successfully to NALT and
that T cells adhere to NALT containing high endothelial venules
[28,29]. These results indicate the possibility that T cells in Peyer’s
patch when activated by oral administration of SST migrate to NALT and
enhance the production of influenza virus-specific IgA Ab from
antibody-secreting cells in NALT through CMIS. In the present study,
oral administration of the mixture of synthesized pinellic acid
stereoisomers showed potent adjuvant activity in the nasal cavity by
enhancing the IgA response to nasal influenza HA vaccine. Furthermore,
in this study, histopathological examination of lung tissue of mice given oral PAM as adjuvant with vaccine followed by
influenza virus infection showed attenuated infiltration of inflam-
matory cells as evidenced by decreases in the alveolar spaces and
increases in the alveolar septa when compared to lung tissues of
control mice. In addition, influenza virus induced mucus secretion
was significantly abated in the lung tissues of mice orally adminis-
tered the PAM compared to vehicle controls. Pinellic acid from
P. ternata is an active component of SST, and the mechanism by which
SST acts as an oral adjuvant may follow a same mechanism when
mixtures of pinellic acid stereoisomers are employed by themselves.
The mechanism by which the PAM defined in this study act as an oral
adjuvant and structure–activity relationship in molecular level are
presently under investigation.
Acknowledgements

We would like to thank Dr. K.K. Yabusaki for critical reading of the manuscript, and Dr. S. Nunome, Mrs. M. Emori Matsuki and Mrs. T. Narikawa Noguchi for their technical assistance. A part of this work was supported by the 21st Century COE Program, Grant-in-Aid for Scientific Research (C) (Kakenhi) [17590601 for T.N.], and Quality Assurance Framework of Higher Education from Ministry of Education, Culture, Sports, Science and Technology in Japan.

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