



Research Paper

Transcutaneous immunotherapy of pollinosis using solid-in-oil nanodispersions loaded with T cell epitope peptides



Qingliang Kong^a, Momoko Kitaoka^a, Rie Wakabayashi^{a,b}, Noriho Kamiya^{a,b,c}, Masahiro Goto^{a,b,c,*}

^a Department of Applied Chemistry, Kyushu University, 744 Moto-oka, 819-0395, Fukuoka, Japan

^b Advanced Transdermal Drug Delivery Center, Kyushu University, 744 Moto-oka, 819-0395, Fukuoka, Japan

^c Center for Future Chemistry, Kyushu University, 744 Moto-oka, 819-0395, Fukuoka, Japan

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ABSTRACT

Pollinosis, a typical seasonal allergy, is a serious public health problem. Limited numbers of patients receive curative immunotherapy instead of symptomatic therapy; however, there are still some concerns about the inconvenience and side effects of subcutaneous injections and sublingual administration caused by immunotherapy. Here, we propose a simple and safe transcutaneous immunotherapy using solid-in-oil (S/O) nanodispersions loaded with vaccine T cell epitope peptides derived from pollen allergen. S/O nanodispersions are oil-based dispersions of antigens coated with hydrophobic surfactants. They have a high potential to deliver biomolecules including peptides or proteins to immune cells in the skin, and to induce an immune response. The result of quantitative and qualitative analysis by *in vitro* permeation experiments demonstrated the effective permeation of T cell epitope peptides into the skin. Furthermore, *in vivo* experiments using a pollinosis mouse model indicated that the S/O nanodispersions loaded with T cell epitopes suppressed serum antibody IgE and cytokine production, and alleviated allergic symptoms to a similar therapeutic level to that observed for subcutaneous injection. These results indicate the potential of transcutaneous immunotherapy using S/O nanodispersions for the future treatment of pollinosis.

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1. Introduction

Pollinosis, also known as allergic rhinitis and hay fever, is a symptomatic disorder induced after pollen exposure, and classified as an immunoglobulin E (IgE)-mediated allergy. It has become a major public health problem in Japan; in particular, Japanese cedar (*Cryptomeria japonica*) pollinosis, which has a prevalence of up to 26.5% (Okamoto et al., 2009; Yamada et al., 2014). Allergen immunotherapy (AIT) is the only effective treatment for pollinosis

patients providing a sustained immunological and clinical tolerance to the pollen (Pfaar et al., 2014). Repeated and increasing doses of whole pollen extracts by subcutaneous injections (subcutaneous immunotherapy SCIT) or sublingual application using tablets or drops (sublingual immunotherapy SLIT) are used in the clinical treatment of AIT (Canonica et al., 2009). Although SCIT and SLIT using whole pollen extracts are effective for pollinosis therapy, low compliance and persistence for patients (SCIT, 23%; SLIT, 7%) were reported in both clinical practice and real-life studies (Kiel et al., 2013). Patients are required to receive subcutaneous injection of pollen extracts in hospital for more than 3 years. Moreover, local (SLIT, mouth and tongue) or systemic (SCIT, anaphylaxis) side effects also significantly decrease the levels of compliance and persistence (Anthony and Frew, 2008; Werfel, 2009).

Recently, transcutaneous immunotherapy (TCIT) has attracted much attention not only as an alternative convenient method of SCIT and SLIT to improve compliance and persistence, but also because of the important immunological functions of the skin. There are abundant antigen presenting cells (APCs) including Langerhans cells and dermal dendritic cells in the skin, and these

Abbreviations: IgE, Immunoglobulin E; AIT, Allergen immunotherapy; SCIT, Subcutaneous immunotherapy; SLIT, Sublingual immunotherapy; TCIT, Transcutaneous immunotherapy; SC, Stratum corneum; S/O, Solid-in-oil; FITC, Fluorescein-4-isothiocyanate; Rho-DOPE, Rhodamine-dioleoyl phosphatidylethanolamine; IPM, Isopropyl myristate; L-195, Sucrose laurate; YMP, Yucatan micropig; W/O, Water-in-oil; PBS, Phosphate buffered saline; SEM, Scanning electron microscopy; ELISA, Enzyme-linked immunosorbent assay; DLS, Dynamic light scattering; CLSM, Confocal laser scanning microscopy; Th1, Type 1 T helper; Th2, Type 2 T helper; Treg, Regulatory T.

* Corresponding author at: Graduate School of Engineering, Kyushu University, 744 Moto-oka, 819-0395 Fukuoka, Japan.

E-mail address: m-goto@mail.cstm.kyushu-u.ac.jp (M. Goto).

induce an effective immune responses (Heath and Carbone, 2013; Tay et al., 2014). Moreover, the use of TCIT might avoid contact with the circulation to reduce the risk of side effects. However, the topmost layer of the skin, the stratum corneum (SC), has a hydrophobic structure and provides a strong barrier to prevent the intrusion of extraneous molecules. Therefore, it is difficult for vaccines containing peptides and proteins to permeate into the skin (Bal et al., 2010; Matsuo et al., 2013). To overcome this issue, a solid-in-oil (S/O) nanodispersion technique was developed for transcutaneous drug delivery (Kitaoka et al., 2016; Tahara et al., 2012). S/O nanodispersions are oil-based dispersions of nano-sized particles with a drug formed by coating them with hydrophobic surfactant molecules. This hydrophilic drug can permeate the SC via the lipophilic surfactants and oil vehicle. A previous report demonstrated that S/O nanodispersions were effective for transcutaneous drug delivery and TCIT (Kitaoka et al., 2013, 2015).

Despite whole pollen extracts used as an antigen molecule for effective treatment of pollinosis, there are still some concerns about its safety (Prickett et al., 2015). The complexity of whole pollen extracts has a risk of side effects caused by its cross-linking ability with allergen-specific IgE antibodies bound to the surface of effector cells (mast cells and basophils). These side effects lead to dose limitations and a treatment duration of 3–5 years. The use of T cell epitope peptides from pollen was recently proposed to reduce the side effects of whole pollen extracts, without reducing the immunoregulatory activity (O'Hehir et al., 2016). T cell epitope peptides are short antigen-derived molecules recognized by T cells. Because they do not cross-link with allergen-specific IgE bound to the surface of mast cells, treatment with T cell epitope peptides do not pose a potential risk of side effects.

A promising method using a mixture of T cell epitope peptides (O'Hehir et al., 2016; Prickett et al., 2015; Worm, 2015) was successfully used for cat allergy (Couroux et al., 2015; Patel et al., 2013). Short T cell epitope peptides can be used for transcutaneous administration because of the low molecular mass of the peptides. To the best of our knowledge, no studies have focused on the immunotherapy of pollinosis by using a mixture of short T cell epitope peptides. In this study, a mixture of short T cell epitope peptides derived from two Japanese cedar pollen allergen molecules (Cry j 1 and Cry j 2) were used as the vaccine antigen.

We investigated the potential of transcutaneous immunotherapy by using S/O nanodispersions loaded with T cell epitope peptides derived from Japanese cedar pollen. We evaluated whether transcutaneous immunotherapy with S/O nanodispersions loaded with short T cell epitope peptides was effective for the treatment of pollinosis compared to that of subcutaneous injection. We found that transcutaneous administration of S/O nanodispersions loaded with short T cell epitope peptides achieved a similar therapeutic effect compared with subcutaneous injection and could be used for the future treatment of pollinosis.

2. Materials and methods

2.1. Materials

Seven T cell epitope peptides derived from Japanese cedar pollen (Table 1) were custom-synthesized at Medical & Biological Laboratories (MBL) (Nagoya, Japan). Fluorescein-4-isothiocyanate (FITC) and Rhodamine-dioleoyl phosphatidylethanolamine (Rh-DOPE) were purchased from Dojindo (Kumamoto, Japan) and Avanti Polar Lipids (Alabaster, AL, USA), respectively. Cyclohexane and Isopropyl myristate (IPM) were obtained from Wako Pure Chemical Industries (Kyoto, Japan) and Tokyo Chemical Industry (Tokyo, Japan), respectively. A surfactant sucrose laurate (L-195)

Table 1

Amino acid sequences of seven T cell epitope peptides derived from the Japanese cedar pollen allergen (Cry j 1 and Cry j 2).

Epitope peptide	Sequence	Molecular weight
A (Cry j 1)	SMKVTVAFNQFGP	1425.68
B (Cry j 1)	YGLVHVANNNDYP	1475.59
C (Cry j 1)	SGKYEGGNITKKEAFNVE	2134.35
D (Cry j 2)	GIIAAYQNPAWSK	1418.63
E (Cry j 2)	QFAKLTGFTLMG	1313.59
F (Cry j 2)	DIFASKNFHLQKN	1561.77
G (Cry j 2)	KLTSGKIASCLN	1234.49

was kindly provided by Mitsubishi-Kagaku Foods (Tokyo, Japan). Cedar Pollen Extract-Cj was purchased by Cosmo Bio (Tokyo, Japan), and Imject Alum was from Thermo Scientific (Waltham, MA, USA). Cry j 1, Biotin-conjugated Cry j 1 and Biotin-conjugated Cry j 2 were obtained from Hayashibara (Okayama, Japan). Histamine dihydrochloride was provided by Nacalai Tesque (Kyoto, Japan). Yucatan micropig (YMP) skin was purchased from Charles River (Burlington, MA, USA).

2.2. Preparation of S/O nanodispersions containing t cell epitope peptides

The S/O nanodispersions were prepared as previously reported (Kitaoka et al., 2016). A water-in-oil (W/O) emulsion was prepared from an equimolar mixture of an aqueous solution containing seven T cell epitope peptides (total 0.5 mg/mL) and a cyclohexane solution of surfactant L-195 (12.5 mg/mL) using a polytron homogenizer PT2500E (Kinematica AG, Luzern, Switzerland) at 26,000 rpm for 2 min (Fig. S1). The W/O emulsion was flash-frozen in liquid nitrogen for 20 min, and then lyophilized for 24 h with a lyophilizer FDU-1200 (Eyela, Tokyo, Japan). Finally, the resultant viscous surfactant-peptide complex was dispersed in IPM to yield the S/O nanodispersions.

The size distribution of the S/O nanodispersions was analyzed with a Zetasizer Nano ZS light scattering instrument (Malvern, Worcestershire, UK). The morphological appearance of the surfactant-peptide complex was observed by scanning electron microscopy (SEM) using Helios NanoLab 600i (FEI, USA). The surfactant-peptide complex was dispersed in cyclohexane (peptide concentration 0.1 mg/mL). A droplet (10 μ L) placed onto a grid was sputter-coated with a gold/palladium alloy and analyzed at an acceleration voltage of 2 kV with a beam current of 5.4 pA.

2.3. In vitro skin permeation study

Labeled S/O nanodispersions were prepared from epitope peptides labeled with FITC (FITC-peptides). An *in vitro* peptide permeation test was performed as previously described (Kitaoka et al., 2015), using Franz diffusion cells (effective diffusion area, 0.785 cm²; receptor volume, 5 mL). A piece of YMP back skin kept in a freezer (−80 °C) was allowed to thaw at room temperature. The skin was cut into 2 cm² pieces and immediately mounted on the Franz diffusion cell. S/O nanodispersions containing FITC-peptides (1 mg/mL, 200 μ L) were applied onto the skin at 32.5 °C. For the control, FITC-peptides in phosphate buffered saline (PBS) (1 mg/mL, 200 μ L) were also placed on the skin under the same conditions. After 24 or 48 h, the skins were washed with an extraction solution, a mixture of PBS, methanol, and acetonitrile (2:1:1, v/v/v). Then, the skin sections were cut into pieces and peptides were extracted with 1 mL of extraction solution for 24 h. The cumulative amounts of FITC-peptides were calculated from the fluorescence intensities measured with a

fluorescence spectrometer LS55 (PerkinElmer, Waltham, Ma, USA).

2.4. Histological observation of YMP back skin treated with S/O nanodispersions

S/O nanodispersions containing FITC-peptides (1 mg/mL, 200 μ L) and FITC-peptides in PBS (1 mg/mL, 200 μ L) were applied onto the YMP back skin as described as 2.3. After 24 or 48 h, the skin was washed with an extraction solution. The diffusion area of the skin was embedded in Tissue-Tek[®] O.C.T compound (Sakura Finetek, Tokyo, Japan) and frozen in liquid nitrogen. The frozen skin sections (20 μ m) were prepared using a CM1510 cryostat microtome (Leica, Wetzlar, Germany). Fluorescence images were observed using a fluorescence microscope BZ-9000 with a $\times$ 20 objective lens (Keyence, Osaka, Japan).

2.5. Permeation pathway study

S/O nanodispersions containing FITC-peptides (1 mg/mL) and Rho-DOPE (50 μ g/mL) were prepared as previously described (Kitaoka et al., 2014). Mouse ear auricles were collected and stored at -80°C until use. S/O nanodispersions (10 μ L) were pipetted onto the top surface of the skin. After 1 h of incubation at room temperature, the excess solution was removed and the ear auricles were immediately imaged on an LSM 700 confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

2.6. Sensitization and immunotherapy

Female B10.S mice (5–6 weeks old) were purchased from SLC (Shizuoka, Japan) a week prior to experimentation, and maintained under standard conditions. Animal experiments were carried out with the approval of the Ethics Committee for Animal Experiments in Kyushu University, and in accordance with the Guide for the Care and Use of Laboratory Animals (Science Council of Japan).

Mice were sensitized to Cj pollen extract according to our previous report (Kitaoka et al., 2015). Briefly, mice were sensitized with a mixture of Cj pollen extract (10 μ g) and Imject Alum (4 mg) by subcutaneous injection once a week for three consecutive weeks. Six days after the final subcutaneous injection, histamine dihydrochloride in PBS (2 μ g/mL) was administrated into each nostril (5 μ L each). Subsequently, a pollen extract diluted with PBS (100 μ g/mL) was challenged into each nostril (5 μ L) for five days from the day after the histamine administration. Mice showing high total IgE levels in sera after the pollen challenge were considered the pollinosis model.

Patches (0.5 cm $\times$ 1.0 cm) of S/O nanodispersions loaded with peptides were put onto both auricles (0.2 or 1 mg/mL, 25 μ L each) once a week for three consecutive weeks ($n=6$). The peptide PBS solution was administrated with a patch onto both auricles (1 mg/mL, 25 μ L each) as a negative control or with subcutaneous injection (1 mg/mL, 50 μ L) as a positive control. Six days after the final peptide administration, histamine dihydrochloride in PBS (2 μ g/mL) was administrated into each nostril (5 μ L each). Subsequently, the pollen extract diluted with PBS (100 μ g/mL) was dropped into each nostril (5 μ L each) for five consecutive days. Blood samples were collected for serum three days after the final Cj pollen extract challenge. Finally, the sera were obtained by centrifugation of the blood samples for 30 min at $3000 \times g$ at 4°C , and antibody levels were measured.

2.7. Measurement of serum antibody levels

Serum total IgE and Cry j 1-specific IgE levels were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA) using a kit Mouse IgE Ready-SET-Go! (eBioscience, San Diego, CA, USA) as previously described. Serum standards were obtained from the mice subjected to s.c. injection of a mixture of Cj pollen extract and Imject Alum once a week for three weeks while the other mice received therapy with S/O nanodispersions. Levels of Cry j 1-specific IgE in standard serum was assigned an arbitrary value of 10,000 relative units (RU)/mL.

2.8. Clinical symptoms of pollen allergy

Nasal symptoms were evaluated by counting the number of nose rubs observed in 5 min after the last intranasal Cj pollen extract challenge.

2.9. Cytokine assay

Mice were sacrificed and their spleens excised two weeks after the final Cj pollen extract challenge. Spleens were minced and passed through a 70- μ m cell strainer. The dispersed spleen cells were plated at 2×10^6 cells per mL in 96-well plates and stimulated with the Cj pollen extract at a final concentration of 20 μ g/mL. The cells were incubated for 5 days in an incubator (5% CO_2 , 37°C) and the supernatants were collected. The amounts of IFN- γ , IL-4, IL-10 and IL-13 in the supernatants were analyzed by sandwich ELISA using Ready-SET-Go kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

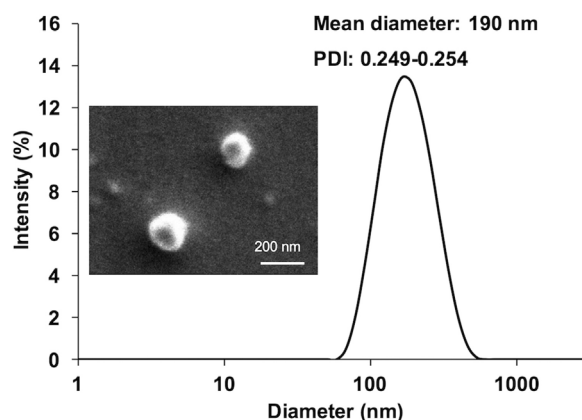


Fig. 1. Size distribution of solid-in-oil nanodispersions and SEM image of solid-in-oil nanoparticles at a magnification of $\times$ 35,000.

2.10. Statistical analysis

The data were expressed as mean $\pm$ standard deviation of the mean (S.D.). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using Graph Pad Prism software (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3. Results

3.1. Characterization of the S/O nanodispersions loaded with t cell epitope peptides

The physical appearance of S/O nanodispersions containing T cell epitope peptides was transparent and homogeneous while the peptides formed a precipitation when they were directly dispersed in IPM. The size distribution of S/O nanodispersions measured by dynamic light scattering (DLS) suggested a mean diameter of 190 nm with a polydispersity index of 0.249–0.254 (Fig. 1). Spherical structures of the surfactant-peptide complex with a similar diameter determined by DLS were observed by SEM. Moreover, the peptide release efficiency of S/O nanodispersions was evaluated. A burst release of epitope peptides was observed within the first 3 h, then the epitope peptides encapsulated into S/O nanodispersions were released gradually (Fig. S2).

3.2. In vitro skin permeation study

A skin permeation study of S/O was conducted by using FITC-labeled epitope peptides (FITC-peptides). Skin sections treated with FITC-peptides in a PBS solution or in S/O nanodispersions were observed using fluorescence microscopy after 24 h and 48 h. When the FITC-peptides in the PBS solution were applied to the skin sections, green fluorescence was observed only on the top of the skin at 24 h (Fig. 2a). Even at 48 h, no fluorescence signal was detected in the skin although some signals were observed in the SC. However, a strong fluorescence signal was observed in skin treated with the FITC-peptide S/O nanodispersions. Moreover, some signals in the epidermis were observed at 48 h. The cumulative amounts of FITC-peptides in the YMP skins were quantified. The S/O nanodispersions facilitated the permeation of the peptides more than 4 times compared with that of the PBS solution at 24 h and 48 h, respectively (Fig. 2b).

To investigate the permeation route of the peptides and the surfactant in the skin, mouse ears treated with S/O nanodispersions loaded with FITC-peptides and Rho-DOPE were analyzed by confocal laser scanning microscopy (CLSM). The green and red fluorescence signals from FITC-peptides and Rho-DOPE, respectively, were observed at the intercellular spaces (Fig. 3). The permeation depth of the peptides and the surfactant was observed by CLSM. Green fluorescence from FITC-peptides

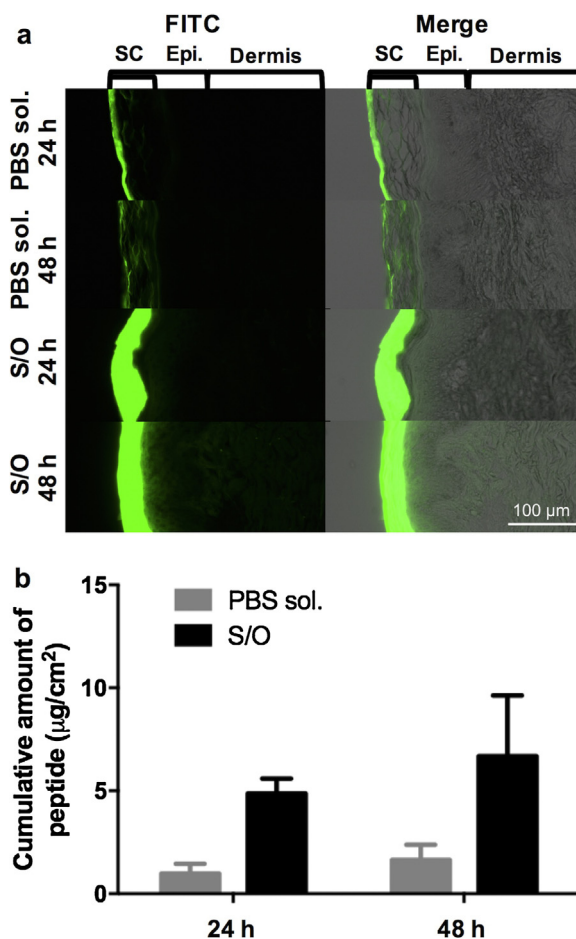


Fig. 2. In vitro skin permeation study of FITC-peptides in the Yucatan micropig (YMP) back skin after application of PBS solution (PBS sol.) and solid-in-oil nanodispersions (S/O) at 24 h and 48 h. (a) Histological analysis of YMP skin sections was observed by fluorescence microscopy with a $\times 20$ objective lens (SC: stratum corneum; Epi.: epidermis). (b) Comparison of the permeation of FITC-peptides. The data are expressed as the mean $\pm$ SD ($n = 3$).

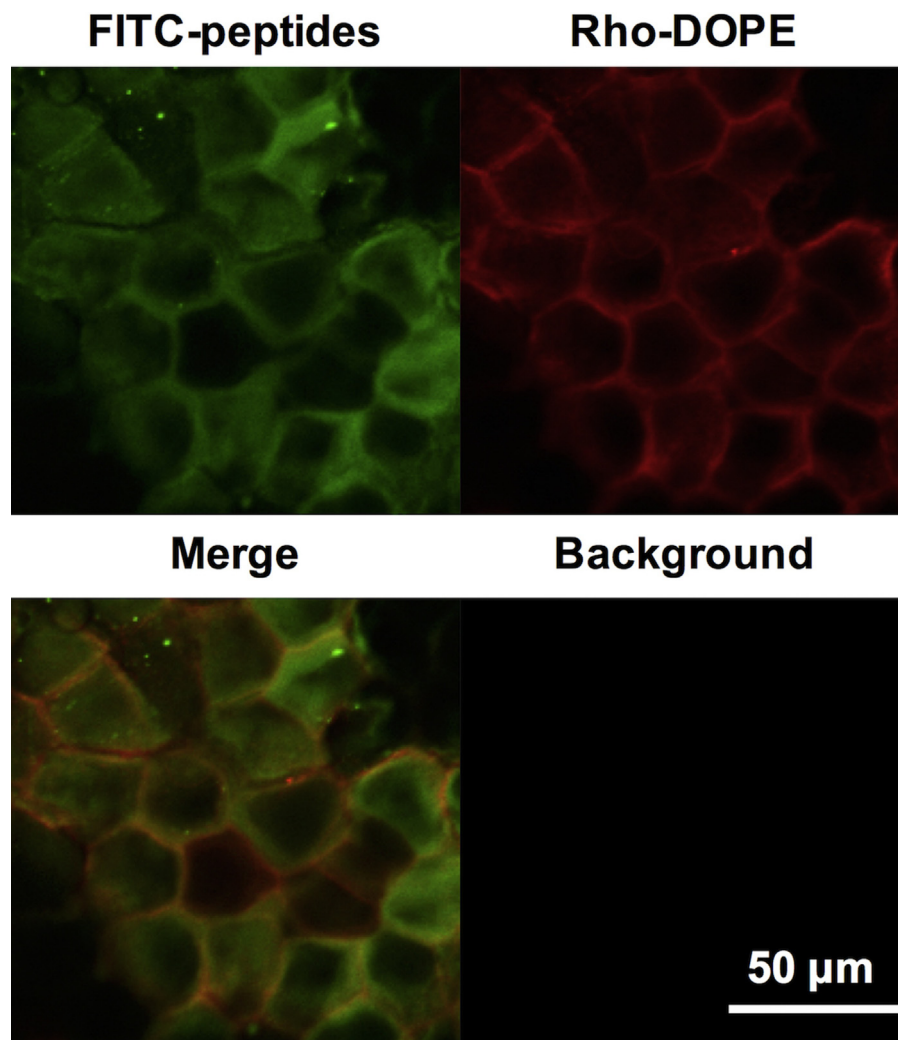


Fig. 3. Histological analysis of mouse ear skins treated with FITC-peptide solid-in-oil nanodispersions. Confocal images are of mouse ears subjected to solid-in-oil nanodispersions containing FITC-peptides and Rhodamine-labeled dioleoyl phosphatidylethanolamine (Rho-DOPE) for 1 h.

was observed relatively deep in the skin while the red fluorescence from Rho-DOPE was only concentrated on the top of skin (Fig. S3).

3.3. *In vivo* skin permeation study

After the treatment with S/O nanodispersions loaded with T cell epitope peptides, both the total IgE and Cry j 1-specific IgE levels in sera were reduced compared with mice treated with a patch of peptide PBS solution (Fig. 4). S/O nanodispersions loaded with epitope peptides demonstrated corresponding efficacies in lowering IgE antibody levels similar with the subcutaneous injection. The total and Cry j 1-specific IgE levels were significantly reduced with the S/O nanodispersions, particularly when the S/O nanodispersions contained 10 μ g of epitope peptides (47% and 45% suppression, respectively; $p < 0.05$). Cry j 2-specific IgE levels from mice administrated S/O nanodispersions were significantly lower than that in mice treated with a patch of peptide PBS solution (54% suppression; $p < 0.001$) (Fig. S4).

To examine the effect of transcutaneous treatment with epitope peptides on the development of clinical symptoms of pollinosis, the number of nose rubs was counted. The number of nose rubs in mice treated with S/O nanodispersions was reduced compared with that in the group of mice treated with a patch of peptide PBS

solution, particularly when the S/O nanodispersions contained 10 μ g of epitope peptides (51% suppression; $p < 0.05$) (Fig. 5).

To further demonstrate the effect of TCIT with epitope peptides on the inhibition of allergen-specific T cell responses, the production of IFN- γ , IL-4, IL-10 and IL-13 in splenocytes from immunized mice was investigated. In the control groups of mice treated with the patch containing a peptide PBS solution, high quantities of IFN- γ , IL-10 and IL-13 cytokine were produced (Fig. 6). However, in the groups of mice treated with the patch of S/O nanodispersions containing epitope peptides, levels of IFN- γ , IL-10 and IL-13 cytokines were significantly lower than those of the control groups (61%, 79%, and 59% suppression, respectively; $p < 0.05$), which was a greater reduction compared with the subcutaneous injection group. Although IL-4 levels were not decreased significantly, their levels were also decreased in mice treated with the S/O nanodispersions (65% suppression).

4. Discussion

Pollinosis has been studied for more than half century because of its universality and severity (Yamada et al., 2014). Although two major allergens Cry j 1 and Cry j 2 from Japanese cedar pollen were identified and thought to be useful for pollinosis vaccines, there are

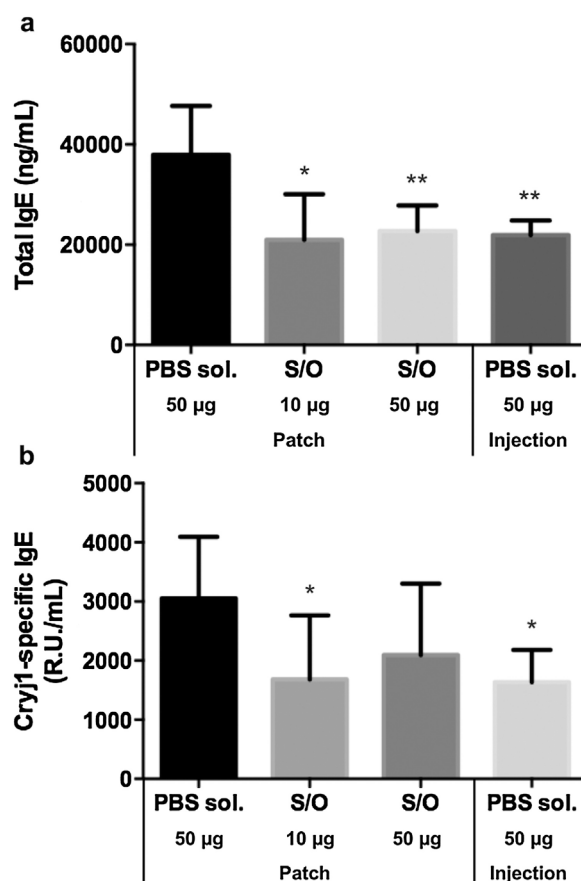


Fig. 4. Serum antibody responses after immunotherapy using T cell epitope peptides in a pollinosis model mice. T cell epitope peptides in solid-in-oil nanodispersions (S/O) and PBS solution (PBS sol.) were administrated once a week for 3 weeks, and (a) serum total IgE and (b) Cry j 1-specific IgE levels in mice were measured by ELISA. Data are expressed as the mean $\pm$ SD ($n=6$). * $p < 0.05$; ** $p < 0.01$.

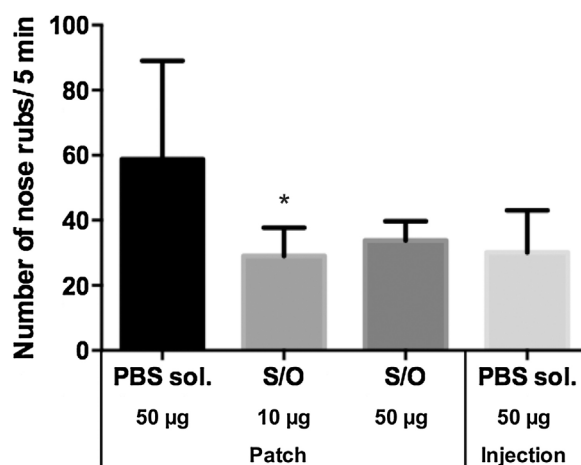


Fig. 5. Reduced number of nose rubs in pollinosis model mice treated with T cell epitope peptides in solid-in-oil nanodispersions (S/O) and PBS solution (PBS sol.). The number of nose rubs was counted in the 5 min after the last intranasal antigen challenge. Data are expressed as the mean $\pm$ SD ($n=6$). * $p < 0.05$.

still some risks of serious side effects induced by the administration of whole antigen molecules. To overcome this issue, T cell epitope peptides that avoid causing side effects were developed as a new vaccine for the treatment of pollinosis. The hybrid peptide 7 Crp contains seven immunodominant human T cell epitope peptides from Japanese cedar pollen allergens Cry j 1 and Cry j 2 (Table 1, peptide A, B and C from Cry j 1; peptide D, E, F and G from

Cry j 2) and was proven to be effective for humans (Hirahara et al., 2001). However, it has a relatively high molecular mass (10 kDa); therefore, the transcutaneous delivery of 7 Crp might be difficult. Separated T cell epitope peptides containing 12–19 amino acids are short (1.2–2.1 kDa) and can more easily permeate the skin when combined with S/O technology. Therefore, we used a mixture of seven short T cell epitope peptides from 7 Crp as a vaccine. In this

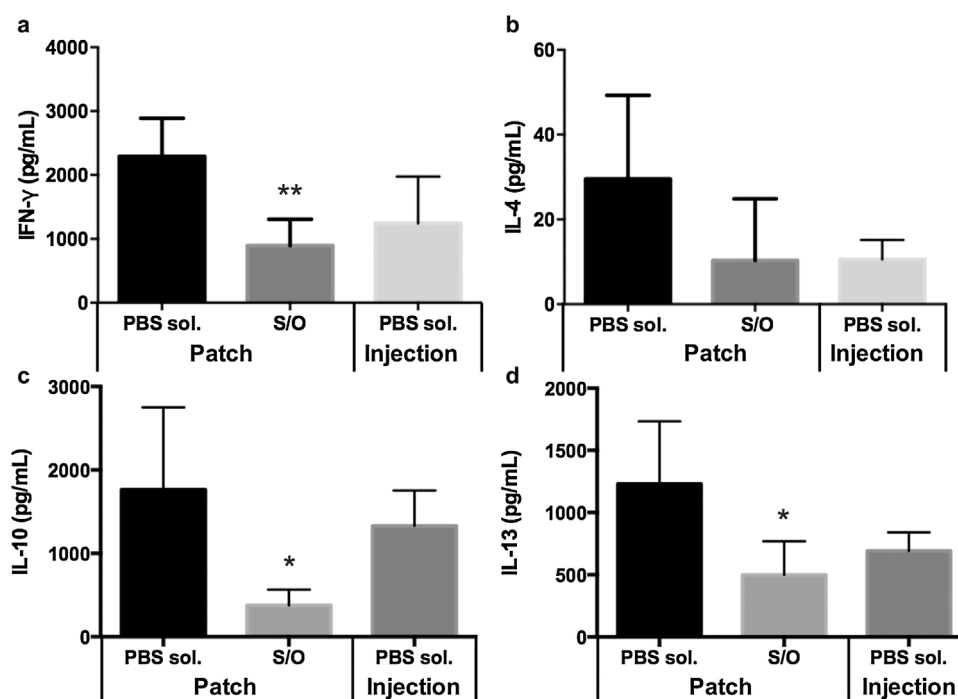


Fig. 6. Inhibition of allergen induced Th1 and Th2 cytokine production by splenic T cells isolated from mice. Levels of (a) INF- γ , (b) IL-4, (c) IL-10, and (d) IL-13 were measured by ELISA. Splenocytes isolated from B10.S mice immunized by the transcutaneous administration of T cell epitope peptides in solid-in-oil nanodispersions (S/O) and PBS solution (PBS sol.) and subcutaneous injection of PBS sol. Splenocytes were stimulated with Cj pollen extract (20 μ g/mL) for 5 days. Data are expressed as the mean $\pm$ SD (n = 4). *p < 0.05; **p < 0.01.

study, although an equimolar mixture of seven T cell epitope peptides was used, the ratio of peptides could be adjusted for individual therapy in the future.

The mixture of peptides did not dissolve in IPM and formed a precipitation. In contrast, S/O nanodispersions with the T cell epitope peptide mixture were transparent and homogeneous. A main peak with an average diameter of 190 nm was observed in the DLS analysis (Fig. 1). The image of the surfactant-peptide complex by SEM suggested a spherical shape with a diameter around 190 nm, similar to that observed by DLS. These results demonstrated that the mixture of T cell epitope peptides was dispersed into IPM successfully as nano-order particles. Moreover, the result of *in vitro* peptide release efficiency of S/O nanodispersions showed that the peptides were released gradually from S/O nanodispersions (Fig. S2).

We performed an *in vitro* skin permeation study of the peptide in S/O nanodispersions using FITC-labeled peptides. The results of quantitative and qualitative analysis showed the S/O nanodispersions improved the permeation of peptides compared with the PBS solution (Fig. 2). Moreover, the skin permeation pathway of the FITC-peptides and surfactants mixed with Rho-DOPE in S/O nanodispersions was determined. Confocal fluorescence microscopic images of mouse ears treated with labeled S/O nanodispersions indicated they permeated the skin via an intercellular pathway (Fig. 3). The skin penetration enhancer IPM, may have affected the intercellular lipid bilayers in the SC and decreased the barrier function of SC (Hirakawa et al., 2015). In addition, Z section images of CLSM suggested that FITC-peptides permeated more deeply than Rho-DOPE, which was used as a marker of surfactant in this study (Fig. S3). This corresponds with our previous study showing S/O nanoparticles collapsed in SC, and that the released drugs permeated the hydrophilic viable epidermis and dermis in the skin (Tahara et al., 2008).

Pollinosis is a typical Type I allergy, which has distinctive symptoms such as sneezing and nasal rubs caused by the release of histamine molecules induced by the enhanced production of IgE antibodies in sera. Therefore, serum total IgE, antigen-specific IgE level and the above mentioned symptoms are often used as markers of pollinosis (Suzuki et al., 2010; Takagi et al., 2005). After the transcutaneous treatment of the pollinosis mouse models, the total IgE and the Cry j 1-specific IgE levels in the sera were decreased, similar to that in allergy mouse models treated by subcutaneous injection (Fig. 4). Moreover, the decrease in the number of nose rubs after S/O nanodispersion treatment suggests the alleviation of allergy (Fig. 5). The decrease in total IgE, specific IgE levels, and nose rubs may be a result of the successful delivery of epitope peptides, which traffic through the SC aided by the S/O nanodispersions, to the APCs in the epidermis. In our previous study, S/O nanodispersions loaded with hybrid peptide 7 CrpR, an arginine-modified 7Crp, showed an unsatisfactory therapeutic effect for pollinosis (Kitaoka et al., 2015). The difference between studies might be because of the difference in molecular mass between short peptides (1.2–2.1 kDa) and the hybrid peptide (14.8 kDa). Short peptides are small and easily permeate the skin compared with the hybrid peptide, which has a relatively high molecular mass. Moreover, the difference in skin permeation depth between insulin (6 kDa), protein EGFP (27 kDa) and protein HRP (40 kDa) also showed that drugs with a low molecular mass permeate more deeply into skin when the S/O nanodispersion system is used (Tahara et al., 2008). Interestingly, S/O nanodispersions loaded with a low amount of epitope peptides (10 μ g) induced a significant immune response measured by IgE levels and a decrease in nose rubs. This result was similar to our previous research, which used the hybrid peptide 7 CrpR (Kitaoka et al., 2015). A reason for this might be related to the surfactant amount. Higher amounts of peptides need more surfactant to form S/O

nanodispersions, but excess surfactant may block the permeation of peptides into the skin because of the hydrophobicity of the surfactant L-195. Therefore, the dose of epitope peptide and surfactant-peptide should be optimized to further increase drug delivery efficiency.

To understand the effect of the transcutaneous administration of S/O nanodispersions on the inhibition of allergic-specific T cell responses, we assessed the immune responses of Type 1 T helper (Th1) cell, Type 2 T helper (Th2) cell and regulatory T (Treg) cells by measuring cytokine levels. Th1 cells produce IFN- γ , which mediates cell-mediated immunity. By contrast, Th2 cells produce IL-4 and IL-13 cytokines that induce humoral immune responses by switching B cells to produce IgE antibodies. Pollinosis is an IgE mediated allergy and is strongly influenced by Th2 cell responses. Moreover, Treg cells secrete the immunosuppressive cytokine IL-10 that modulates the immune system (Sakaguchi et al., 2008). Lower levels of IFN- γ , IL-4, IL-10, and IL-13 levels were observed after the administration of S/O nanodispersions compared with the PBS solution of peptides (Fig. 6). This demonstrated that immune responses were inhibited by the effective delivery of T cell epitope peptides using S/O nanodispersions. Although further studies are required to understand the underlying immune mechanism of TCIT using T cell epitope peptides, our data suggest that S/O nanodispersions loaded with T cell epitope peptides induce specific tolerance of pollinosis more effectively compared with the transcutaneous administration of a peptide PBS solution and even better than the subcutaneous injection of a peptide PBS solution.

5. Conclusion

The quantitative and qualitative analysis of *in vitro* permeation results demonstrated the effective permeation of T cell epitope peptides to the skin. Moreover, an *in vivo* study using a mouse model of pollen allergy indicated that S/O nanodispersions loaded with T cell epitope peptides had a similar therapeutic effect compared with subcutaneous injection. Our results suggest that TCIT using S/O nanodispersions loaded with T cell epitope peptides might be an easier and simpler alternative to the injection method for the future therapeutic treatment of cedar pollinosis.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpharm.2017.07.020>.

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