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Research Article

Transcutaneous Peptide Immunotherapy of Japanese Cedar Pollinosis Using Solid-in-Oil Nanodispersion Technology

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Abstract. Peptide immunotherapy is an attractive approach to relieve allergic symptoms such as rhinitis and asthma. Treatment of Japanse cedar pollinosis (*Cryptomeria japonica*; Cj), from which over one quarter of Japanese population suffer, is becoming a great concern. Recently, oral feeding of a peptide (7crp) consisting of seven immunodominant human T cell epitopes derived from two enzymes present in Cj pollen was demonstrated to have a benefit in treating Cj pollinosis. In this work, we aimed to apply a novel transcutaneous administration system as a simple and easy peptide delivery for an immunotherapy using a T cell epitope peptide. A modified 7crp peptide (7crpR) which contained triarginine linkers between each epitopes was designed to increase water solubility and was encapsulated in a unique solid-in-oil (S/O) nanodispersion. The S/O nanodispersion consists of a nano-sized peptide-surfactant complex dispersed in an oil vehicle. The S/O nanopartilces having an average diameter of 230 nm facilitated the permeation of the peptide 7crpR into the skin and suppressed serum total IgE and antigen-specific IgE levels in a Cj pollinosis mouse model. Transcutaneous administration of the T cell epitope peptide using the S/O nanodispersion system has potential for future simple and easy immunotherapy of Cj pollinosis.

KEY WORDS: epitope peptide; immunotherapy; pollinosis; solid-in-oil nanodispersion; transdermal drug delivery.

INTRODUCTION

Allergic symptoms caused by environmental aeroallergens, such as house dust mites, spores, and pollens, are social problems in industrialized countries. Above all, seasonal allergic rhinitis caused by Japanese cedar (*Cryptomeria japonica*; Cj) pollen is a significant concern in Japan (1). The prevalence of Cj pollinosis is increasingly affecting over one quarter of the Japanese population (2). Pharmacotherapy is the main approach to treat Cj pollinosis and reduces allergic symptoms; however, symptomatic treatment does not cure allergy. Since Cj pollinosis is a type I allergy mediated by immunoglobulin (Ig) E, antigen-specific immunotherapy is anticipated to be curative. Although conventional immunotherapy using antigen molecules has a risk of acute anaphylaxis, especially when administered by injection, sublingual immunotherapy has been demonstrated to be a safer approach (3,4), with long-lasting effects after discontinuation (5,6), and is supported by health insurance in Japan. However, risks of adverse side effects remain when whole allergen molecules are used for immunotherapy.

Recently, the use of short, antigen-derived peptides was shown to be an effective immunotherapy (7). The peptides contain only T cell epitope domains from antigen molecules, thus lack the ability to cross-link IgE molecules which subsequently bind to IgE receptors on the surface of mast cells or basophils. Consequently, the T cell epitope peptide leads to suppression of IgE-mediated allergic reactions including anaphylaxis. Immunotherapy using T cell epitope peptides was successfully demonstrated to induce immune tolerance in allergy models of house dust mites and Birch pollens (8,9). For the Cj pollinosis, two enzymes (Cry j 1 and Cry j 2) present in Cj pollen are considered the major allergens (10), and several immunodominant human T cell epitopes were determined and evaluated using blood samples from Cj pollinosis patients (11–13). Peptides comprised of multiple T cell epitopes administered orally effectively reduced allergic responses represented by allergen-specific antibody responses, T cell proliferation, and number of sneezing events (14–17). Oral administration of protein/peptide drugs including edible vaccines is needle free and convenient and utilizes the whole length of the gastrointestinal tract; however, dosage and vaccine-food interactions are the concerns (18).

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Nanoparticle- and microparticle-based drug delivery systems are recognized as beneficial techniques because of their stability, increased bioavailability, and modulated drug release (19). Previously, our group proposed an original transcutaneous vaccine delivery technique based on a solid-in-oil (S/O) nanodispersion system. Using an antigen model protein, ovalbumin (OVA), the S/O nanodispersion system efficiently induced antigen-specific antibodies in murine models using a simple patch (20,21). The S/O nanodispersion is an oil-based dispersion of nano-sized particles containing protein/peptide molecules coated with surfactant molecules, which is formed by lyophilization of a water-in-oil emulsion and re-dispersion in an oil-vehicle. Hydrophilic biomolecules such as proteins and peptides can penetrate the hydrophobic stratum corneum (SC), the outermost layer of the skin, assisted by surfactants and oil-vehicle. Transcutaneous drug administration has advantages compared with conventional needle-based administration: reduced pain, avoidance of first pass metabolism, and effective immunization by utilizing the skin immune system. In addition, there are no risks of needle-related injuries or transmission of pathogens.

Here, we first report a transcutaneous administration of T cell epitope peptide for immunotherapy using the S/O nanodispersion system. For therapeutic treatment, we used a recombinant peptide comprising seven human T cell epitopes derived from Cry j 1 and 2 with modification to enhance water solubility (11). An integrated peptide of seven T cell epitopes containing triarginine spacers between the epitopes (7crpR) was produced in *Escherichia coli* and coated with surfactants. Size and skin permeation efficiency of the S/O nanodispersion containing 7crpR was characterized; then, the serum total and antigen-specific IgE levels in a mouse model of Cj pollinosis were evaluated after transcutaneous administration.

MATERIALS AND METHODS

Animals

Female B10.S mice (7 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and housed in a temperature-controlled room (23°C) with a 12-h light/dark cycle. Animal experiments were performed with approval of the Ethics Committee for Animal Experiments (A26-267-0, Kyushu University) and in accordance with the Guide for the Care and Use of Laboratory Animals (Science Council of Japan).

Materials

Cyclohexane, dithiothreitol, ethanol (99%), acetonitrile, and imidazole were purchased from Wako Pure Chemical Industries (Kyoto, Japan). Isopropyl myristate (IPM) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). A surfactant sucrose laurate (L-195) was kindly provided by Mitsubishi-Kagaku Foods (Tokyo, Japan). Cj pollen extract (Cedar Pollen Extract-Cj) was purchased from Cosmo Bio (Tokyo, Japan). Imject Alum was sourced from Thermo Fisher scientific (Waltham, MA, USA). Histamine was from Nacalai Tesque (Kyoto, Japan).

Methods

Preparation of the T Cell Epitope Peptide 7crpR

The peptide 7crpR design was based on a previously reported artificial peptide (7crp) containing human T cell epitope determinants from Cry j 1 (212–224, 235–247, 312–330) and Cry j 2 (74–89, 96–107, 192–204, 356–367) (11,17). 7crpR additionally contained triarginine linkers between determinants and hexahistidine-tag at the C-terminus (Fig. 1). A double-stranded DNA encoding the peptide 7crpR with an optimized codon for *E. coli* expression was custom-synthesized at Medical and Biological Laboratories (Nagoya, Japan). The 7crpR gene was cloned into the pET22b(+) plasmid vector, which was transformed into *E. coli* strain BL21(DE3). The peptide 7crpR was produced by *E. coli*, purified, and stored at 4°C until use (see Electronic supplementary material for the production condition of 7crpR).

Preparation of S/O Nanodispersions Containing 7crpR

A S/O nanodispersion was prepared as previously reported (20). A 2.0-mL 7crpR aqueous solution (0.5 mg/mL) and a 4.0-mL L-195 cyclohexane solution (12.5 mg/mL) were homogenized with a polytron homogenizer (Kinematica AG, Luzern, Switzerland) at 26,000 rpm for 2 min to obtain a water-in-oil (W/O) emulsion. The W/O emulsion was incubated in liquid nitrogen for 20 min, and then lyophilized for 24 h with a freeze-dryer (Eyela, Tokyo, Japan). The resultant viscous surfactant-protein complex was dispersed in IPM to prepare a S/O nanodispersion.

Particle Size

The mean diameter and polydispersity index for the S/O nanodispersion was measured by dynamic light scattering (DLS) using Zatasizer NanoZS (Malvern, Worcestershire, UK).

Scanning Electron Microscopy

Morphological appearance of the surfactant-peptide complex was observed by scanning electron microscopy (SEM) using Inspect S50 (FEI, Hillsboro, OR, USA). The complex was dispersed in cyclohexane (7crpR concentration 0.1 mg/mL). A droplet (5 μL) was placed onto a carbon conductive double-sided adhesive tape and dried overnight. After being sputter-coated with gold/palladium alloy, the sample was analyzed at an acceleration voltage of 5 kV.

In Vitro Skin Permeation Test

The peptide was labeled with Cy3 using a kit (Cy3 Mono-Reactive Dye Pack, GE Healthcare) according to the manufacturer's instructions. *In vitro* peptide permeation test was performed as previously described (21), using custom-fabricated Franz diffusion cells (effective diffusion area, 0.785 cm^2; receptor volume, 5 mL). Yucatan micropig (YMP) back skin was cut into 3-cm^2 pieces and was set on a Franz diffusion cell. The S/O nanodispersion containing Cy3-7crpR (1 mg/mL, 200 μL) was applied onto the skin for 24 or 48 h at

MGIIAAYQNPASWKRRRSMKVTVAFNQFGPRRRDIFASKNFHLQKNRRRKLTSGKIASCLNRRR
Cry j 2(74-89) Cry j 1(212-224) Cry j 2(192-204) Cry j 2(356-367)

YGLVHVANNNYDPRRRSGKYEGGNIYTKKEAFNVERRRQFAKLTGFTLMGIEGRGSHHHHHH
Cry j 1(235-247) Cry j 1(312-330) Cry j 2(96-107)

Fig. 1. Amino acid sequences of 7crpR. *Underlined sequences* represent the T cell epitope domains derived from the enzymes Cry j 1and Cry j 2. *Numbers* in parenthesis denote amino acid positions in the enzymes

32.5°C. Cy3-7crpR in PBS (1 mg/mL, 200 μL) was also put on the YMP skin under the same conditions as a control. Before the extraction of the peptide, the skin was washed with an extraction solution, a mixture of water, ethanol, and acetonitrile (2:1:1, *v/v/v*). Then, the skin was cut into pieces and the peptide was extracted with the 0.5 mL of extraction solution for 12 h under dark. The cumulative amounts of Cy3-7crpR were calculated from the fluorescence intensities measured with a fluorescence spectrometer LS55 (PerkinElmer, Waltham, Ma, USA) at Ex/Em=540 nm/570 nm.

Histological Observation of the Mouse Ear Skin

Tissue papers impregnated with the S/O nanoparticle containing Cy3-7crpR (1 mg/mL, 525 μL each) was patched onto mouse ears for 24 h. After removal of patches, ear tissues were washed with 99% ethanol, followed by a Milli-Q wash. Tissues were immersed in 4% paraformaldehyde for 6 h then frozen in Tissue-tek O.C.T. compound (Sakura Finetek, Tokyo, Japan). Tissue sections (16 μm thick) were prepared with a cryostat microtome (Leuca, Wetzlar, Germany). Fluorescence images were observed using a fluorescence microscope BZ-9000 with a ×20 objective lens (Keyence, Osaka, Japan).

Sensitization and Peptide Administration Procedures

Mice (9 weeks old) were sensitized as previously described (16,22). Briefly, a mixture of 10 μg of Cj pollen extract and 4 mg of Imject Alum was subcutaneously injected to mice three times with 1-week intervals. Six days after the final sensitization, 5 μL of histamine dihydrochloride in PBS (2 μg/mL) was dropped into each nostril. Subsequently, 5 μL of Cj pollen extract diluted with PBS (400 μg/mL) was dropped into each nostril for five consecutive days from the day after histamine administration.

For therapeutic treatment, tissue papers impregnated with S/O nanodispersion containing 7crpR were patched onto both auricles (0.2 or 1 mg/mL, 25 μL each) at 6 weeks after intranasal antigen challenge. S/O nanodispersions were administered once a week for three consecutive weeks. The peptide 7crpR PBS solution was also administered with patches (1 mg/mL, 25 μL each) or by subcutaneous injection (1 mg/mL, 50 μL) as a negative or a positive control. Two weeks after the last peptide administration, 5 μL of Cj pollen extract in PBS (400 μg/mL) was challenged into both nostrils for five consecutive days. Blood was collected for serum 3 days after final antigen challenge.

Measurement of Total IgE and Cry j 1-Specific IgE

Total serum IgE was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Mouse IgE ELISA Ready-SET-Go; eBioscience, San Diego, CA, USA). Briefly, plates were coated with anti-mouse IgE at 4°C overnight, washed, and blocked. Then, diluted serum (1:500) was added and detected with biotin-labeled anti-mouse IgE. After coloring reaction with tetramethylbenzidine at room temperature for 15 min in the dark, the reaction was stopped by adding 1 M sulfuric acid. Optical density at 450 nm was measured by microplate reader (BioTek Instruments, Winooski, VT, USA). Serum Cry j 1-specific IgE was measured according to the previous report (23). Briefly, plates were coated with anti-mouse IgE, washed, and blocked. Diluted serum (1:30) was added to the plates, and Cry j 1-specific IgE was detected with 100 μL (2.5 μg/mL) of biotin-labeled Cry j 1 (Hayashibara, Okayama, Japan). Serum standards were obtained from mice to which Cj pollen extract was administered by intraperitoneal injection eight times with 1-week intervals, and arbitrarily assigned to a value of 10,000 relative units (RU)/mL of Cry j 1-specific IgE. Samples were assayed in

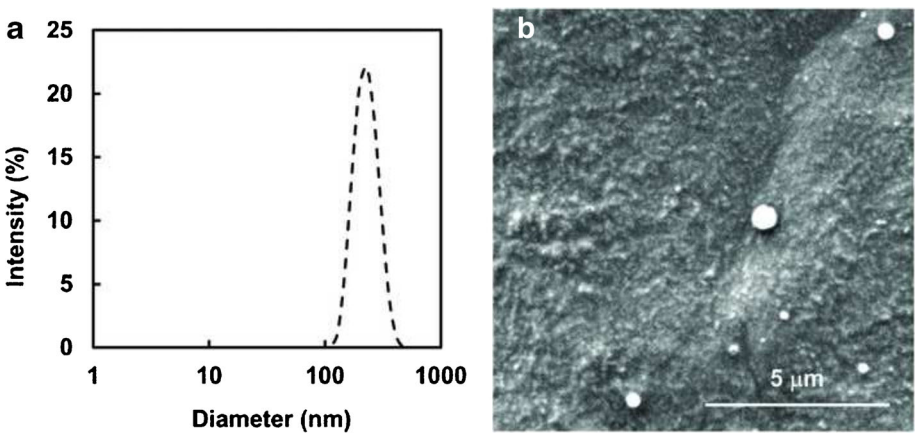

Fig. 2. Size distribution of S/O nanodispersion containint 7crpR (**a**) and SEM images of S/O nanoparticles at a magnification of ×10,000 (**b**)

triplicate, and results were expressed as the mean±standard deviation (SD) (n=4–6). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (* $p<0.05$, ** $p<0.01$, *** $p<0.0001$).

RESULTS

Preparation of S/O Particles Containing a T cell Epitope Peptide

The recombinant peptide 7crpR expressed using an *E. coli* system was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the result showed a single band at around 15 kDa after purification (Supplementary Fig. S1). The S/O nanodispersion containing 7crpR was prepared following to our standard protocol (20,22), and was subjected to DLS analysis. As a result, the nanoparticle had a mean diameter of 230 nm with a polydispersity index of 0.250–0.281 in IPM (Fig. 2a). SEM images of the surfactant-peptide complex showed particles of a round shape, smaller than 500 nm (Fig. 2b, c). The physical appearance of the S/O nanodispersions was slightly translucent, although the peptide itself was insoluble in IPM and formed a precipitation (Supplementary Fig. S2).

In Vitro and *In Vivo* Skin Permeation Efficiencies of 7crpR-Containing S/O Nanodispersion

In vitro and *in vivo* skin penetration efficiencies of the peptide were examined using a fluorescence Cy3-labeled 7crpR. Employing a Cy3-dye labeling kit, approximately 30% of 7crpR was labeled with Cy3 by a calculation based on UV absorbance. Figure 3a shows cumulative amounts of Cy3-7crpR in the YMP skins treated with a peptide PBS solution and a S/O nanodispersion. The S/O nanodispersion system facilitated permeation of the peptide 4.8-fold compared to the PBS solution in 48 h. Mouse auricle sections processed with 7crpR PBS solution for 24 h had weak fluorescence in the SC layer; however, auricle sections treated with the S/O nanodispersion exhibited strong fluorescence at SC layer with a weak signal in epidermis (Fig. 3b). No fluorescence image was observed in the mouse auricle sections treated with PBS without peptide. Thickening of SC layer was observed by applying the S/O nanodispersion patch for another 24 h (Supplementary Fig. S3).

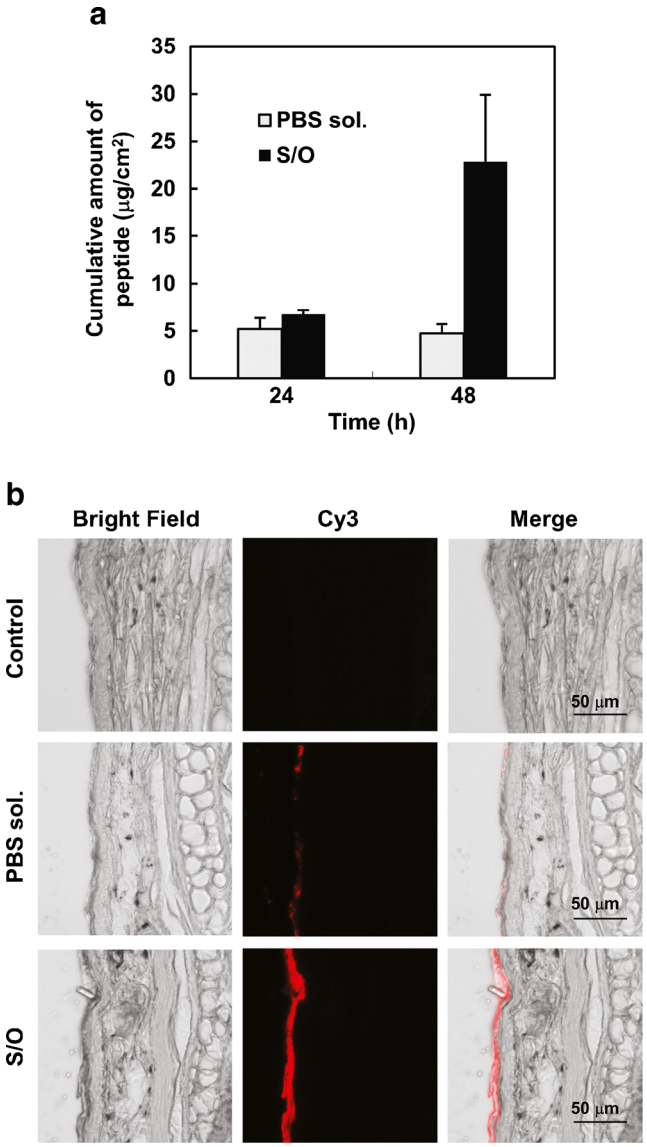

Fig. 3. *In vitro* skin permeation test of Cy3-7crpR in the Yucatan micropig (YMP) back skin after application of the S/O nanodispersions (**a**) and *in vivo* skin permeation of Cy3-7crpR in mouse auricle (**b**). Data expressed as the mean±SD (n=3). Auricle sections were prepared from mouse auricles subjected to the S/O nanodispersion for 24 h and analyzed by fluorescence microscopy with an objective lens of ×20

Therapeutic Treatment of Pollinosis Mouse Models

We developed a B10.S mouse model of allergic rhinitis responding to Cj pollen antigen following the previous description (Fig. 4a) (16). Sensitization was confirmed by induction of serum total IgE which was not detected in the serum of an unprimed mouse. The mouse model was reported to be suitable as a mouse model developing Japanese cedar pollinosis with nasal symptoms (24), and its symptoms to be reduced by inoculation of peptide 7crp (16,17). We examined the therapeutic effect of transcutaneous administration of 7crpR to a rhinitis mouse model. A PBS solution of 7crpR (50 μg) was transcutaneously administered three times as a control. Total serum IgE levels from mice three times transcutaneously administered S/O nanodispersions containing 7crpR were lower than that from control mice (Fig. 4b). A significant reduction of Cry j 1-specific IgE level was observed when a S/O nanodispersion containing 10 μg of 7crpR was administered three times (Fig. 4c). Both total IgE levels and Cry j 1-specific IgE levels in sera from mice to which 50 μg of 7crpR PBS solution were administered by subcutaneous injection were significantly lower than those from control mice.

DISCUSSION

We prepared a nano-sized carrier for the transcutaneous administration of a T cell epitope peptide for immunotherapy of Cj pollinosis mouse models. The previously reported peptide 7crp was a simple combination of T cell epitope

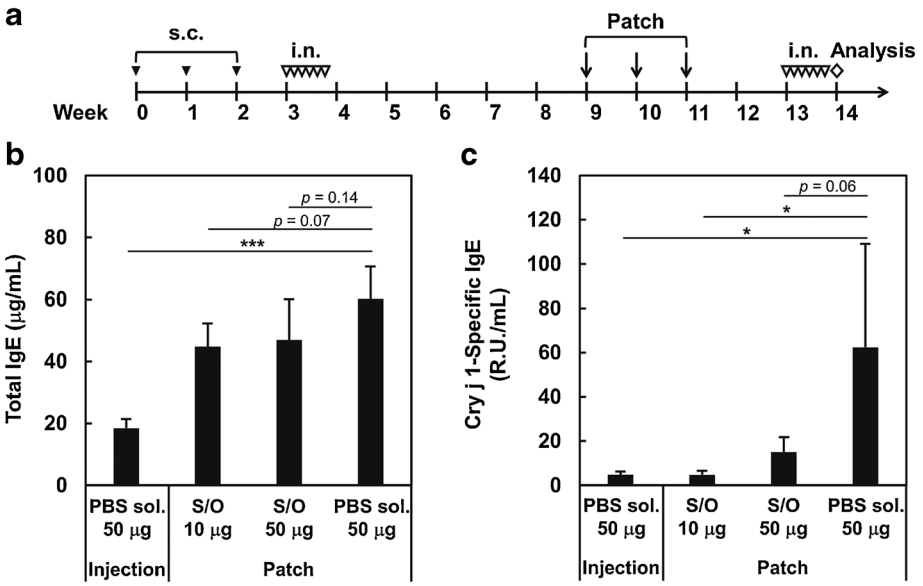

Fig. 4. Sensitization and peptide administration schedule (**a**) to B10.S mice and serum total IgE (**b**) and Cry j 1-specific IgE (**c**) levels of the mice after therapeutic treatment with 7crpR. Mice were sensitized to Cj pollen extract by subcutaneous injection (s.c.). Pollinosis symptoms were generated by intranasal (i.n.) challenge with the Cj pollen extract. The peptide 7crpR was transcutaneously administered with patches on both auricles. Data show the mean and SD (n=4–6). *p<0.05; ***p<0.0001

determinants and, thus, was hydrophobic and intrinsically had a low solubility in water. Therefore, 7crp was not produced in large quantity in *E. coli* unless it was fused to a solubility-enhancing glutathione S-transferase (GST)-tag as reported previously (16). Since we assumed that a rather higher solubility in body fluid (more than 1 mg/mL) was desirable for administration by injection or by S/O nanodispersion system, we introduced small linkers (triarginine) between the epitopes in 7crp and designed a new peptide 7crpR (Fig. 1). The 7crpR was composed of 126 amino acids (14.8 kDa), however, smaller than 7crp-GST conjugate (35 kDa). The T cell receptor is known to recognize linear epitopes, and peptide linkers actually had no effect on peptide activity in T cell proliferation assay in the previous report (11). After production of 7crpR in *E. coli*, the peptide size was confirmed by SDS-PAGE. The mobility of 7crpR was slightly slower than estimated because of positively charged arginine likers. The peptide 7crpR was soluble in Milli-Q water (6 mg/mL) and no precipitation was observed after storage for 2 month at 4°C, indicating that the water solubility of 7crp was successfully increased by the introduction of triarginine linkers.

Then, we prepared a S/O nanodispersion using sucrose laurate (L-195) as a surfactant surrounding the peptide. A single-dispersed S/O nanodispersion with an average diameter of 230 nm was obtained according to the result of DLS analysis (Fig. 2a). The surfactant-protein complexes in SEM images had a spherical shape with a smooth surface. The sizes of particles in the SEM images were less than 500 nm (Fig. 2b), showing a good correspondence with the DLS result.

In vitro skin permeation efficiency of the peptide in S/O nanodispersion was examined using Cy3-labeled 7crpR. The S/O nanodispersion system assisted the permeation of peptide 4.8-fold higher than did PBS solution in 48 h; however, the skin permeation efficiency of 7crpR was slower compared to that of OVA. In the previous experiment delivering OVA, the S/O nanodispersion system increased the protein permeation by10-fold in 24 h (25). This difference may be relevant to the hydrophobicity of the peptide/protein. The peptide 7crpR does not have specific conformation and composed of 38% hydrophobic amino acids. Consequently, 7crpR had a higher affinity for sucrose laurate which hydrophile-lipophile balance value is 1. Our previous investigations demonstrated a mechanism of protein permeation through the S/O nanodispersion system: surfactant molecules were removed in the outermost SC layer and hydrophilic molecules only permeate into the hydrophilic layers in skin (20). Thus, inefficient removal of surfactant depressed the permeation of 7crpR into epidermis. Further introduction of hydrophilic peptide linkers may improve the skin penetration efficiency of 7crpR. Fluorescence microscopic images of mouse auricle sections more clearly revealed that the S/O nanodispersion system facilitated permeation of Cy3-7crpR. Since the mouse auricle skin is thinner than the YMP back skin, the difference in peptide permeation was obvious. In addition, the SC layer in mouse ears subjected to S/O nanodispersion became thicker than that subjected to peptide PBS solution after 48 h of patches. These results implied that the SC layer could act as a reservoir for S/O nanodispersion to help the peptide being released after the removal of patches.

Serum total IgE and Cry j 1-specific IgE levels were decreased by subcutaneous injection of 7crpR PBS solution. Thus, we assumed that the combined seven T cell epitope peptides held a curative effect on the pollinosis symptoms in this model after introduction of triarginine linkers. Declines in total IgE and Cry j 1-specific IgE levels also occurred in mouse sera receiving S/O nanodispersions containing 7crpR via the auricle skin. Transcutaneous administration of 7crpR using the S/O nanodispersion system reduced serum total IgE level

little compared to the subcutaneous administration by injection, probably because the technique induced a slow antibody response compared to the injection method. Serum Cry j 1-specific IgE level decreased significantly by the administration of the S/O nanodispersion containing 10 μg of 7crpR. Previously, smaller amount of 7crp fused with chicken egg white lysozyme (0.3 μg per day, 5 days a week, 4 weeks) applied orally as an edible vaccine reduced serum total IgE and Cry j 1-specific IgE levels similar to the experiments described here (16). Moreover, administration of a higher concentration of 7crpR in PBS (4 mg/mL, the amount of 7crpR: 200 μg) by subcutaneous injection did not reduce serum total IgE level in our preliminary study. Therefore, an optimal amount of 7crpR-containing S/O nanodispersion for therapeutic treatment of Cj pollinosis might be less than those examined here. In the present study, optimizations of amino-acid linker length, surfactant-peptide ratio, along with the peptide dose have not yet examined. Investigations on these factors, along with the effects of adjuvants and skin-permeation enhancing peptides would improve the efficacy of transcutaneous administration of the peptide vaccine for Cj pollinosis using the S/O nanodispersion system.

CONCLUSION

We developed a novel transcutaneous peptide administration system for therapeutic treatment of pollinosis. A S/O nanodispersion containing an integrated T cell epitope peptide 7crpR was prepared for transcutaneous administration to a mouse model of Japanese cedar pollen allergy. Size, morphology, and skin-permeation efficiency of the nanoparticle were characterized. A significant reduction of antigen-specific IgE in the serum was observed by the transcutaneous administration of the S/O nanodispersion containing 7crpR, indicating that the S/O nanodispersion system could be used for the future treatment of Japanese cedar pollinosis.

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Conflict of Interest The authors declare that they have no competing interests.

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