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RESEARCH PAPER

Transdermal Immunization using Solid-in-oil Nanodispersion with CpG Oligodeoxynucleotide Adjuvants

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ABSTRACT

Purpose Simple and noninvasive vaccine administration alternatives to injections are desired. A solid-in-oil (S/O) nanodispersion system was able to overcome skin barriers and induce an immune response; however, antibody levels remained low. We applied an immune potentiator, CpG oligodeoxynucleotide (ODN), to enhance the immune response by controlling the T helper 1 (Th1)/T helper 2 (Th2) balance.

Methods S/O nanodispersions containing ovalbumin (OVA) and CpG ODN (CpG-A or CpG-B) were characterized by size distribution analysis and a protein release test. The skin permeation of fluorescence-labeled OVA was observed by fluorescence microscopy. Antigen-specific IgG, IgG1, and IgG2a responses were measured by enzyme-linked immunosorbent assay.

Results Co-encapsulation of CpG ODNs in S/O nanodispersions enhanced induction of OVA-specific IgG. S/O nanodispersion containing OVA and CpG-A had a smaller mean particle size and permeated the skin more efficiently. In contrast, CpG-B showed the highest protein release and induction of OVA-specific IgG. IgG subclass analysis revealed that OVA induced a Th2-dominant immune response, while the S/O nanodispersion containing CpG-A skewed the immune response toward a Th1-bias.

Conclusions In combination with CpG ODN, the S/O nanodispersion system efficiently induced an antigen-specific antibody response. The Th1/Th2 immune balance could be controlled by the selection of CpG ODN type.

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KEY WORDS CpG oligodeoxynucleotide · solid-in-oil nanodispersion · transcutaneous immunization · transdermal drug delivery · vaccine

ABBREVIATIONS

BCA	Bicinchoninic acid
BSA	Bovine serum albumin
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
IFN	Interferon
IPM	Isopropyl myristate
L-195	Sucrose laurate surfactant
LC	Langerhans cell
OD	Optical density
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PBS	Phosphate-buffered saline
SC	Stratum corneum
S/O	Solid-in-oil
Th	T helper
Th1	T helper type 1
Th2	T helper type 2

INTRODUCTION

Vaccination is the most effective prophylaxis for infectious diseases, and herd immunity has successfully reduced transmission of disease worldwide [1]. Immunization aims to produce specific and long-lasting immunity to prevent or treat disease by administration of antigens, *i.e.*, killed or attenuated pathogens, proteins, or peptides [2]. In addition to use with infectious diseases, novel research focuses on the use of the vaccines to treat existing cancers [3]. Although some vaccines are administered by mouth and by aerosol, the majority are administered by injection, which raises several concerns including pain, needle-related injuries, and a requirement for

trained medical professionals to administer them. Therefore, recent research aims to develop safe, simple, and non-invasive vaccine administration methods via oral [4], nasal [5, 6], and transcutaneous [7, 8] routes. The transcutaneous route is advantageous for immunization because it can simulate Langerhans cells (LC) and dermal dendritic cells (dDC). Efficient immunizations with fewer antigen molecules can be expected via this route because LC and dDC are antigen-presenting cells abundant in the epidermis [9]. However, the outermost hydrophobic layer, stratum corneum (SC), poses a barrier meant to interrupt the invasion of bacteria and viruses, which also prevents permeation of hydrophilic molecules with a molecular mass >500 Da into the lower layers [10].

In a previous study, we developed a transcutaneous immunization method using a unique solid-in-oil (S/O) nanodispersion system containing ovalbumin (OVA) as a model antigen protein [11, 12]. For the preparation of the S/O nanodispersion, a water-in-oil emulsion containing OVA is lyophilized and the residual paste is re-dispersed in an oil vehicle, which provides an oil-based dispersion of OVA particles coated with hydrophobic surfactants. The hydrophobicity of the vehicle carrying the S/O nanodispersions allows these hydrophilic biomolecules to permeate into the epidermis without any skin pretreatment. Moreover, an improved immunization was obtained by co-encapsulation of hexa-arginine (R6) and OVA in a single S/O nanodispersion [13]. However, the mean antigen-specific antibody level induced by the S/O nanodispersion was still less than that induced by injection. Furthermore, an IgG subclass analysis indicated that the OVA administered by the transcutaneous route was prone to induce a T helper type 2 (Th2) immune response [13, 14]. Among the T helper (Th) cell subsets T helper type 1 (Th1) and Th2, Th1 cells enhance cellular immunity while Th2 cells are involved in humoral immunity. Because the Th cell subsets regulate the function of each other, a balanced immune response is preferable for prophylactic or therapeutic treatment [15].

Short, synthetic, and unmethylated oligodeoxynucleotides (ODNs) having CpG motifs were found to interact with toll-like receptor 9 (TLR9), and to induce a Th1-biased immune milieu [16]. Mice are known to express TLR9 in LC, keratinocytes, and immune cells of the myeloid lineage [17]. CpG ODNs are classified into multiple types according to their structural features and immunomodulatory functions [18]. Type A CpG ODNs (CpG-A) have a central palindromic motif and a 3'-phosphorothioate poly-G tail, and induce secretion of interferon (IFN)-α from plasmacytoid dendritic cells (pDCs). Type B CpG ODNs (CpG-B) contain phosphorothioate backbones and activate B cells to enhance the induction of proinflammatory cytokines, such as interleukin (IL)-6. Inducing strong Th1-like immunity, the CpG ODNs have potential as an adjuvant in vaccines against infectious diseases [19], cancer [20], and allergies [21].

In the present study, we incorporated 2 types of CpG ODNs, ODN 1585 (CpG-A) or ODN 1668 (CpG-B) with OVA into the S/O nanodispersions, characterized the nanodispersions, and investigated their effects on the enhanced and balanced transcutaneous immunization using the S/O nanodispersion system.

MATERIALS AND METHODS

Materials

OVA and 3,3',5,5'-tetramethyl benzidine solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isopropyl myristate (IPM) and cyclohexane were purchased from Tokyo Kasei (Tokyo, Japan) and Wako (Kyoto, Japan), respectively. Sucrose laurate (L-195) was kindly provided by Mitsubishi-Kagaku Foods (Tokyo, Japan). CpG-A (ODN 1585; 5′-ggGGTCAACGTTGAgggggg-3′) and CpG-B (ODN 1668; 5′-tccatgacgttcctgatgct-3′), were obtained from Invitrogen (Carlsbad, CA, USA) (Capital letters and lower case letters indicate phosphodiester linkage and phosphorothioate linkage, respectively). Horseradish peroxidase-labeled rabbit anti-mouse IgG, IgG1, and IgG2a were products of Rockland Immunochemicals (Gilbertsville, PA, USA).

Animals

Male 6-week-old ddY mice were purchased from Kyudo (Saga, Japan) and maintained under standardized conditions. Animal experiments were carried out with the approval of the Ethics Committee for Animal Experiments (Kyushu University) and in accordance with the Guide for the Care and Use of Laboratory Animals (Science Council of Japan).

Preparation of S/O Nanodispersions Containing OVA and CpG ODN

The S/O nanodispersions were prepared according to a previously described method [11]. Briefly, a 1.2 ml OVA aqueous solution (0.5 mg/ml) containing CpG (0 or 0.05 mg/ml) and a 2.4 ml L-195 cyclohexane solution (12.5 mg/ml) were added in a 10 ml glass vial, and a water-in-oil emulsion was formed using a polytron homogenizer (Kinematica AG, Luzern, Switzerland) at 26,000 rpm for 2 min. The emulsion was frozen in liquid nitrogen for 20 min and then lyophilized for 24 h using a freeze dryer (EYELA, Tokyo, Japan) to obtain a surfactant-protein complex. IPM (0.3 ml) was added to the surfactant-protein complex and vortexed for 15 min at room temperature to yield an S/O nanodispersion.

The average diameter and the polydispersity index of the particles were measured via dynamic light scattering (DLS) using a Zetasizer NanoZS (Malvern, Worcestershire, UK).

Determination of Protein Release Efficiency

The protein was released into PBS from the S/O nanodispersions as previously described [11]. An S/O nanodispersion solution (2 mg/ml, 0.35 ml) was added to 1.5 ml PBS and gently stirred for 24 h at 37°C. Aliquots (100 μl) were collected at various intervals (0, 1, 2, 3, 6, 12, and 24 h) and the OVA concentration was determined by means of a bicinchoninic acid assay using a Bicinchoninic Aid Kit according to the manufacturer's instructions (Sigma-Aldrich).

Permeation of the S/O Nanodispersion into the Skin

In vitro skin permeation of S/O nanodispersion was performed using the Yucatan micropig (YMP) back skin and Cy3-labeled OVA on custom fabricated Franz diffusion cells (effective diffusion area; 0.785 cm^2, receptor volume; 5 ml), according to a previous description [22]. Cy3-labeled OVA (labelling ratio =1:1) was prepared using a kit Cy3 Mono-Reactive Dye Pack (GE Healthcare, Buckinghamshire, England). A 0.25 ml S/O nanodispersion containing 1 mg/ml of Cy3-OVA was put on epidermis of the YMP skin. The receptor was filled with PBS (pH 7.4) and stirred constantly at 32±1°C. As a control, Cy3-OVA in PBS solutions was put on the YMP skins under the same conditions. After 24 h, the samples were removed with tissue papers, and the skin was cut into pieces. The OVA permeated into the skin was extracted with a 0.5 ml extraction liquid (Milli-Q : ethanol : acetonitrile =2:1:1). The cumulative amounts of Cy3-OVA in the YMP skins were calculated from the fluorescence intensities from the extraction liquids measured with a fluorescence spectrometer LS55 (PerkinElmer, Waltham, MA, USA) at Ex/Em =540 nm/568 nm.

In vivo permeation of fluorescence-labeled OVA into mouse ear skin was observed using a fluorescence microscope. Fluorescence Cy5 dye was labeled on OVA using Cy5 Mono-Reactive Dye Pack (GE Healthcare), according to the manufacturer's instructions. Two tissue papers were folded into squares (5 mm×10 mm) and impregnated with the S/O nanodispersions containing Cy5-labeled OVA with CpG-A or CpG-B. The pair of papers with the same S/O nanodispersion were then patched onto the posterior auricle skin (100 μg OVA per mouse). The mice (n=2 per sample) were sacrificed after 24 h and the patches were removed. Next, the auricle tissue was washed with 99% ethanol, followed by a wash with PBS. The auricle tissues were immersed in 4% paraformaldehyde for 6 h and frozen in Tissue-tek O.C.T. compound (Sakura Finetek, Tokyo, Japan). Approximately 20 tissue sections (16 μm) were prepared from each auricle tissue using a cryostat microtome (Leica, Wetzlar, Germany). Fluorescence images were observed using a fluorescence microscope BZ-9000 equipped with a 20× objective lens and BZ-II software (Keyence, Osaka, Japan).

Transcutaneous Immunization of Mice

Male, 7-week-old, ddY mice were immunized with OVA. A total of 100 μg of OVA in the S/O nanodispersion or in PBS was impregnated into tissue papers, and the tissue papers were patched three times (day 0, 7, and 14) onto both mouse posterior auricles without any skin pretreatment. The patches were removed after 24 h. For a control experiment, 100 μg of OVA in a PBS solution (1.0 mg/ml) was subcutaneously injected into the abdomen three times (day 0, 7, and 14). Blood was collected from a tail vein 1 week after every administration.

Measurement of Specific Antibody Titers

Anti-OVA IgG, IgG1, and IgG2a titers were determined using an enzyme-linked immunosorbent assay (ELISA) following a previously described protocol [13]. Briefly, a 96-well Nunc MaxiSorp microplate (Thermo Fisher Scientific, Waltham, MA, USA) was coated with OVA by incubation with an OVA aqueous solution (5.0 mg/ml, 100 μl/well) at 4°C overnight. After washing the plate five times with PBS-T (1% Tween-20 in PBS), 200 μl of PBS containing 2% BSA was added to each well. The plate was incubated for 2 h at 37°C, then washed with PBS-T. Next, 20 μl of diluted serum sample was added to each well. The plate was incubated for 2 h at 37°C, then washed with PBS-T. The specific antibody was detected by incubation with 100 μl of a 20,000 dilution (in PBS containing 2% BSA) of horseradish peroxidase-labeled rabbit anti-mouse IgG, IgG1, or IgG2a. The plate was incubated for 2 h at 37°C, then washed with PBS-T. TMB solution (100 μl) was added to each well and the color-change reaction was performed for 30 min at 37°C in darkness. After the reaction was stopped with 1 M sulfuric acid, the optical density (OD) at 450 nm was measured using a microplate reader (BioTek Instruments, Winooski, VT, USA). Antibody titers were defined as the dilution that yielded an OD equal to the OD of a 50-fold dilution of serum sample obtained from a non-immunized mouse. The data were expressed as the mean ± standard deviation of five experiments. Significant differences were determined using the Student's *t*-test.

RESULTS

Characterization of the S/O Nanodispersions

The particle size and polydispersity index of the S/O nanodispersion containing OVA alone or OVA and CpG ODN are shown in Table 1 and Fig. 1. The all surfactant-protein complexes were uniformly dispersed, and the S/O nanodispersion containing OVA and CpG ODN displayed

Table I Size Distribution Measurements of S/O Nanodispersions

Sample	Z-Average (nm)	Polydispersity Index
OVA alone	168	0.10–0.19
OVA + CpG-A	109	0.17–0.22
OVA + CpG-B	107	0.18–0.25

Data represent the mean ± standard deviation of three measurements

lower mean particle sizes than the S/O nanodispersion containing OVA alone. An *in vitro* OVA release test exhibited a burst of OVA in the first 6 h, followed by a sustained release in all three experiments (Fig. 2). The S/O nanodispersions containing CpG-A or CpG-B displayed similar release curves for the initial burst. After 6 h, the accumulative amount of OVA released from the S/O nanodispersions containing CpG ODNs were 1.8-fold higher than that from the S/O nanodispersion containing OVA alone. Approximately 29, 25, and 19% of OVAs were released after 24 h from the S/O nanoparticles carrying OVA and CpG-B, OVA and CpG-A, and OVA alone, respectively. *In vitro* skin permeation study revealed that cumulative amount of the Cy3-labeled OVA applied in the S/O nanodispersion and in the PBS solution in the YMP skins after 24 h were 4.4 ± 0.8 and 0.4 ± 0.1 μg/cm^2, respectively.

In vivo Skin Permeation

To test the *in vivo* permeation efficiencies of S/O nanodispersions, additional S/O nanodispersions containing Cy5-OVA with CpG ODN were created and observed via fluorescence microscopy. As shown in Fig. 3, no signal was observed in the skin section to which the Cy5-OVA PBS solution was applied. In contrast, blue fluorescence signals from Cy5 were observed in the epidermis of skin sections

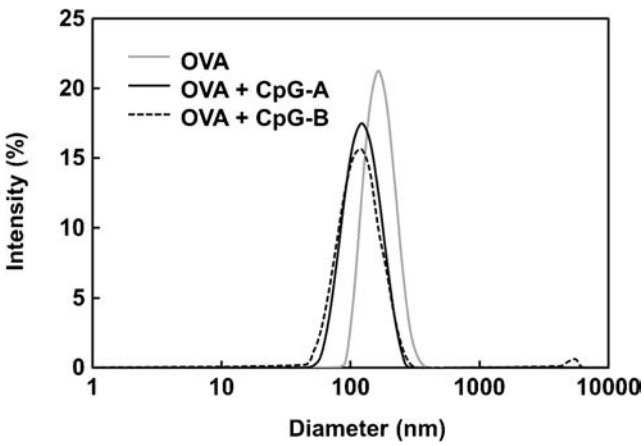

Fig. 1 Dynamic light scattering measurement of S/O nanodispersions. The S/O nanodispersions containing OVA alone, OVA and CpG-A, and OVA and CpG-B are shown.

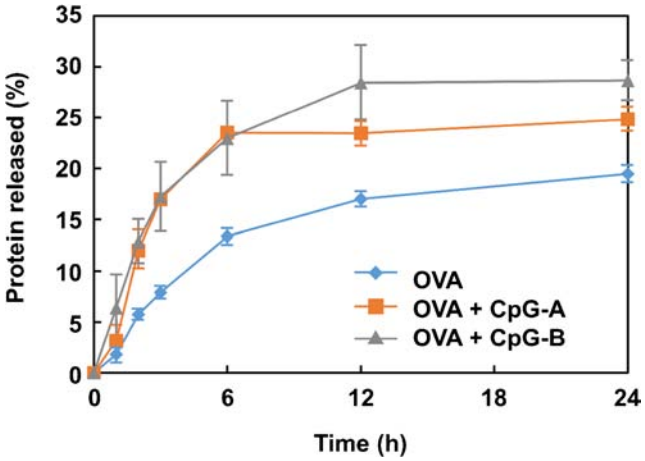

Fig. 2 *In vitro* protein release efficiency from S/O nanodispersions. The cumulative amount of proteins released from S/O nanodispersions containing OVA alone, OVA and CpG-A, and OVA and CpG-B were measured. Data shown as mean ± SD from three experiments.

subjected to S/O nanodispersions. The S/O nanodispersion containing CpG-A permeated the epidermis more efficiently than the nanodispersion containing CpG-B.

OVA-specific Antibody Responses

To determine the antibody response to transcutaneous administration of our S/O nanodispersions, the anti-OVA antibody levels in sera from the immunized mice were measured by sandwich ELISA (Fig. 4). After three administrations, OVA encapsulated with CpG ODNs induced a higher level of specific antibody than OVA alone. The mean OVA-specific IgG levels were increased 6- and 9-fold by the addition of CpG CpG-A and CpG-B, respectively. However, OVA administered by subcutaneous injection induced OVA-specific IgG the most efficiently, leading to antibody levels 16-fold higher than those induced by OVA alone in the S/O nanodispersions.

Levels of IgG subclass antibodies, IgG1 and IgG2a, were also analyzed (Fig. 5). IgG1 levels were higher than IgG2a levels in the sera of all immunized mice, indicative of Th2 dominant immune responses. Induction of both IgG1 and IgG2a were increased by the addition of CpG ODN to the S/O nanodispersion system. The mean IgG1 level was increased 10- and 14-fold by the addition of CpG-A and CpG-B, respectively. The mean IgG2a level was also increased 10-fold by the addition of CpG-A in the S/O nanodispersion; however, it was increased only 7-fold by the co-encapsulation of CpG-B into the S/O nanodispersion. The mean IgG1/IgG2a ratio was diminished by co-encapsulation of CpG-A, while the ratio was increased by the co-encapsulation of CpG-B into the S/O nanodispersion. The IgG1 to IgG2a ratio was similar whether OVA alone was administered by injection or via the S/O nanodispersion.

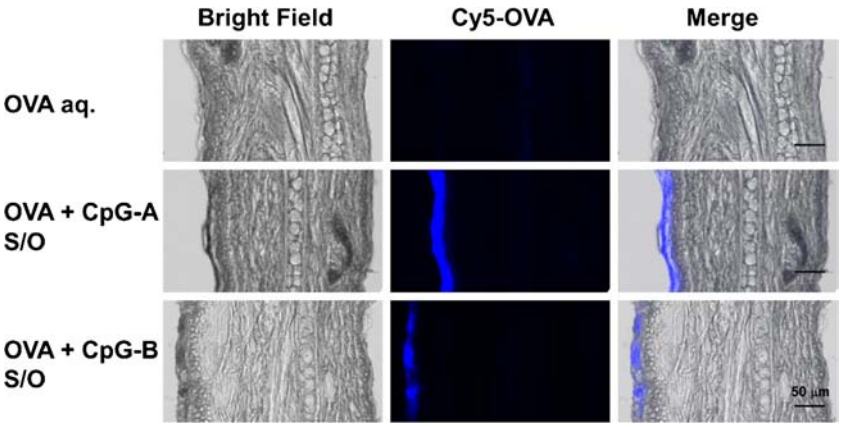

Fig. 3 Bright field and fluorescent microscopic images of mouse ear skin sections. Cy5-OVA in PBS solution (OVA aq.) and Cy5-OVA in S/O nanodispersion with CpG-A (OVA + CpG-A S/O) or CpG-B (OVA + CpG-B S/O) were patched on mouse ears for 24 h. Representative images from 20 sections per auricle tissue are shown.

DISCUS?SION

In the present study, we investigated an enhanced transcutaneous immunization system using S/O nanodispersions with CpG ODNs to control the Th1/Th2 balance. Naked OVA formed a white precipitate in IPM, however, surfactant-OVA complexes formed translucent dispersions in IPM. Mean particle size and size distribution analyses of the surfactant-protein complexes with or without CpG ODNs indicated that the particles were in the nano size range and uniformly dispersed whether CpG ODNs were present or not. The results from the *in vitro* skin permeation study indicated that the S/O nanodispersion system facilitated the permeation of OVA by 10-fold when compared to that of the naked OVA in PBS solution. Next, we examined the *in vitro* OVA release efficiency from S/O nanodispersions into PBS. The initial bursts from all the S/O nanodispersions in the first 6 h were similar to our previous results [11, 13]. The protein release rate was increased by the co-encapsulation of CpG ODNs, suggesting that the electrostatic repulsion between the ODN molecules might have helped collapse the S/O nanoparticles. The S/O nanodispersions containing CpG-A, which is capable of forming a compact construct (stem-loop and guanine-stacking shapes), showed a lower release efficiency after 24 h than the S/O nanodispersion containing CpG-B. The skin permeability of the S/O nanodispersion containing CpG-B was lower than that containing CpG-A, indicating that the stability of the nanoparticles affected the skin permeation efficiency.

An improved induction of the OVA-specific IgG was observed in the presence of CpG ODNs. Permeation efficiency of the S/O nanodispersion containing OVA and CpG-B was low in the fluorescence microscopic image; however, the effect of CpG-B was still strong enough to induce a similar antibody response as the one induced by the S/O nanodispersion containing OVA and CpG-A. Notably, the mean OVA-

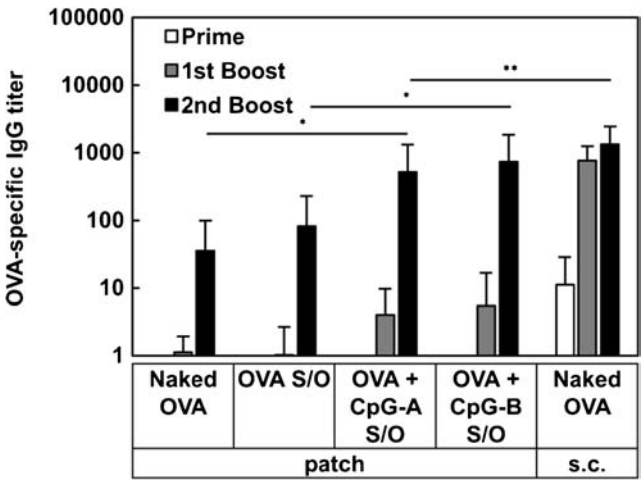

Fig. 4 Serum IgG responses to OVA. OVA-specific IgG titers 1 week after prime (white bar), 1st boost (gray bar), and 2nd boost (black bar) were determined by ELISA. Naked OVA or OVA encapsulated in S/O nanodispersions were administered to mice with patches. Naked OVA was also administered by subcutaneous (s.c.) injection as a positive control. Data shown as mean + SD (n =4–5, *p < 0.05 and **p < 0.01).

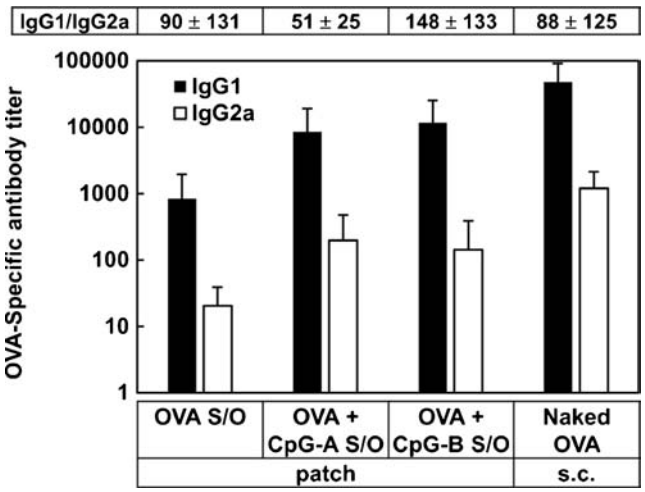

Fig. 5 OVA-specific IgG1 and IgG2a in serum responses after 2nd boost. OVA-specific IgG1 (black bar) and IgG2a (white bar) titers were measured a week after the 2nd boost. OVA encapsulated in S/O nanodispersions were administered with patches. Naked OVA was also administered by subcutaneous (s.c.) injection as a positive control. Data shown as mean + SD (n = 4–5). The IgG1/IgG2a ratios were calculated for individual mice and are shown as mean ± SD.

specific IgG titer induced by OVA in the presence of CpG-B after the 2nd boost was close to that induced by subcutaneous injection of OVA. Compared with the subcutaneous injection method, transcutaneous immunization induced antigen-specific antibody slowly because of its slow permeation efficiency into the skin. This delay may be useful for monitoring and treating acute adverse events during vaccination.

We further analyzed the serum IgG for subclass antibody (IgG1 and IgG2a) responses, as marker molecules of Th2- and Th1-type immune responses, respectively. As previously shown, OVA induced Th2-biased immunity in mice whether it was administered by subcutaneous injection or via the S/O nanodispersion system. Previous investigations of IgG subclass and cytokine responses to CpG ODNs indicated that they induce a Th1-like immune response [23–25]. These studies, where OVA and ODN 1555 were intraperitoneally administered to BALB/c mice [26], and OVA and ODN 1668 were transcutaneously administered to NC/Nga mice after a tape-stripping pretreatment [27], additionally found a reduction in the IgG1/IgG2a ratio. Our experiments in this study also indicate that ODN 1585 (CpG-A) skews the immune balance toward a Th1-biased response.

In contrast, the effect of CpG-B as an adjuvant known to induce Th1-type immunity appeared to be suppressed in the S/O nanodispersion system. An increase in the IgG1/IgG2a ratio was observed, implying the Th2-like immunity was actually enhanced. It is known that CpG-A and CpG-B differentially stimulate antigen-presenting cells; CpG-A directly stimulate the IFN-α induction from pDCs, whereas CpG-B trigger differentiation of antigen-presenting cells, stimulates B cells to induce proinflammatory Th1 cytokines, in addition to pDC maturation [28, 29]. Such differences in immune activation pathways could be one reason that the Th2-biased immune response was enhanced by CpG-B in the S/O nanodispersions. Recently, Bouwstra *et al.* reported that the effect of CpG-B (ODN 2006) as an adjuvant was changed by its administration routes or its degree of colocalization with antigens [30]. OVA and CpG ODN, which were co-encapsulated in liposomes and transcutaneously administered to mice with microneedle arrays, caused a reduction in the IgG1 level and an increase in the IgG2a level, indicating a shift towards a Th1-type immune response. Meanwhile, a mixed solution of OVA and CpG ODN, in which they were not co-encapsulated, did not cause the apparent shift after administration in the same manner. They suggested that colocalization of OVA and CpG in the lymph nodes was key to inducing a strong Th1-type immunity. In our previous examination observing the permeation of the S/O nanodispersions into the mouse ear skin with a confocal laser scanning fluorescence microscope, we concluded that the most surfactants were removed in the hydrophobic SC layer, and only protein molecules permeated into the hydrophilic layers beneath the SC [11, 31]. The protein release efficiency of S/O nanodispersions containing CpG-B was high, and the particles could have collapsed before arriving at pDCs which are located in lymphoids. Still, the S/O particles containing OVA and CpG-B most efficiently induced the serum total IgG, indicating that CpG-B permeated the skin where LCs and dDCs exist. An *in vitro* investigation by another group demonstrated that a CpG-B (ODN 1826) could dose-dependently upregulate the antigen-presenting ability of LCs in the existence of keratinocytes [18].

CONCLUSIONS

The studies presented here show that an improved immunization by the transcutaneous route was attained by the addition of CpG ODNs into the S/O nanodispersions. Additionally, these studies indicate that the Th1/Th2 polarization of the immune response can be controlled through the selection of specific CpG ODN adjuvants. Further investigation is still needed to fully characterize the immune response to S/O nanodispersions with CpG ODNs, including cytokine profiling, characterization of other antibody responses, and immunization with other antigens and CpG ODNs to characterize the mechanism of enhanced induction of IgG and IgG subclasses. However, our data contribute to the progress towards a simple and non-invasive transcutaneous vaccination method that induces a balanced immunity.

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