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Research Paper

Iontophoresis-assisted transdermal immunization induces anti-histone H1 antibody production and immunosuppressive activity

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ABSTRACT

Most monoclonal antibodies and vaccines require injection by healthcare professionals. During the COVID-19 pandemic, vaccination programs faced delays due to shortages. Transdermal delivery has attracted interest to reduce workload. However, many vaccines and monoclonal antibodies show poor skin absorption due to high molecular weight, requiring permeation strategies. This study examined iontophoresis (IP), a method enhancing transdermal drug delivery, for vaccination. SSV-KLH, a peptide–carrier conjugate vaccine designed to induce anti-histone H1 antibody production, was applied to mouse skin using IP. Immune responses were compared with intraperitoneal injection and transdermal delivery without IP. IP increased antibody production compared with non-IP delivery, although the difference was not significant. Antibody levels in the IP group were not significantly different from those in the intraperitoneal injection group. Both IP and injection induced similar immunosuppressive effects, unlike non-IP delivery. These results suggest IP enables effective transdermal vaccine delivery and may reduce reliance on healthcare professionals.

Key words: iontophoresis, vaccine, transdermal delivery, antibody production, immune response

1. Introduction

In organ transplantation, the administration of immunosuppressive agents is essential to prevent immunological rejection following transplantation. However, existing immunosuppressive drugs may cause adverse effects such as nephrotoxicity, increased susceptibility to infections, and a higher risk of tumor development with long term use. In addition, it has been pointed out that transplant recipients must continue taking immunosuppressive agents throughout their lifetime, which poses a lifelong clinical challenge for transplant recipients (Cajanding, 2018; Katabathina et al., 2016; Taylor et al., 2005). Such adverse effects can considerably reduce patients' quality of life (QOL), making the establishment of new immunosuppressive strategies clinically essential.

Nakano et al. discovered in a rat orthotopic liver transplantation model that, following liver transplantation, a substance suppressing allogeneic rejection namely, an inducer of immune tolerance is produced in blood, and identified this substance as an anti-histone H1 antibody (Nakano et al., 2004). Furthermore, Chiang et al. employed a phage display method to develop a mimetic epitope peptide consisting of 12 amino acid residues (sequence: SSVLYGGPPSAA), which is recognized by the anti-histone H1 monoclonal antibody (SSV). This peptide, hereafter referred to as the SSV peptide, was found to be immunologically useful. However, due to its short peptide chain, the SSV peptide exhibited low immunogenicity *in vivo* and failed to induce a sufficient immune response. Therefore, some of the authors of this study (T.M., N.O., K.M., T.G., and S.S.) prepared SSV-KLH, in which the SSV peptide was conjugated to Keyhole Limpet Hemocyanin (KLH), a large carrier protein with a molecular weight of approximately 5 million, to enhance its immunogenicity. When SSV-KLH was administered to rats, anti-histone H1 antibodies were induced, and immunosuppressive effects were observed in an allogeneic heterotopic transplantation model (Chiang et al., 2009). These findings suggested the potential of a novel vaccine-based immunosuppressive therapy that does not rely on conventional immunosuppressive drugs.

There are still many limitations regarding the administration methods of peptide-based formulations and vaccines. At present, with a few exceptions, most vaccines are administered through invasive injections. Although injections are an established method in terms of efficacy, they can cause local reactions such as pain and swelling at the injection site and impose a significant psychological burden, particularly on children. Moreover, during the COVID-19 pandemic, large-scale vaccination programs were implemented worldwide; however, in countries such as Japan and Indonesia, delays in vaccination were reported despite sufficient vaccine supply, due to a shortage of healthcare professionals qualified to administer injections (Fuady et al., 2021; Okubo et al., 2021). These considerations highlight the strong need for the development of simpler and non-invasive vaccine delivery methods. Alternative routes to injection, such as oral or sublingual administration, have been explored; however, it is well known that antigens delivered via these routes can be degraded by gastric acid, digestive enzymes, or enzymes in the bloodstream, preventing them from eliciting sufficient immunological effects (Hasija et al., 2016; Kim et al., 2024). Thus, in recent years, transdermal vaccine delivery has been actively investigated as a novel alternative to injection (Ito et al., 2010; Nguyen, 2025). While transdermal administration is advantageous for being non-invasive and convenient, a major limitation is that most peptide-based formulations and vaccine molecules are large in size, making it difficult for them to penetrate the epidermal barrier and elicit a sufficient immune response (Bos et al., 2000). To overcome this challenge, numerous approaches have been investigated, including physical enhancement methods (Aldawood et al., 2021; Courtenay et al., 2018; Kougkolos et al., 2024; Tang et al., 2025; Yu et al., 2022) and chemical enhancement methods (Oshizaka et al., 2023; Sloan et al., 1983; Takeuchi et al., 2022). Recently, microneedle systems have been widely investigated as a useful approach to enhance the transdermal delivery of macromolecules (Balde et al., 2025). However, microneedles are still considered minimally invasive and may involve risks such as infection. In addition, experimental studies have reported that only about 46% of dissolving microneedles are successfully inserted into the skin, suggesting limitations in insertion reproducibility and depth control (Lahiji et al., 2015). Therefore, alternative non-invasive strategies to enhance transdermal antigen delivery are still needed.

In this study, as a first step toward the major goal of developing a non-invasive and sustainable immunosuppressive vaccine delivery method, we selected iontophoresis (IP), a physicochemical enhancement technique (Dhote et al., 2012; Mori et al., 2023; Sugibayashi et al., 2021; Tari et al., 2021), to enable transdermal administration of SSV-KLH. Its effectiveness was evaluated by measuring anti-SSV antibody titer and mixed lymphocyte reactions (MLR) as indicators. Notably, immune responses correlate with antigen dose but are not proportional to it (Rhodes et al., 2019). Therefore, the transdermal permeation amount of SSV-KLH was not measured in this study. In this approach, iontophoresis was used not only to enhance antigen transport across the skin barrier but also to provide mild electrical stimulation to the skin, which may support immune activation through local antigen-presenting cells (Toyoda et al., 2015).

Iontophoresis may cause skin irritation or inflammation under certain conditions. In general, current densities of approximately 0.3–0.5 mA/cm^2 are commonly used (Alkilani et al., 2022; Yeasmin et al., 2022). In this study, we also evaluated whether anti-SSV antibody production and MLR responses change depending on current density. Therefore, current densities higher than those typically used in clinical settings were included for exploratory evaluation.

2. Materials and Methods

2.1. Materials

SSV-KLH, in which the SSV peptide a mimotope composed of 12 amino acid residues was conjugated to the carrier protein Keyhole Limpet Hemocyanin (KLH), was obtained from Peptide Institute, Inc. (Ibaraki, Osaka, Japan). Freund's complete adjuvant was purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). The Ag foil used as the electrode for IP was purchased from Murata Yohaku & Co. (Tokyo, Japan). Tacrolimus was purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). All other reagents and solvents were used without further purification.

2.2. Animal Experiments

Male BALB/c mice were purchased at 4 weeks of age from Charles River Laboratories Japan (Yokohama, Japan), and dosing was initiated at 5 weeks of age after one week acclimation period. This study was approved by the Animal Experimentation Ethics Committee of Josai International University (Approval No. 36) and was conducted in accordance with both international and domestic guidelines for the care and use of laboratory animals.

2.3. Administration of SSV-KLH and Blood Collection

As a positive control, 50 µL of an emulsion consisting of SSV-KLH solution (650 µg/mL) and Freund's complete adjuvant (1:1, v/v) was administered intraperitoneally to BALB/c mice. Blood samples were collected before SSV-KLH administration and on days 14, 28, 42, and 65 after administration. The same mice were used throughout the experimental period, and blood samples were collected at each time point.

For blood collection, the tip of the mouse tail was gently incised with a sterile scalpel blade, and more than three drops of blood were collected. Hemostasis was achieved by

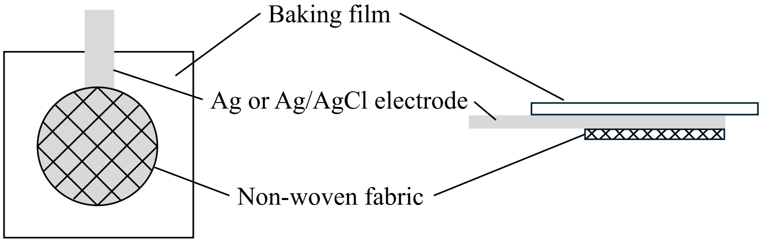

Figure 1. Illustration of the applied support, electrode, and non-woven fabric.

applying sterile gauze to the incision site (tail nick method). The collected blood was kept at approximately 4°C for 1–2 h to allow natural coagulation, followed by centrifugation at 3,000 rpm for 15 min. The obtained serum was stored at −80°C until analysis.

2.4. Skin permeation using iontophoresis

Figure 1 shows the arrangement of the support, electrodes, and nonwoven fabric used for skin permeation by IP. The anode electrode was made of Ag foil cut into a 3.14 cm^2 piece, and the cathode electrode was prepared by electrolyzing an Ag foil of the same size in saline to form Ag/AgCl foil. The back side of the mouse was used as the anode side, and the abdominal side as the cathode side.

The back and abdominal regions of the mice, corresponding to the electrode application sites, were shaved and depilated using clippers and a hair removal cream. A mixture of 200 μL of SSV-KLH (650 μg/mL) and 10 μL of citrate buffer (20 mM, pH 3.0) was applied to the non-woven pad on the anode side, while a mixture of 200 μL of SSV-KLH (650 μg/mL) and 10 μL of Tris-HCl buffer (20 mM, pH 8.79) was applied to the non-woven pad on the cathode side, and both were affixed to the respective regions of the mice. After securing the electrodes with surgical tape, a direct current was applied for 30 minutes using a direct current device (VI1002, Precise Gauges Co., Hamamatsu, Japan) at current densities of 0.314, 0.628, or 1.236 mA/cm^2. The current densities and application duration were selected based on previously reported iontophoresis conditions in transdermal delivery studies (Dhote et al., 2012; Sugibayashi et al., 2021). These conditions were chosen to explore the relationship between current density and immunological response. As a control (IP non-applied group), SSV-KLH was applied in the same manner, but no current was applied. Skin permeation experiments were conducted four times with 2 weeks-intervals, the same as for intraperitoneal injection. In each session, SSV-KLH containing nonwoven fabric was applied to the mouse skin, IP was performed for 30 min, and then the electrodes and fabric were removed. Blood collection and serum preparation were carried out in the same manner as for the intraperitoneal injection.

The IP experiment was conducted once for each animal group, and serum samples obtained from each mouse were used for ELISA and MLR assays. ELISA measurements were performed in duplicate, and MLR assays were performed in triplicate as technical replicates. The mean value for each mouse was used for statistical analysis.

2.5. Measurement of anti-SSV antibodies

The anti-SSV antibody titer in serum was measured using a standard indirect ELISA. 96 well microplates were coated with SSV-OVA solution, and after blocking, diluted serum samples were added and incubated at room temperature. The colorimetric reaction was carried out using an HRP conjugated secondary antibody, and the absorbance at 405 nm was measured using a microplate reader (Multiskan Ascent; Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Measurement of Mixed Lymphocyte Reaction

MLR was evaluated using a cell proliferation ELISA kit (Cell Proliferation ELISA, BrdU [colorimetric]; Roche Diagnostics, Tokyo, Japan). Splenocytes were isolated from Dark agouti rats (DA rats; $RT1^a$) and Lewis rats (LEW rats; $RT1^l$), with LEW-derived cells used as responder cells and DA-derived cells treated with mitomycin C used as stimulator cells. This combination represents an allogeneic mismatch model with differing major histocompatibility antigens, suitable for assessing immunosuppressive effects. The 2 cell populations were co-cultured in 96 well plates, and mouse serum obtained 3 weeks after the 4 administration was added at a final concentration of 0.5%. After 72 h-culture, cells were labeled with BrdU, and proliferation was measured by ELISA. Absorbance at 492, 650 nm was read using a microplate reader.

2.7. Statistical Analysis

ELISA and MLR measurements were performed multiple times using the same samples, and the mean values of each measurement were used for analysis. Each group consisted of 3–8 mice (biological replicates). ELISA and MLR assays were performed in triplicate (technical replicates). The results are presented as the mean ± standard error (S.E.). Statistical comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. When comparisons between two groups were required, Student’s t-test was used. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

Figure 2 shows the effects of the different SSV-KLH administration groups on anti-SSV antibody levels. In the intraperitoneal injection group, anti-SSV antibody production was observed from day 14 onward, with significantly higher antibody levels detected on all measured days ($p < 0.05$). For transdermal administration using IP, anti-SSV antibody production was observed from day 28 onward at all current densities. In the IP-non-applied group, antibody production was detected from day 42 onward. Antibody levels on day 65 tended to be higher in the IP groups than in the patch-only group, although the differences were not statistically significant. Antibody levels in the IP groups were also not significantly different from those observed after intraperitoneal injection. However, no significant differences in antibody levels were observed among the different current densities on day 65. Statistical differences were analyzed using one-way ANOVA followed by Tukey’s multiple comparison test.

Figure 3 shows the evaluation of immunosuppressive activity using the MLR. The vertical axis represents the absorbance (OD value) measured by ELISA as an indicator of the number of lymphocytes that proliferated and were activated in the MLR. Higher OD values indicate a greater number of lymphocytes and correspondingly weaker immunosuppressive activity. The intraperitoneal injection group and the groups receiving IP at any current density (Figure 3E, F, and G) showed significantly lower OD values compared to the allogeneic transplant rejection model, DA/LEW (Figure 3B) ($p < 0.01$). No differences in OD values were observed among the different IP current densities. Furthermore, the patch-only group (Figure 3H) showed OD values comparable to DA/LEW (Figure 3B). On the other hand, the OD values in the intraperitoneal injection and IP-assisted transdermal groups were higher than those in the tacrolimus-treated group (2 nM), which suppresses T cell activity and cytokine production ($p < 0.01$).

Table 1 shows the presence or absence of anti-SSV antibody production and MLR activity. Skin permeation of SSV-KLH via IP induced anti-SSV antibody production, although the antibody titer was lower than those observed with intraperitoneal injection. On the other hand, MLR results indicated that skin permeation of SSV-KLH with IP elicited immunosuppressive effects comparable to those of intraperitoneal injection at all current densities. In the patch only group (without IP), antibody production was observed, but no immunosuppressive activity was detected.

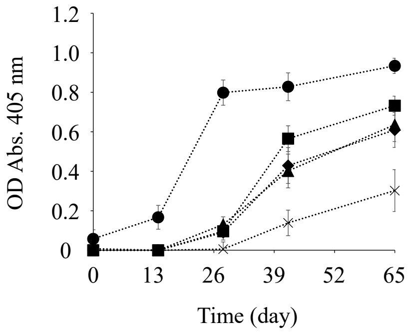

Figure 2. Changes in anti-SSV antibody expression levels following SSV-KLH administration using IP. Symbols: ● Intraperitoneal, ■ IP (1.236 mA/cm^2), ▲ IP (0.628 mA/cm^2), ♦ IP (0.314 mA/cm^2), and × Topical application without IP. Each data point represents the mean ± S.E. (n = 3-6). Statistical differences were analyzed using one-way ANOVA followed by Tukey’s multiple comparison test.

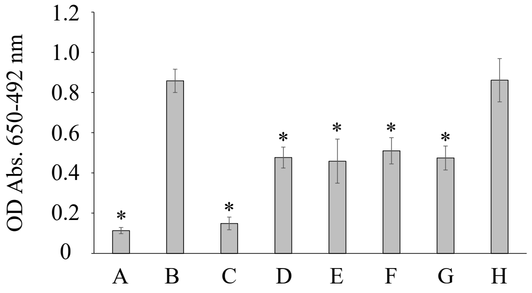

Figure 3. Assessment of pharmacological efficacy through a MLR. A: LEW/LEW, B: DA/LEW, C: 2 nM Tacrolimus, D: Intraperitoneal, E: IP (1.236 mA/cm^2), F: IP (0.628 mA/cm^2), G: IP (0.314 mA/cm^2), and H: Only application. Each bar shows the mean ± S.E. (n = 4-8). Statistical differences were analyzed using one-way ANOVA followed by Tukey’s multiple comparison test. (*; $p < 0.01$)

Table 1. Evaluation of the presence or absence of anti-SSV antibody production and MLR activity.

	Production of anti-SSV antibodies	**MLR**
Intraperitoneal	+	(−)
Iontophoresis	+ With potential difference	(−) Without potential difference
Only application	+	(+)

+: antibody production, −: no antibody production
(+): immune response present, (−): no immune response

4. Discussion

In this study, we evaluated antibody production and immunosuppressive activity following skin permeation of SSV-KLH using IP. In the intraperitoneal injection group, anti-SSV antibody production was observed from day 14 onward, with significantly higher antibody titers at all time points, demonstrating that intraperitoneal injection induces antibodies rapidly and efficiently. This effect is likely due to the direct delivery of antigen into the body, allowing prompt access to antigen presenting cells and lymphoid tissues. On the other hand, in the transdermal administration groups, antibody production was observed from day 28 onward in the IP applied groups, whereas in the IP non-applied group, antibodies were detected only from day 42. These findings suggest that IP temporarily disrupts the skin barrier, enhancing antigen permeability and improving antigen presentation efficiency. In addition to enhancing transdermal transport, electrical stimulation associated with iontophoresis may induce mild local skin irritation. The skin is known to contain abundant antigen-presenting cells, including Langerhans cells and dermal dendritic cells, which play important roles in initiating immune responses. Mild inflammatory stimulation of the skin may therefore facilitate antigen uptake and presentation to immune cells. In this context, iontophoresis may function not only as a physical permeation enhancement method but also as a stimulus that promotes local immune activation in the skin. Notably, in the groups subjected to IP at 0.628 and 1.236 mA/cm^2, antibody titer on day 65 was significantly higher than that in the IP non-applied group, confirming that immune responses can be effectively induced even via transdermal administration. On the other hand, no significant differences in day 65 antibody titer were observed among the different current intensities, suggesting that within a certain range, increasing the current does not markedly affect the final antibody production. This immunological characteristic of the skin has been utilized in several transdermal vaccination approaches (Rhodes et al., 2019). Molecular transport by iontophoresis is generally current-dependent; however, the relationship between antigen delivery and the resulting immune response is not necessarily linear (Rhodes et al., 2019). Antigens are taken up by antigen-presenting cells to elicit an immune response, and antibody titers typically reach a plateau after booster immunization. Therefore, even if transdermal transport increases to some extent at higher current densities, the immune response itself may already be close to the maximal level at 0.314 mA/cm^2.

In the MLR results, both the intraperitoneal and IP groups exhibited significantly lower OD values compared to the allogeneic graft rejection model (DA/LEW), indicating suppression of T cell proliferation and acquisition of immunosuppressive function. This suggests that skin permeation of IP allowed sufficient antigen delivery to lymphoid tissues, achieving immune response suppression. In contrast, in the IP non-applied group, although antibody production was observed, no suppressive effect was detected in the MLR, suggesting insufficient antigen penetration through the skin. Furthermore, the OD values in the intraperitoneal and IP groups were slightly higher than those in the tacrolimus group, which directly acts on T cells to suppress cytokine production. This indicates that while transdermal IP administration does not achieve as strong an immunosuppressive effect as chemical immunosuppressants, it can still modulate T cells within a physiological range. In other words, transdermal administration is a promising non-invasive approach that can balance antibody induction with immune regulation while avoiding excessive immunosuppression.

This study provides fundamental evidence that IP mediated enhancement of antigen skin permeation can serve as a novel administration route for immunosuppressive vaccines. Skin permeation is non-invasive and suitable for long term and repeated administration, which is expected to reduce patient burden and improve safety. Future studies should focus on optimizing administration parameters and investigating how differences in antigen type or carrier affect immune responses, a crucial step toward future clinical translation. In this study, mice and rats were used. In transdermal permeability studies using iontophoresis with tramadol, no species differences were reported among pig, guinea pig, and mouse skin (Takasuga et al., 2011). However, since no data are available for human skin, future studies will be needed to evaluate the transdermal permeability of SSV-KLH using human skin.

Based on the results of this study, an immune response was observed following transdermal administration, suggesting that SSV-KLH was absorbed into the body in its conjugated form. KLH is widely used as a carrier protein in vaccine formulations and is known to provide structural stability to conjugated peptides (Kafi et al., 2008). However, the stability of SSV-KLH within the skin has not been evaluated in this study; therefore, further investigation will be necessary. Furthermore, iontophoresis may potentially induce skin damage as a result of the applied electrical current. Previous studies have reported that iontophoresis at moderate current densities does not cause significant structural damage to the skin (Alkilani et al., 2022; Yeasmin et al., 2022). In the present study, no visible signs of severe skin damage were observed during the experiments. Further histological evaluation will be considered in future studies.

5. Conclusion

This study demonstrated that skin permeation of SSV-KLH combined with IP tended to increase antibody production compared with patch application alone and exhibited immunosuppressive effects comparable to those of intraperitoneal injection, and exhibited immunosuppressive effects comparable to those of intraperitoneal injection. Differences in current intensity had only a limited impact on antibody production and immunosuppressive activity, indicating that sufficient immune responses can be induced with electrical stimulation within a certain range.

These findings support the feasibility of developing a non-invasive, sustainable, and clinical practical transdermal immunosuppressive vaccine delivery system and provide foundational data that could guide future clinical applications and dosing strategies.

In clinical settings, the optimal transdermal delivery method may vary depending on the physicochemical properties of the formulation, the required dose, the target patient population, and the anticipated clinical situation. In future studies, evaluating the transdermal permeability of SSV-KLH using alternative enhancement methods, such as microneedles, may enable the use of multiple transdermal delivery technologies and could be beneficial for expanding its potential clinical applications.

Conflicts of Interest

The authors declare that they have no known competing interests.

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