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Original Article

Transcutaneous delivery and thermostability of a dry trivalent inactivated influenza vaccine patch

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A patch containing a trivalent inactivated influenza vaccine (TIV) was prepared in a dried, stabilized formulation for transcutaneous delivery. When used in a guinea pig immunogenicity model, the dry patch was as effective as a wet TIV patch in inducing serum anti-influenza IgG antibodies. When the dry TIV patch was administered with LT as an adjuvant, a robust immune response was obtained that was comparable with or better than an injected TIV vaccine. When stored sealed in a nitrogen-purged foil, the dry TIV patch was stable for 12 months, as measured by HA content, under both refrigerated and room temperature conditions. Moreover, the immunological potency of the vaccine product was not affected by long-term storage. The dry TIV patch was also thermostable against three cycles of alternating low-to-high temperatures of −20/25 and −20/40°C, and under short-term temperature stress conditions. These studies indicate that the dry TIV patch product can tolerate unexpected environmental stresses that may be encountered during shipping and distribution. Because of its effectiveness in vaccine delivery and its superior thermostable characteristics, the dry TIV patch represents a major advance for needle-free influenza vaccination.

Keywords Influenza vaccine, thermostability, transcutaneous immunization, vaccine patch.

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Introduction

Transcutaneous immunization (TCI), the delivery of vaccine antigens to the skin, is most reliably performed using a patch applied on the skin.[1] Antigens must pass through the stratum corneum into the viable epidermis where there is an abundance of immune surveillance cells, known as Langerhans cells. These specialized cells detect antigens in the skin, take up and process the antigens, and migrate to the nearest draining lymph node via the lymphatic system. Upon arrival, the Langerhans cells present the processed antigens to the T and B cells, which in turn trigger a highly specific immune response to the vaccine antigen.[2–4] TCI induces systemic and mucosal immune responses to pathogen-derived antigens and confers robust cell-mediated immunity to the delivered antigen.[2–6]

We, and others, showed that a potent and robust immune response to TCI immunization can be obtained by delivering the vaccine antigen with an adjuvant.[7–9] Adjuvants are immunostimulating compounds that are often used to amplify the immune response to the vaccine antigens. TCI studies have shown that a potent adjuvant, such as heat-labile enterotoxin from *Escherichia coli* (LT), when administered in conjunction with vaccine antigens, induces functional antibodies to higher levels than the antigens alone.[10,11] In a recent human clinical trial, the transcutaneous LT patch, when used both as an antigen and adjuvant, was shown to induce long-lasting serum anti-LT IgG and IgA antibodies, anti-LT IgG and IgA antibody-secreting cells, as well as fecal stool IgA antibodies that mitigated the severity of enterotoxigenic *E. coli* illness.[12]

Most early preclinical and human TCI studies conducted in our laboratory used a 'wet patch' format. Typically, the wet patch approach entails pipetting liquid solutions of antigen and adjuvant onto a gauze patch that is placed over a pre-treated skin area, where the stratum corneum has been minimally disrupted with a mild abrasive device, such as adhesive tape, emery paper or pumice swab.[3,13] After application of the liquid antigen and adjuvant mixture, the wet patch is covered with a protective adhesive overlay and worn for several hours. The wet patch has been used to establish proof-of-principle that a specific vaccine antigen co-administered with an adjuvant can be delivered to the skin to induce a robust immune response.[6,7]

Our success in eliciting immune responses transcutaneously has prompted us to formulate a stabilized dry patch

for vaccine delivery. A dry patch is appealing in terms of its ease of use in a clinical setting (i.e. less manipulative steps required for administration). A vaccine antigen in a dry patch format is also expected to exhibit a better stability profile due to the significant reduction in physical and chemical degradation processes in a dried, solid-state product. Recently, we developed a formulation process to prepare vaccine antigens and adjuvants in dried solid-state patches. We have used this formulation process to prepare a trivalent inactivated influenza split virus vaccine (TIV) patch for transcutaneous delivery. The viral composition of the vaccine consisted of two representative type A viruses, A/New Caledonia/20/99 (H1N1) and A/Wyoming/3/2003 (H3N2), and one type B virus, B/Jiangsu/10/2003. The dried formulated TIV vaccine can be administered on pretreated skin with LT as an adjuvant. The LT can be administered as a separate dry patch in combination with the TIV patch or added to a TIV blend mixture to make the dry patch. The dry patch is then covered with an occlusive backing and an adhesive overlay and worn overnight.

The aim of this paper is to address the characteristics of the dry TIV patch with respect to: (1) vaccine delivery when compared with a wet TIV patch and with an injected TIV vaccine in guinea pigs; (2) stability under real-time and accelerated conditions including air versus nitrogen environments; and (3) tolerance to temperature excursions (thermal cycling) and short-term stress conditions that may be encountered during shipping and distribution.

Materials and methods

Concentrated TIV bulk blend

A concentrated TIV blend was prepared in phosphate-buffered saline (PBS) from the 2005 monovalent virus bulks obtained from Solvay Pharmaceuticals. TIV consists of A/Wyoming/3/2003 X-147 (H3N2), A/New Caledonia/20/99 (H1N1), and B/Jiangsu/10/2003), and was prepared at approximately 3 mg/ml of total hemagglutinin (HA) content, or 1 mg/ml of each strain.

TIV dry patch preparation and analysis

A proprietary customized bulk stabilizing (PCBS) solution was used both to dilute the concentrated TIV bulk blend and to prepare the final blend for patch manufacturing. All excipients were of reagent grade and are on the list of 'generally regarded as safe' (GRAS) excipients. The final formulation was prepared by using a combination of required volumes of PCBS, concentrated TIV and/or LT bulk. The dry patch was prepared by adsorbing the final formulated blend onto a non-woven polymeric matrix disc (3 cm^2), followed by drying under moderate condition (45°C) in a convection oven. Following the drying process, the dry patches were assembled between two occlusive release liners, sealed in nitrogen-purged foil pouches, and stored under different temperature conditions. Typically, each 3-cm^2 patch contained approximately 45 μg of total HA at the recommended ratio of 15 μg of HA of each viral strain, which corresponds to the intramuscular (i.m.) dose of commercial influenza vaccines. For animal immunogenicity studies, a smaller patch (1 cm^2) containing about 15 μg of total HA (i.e. 5 μg of HA of each virus strain) was pre-cut (punched-out) from the original 3-cm^2 dry patch by using a metal die tool.

To perform single radial immunodiffusion (SRID) analysis, HA was extracted from dry TIV patches with Dulbecco phosphate-buffered solution for 60 minutes on an orbital shaker at room temperature. Typically, three separate extracts were prepared at each stability time point and analyzed within 4 hours of extraction as described by Wood *et al.*[14] The average HA content is expressed in micrograms of viral strain HA per patch.

Stability studies

The dry TIV patches (in nitrogen- and air-filled pouches) were placed under real-time (2–8°C) and accelerated conditions (25 and 40°C) in controlled temperature–humidity chambers. Patches were pulled at monthly intervals over a 1-year period. Testing was performed on the sample patch extracts (prepared as described previously) using the SRID assay for HA content. The lower SRID specification requirement was set at 11.3 μg per patch for each virus strain, which represents 75% of the targeted HA dose (15 μg) per patch.

Thermal cycling studies were performed according to the 1998 FDA draft guideline for stability testing of packaged drug products[15,16] to gain insight into the tolerance (stability) of the dry TIV patch to temperature excursions that may be encountered during shipping and distribution. The product was cycled from low to high temperatures for several days, and the exposure was repeated in three cycles.[15,16] For each thermal cycle, six TIV patches were used, and the average HA content was reported.

The dry TIV patch was also exposed to short-term temperature stress conditions as suggested by the 2003 ICH guidance for stability testing of new drug substances and products.[17,18] The TIV patch product intended for room temperature storage conditions was subjected to two short-term temperature stress studies of 50°C for 2 days and 60°C for 2 days.

Guinea pig immunogenicity studies

Female Hartley guinea pigs were used as an evaluation model for immunogenicity of dry and wet TIV patches. All animals, including a control group, were sensitized to influenza antigens by i.m. injection of 0.5 μg of TIV (~0.17 μg of HA of each strain) 3 weeks prior to patch administration. Guinea pigs were shaved on the abdomen

and the shaved areas were pre-treated with a skin abrasion device to disrupt the stratum corneum. For the wet patch group, a 1-cm^2 rayon disc was placed over the pre-treated area and hydrated with a liquid TIV formulation with and without LT. For the dry patch group, dry 1-cm^2 TIV patches with and without LT were applied directly over the pre-treated area. To ensure proper patch adherence, patches were covered with a Tegaderm™ overlay. Patches were applied for 18 hours, removed, and the skin was rinsed with warm water. The control group of guinea pigs was immunized by bolus i.m. injection (right thigh muscle) of a liquid TIV vaccine composed of an equivalent HA dose. For all groups, serum samples were collected before patch administration and 2 weeks after immunization and evaluated for Ag-specific Ig (IgG) titers by an ELISA as previously described.[19] Antibody titers are reported as ELISA units (EU), which correspond to the inverse dilution of the serum that yielded an OD of 1.0 at 405 nm.

Additionally, serum samples were analyzed for neutralizing antibodies (Abs) using a hemagglutination inhibition (HAI) assay as previously described.[20] HAI titers are expressed as the highest serum dilution factor causing complete inhibition of HA.

Comparison of Ab titers among groups of animals immunized by TCI patches and i.m. injections was performed using *t*-test. Differences were considered as statistically significant, if $P < 0.05$.

Results

Delivery of TIV antigens: wet versus dry formulations

A TIV was delivered in a wet or dry patch and induced serum IgG antibodies against each of the three virus strains (Figure 1, top panel). Both patch formats consistently induced significant IgG titers when compared with pre-vaccination levels (typically 200–400 ELISA units, data not shown). The dry patches induced higher IgG titers than the wet patches, although the anti-influenza IgG responses were augmented when LT was co-administered as an adjuvant. Figure 1 (bottom panel) shows the HAI antibody titers for the same serum samples. All HAI titers measured prior to patch administration were below the limit of detection (<2.3 log 2). LT appears to augment the HAI antibody responses across the three virus strains in a manner similar to the ELISA titers.

Real-time and accelerated stability of dry TIV patches

The stability of a dry TIV patch lot at 5°C (real-time) and at 25 and 40°C (accelerated conditions) is shown graphically in Figure 2 with respect to HA content as measured by the SRID assay. For patch products stored at 5 and 25°C, the HA content trended above the specification requirement, thus indicating that the dry TIV patch is stable for 12 months. At the elevated temperature of 40°C, the HA potency for all three influenza strains met specification after 1-month storage; however, the loss of HA was significant thereafter, especially for B/Jiangsu and A/New Caledonia virus strains.

Effect of air versus nitrogen on stability

The impact of storing the pouched, dry TIV patch in air versus nitrogen is listed in Table 1. While patches were analyzed at regular time intervals, only the 12-month time point data at 25°C is presented. Under a nitrogen atmosphere, the HA content for all three strains met the specification. On the other hand, a significant loss in HA was observed for all three virus strains when the dry TIV patches were stored in air. The losses appeared to be greater for the B strain than for the two type A virus strains. It should be pointed out that the dry TIV patches, when stored at 2–8°C for 12 months, were not affected by air (data not shown); thus, the effect of air is observed only at the elevated temperature.

Thermal cycling study results

The thermal cycling studies were performed on a clinical TIV patch lot that had been previously stored for 6 months at 2–8°C. The thermal cycling test results (Figure 3) showed that the TIV patches passed the lower SRID specification of 11.3 μg per patch for an intended refrigerated product for three thermal cycles of −20 to 25°C as well as three thermal cycles of −20 to 40°C intended for a room temperature storage product. The thermocycling data indicated that the TIV patch could tolerate subfreezing and elevated temperatures that may be encountered during shipping, handling and distribution.

Short-term temperature stress results

Two short-term temperature stress studies were performed. One study involved the exposure of TIV patches, which had been through one −20°C/25°C cycle, to 50°C for 2 days. The second study involved the exposure of TIV patches, which had been through one −20°C/40°C cycle, to 60°C for 2 days. As indicated in Table 2, the mean HA content of TIV patches ($n = 3$) for both studies passed the lower specification limit (≥11.3 μg per patch). However, a significant trend in HA degradation for A/New Caledonia and B/Jiangsu was evident at 60°C when compared with 50°C exposure. Of the three virus strains, the A/Wyoming strain appeared to be the most thermostable as little, if any, loss in HA content was observed.

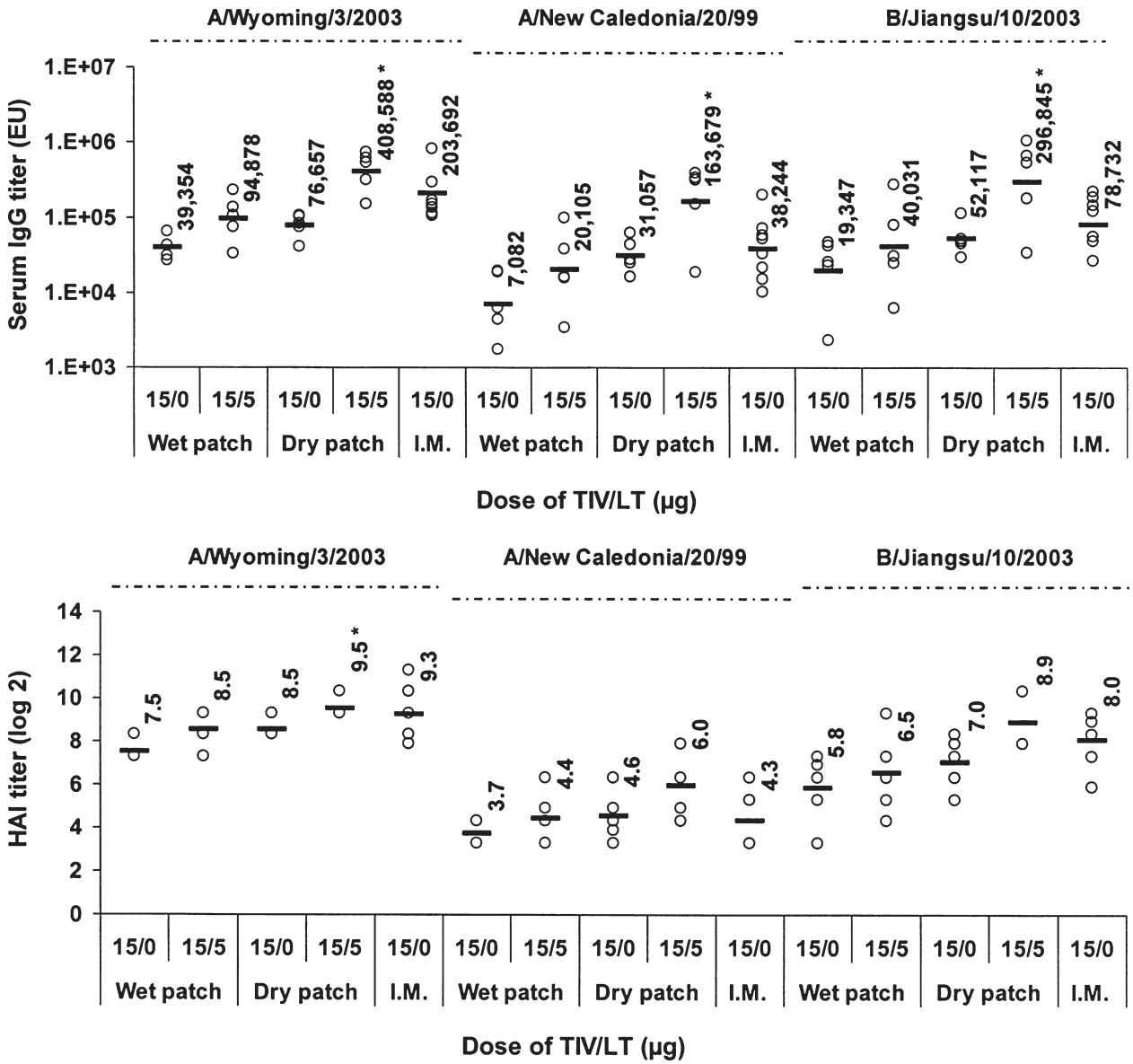

Figure 1. Transcutaneous immunization delivery of TIV antigens via wet and dry formulated patches compared with i.m. injection. Top panel: serum IgG titers determined by an ELISA. Bottom panel: HAI titers. Individual titers are represented by open circles. The geometric mean for each viral strain is represented by a horizontal bar. Numerical values are geometric means of each group. Guinea pigs (five animals per group) were immunized with freshly prepared wet and dry TIV patches containing 15 μg of HA (5 μg of HA of each strain) with and without 5 μg of LT. A control group of guinea pigs was immunized i.m with an injectable TIV vaccine (15 μg of HA). Dry TIV patches containing LT induced higher IgG response than in groups received dry TIV patches without LT (* indicates statistically significant difference). In addition, immunization with the dry TIV/LT patch provided consistently higher geomean values compared with those observed from i.m. injection, but the difference was not statistically significant.

Aged TIV patch in guinea pig immunogenicity study

The effect of long-term storage of the patch at 2–8 and 25°C was evaluated in a guinea pig immunogenicity study. Figure 4 shows the ELISA IgG (top panel) and HAI titers (bottom panel), respectively, for guinea pigs immunized with, 12-month-old dry TIV patches (stored under nitrogen at 5 and 25°C) compared with a group immunized with freshly prepared wet TIV patches as well as a control group receiving an i.m. injection of TIV with the same HA dose. Only the IgG and HAI titers against A/Wyoming strain are shown for illustrative purposes. Similar trends were observed for the other two virus strains.

The aged TIV patches, stored at 5 and 25°C, induced comparable IgG and HAI titers and were as effective as the

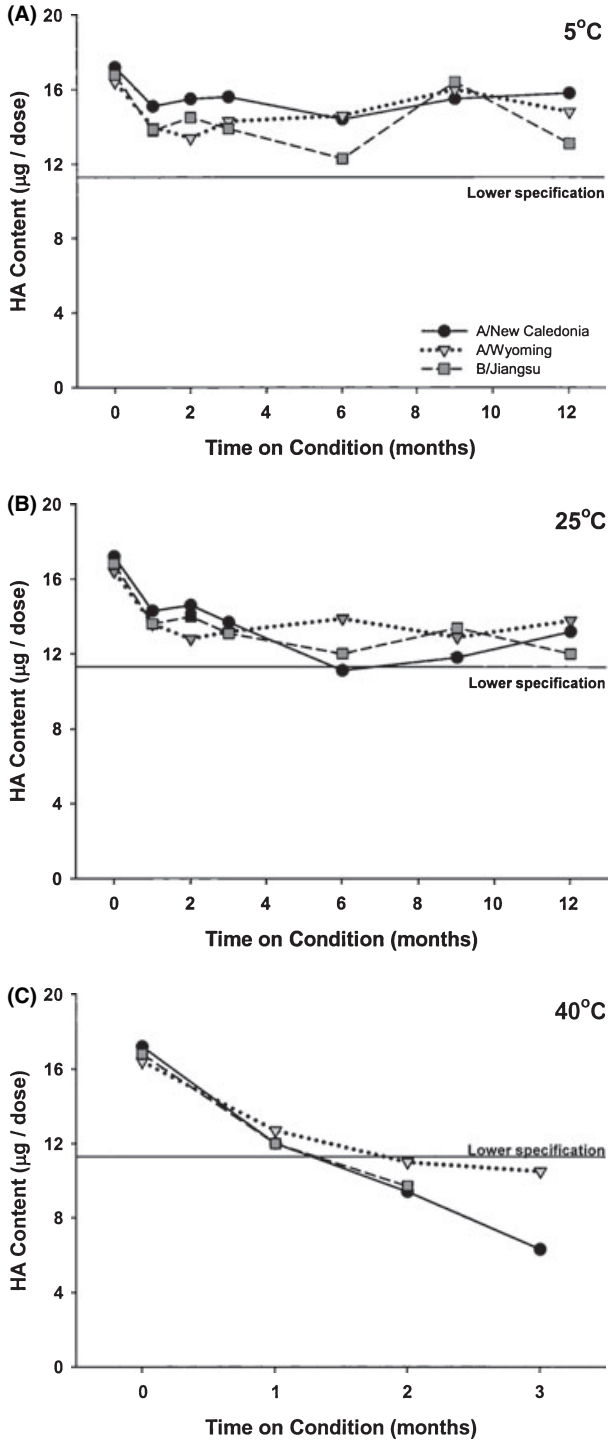

Figure 2. Stability of HA content in 45 μg TIV patches stored at 5°C (panel A), 25°C (panel B) and 40°C (panel C) for the indicated times. HA was extracted from the dry patches prior to analysis. The HA content for each viral strain is reported relative to the respective NIBSC reference virus standards used in the assay. The A/New Caledonia strain is represented by the —●— line, the A/Wyoming strain by ···▼···, and B/Jiangsu by —■—.

Table 1. Mean hemagglutinin content (μg/patch; n = 3) of dry TIV patches following 12 months of exposure to atmospheric air versus nitrogen at 25°C

Stress conditions	Nitrogen	Atmospheric air
A/New Caledonia	13.2	8.5
A/Wyoming	13.8	9.5
B/Jiangsu	12.0	4.5

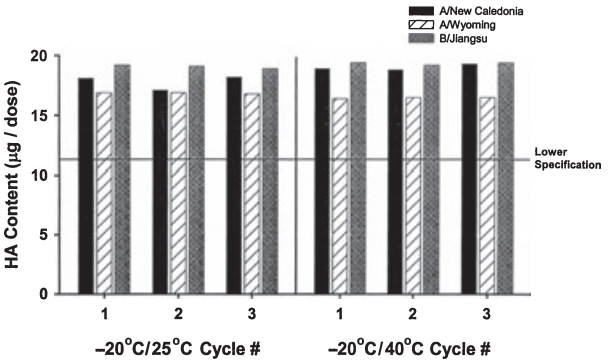

Figure 3. Thermal cycling studies for 45 μg TIV patches after three thermal cycles from −20 to 25°C (left panel), and after three thermal cycles from −20 to 40°C (right panel). The TIV patches were stored at 2–8°C for 6 months prior to their use for the thermal cycling studies. The HA content for each viral strain is reported relative to the respective NIBSC reference virus standards used in the assay.

Table 2. Hemagglutinin content (μg/patch; n = 3) of dry TIV patches following short-term temperature exposure

Stress conditions	50°C for 2 days (study 1) after 1 cycle of −20°C/25°C	60°C for 2 days (study 2) after 1 cycle of −20°C/40°C
A/New Caledonia	16.3 (15.3–17.4)*	12.5 (11.4–13.6)
A/Wyoming	16.3 (15.6–17.0)	16.1 (15.1–17.1)
B/Jiangsu	14.6 (13.4–15.8)	11.9 (8.1–15.7)†

*The values in parenthesis represent the lower and upper 95% confidence limits.
†The stain intensity of precipitation rings was significantly diminished.

freshly prepared wet TIV patches. Thus, even after long-term aging for 12 months at 25°C, the immunological potency of the dry TIV patches was preserved. Additionally, an enhanced immune response was obtained when LT was co-administered with the dry and wet TIV patches. The

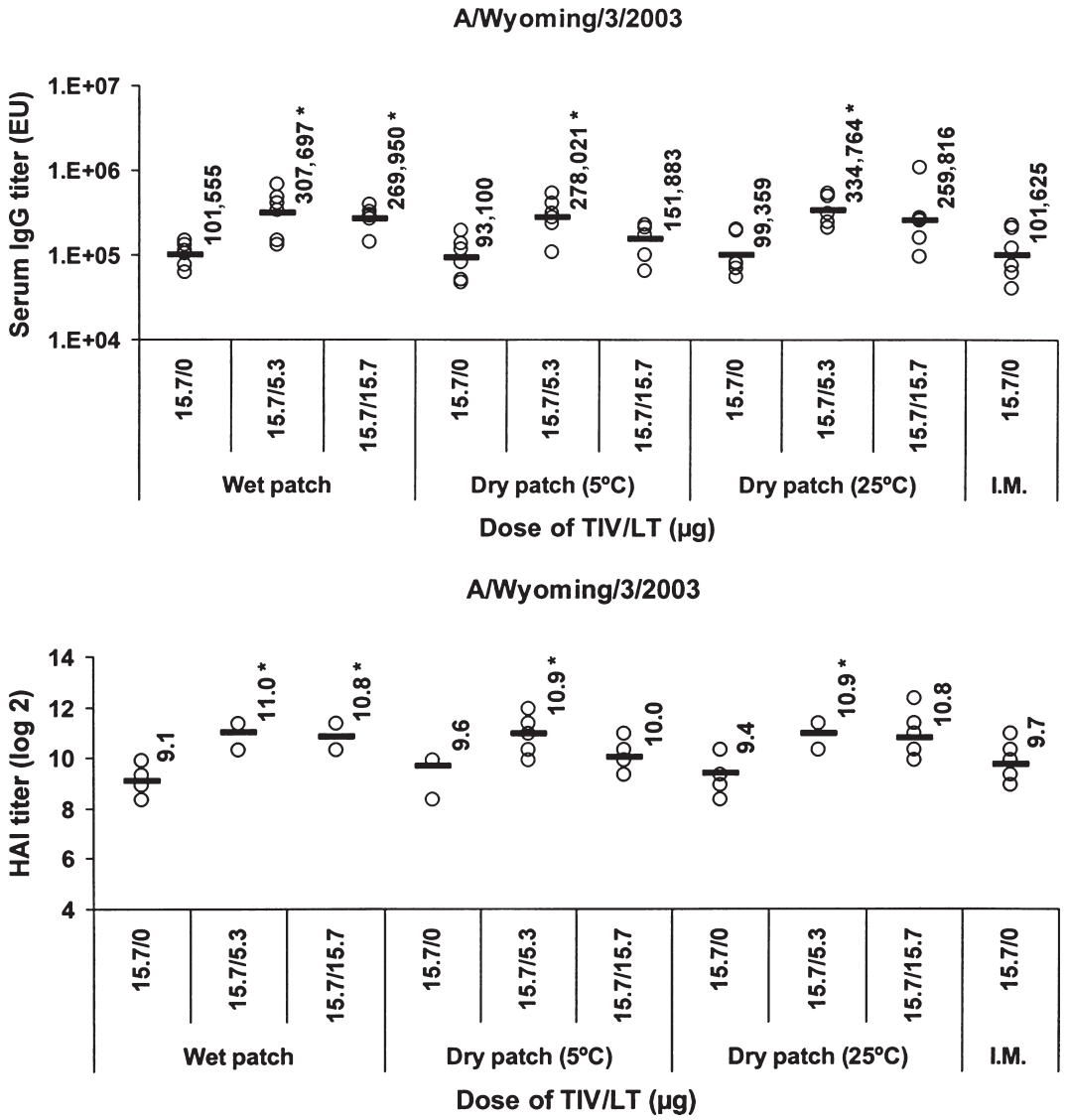

Figure 4. The effect of 5 and 25°C aging of TIV patches on immunological potency in the guinea pig model. Three groups of guinea pigs, six animals each, were immunized with 12-month-old dry TIV patches (stored at 5 and 25°C) containing 15.7 μg HA (total) with and without LT at 0, 5.3 or 15.7 μg. Three other groups were immunized with freshly prepared wet TIV patches with equivalent HA and LT doses. A group of guinea pigs immunized by the i.m. route with an injectable TIV vaccine (15.7 μg of HA) served as a control. Only serum IgG titers (top panel) and HAI titers (bottom panel) to A/Wyoming are shown. Individual titers are represented by open circles. The geometric mean for each viral strain is represented by a horizontal bar and shown numerically. IgG and HAI titers induced by wet patches and aged dry TIV/LT patches were consistently higher than the titers observed after i.m. injection (* indicates statistically significant difference between the i.m. group and all other groups).

augmentation due to LT is consistent with previous TCIs. The ELISA IgG and HAI titers achieved with the adjuvanted TIV patches were comparable with or higher than those achieved with the i.m. injected control.

Discussion

In earlier transcutaneous studies, we used wet patches to demonstrate that the skin epidermis is an accessible and competent immune environment suitable for vaccine delivery. As biological vaccine products can undergo physical (e.g. denaturation and aggregation) and chemical (e.g. deamidation, oxidation and hydrolysis) changes during storage in aqueous solutions, and the rates of these degradation pathways can be unacceptably rapid, a TCI delivery system involving liquid dosing of products onto patches is not practical in terms of long-term stability, storage and handling. Thus, a dry and stable patch product is appealing with respect to ease-of-use, safety and patient compliance.

Towards this goal, our laboratory formulated a trivalent liquid vaccine blend consisting of the 2005/2006 monovalent inactivated influenza bulks with a proprietary stabilizing solution, and dried the resulting mixture on a matrix disc. Using mild evaporative drying conditions, we converted the TIV patch formulation into a hydroscopic matrix with low moisture content. We maintained the patch dryness by storing the dry TIV patch in a sealed, nitrogen-purged foil pouch.

Hartley guinea pigs provide an attractive animal model for studying transcutaneous antigen delivery due to their skin properties (thickness, concentration of Langerhans cells, etc.).[3] However, naïve animals demonstrate a poor response to influenza antigens and low frequency of seroconversion as determined by HAI assay, even at high doses of TIV delivered by a single i.m. injection (15 μg of total HA) (data not shown). We have found that by priming the animal with 0·5 μg of TIV, the sensitized guinea pigs generate robust immune responses following a boost with either a TCI patch or an i.m. injection. Additionally, this sensitizing procedure may be relevant to the fact that most humans have been exposed to currently circulating influenza strains either by prior vaccination or infection.

The dry patch was as effective as or better than the wet patch in delivering TIV antigens to stimulate serum anti-influenza IgG antibodies in the guinea pig immunogenicity model. Moreover, an augmentation in the antibody levels was obtained when the LT adjuvant was also used in the patch administration, which is consistent with our previous findings.[3,4,7,8,11] With respect to the dry adjuvanted TIV patch, a more robust immune response was obtained than with the standard i.m. injected TIV vaccine.

The animal immunogenicity data confirm that transcutaneous delivery of high molecular weight, complex particulate antigens, such as trivalent split-inactivated influenza vaccine used in this study is feasible. We hypothesize that the effective delivery of antigens from the dry patch into the epidermis is facilitated by several factors. First, the dried solid state of the patch formulation is very hydroscopic and readily takes up moisture. Second, effective solubilization of dry antigens occurs in the patch due to increased transepidermal water loss caused by skin pretreatment (i.e. mild disruption of the stratum corneum). Third, the solubilized antigens in the patch are highly concentrated, which provides a strong chemical gradient for passive diffusion of the vaccine antigens from the patch into the skin.

Because the patch formulation was prepared in a dry, solid state, one would expect a stability profile comparable with those obtained by lyophilization[21] and spray-drying.[22] Our expectation of a stable, dry TIV patch vaccine was confirmed. There was no significant deterioration of HA content throughout 12-month storage at 2–8 and 25°C. The immunological potency, as tested in the guinea pig model, was not compromised by 12-month storage at these two temperatures. More importantly, the dry TIV patch administered with LT induced an anti-influenza immune response equal to or even higher than that obtained with a traditional, intramuscularly injected TIV vaccine.

The thermostability of the dry TIV patch was further confirmed by thermal cycling studies between −20/25 and −20/40°C in which little, if any, decrease in HA content was observed. The thermostability was maintained under room temperature conditions as long as the patch was stored in a nitrogen-purged pouch. When stored under atmospheric air conditions, the patch HA content fell below specification following 12-month storage at 25°C. Air, however, had little impact on product stored in a refrigerator.

The dry TIV patch tolerated a 2-day stress exposure at 50°C, but, when exposed to 60°C for 2 days, a significant trend in HA degradation of B/Jiangsu was evident, indicating that, for this strain, critical transition temperature of HA denaturation occurs between 50 and 60°C. The HA antigens of the other strains, A/New Caledonia (H1N1) and A/Wyoming (H2N3), appeared more stable, suggesting that their critical transitional temperatures must be greater than 60°C.

Our short-term temperature stress data are consistent with observations made by others[23,24] who have monitored structural conformational changes of HA in monovalent vaccine bulks of influenza virus strains. In one study using a combination of fluorescence and circular dichroism spectroscopy, investigators found that 60°C incubation of a monovalent B/Guangdong/120/2000 strain resulted in denaturation of the HA protein[24]. In addition, their study indicated that a dramatic change in the fluorescence spectrum of the B-type virus was due to the (air) oxidation of trypthophan within the HA protein molecule. Another laboratory,[23] using differential scanning calorimetry (DSC) to analyze purified influenza viruses, showed major and minor DSC thermal transitions corresponding to unfolding of HA and other influenza viral proteins respectively. The major HA unfolding transitions occurred at 61.5, 62.3 and 68.9°C, respectively, for the A/Panama (H3N2), B/Shangdong and A/New Caledonia (H1N1) viruses; while the minor unfolding temperatures occurred at 55.6 and 62.5°C, respectively, for B/Shangdong and A/New Caledonia (H1N1). Their physiochemical findings and our short-term temperature stress data are consistent in showing that the B-type influenza virus is less stable than the A-type strains, and that the critical transition temperatures for influenza viruses could range from 55 to 70°C.

In summary, the dry TIV patch formulation is a promising option for vaccine delivery by the transcutaneous route. In animals, the dry patches were as effective

as wet patches in delivering TIV antigens to induce anti-influenza antibodies. When administered with LT, the dry TIV patch could induce antibody titers at levels higher than those obtained with an injected influenza vaccine. When sealed in a nitrogen-purged foil pouch, the dry TIV patch exhibited 12-month stability in a refrigerator or at room temperature. In comparison, a shelf life of 1 year in the refrigerator is typically claimed for commercial, liquid influenza vaccine formulations. The dry TIV patch product is thermostable, and can withstand unexpected environmental stresses that may be encountered during shipping and distribution of the product. In addition to effective vaccine delivery and superior thermostable characteristics, other inherent advantages offered by the dry TIV patch include: (1) effective flu vaccination without the use of needles; (2) increased patient compliance; and (3) ease of administration and disposal.

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Conflicts of interest

All authors' work was performed as full-time employees on behalf of Iomai Corporation. All authors hold shares of Iomai common stock and/or options to purchase shares of Iomai common stock.

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