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Fu-Shi Quan, Kyung Hee University
Yeu-Chun Kim, Korea Adv Inst Sci & Technol
Jae-Min Song, Sungshin Women's University
Hye Suk Hwang, Georgia State University
Richard W Compans, Emory University
Mark R. Prausnitz, Emory University
Sang-Moo Kang, Georgia State University

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Fu-Shi Quan, a Yeu-Chun Kim, b Jae-Min Song, c Hye Suk Hwang, d Richard W. Compans, e Mark R. Prausnitz, e Sang-Moo Kang f

Department of Medical Zoology, Kyung Hee University School of Medicine, Seoul, South Korea; Department of Chemical and Biomolecular Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, South Korea; Department of Global Medical Science, Sungshin Women’s University, Seoul, South Korea; Department of Microbiology and Immunology and Emory Vaccine Center, Emory University School of Medicine, Atlanta, Georgia, USA; School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, Georgia, USA; Center for Inflammation, Immunity and Infection, and Department of Biology, Georgia State University, Atlanta, Georgia, USA.

Skin vaccination with influenza virus-like particles (VLPs) using microneedles has been shown to induce protection similar to or better than that induced by intramuscular immunization. In this study, we examined the long-term protective efficacy of influenza (H1N1 A/PR/8/34) VLPs after skin vaccination using microneedle patches coated with the vaccine. Microneedle vaccination of mice in the skin induced 100% protection against lethal challenge infection with influenza A/PR/8/34 virus 14 months after a single vaccine dose. Influenza virus-specific total IgG response and hemagglutination inhibition (HAI) titers were maintained at high levels for over 1 year after microneedle vaccination. Microneedle vaccination also induced substantial levels of lung IgG and IgA antibody responses, and antibody-secreting plasma cells from spleen and bone marrow, as well as conferring effective control of lung viral loads, resulting in complete protection 14 months after vaccination. These strong and long-lasting immune responses were enabled in part by stabilization of the vaccine by formulation with trehalose during microneedle patch fabrication. Administration of the stabilized vaccine using microneedles was especially effective at enabling strong recall responses measured 4 days after lethal virus challenge, including increased HAI and antibody-secreting cells in the spleen and reduced viral titer and inflammatory response in the lung. The results in this study indicate that skin vaccination with VLP vaccine using a microneedle patch provides long-term protection against influenza in mice.

Influenza is a serious respiratory disease spreading around the world, causing seasonal epidemics and recurrent outbreaks, resulting in more than 220,000 hospitalizations. Approximately 36,000 people die in the United States every year (1, 2). The experience with the 2009 H1N1 pandemic demonstrated that conventional vaccination showed a significant delay in controlling the new pandemic spread. Significant shortages and delays happened in the supply of the 2009 pandemic vaccine, due in part to lower growth in egg substrates compared to those observed with seasonal vaccines. New approaches are therefore needed to develop an effective influenza vaccine that can be rapidly produced on a large scale with low production costs.

Virus-like particles (VLPs) are noninfectious, thus requiring no exceptional biosafety containment, and can be manufactured rapidly. They present structurally native, immunologically relevant viral antigens. Influenza VLPs, as a promising vaccine candidate, have been shown to induce high neutralizing antibody titers and strong protective immunity (3–7). Influenza VLP vaccines were shown to be more immunogenic and to provide better protection than a commercial split vaccine in ferrets (8) or a soluble hemagglutinin (HA) protein vaccine (9), indicating the possibility that influenza VLPs could be a new vaccine platform (10, 11).

Skin is considered an important peripheral immune organ rich in potent immune-inducing cells, including Langerhans cells (LCs), dermal dendritic cells (DCs), and keratinocytes (12–15). Thus, vaccine delivery via skin has been suggested to be an attractive approach for vaccination, especially using a microneedle patch (16–23). Microneedles are micrometer-scale needles that can be coated with vaccine for simple, painless, and targeted delivery of the vaccine to the skin (24). It was also reported that microneedle vaccination induces protective immunity at a lower dose and provides vaccine dose-sparing effects (25). In addition, skin immunization with microneedles coated with influenza VLPs or inactivated viral vaccines in the presence of a stabilizer, trehalose, was shown to induce better protection than intramuscular immunization (19, 20, 26, 27). However, protective immunity longer than 6 months has received only limited attention after microneedle vaccination (28).

In this study, we determined the protective efficacy of influenza VLP vaccine delivered to the skin using coated microneedles. Microneedle vaccine effects after over a year of immunization were compared in formulations with and those without trehalose as a stabilizer. We found that stabilized microneedle vaccination in the skin provided improved efficacy of protection after 14 months of vaccination.
purified from allantoic fluids by using a discontinuous sucrose gradient (15%, 30%, and 60% layers) and ultracentrifugation (at 28,000 rpm for 60 min). The purified virus was inactivated by incubation overnight at 37°C and then incubated for 30 min at 56°C. Sera were serially diluted, mixed with 4 HA units (HAU) of influenza A/PR8 virus, and incubated for 30 min at room temperature prior to adding 0.5% chicken red blood cells. The reciprocal of the highest serum dilution preventing hemagglutination was scored as the HA titer.

**Lung viral titer and lung inflammatory cytokine assays.** Lung viral titers at day 4 postchallenge were determined by counting plaques formed on the MDCK cells as described previously (6). The whole-lung extracts prepared as homogenates using frosted glass slides were centrifuged at 1,000 rpm for 10 min to collect supernatants. The lung supernatants were frozen and kept at −70°C until used for immunoglobulin and virus titers and cytokine assays. Influenza cytokines gamma interferon (IFN-γ), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) in lungs and bronchoalveolar lavage fluid (BALF) collected at day 4 postchallenge were analyzed by the Ready-Set-Go cytokine kit (eBioscience) according to the manufacturer’s procedure as previously described (31, 32).

**Analysis of antibody-secreting cell response in vitro and antibody responses postchallenge.** Virus-specific antibody responses were determined from serum, lung, bone marrow, and spleen at day 4 postchallenge using ELISA (6, 20, 29). To determine antibody-producing cell responses in vitro, bone marrow and spleen cells were cultured in multiscreen 96-well filtration plates (Millipore) coated with inactivated A/PR8 viral antigen with RPMI medium for 2 and 4 days (5 × 10^5 cells per well), and levels of virus-specific antibodies secreted into the culture medium were determined (6, 20, 29).

**Histopathology.** Mice were challenged with A/PR8/34 virus after 6 months of immunization with 4 μg of influenza VLPs. For histological analysis of lung tissue, mice were anesthetized with isoflurane and exanguinated from the abdominal aorta. Lung samples were fixed in 10% neutral buffered formalin for 48 h, transferred to 70% ethanol, embedded in paraffin blocks, sectioned into a thickness of 5 μm, and stained with hematoxylin and eosin (H&E) as described previously (33, 34).

**RESULTS**

**High levels of long-lasting virus-specific antibodies were achieved after microneedle VLP vaccination.** Our previous studies demonstrated that trehalose-stabilized microneedle influenza vaccines were effective in inducing higher recall immune responses and enhanced immunity (20, 26, 30). In the present study, to determine long-term antibody responses after skin delivery of vaccines, groups of mice were immunized in the skin with influenza VLPs using coated microneedles with and without trehalose stabilizer. Levels of total IgG antibody titers specific to influenza virus (A/PR8) were determined in serum samples collected at 1 and 2 weeks and 1, 2, 3, 7, 10, and 13 months after a single vaccination and at month 14 after challenge (Fig. 1). At an early time point of 1 week, virus-specific IgG antibody levels were detected at very low levels (Fig. 1A). At week 2 after microneedle vaccination, IgG antibodies were detected at increased levels (Fig. 1A; **, P < 0.05). At month 1 after microneedle vaccination, both the trehalose-stabilized microneedle group (VT) and the trehalose-free microneedle group (V) showed high levels of virus-specific antibodies compared to that at week 2 (Fig. 1A; ***, P < 0.01) and then maintained high levels at months 2, 3, 7, and 10 and up to month 13 after microneedle vaccination (Fig. 1A and B). IgG titers in the VT group were significantly higher than those in the V group at all
time points except week 1 (*, P < 0.05). Interestingly, the trehalose-stabilized VT group showed a trend of inducing higher levels of IgG2a isotype antibody levels (see Fig. S1 in the supplemental material).

Groups of mice were challenged 14 months after microneedle vaccination. A significant increase in levels of virus-specific antibodies was observed at day 4 postchallenge in both stabilized (VT) and nonstabilized (V) microneedle groups over that prior to challenge (Fig. 1B; **, P < 0.01). In particular, we observed a greater increase in virus-specific antibody titters from the trehalose-stabilized microneedle group (VT) compared to that in the trehalose-free microneedle group (V) as well as higher levels of IgG2a isotype antibodies in the VT group at day 4 postchallenge (see Fig. S1 in the supplemental material). Taken together, stabilized microneedle immunization could induce effective memory B cells that differentiate rapidly into antibody-secreting cells upon exposure to viral infection. The results also suggest that microneedle immunizations in the skin with VLP vaccines (VT and V) were effective in inducing virus-specific antibodies for a long period of time.

Stabilized microneedle vaccine contributes significantly in enhancing recall HAI activity compared to trehalose-free microneedle vaccine. Titters of hemagglutination-inhibiting activity are used as a criterion in assessing vaccine efficacy. We determined HAI titters in serum samples collected at different time points after microneedle vaccination. At week 2 after microneedle vaccination, an increased HAI titer was observed (Fig. 2; *, P < 0.05) compared to week 1 and week 0. At 1 month after microneedle vaccination, an HAI titer of approximately 80 was observed and maintained up to 13 months (Fig. 2; *, P < 0.05). No significant differences were found between the trehalose-stabilized (VT) and nonstabilized (V) microneedle influenza VLP vaccines. However, when serum samples were collected at day 4 postchallenge, significant increases of HAI titters were detected in both trehalose-stabilized and nonstabilized groups (Fig. 2; *, P < 0.05, and **, P < 0.01). Interestingly, the trehalose-stabilized group showed much higher increases in HAI titer (Fig. 2; *, P < 0.05) than did the nonstabilized group. These results suggest that stabilization of microneedles coated with influenza vaccines using trehalose may be important in inducing a high level of recall HAI activities, which are functional and protective antibody responses to influenza virus.

Stabilized microneedle influenza VLP vaccine confers improved protection. To determine and compare the long-term protective efficacies after delivery of influenza VLP vaccines, groups of mice, including those microneedle treated with (VT) or without (V) trehalose, were challenged with a lethal dose of A/PR/8 virus (10 LD50) 14 months after a single microneedle vaccination. All mice in the mock-vaccinated negative-control group rapidly lost body weight and were euthanized when body weight loss was over 25%. Mice vaccinated with nonstabilized microneedle vaccine exhibited substantial body weight losses up to...
approximately 10%, whereas mice vaccinated with stabilized microneedle vaccine showed no body weight loss (Fig. 3A). Both groups of mice showed 100% survival protection (Fig. 3B).

To better assess the efficacy of vaccines, we determined viral titers in lungs at day 4 postchallenge. The mock control group showed the highest viral titers of $5 \times 10^5$ PFU. Virus titers were below the limit of detection in the mice vaccinated with stabilized microneedle vaccine (Fig. 4A). The nonstabilized microneedle vaccination group showed $2 \times 10^3$ PFU/ml (Fig. 4A; *, $P < 0.01$), which is 200-fold less than the mock-vaccinated control group. To determine whether microneedle vaccination would diminish an inflammatory response due to influenza viral replication in lungs, we measured levels of inflammatory cytokines, IFN-γ and IL-6, in lung extracts at day 4 postchallenge (Fig. 4B). IFN-γ was not detected in lung extracts of mice vaccinated with stabilized microneedle vaccine, while mice immunized with nonstabilized microneedle vaccine and the mock-vaccinated control group showed 200 and 500 pg/ml IFN-γ, respectively (Fig. 4B; *, $P < 0.01$). IL-6 levels showed a similar patterns of IFN-γ (Fig. 4C). These results indicate that stabilized microneedle influenza VLP vaccination was more effective in inducing protection and in controlling lung viral replication than nonstabilized microneedle VLP vaccination.

Levels of lung IgG antibodies were detected at higher levels than IgA antibodies (Fig. 5A and B). In particular, the stabilized microneedle group showed significantly higher levels of virus-specific lung IgG and IgA antibodies than those in the nonstabilized microneedle group (Fig. 5; *, $P < 0.01$). Thus, these results further indicate that trehalose-mediated stabilization of microneedle influenza VLP vaccines is important for improving long-term protective immunity.

**Microneedle vaccination induces long-lived antibody-secreting cell responses.** In general, vaccine antigen-specific cells such as memory B cells and plasma cells are present at low levels at the memory state after vaccination. Therefore, we determined recall antibody-secreting cell responses shortly after challenge. Spleen and bone marrow cells were harvested at day 4 postchallenge, which was a time point of 14 months after microneedle vaccination. The stabilized microneedle vaccination elicited higher levels of recall antibody-secreting cells from spleens after 2 and 4 days of *in vitro* cultures in the presence of inactivated A/PR/8 viral antigen than did nonstabilized microneedle or placebo vaccination (Fig. 6A; $P < 0.05$). Similar levels of antibody-secreting cell responses were detected from bone marrow cell cultures in both microneedle vaccine groups (Fig. 6B). These results indicate that microneedle vaccination in the skin induces long-lived cells capable of producing antibodies specific to virus.

**FIG 3** Body weight changes and survival. At month 14 after microneedle vaccination, mice were challenged with a lethal dose (A/PR8 virus, 10 LD$_{50}$) and were monitored daily to record body weight changes (A) and survival (B). V, microneedle vaccine without trehalose; VT, microneedle vaccine with trehalose; Mock, microneedles with coating buffer only. Data show averages ± standard errors of the means from 6 mice out of 12 mice per group.

**FIG 4** Lung virus titers and lung IFN-γ and IL-6 responses. Lungs from individual mice in each group were collected on day 4 postchallenge, and lung virus titers (PFU) and lung IFN-γ and IL-6 responses were determined in the lung extracts at day 4 postchallenge. V, microneedle vaccine without trehalose; VT, microneedle vaccine with trehalose; Mock, microneedles with coating buffer only; Naive, normal mice. Either no virus or lower virus titers were detected in VT and V groups (*, $P < 0.01$). No inflammatory cytokines IFN-γ and IL-6 were determined in the VT group compared to the V or mock control group (*, $P < 0.01$). Data show averages ± standard errors of the means from 6 mice.
DISCUSSION

Our previous studies showed that skin vaccination with influenza VLP-coated microneedles provided higher short-term efficacy of protection than did intramuscular immunization. Thus, the capability of inducing long-term protective immunity by vaccination using VLP-coated microneedles would be an important added strength of this approach. In this study, we focused on long-term protective immunity after single microneedle vaccination with influenza VLPs in the presence and absence of a trehalose stabilizer. Results indicate that complete virus clearance was found 14 months after stabilized microneedle vaccination without any body weight loss.

Immune parameters such as IgG response, HAI titer, lung virus titer, lung IgG or IgA response, lung inflammatory cytokine IFN-γ response, cellular response or memory response, body weight change, and survival rate are informative in assessing the influenza vaccine-induced efficacy. All these immune parameters are likely contributing to clearing lung viral loads and protection against lethal influenza virus challenge. Influenza vaccines were administered to mice by subcutaneous or intranasal routes, and various immune responses were observed. For example, significant increases in IgG and IgA responses were observed in mice vaccinated with influenza VLPs in the presence of trehalose. These responses were higher than those observed in mice vaccinated with influenza VLPs alone or in the absence of trehalose.

Immune responses to viral infection. The levels of inflammatory cytokines in lung extracts were highest in unvaccinated naive mice that were infected with A/PR8 virus (see Fig. S2) in contrast to those in the VLP-vaccinated and A/PR8 virus-infected mice (vaccine plus virus; see Fig. S2). Peripheral bronchioles of lung sections after H&E staining showed necrotizing bronchiolitis, and alveolar space contained infiltrates of mixed inflammatory cells after A/PR8 virus infection (see Fig. S3). There was a correlation between levels of IFN-γ in lung homogenates and infiltrates in H&E staining of lung histology (see Fig. S3).

In particular, clearance of virus in lungs is considered to be an important and sensitive barometer for evaluating vaccine efficacy. Different types of microneedle influenza vaccines and efficacies have been demonstrated. Substantial amounts of viral loads with a range of $10^4$ to $10^5$ PFU titers were detected in mice that were immunized on the skin using microneedles coated with 0.4 to 2 μg H5 HA VLPs derived from influenza A/Vietnam/1203/04 virus (21, 35). Also, $10^2$ to $10^3$ PFU lung viral titers and weight losses of 5 to 10% were observed in mice immunized with microneedle vaccines coated with 3 μg of split A/Brisbane/59/2007 vaccine, which are approximately 10- to 50-fold less than the infected naive control group (36). A low level of lung viral titers ($10^2$-PFU titers) was reported in mice after 5 weeks of microneedle vaccination with 0.4 μg inactivated A/PR/8/34 H1N1 virus (20). In summary, previous studies demonstrated a certain level of lung virus titers and body weight loss. Also, microneedle vaccine-induced efficacies in mice are different depending on influenza virus strains,
vaccine doses, prime-boost immunizations, and timing of postimmunization at the time of challenge. In this study, immunization with a high dose (4 μg) of microneedle vaccines induced high levels of IgG antibody responses (before challenge) regardless of trehalose stabilization, which is consistent with an early study of microneedle vaccination without trehalose (23). Dosage effects and higher immunogenicity of microneedle VLP vaccines were previously demonstrated when a low dose of 0.4 μg of vaccines was used to immunize mice (20, 25, 26). Nevertheless, we observed that stabilized microneedle vaccination with 4 μg HA VLPs (derived from A/PR/8/34) was able to provide lung virus clearance and prevent weight loss in lethal challenge infection for a long period of 14 months.

Influenza hemagglutinin (HA) stability is likely to play an important role in providing long-term protective immunity. Our previous studies demonstrated that inclusion of 15% trehalose in the microneedle coating formulation resulted in retaining the HA activity of VLPs over 60% after a 1-day drying process in contrast to less than 10% HA activity of VLPs from coated microneedles without trehalose (26, 30). HA titers were maintained at similar levels for 13 months after vaccination between stabilized and nonstabilized microneedle vaccination groups. Interestingly, HA titers were found to increase rapidly in the stabilized microneedle vaccine group upon challenge. Differences in host protective immune responses of two microneedle vaccine groups were found to be more evident after challenge. The experimental data for improved protection include no detectable lung viral titers, no loss in body weight, and higher levels of protective host responses such as significant increases in HA titers and vaccine antigen-specific recall antibody-secreting cell responses. We have shown that IgG2a isotype antibody and Th1 dominant immune responses were due to increased stability of the HA in the trehalose-stabilized microneedle vaccination (20). There was no significant difference in bone marrow antibody-secreting cell responses between stabilized VT and nonstabilized V groups. However, higher levels of splenic B cells secreting antibodies were detected in the stabilized microneedle vaccination at an early time point after challenge, which was also reflected by enhanced serum total IgG antibodies (Fig. 1) and IgG2a isotype antibody levels (see Fig. S1 in the supplemental material). Stabilized microneedle vaccination was highly effective in inducing improved protection. Therefore, maintaining influenza virus HA antigen stability in vaccines is important in providing long-term protective immunity.

The present study implies an important aspect in evaluating the efficacy of experimental vaccines. Immunogenicity itself may not reflect a good correlation with protective efficacy. At month 13 after vaccination and at an early time point of challenge, levels of binding antibodies did not show obvious differences between the two groups of microneedle-vaccinated mice. Even the HA titers, a measure of functional antibodies for predicting protection against influenza virus, did not show a significant difference at 13 months after vaccination. However, protective efficacy was significantly improved in the stabilized microneedle group after challenge (Fig. 3). It is likely that applying influenza vaccines as a coating onto microneedles in the absence of stabilizer might have resulted in exposing nonneutralizing domains of influenza virus HA proteins in a denatured conformation. These conformational changes are inferred from a result of decreased hemagglutination activity of coated microneedle vaccines (25, 27). This might explain similar levels of binding antibodies in both stabilized and nonstabilized groups. An insignificant difference in qualitative antibody levels was rapidly expanded upon infectious virus challenge, as shown by a significant increase in HAI titers. Therefore, developing an appropriate challenge-protection animal model should be an essential feature of evaluating the vaccine efficacy.

Another important arm of host immunity to pathogens is T cell immune responses. Although we have not investigated T cell immunity in this study, there is a possibility that T cell immune responses induced by stabilized microneedle vaccination might have contributed to improved protection observed. In previous studies, we observed enhanced T cell responses after stabilized microneedle vaccination compared to intramuscular immunization or nonstabilized microneedle vaccines (20, 26). Also, intradermal vaccination in the elderly population was reported to be more effective in inducing protective immune responses, including CD4 or CD8 T cell responses (37–42). As a future direction, it is important to study long-term T cell immune responses after microneedle skin vaccination.

Overall, the literature suggests that microneedle skin vaccination can offer important advantages. Influenza VLP microneedle vaccination generated dose-sparing effects and robust HAI titers (20, 26, 43). Microneedle skin delivery of inactivated influenza vaccine induced better control of viral replication and reduced inflammatory responses in the lungs (43). In addition, skin microneedle vaccination provides important logistic advantages. Microneedle skin vaccination can probably be administered by patients themselves, significantly increasing the coverage of vaccination. It can also eliminate or reduce the risk of needle-associated injury and reuse of needles and syringes (43). These advantages from both immunologic and logistic benefits, combined with long-term protective immunity as presented in this study, indicate that microneedle delivery to the skin may offer a strategy for improved influenza vaccination.

In conclusion, this study demonstrated that highly effective long-term immunity to influenza was induced by skin vaccination using microneedles coated with VLPs. Stabilized microneedle VLP vaccine conferred rapid increases in functional antibody responses and effective viral clearance resulting in improved long-term protection after lethal challenge. Thus, microneedle delivery to the skin using nonreplicating influenza VLPs has the potential to become an effective vaccine for inducing long-term protective immunity.

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