Microneedle Vaccination with Stabilized Recombinant Influenza Virus Hemagglutinin Induces Improved Protective Immunity

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The emergence of the swine-origin 2009 influenza pandemic illustrates the need for improved vaccine production and delivery strategies. Skin-based immunization represents an attractive alternative to traditional hypodermic needle vaccination routes. Microneedles (MNs) can deliver vaccine to the epidermis and dermis, which are rich in antigen-presenting cells (APC) such as Langerhans cells and dermal dendritic cells. Previous studies using coated or dissolvable microneedles emphasized the use of inactivated influenza virus or virus-like particles as skin-based vaccines. However, most currently available influenza vaccines consist of solubilized viral protein antigens. Here we test the hypothesis that a recombinant subunit influenza vaccine can be delivered to the skin by coated microneedles and can induce protective immunity. We found that mice vaccinated via MN delivery with a stabilized recombinant trimeric soluble hemagglutinin (sHA) derived from A/Aichi/2/68 (H3) virus had significantly higher immune responses than did mice vaccinated with unmodified sHA. These mice were fully protected against a lethal challenge with influenza virus. Analysis of postchallenge lung titers showed that MN-immunized mice had completely cleared the virus from their lungs, in contrast to mice given the same vaccine by a standard subcutaneous route. In addition, we observed a higher ratio of antigen-specific Th1 cells in trimeric sHA-vaccinated mice and a greater mucosal antibody response. Our data therefore demonstrate the improved efficacy of a skin-based recombinant subunit influenza vaccine and emphasize the advantage of this route of vaccination for a protein subunit vaccine.

The skin acts as a mechanical barrier against the environment and provides the first line of defense against pathogens. The skin-associated lymphoid tissue (SALT), which was first described by Streilein (34), represents an ideal target for skin-based vaccinations because it contains keratinocytes, Langerhans cells (LC), dermal dendritic cells (DDC), and T cells. Skin-associated antigen-presenting cells (APC) and keratinocytes have been shown to express several pattern recognition receptors (PRRs), including TLR9, TLR2, and TLR3 (19, 20), which are important enhancers of the immune response. The LC and DDC present in the epidermis and dermis, respectively, have been shown to take up antigen, migrate to draining lymph nodes, and induce an antigen-specific adaptive immune response (36). Therefore, targeting vaccine to the skin has been shown to enhance immunogenicity (2, 3, 15, 21).

The 2009 swine-origin influenza pandemic illustrates the need for rapid and effective vaccination. Skin-based influenza vaccines have utilized approaches including tape stripping (32), epidermal powder immunization (4), and microneedles (MNs) (9). These strategies have used diverse antigens including virus-like particles (VLP) (25), inactivated influenza virus (24), and hemagglutinin (HA) DNA vaccines (1). However, a recombinant HA subunit vaccine, which has the advantage of rapid, high-yield production in an expression system with a high level of purity, has not been evaluated.

Microneedle arrays are designed to penetrate the stratum corneum, the outer layer of the skin, and deposit vaccine or drug into the epidermis and dermis. Using this approach, vaccine is applied as a coating to the surface of metal microneedles or encapsulated in a polymer. Delivery of soluble protein via coated microneedles suggests that antigen can be delivered quickly into the skin. Furthermore, this immunization method generated an antigen-specific antibody response that was superior to those induced by subcutaneous (s.c.) and intramuscular (i.m.) routes (17, 18, 35, 41).

We previously demonstrated that a modified form of soluble HA (sSHA) derived from the H3N2 influenza virus A/Net Relief/2/68 containing the GCN4pII trimerization repeat stabilized the trimeric structure of the HA protein (20). In the current study, we tested the hypothesis that DN delivery of the recombinant vaccine would induce levels of protective immune responses superior or at least equivalent to those induced by subcutaneous immunization. Specifically, we investigated the efficacy of skin delivery of stabilized trimeric influenza virus HA from the H3 virus A/Net Relief/2/68 via coated microneedles. In addition we determined whether the stabilized trimeric sSHA microneedle vaccination induces improved humoral and cellular responses compared with those induced by s.c. immunization. To compare the effects of immunization on postchallenge virus clearance, we determined virus lung titers after challenge infection. The work presented here illustrates the first analysis of trans-
dermal delivery of a recombinant influenza virus subunit H5 vaccine using microneedle technology.

MATERIALS AND METHODS

Recombinant trimeric soluble influenza virus hemagglutinin (sHA). The HA gene derived from the H3N2 influenza virus A/Aichi/2/68 was truncated, and the trimeric GCN4pI sequence from Saccharomyces cerevisiae, encoding the tri- merization motif, was fused to the C terminus and cloned into the recombinant baculovirus (rBV) pFastBac1 expression vector as previously described (37). rBVs carrying genes for the sHA and SHAGCN4pI proteins were generated, and recombinant proteins were expressed and purified as previously described (37). For purification, a His tag was added to the C terminus of each protein construct.

Microneedle fabrication and coating. Microneedles were fabricated from stainless steel sheets (Trinity Brand Industries, Georgia; SS304; 50 μm thick) by wet etching. Individual microneedles had a length of 750 μm and a width of 200 μm.

The coating solution was composed of 1% (wt/vol) carboxymethyl cellulose sodium salt (low viscosity, USP grade; Carbo-Mer, San Diego, CA), 0.5% (wt/vol) Lutrol F-68 NF (BASF, Mt. Olive, NJ), and soluble HA protein at 5 mg/ml. In order to reduce protein coating concentration in coating buffer, sera were evaporated for 5 to 10 min at room temperature (+23°C) at the final step of preparation (Vacufuge; Eppendorf, New York). The coating step was performed by a dip coating process (12). The apparatus had a chamber with coating solution and a microneedle holder which was attached to a linear stage that allowed the microneedle array to move in two dimensions with 0.4-μm accuracy. The coating was performed automatically and was monitored by a video camera (Prosilica, Massachusetts) attached to a computer.

To measure the amount of vaccine applied as a coating per row of microneedles, three rows out of each batch of coated microneedles were each submerged into 200 μl of phosphate-buffered saline (PBS) buffer for 5 min. The concentration of protein in the solution was measured by bicinchoninic acid (BCA) protein assay and was consistent within each batch (Pierce, Rockford, IL).

Bioreactor cross-linking. The oligomeric status of purified recombinant proteins was determined using the water-soluble BS3 (bis[sulfosuccinimidyl] suberate) cross-linker (Pierce, Rockford, IL). Cross-linking was performed as described by De Fillette et al. (8), with the following modifications. Briefly, 1 μg of recombinant protein was incubated at room temperature in the presence of BS3 (final concentration, 3 mM) for 30 min. Cross-linking was stopped by the addition of 1 M Tris-HCl, pH 8.0, to a final concentration of 50 mM. After cross-linking, proteins were separated on a 5 to 15% SDS-PAGE gel under reducing conditions (1% mercaptoethanol) and then blotted and analyzed by Western blotting using anti-six-His antibody and developed using ECL-Plus.

Vaccinations. Female BALB/c mice (6 to 8 weeks old) were anesthetized with a combination of 25% was used as the endpoint at which mice

HAI and microneutralization. Hemagglutination inhibition (HAI) tests were performed on vaccinated animal sera based on the WHO protocol (38). Briefly, sera were treated with receptor-destroying enzyme (Denka Seiken Co. Ltd., Tokyo, Japan) for 16 h at 37°C and then heat inactivated for 30 min at 56°C. Treated sera were diluted to a final concentration of 1:10 in PBS and incubated with packed chicken erythrocytes (RBC) for 1 h at 4°C to remove cryoglobulins. Treated sera were serially diluted and incubated with 4 HA units of A/Aichi/2/68 virus for 30 min at room temperature. An equal volume of 0.5% chicken RBC was added to each well and incubated for 30 min at room temperature. The HAI titer was read as the reciprocal of the highest dilution of serum that inhibited hemagglutination. Values were expressed as the geometric mean with a 95% confidence interval.

The microneutralization assay was performed as described by Rowe et al. (29) with modifications. Briefly, mouse sera were heat inactivated for 30 min at 56°C and serially diluted in virus diluent (DMEM plus 1% BSA) in a 96-well tissue culture plate. Virus (200 50% tissue culture infective doses [TCID₅₀]) was added to diluted serum in virus diluent supplemented with 2 μg/ml of tosylsulfonphenylalanilyl chloromethyl ketone (TPCK)-treated trypsin and incubated for 2 h at 37°C. Freshly trypsinized MDCK cells were added to all wells and incubated overnight at 37°C. Cells were then fixed in 80% acetone-PBS and washed 3 times with PBS-Tween (0.05% Tween 20). A 1:2,000 dilution of biotin-conjugated anti-influenza A virus nucleoprotein (clone A3) (Milpore, Billerica, MA) and streptavidin-HRP was added for the detection of infected cells. For each plate, the 50% specific signal value was calculated as follows: [(average of virus-only wells) − (average of cell-only well)]/2 + average of cell-only well. The 50% endpoint neutralization titer is reported as the last dilution to score below the 50% specific signal value.

Cellular immune responses. Single-cell suspensions were prepared from spleens of mice 14 days postvaccination or from those of naïve mice, by mincing the tissue through a 70-μm cell strainer (BD Falcon), followed by incubation in red blood cell lysis buffer (Sigma) to remove red blood cells. CD4⁺ T cells were purified by negative selection using BD IMag magnetic cell separation (BD Biosciences, San Jose, CA). Naïve splenocytes were treated with mitomycin C for 30 min and used as accessory cells after incubation with 20, 5, or 0 μg of vaccine in complete RPMI medium overnight at 37°C in a 5% CO₂ incubator. Purified CD4⁺ T cells were added to accessory cells at a ratio of 2:1 (responder/accessory cells) in complete RPMI medium supplemented with 30 U of recombinant human interleukin-2 (IL-2; BD Biosciences, San Jose, CA). In vitro antigen-specific stimulation was measured after incubation for 5 days by intracellular cytokine staining.

Antibodies and flow cytometry. Cells were washed with PBS-1% BSA buffer and surface stained with fluorescein-conjugated antibodies to CD4 and CD3, followed by intracellular staining of gamma interferon (IFN-γ) and IL-4. Antibodies were purchased from eBiosciences and BD Biosciences. For intracellular cytokine staining, cells were fixed and permeabilized using the BD Cytofix/ Cytoperm manufacturer’s protocol and reagents (BD Biosciences, San Jose, CA). The data were acquired on a BD LSR-II flow cytometer and analyzed with FlowJo Software (Tree Star, Inc.; v7.6.1).
induced approximately 3.7-fold-higher A/Aichi/2/68-specific form. In addition, 28 days later, boosting with sHA.GCN4pII enhanced immunogenicity of the HA when in its trimeric native than did mice boosted with sHA (approximately 2.6-fold-higher A/Aichi/2/68-specific serum IgG titers).

After priming, mice vaccinated with sHA.GCN4pII had approximately 2.6-fold-higher A/Aichi/2/68-specific humoral responses. To test the immunogenicity of recombinant soluble HA delivered to the skin by coated microneedles, female BALB/c mice (6 to 8 weeks old) were vaccinated with 3 μg of sHA or sHA.GCN4pII on day 0 and boosted on day 28. After priming, mice vaccinated with sHA.GCN4pII had approximately 2.6-fold-higher A/Aichi/2/68-specific serum IgG titers than did mice boosted with sHA (P = 0.0032), indicating the enhanced immunogenicity of the HA when in its trimeric native form. In addition, 28 days later, boosting with sHA.GCN4pII induced approximately 3.7-fold-higher A/Aichi/2/68-specific serum IgG titers than did boosting with sHA (P < 0.0001).

The influenza virus-specific IgG subtype profile in the serum of MN-vaccinated mice indicates that sHA.GCN4pII induced higher IgG1 (1.4-fold, P < 0.0001), IgG2a (2.2-fold, P = 0.0053), and IgG2b (2-fold, P = 0.0148) levels than did the use of sHA or uncoated microneedles (Fig. 3). No significant differences were found in the proportions of IgG1 to IgG2a/b between the groups, which indicates that the nature of the antigen did not influence the expression of the IgG subtype. There was no detectable antigen-specific IgG3 in either vaccinated group (data not shown). The serum immunoglobulin levels indicate that the transdermal delivery of recombinant trimeric sHA induces a robust IgG response and enhances the levels of IgG2a, IgG2b, and IgG1 subtypes over those in sHA-vaccinated mice.

sHA.GCN4pII induces improved HAI and neutralizing antibody responses. The HAI test is used to measure antibodies which bind to the HA receptor binding domain, blocking binding of HA to sialic acid receptors. In general, an HAI titer of ≥40 is correlated with vaccine-induced protection in humans (13). Therefore, serum from microneedle-vaccinated mice was tested for HAI titers. At 21 days after priming, no detectable HAI titer was observed in the vaccinated mice. However, after boosting, the sHA.GCN4pII-vaccinated mice had an HAI titer of 190, while sHA-vaccinated mice had no detectable HAI titer (P < 0.0001) (Fig. 4A). We previously observed similar enhancement of HAI titers when the stabilized trimeric HA was administered by subcutaneous immunization (37).

In order to measure the ability of serum antibodies to neutralize virus infectivity, we performed a microneutralization assay. sHA.GCN4pII-vaccinated mice had an approximately 70-fold-higher neutralization endpoint titer than did mice vaccinated with sHA (P < 0.0001) (Fig. 4B). Therefore, MN vaccination with the sHA.GCN4pII protein induced high levels of functional antibodies but similar immunization with the sHA protein did not induce such responses.

Microneedle vaccination with sHA.GCN4pII induces a robust antibody response at mucosal sites. Influenza virus is a respiratory pathogen that infects the airway epithelia. A strong...
mucosal antibody response has been associated with prevention of viral pathology in the upper respiratory tract of mice (27). Previous studies using intradermal or transdermal influenza vaccinations have investigated the induction of secretory IgA (sIgA) at mucosal sites (32). Therefore, to determine the induction of secretory IgA by microneedle vaccination with recombinant soluble HA, we measured sIgA in vaginal washes by ELISA. On day 28 after boosting, sHA.GCN4pII-vaccinated mice had approximately 6.7-fold-higher sIgA levels than did mice vaccinated with sHA (*P*/H11005 0.0105) (Fig. 5A). When serum IgA levels were measured, we found that the levels in the sHA.GCN4pII microneedle-vaccinated mice were approximately 3.3-fold higher than those in the sHA-vaccinated mice (*P* < 0.0001), correlating with the higher mucosal IgA levels that we observed (Fig. 5B).

It has been demonstrated previously that IgG antibodies have an important role in neutralizing viruses after infection has been established in addition to preventing lung pathology (27). Therefore, we measured the total virus-specific IgG in the lung homogenates of microneedle-vaccinated mice. Lung homogenates from mice vaccinated with sHA.GCN4pII had 1.4-fold-higher levels of virus-specific IgG than did mice vaccinated with sHA (*P* = 0.0002) (Fig. 5C). These data suggest that skin delivery by microneedle vaccination with the trimeric HA, sHA.GCN4pII, induces improved humoral mucosal immune responses compared to those induced by sHA.

**Microneedle vaccination with sHA.GCN4pII induces complete protection against challenge infection.** To determine the efficacy of microneedle vaccination with recombinant soluble HA, we challenged vaccinated mice intranasally with 5 LD$_{50}$ (lethal) or 0.1 LD$_{50}$ (sublethal) of mouse-adapted A/Aichi/2/68. Body weights and survival were monitored for 14 days postchallenge. Following lethal challenge, mice vaccinated with sHA.GCN4pII maintained their body weight for the duration of the challenge while mice vaccinated with sHA lost approximately 20% of their body weight (Fig. 6A). Microneedle vaccination with sHA.GCN4pII maintained their body weight for the duration of the challenge while mice vaccinated with sHA lost approximately 20% of their body weight (Fig. 6A). Microneedle vaccination with sHA.GCN4pII maintained their body weight for the duration of the challenge while mice vaccinated with sHA lost approximately 20% of their body weight (Fig. 6A). Microneedle vaccination with sHA.GCN4pII maintained their body weight for the duration of the challenge while mice vaccinated with sHA lost approximately 20% of their body weight (Fig. 6A). Microneedle vaccination with sHA.GCN4pII maintained their body weight for the duration of the challenge while mice vaccinated with sHA lost approximately 20% of their body weight (Fig. 6A). Microneedle vaccination with sHA.GCN4pII maintained their body weight for the duration of the challenge while mice vaccinated with sHA lost approximately 20% of their body weight (Fig. 6A). Microneedle vaccination with sHA.GCN4pII maintained their body weight for the duration of the challenge while mice vaccinated with sHA lost approximately 20% of their body weight (Fig. 6A).
Microneedle vaccination induces improved viral clearance after lethal challenge. As a sensitive approach to determine the ability of vaccinated mice to reduce viral replication following lethal challenge, we examined the viral lung titers 4 days postchallenge. Mice vaccinated with sHA.GCN4pII-coated microneedles had no detectable viral lung titers at 4 days postchallenge, representing a reduction of over 10^6-fold compared with the unimmunized group (P < 0.001). In contrast, mice vaccinated subcutaneously with sHA.GCN4pII had a residual virus titer of about 10^2 PFU/g, demonstrating less efficient clearance. Independent of the route of immunization, the mice immunized with sHA-coated microneedles had less than a 1-log reduction in viral lung titers compared to those in unimmunized control mice (Fig. 7). These data suggest that the microneedle route of vaccination induces improved clearance of replicating virus for both sHA and sHA.GCN4pII compared to the s.c. route.

sHA.GCN4pII induces a type 1 helper T cell population after microneedle vaccination. The effector phenotype of the helper T cell compartment can influence the B cell response and the CD8+ T cell response (14, 26). Therefore, to determine the helper T cell phenotype, we purified CD4+ T cells from vaccinated mice and restimulated the cells with vaccine being presented by accessory cells. The cytokines IFN-γ and IL-4 are known to be produced by helper T cell type 1 and helper T cell type 2, respectively. The frequency of IFN-γ+ CD4+ T cells was assayed by intracellular cytokine staining after in vitro restimulation. We observed that mice vaccinated with sHA.GCN4pII had an approximately 2-fold-higher frequency of IFN-γ+ CD4+ T cells than did mice vaccinated with sHA at both antigen concentrations (20 μg, P = 0.117, and 5 μg, P = 0.0262) (Fig. 8A). Conversely, mice vaccinated with
sHA had an approximately 1.3-fold-higher frequency of IL-4+ CD4+ T cells than did mice vaccinated with sHA.GCN4pII at both antigen concentrations, although this difference was not significant (Fig. 8B). These results indicated that microneedle vaccination with sHA.GCN4pII induced a more robust Th1 response in mice. The ratio of IFN-γ+ CD4+ T cells to IL-4+ CD4+ T cells suggests that the helper T cell phenotype is dominated by Th1 cells in mice vaccinated with sHA and sHA.GCN4pII (20 μg, P = 0.0563, and 5 μg, P = 0.0323) (Fig. 8C). These results indicated that microneedle vaccination with sHA.GCN4pII induced a more robust Th1 response in mice.

**DISCUSSION**

The recent H1N1 influenza pandemic has illustrated the need for convenient alternatives to traditional influenza vaccine. Previous studies from our lab and others have demonstrated the successful use of both coated and dissolving microneedles for transdermal delivery of influenza vaccines using virus-like particles or inactivated virus (17, 23, 35). However, little information is available on using this approach to deliver a protein subunit vaccine. Microneedles have the advantage of delivering vaccine to an area rich in APC such as the epidermis and dermis without causing pain (11). These vaccinations induced robust antibody and cellular responses, resulting in protection against lethal challenge with several subtypes of influenza virus.

We have previously demonstrated that the influenza virus HA protein modified at the C terminus with the trimerization repeat, GCN4pII, generates stable trimeric soluble HA. Following subcutaneous vaccination, the modified trimeric sHA was able to induce higher virus-specific serum IgG and HAI titers (37). These enhanced humoral responses translated to a greater vaccine-induced protection following challenge with homologous virus. In the present study, we have tested the hypothesis that MN-mediated delivery via the transdermal route would be effective in enhancing the immune response to an influenza virus subunit vaccine. We demonstrated that the structure of the recombinant trimeric sHA was preserved when 15% (wt/vol) trehalose was included in the coating formulation. However, the unmodified sHA remained as a mixture of trimers, dimers, and monomers in the presence or absence of 15% trehalose. A stabilizing effect of trehalose on the HA activity of influenza virus was previously observed when coating microneedles with the whole inactivated virus (24). Microneedle vaccination with the modified trimeric sHA induced an improved humoral systemic response compared with that induced by unmodified sHA as determined by ELISA. This improved response to trimeric sHA over that to unmodified sHA was particularly evident in HAI and micro-neutralization titers. These results strongly support the view that the trimeric sHA induces higher levels of functional antibodies than does the unmodified sHA, while both recombinant proteins induce binding antibodies as measured by ELISA. This difference in induction of functional antibodies could be attributed to the better presentation of native epitopes present in the stabilized trimeric structure of the modified sHA corre-
sHA.GCN4pII induced a higher frequency of IFN-γ and IL-4 by intracellular cytokine staining. We compared the postchallenge viral lung titers observed in mice vaccinated with recombinant soluble HA-coated microneedles. Our results showed that the soluble trimeric HA induced immune responses that were efficient at clearing replicating virus. In addition, the differences observed at the mucosal site could be the result of lower immunogenicity of sHA, or perhaps sHA results in a homing pattern different from that of sHA.GCN4pII. These results demonstrate the efficacy of transdermal vaccinations using recombinant sHA derived from the A/Aichi/2/68 (H3N2) virus. Microneedle vaccination with the stabilized HA trimers induced protective immune responses in mice. It is noteworthy that in comparison to our previous work with subcutaneous vaccination, the MN immunization with the trimeric HA induced similar serum IgG and HAI titers and induced the same level of protection against lethal challenge as that in the s.c. vaccinated mice. Comparisons between intramuscular, intranasal, subcutaneous, and microneedle vaccinations using virus-like particles (VLP) and inactivated virus antigens have all demonstrated the enhanced immunogenicity of the transdermal route (16, 17, 23). Taken together, the results emphasize the conclusion that the delivery route as well as the nature of the vaccine antigen is important in determining the efficacy of an influenza vaccine.

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M.R.P. serves as a consultant and is an inventor on patents licensed to companies developing microneedle-based products. This possible conflict of interest has been disclosed and is being managed by Georgia Tech and Emory University.

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