



Short communication

Improved protection against avian influenza H5N1 virus by a single vaccination with virus-like particles in skin using microneedles

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ABSTRACT

To develop a more effective vaccination method against H5N1 virus, we investigated the immunogenicity and protective efficacy after skin vaccination using microneedles coated with influenza virus-like particles containing hemagglutinin derived from A/Vietnam/1203/04 H5N1 virus (H5 VLPs). A single microneedle vaccination of mice with H5 VLPs induced increased levels of antibodies and provided complete protection against lethal challenge without apparent disease symptoms. In contrast, intramuscular injection with the same vaccine dose showed low levels of antibodies and provided only partial protection accompanied by severe body weight loss. Post-challenge analysis suggested that improved protection was associated with lower lung viral titers and enhanced generation of recall antibody secreting cells by microneedle vaccination. Thus, this study provides evidence that skin delivery of H5 VLP vaccines using microneedles designed for self-administration induces improved protection compared to conventional intramuscular immunization.

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Avian H5N1 influenza viruses cause sporadic zoonotic infections of humans with high fatality rates of 60% (Sims et al., 2005; Webster et al., 2005). Furthermore, the pandemic potential of these viruses poses a serious threat to public health. The influenza pandemic caused by the 2009 H1N1 virus provided an opportunity to examine the performance of current vaccination. The available evidence indicates that the second wave of infection spread through the US population in the early Fall of 2009, before the vaccine became available to the majority of targeted high-risk population groups (Litchfield, 2009; Loeb et al., 2010). This experience indicated that development of new and faster methods of vaccine manufacturing and immunization should be a priority.

The skin has been suggested as an attractive site for immunization due to the presence of potent antigen-presenting cells such as Langerhans and dermal dendritic cells (Glenn and Kenney, 2006;

Hammond et al., 2001). To improve protective efficacy while reducing the antigen mass by targeting influenza antigens to the skin, intradermal (ID) immunization has been evaluated in clinical trials (Auewarakul et al., 2007; Belshe et al., 2004; Kenney et al., 2004; Khanlou et al., 2006; Van Damme et al., 2009). However, the conventional ID injection procedure requires highly trained medical personnel and is not well tolerated by vaccinees due to pain and discomfort at the site of injection (Auewarakul et al., 2007; Belshe et al., 2004; Kenney et al., 2004). Recent studies have demonstrated a promising alternative method that delivers inactivated whole-virion vaccines to the skin using microneedles, penetrating the outer layer of the skin (Kim et al., 2010, 2009; Quan et al., 2009; Zhu et al., 2009). This simple design could permit self-administration of vaccine by patients, possibly enabling vaccination campaigns to rapidly reach large populations (Prausnitz et al., 2009).

Conventional inactivated vaccines are produced from virus propagated in eggs. A new vaccine platform, virus-like particles (VLPs) produced in cell culture, has been shown to confer protection against highly pathogenic avian-origin influenza viruses in animal models, and can be manufactured without handling pathogenic live viruses (Bright et al., 2008; Haynes et al., 2009; Kang et al., 2009). In the present study, we investigated the immunogenicity and protective efficacy after a single vaccination using microneedles coated with dried H5 VLPs, in comparison with conventional intramuscular injection.

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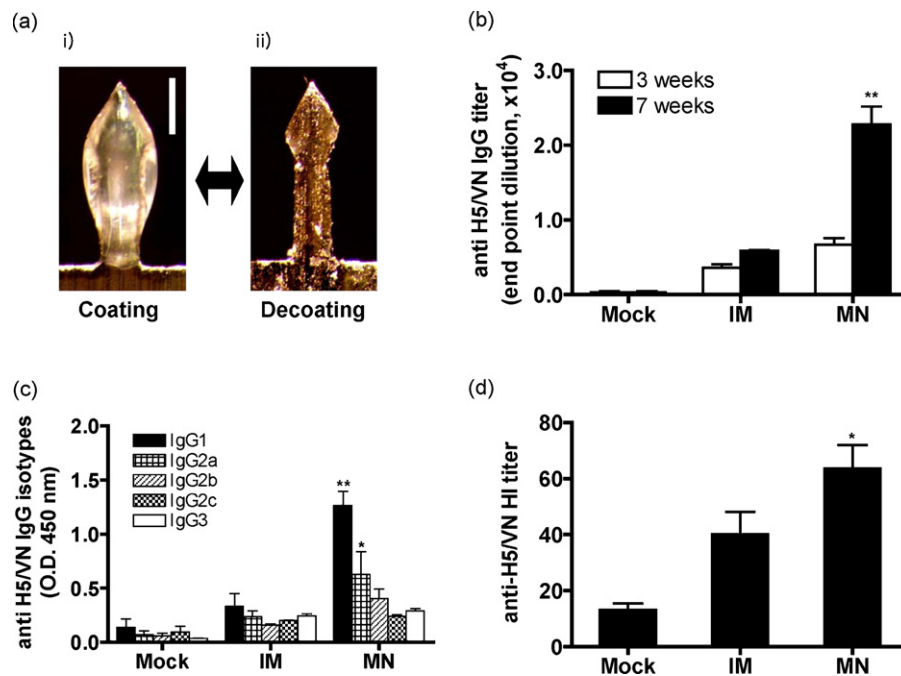


Fig. 1. Immunogenicity of influenza H5 VLP vaccination in the skin using microneedles. (a) A microneedle coated with H5 VLPs before and after dissolution in PBS. A microneedle is shown as observed by bright field microscopy after coating with influenza H5 VLPs for skin vaccination (i) and after dissolution of the coating from the microneedle in PBS (ii). Bar = 250 μ m. (b) H5 HA-specific IgG titers at weeks 3 and 7 after a single vaccination. Titers of antibodies specific to inactivated H5 A/Vietnam/1203/04 virus are expressed as the highest dilution of sera having a value of optical density at 450 nm (OD450) greater than the mean plus 2 standard deviations of similarly diluted naïve sera as described previously (Quan et al., 2007). (c) H5 HA-specific isotype antibodies as presented in OD450 values of 100 \times diluted sera at week 7. BALB/c mice ($n = 11$ per group) were immunized once with H5 VLPs using microneedles (MN), intramuscular injection (IM), or uncoated placebo microneedles (Mock). Bars indicate means \pm S.E.M. (d) Hemagglutination inhibition (HI) titers determined at week 7 after vaccination. Asterisk indicates significance between MN and IM groups (** $p < 0.01$, * $p < 0.05$, Student's 2-tailed t -test).

H5 VLPs derived from influenza A/Vietnam/1203/04 (A/VN/04) virus were produced in insect cells using recombinant baculovirus expression as previously described (Kang et al., 2009). Stainless steel microneedles were fabricated as arrays of 5 needles (Kim et al., 2010). The 700 μ m length of microneedles used in this study is suitable for effective delivery of vaccine into mouse skin with a thickness of 500–600 μ m (Azzi et al., 2005), because the whole microneedle is not fully inserted into the skin due to skin deformation during insertion. For vaccination in the skin, microneedles were coated on their surfaces with H5 VLPs in coating solution (1% carboxymethylcellulose (CMC) sodium salt as viscosity enhancer, 0.5% (w/v) Lutrol F-68 NF as surfactant, and 15% trehalose as stabilizer) and then air dried (Kim et al., 2010). A change in thickness of the microneedle was observed by bright field microscopy after coating with H5 VLPs and dissolution of coated H5 VLPs into PBS buffer (Fig. 1a). The amount of H5 VLPs coated onto each 5-microneedle array was 2.0 ± 0.15 μ g total proteins (approximately 0.2 μ g HA) as determined after elution into PBS using a protein assay kit (Quan et al., 2009).

Groups of mice (BALB/c, 6–8 weeks old, $n = 11$ per group) were immunized using either (i) microneedles without antigen (mock), (ii) microneedles coated with 2 μ g of H5 VLPs (MN), or (iii) 2 μ g of H5 VLPs in PBS buffer solution dissolved from coated microneedles given by intramuscular injection (IM). At weeks 3 and 7 after a single dose vaccination, antibody responses in sera were determined by quantitative ELISA using recombinant H5 HA protein as a coating antigen (Fig. 1b). Interestingly, at week 7 after a single immunization, 5-fold higher levels of H5 HA specific antibodies were observed in the microneedle vaccination group compared to the intramuscular control, which is significantly higher than those at week 3 (Fig. 1b).

The pattern of antibody isotypes may provide informative insight into the T helper type 1 or 2 immune responses (Hocart

et al., 1989). Therefore, we determined antibody isotypes after a single microneedle or intramuscular vaccination. IgG1 isotype antibody was induced at significantly higher levels than IgG2a antibody after microneedle delivery ($p = 0.031$, Fig. 1c). This IgG1 isotype-dominant pattern following H5 VLP microneedle immunization is similar to that observed with microneedle vaccination using whole inactivated A/Aichi/68 virus (H3N2) (Koutsouanos et al., 2009), but different from results obtained from 0.4 μ g low vaccine dose of the A/PR/8/34 (H1N1) virus or VLPs (Quan et al., 2010, 2009), which predominantly induced IgG2a isotype antibody after microneedle vaccination of BALB/c mice. Interestingly, the IgG1 isotype antibody was previously shown to have higher neutralizing and similar HI titers, respectively, compared to the IgG2a isotype in immune sera of BALB/c mice (Hocart et al., 1989). Although it is not clear what factor(s) influences IgG1/IgG2a ratios, there are several potential parameters affecting the antibody isotype pattern, which include mouse strains and types of vaccines (Hocart et al., 1989), stability and integrity of vaccine antigens (Quan et al., 2010, 2009), routes and doses of vaccines (Bright et al., 2008), and intrinsic immunogenicity properties of vaccines such as H5 VLPs of A/Vietnam/1203/04 observed in this study.

Next, we determined hemagglutination inhibition (HI) antibody responses using 1% horse red blood erythrocyte (Fig. 1d). HI titers over 60 were detected in the microneedle vaccination group, which is 4-fold higher than the titers in the mock control and 1.5-fold higher than the IM group ($p = 0.03$). As high doses of H5 vaccine were reported to be required in humans for moderate immunogenicity, this observation suggests that a MN H5 vaccine could bring significant improvement compared to other non-adjuvanted vaccines tested thus far in clinical trials. The low antibody response reported in a study by Patel et al. (2010) is likely related to the small doses of antigen injected intradermally. In other clinical trials indicating low immunogenicity of H5 vaccines, prime-boost immu-

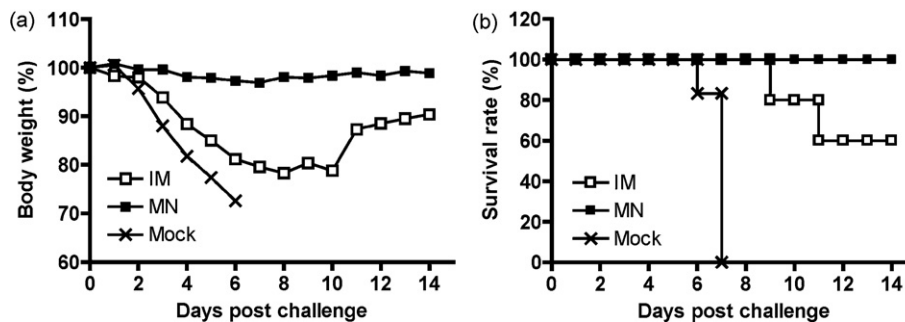


Fig. 2. Protection by a single-dose microneedle vaccination with H5 VLPs. Groups of BALB/c mice that had been vaccinated (Mock, IM, MN) were intranasally challenged with a lethal dose ($20 \times LD_{50}$) of wild type A/Vietnam/1203/04 (H5N1) virus at week 20 post vaccination. (a) Daily mean body weight ($n = 5$ out of 11 vaccinated mice). Significant morbidity indicated by greater than 15% weight loss as well as ruffling fur and inactivity between days 6 and 10 post challenge was observed in the IM group. In contrast, the MN group had no weight loss. (b) Daily proportion of mice alive ($n = 5$ out of 11 vaccinated mice).

nizations with $90 \mu\text{g}$ HA doses of baculovirus-expressed or $30 \mu\text{g}$ of inactivated subunit vaccines produced in eggs were needed to induce antibody responses that were expected to be protective in 54–58% of individuals vaccinated (Nicholson et al., 2001; Treanor et al., 2006, 2001).

For challenge studies to determine protective efficacy against lethal infection at 20 weeks after a single vaccination, mice ($n = 5$ out of 11 vaccinated mice) were inoculated with $20 \times LD_{50}$ of wild type A/Vietnam/1203/04 (H5N1) by intranasal instillation. All mice in the mock control group showed severe losses in weight and reached the euthanasia endpoint between 6 and 7 days after challenge (Fig. 2a). Mice in the IM group also experienced severe weight losses and showed a significant delay in recovering body weight after lethal challenge infection (Fig. 2b). In contrast, the H5 VLP microneedle group (MN) did not lose body weight and showed 100% protection compared to partial protection (60%) by IM immunization. Therefore, microneedle vaccination in the skin with H5 VLPs induced enhanced protective efficacy compared to intramuscular injection.

To better characterize the protection afforded by microneedle vaccination with H5 VLPs, lungs were harvested at week 20 post vaccination from additional groups of mice ($n = 6$ out of 11 vaccinated mice) euthanized at day 4 post lethal challenge with reassortant A/Vietnam/1203/04 (H5N1) virus ($20 \times LD_{50}$), homogenized and titrated by plaque assay (Fig. 3a) as described previously (Quan et al., 2007). The reassortant A/Vietnam/1203/04 virus was generated to contain HA and NA genes from A/Vietnam/1203/04 (H5N1) and internal genes from A/PR/8/34 as described previously (Bright et al., 2008; Hoffmann et al., 2002). The H5 VLP microneedle vaccination group showed lung viral titers that were 20-fold lower

than the mock control and 6-fold lower than the intramuscular immunization group. Thus, these results suggest that the improved protection by microneedle vaccination with H5 VLPs was partially due to more effective early control of viral replication in the lungs.

One of the main goals of vaccination is to induce long-lasting immune memory that mediates protection upon exposure to infectious agents. We tested whether a single microneedle vaccination in the skin with H5 VLPs could induce increased levels of memory B cells that rapidly differentiate into antibody secreting plasma cells upon viral infection (Fig. 3b). Spleen cells were collected from the same groups of mice at day 4 after challenge as described for assay of lung viral titers. Single cell suspensions from spleens were subjected to in vitro culture with inactivated H5N1 A/Vietnam/1203/04 viral antigen coated on the plates. Importantly, with antigenic stimulation by viral antigen coated on the plates, spleen cells collected from the group of mice that were immunized in the skin using microneedles showed significantly higher levels of virus-specific antibodies secreted into culture supernatants compared to those by intramuscular immunization after 6 days but not after 2 days, of culture (Fig. 3b). Therefore, these results suggest that a single microneedle vaccination with H5 VLPs is more effective in generating memory B cells that can differentiate into antibody-secreting cells compared to conventional intramuscular immunization. Regarding the cytokine-secreting splenocyte responses, intramuscular immunization with H5 VLPs showed higher IL-4 and similar levels of IFN-secreting cells, respectively, compared to those obtained after microneedle vaccination (data not shown), indicating that IL-4 and IFN- γ secreting cellular responses have less correlation with improved protection by microneedle vaccination in the skin with H5 VLPs.

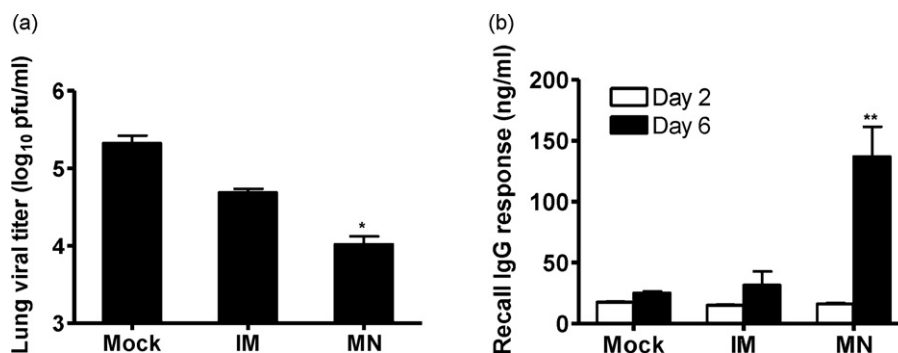


Fig. 3. Lung viral titers and virus-specific antibody-secreting cell responses. Six out of eleven vaccinated mice (Mock, IM, MN) were sacrificed for analysis of lung viral titers and antibody secreting cell responses at day 4 post challenge as described (Quan et al., 2007). (a) Lung viral titers were determined by a plaque assay with lung homogenate obtained from 6 individual mice. (b) Recall antibody secreting cell responses of spleen cells. Antibody levels were presented as concentrations in culture supernatants (expressed in ng/ml). Values are means \pm S.E.M. of individual mice ($n = 6$) after in vitro culture. Asterisk indicates significance between MN and IM groups ($*p < 0.05$, $**p < 0.01$, Student's 2-tailed t -test).

In conclusion, this study demonstrates that a single vaccination in the skin using microneedles coated with dried H5 VLP vaccine induces improved protective immunity against highly pathogenic H5N1 virus as compared to intramuscular injection, and induces IgG1 isotype predominant antibody responses that inhibit hemagglutination. The results from this study provide evidence that microneedle vaccination with H5 VLPs in the skin is a promising vaccine delivery method. Thus, microneedle skin vaccination with H5 VLPs enables improved protection against influenza virus with pandemic potential and offers the potential for self-administration, which could dramatically expedite vaccination delivery.

Conflict of interest

M.R.P. is a consultant and inventor on patents licensed to companies with an interest in microneedles. R.W.C., S. M.K., and Emory University have equity in Zetra Biologicals which is developing VLP technology under license from Emory University. The associated conflict of interest is being managed by Georgia Tech and Emory University.

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