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Skin immunization with influenza vaccines

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Abstract

Problems with existing influenza vaccines include the strain specificity of the immune response, resulting in the need for frequent reformulation in response to viral antigenic drift. Even in years when the same influenza strains are prevalent, the duration of immunity is limited, and results in the need for annual revaccination. The immunogenicity of the present split or subunit vaccines is also lower than that observed with whole inactivated virus, and the vaccines are not very effective in high risk groups such as the young or the elderly. Vaccine coverage is incomplete, due in part to concerns about the use of hypodermic needles for delivery. Alternative approaches for vaccination are being developed which address many of these concerns. Here we review new approaches which focus on skin immunization, including the development of needle-free delivery systems which use stable dry formulations and induce stronger and longer-lasting immune responses.

1 Introduction

Over 200 years ago, Edward Jenner demonstrated that delivery of live cowpox virus to human skin resulted in transient skin lesions, and gave rise to protective immunity against subsequent exposure to smallpox virus (Riedel 2005). This procedure was further developed over the subsequent centuries, and the term **vaccination** was adapted, reflecting the use of the cowpox-related virus **vaccinia** for smallpox immunization. A global vaccination campaign against smallpox resulted in its eradication, which was declared by the WHO in 1980, and is recognized as one of the greatest achievements in medicine (Riedel 2005). During the course of this project, a variety of devices was developed and used for delivery of the smallpox vaccine to the skin, and they have been described in detail in other recent reviews (Weniger and Glenn 2013). In addition to smallpox vaccination, skin immunization has been widely used for delivery of the Bacille Calmette-Guerin (BCG) vaccine for tuberculosis (Hoft et al. 2008). Experimental studies of intradermal vaccine delivery have also been carried out with a number of other vaccines for infectious disease prevention, including clinical trials as well as studies in experimental animals, and have been recently reviewed (Kim et al. 2011). The high current interest in this approach for vaccination reflects its potential immunological as well as logistical advantages, as discussed in this review for

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influenza immunization. In addition to improving immune responses, acceptability of vaccination by the public is likely to be enhanced by avoiding the use of hypodermic needles. A more complete overview of research on intradermal immunization has been recently published as Volume 351 of this series (Teunissen et al. 2012).

A number of early studies were carried out with inactivated influenza vaccines using intradermal delivery with the goal of using reduced doses of vaccine (Bruyn et al. 1949b; Glazier et al. 1956; Hilleman et al. 1958; Tauraso et al. 1969; Weller et al. 1948) and in several studies it was observed that lower doses were sufficient to elicit the same immune response when compared with subcutaneous injection. The approach for intradermal delivery used in these studies was developed by Mantoux (Mantoux 1909), who used a hypodermic needle inserted at an angle to deliver antigen just beneath the dermis. However this approach is technically difficult and requires trained personnel, and it has not been widely employed in many recent vaccination programs.

In this review, we have focused on recent studies using alternative approaches for skin delivery of influenza vaccines. These studies have employed a number of different methods for vaccine delivery, examples of which are listed in Table 1. We have included studies using whole inactivated virus, which was widely used until the 1970s, as well as split or subunit vaccines which were subsequently developed using detergent-disrupted virus. Influenza vaccine has been an antigen of choice for many studies of intradermal vaccination, in part because the vaccine is well characterized and widely used, and because animal models are available to evaluate the resulting immune responses as well as the protective efficacy. The results have revealed significant immunological advantages for this route of vaccine delivery. In addition, skin immunization could provide an approach to overcome some of the limitations and problems with current influenza vaccines, including the limited duration of immunity and the problems in conferring effective protection to high risk groups, such as young children and adults over 65 years old (Osterholm et al. 2012).

2 Human studies

The first studies that explored alternative vaccination routes with inactivated influenza vaccine were carried out in human volunteers by Francis and Magill in 1937, in a series of experiments designed to induce neutralizing antibody production against type A influenza virus grown in tissue culture. The authors showed a significant rise in circulating strain-specific antibody, a booster effect in case of pre-existing immunity and persistence of immune responses up to 5 months (Francis and Magill 1937). Van Gelder et al reported that intracutaneous delivery of 1/10 of the normal dose of concentrated inactivated influenza type A and B viruses resulted in a rapid and considerable rise in the serum anti-hemagglutinin titers, which was greater than that observed with a subcutaneous dose, and proposed that this vaccine delivery approach should be further studied to determine its protective effect against influenza (Van Gelder et al. 1947). Importantly the authors also observed lower incidence of generalized reactions to the vaccine in the group which received intradermal inoculations. In the spring of the same year an influenza epidemic occurred in Boston, which led Weller et al to carry out intradermal vaccination in a group of adult hospital personnel. The investigators could not reproduce the dose-sparing effect of intradermal inoculation or the increasing titers

reported by Van Gelder, suggesting that these results may be due to differences in vaccine concentration. They confirmed that the local reactions to intradermal vaccination were mild and that systemic reactions were rare (Weller et al. 1948). The need for a single vaccine dose, important for mass vaccinations, was emphasized by Bruyn et al who did not find significant differences between one or two doses of influenza A and B vaccines delivered intradermally in adults or children. Their data did not show differences between responses to intradermal vs. subcutaneous immunization (Bruyn et al. 1949a). Subsequent studies by Glazier et al on intradermal vaccination of children with inactivated influenza A and B viruses demonstrated effectiveness and dose sparing compared to subcutaneous immunization. The authors also observed rises in titers with booster doses either one week or 5–7 months after primary immunization (Glazier et al. 1956).

The emergence of the Asian flu pandemic (1957–58) and the urgent need for mass vaccination provided a reason for re-visiting the issue of dose sparing. Boger and Liu reported that intradermal vaccination did not offer any advantages in terms of dose sparing and suggested that subcutaneous immunization in humans should be the recommended vaccination route (Boger and Liu 1957). In contrast Hilleman et al demonstrated that intradermal delivery of 1/10 dose of egg grown A/Japan/305/57 vaccine induced similar antibody responses in human volunteers as the full dose given subcutaneously. The seasonal polyvalent vaccine did not induce cross-reactive responses to A/Japan/305/57 virus, but cross-reactive antibody could be observed with high vaccine doses against A/Swine/30 and A/Hawaii/305/56 suggesting sharing of common antigens. Based on the seroconversion rates elicited after intradermal vaccination, the authors suggested that this route offers considerable advantage in conserving vaccine during periods of shortages as well as reducing the cost for vaccination (Hilleman et al. 1958). The difference between the 1957 and 1958 studies may be related to age differences of vaccinees. Boger and Liu recruited volunteers aged over 70 in order to determine effects of pre-existing immunity that could offer cross-protection against the novel H3N2 influenza subtype, whereas Hilleman et al carried out their studies in healthy adults.

Ten years later the appearance of the Hong Kong pandemic (1968–69) resulted in a subsequent vaccine shortage, and caused re-evaluation of humoral immune responses following subcutaneous and intradermal inoculation vaccination. Tausaro et al used vaccine purified by zonal centrifugation for the first time, and tested vaccine efficacy in healthy adult volunteers. Based on their findings of a significant dose sparing (1/5 dose when compared to the subcutaneous dose) the authors suggested that intradermal inoculation would be a reasonable alternative for mass vaccinations. Additional interesting results from this study were the finding of a 4-fold seroconversion after 2 vaccine doses, and an inverse relationship between antibody response and pre-immunization titers; also, individuals 65 years or older developed antibody responses more rapidly than younger age-groups based on a 4-fold or greater rise in antibody and the geometric mean ratio in sera at intervals after vaccination (Tausaro et al. 1969).

With the emergence of a swine flu variant infecting humans in 1976, a national vaccination campaign was initiated in the U.S. Brown et al reported that among the 18–24 year-old age group persons who did not have pre-existing immunity against the A/New Jersey/31/76

(HswINI), when vaccinated intradermally with whole-virus vaccine at 1/5 dose than those immunized intramuscularly (40 HA units/0.1-ml dose vs 200 HA units/0.5-ml dose) exhibited lower HAI titers, and repeated vaccinations did not offer an immunological advantage. In contrast, vaccinees over the age of 24 without detectable homologous immunity had comparable serologic responses to intradermal or intramuscular vaccination. Persons infected with influenza virus prior to vaccination exhibited an immediate antibody response indicative of a secondary type of response. The antibody responses after intradermal vaccination were lower for those vaccinees with pre-existing immunity, and in a younger age group. In older age groups who were vaccinated either intradermally or intramuscularly, only the ID group needed a booster dose (Brown et al. 1977). As a result of mild side effects of intradermal influenza vaccine delivery and the lack of systematic advantages of this route compared to conventional intramuscular inoculation, such as dose sparing, the intradermal route lost its appeal. In addition, other factors such as the inadequate purification of whole inactivated influenza virus, uncertainties about the inactivation process and the termination of the vaccination program due to a media frenzy related to Guillain-Barré syndrome (GBS) among persons receiving swine flu immunizations, attention shifted to newer and safer vaccine formulations such as subunit and split inactivated influenza vaccines (Sencer and Millar 2006). Currently, inactivated trivalent influenza vaccines (TIVs) include the unadjuvanted whole virion, split virus, subunit, virosomes containing subunit antigens, and adjuvanted subunit vaccines.

In 1960 Aaron Ismach designed jet injectors for mass vaccinations aiming to controlled vaccine or drug delivery in the skin (Ismach 1960). Jet injectors are a type of injecting syringe using a high-pressure narrow jet of an injected liquid instead of a hypodermic needle to penetrate the epidermis. Studies in healthy young adults showed that the device caused higher levels of pain and local reactions following vaccination, while there was no dose sparing or improvement of humoral responses (Jackson et al. 2001). A similar approach was attempted by Chen using the PowderJect ND5.2 delivery system for powdered trivalent influenza vaccine delivery in the epidermis, targeting Langerhans cells (Chen et al. 2004). A phase I clinical trial comparing intramuscular and epidermal (EPI) immunization reported no differences in systemic reactogenicity or any severe site reactions, and equivalent or superior immune responses were elicited in EPI-immunized groups as compared to groups immunized intramuscularly (Dean and Chen 2004). Due to potential risks of pathogen transmission during applications, the World Health Organization no longer recommends jet injectors for vaccination ((WHO) 2005). As of today no influenza vaccines are licensed in the United States for administration via jet-injector ((FDA) 2011).

Several studies have been carried out using intradermal injection of trivalent subunit vaccines with hypodermic needles (Auewarakul et al. 2007; Belshe et al. 2004; Beran et al. 2009), and a stronger immune response or dose-sparing effect was observed in some of the studies. Subsequently, a device was developed by Becton Dickinson (Soluvia™) for intradermal immunization using a prefilled syringe and a 30 gauge, 1.5mm-long needle, which limits the depth of penetration of the needle (Laurent et al. 2007). A number of clinical trials have been carried out using this device to deliver trivalent detergent-split vaccine, and have been recently reviewed (Icardi et al., 2012; (Ansaldi et al. 2012). The device offers improved reproducibility and ease of delivery compared with the Mantoux

technique. BD Soluvia™ has been licensed for use in Europe and the U.S for Sanofi Pasteur's Fluzone® for active immunization of adults aged between 18 and 64 years. The reduced-antigen-content ID vaccine (9 µg hemagglutinin per strain) is as immunogenic as standard-dose IM influenza vaccine; it is safe and well tolerated by patients, which could result in an improvement in vaccine coverage by including individuals who fear injection with standard hypodermic needles (Icardi et al. 2012).

NanoPass Technologies LTD developed a single-use device for painless intradermal delivery (MicronJet) to deliver drugs and vaccines. Microneedles are manufactured by using MEMS (Micro Electro Mechanical Systems) technology and are made of pure silicon crystals in the shape of micro-pyramidal needles. Prospective randomized trials in healthy adults who received a 6 µg or a 3 µg dose of hemagglutinin per strain by intradermal injection of seasonal influenza vaccine with the MicronJet device demonstrated humoral immune responses similar to those elicited by the full-dose 15 µg intramuscular vaccination. The microneedle injection device used in this study was found to be effective, safe, and reliable (Van Damme et al. 2009). A follow up prospective randomized trial on elderly and chronically ill adults delivering reduced dose ID TIV (3 µg or 9 µg of hemagglutinin (HA) per strain) by MicronJet600™ demonstrated dose sparing and improved seroconversion and seroprotection when compared to the full vaccine dose (15 µg) delivered intramuscularly as measured by hemagglutination inhibition (HAI) and neutralizing antibody titers (Hung et al. 2012). The data support further investigation of this approach for intradermal immunization with vaccines of low immunogenicity.

Most of the above studies in humans have been focused on the issue of dose sparing, which has become relatively less important because of the availability of a more reliable vaccine supply. Relatively little information has been obtained on other potential advantages such as quality and duration of the immune response, breadth of immunity, or induction of mucosal or cellular responses. Recently, Combadiere et al demonstrated the superiority of transcutaneous immunization (TC) with inactivated influenza vaccine in the induction of CD8+ T cell responses in randomized Phase I Clinical Trials (Combadiere et al. 2010).

All human vaccine studies described above have employed intradermal delivery of influenza vaccines with needle-based devices but it is generally accepted that there are many concerns related to unsafe practices and pathogen transmission involving needles. In addition there is a large percentage of children and adults who suffer from distress or fear of injections (Giudice and Campbell 2006), resulting in reluctance to receive immunizations. Another major hurdle in expanding vaccination coverage is the need to maintain vaccines at low temperatures. The need for *cold chain* to preserve vaccine potency mandates a 2 to 8°C temperature range for vaccine storage from the time that they are manufactured until they are given to patients (Atkinson et al. 2002) (Weir and Hatch 2004). Needle-phobia, discomfort and reactogenicity of particular vaccines (Moylett and Hanson 2004) directed research to discovery of novel immunization approaches such as lyophilized vaccines delivered in the form of patches (Glenn et al. 2003). An alternative vaccine formulation independent of the *cold chain* is a promising approach for rapid distribution to remote areas of the world without the appropriate infrastructure to establish effective mass vaccination strategies and lead to a reduction in the costs of vaccine distribution.

3 Animal studies

3.1 Mice

3.1.1 Potency of immune responses and dose sparing

3.1.1.1 Inactivated, subunit and VLP vaccines: Delivery of inactivated whole influenza virus with topical application on the skin was demonstrated in the mouse model (Skountzou et al. 2006). Despite its large size the virus could transverse the skin after mild pre-treatment and induce influenza-specific hemagglutination inhibition and neutralizing antibody titers in serum as well as salivary and fecal IgA. Lung viral titers were assessed as an indicator of protection after intranasal challenge with mouse adapted homologous virus. Effective clearance of virus in vaccinated animals as compared to unvaccinated infected controls within 8 days was attributed to robust humoral and cellular immune responses. Despite the encouraging data it was evident that this approach for transcutaneous immunization required higher vaccine doses and multiple immunizations, suggesting the need for improved delivery of antigen for dose sparing.

To overcome the limitations of non-invasive skin vaccination methods, while avoiding the concerns about hypodermic needles, minimally invasive methods to administer vaccine to the skin have been developed, primarily using very small hollow or solid needles. Most research on minimally invasive skin vaccination has involved the use of microneedles, which are long enough to cross the stratum corneum barrier and to reliably remain within the skin for targeted delivery, but short enough to avoid pain (Gill et al. 2008). There are four different types of microneedles that have been studied for vaccine delivery: hollow, solid, coated, and dissolving microneedles. Because this approach directly and actively deposits vaccine in the skin, it can deliver vaccine doses faster and more reliably than non-invasive vaccinations (Kim et al. 2012).

Metal microneedle arrays coated with low (3 µg) or higher (10 µg) doses of inactivated A/Aichi/2/68 (H3N2) influenza virus elicited substantial influenza-specific neutralizing antibodies in BALB/c mouse sera after a single immunization. Interestingly, mice vaccinated cutaneously or intramuscularly with the low vaccine dose showed similar titers whereas humoral responses to cutaneous immunization were significantly higher with increased vaccine doses. All vaccinated groups were fully protected upon challenge with 5×LD₅₀ of mouse adapted Aichi virus. Virus clearance from the lungs was complete by day 4 in mice that received the high vaccine dose. Microneedle vaccination induced a broad spectrum of immune responses including CD4⁺ and CD8⁺ responses in the spleen and draining lymph nodes, a high frequency of antibody-secreting cells in the lung and induction of virus-specific memory B-cells. The similarity in results observed after IM and MN immunization demonstrated that microneedle immunization was as effective as intramuscular vaccination (Koutsonanos et al. 2009). Similarly, Zhu et al observed that mice immunized by a single dose of H1N1 inactivated influenza virus (A/PR/8/34) coated on MNs were effectively protected against lethal challenge by a very high dose (100×LD₅₀) of mouse-adapted influenza virus A/PR/8/34 (Zhu et al. 2009).

Skin immunization with microneedles fabricated using a biocompatible polymer (PVP, polyvinylpyrrolidone) encapsulating 6µg of inactivated whole influenza virus vaccine (A/PR/8/34) with trehalose for insertion and dissolution in the skin within minutes with at least 80% antigen delivery. Challenge of mice with 5×LD₅₀ of homologous virus showed a 10³-fold-decrease in lung viral titers in intramuscularly immunized mice compared to unimmunized infected mice, whereas microneedle-immunized mice showed a dramatic 10⁶-fold decrease in lung viral titers. Levels of IFN-γ, IL-12p70 and IL-21 in the lung induced after polyclonal re-stimulation were higher in the intramuscular compared to the microneedle group, suggesting stronger local Th1 response upon challenge. In contrast, influenza virus MHC Class I and II restricted T cell responses were increased in spleen of microneedle-immunized groups, indicative of increased recall CD4+ and CD8+ T cell responses (Sullivan et al. 2010)

Even without causing cell death in the epidermis, skin immunization is superior to intramuscular delivery in dose sparing. A very low dose of (A/PR/8/34 H1N1) VLPs (0.3µg) delivered with metal microneedles induced significantly superior protective immunity, which included binding and functional antibodies as well as complete protection against a high dose lethal infection with the homologous virus, whereas IM immunization provided only partial (40%) protection (Quan et al. 2010a). Furthermore a single immunization with metal microneedles coated with a very low dose (0.2 µg) of A/Vietnam/1203/04 H5N1 virus (H5 VLPs) induced high levels of antibodies and provided complete protection against 20×LD₅₀ lethal challenge without apparent disease symptoms. In contrast, intramuscular injection with the same vaccine dose showed low levels of antibodies and provided only 60% protection accompanied by severe body weight loss (Song et al. 2010a).

Influenza vaccines have a limited shelf-life even when stored at 4°C. Quan et al found that the stability of A/PR/8/34 influenza virus, as measured by hemagglutination activity, was significantly damaged during metal microneedle coating at room temperature. The addition of trehalose to the vaccine formulation resulted in retention of functional integrity and vaccine potency as shown in mouse studies. Both intramuscular and microneedle skin immunization with un-stabilized vaccine yielded weaker protective immune responses including viral antibodies, protective efficacies, and recall immune responses to influenza virus, whereas a single microneedle-based vaccination using stabilized influenza vaccine was superior to intramuscular immunization in controlling virus replication as well as in inducing rapid recall immune responses post-challenge (Quan et al. 2009). Addition of trehalose to the coating formulation was found to protect the antigen and retention of 48–82% antigen activity for all three major subtypes of seasonal influenza: H1N1, H3N2 and B. Influenza vaccine coated in the presence of trehalose also exhibited thermal stability, such that activity loss was independent of temperature over the range of 4°–37°C for 24h (Kim et al. 2010a). In the absence of trehalose hemagglutinin activity decreased below 10% after 1 h and was not detected after 1 month of drying, respectively. Addition of trehalose maintained HA activity above 60% after drying and above 20% after 1 month storage at 25°C. Loss of HA activity generally correlated with increased virus particle aggregation. Administration of microneedles coated with trehalose-stabilized influenza vaccine yielded high serum IgG antibody titers even after 1 month storage, and all animals survived lethal challenge infection with minimal weight loss (Kim et al. 2011). Choi et al reported that crystallization and phase

separation of the dried coating matrix are important factors affecting long-term stability of influenza vaccine-coated microneedles (Choi et al. 2012). The partial viral activity loss observed in the trehalose-containing formulation was hypothesized to result from osmotic pressure-induced vaccine destabilization. Inclusion of a viscosity enhancer, carboxymethyl cellulose, overcame this effect and full vaccine activity was retained on washed or plasma-cleaned titanium surfaces (Choi et al. 2013).

Similarly, addition of trehalose in vaccine coating formulations stabilized influenza VLPs containing HA and M1 proteins from A/PR/8/34 virus, demonstrating preservation of hemagglutinin antigen. A single dose of stabilized influenza VLPs induced 100% protection against challenge infection with a high lethal dose ($20 \times LD_{50}$) of homologous virus. In contrast, unstabilized influenza VLPs, as well as intramuscularly injected vaccines, provided inferior immunity and only partial protection ($\leq 40\%$). These results correlated with influenza specific neutralizing antibody titers and antibody secreting cells detected in spleen and bone marrow (Quan et al. 2010b). Optimizing the coating formulation required balancing the factors affecting the coating dose and vaccine antigen stability. Vaccine stability, as measured by an *in vitro* hemagglutination assay, was increased by formulation with increased concentration of trehalose or other stabilizing carbohydrate compounds and decreased concentration of carboxymethylcellulose (CMC) or other viscosity-enhancing compounds (Kim et al. 2010b).

Matsuo et al developed dissolving microneedle arrays, called MicroHyal (MH), as a transcutaneous immunization device, that co-polymerized sodium hyaluronate with influenza antigens dissolved in aqueous solution. The length of microneedles was either 300 μm (MH300) or 800 μm (MH800), and the arrays contained over 200 microneedles/ cm^2 . TCI vaccination efficacy was compared to that of conventional immunization systems, such as subcutaneous immunization (SCI), intradermal immunization (IDI), intramuscular immunization (IMI), and intranasal immunization (INI). Influenza-specific IgG or HAI titers elicited by TCI delivery of unadjuvanted vaccine were similar to intramuscular or intradermal immunization in the presence or absence of Alum as an adjuvant. These data were correlated with survival studies where all ID, IM and TCI groups survived lethal challenge with homologous virus and showed no lung pathology (Matsuo et al. 2012).

The skin epidermis consists mainly of keratinocytes, melanocytes, and Langerhans cells (LCs) (Skountzou and Kang 2009). Langerhans cells are present in all layers of the epidermis and are in close proximity to the *stratum corneum* (Banchereau and Steinman 1998). These are immature APCs produced from bone marrow precursors that reach and populate the skin through the peripheral circulation (Romani et al. 1985). Fernando et al took advantage of the densely packed cell layer and designed a Nanopatch, which contains an array of densely packed projections ($21025/\text{cm}^2$) (110 micron in length), that were dry-coated with vaccine. They observed that the Nanopatches could deliver a seasonal influenza vaccine (Fluvax® 2008) in 2 minutes and that by physically targeting vaccine directly to these antigen presenting cells they induced protective levels of functional antibody responses in mice, comparable to the vaccine delivered intramuscularly with a needle and syringe, but with less than $1/100^{\text{th}}$ of the delivered antigen (Fernando et al. 2010; Raphael et al. 2010). The impressive dose sparing effect observed with the Nanopatch suggested that it resulted

from cell death in proximity to sites of antigen deposition in the skin, resulting in enhancement of immunogenicity (Depelsenaire et al. 2014).

3.1.1.2 Vectored vaccines: Few studies have been done using skin immunization with recombinant vectored vaccines for influenza virus. However, a replication-defective adenovirus recombinant encoding the influenza A/PR/8/34 HA has been evaluated in human volunteers (Van Kampen et al. 2005). The recombinant vaccine was administered intranasally or applied to the abdominal skin after shaving, followed by gentle brushing with a soft-bristle toothbrush for 30 strokes. After the topical application of vaccine, the administration site was covered with a Tegaderm™ patch. The adenovirus-vectored nasal and epicutaneous influenza vaccines were both well tolerated by human volunteers. However the intranasally administered vaccine induced stronger immune responses than those observed in the cohort which received the epicutaneous vaccination.

3.1.1.3 Universal influenza vaccines: Current influenza vaccines induce immune responses which are highly strain-specific, resulting in the need to change the composition in order to protect against variant strains which emerge to escape from preexisting immunity. In addition, current vaccines would provide little or no protection against emergence of a new antigenic subtype, which could result in a global pandemic. It is therefore of high interest to identify conserved antigens and use them to develop vaccines which are broadly cross-reactive with multiple subtypes of influenza A viruses. It is known that the viral internal proteins, such as NP and M1, are more highly conserved. They do not elicit neutralizing antibodies, but can contribute to protective immunity by eliciting cross-reactive T-cell responses (Yewdell et al. 1985). However, most efforts to develop universal vaccines have focused on conserved epitopes on the external surface of the virion. The membrane-proximal HA stalk domain is highly conserved, and is a promising target for universal vaccine design. A growing body of evidence has emerged to support the idea that a stalk-based vaccine is capable of eliciting neutralizing antibodies with broad-cross protective efficacy (Wrammert et al. 2011). However it is not normally exposed to the immune system because it is covered by the globular HA head domain.

A second conserved antigen on the viral surface is the short external domain of the M2 protein, a trans-membrane protein which serves as an ion channel and also functions in viral assembly. This protein is present at low levels in the virion, and does not usually elicit an immune response by vaccination or infection. However it has been possible to design modified forms of the M2 external domain (M2e), some of which elicit protective immune responses when used as vaccines. A fusion protein of the TLR5-agonist domains of the flagellin protein from *Salmonella typhimurium* and multiple repeats of influenza M2e has been constructed, and evaluated as a vaccine in a mouse model using alternative immunization routes (Wang et al. 2014). It was found that skin immunization using metal microneedles coated with the fusion protein elicited strong systemic as well as mucosal immunity, and the protective efficacy induced by microneedle immunization was found to be much better than that observed with IM immunization.

3.1.1.4. Recombinant protein vaccines: Since most currently available influenza vaccines consist of solubilized viral protein antigens, recombinant subunit influenza vaccine was also

tested for its immunogenic properties when delivered to the skin by coated microneedles. BALB/c vaccinated via metal microneedle 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 and they were fully protected against a lethal challenge with influenza virus. Analysis of post-challenge 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 (Weldon et al. 2011).

3.1.1.5. DNA Vaccines: Early studies of immunization using DNA plasmids to elicit immune responses to the encoded proteins employed coating the plasmids onto gold particles, which were administered by various routes including the delivery into skin by electroporation (Wolff et al. 1990) or by high velocity gene gun injection (Jackson et al. 2001). In studies comparing different immunization routes using a DNA plasmid encoding the influenza HA protein (Fynan et al. 1993a; Fynan et al. 1993b), it was observed that using a gene gun to deliver DNA-coated gold beads to the epidermis of mice was a more effective route than intramuscular injection. Gene gun delivery of DNA vaccines encoding proteins of H1N1 or H3N2 influenza viruses induced protective immune responses in ferrets against challenge infection with heterologous influenza drift strains (Bragstad et al. 2011). Influenza DNA vaccines have also been shown to induce protective immunity in a phase 1 clinical trial (Jones et al. 2009).

Because of the high molecular weight and viscosity of seasonal or avian influenza DNA vaccines, they can also be used for coating of microneedles in the absence of other excipients such as carboxymethylcellulose or detergents (Kim et al. 2013; Song et al. 2012). It was found that delivery of microneedle-coated seasonal influenza HA-encoding DNA vaccines to the skin conferred dose sparing effects and induced improved protection in mice against subsequent virus challenge, when compared to IM immunization with the same DNA plasmids (Song et al. 2012).

3.1.2 Duration of immunity—A concern about the present seasonal influenza vaccines is that they elicit a relatively short duration of immunity, which wanes 6 to 8 months after immunization (Albrecht et al. 2014). Therefore, it is of interest to determine whether an alternative vaccination route could confer improved duration of immunity.

Prime-boost skin vaccination with metal microneedles coated with H5 VLPs containing the hemagglutinin (HA) of A/Vietnam/1203/04 demonstrated higher levels of virus specific neutralizing antibodies as well as B and T cell responses up to 8 months after vaccination compared to the same antigen delivered intramuscularly and conferred 100% protection against lethal challenge with the wild-type A/Vietnam/1203/04 virus 16 weeks after vaccination (Song et al. 2010b). In the case of seasonal influenza (H1N1 A/PR/8/34) VLPs a single dose was sufficient to confer long-term protective efficacy of influenza 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 for at least 14 months, that correlated with humoral systemic and mucosal

influenza virus-specific immune responses that were maintained for over a year (Quan et al. 2013).

In another study 5 µg of pandemic swine origin inactivated A/California/04/2009 influenza virus delivered once to BALB/c mice either with metal microneedles or subcutaneously elicited similar serum IgG and hemagglutination inhibition titers and 100% protection against lethal viral challenge 6 weeks after vaccination. However, six months after vaccination, the subcutaneous group exhibited a 60% decrease in functional antibody titers and extensive lung inflammation after challenge with 10×LD₅₀ of homologous virus, whereas the microneedle group maintained high functional antibody titers and IFN-γ levels, inhibition of viral replication, and no signs of lung inflammation after challenge. Microneedle vaccination conferred complete protection against lethal challenge as well as high numbers of bone marrow plasma cells and spleen antibody-secreting cells (Koutsonanos et al. 2011).

A single 3 µg dose of the subunit A/Brisbane/59/2007 vaccine with metal microneedles conferred complete protection against 5×LD₅₀ of mouse-adapted virus at 4, 12 and 36 weeks post-vaccination whereas intramuscular immunization conferred completed protection only at 4 and 12 weeks post vaccination (Koutsonanos et al. 2012). The decreased protection at the later time correlated with lower IgG2a titers and a 38% drop of HAI titers below 40 in the intramuscularly vaccinated group after 9 months, whereas all mice in the microneedle vaccinated group retained HAI titers above protective levels (HAI>40). The maintenance of these high levels of functional antibody titers throughout a period of nine months can be attributed to the higher number of influenza-specific antibody secreting cells (long-lived bone marrow plasma cells) detected in the microneedle immunized group.

3.1.3 Breadth of immunity—The continuous antigenic drift of influenza viruses mandates frequent changes of the vaccine composition. The need for annual revaccination against influenza is a burden on the healthcare system, and leads to low vaccination coverage rates, and makes timely vaccination difficult against pandemic strains, such as during the 2009 H1N1 influenza pandemic. Using a DNA vaccine encoding A/PR/8/34 influenza hemagglutinin that increases the viscosity of a coating formulation together with inactivated virus vaccine of the same strain, Kim et al succeeded in generation of robust immune responses and cross-protective immunity in mice (Kim et al. 2013).

Microneedles coated with a recombinant fusion protein comprised of *S. typhimurium* Phase I flagellin (FliC) and the conserved extracellular domain of the membrane-bound matrix protein 2 (M2e) from human influenza A viruses elicited strong humoral as well as mucosal antibody responses, and conferred complete protection against homo- and heterosubtypic lethal virus challenges (A/PR8, H1N1 and A/Philippines, H3N2) three months after vaccination (Wang et al. 2014). These results provide encouraging evidence that skin immunization can be effective in enhancing the breadth of immunity to influenza viruses.

3.1.4 Target cells for skin immunization—As discussed above, the skin epidermis contains densely packed LCs and keratinocytes, whereas dermis which lies beneath the epidermis is largely populated by dermal dendritic cells (DDCs) that are distinct from the

epidermal Langerhans cells populations based on their surface markers. LCs express differential levels of CD11b, CD205 int/high and more specifically CD207 (Langerin) while DCs express CD11b high, and CD205 low/int and are CD207 negative (Itano et al. 2003; Valladeau et al. 2000). Additionally, these two populations are characterized by differences in chemokine receptor expression, especially during the maturation and migration of LCs from tissues to draining lymph nodes (Locati et al. 2000; Sozzani et al. 2000). The presence of two types of antigen presenting cells, LCs and DDCs, classify the skin as an immunological organ (Romani et al. 2012). Additionally, the expression of Toll-like receptors (TLRs) on LCs, DDCs, and keratinocytes make it an ideal target for vaccine delivery (Abdelsadik and Trad 2011). These two types of APCs, in combination with other immunologically active cells residing in the skin including LC-like DCs, monocytes, and macrophages (Dupasquier et al. 2004), recognize and take up the antigen upon delivery in the skin, and migrate while undergoing maturation to the proximal lymph nodes where they prime naïve T and B cells thus initiating and shaping the adaptive immune responses. Both LCs and DDCs are involved in the process of T cell activation. Studies have demonstrated that in the absence of a stimulus, epidermal LCs and dermal DCs express low levels of major histocompatibility antigens (Banchereau and Steinman 1998).

Epidermal powder immunization (EPI) of mice with an influenza vaccine elicited consistently a higher hemagglutination inhibition (HAI) antibody titers than intramuscular (IM) injection using the same dose of vaccine. Vaccine controlled delivery to skin antigen-presenting cells increases the local innate immune responses and facilitates antigen presentation to naïve T and B lymphocytes which reside in the draining lymph nodes adjacent to site of delivery. The epidermal Langerhans cells (LCs) at the site of EPI were found to play an important role in the immune responses because depletion of LCs from the immunization site prior to EPI caused a significant reduction in the antibody response. Transfer of LCs isolated from the EPI sites to naïve mice induced a robust antigen-specific antibody response (Chen et al. 2004). In ex vivo experiments using freshly excised human skin, Pearton et al delivered H1 (A/PR/8/34) and H5 (A/VietNam/1203/04) VLPs with solid metal microneedles to the skin epidermis and they demonstrated 80% deposition of the coating within 60s of insertion. They further showed a dramatic reduction of CD207⁺ (Langerin⁺) cells in the epidermal sheaths within 48h of vaccine delivery, which simulated activation and migration of Langerhans cells (LCs) in the human skin environment (Pearton et al. 2010a; Pearton et al. 2010b). Gene expression mapping, ontological analysis, and qPCR revealed up-regulation of a host of genes responsible for key immunomodulatory processes and host responses, including cell recruitment, activation, migration, and T cell interaction following either ID or microneedle injection of VLPs; the response from the microneedles being more subtle (Pearton et al. 2013).

Detailed *in vivo* studies in the mouse model showed that metal microneedle delivery of inactivated influenza virus (A/PR/8/34) significantly increased interleukin 1 β (IL-1 β), macrophage inflammatory protein 1 alpha (MIP-1 α), macrophage inflammatory protein 2 (MIP-2), tumor necrosis factor alpha (TNF- α), and monocyte chemoattractant protein 1 (MCP-1) (del Pilar Martin et al. 2012). Although these cytokines were also induced upon insertion of blank microneedles, their levels were further increased by immunization using microneedles coated with influenza vaccine. Dendritic (CD11c⁺) cells that emigrated from

the vaccinated skin showed high expression levels of the cell surface markers MHC II and CD205 and, more than 50% had increased expression of the co-stimulatory markers CD40 and CD86 suggesting activation and maturation of skin-derived dendritic cells (Sparber et al. 2010). This phenotype is required for T cell priming, and such cells are likely to contribute to the strong immune response observed with microneedle vaccination.

3.2 Guinea pigs

Guinea pigs are an attractive model for skin immunization studies due to the similar thickness of guinea pig and human skin. Transcutaneous immunization of guinea pigs on the abdomen after skin pre-treatment with abrasion device to disrupt the stratum corneum with a patch containing a trivalent inactivated influenza vaccine in a wet fresh formulation or a dried, stabilized formulation showed that the dry patch was as effective as a wet patch in inducing serum anti-influenza IgG and HAI antibodies. The immunological potency of the vaccine product was not affected by one-year storage at 4°C or 25°C (Frolov et al. 2008). Moreover the dry patch could tolerate unexpected environmental stresses such as those that may be encountered during shipping and distribution (low-to-high temperatures of -20/25 and -20/40°C). Because of its effectiveness in vaccine delivery and its superior thermostability characteristics, the dry TIV patch represents a major advance for needle-free influenza vaccination.

Using 3M's solid Microstructured Transdermal System (sMTS), Kommareddy et al vaccinated guinea pigs with the 2008/2009 seasonal vaccine. The antibody titers to influenza virus were comparable to those observed with trivalent vaccine administered intramuscularly. The coated antigens were stable for at least 8 weeks upon storage at 4°C and room temperature, and also when subjected to freeze-thaw conditions (Kommareddy et al. 2013).

3.3 Rats

Influenza vaccine dose sparing was demonstrated with a delivery system from BD Technologies using hollow microneedles 1 mm long, affixed to a conventional syringe. Rats were selected for the study because of their thicker skin, which ensured reliable deposit of vaccine intradermally. Delivery of the whole inactivated virus provided up to 100-fold dose sparing compared to intramuscular injection. Intradermal delivery of the trivalent human vaccine also enabled at least 10-fold dose sparing for the H1N1 strain, and elicited levels of response across a dose range similar to those of intramuscular injection for the H3N2 and B strains. Furthermore, at least fivefold dose sparing from intradermal delivery was shown in animals immunized with multiple doses of DNA plasmid vaccine (Alarcon et al. 2007).

3.4 Chickens

The continuing outbreaks of highly pathogenic avian influenza (HPAI) H5N1 in avian species increase the risk of reassortment and adaptation of the virus to humans, and limit the capacity of egg-based vaccine production with severe economic repercussions. In chickens, protection was observed against heterologous strains of HPAI H5N1 after vaccination with a trivalent H5 serotype DNA vaccine with doses as low as 5 µg DNA given twice or 3 times by a needle-free (Agro-Jet®) device. The vaccination elicited comparable responses to

intramuscular immunization with hypodermic needles (Rao et al. 2008). Comparable immune responses were also observed between subcutaneous and Agro-Jet injector delivery of a water-in-oil emulsion of inactivated, recombinant H5N3 virus suspended in a proprietary adjuvant mixture (Poulvac Flufend I AI H5N3 RG) (Ogunremi et al. 2013).

3.5 Non-human primates

Rhesus macaques, which have an immune system and skin structure similar to humans, were used to further evaluate the immunogenicity of the influenza vaccine following epidermal powder immunization (EPI) with an influenza vaccine. Administration of unadjuvanted vaccine by EPI elicited HAI titers in the monkeys which were comparable to intramuscular injection. EPI with an influenza vaccine and QS-21 adjuvant significantly improved serum hemagglutination inhibition (HAI) titers when compared with antigen alone administered by EPI or by intramuscular (IM) injection using a needle and syringe (Chen et al. 2003).

4 Skin immunization with adjuvanted vaccines

Both circulating and mucosal antibodies are considered important for protection against infection by influenza virus in humans and animals. However, current inactivated vaccines administered by intramuscular injection using a syringe and needle elicit primarily circulating antibodies. As discussed above, a number of recent studies have demonstrated that skin immunization with influenza vaccines, using a variety of devices, results in immune responses which are superior to those observed using unadjuvanted vaccines administered by other immunization routes. However there has been limited information on the effects of adjuvants using this route of immunization.

Serum antibody responses to influenza vaccine following epidermal powder immunization (EPI) of mice were enhanced by co-delivery of cholera toxin (CT), a synthetic oligodeoxynucleotide containing immunostimulatory CpG motifs (CpG DNA), or the combination of these two adjuvants. A single dose of influenza vaccine containing CT or CT and CpG DNA conferred complete protection against lethal challenges with A/PR/8/34, whereas a prime and boost immunizations were required for protection in the absence of an adjuvant (Chen et al. 2001b). Co-delivery of influenza vaccine with CpG-DNA as adjuvant elicited Th1 type response whereas EPI with alum adsorbed DT promoted a predominantly IgG1 subclass antibody response, indicating that the use of appropriate adjuvants can produce an augmented antibody response and desirable cellular immune responses (Chen et al. 2001a). Depletion of LCs from the immunization site prior to EPI caused a significant reduction in the antibody response. Chen et al reported that skin immunization with EPI induced cytokine production by the target site cells important for the augmented immune responses and that LTR72, a genetically detoxified heat-labile toxin from *Escherichia coli* with a strong adjuvant effect in EPI, was found to bind to the keratinocytes of the epidermis, but not the LCs, and caused the production of elevated TNF-alpha and IL-12 cytokines in emigrating epidermal cells (Chen et al. 2004).

EPI of rhesus macaques with the 2000–2001 seasonal influenza vaccine and QS-21 adjuvant elicited significantly higher serum hemagglutination inhibition (HI) titers than antigen alone

administered by EPI or by intramuscular (IM) injection using a needle and syringe (Chen et al. 2003).

Application of an adjuvant to the skin has also been used to enhance the response to an injected influenza vaccine in young and aged mice (Guebre-Xabier et al. 2004) as well as in elderly human vaccinees (Frech et al. 2005). A trivalent liposomal vaccine was administered to patients by IM injection, and a skin patch containing the heat labile enterotoxin from *E. coli* was applied to stratum corneum-disrupted skin near the site of immunization in a subgroup of the patients. The adjuvant patch enhanced the seroconversion rate in the elderly patients to a level similar to that observed in healthy adult vaccinees.

Transcutaneous immunization of mice with whole inactivated influenza virus in the presence of cholera toxin (CT), a potent adjuvant for TCI, significantly enhanced immune responses against influenza virus antigen. Pretreatment of mouse skin with permeability enhancers/immunomodulators oleic acid (OA) and retinoic acid (RA) elicited increased levels of influenza virus-specific binding and neutralizing antibodies to levels equivalent to those induced by intranasal immunization with inactivated influenza virus. Oleic acid and particularly retinoic acid treatments differentially affected the pattern of cytokine production upon stimulation with influenza viral antigen and provided enhanced long-lasting protection (Martin Mdel et al. 2010; Skountzou et al. 2006).

Transdermal immunization by use of the PassPort system that creates 80 micropores within a 1-cm² area with a disposable filament attached to an applicator containing 3 µg of baculovirus-expressed H5 rHA protein from the A/Hong Kong/156/97 (H5N1; HK/156) virus either alone or with 25 µg of CpG oligodeoxynucleotide (TLR9 ligand) demonstrated complete protection following challenge with 10×LD₅₀ or 50×LD₅₀ H5N1 virus. In contrast 30 µg of R-848 (resiquimod hydrochloride, a TLR7 ligand) did not enhance the protective immune responses (Garg et al. 2007).

Some currently used adjuvants are lipid-based, and would be expected to interfere with formulations used to produce coated or dissolving microneedles. The use of toll-like receptor (TLR) ligands as adjuvants was investigated using microneedle delivery of an influenza subunit vaccine in mice (Weldon et al. 2012). The subunit vaccine alone was compared with vaccine coadministered with the TLR ligands, imiquimod or poly(I:C), individually or in combination. The inclusion of poly(I:C) did not enhance immune responses when compared to the non-adjuvanted vaccine. However the imiquimod-adjuvanted vaccine elicited higher levels of virus-specific antibodies and increased hemagglutination inhibition titers, as well as inducing a robust IFN-γ cellular response. These responses correlated with improved protection to challenge infection when compared to microneedle immunization with influenza subunit vaccine alone, as well as reduced viral replication levels in the lungs.

In another study, the plant-derived saponin Quil-A was used as an adjuvant with influenza vaccine in mice. The anti-influenza IgG and HAI responses induced by very low antigen doses, 6.5 ng of vaccine, were similar to responses induced by IM injection using 900-fold

higher doses of vaccine, demonstrating a potent dose-sparing effect by delivery of the antigen and adjuvant to skin (Fernando et al. 2012).

5 Conclusions and future directions

Skin immunization has generated a high level of interest because of its immunological advantages, including the induction of stronger and longer-lasting immunity when compared with conventional intramuscular immunization. A number of delivery systems are at various stages of development, and studies are in progress in various animal models and to a lesser extent, in clinical trials. In addition to its immunological advantages, skin vaccination also offers potential logistical advantages. Dried vaccine formulations are used in many approaches, and provide enhanced vaccine stability which can enable global vaccine distribution without the need for maintaining a cold chain, with a resulting decrease in cost. A particularly noteworthy feature is the avoidance of hypodermic needles, thus enhancing the acceptability of vaccination by subjects who presently remain unvaccinated because of fear of the pain associated with needle sticks. Devices are also being developed which would be suitable for self-administration of vaccines, and a recent study (Norman et al 2014) has provided evidence that this approach could significantly enhance influenza vaccination coverage by improving patient acceptability.

Whereas there have been many studies of new approaches in animal models, only a limited number have been advanced to clinical studies. More rapid progress in such studies is anticipated in future. Other aspects which remain largely undeveloped include the use of adjuvants and identification of novel adjuvants which are specifically designed to enhance responses to skin immunization. There is also very limited information on the ability of skin immunization to elicit mucosal immune responses, which are known to provide the most effective means of preventing infection by influenza viruses.

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Figure 1. Lung histopathological examination after lethal infection

Lung tissue sections from naïve, subcutaneously immunized and microneedle immunized mice challenged with $10 \times LD_{50}$ of live virus six months after immunization were stained with hematoxylin and eosin stain. The histopathology of the lung tissues collected upon challenge of immunized or unimmunized mice 6 months post immunization showed clear signs of profound pulmonary inflammation in the unimmunized infected (A) or subcutaneously immunized animals (B). Peribronchial and intra-alveolar inflammation was accompanied by significant pulmonary edema and cellular infiltration mainly consisting of neutrophils. The group of microneedle immunized mice did not show any signs of inflammation (C).

Figure 2. Protective efficacy against H1N1 A/Brisbane/59/2007 lethal infection

Body weight changes and survival rates were recorded after lethal challenge with $5 \times LD_{50}$ of live A/Brisbane/59/2007 virus in microneedle (MN) and intramuscularly (IM) immunized and naïve mice at 3 months (A,B) and nine months (C,D) post-vaccination. N: naïve mice. Data represent the mean $\pm$ SEM.

Table 1

Examples of delivery systems for skin immunization with influenza vaccines in various species

Delivery System	Vaccine	Reference
HUMAN		
Needle-free jet injector	Trivalent split vaccine	(Jackson et al. 2001)
Epidermal powder injection	Trivalent split vaccine	(Dean and Chen 2004)
Hypodermic needle (Mantoux method)	Trivalent split vaccine	(Belshe et al. 2007; Kunzi et al. 2009)
Hollow microneedle (30 gauge) (br/)(BD Soluvia)	Trivalent split vaccine	(Laurent et al. 2007) (Icardi et al. 2012)
Epidermal (topical) application	Tetragrip (Tetanus-influenza)	(Combadiere et al. 2010)
MOUSE		
Coated metal microneedles	Monovalent subunit vaccine	(Koutsonanos et al. 2012)
	H1N1 VLPs	(Quan et al. 2010a)
	H5N1 VLPs	(Song et al. 2010a)
	Whole inactivated H3N2	(Koutsonanos et al. 2009)
	Whole inactivated H1N1	(Zhu et al. 2009)
Nanopatch™	Trivalent subunit vaccine	(Fernando et al. 2010)
	Dissolving microneedles	Whole inactivated H1N1 (Sullivan et al. 2010)
		Trivalent subunit vaccine (Kommareddy et al. 2012)
	Permeability enhancers	Whole inactivated H1N1 (Skountzou et al. 2006)
	Blank microneedles and topical application	Influenza subunit vaccine (Ding et al. 2009)
	Electroporation	Recombinant H5 HA (Garg et al. 2007)
	Epidermal powder injection	Trivalent split vaccine (Chen et al. 2003)
GUINEA PIG		
	Coated microneedles	Trivalent subunit vaccine (Kommareddy et al. 2013)
	Dry skin patch	Trivalent split vaccine (Frolov et al. 2008)
RAT		
	Microneedles	Trivalent split vaccine (Alarcon et al. 2007)
CHICKEN		

Delivery System	Vaccine	Reference
HUMAN		
	Jet injector vaccine recombinant	H5 DNA Inactivated vaccine
NON-HUMAN PRIMATE		
	Epidermal powder injection	Trivalent vaccine (Chen et al. 2003)