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DNA Vaccine Delivery and Improved Immunogenicity

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Abstract
The promise of DNA vaccines is as compelling today as it was more than a decade ago. Ease of manufacture, stability at ambient temperatures without the need for a cold chain and its ability to mimic natural infections and elicit appropriate immune responses makes this vaccine platform extremely attractive. Although, human clinical trials of DNA vaccines have yielded less than optimal results, the approval and licensing of a few veterinary vaccines is testimony to the proof-of-concept and the hope that licensed DNA vaccines for human use may not be too far away. Delivery and targeting of immunologically relevant cells appears to be the major hurdle in maximizing the immunogenicity of DNA vaccines. Several different approaches that are currently pursued in achieving this objective are discussed.

Introduction

The use of naked DNA is one of many approaches to developing vaccines to protect humans and animals against infectious pathogens. To date there are no DNA vaccines licensed for human use, but there are several that are licensed for fish and animals (Kutzler and Weiner, 2008). The demonstrated efficacy of DNA vaccines in animals provides prospects that this platform could eventually lead to a licensed DNA vaccine for human use.

DNA immunization has many advantages over other vaccine platforms including product stability, ease of manufacturing and ease of modification to match any mutations or genetic shifts that change the antigenic profile of the target organism. DNA immunization is used also in combating some malignancies. Despite these advantages together with successful demonstrations of protective efficacy in small and large animal models, DNA vaccines for a variety of infectious pathogens yielded somewhat disappointing results in human clinical trials (Kutzler and Weiner, 2008). There are a number of reasons for this poor performance but a major reason is thought to be because of insufficient antigenic load due to inefficient expression of the target antigenic proteins in immunologically relevant cells and tissues. The low levels of protein expression are largely due to poor uptake of the DNA vaccine plasmids by antigen presenting cells (APC). To facilitate better uptake, the route of DNA vaccine delivery may be important. Accordingly, scientists have studied alternate routes and methods of delivering DNA vaccines *in lieu* of the usual subcutaneous and intramuscular routes using hypodermic needles. Transcutaneous, mucosal and microneedle application, along with jet injection and electroporation are all methods that have been tested to improve the immunogenicity of DNA vaccines. The aim of this chapter is to review the various DNA vaccine delivery strategies used to improve immunogenicity. The use of electroporation to deliver and improve immune responses to DNA vaccines will be covered elsewhere. Due to paucity of *in vivo* data, efforts in the development of lipopolyplexes as efficient vehicles for delivery of DNA (Rezaee et al., 2016) is not included.

Transcutaneous Delivery

The skin and mucosal surfaces are rich in antigen presenting Langerhans cells (LC) that serve as a first line of defense against infectious pathogens. A form of dendritic cell (DC), LC are particularly important in generating immune responses against vector borne viral pathogens

like dengue and chikungunya where the infectious agent is inoculated into the skin by mosquitoes during the blood feeding process. LC take up the infectious pathogen, process the pathogen-specific antigens and then travel to regional lymph nodes where an immune response is initiated by the presentation of the MHC-complexed foreign antigens to resident T cells.

While there are many examples of medications that can be effectively delivered transcutaneously, there are no vaccines that are FDA-licensed for delivery via the skin. Using drug delivery as a model, researchers have begun to explore ways to take advantage of the APC-rich skin and mucosal surfaces as a way to elicit protective immune responses against infectious agents.

Delivery by patch

The stratum corneum poses a significant obstacle with regard to the penetration of the vaccine to the epidermal and dermal layers where keratinocytes and LC reside. Therefore, transcutaneous immunization often involves an initial step where mild abrasion of the stratum corneum occurs prior to application of the vaccine. This is followed by an occlusive dressing that allows time for the vaccine to absorb into the skin. In a recent study, delivery of influenza nucleoprotein DNA vaccine using a dry coated densely packed microprojection array (nanopatch) was shown to induce potent antibody and CD8+ T cell responses in mice (Fernando et al., 2016).

DNA Tattooing

A novel approach to transcutaneous (TQ) administration of DNA vaccines is tattooing. DNA tattooing produced improved T cell responses to an HIV DNA vaccine in a non-human primate model. This method of delivery involves applying the DNA vaccine directly to the shaved skin surface and using a rotary tattoo machine to introduce the plasmid immunogen into the dermis. Compared to IM delivery by needle, DNA tattooing generated 24-fold higher levels of antigen specific IFN-γ responses four weeks following the first application. Similar increases were noted four weeks after the second injection (Verstrepen et al., 2008). Whether the improved immunogenicity resulted in improved protective efficacy was not studied. Pokorna et al. demonstrated both enhanced cellular and humoral immune responses to a papillomavirus type 16 DNA vaccine delivered by tattooing in a murine model (Pokorna et al., 2008).

But while TQ delivery of DNA vaccines by tattooing of the naked DNA to the skin produced promising results in animal models, there are no published human clinical studies demonstrating improved immunogenicity compared to parenteral delivery. There are however several studies using particle-mediated delivery of DNA where colloidal gold particles coated with the DNA vaccine are injected into the epidermis by a ballistic gene gun. Delivery by this method enhances vaccine uptake and antigen presentation by LC and DC (Porgador et al., 1998).

Intradermal Delivery of DNA Vaccines

Gene Gun

There are several published human clinical trials evaluating gene gun delivery of DNA vaccines targeting hepatitis B, influenza, malaria and hantavirus (Fuller et al., 2006). A recent clinical trial involved Hantaan virus (HTN) and Puumala virus (PUUV) DNA vaccines coding for M segment-derived glycoproteins Gn and Gc (Boudreau et al., 2012). The vaccines were administered alone (8μg DNA/4μg gold) and in combination (4μg HTN+ 4μg PUUV/4 μg gold particles). Both the HTN and PUUV vaccines were well tolerated with no vaccine-related serious adverse events observed. Despite high neutralizing antibody levels being achieved in some individuals, the seroconversion rates were less than 50%. Because of dose limitations and the difficulty of adapting gene gun to routine use, use of the this methodology has not been aggressively pursued.

Delivery By Microneedles

Microneedles (MN) are another effective way of achieving transdermal delivery of vaccines. This method of vaccine delivery has attracted a lot of attention in recent years due mainly to advances in fabrication and material engineering to produce precise MN arrays. However, the only commercial application of MN for vaccine delivery has been an influenza vaccine delivered by a single, 1.5 mm long, hollow MN (Atmar et al., 2010; Eizenberg et al., 2011). Using this device, superior immunogenicity in elderly subjects was reported with lower than standard dose of influenza vaccine (Holland et al., 2008). The device's single needle, which is much longer

than most current MN arrays, makes it arguable if this is truly MN technology. A recent review summarizes current methods and applications of MN technology and various MN platforms (Vrdoljak, 2013). Although MN vaccination in a number of animal models for vaccines based on proteins and inactivated viruses (Matsuo et al., 2012; Prow et al., 2010; Weldon et al., 2012), virus-like-particles (Corbett et al., 2010; Quan et al., 2010), live viruses (Bachy et al., 2013; Dean et al., 2005; Edens et al., 2013) and bacterial agents and toxins (Bal et al., 2010; Mikszta et al., 2002) has been reported, we will limit our discussion here to applications with DNA vaccines. To date, MN methodology has been applied only in clinical trials of influenza vaccines. In a safety and efficacy study of influenza vaccine delivered by MN to healthy adults, Van Damme et al. (2009) reported dose sparing, wherein similar antibody responses were observed using a tenth of the standard dose delivered intramuscularly.

Early work with MN delivery of DNA vaccines involved two stages; pretreatment of skin with solid metal MN array followed by topical application of vaccine to pretreated area of skin. Using this technique with hepatitis-B DNA vaccine in mice, Mikszta et al. (2002) reported better humoral and cellular immune responses compared to IM or ID vaccination. More recent studies have used MN arrays coated with dry DNA vaccine. Although hollow and dissolving MN arrays have been used to deliver live virus or subunit vaccines, solid coated MN arrays have become the mainstay of DNA vaccines. MN arrays used vary widely from a row of 5 needles 700 μM long (Gill et al., 2010) to polycarbonate or silicone patches (0.5 to 1 cm^2) containing greater than 3000 projections (Chen et al., 2010; Kim et al., 2014). Use of frozen living human skin explants (Pearton et al., 2012) can be very effective in optimizing various parameters of MN fabrication and methods of vaccine delivery.

Percutaneous injection of 3 μg of a DNA plasmid encoding influenza hemagglutinin (HA) into mice using a solid coated array of five microneedles, resulted in superior immune responses and protective efficacy compared to IM delivery of 10 μg DNA (Song et al., 2012). Increasing the MN vaccination dose to 10 μg DNA further enhanced immune responses and protective efficacy. For coating solid MN with protein or virus based vaccines, it is necessary to use viscosity enhancing agents such as carboxymethyl cellulose in the formulation. These agents could potentially lead to loss of activity of the antigen coated. In one study, Kim et al. (2013) took advantage of the viscosity of DNA and coated MN arrays with a mixture of inactivated influenza virus vaccine and influenza HA DNA vaccine, the DNA vaccine replacing CM cellulose. Co-immunization of mice with this inactivated and DNA vaccine mixture against A/PR8 influenza strain generated robust antibody responses to A/PR8. In addition, such co-immunization also led to generation of heterologous responses and cross-protection against the 2009 H1N1 strain. Kask and coworkers (Kask et al., 2010) compared IM delivery of a herpes simplex virus type 2 (HSV-2) DNA vaccine to delivery using a nanopatch of densely packed DNA vaccine coated silicone MN array. After 2 vaccinations of a 1 μg dose of DNA, 50% and 92% of the mice seroconverted by IM and MN delivery respectively. Protection from challenge was comparable when 10 μg DNA was delivered by IM injection or MN vaccination, but at 1 μg dose, MN vaccination resulted in better survival. A hepatitis C virus DNA vaccine delivered by MN was shown to be just as effective as more invasive needle injection or gene-gun delivery (Gill et al., 2010). To increase cellular uptake of DNA, Yin et al. (2013) used a hepatitis B virus DNA vaccine complexed with P123-modified polyethyleneinine (P123-PEI). Mice vaccinated with this complex using a silicone MN array responded with significantly higher HBV antibody and T cell responses compared to traditional needle injected mice.

Mucosal Immunization

Mucosal surfaces offer an advantage over transcutaneous immunization due to the absence of a stratum corneum. Potential access routes for muscosal immunization include the oral cavity, respiratory and intestinal tracks. The surfaces of these areas differ in structure and antigen-presenting cellular composition, and therefore the strategies to induce protective immune responses differ. The stratified squamous epithelial cell layer of the oropharynx contains mobile DC, which take up and process antigens from the mucosal surface and transport them to nearby lymphoid tissues to initiate the pathogen-specific immune response. In contrast, respiratory and intestinal mucosa are composed of a single epithelial layer that allows antigens to move from the mucosal surface to submucosal

sites via tight junctions between cells. The microfold cells (M cells) present in the epithelium are responsible for taking up and processing foreign antigens for presentation to T and B cells resident in respiratory and gut lymphoid tissues (Peyer's patches).

Mucosal immunization is well suited for pathogens that invade and infect via the mucosal route. Neutralizing the infectious agent by preventing attachment and entry across the mucosal surface is a key component of protection. Current licensed vaccines approved for mucosal immunization include, poliomyelitis, cholera, typhoid, rotavirus and influenza. Mucosal immunization with DNA vaccines was not widely pursued given the tremendous inefficiency by which the DNA itself can transit to resident antigen presenting cells for processing and protein expression. DNA immunization via the mucosal route is however being explored using bacterial vectors.

Delivery using bacterial vectors

Attenuated bacteria, because of their innate ability to penetrate mucosal surfaces, were used as vectors to enhance the delivery of DNA vaccine plasmids to antigen presenting cells (Daudel et al., 2007). Organisms employed for this purpose include *Salmonella*, *Shigella*, *E. coli, Lactobacilli, Lactococci*, *Vibrio cholera* and *Listeria*. Using this mechanism, the bacteria carrying the vaccine plasmid traverse the mucosa and are ingested by phagocytes and antigen-processing DC cells, with the subsequent release of the DNA plasmid into the cytosol. The plasmid then makes its way to the nucleus and is expressed resulting in endogenous antigens that are complexed with MHC class I molecules. This results in the generation of CD8 effector cells. Cross priming with the production of both CD8 and CD4 responses that gives rise to cellular and humoral immunity occurs when DNA vaccine expressed antigens from other bacteria vector-infected cells are taken up by antigen presenting cells and processed via both the MHC class I and II pathways.

Bacteria licensed for human use are *Salmonella enterica* serovar Typhi Ty21a (oral typhoid vaccine) and *Vibrio cholera* CVD103 HgR, with most work done with *Salmonella enterica*. Using *Salmonella* as a delivery vector is logical given the safety profile and effectiveness of the oral *Salmonella* vaccine. Fraillery et al. reported on the use of this typhoid vaccine strain along with two other *Salmonella* strains to deliver DNA vaccine plasmids expressing the HPV16 L1 capsid protein to mice (Fraillery et al., 2007). Following intranasal administration of the vaccines, high levels of anti-HPV neutralizing antibodies were detected both in serum and in genital secretions using the vaccine *Salmonella* strain, while the other strains generated low level responses. Strong CD4+ T cell responses were seen as well.

Improved immunogenicity of an influenza DNA vaccine expressing the H5N1 HA protein was demonstrated using *Salmonella enterica* compared to DNA alone. Following a single dose of vaccine administered orally by gavage, chicks developed significantly higher HI antibody titers compared to DNA alone given IM (Jazayeri et al., 2012). In an earlier study by Jiao et al., a *Salmonella enterica* vectored DNA vaccine against infectious bronchitis virus (IBV) was evaluated in chickens and shown to elicit both systemic and mucosal antibody responses that provided protection against IBV comparable to the live attenuated IBV vaccine H120 (Jiao et al., 2011).

To enhance the safety and immunogenicity of this DNA vaccine delivery platform, Kong et al. introduced self-destructing genetic modifications into the *Salmonella* Typhimurium bacteria that resulted in delayed attenuation. Additional changes were made to increase the organism's host invasiveness and ability to escape from the endosome and release the DNA vaccine into the cytosol. Further genetic modifications were made to enhance nucleus targeting and decrease the organism's ability to induce apoptosis, thus increasing the protein expression efficiency of the released DNA vaccine plasmid (Kong et al., 2012). The improved, recombinant attenuated *Salmonella* vaccine strains (RASV) were used to evaluate the protective efficacy of a DNA vaccine that encoded the HA antigen of influenza A/WSN/33 virus. Following oral administration of the vaccines, mice elicited both humoral (systemic and mucosal) and cellular responses that provided significant protection against intranasal challenge with 100 LD_{50} of rWSN influenza virus.

To date, the testing of this delivery platform in humans has not been met with much success. Frey and others conducted a Phase 1 dose-escalation study of three genetically attenuated self destructing *Salmonella enterica*-vectored vaccines that express the *Streptococcus pneumonia* surface protein A (PspA) (Frey et al., 2013). The study design involved four dosage arms (10^7, 10^8, 10^9, 10^{10} CFU), with each arm consisting of three groups of five volunteers. While the vaccines were demonstrated safe and well tolerated, only one volunteer in the highest dose group showed a four-fold rise in ELISA antibody titer against PspA. The reason for the limited responses was not known, but pre-existing cross-reactive antibodies to bacterial components were postulated to have masked responses to the vaccine.

Another bacteria-based vaccine delivery platform used to enhance the immunogenicity of DNA vaccines is the bacterial ghost (BG) system (Langemann et al., 2010; Mayr et al., 2005b). This system uses non-living bacterial envelopes generated by protein E lysis of gram-negative bacteria. Because the cell surface structures on the envelopes remain intact, the BG retain the capability to adhere to mucosal cell surfaces. The BG can be engineered to express foreign antigens for use as oral or parenteral vaccines. Wen et al. described the use of this platform to develop an HIV vaccine. *Salmonella typhi* Ty21a bacterial ghost were generated and loaded with a DNA vaccine that expresses full-length gp120 and the ectodomain of gp41 (Wen et al., 2012). The results demonstrated a significantly higher systemic and mucosal anti-gp120 antibody response compared to naked DNA. Further work is needed to evaluate the protective nature of the enhanced antibody responses. In another murine study, Mayr et al. protected 93% of mice against lethal challenge with enterohemorrhagic *E. coli* (EHEC) using an orally administered EHEC-derived BG vaccine (Mayr et al., 2005a).

DNA Vaccine Delivery Using Nanoparticles

Delivering DNA by way of particles other than colloidal gold was explored with limited success. Nanoparticles are being developed as carriers for vaccines and drugs and offer advantages such as stability of the biologic, sustained release, modulation of APC and adjuvant effect among others (recently reviewed by Torres-Sangiao et al., 2016; Shah et al., 2015) and have received attention from DNA vaccinologists as a method to enhance the immunogenicity of DNA vaccines. Poly(lactic-co-glycolic acid) (PLGA) nanoparticles containing DNA vaccine plasmids expressing antigens of lymphocystis disease virus given orally to Japanese flounder, demonstrated systemic expression of the antigen as far out as 90 days (Tian and Yu, 2011). Lipodine liposomal particles expressing hepatitis B antigens were administered orally in mice and induced greater levels of secretory anti-hepatitis IgA compared to levels generated by IM administration of naked DNA plasmids expressing the same antigen (Perrie et al., 2002). More recently, Poecheim et al. (2016) reported increased antibody and T cell response (Th1 type) in animal model when a *Mycobacterium tuberculosis* antigen 85A DNA vaccine was formulated with trimethyl chitosan (TMC) nanoparticles. Significant additional T cell responses were reported when muramyl dipeptide, an immunostimulatory agent, was also included. A TMC formulated anti-caries DNA vaccine elicited significantly higher IgG antibody in rats compared to unformulated naked DNA, and that intranasal immunization resulted in higher IgG and IgA antibody compared to intramuscular immunization (Li et al., 2016). Zhao et al. (2016) have reported an initial burst release followed by an extended sustained release of a Newcastle disease virus DNA vaccine formulated with silver and silicon dioxide (Ag@SiO2) nanoparticles. Intranasal immunization of chickens with this formulation induced high titer serum antibody. A botulinum neurotoxin (BoNT) DNA vaccine delivered using PLGA nanoparticles induced higher antibody response compared to delivery of naked DNA and also protected vaccinated mice from a lethal dose of BoNT (Ruwona et al., 2016).

Needle-free Jet Injection of DNA Vaccines

The popularity of needle-free delivery of drugs and vaccines during the Second World War came to pass quickly because multi-dose syringes were used for mass vaccinations and it was soon realized that blood borne pathogens could spread by the practice. In recent years, this method of delivery has made a comeback due to sophisticated designs of single use syringes. Both spring activated and compressed gas mechanisms for delivery are in use. Although devices from Bioject and Pharmajet are approved for clinical use, and are widely used in certain scenarios, use of these devices for delivering DNA vaccines has been limited to

Table 1. DNA delivery methods other than electroporation

DNA Delivery Method	Route*	Animal Testing**	Clinical Trial**	Immunogenicity
Patch Application	TQ	1	NK	Stratum corneum decreases effectiveness
DNA Tattoing	TQ	2	NK	Clinical utility remains to be seen
Gene Gun/Colloidal Gold	ID	3	9	Dose limiting
Nanoparticles	MC	4	NK	Clinical utility remains to be seen
Attenuated Bacteria	MC, IM	5	10	Evaluated Clinically
Bacterial Ghosts	MC, IM	6	NK	Less endotoxin effect because of cell association
Microneedle	ID	7	11	Evaluated Clinically
Needless Jet Injection (Biojector, Pharmajet, Zetajet)	ID, IM	8	12	Evaluated clinically

* TQ, transcutaneous; ID, intradermal; MC, mucosal; IM.
** References related to the indicated delivery method are listed; 1) Fernando et al. 2016; 2) Verstrepen et al. 2008, Pokorna et al. 2008; 3) Porgador et al. 1998; 4) Tian et al. 2011, Perrie et al. 2002, Poecheim et al. 2016, Li et al. 2016, Zhao et al. 2016, Ruwona et al. 2016; 5) Daudel et al. 2007, Fraillery et al. 2007, Jazayeri et al. 2012, Jiao et al. 2011, Kong et al. 2012; 6) Langemann et al. 2010, Mayr et al. 2005a/b, Wen et al. 2012; 6) Atmar et al. 2010, Eizenberg et al. 2011, Matsuo et al. 2012, Prow et al. 2010, Weldon et al. 2012, Corbett et al. 2010, Quan et al. 2010, Bachy et al. 2013, Dean et al. 2005, Edens et al. 2013, Bal et al. 2010, Mikszta et al. 2002, Gill et al. 2010, Chen et al. 2010, Kim et al. 2013, 2014, Pearton et al. 2012, Song et al. 2012, Kask et al. 2010, Yin et al. 2013; 8) Rao et al. 2006, Ault et al. 2012, Gorres et al. 2011, Borggren et al. 2016; 9) Fuller et al. 2006, Boudreau et al. 2012; 10) Frey et al. 2013; 11) Holland et al. 2008, Van Damme et al. 2009; 12) Cattamanchi et al. 2008, Bakari et al. 2011, Sandstrom et al. 2008, Resik et al. 2015, Soonawala et al. 2013, Ledgerwood et al. 2012; NK) none known.

animal studies and a few early phase clinical trials. Delivery by this method is thought to lead to a wider tissue distribution of vaccine leading to increased uptake and immunogenicity. However, mixed results have been reported. Rao et al. (2006) compared immunogenicity of a HIV1 DNA vaccine delivered by needle, needle-free Biojector (Bioject Medical Devices) and Miniject (Valeritas). Cynomolgous macaques immunized by Biojector or Miniject elicited similar immune responses to target antigens as animals vaccinated by needle and syringe. Likewise, similar immune responses and protection from challenge were observed in mini pigs vaccinated with pandemic and swine influenza virus DNA vaccine or ponies vaccinated with equine influenza virus DNA vaccine using either the needle and syringe or the Pharmajet system (Ault et al., 2012; Gorres et al., 2011). In a clinical trial, a HSV-2 glycoprotein D-2 (gD2) based DNA vaccine was administered at 100 µg to 3 mg doses to volunteers via the IM route using Biojector. Only a small number of volunteers developed gD2-specific CTL and lymphoproliferative responses indicating that a much higher dose of DNA may be needed (Cattamanchi et al., 2008). In another clinical trial of an HIV1 vaccine using DNA to prime and a recombinant MVA (modified vaccinia Ankara) to boost (heterologous prime-boost study), DNA delivery via the IM and ID routes using Biojector were investigated. Ninety-seven percent (37/38) of subjects mounted HIV1-specific IFN-gamma and/or lymphoproliferative response. A low ID dose of DNA was as effective as the high IM dose in priming for MVA boosting (Bakari et al., 2011; Sandstrom et al., 2008). There have been suggestions that intradermal delivery of DNA vaccines by jet injection may result in improved immune responses. Significant dose sparing has been reported by intradermal administration of licensed non-DNA vaccine (inactivated polio virus vaccine) using the Biojector 2000 or the new Biopen (both from Bioject Medical Devices) and Tropis (Pharmajet) systems in clinical trials (Resik et al., 2015; Soonawala et al., 2013). Cross-reactive humoral and cellular immune responses to multiple strains of influenza strains were reported in young pigs vaccinated by needle-free intradermal delivery of a polyvalent

influenza DNA vaccine (Borggren et al., 2016). However, in a clinical study of H5 influenza virus DNA vaccine, no such benefit was reported. Similar immune responses were reported when the same dose of vaccine was administered intramuscularly (Biojector) or intradermally (needle or Biojector) (Ledgerwood et al., 2012).

Conclusions and the Way Forward

DNA immunization has many advantages over conventional immunization platforms, including ease of production and DNA vaccine stability. While DNA vaccines show great promise in small and large animal studies, to date there are none licensed for use in humans. With increased understanding of the mechanisms that lead to immune responses to DNA vaccines, improved humoral and cellular immunity in humans after DNA vaccine immunization remains a possibility. Efficiently delivering the DNA vaccine plasmid to appropriate immune cells for expression and processing of the immunogen is germane to optimizing this platform for developing a product that meets FDA licensure guidelines for use in humans. While the vaccine delivery platforms and methodologies described in this chapter (Table 1) show improved targeting of antigen presenting cells and increased immunogenicity, continued work is needed to advance them to early and late phase human clinical testing.

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