Using Plasmids as DNA Vaccines for Infectious Diseases

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ABSTRACT DNA plasmids can be used to induce a protective (or therapeutic) immune response by delivering genes encoding vaccine antigens. That naked DNA (without the refinement of coat proteins or host evasion systems) can cross from outside the cell into the nucleus and be expressed is particularly remarkable given the sophistication of the immune system in preventing infection by pathogens. As a result of the ease, low cost, and speed of custom gene synthesis, DNA vaccines dangle a tantalizing prospect of the next wave of vaccine technology, promising individual designer vaccines for cancer or mass vaccines with a rapid response time to emerging pandemics. There is considerable enthusiasm for the use of DNA vaccination as an approach, but this enthusiasm should be tempered by the successive failures in clinical trials to induce a potent immune response. The technology is evolving with the development of improved delivery systems that increase expression levels, particularly electroporation and the incorporation of genetically encoded adjuvants. This review will introduce some key concepts in the use of DNA plasmids as vaccines, including how the DNA enters the cell and is expressed, how it induces an immune response, and a summary of clinical trials with DNA vaccines. The review also explores the advances being made in vector design, delivery, formulation, and adjuvants to try to realize the promise of this technology for new vaccines. If the immunogenicity and expression barriers can be cracked, then DNA vaccines may offer a step change in mass vaccination.

INTRODUCTION: HISTORY, ADVANTAGES, REGULATION, AND LICENSED PRODUCTS

The observation that antigens encoded by DNA could induce an immune response when injected in vivo was first made in the 1960s using DNA from mouse tumors (1). Humoral (antibody) responses were subsequently observed following injection of DNA from polyoma (2) and hepatitis B viruses (3). The first study to specifically look at DNA as an immunogen was performed in the early 1990s using human growth hormone as a model antigen, delivered by a biolistic transfer device (gene gun) (4). This was quickly followed by studies showing that not only antibody, but also cytotoxic T cells could be induced by DNA delivery (5–7).

Since then, DNA vaccines have been proposed for a diverse range of diseases, from cancer (8) to allergy (9) and autoimmunity (10), but this review will focus on prophylactic DNA vaccines for infections. Recently, a database (DNAVAXDB) was developed that encompasses the wide range of approaches tested (http://www.violinet.org/dnavaxdb/index.php) (11). This broad range of targets and diseases is reflective of the fact that DNA vaccines are significantly easier and cheaper to develop for any desired antigen than protein vaccines. The speed of development is a key advantage of DNA vaccines, especially for pandemic outbreaks. The switch of many labs from cloning target genes directly from the pathogen to custom synthesis (12) has further hastened the process. In addition, it has made it safer and easier. DNA vaccines are widely described as being more thermostable than other vaccine approaches. This assumption is based on...
characteristics of the DNA molecule, but no studies have been published that formally demonstrate this. DNA vaccines should have a better safety profile than live attenuated vaccines, with no risk of reversion to virulence, as they only encode individual genes from the pathogen. There is also little reported inflammation following DNA vaccination in clinical trials (Table 1). Expression from eukaryotic cells means DNA vaccines may mimic the posttranslational modifications that viral proteins undergo, potentially inducing a more appropriate immune response. This is advantageous in the induction of antibodies that recognize the tertiary structure from bacterial pathogens. With the correct targeting sequences, DNA vaccines may also be effective for the expression of membrane proteins, which are traditionally hard to express by conventional recombinant protein approaches.

Three DNA vaccines have been successfully licensed in veterinary practice. The first licensed DNA vaccine was against West Nile virus (Fort Dodge Animal Health, part of Wyeth/Pfizer), approved for use in horses by the USDA (13), though it is not commercially available. Another DNA vaccine, for infectious hematopoietic necrosis virus (Apex-IHN, Novartis Animal Health), has been approved for use in farmed salmon and trout (14) in Canada, but not in the United States or Europe. Apex-IHN is delivered by a single intramuscular injection of a small volume (50 μl). A therapeutic cancer vaccine expressing human tyrosinase (Oncept, Merial), used for the treatment of oral canine melanoma, was licensed by the USDA in 2010 (15), but a randomized, placebo-controlled clinical trial is required to confirm efficacy in improving postsurgery survival times (16). Oncept is delivered using a transdermal/needle-free system and requires four shots at 2-week intervals.

Initially, additional safety concerns with DNA vaccines led to specific regulatory requirements compared to other vaccines. These included concerns over the generation of an anti-DNA autoimmune response, the induction of tolerance to the vaccine antigen, and the transfer of antibiotic resistance. One other consideration about DNA vaccines is integration into the host genome. This has been a potential safety concern, as integration may induce tumors by activating oncogenes, but this appears to be a rare event (17). The FDA guidelines only require preclinical integration studies when more than 30,000 copies of plasmid are detected per microgram of host DNA. While some studies suggest that integrative DNA vaccine constructs can improve the T cell response (18), constitutive expression of vaccine antigen may actually be counterproductive for the optimum immune response, especially memory responses that require a contraction of the effector pool (19). The success of phase I clinical trials in demonstrating DNA vaccines to be safe and well tolerated has led to the relaxation of the initial safety concerns (Table 1). Specific guidelines for DNA vaccines have been published by the FDA (20) and the WHO (21). These guidelines cover issues of good manufacturing practice (GMP) specific to DNA vaccines, including sequencing of the plasmid, the required degree of supercoiling, and removal of other macromolecules from the bacterial preparation used to generate the plasmid, particularly lipopolysaccharide. The preclinical checks for DNA vaccines are very similar to other vaccine types including toxicity and immunogenicity and, specifically, examining the effect of any genetic adjuvant included. We would argue for the further relaxation of regulation of DNA vaccines that come from the same manufacturer targeting variants of the same antigen, e.g., influenza hemagglutinin or the HIV envelope protein, being treated as a single product for preclinical toxicity. This would allow for the rapid development to market of a vaccine in response to a pandemic (influenza) or multiple iterations of similar antigens for experimental human vaccine studies (HIV).

In spite of the relaxation of FDA guidelines around DNA vaccines, there is still a drive to improve DNA vaccine manufacture. There are some safety concerns about the presence of antibiotic-resistance genes needed for selection and growth in bacteria, particularly related horizontal transfer to commensals (22). Additionally, antibiotic selection can reduce the plasmid yield (23). While large-scale, cost-effective production of DNA vaccines through bacterial fermentation is relatively simple, a limiting step in production lies with the purification of the DNA, particularly supercoiled DNA, which is similar in size to many contaminants (24). An additional concern is that bacterial fermentation steps currently used in plasmid preparation introduce endotoxins, and stringent certified quality control checks are required to produce a GMP-quality product (25). A number of strategies have been proposed to overcome these issues (26) and are being incorporated into the current generation of DNA vaccines. New methods based either on cellular production of expression cassettes or using alternative selection methods have been developed to avoid antibiotic resistance (Table 2).

In spite of the perceived advantages of DNA vaccines, the enormous amount of preclinical research (>55,000 articles [27]), and the limited successes in veterinary vaccines, DNA vaccines have not translated into human
vaccines. As of July 2014, there were 121 registered phase I clinical trials, 38 registered phase II clinical trials, and no phase III clinical trials listed on www.clinicaltrials.gov and 3 phase I and 9 phase II clinical trials listed on www.clinicaltrialsregister.eu (using the search term “DNA vaccine”). Some of this data is captured by a database of gene therapy clinical trials (http://www.abedia.com/wiley/). Of the registered trials, 66 use HIV antigens and 15 use influenza antigen, the others comprising a mixture of other pathogens, including dengue, malaria, and hepatitis B and C viruses. The trials using infectious disease antigens that have published data are described in Table 1. In spite of good safety and tolerability, the clinical trial results have been disappointing, showing limited immunogenicity. Where immunogenicity has been reported, it has mainly been the development of T cell responses and very limited antibody responses.

**DISADVANTAGES: WHY DON'T THEY WORK**

A number of issues have limited the translation of DNA vaccines from successful preclinical studies into the clinic, and a better understanding of these issues will greatly help the development of a clinically effective DNA vaccine. One of the simpler issues is scale: the original volumes and doses used in murine studies (often 50 μg in a 50-μl volume) were large relative to the size of the muscle. The delivery of such a large bolus of DNA would have a biophysical effect on the delivery of the DNA, creating shear forces on the cells, inducing inflammation, and generating hydrodynamic pressure increasing DNA uptake (28). Scaling this up into the human muscle (based on values of muscle size from reference 29), an equivalent dose of 19.4 mg in a volume of 19.4 ml would be required, equating to approximately 40 times the normal vaccine volume. Second, the immune response required to protect a small rodent against infection may not be the same as that required to protect a human. Furthermore, the induction of immune responses, particularly the innate immune recognition of cytosolic and extracellular DNA, may differ among species (30). Inadequate animal models are an ongoing issue with all preclinical vaccine development and not limited to DNA vaccines. Third, the way in which DNA is taken up, processed, and expressed appears to be different in different species; for example, the DNA scavenger serum amyloid P is more active in humans than mice (31). Interestingly, inhibition of serum amyloid P is being targeted in an ongoing clinical trial to improve DNA vaccine responses (project ref: MR/J008605/1 on http://gtr.rcuk.ac.uk/).

Steps have been made in recent years to overcome these issues for clinical use. Before exploring the approaches used to optimize DNA vaccines for clinical use, it is necessary to understand how they work, both as gene delivery systems and as vaccine immunogens.

**HOW DNA VACCINES WORK: EXPRESSION**

To induce an immune response, the gene encoded on the DNA plasmid needs to be expressed in the host cell, and there are a number of barriers to this (32). While a large amount of DNA that is injected into the animal enters the blood circulation and is degraded (33, 34), the remainder enters cells that are local to the injection site, for example, myocytes after intramuscular injections or keratinocytes after subcutaneous injections. Some of the DNA directly enters and is expressed by local antigen-presenting cells (APCs) (35). It is not entirely clear how naked DNA enters the cell, but caveolae-dependent endocytosis (36), macropinocytosis (37), and clathrin-mediated endocytosis (38) have all been proposed as mechanisms. There are differences depending on the cell type that is taking up the material and whether the DNA is packaged chemically or naked. Having entered the cell, the DNA travels to the nucleus (39); movement to the nucleus has been suggested to occur either as free DNA (40) or in vesicles associated with the cytoskeleton (41), and progression through the cytoplasm can be associated with some degradation of the DNA by nucleases (42).

The nuclear membrane is a significant barrier to the expression of DNA: less than 0.1% of plasmid DNA that enters the cytosol is transcribed (43). Nuclear entry of plasmid DNA occurs either during nuclear envelope breakdown and reformation in mitosis (44) or via nuclear pores (45). Due to its small size, supercoiled plasmid DNA can transit through nuclear pores, but it is more likely that the plasmid DNA piggybacks onto proteins with nuclear localization signals (46), for example, transcription factors. This process can be optimized by encoding sequences in the DNA vaccine that increase binding to these proteins, for example, the enhancer region from simian virus 40 (SV40) (47). Finally, the gene(s) encoded on the plasmid need to be transcribed and translated. Expression level is a function of a number of features including the promoter used, the presence of a Kozak consensus sequence, and codon usage.

Transit into and across the cell also has an impact on the activation of the innate immune response to DNA vaccines and therefore the immunogenicity of the vaccine.
<table>
<thead>
<tr>
<th>Trial identifier</th>
<th>Date posted</th>
<th>Phase</th>
<th>Pathogen</th>
<th>Antigen</th>
<th>Additional protocols</th>
<th>Ref</th>
<th>Safe?</th>
<th>Immunogenic?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT00047931</td>
<td>Oct 2002</td>
<td>I</td>
<td>HIV</td>
<td>Clade B Gag-Pol-Nef fusion protein and modified envelope (Env) (VRC-HIVDNA009-00-VP)</td>
<td></td>
<td>128</td>
<td>Safe</td>
<td>Some antibody and T cell responses</td>
</tr>
<tr>
<td>NCT00054860</td>
<td>Feb 2003</td>
<td>I</td>
<td>HIV</td>
<td>Poly epitope</td>
<td></td>
<td>129</td>
<td>Safe</td>
<td>Not immunogenic</td>
</tr>
<tr>
<td>NCT00069030</td>
<td>Sep 2003</td>
<td>I</td>
<td>HIV</td>
<td>Gag-Pol-Nef-multiclade Env (VRC-HIVDNA009-00-VP) IL-2 DNA</td>
<td>Needle-free (Bioject)</td>
<td>116</td>
<td>Safe</td>
<td>Timing of IL-2 boost mattered, 2 day delay increased response</td>
</tr>
<tr>
<td>NCT00109629</td>
<td>Apr 2005</td>
<td>I</td>
<td>HIV (clades A–C)</td>
<td>Env (A–C), Gag, Pol, Nef (VRC-HIVDNA016-00-VP and VRC-HIVDNA014-00-VP)</td>
<td></td>
<td>130</td>
<td>Well tolerated</td>
<td>Needle free delivery improved immune response</td>
</tr>
<tr>
<td>NCT00111605</td>
<td>May 2005</td>
<td>I</td>
<td>HIV</td>
<td>Gag</td>
<td>IL-12 DNA</td>
<td>123</td>
<td>Well tolerated</td>
<td>No detectable effect of cytokines</td>
</tr>
<tr>
<td>NCT00115960</td>
<td>Jun 2005</td>
<td>I</td>
<td>HIV</td>
<td>Gag</td>
<td>IL-12 and/or IL-15 DNA</td>
<td>131</td>
<td>Safe</td>
<td>No detectable effect of cytokines</td>
</tr>
<tr>
<td>NCT00125099</td>
<td>Jul 2005</td>
<td>I/II</td>
<td>HIV</td>
<td>Gag, Pol, Nef, Env (VRC-HIVDNA009-00-VP) IL-2 DNA</td>
<td>Needle-free (Bioject)</td>
<td>116</td>
<td>Safe</td>
<td>Poorly Immunogenic</td>
</tr>
<tr>
<td>NCT00141024</td>
<td>Aug 2005</td>
<td>I</td>
<td>HIV</td>
<td>Poly epitope</td>
<td></td>
<td>132</td>
<td>Safe</td>
<td>Modest but transient</td>
</tr>
<tr>
<td>NCT00249106</td>
<td>Nov 2005</td>
<td>I</td>
<td>HIV clade C/B’</td>
<td>Env, Gag, Pol, Nef, and Tat (ADVAX)</td>
<td></td>
<td>133</td>
<td>Safe</td>
<td>Modest but transient</td>
</tr>
<tr>
<td>NCT00270465</td>
<td>Dec 2005</td>
<td>I</td>
<td>HIV</td>
<td>Env (A–C), Gag, Pol, Nef (VRC-HIVDNA016-00-VP and VRC-HIVDNA014-00-VP)</td>
<td>Needle-free (Bioject)</td>
<td>134</td>
<td>Safe and well tolerated</td>
<td>Increased T cell breadth and magnitude. No effect on viral control</td>
</tr>
<tr>
<td>NCT00290147</td>
<td>Feb 2006</td>
<td>I</td>
<td>Dengue virus</td>
<td>Env</td>
<td>Needle-free (Bioject)</td>
<td>135</td>
<td>Some local pain/swelling</td>
<td>Modest: 5 of 12 subjects in high dose group had neutralizing antibody. 10/12 had Interferon-γ (IFN) positive responses</td>
</tr>
<tr>
<td>NCT00301184</td>
<td>Mar 2006</td>
<td>I</td>
<td>HIV</td>
<td>VLP (pGAG2/J57) Modified vaccinia Ankara (MVA) boost</td>
<td></td>
<td>136</td>
<td>Mild or moderate local responses</td>
<td>Modest responses, different patterns of response according to prime boost regime</td>
</tr>
<tr>
<td>NCT00321061</td>
<td>May 2006</td>
<td>I</td>
<td>HIV (clades A–C)</td>
<td>Env (A–C), Gag, Pol, Nef (VRC-HIVDNA016-00-VP)</td>
<td>Ad boost</td>
<td>60</td>
<td>Local responses after i.d. or s.c.</td>
<td>No difference by route of administration</td>
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<td>NCT00384787</td>
<td>Oct 2006</td>
<td>I</td>
<td>HIV</td>
<td>Env (A–C), Gag, Pol, Nef (VRC-HIVDNA009-00-VP)</td>
<td>Ad boost</td>
<td>137</td>
<td>Safe</td>
<td>Modest</td>
</tr>
<tr>
<td>NCT00428337</td>
<td>Jan 2007</td>
<td>I</td>
<td>HIV</td>
<td>CTL epitopes</td>
<td>MVA boost</td>
<td>138</td>
<td>Safe</td>
<td>Ineffective</td>
</tr>
<tr>
<td>NCT00536627</td>
<td>Sep 2007</td>
<td>I</td>
<td>HBV</td>
<td>Env</td>
<td></td>
<td>139</td>
<td>Well tolerated</td>
<td>Induction of antigen specific T cells, no protection from HBV reactivation after cessation of anti-viral therapy</td>
</tr>
<tr>
<td>NCT00545987</td>
<td>Oct 2007</td>
<td>I</td>
<td>HIV clade C/B’</td>
<td>Env, Gag, Pol, Nef, and Tat (ADVAX)</td>
<td>Electroporation (Ichor)</td>
<td>95</td>
<td>Safe</td>
<td>Electroporation increased T cell responses</td>
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<tr>
<td>NCT00694213</td>
<td>Jun 2008</td>
<td>I</td>
<td>Influenza H5</td>
<td>HA</td>
<td>Lipid-based adjuvant (Vaxfectin) and needle-free injection (Bioject)</td>
<td>76</td>
<td>Safe</td>
<td>No difference to needle delivery above</td>
</tr>
<tr>
<td>NCT Reference</td>
<td>Start Date</td>
<td>Design</td>
<td>Disease</td>
<td>Antigen</td>
<td>Adjuvant</td>
<td>Study Design</td>
<td>Outcome</td>
<td></td>
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<tr>
<td>NCT00709800</td>
<td>Feb 2009</td>
<td>I</td>
<td>Influenza H5</td>
<td>HA</td>
<td>Lipid-based adjuvant (Vaxfectin)</td>
<td>Safe</td>
<td>Antibody responses induced</td>
<td></td>
</tr>
<tr>
<td>NCT00870987</td>
<td>Mar 2009</td>
<td>I</td>
<td>Malaria</td>
<td>Circumpsorozoite protein (PICSP) and apical membrane antigen-1 (AMA1)</td>
<td>Ad boost</td>
<td>Safe</td>
<td>4/15 volunteers protected against malaria challenge</td>
<td></td>
</tr>
<tr>
<td>NCT00865566</td>
<td>Mar 2009</td>
<td>II</td>
<td>HIV</td>
<td>Clade B Gag, Pol, and Nef and Env from clades A, B, and C (VRC-HIVDNA016-00-VP)</td>
<td>Ad boost</td>
<td>Tolerated</td>
<td>No effect on HIV acquisition or viral set point</td>
<td></td>
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<tr>
<td>NCT00988767</td>
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<td>I</td>
<td>HBV</td>
<td>Env</td>
<td>Inactivated virus boost</td>
<td>Safe</td>
<td>Changes in NK population</td>
<td></td>
</tr>
<tr>
<td>NCT01086657</td>
<td>Mar 2010</td>
<td>I</td>
<td>Influenza H5 strain</td>
<td>HA</td>
<td>Lipid-based adjuvant (Vaxfectin)</td>
<td>Safe</td>
<td>Higher titer antibody compared to inactivated only groups (144). Increased antibody repertoire in DNA primed groups (145). Interval in boost important in effect (144).</td>
<td></td>
</tr>
<tr>
<td>NCT00776711</td>
<td></td>
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<tr>
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<td>Feb 2009</td>
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<td>Mar 2009</td>
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<td>Mar 2010</td>
<td></td>
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</tr>
</tbody>
</table>

Abbreviations: i.m., intramuscular; s.c., subcutaneous; i.d., intradermal; Env, envelope; HA, hemagglutinin; IL, interleukin; IFN, interferon; HBV, hepatitis B virus; CSP, circumsporozoite protein; TRAP, thrombospondin-related adhesive protein; Ad, adenovirus; MVA, modified vaccinia Ankara; GM-CSF, granulocyte macrophage colony-stimulating factor.
TABLE 2 Novel DNA vaccine technologies to remove antibiotic resistance

<table>
<thead>
<tr>
<th>Name of approach</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternate vectors</td>
<td></td>
<td>158</td>
</tr>
<tr>
<td>Mini-circle DNA vaccines</td>
<td>Produced by a site-specific recombination event of a parental plasmid producing a mini-circle containing an expression cassette and a mini-plasmid comprised of backbone regions.</td>
<td></td>
</tr>
<tr>
<td>Minimalistic immunogenically defined</td>
<td></td>
<td>159</td>
</tr>
<tr>
<td>gene expression (MIDGE) vectors</td>
<td>Use a restriction digestion system to excise the backbone, resulting in a linear expression cassette that is capped by hairpin loop. Capped linear DNA constructs made by a novel one-step enzymatic process where antibiotic resistance genes are first included in parental plasmids and then eliminated once capped linear constructs are produced. Can be generated independently of a bacterial fermentation step.</td>
<td></td>
</tr>
<tr>
<td>Doggybones</td>
<td></td>
<td>160</td>
</tr>
<tr>
<td>Alternative selection systems (22)</td>
<td>Essential gene under repressor control is incorporated into bacterial host chromosome. Plasmid encodes several operator cassettes, e.g., Lac or RNAII; these titrate out the repressor, allowing gene expression.</td>
<td></td>
</tr>
<tr>
<td>Operator-repressor-titration systems</td>
<td></td>
<td>161, 162</td>
</tr>
<tr>
<td>Auxotrophic complementation</td>
<td>Rely on deletion of an essential or conditionally essential gene where bacterial growth is only restored upon introduction of a plasmid containing the altered gene.</td>
<td>163</td>
</tr>
<tr>
<td>Toxin-antitoxin-based systems</td>
<td>Toxins remain in bacterial cells and lead to cell death after loss of plasmid.</td>
<td>164</td>
</tr>
<tr>
<td>RNA-out</td>
<td>Use antisense RNA on the plasmid to silence toxin genes expressed by the bacteria.</td>
<td>165</td>
</tr>
<tr>
<td>Overexpression of growth genes</td>
<td>Certain bacterial growth genes circumvent toxic effects of antibacterials when overexpressed, e.g., the enoyl ACP reductase encoded by the fabI gene.</td>
<td>166</td>
</tr>
</tbody>
</table>

HOW DNA VACCINES WORK: IMMUNE RESPONSE

Innate Response to DNA Vaccines

DNA sensing by pattern recognition receptors has been shown to be essential for vaccine responses (30). Many of the pattern recognition receptors used by cells to detect infection are based on the recognition of chemicals that are outside their usual location, so the presence of naked DNA outside of the nucleus is inherently inflammatory. Transfected immune and somatic cells are able to sense the presence of “foreign DNA” in the cytosol, using a range of intracellular pattern recognition receptors including RIG-I, AIM2, ZBP1/DAI, and HB2 histones (48). However, their role in the initiation of an innate signaling cascade to DNA vaccination is unclear, as knockout studies showed minimal contributions, suggesting redundancy (30). The detection of unmethylated CpG motifs inherently present in DNA vaccines by toll-like receptor (TLR) 9 is assumed to have a role in the detection of DNA vaccines, but TLR9−/− mice had immune responses to DNA vaccines similar to control mice (49), again suggesting some redundancy. Studies have shown that the downstream signaling molecules STING and TBK1 are essential for instigation of an innate immune response to DNA vaccines, as their deletion abrogated type I interferon production (50, 51). The induction of an innate immune response to the DNA vaccine is critical in activating the APCs that present the expressed antigen to the T cells.

Adaptive Responses to DNA Vaccines

The adaptive immune response to a DNA vaccine is heavily influenced by the cell that is transfected by the DNA. As described above, antigen is either expressed by non-antigen-presenting cells such as myocytes or keratinocytes or by APCs that have taken up the DNA. Which cell expresses the DNA is influenced by a number of factors including the route of delivery, the device used to deliver the vaccine, the formulation of the vaccine, and the use of adjuvants. Speculatively, if APCs are directly transfected, they are most likely to present antigen on major histocompatibility complex class I (MHC-I) molecules, thereby initiating a CD8+ T cell response. If somatic cells are transfected, the antigen will be either displayed on MHC-I, secreted as processed antigen, or released upon cell death. Antigen that is displayed upon MHC-I acts as a trigger for activated CD8+ T cells to kill the transfected cell, reducing the expression of the vaccine and potentially dampening the immunogenicity (52). Secreted antigen is either taken up by APCs and presented on MHC-II molecules to CD4+ T cells or interacts with B cells, inducing an antibody response. Finally, antigen released after cell death most likely enters APCs and is loaded onto MHC-I by the cross-presentation pathway. Cross-presentation of DNA vaccine derived antigen has been demonstrated in vitro (53), and in vivo studies suggest that antigen expressed from non-antigen-presenting cells is more important in the induction of the immune response to DNA vaccines than antigen from APCs (54).
The clinical trial experience (Table 1) suggests that DNA vaccines are better at inducing T cell responses than B cell responses. There are a number of reasons why this might be the case, including incorrectly folded antigen, low expression levels, poor activation of the innate response via pattern recognition receptors, and differences in MHC-I and MHC-II loading. Our understanding of the immune response to expressed antigen can be used to target either the DNA vaccine or the expressed antigen to different cells to alter the immune response (55). Fusing the antigen to an anti-MHC-II single chain fragment variable molecule that targets it to APCs has been shown to increase the response (56). Optimizing the expression, presentation, and secretion of the antigen to engage different arms of the adaptive response is critical for improving immunogenicity.

OPTIMIZING FOR THE CLINIC

Route of Delivery
Altering the route of delivery can have a marked effect on the downstream immune response to DNA vaccines. Various routes have been proposed for DNA vaccine delivery including intramuscular, subcutaneous, intranasal, and oral (57). Ultimately, route selection is a balance between optimum expression, immunogenicity, cost, and acceptability for patients. Considerations of route and delivery are dependent upon the target of the vaccine, cancer vaccines because of the individual nature of the treatment may be able to exploit more niche approaches than infectious disease vaccines that need to be cheap and easy to deliver. Conventionally, DNA vaccines are delivered intramuscularly, which has the advantage of matching currently available vaccines and therefore requiring less training of staff administering the vaccine. Expression of DNA following intramuscular delivery is mostly by myocytes, which because they are energy rich, low turnover cells, may lead to increased expression levels. The subcutaneous route is also relatively easy for vaccine delivery and may be advantageous because of the high numbers of APCs in the skin (58). Intradermal immunization is slightly more challenging, requiring specific needles and training of healthcare staff, but may be advantageous because of the specific dendritic cell subsets present in the dermis (59). While small animal studies suggest a switching in the immune response when different routes are compared (57), a recent clinical trial comparing DNA vaccines delivered by these routes found no difference in immunogenicity (60) but found mildly greater reactogenicity after intradermal or subcutaneous delivery. In addition to delivering vaccines via these routes by injection, a number of innovative approaches have been proposed including tape stripping of the hair follicles and “painting” the vaccine on (61).

Other more exotic routes have been explored to improve DNA vaccine immunogenicity. One method proposed is hydrodynamic delivery (62). This method is based on the delivery of high volumes of DNA solution intravenously at high pressure, which effectively forces the DNA into the cells and mainly targets the liver (63). Beyond the complexity of the procedure, there is an additional scale-up complication: a dose in humans equivalent to that used in mice would be 7 liters, based on 100 ml/kg in mice (63). While this approach has been demonstrated to be effective in nonhuman primates (64), to date no clinical trials have been performed using this route.

Another, more attractive, prospect is the targeting of mucosal surfaces because of the high incidence of infections at mucosal sites and the potential for generating local immunity. DNA vaccines have been tested in small animal models using the nasal (65), vaginal (66), rectal (67), sublingual (68), and oral routes. Of these only an orally delivered cancer vaccine has made it to a phase I clinical trial (69). Mucosally delivered DNA vaccines are particularly sensitive to degradation because the tissues have a number of chemical and enzymatic barriers including nucleases and mucus. Therefore, one of the considerations for DNA vaccine delivery by the mucosal route is how the DNA is packaged, but formulation of DNA vaccines is also important for other routes.

Packaging for Delivery
A lot of research has focused on the packaging of DNA to optimize delivery and expression. Formulation of the DNA vaccine can alter the routes of cellular entry and intracellular trafficking, increasing uptake and expression. Two broad approaches have been used: compaction with cationic polymers and liposomes (70). The phosphate backbone of DNA imparts a significant negative charge to the macromolecule, which enables the formation of complexes with cationic polymers. A number of different molecules have been proposed including polyethylenimine, chitosan, and cationic poly(lactic-co-glycolic acid) nanoparticles. Interestingly, both polyethylenimine (71) and chitosan (72) have an additional adjuvant effect, causing local inflammation. Of these agents, poly(lactic-co-glycolic acid) has been tested in a clinical trial (73), with a good safety profile but limited immunogenicity. Manipulating the chemistry of the polymer can alter the immune response to these carriers, and
the ratio of polymer to DNA can be altered, affecting particle size, which may have an effect on the immune response to the vaccine, though the effect of particle size on immune response is mixed (74). In addition to stabilizing the plasmid in the extracellular space, formulating appears to alter the route of uptake of the plasmid and transit from the endosome into the cell (32). Alternatively, DNA vaccines can be packaged in phospholipid bilayers (liposomes), with the aim being to increase fusion with the cell membrane and enable cellular entry of the DNA (75). A number of different synthetic lipids have been suggested for this approach, but most commonly they include DOPE/DOTAP. DNA can either be bound to the surface through electrostatic forces or can be encapsulated within the liposome. A lipid formulation, Vaxfectin (Vical), has been tested in a number of clinical trials of DNA vaccines (76). There are issues with the stability and quality assurance of liposomes, limiting their translation to the clinic.

Another approach is to use virosomes, which have viral proteins studded in the lipid membrane, enabling the targeting of particles to specific cell types (77). However, the virosome approach would negate a key advantage of DNA vaccines—their low manufacturing cost—because the addition of proteins would increase the price significantly. Alternatively, the use of attenuated bacteria as carriers of DNA vaccines (78) has been proposed, as some bacteria are able to transfer the plasmid DNA across phylogenetic borders. There are issues with the development of a GMP-quality, live attenuated, genetically modified vaccine, which again may limit its use as a prophylactic vaccine. While these approaches are often described as DNA or genetic vaccines, they should be considered vectored vaccines.

Much of the thinking behind formulation approaches comes from transfection of mammalian cells in vitro, which may not translate in vivo. The inclusion of extra chemical components may delay the licensure of the vaccine due to concerns over the inflammatory profile of the agents used. Rather than chemically modifying each DNA vaccine to enhance uptake, a more universal approach is to use alternative delivery devices to enhance the uptake and immunogenicity.

**Device-Mediated Delivery**

DNA vaccines have to enter cells and be expressed prior to inducing an immune response. This is fundamentally different from conventional vaccines, which exploit the normal surveillance and uptake processes of the immune response. This necessity for antigen expression prior to immunogenicity means that alternative delivery approaches may be required to achieve optimal responses. To this end, a number of different devices have been developed and tested to improve DNA vaccine delivery. The acceptability profile of these devices may be very different from conventional needle-delivered vaccines, as they can be perceived to be more invasive.

One of the earliest techniques proposed is biolistic delivery (also referred to as a gene gun) (79), where DNA is coated onto inert microscopic particles (often made of gold) that are then “fired” at the skin by the biolistic device. This was literally the case in the initial devices, as gunpowder was used to power the transit of the particles, though helium is now used. These approaches have been licensed by PowderJect (Chiron/Novartis) and PowderMed (Pfizer). DNA vaccines delivered by these devices have gone into the clinic with moderate efficacy but a key advantage of dose sparing (80–83). Alternatively, liquid jet systems that use compressed gas or springs to force liquid through a small orifice (0.1 to 0.5 mm) are also able to force DNA into cells (84), with systems made by a number of companies (including Bioject and Pharmajet). A clinical trial comparing forced DNA vaccine delivery with and without microparticles (using the PowderMed and Bioject devices, respectively) showed no difference in immunogenicity between the two approaches (85). Alternatively, the use of tattooing has been suggested as an approach (86), with some efficacy in preclinical studies (87), though again, scale-up and acceptability may be an issue; for example, in a recent study using rhesus macaques a 30 cm² area was used (88). Finally, some of the formulation approaches described above can be combined with delivery approaches, for example, microneedles, which are soluble patches of multiple needles approximately 100 μm long coated in DNA (89). Microneedles have a number of advantages, including controlling the depth of vaccine delivery, combining adjuvant and antigen in the same device, stability, and reduced risk to the physician or patient. These are all conceptually interesting ideas and have been demonstrated in a number of preclinical studies, but whether they can be translated to efficacious clinical approaches remains to be seen.

The biggest advance in DNA vaccine delivery has been the use of electroporation (also described as electroporation or electropermeabilization) to improve uptake and transfection efficiency (90, 91). Electroporation has long been used for in vitro transformation of cells (both bacterial and mammalian) and was first proposed for use in vivo in 1996 (92) and for the delivery of DNA vaccines in 2000 (93). Though not entirely understood, electroporation is believed to work mainly by inducing and stabilizing
pores in the cell membrane and then moving the DNA along the gradient of the applied current (toward the positive electrode). It probably also induces local inflammation, boosting the immune response (94). A range of variations on the theme of electroporation have been explored, including changing the parameters of the electric pulse (length, voltage, and current) delivered and the type of electrode used. It has been used in a number of clinical trials (Table 1) and is well tolerated, with a short muscle contraction that can cause some discomfort (95). These approaches all require the development of practical and affordable devices that can be used in the community, particularly in resource-poor settings.

**OPTIMIZING EXPRESSION**

Trials have shown that the primary problem with the use of DNA vaccines is the lack of immunogenicity in humans and primates. There is some evidence that immune responses to DNA vaccines increase with dose (96, 97), but there is an upper dose limit that is feasible for administration to humans. Therefore, optimizing the expression of antigen from current DNA vaccines is key to improving performance (98). All aspects of the transcription and translation of the encoded gene can be optimized to improve expression and can be combined for additive effect. Promoter elements placed upstream of the open reading frame in the plasmid backbone have been optimized, with the majority containing the cytomegalovirus promoter, which allows for constitutive expression in many mammalian cell lines, and being more effective than tissue-specific promoters (99, 100). Protein expression is increased several-fold after codon optimization, when the codon use of the gene of interest is matched to the species vaccinated (101). The manipulation of the termination sequence (e.g., adding a polyA tail) has been shown to increase the amount of mRNA exported out of the nucleus by allowing efficient termination of transcription (102). Relaxing of the supercoiled form of the plasmid DNA upon delivery makes it more susceptible to endonucleases that degrade the DNA, and any DNA secondary structures exacerbate this degradation, so they are now avoided (103). Other molecular approaches to increasing expression include the addition of signal sequences that target the antigen to intracellular compartments involved in MHC-I or MHC-II processing (104). Nuclear localization signals have also been tested, aiming to direct DNA to the nucleus for transcription (105). In addition to improving the transfection efficiency of DNA vaccines, various strategies have been evaluated to improve their immunogenicity.

**OPTIMIZING IMMUNOGENICITY**

Electroporation and biolistic and liquid jet delivery all have the additional effect of causing cell damage, which induces a local inflammatory immune response (94), which in turn may potentiate the immunogenicity of the delivered DNA. Boosting the immunogenicity to delivered antigen is critical in maximizing the efficacy of vaccines. Adjuvants are chemicals used to increase the local inflammatory response, thereby increasing the uptake and presentation of the antigen by APCs and also increasing the recruitment of cells to the site. Two broad adjuvant approaches are used in DNA vaccines: the addition of inflammatory agents (chemical adjuvants) and the inclusion of genes that trigger the immune response (genetic adjuvants).

**Chemical Adjuvants**

DNA vaccines by their nature are inflammatory because they introduce extra-nuclear DNA to the cell. Unmethylated CpG oligonucleotides are used as an adjuvant in their own right (106), and the effect of the addition of these motifs has been explored in DNA vaccines. When the CpG motifs were methylated there was a reduction in the immunogenicity of DNA vaccines (107), and the introduction of CpG motifs increased antibody responses in vaccinated fish (108), but other studies have suggested that TLR9-/- mice are still able to respond to DNA vaccines (49). The understanding of other sensors of cytosolic DNA is not as comprehensive as the understanding of the TLR pathway. As yet, apart from the STING pathway (109), little has been done to exploit cytosolic DNA sensing for the development of adjuvants, but conceivably, DNA vaccine plasmids could be engineered to target these receptors. In addition to increasing the inherent immunogenicity of the plasmid vaccine, a number of agents have been coformulated with DNA vaccines, including conventional chemical adjuvants such as alum (110) or MF59 (111) and more immunologically derived adjuvants including TLR7/8/9 ligands (112), resiquimod (113), or extracted material from BCG (114). However, these approaches appear to have had a limited impact on DNA vaccine immunogenicity. This may be due to timing issues since adjuvant induced inflammation is very acute and may not match the peak of antigen expression. Alternatively, the inflammation induced by the adjuvant may lead to the clearance of the transfected cells before sufficient antigen is expressed.

**Genetic Adjuvants**

One elegant strategy to overcome the poor immunogenicity of DNA vaccines is to include genes that boost
or modulate the immune response. This may be advantageous because the timing of expression will match that of the antigen and therefore lead to a coordinated response. The effect may also be more focused than TLR-type adjuvants, which can be reactogenic. A wide range of genes have been tested in this context (described in reference 115). They fit into the following broad categories: (i) cytokines, which are molecules that attract or modulate the responses of cells in the immune response, e.g., IL-2 (116) and IL-12 (117); (ii) factors that enhance APC function, e.g., GM-CSF (118) and Flt3 (119); (iii) costimulatory molecules, e.g., CD86 but not CD80 (119); (iv) adhesion molecules, e.g., ICAM1 (120); (v) bacterial ligands, e.g., flagellin (121); and (vi) molecules in pattern recognition pathways, e.g., RIG-I (122) or DAI (122). A number of these agents have entered clinical trials, including IL-12 (117), IL-15 (123), GM-CSF (118), and IL-2 (116), with acceptable safety profiles but modest immunogenicity.

PRIME BOOST

The apparent failure of DNA vaccines as individual agents in clinical trials has led to a large body of work using them as the priming immunization in various heterologous prime boost regimes (where the initial vaccination is different from subsequent ones) (86). These have usually been associated with a range of recombinant attenuated viruses expressing the vaccine antigen, including adenovirus, modified vaccinia Ankara, and fowlpox (Table 1). The rationale for this approach is that priming with the DNA vaccine may reduce the negative effect of antivector immunity. It has also been suggested that DNA priming can increase the breadth of the immune response (124). An array of combinations has been tried in various orders and sequences, and greater responses have been reported for prime boost combinations than individual immunizations (Table 1). One issue is that the regimes required to achieve the moderate immune response observed with these approaches are often long and complex, again limiting the efficacy in a mass vaccination campaign.

NONCONVENTIONAL VACCINES

Given the limited efficacy of DNA vaccines in humans, there may be alternative uses for DNA vaccines beyond the classic prophylactic vaccine. The first is for experimental vaccines, to screen and identify the best candidate antigens prior to developing costly GMP-quality proteins (125). In this context the use of complex delivery devices or large doses may not be an issue. This may be particularly effective for membrane proteins (for example, HIV envelope), which are extremely difficult to express as proteins in high quantities in the correct confirmation. They may also be more appropriate for epitope screening, particularly in chimeric or mosaic vaccines. Alternatively, the expression of monoclonal neutralizing antibodies by DNA may be used as a form of passive immunization (126), which could be used for at-risk patients, especially when short-term protection is required. A third use may be the generation of monoclonal antibodies, either in mice (127) or in humans. These could be developed postimmunization with a hybridoma approach or with novel sorting and expression techniques. This approach would also benefit from the ease of production of the constructs and the bypassing of the requirement to make a protein antigen. Finally, due to their individual nature and high costs, DNA vaccines may ultimately be better suited to cancer treatment than prophylactic vaccines to infectious diseases.

CONCLUSIONS

DNA vaccines in humans have yet to live up to the excitement generated by the preclinical studies. This is due to issues with scaling up the dose and differences in both the expression of foreign nucleic acids and the initiation of an immune response to DNA between mice and humans. Making the preclinical models more relevant to humans is a key priority, particularly dose reduction, but novel approaches to improve expression levels and immunogenicity in clinical studies are also required. Of the current platforms, electroporation appears to have the largest impact, but current-generation devices do not lend themselves to mass vaccination campaigns. If the immunogenicity and expression issues can be resolved, then DNA vaccines will revolutionize vaccines.

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Using Plasmids as DNA Vaccines for Infectious Diseases


