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# Developing non-viral DNA delivery systems for cancer and infectious disease

Edward G. Spack and Frank L. Sorgi

**Efforts to deliver therapeutic genes are frequently rebuffed by the body's adaptive immune response against viral delivery vectors. Attempts to circumvent this problem using non-viral delivery systems have encountered problems with transient expression and inflammatory responses induced by reaction of the innate immune system reacting against bacterial DNA. However, within the past decade, these barriers to non-viral DNA delivery have been recognized as potential allies in the development of novel vaccines for cancer and infectious disease. This review summarizes preclinical and current clinical studies testing the formulation, delivery route and adjuvant options in the development of novel DNA-based vaccines.**

*Edward G. Spack
InterMune Pharmaceuticals
Burlingame, CA-94010, USA
*tel: + 1 650 409 2055
fax: + 1 650 259 0774
e-mail: tspack@intermune.com
Frank L. Sorgi
OPTIME Therapeutics
Petaluma, CA-94954, USA

## ▼ Reasons for the development of non-viral DNA delivery systems

Gene delivery to somatic cells holds great promise as a means to supplement genetic deficits. Unfortunately, the most efficient viral vectors used to deliver genes to target tissues trigger immune responses that limit the duration of gene expression and the ability to re-administer the genes[1]. Many patients, by natural exposure to viruses, have pre-existing antibodies against viral-coat proteins that block the uptake of viral vectors and, therefore, the expression of the packaged genes. In other cases, the viral proteins stimulate T and B lymphocyte responses that generate antiviral antibodies and cytotoxic T lymphocytes (CTLs), and kill the transfected cells[2]. A potential solution to these problems was the discovery that purified plasmid DNA, devoid of any viral packaging (naked DNA), could mediate the expression of encoded proteins after injection into skeletal muscle[3]. Preparation of plasmid DNA is a simpler manufacturing process than the packaging and purification of modified viruses. Furthermore, plasmids do not share the size constraints imposed by viral packaging, permitting the delivery of large genes or sets of genes. However, subsequent studies revealed that non-viral delivery of naked DNA, or DNA complexed with other molecules such as cationic lipids, encountered a different set of immunological barriers.

## The effect of the innate immune response on non-viral DNA delivery

### *Unmethylated CpG sequences in DNA plasmids*

Among the early attractions of non-viral gene delivery, in addition to its simplicity, was the hope that it would evade the adaptive immune response that suppresses viral delivery systems. However, it soon became apparent that the duration of expression of genes delivered by plasmids was often significantly shorter than that of genes delivered by viruses.

Several laboratories discovered in the mid-1990s that a main source of these problems was the immune response to bacterial DNA sequences in the delivery plasmids. CpG (cytidine-phosphate-guanosine) dinucleotides occur at the expected random frequency of one-in-sixteen in bacterial DNA, but this nucleotide combination is suppressed in vertebrate DNA, occurring at a four–fivefold lower frequency. Furthermore, 75% of the rare CpG dinucleotides in vertebrate DNA are methylated to 5-methylcytosine, but bacterial sequences containing CpG are not methylated[4]. These sequence differences in bacterial DNA trigger components of the innate immune system, which is an evolutionarily ancient host defense based on invariant receptors encoded in the germline that recognizes conserved molecules unique to microorganisms[5].



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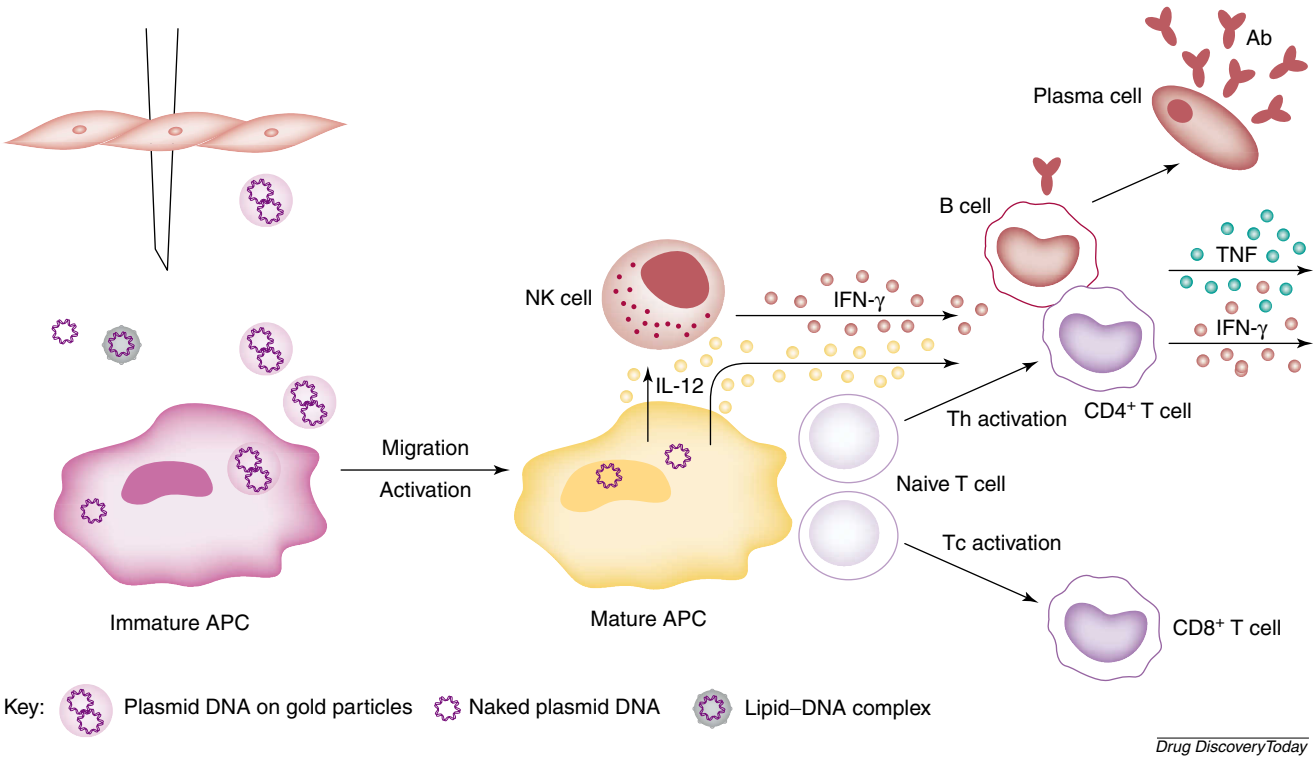


**Figure 1.** Immune interactions in DNA vaccination: delivery of plasmid DNA leads to plasmid uptake by antigen presenting cells (APCs: macrophages, dendritic cells, Langerhan's cells). Local trauma and internalized plasmid DNA induce maturation and migration of APCs to regional lymph nodes. Unmethylated CpG sequences within the internalized plasmids activate inflammatory cytokines, including interleukin-12 (IL-12). IL-12 is a potent activator of natural killer (NK) cells, which secrete interferon-γ (IFN-γ). In the lymph nodes, mature APCs process antigen encoded by the plasmid, and present peptide portions of the antigen on their surface bound to molecules of the major histocompatibility complex (MHC). T cells bearing receptors for this MHC–peptide combination are activated to form either $CD8^+$ cytotoxic T cells that kill infected and tumor cells (cellular immunity) or $CD4^+$ helper cells that activate B cells to secrete antibodies (humoral immunity). The strong INF-γ response also induces $CD4^+$ T cells to secrete Th1 cytokines (e.g. IL-2, tumour necrosis factor-α and interferon-γ) with anti-viral and anti-tumor properties.

Viral DNA does not provoke this response because it replicates in the host cell and shares the host methylation pattern. Because plasmids used for non-viral gene delivery are made in bacteria, their CpG sequences are unmethylated and therefore trigger the innate immune response. Initial observations that bacterial DNA activates natural killer (NK) cells and B cells[6,7] were largely ignored. Later studies confirmed these findings, and showed that palindromic sequences containing the unmethylated CpG motif were responsible for this activation[8]. Uptake of DNA containing these sequences triggers rapid intracellular signaling in B cells, macrophages and dendritic cell (DCs), leading to upregulation of several molecules involved in T cell activation and secretion of Th1 cytokines (e.g. interleukins IL-12 and IL-18; Fig. 1)[9]. IL-12 is a potent stimulator of NK cells, thus inducing the release of substantial quantities of interferon-γ (IFN-γ)[10]. Among the pleiotropic effects of IFN-γ is its antimicrobial activity, attributed to its suppression of viral promoters.

Although mammalian promoters are occasionally incorporated in the DNA plasmids used for gene delivery, most experiments and clinical trials utilize more active viral promoters. The viral promoters most commonly used in plasmid expression vectors are derived from cytomegalovirus (CMV), Simian virus 40 (SV40), Moloney murine leukemia virus (MOMLV) and Rous sarcoma virus (RSV)[11]. Although these promoters have high transcription rates *in vitro*, they are inhibited by IFN-γ and tumor necrosis factor-α (TNF-α), reducing transcription and lowering expression levels[12,13]. Many of the disease targets for gene therapy (e.g. cancer, AIDS, ischemia–reperfusion) are characterized by an elevation in local or systemic levels of inflammatory cytokines, including IFN-γ and/or TNF-α. Introduction of plasmids into these environments can reduce promoter function. Furthermore, the strong IFN-γ response induced by unmethylated CpG sequences in the plasmid-DNA backbone further suppresses these viral promoters, rapidly attenuating expression for several days after delivery. Therefore, the use

of a viral promoter in many gene delivery plasmids probably contributes to the limited duration of expression. The persistence of inflammatory cytokines, including IFN-γ, also prevents expression of re-administered plasmids for up to several weeks following the initial administration. This refractory period hinders any attempts to sustain gene expression by redosing.

*Toxicity associated with non-viral delivery systems*

The strong Th1 cytokine response provoked by plasmid DNA could also account for much of the toxicity seen with some non-viral gene delivery systems. This toxicity can be augmented by the use of lipid formulations. Several clinical trials have tested gene delivery to the respiratory tract aimed at correcting genetic defects such as α1-antitrypsin deficiency and cystic fibrosis. Although *in vivo* expression was achieved, lung inflammation was elevated[14]. Several studies in mice have shown that intra-tracheal administration of cationic lipid–plasmid complexes induced an influx of leukocytes (predominantly neutrophils) and an increase of inflammatory cytokines (IL-6, TNF-α, IFN-γ) in the bronchoalveolar lavage fluid[15–17]. The inflammatory effects of the cationic lipid might arise from both a direct response to the lipid and an increased internalization of the immunostimulatory CpG sequences.

Intravenous delivery of lipid–DNA complexes can also induce strong toxicity and result in considerable mortality[18–20]. Intraperitoneal administration of cationic liposome–DNA complexes induced proinflammatory cytokines (TNF-α, IL-1β) and serum markers of hepatic toxicity (amyloid A, amyloid P, seromucoid) in healthy animals, and increased mortality in animals with induced pancreatitis[21]. These studies raise concerns that in some clinical settings, certain non-viral DNA delivery formulations might have the pharmacological effect akin to trying to extinguish a fire using kerosene. Recent work using animal models suggests that eliminating some of the unmethylated CpG sequences in the plasmid might reduce the inflammatory response[22], and that optimizing the composition and ratio of lipids can also improve the therapeutic index[23].

The barriers of low expression, limited persistence, refractory period and toxicity have thwarted many attempts at gene therapy. Although non-viral delivery has circumvented some of the problems caused by anti-viral immunity, the inflammatory immune response to bacterial DNA and the sensitivity of current viral promoters to suppression by cytokines has raised new barriers to prolonged gene expression. However, the cytokine storm provoked by unmethylated CpG sequences and the limited duration of expression could actually prove to be advantageous for vaccine applications. Two years after the initial demonstration of gene expression following plasmid inoculation, the first report of an antigen-specific antibody response induced by injection of plasmid DNA encoding a foreign protein was published[24]. Shortly thereafter, a pivotal study by Ulmer and coworkers demonstrated that immunization with a plasmid encoding influenza A nucleoprotein established protective immunity in mice, featuring both cellular and humoral responses against influenza A, and cross-protection against another influenza strain[25]. These studies, and many others that followed, have demonstrated that non-viral delivery of plasmid DNA holds great promise for vaccine development.

## Potential for non-viral DNA delivery systems for vaccines

*Disadvantages of current vaccines*

Although vaccination is one of the great success stories of modern medicine, many pathogens are still resistant to current vaccine approaches. In many cases, current vaccination strategies do not generate an effective form of immune response. Vaccines composed of protein subunits or killed pathogens are primarily processed by the immune system as exogenous antigens. They are internalized by antigen presenting cells (APCs), including macrophages and dendritic cells, and presented to Th lymphocytes by the major histocompatibility complex (MHC) class II pathway, which provokes a predominantly humoral (antibody) response. This antibody response is effective against some pathogens, but provides inadequate protection against others.

The CTL response recognizes endogenous antigens, such as viruses replicating within host cells, that are presented to T lymphocytes by the MHC class I pathway. Attenuated viruses can replicate in the host and provoke both a humoral and cellular response. However, a strong CTL response often requires additional stimulation by Th1 cytokines. In vaccines, these Th1 cytokines can be provoked by an adjuvant. Complete Freunds adjuvant (CFA), a mixture of mineral oil and fractionated mycobacteria, provokes a strong Th1 response in animals, but it is not approved for human use. Alum ($Al_2O_3$), the only adjuvant currently approved for human vaccines, does not induce a robust Th1 response, and consequently favors the development of humoral immunity.

*Potential advantages of DNA vaccines*

One of the advantages of non-viral DNA delivery for vaccination (Table 1) is that it comes with its own built-in adjuvant. Mycobacterial DNA is responsible for the adjuvant properties of CFA, although ironically this was not appreciated until half a century after its formulation[26].

**Table 1. Potential advantages of DNA vaccines**

| Structural feature | Consequence |
|---|---|
| Unmethylated CpG sequences | Adjuvant, boosts cellular immunity |
| Can encode cytokines/signaling molecules | Enhances activation, regulates Th1/Th2 |
| Endogenous antigen expression | MHC I[a] presentation, cellular immunity |
| Replication in bacterial culture | Simpler and cheaper manufacture |
| Stability in lyophilized form | Easier storage: no refrigeration required |
| No attenuated virus | No danger of reversion |

[a]MHC I, major histocompatibility complex class I molecule.

Oligodeoxynucleotides (ODNs) containing non-methylated CpG sequences can substitute for CFA as an adjuvant in immunizations of mice with recombinant proteins[27,28]. The consensus motif for the stimulation of mouse immune cells, 5′-purine-purine-CpG-pyrimidine-pyrimidine-3′ (Ref. 29), is a relatively weak stimulator of primate leukocytes. The recent identification of an ODN with optimal immunostimulatory activity for human, chimpanzee and rhesus monkey leukocytes[30] should provide new opportunities for the development of rational human adjuvants. These stimulatory motifs might occur naturally in some plasmid-DNA backbones, or could be added to the plasmids to boost the stimulation of Th1 cytokines.

The adjuvant properties of CpG motifs can be supplemented by the co-administration of genes specifically encoding cytokines or other co-stimulatory factors (Fig. 2). Activation of naïve T cells requires engagement of the T cell receptor and a co-stimulatory signal generated by the interaction between B7 molecules (CD80 and CD86) on the APC, and CD28 on the T cell. Increasing the density of B7 molecules on APCs can increase the efficiency of T cell activation, producing a stronger Th cell response and consequently a higher antibody titer. Co-immunization with DNA encoding B7-1 and/or B7-2 enhanced the antibody response to DNA vaccines encoding bacterial antigens (e.g. *Mycobacterium tuberculosis* hsp65[31]), viral antigens (e.g. hepatitis B surface antigen[32]) and tumor antigens (e.g. carcinoembryonic antigen[33]). Co-immunization with DNA encoding the cytokine, granulocyte-macrophage-colony-stimulating factor (GM-CSF), offers an alternative strategy for supplementing DNA vaccines. GM-CSF is a potent inducer of DC maturation and supports stronger humoral responses and cellular immune responses mediated by

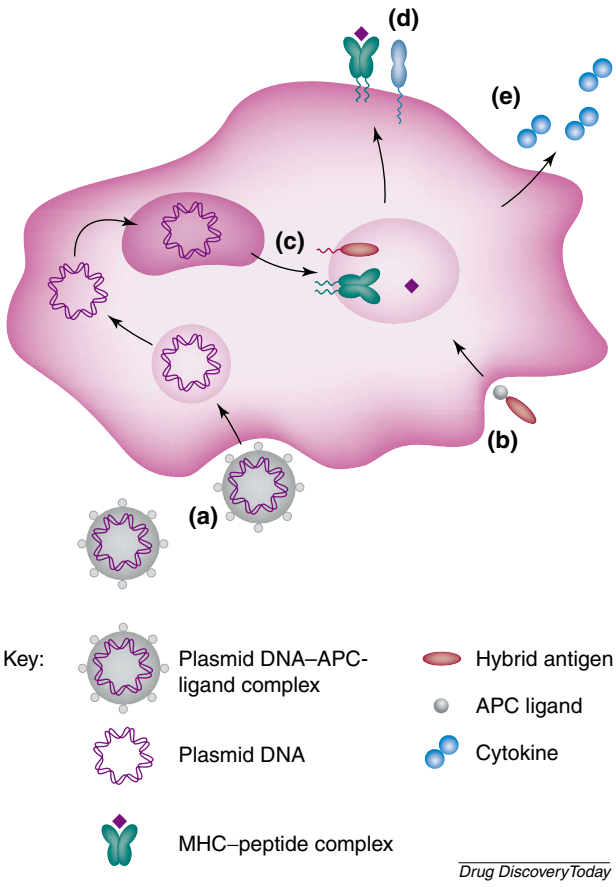


**Figure 2.** Strategies for optimizing DNA vaccines: **(a)** complexing DNA with antigen presenting cell (APC) ligands (e.g. DEAE-dextran, mannosylated polylysine); **(b)** encoding a secreted hybrid antigen containing an APC ligand [e.g. cytotoxic lymphocyte antigen-4 (CTA4)]; **(c)** encoding a hybrid antigen containing an endosome targeting sequence (e.g. lysosome integral membrane protein II); **(d)** encoding additional costimulatory molecules (e.g. B7); **(e)** encoding additional cytokine molecules (e.g. interleukin-2).

both $CD4^+$ and $CD8^+$ T cells[34]. Co-expression of antigen and GM-CSF enhanced the antibody response to DNA vaccines against rabies virus glycoprotein[35] and carcinoembryonic antigen[33], and enhanced the cytotoxic T cell response to hepatitis C virus p21 core-antigen[36] and influenza virus nucleoprotein[37]. Many other genes encoding cytokines and chemokines (e.g. IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, TGF-β and IFN-α) have been co-administered with antigen genes in DNA vaccines, yielding improved humoral and/or cell-mediated immune responses[38–39]. Although the selection of an optimum cytokine gene is still largely empirical, the opportunity to customize the immune response by the co-administration of an appropriate cytokine gene is one of the great theoretical advantages of DNA vaccines.

Non-viral DNA delivery offers several other theoretical advantages over current vaccines (Table 1). The endogenous expression of foreign antigens encoded by DNA vaccines mimics viral infection, accessing the MHC class I processing and presentation pathway and inducing cellular and humoral immune responses. Thus, DNA vaccines share many advantages of live or attenuated viral vaccines without some of the safety concerns, such as reversion to a virulent form. DNA vaccines also offer manufacturing and storage advantages. Vaccines composed of naked DNA or DNA formulated with lipids are more simple and less expensive to manufacture than vaccines containing attenuated virus, recombinant subunits or peptides. Some DNA vaccine formulations can be lyophilized, eliminating the need for continuous cold storage that hinders many vaccine efforts in developing countries. Naked DNA vaccines can often generate effective immune responses, but formulation with charged lipids, peptides or other agents can protect the DNA from serum degradation and could offer the means to target DNA to specific cells.

*Formulation of plasmid DNA with other molecules*

Although injection of naked DNA into the muscle produces transient gene expression, many other routes of administration lead to rapid degradation and inefficient gene transfer[40]. Therefore, considerable effort has been devoted to developing formulations that would protect DNA and improve cellular uptake. Many of the current formulation strategies arose from efforts to improve *in vitro* transfection efficiency. Attempts to package DNA into a conventional liposome by encapsulation into the aqueous core were hindered by the large size of the plasmids[41]. Furthermore, the cellular uptake of these liposomes was minimal. In many cases, liposomes were composed of neutral and anionic lipids, which poses a problem for effective contact with anionic cell membranes. Felgner and colleagues developed a cationic lipid, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium chloride (DOTMA) to overcome this charge repulsion and improve delivery[42]. These cationic lipids are often formulated with a 'helper lipid' that is used to provide stability to the liposome structure or to increase the 'fusogenicity', which might result in increased cellular uptake and expression. The most common 'helper lipids' are dioleoylphosphatidylethanolamine (DOPE) and cholesterol. These lipids are often used in an equimolar ratio with the cationic lipid[43]. Cationic liposomes have now become commonly used, commercially available research reagents and have provided an extremely simple, safe and efficient system for the delivery of DNA into cells.

Following the development of DOTMA, many novel cationic lipids were developed for DNA transport[44,45]. The phosphate backbone of DNA confers a negative charge on the molecule, and cationic liposomes interact with DNA via electrostatic interactions to form condensed DNA with a protective lipid coating[46]. Once inside the cell, the DNA–lipid complexes are transported to endosomes and eventually to lysosomes, where most are eventually degraded[47,48]. By an unknown mechanism, some small portion of DNA–liposome complexes escape this degradative pathway and enter the nucleus, where transcription of transfected DNA occurs[48,49]. Many current efforts to match the efficiency of viral DNA delivery are focused on understanding and optimizing this process. This research effort centers around the incorporation of the viral components that allow for the efficient cellular trafficking of viral vectors. The addition of fusogenic peptides (to promote endosomal escape) and/or nuclear targeting signals (to increase trafficking to the nucleus) to non-viral systems might increase their efficiency, but care must be taken not to incorporate the unwanted immunogenicity that is also associated with these viral components.

The initial success with cationic lipids led to experimentation with other cationic molecules. Cationic polymers composed of repetitive amino acid chains (e.g. polylysine, polyarginine and polyornithine) as well as naturally occurring cationic peptides (e.g. histone, spermadine and protamine) interact with DNA. These peptides and polymers have been used alone or in combination with other cationic lipids as DNA delivery vehicles[50,51]. As these compounds are found in the body, their degradative pathways are well characterized and they are generally regarded as safe. Other synthetic cationic polymers [e.g. polyamidoamine dendrimers[52], polyethyleneimine[53] and diethylaminoethyl (DEAE)[54]] are also effective packaging and transfer agents. However, the polymerization process is difficult to control and generates a heterogeneous mixture of molecules with different sizes and charges that could challenge attempts to standardize manufacturing in drug development. Furthermore, many polymers are not biodegradable, which might lead to accumulation problems, especially in cases of repeat dosing. Other cationic delivery systems include naturally occurring polymers (chitosan[55]) as well as approved drugs that bear a cationic charge, such as protamine sulfate[51] and the local anesthetic bupivacaine[56]. Encapsulation of DNA in poly(lactide-co-glycolide) microspheres also confers sustained protection, and the preferential uptake by APCs could prove advantageous for vaccine delivery[57]. Comparisons of naked DNA and DNA formulations are complicated by many factors, including the size of complexes and the route of administration. Despite considerable effort since the development of Lipofectin® (DOTMA/DOPE) by Felgner in 1987[42], the

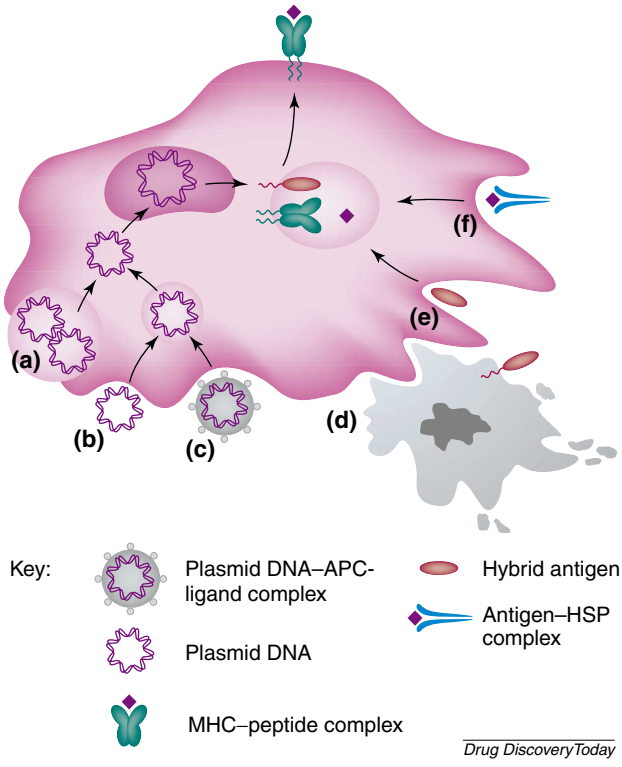


**Figure 3.** Delivery of plasmids and plasmid-encoded antigen to antigen presenting cells (APCs). APCs, such as macrophages, dendritic cells and Langerhans cells, have several antigen uptake strategies that can be exploited by DNA vaccines. Plasmids can be delivered directly by ballistic delivery on gold particles **(a)** or by pinocytosis of naked plasmids **(b)**. Complexing DNA plasmids with certain lipids, peptides and/or carbohydrates can facilitate endocytosis mediated by APC receptors such as the mannose receptor, Fc receptor or apoptosis receptor **(c)**. APCs can acquire antigen from apoptotic cells, including cells expressing antigen encoded by DNA plasmids, a process known as cross-priming **(d)**. Secreted antigen from cells transfected with DNA plasmids can be acquired directly by pinocytosis **(e)**. Transfected cells can also secrete peptide fragments of the encoded antigen associated with heat shock proteins, which are internalized by APCs **(f)**.

formulation of DNA for *in vivo* delivery remains largely empirical.

## Delivery options for DNA vaccines

### *Mechanical delivery options*

Initial studies of DNA vaccines administered the plasmids by intramuscular injection using a hypodermic needle and syringe. Although effective, this might not be the most efficient delivery system. The uptake of injected DNA by muscle cells is variable. Expression is often confined to the needle track, and injection parallel to myofibers yields higher expression than perpendicular injection[58]. The efficiency of uptake and expression is increased tenfold in regenerating muscle damaged by the injection of bupivacaine or cardiotoxin[59,60], but this does not always translate into more effective vaccination[61]. The recent application of electroporation following intramuscular injection offers another means to increase DNA transfer and expression. Electroporation was originally developed as a method to permeabilize cells *in vitro* using short electric pulses to improve DNA transfer efficiency. For *in vivo* gene transfer, a probe is placed above the muscle following an intramuscular injection of plasmid DNA, and a series of low-voltage high frequency electric pulses are generated. Electroporation increases the efficiency of DNA transfer and expression by 100–500-fold over simple needle injection[62,63]. Correspondingly, electroporation after needle injection of the DNA plasmid can increase vaccine potency by 10–100-fold[64].

Although muscle cells can express genes encoded by injected plasmids, they lack the MHC II, co-stimulatory molecules and cytokines required for T cell activation. Therefore, it is unlikely that transfected muscle cells directly induce immunity. Plasmid immunization of bone marrow chimeric mice (mice with immune cells derived from a genetically different donor) demonstrated that 'professional' APCs express the antigen encoded by the injected plasmid and initiate the immune response[65,66]. APCs can acquire antigens, including plasmid-encoded antigens, by several routes (Fig. 3). It is not clear whether these resident APCs are directly transfected by the injected plasmid DNA or whether the APCs acquire antigen from transfected muscle cells. However, because antigen-specific CTLs lyse muscle cells expressing the antigen encoded by the plasmid[67], the release of plasmids and antigen from damaged muscle and their increased uptake by APCs might reinforce the immune response by a process known as cross-priming.

### *Subcutaneous and mucosal DNA vaccination*

The 'gene gun' ballistic delivery of gold particles coated with DNA offers an alternative gene delivery method. A shock wave from compressed helium propels the particles into the cytosol of cells in the epidermis, transfecting up to 20% of the cells in this target area[68]. Subcutaneously delivered DNA transfects Langerhans cells (LCs), a specialized APC in the skin. The transfected LCs rapidly migrate from the epidermis[69], and are detected in draining lymph nodes within 24 h following cutaneous immunization[70]. Other cells, including keratinocytes and fibroblasts, can express antigens encoded by DNA delivered biolistically (ballistic delivery to a biological system) to the skin, but only the transfected MHC $II^+$ LCs can process and present antigenic epitopes that can activate appropriate T cells[71]. Intramuscular immunization with DNA leads to a similar

migration of transfected DCs and macrophages to proximal lymph nodes[72]. Gene gun immunization requires 10–1,000-fold less DNA than muscle injection to generate an equivalent antibody response[73], and antibody titers produced by the gene gun are more consistent among individuals than the titers of needle-injected recipients[74]. However, the characteristics of the immune response generated by each method might differ. Gene gun immunization usually induces a Th2 response, whereas hypodermic inoculation of muscle induces a Th1 response, perhaps because of the higher quantities of DNA, and hence the higher levels of immunostimulatory CpG sequences delivered[75]. However, modifications in the injection regimen or co-delivery of genes encoding Th1 cytokines (IL-2, IL-7 and IL-12) can induce a Th1 response to gene gun immunization[76].

Many pathogens that resist conventional vaccines are transmitted through mucosal surfaces. Although DNA vaccination of muscle and skin induces humoral and cellular immune responses, in most cases it does not induce the secretory antibody isotypes or CTLs in appropriate locations required for effective mucosal immunity. Recent preclinical studies suggest that intranasal or oral delivery of lipid–DNA complexes could enhance mucosal immunity. For example, intranasal administration of cationic liposomes complexed with DNA plasmids encoding luciferase, induced secretory IgA antibodies in the vaginal and rectal tracts, and generated antigen-specific CTLs in the genital and cervical lymph nodes[77]. Furthermore, oral administration of DNA encoding rotavirus antigens encapsulated in poly(lactide-co-glycolide) microparticles, conferred protective immunity in a mouse model of infant diarrhea[78]. These studies demonstrate the importance of the route of vaccination in the development of effective immunity, and show that the correct formulation can enhance the efficacy of mucosal DNA vaccines.

### *Improving DNA plasmid uptake or processing of encoded antigens*

Improving the delivery of plasmids to specific cells, such as APCs, or improving the intracellular trafficking of encoded proteins to the antigen processing and presentation pathway could provide another delivery option for improving DNA vaccines (Fig. 3). For example, complexing plasmid DNA with DEAE-dextran[79] or mannosylated polylysine[80] facilitates *in vitro* uptake by the mannose receptor and improves the transfection of macrophages. Alternatively, plasmids encoding fusion proteins offer an opportunity to improve extracellular or intracellular targeting. For example, plasmids encoding a CTLA4 molecule fused to an antigenic sequence delivered the secreted hybrid molecule to APCs with CTLA4 receptors (B7-1 and B7-2), enhancing uptake and immunity[81]. Plasmids encoding antigens fused to a ubiquitin gene produce intracellular hybrid molecules that are targeted to proteosomes, therefore increasing access of the encoded antigen to the MHC I processing and presentation pathway and enhancing CTL induction[82]. Similarly, a fusion gene encoding an antigen and a sequence that controls intracellular routing to lysosomes could increase access to the MHC II processing and presentation pathway. Furthermore, immunization of mice with a plasmid encoding a microbial protein and the cytosolic tail of lysosome integral membrane protein II (LIMP-II) greatly enhanced the induction of Th1 $CD4^+$ cells[83]. These targeting options are an advantage of DNA vaccines that is currently under exploited.

## Therapeutic applications

### *Infectious disease*

In the past decade, successful DNA vaccination using a variety of formulations and delivery methods has been demonstrated in many animal models of viral and bacterial infection[84,85]. It remains to be seen how many of the lessons learned in these studies will apply to human vaccination. Although many of the preclinical DNA vaccination experiments have been conducted in mice, a growing number of studies in non-human primates support the commencement of clinical trials. As with many current vaccines, establishing a strong, sustained immune response to DNA vaccines often requires more than one injection. In some cases, priming with DNA vaccine and boosting with an alternative delivery mode, or form of the antigen, is required to generate high, sustained antibody titers[86]. The most common booster protocols administer recombinant protein or a replication-defective poxvirus. In rhesus monkeys, vaccination with DNA encoding the HIV envelope protein (env) induced a strong CTL response but a transient antibody response. This weak antibody titer was substantially raised by a protein boost, which conferred protection against an infectious challenge[87]. A dramatic enhancement of protective immunity was also demonstrated in a rhesus model of HIV-1 infection with DNA encoding env and gag (group specific antigen) proteins administered as both a priming dose of DNA plasmids and as a boost of recombinant fowlpox virus[88].

Several recent trials have begun to apply various DNA vaccination strategies to healthy volunteers and patients (Table 2). A dose-escalating study tested the response of healthy volunteers to three intramuscular injections of 500–2500 μg of plasmid DNA encoding *Plasmodium falciparum* circumsporozoite protein given at four-week intervals[89]. Eleven of twenty subjects developed malaria-specific

**Table 2. Recent clinical trials of DNA immunotherapies and vaccines for infectious diseases**

| Principal gene(s) | Formulation | Institute | Sponsor | Phase |
|---|---|---|---|---|
| **Hepatitis B** | | | | |
| HBsAg | Gold particles | UMD | PowderJect[a]/GlaxoWellcome[b] | Ib |
| CpG7909 + Engerix-B® | Oligo + HBsAg/alum | TWH/OH | Coley Pharmaceuticals[c] SmithKline Beecham[d] | I/II |
| **HIV** | | | | |
| *nef*, *rev*, or *tat* | Naked DNA | SIIDC | None | I |
| *gag* (prime), MVA (boost) | NA | UO | IAVI | I |
| *env* and *rev* | Bupivacaine | UPA | | I |
| HIV genes | Naked DNA | | Vical[e]/Merck[f] | I |
| **Influenza** | | | | |
| Influenza genes | Naked DNA | NA | Vical/Merck | I |
| **Malaria** | | | | |
| *P. falciparum* genes (MuStDO 5) + GM-CSF | Naked DNA | NMRC | Vical/Aventis Pasteur[g] Bioject[h] | II |
| Malaria genes | Gold particles | UO | PowderJect | I |

[a]Oxford, UK; [b]Greenford, UK; [c]Wellesley, MA, USA; [d]Welwyn, UK; [e]San Diego, CA, USA; [f]Rahway, NJ, USA; [g]Lyons, France; [h]Bioject Medical Technologies, Portland, Oregon. Abbreviations: GM-CSF, granulocyte-macrophage-colony-stimulating factor; HBsAg, hepatitis B surface antigen; IAVI, International AIDS Vaccine Initiative (New York, NY, USA); MVA, Modified Vaccinia Ankara; NA, no available information; NMRC, Naval Medical Research Center (Forest Glen, MD, USA); *P. falciparum*, *Plasmodium falciparum*; SIIDC, Swedish Institute for Infectious Disease Control (Solna, Sweden); TWH, Toronto and Western Hospital (Toronto, Canada); OH, Ottowa Hospital (Ottowa, Canada); UMD, University of Maryland (Baltimore, MD, USA); UO, University of Oxford (Oxford, UK); UPA, University of Pennsylvania (Philadelphia, PA, USA).

CTLs, with a higher percentage of responsive individuals at the higher doses. Although this study did not determine whether protective immunity against malaria was established, or whether boosts with recombinant protein or poxvirus will be required, it was the first demonstration of a cellular immune response induced in humans by a DNA vaccine.

Long-term survival of seropositive HIV patients is correlated with a strong virus-specific CTL response, suggesting the need for an HIV vaccine that boosts cellular as well as humoral immunity. DNA immunization could fulfil this need, and plasmids encoding env, rev and gag/pol proteins have stimulated immune responses in a number of HIV animal models. DNA immunization conferred protection against HIV infection in chimpanzees[90], and lowered the viral load in HIV-1-infected chimpanzees[91]. Several Phase I clinical trials of HIV DNA vaccines are currently in progress. In a study of nine asymptomatic HIV patients, sets of three patients were injected three times with DNA plasmids encoding HIV-1 *nef*, *rev* or *tat* genes. All of the treated patients developed memory immune responses, and eight developed specific CTL responses[92]. Antibody and CTL responses were also induced in a study of 15 asymptomatic HIV patients receiving three doses of plasmid DNA encoding HIV-1 env and rev in a dose-escalation trial[93].

Several DNA vaccine approaches are currently in clinical trials for protection against hepatitis B, a virus carried by approximately 350 million people worldwide that can lead to liver cirrhosis and liver cancer. PowderJect (Oxford, UK) and GlaxoWellcome (Greenford, UK) are testing multiple biolistic doses of plasmid DNA encoding hepatitis B surface antigen (HBsAg). Coley Pharmaceutical Group (Wellesley, MA, USA) is comparing the potency of Smithkline Beecham's approved HBsAg vaccine (Engerix-B®) when injected with either alum or CpG7909, an oligonucleotide containing a proprietary non-methylated CpG sequence. These examples demonstrate the diversity of DNA vaccine approaches that are currently in Phase I and Phase II clinical trials for immunization against infectious diseases.

### *Cancer*

The induction of tumor immunity by DNA delivery presents a different set of challenges than those encountered during the development of DNA vaccines against infectious diseases because tumors are heterogeneous. Although the list of identified tumor antigens and associated T cell epitopes has grown considerably over the past decade, the expression of these antigens is variable among cancer patients. This heterogeneity complicates efforts to develop tumor vaccines. The expression of MHC molecules on

these tumors is often repressed, and the level of co-stimulatory molecules is frequently low, resulting in poor immunogenicity. Many tumors also secrete immunosuppressive cytokines such as TGF-β, IL-10 and prostaglandin E2, which further suppresses the activation of anti-tumor CTLs. Therefore, attempts to boost anti-tumor immunity face several hurdles.

The first clinical trial of direct tumor injection with plasmid DNA tested whether altering MHC expression on tumors could enhance immunogenicity and increase tumor cell lysis[94]. Tumors were injected with DNA encoding human leukocyte antigen-B7 (HLA-B7), which is a rare MHC allele that would activate alloreactive CTLs (those that recognize foreign HLA alleles). Because alloreactive T cells are more abundant than antigen-specific T cells by several orders of magnitude, the expression of foreign MHC alleles on tumors provokes a more robust CTL response. Vical (San Diego, CA, USA) is currently testing a liposome formulation of plasmid DNA encoding HLA-B7 and β2-microglobulin (Allovectin-7®) in Phase II and Phase III trials for metastatic melanoma (Table 3). Of 60 HLA-B7-negative patients in the three trials, six had tumor reduction and 14 had a stabilized disease after the first treatment cycle[95].

In several tumors, the presence of a strong, well-characterized tumor antigen has encouraged a DNA vaccination approach. In a Phase I/II trial, half of the prostate cancer patients administered with intradermal inoculations of DNA encoding the prostate-specific membrane antigen (PMSA) and the co-stimulatory molecule CD86, developed delayed-type hypersensitivity to PMSA[96]. Boosting with a replication-deficient viral vector encoding PMSA induced immunity in all of the patients, and several responsive individuals showed changes in PMSA levels and distant metastases.

B cell lymphomas bear clonal immunoglobulins, and the idiotypic determinants on these immunoglobulins are unique tumor-specific antigens that could prove suitable targets for DNA vaccination. Inoculation with DNA encoding these idiotypic determinants, particularly sequences from the complementarity-determining regions, can induce an anti-idiotypic immune response that protects against lymphoma challenge in animal models[97]. This type of therapy will require an individualized approach, achieved by cloning the unique sequences from each patient's biopsied lymphoma cells. Vaxid®, an anti-idiotypic plasmid vaccine developed by Vical, is currently in Phase I/II testing for the prevention of relapse in B cell lymphoma patients. Moreover, plasmids encoding colorectal cancer antigens 17-1A, 791Tgp72 and carcinoembryonic antigen are also currently undergoing clinical testing under several sponsors[98].

*Induction and delivery of cytokines in DNA-based cancer immunotherapy*

Before the inflammatory response induced by non-methylated CpG sequences was appreciated, Tokunaga and colleagues demonstrated that intralesional injection of bacterial DNA purified from *Mycobacterium* induced regression of a carcinoma in mice and guinea pigs[99]. In subsequent studies of non-viral DNA tumor therapy, several groups discovered that a negative control consisting of the expression plasmid lacking an inserted therapeutic gene often had anti-tumor activity as well, a phenomenon sometimes referred to as the empty vector effect (EVE). In retrospect, these animal studies pointed to the anti-tumor properties of the inflammatory cytokines induced by the plasmid DNA. This cytokine activation, particularly the release of IL-12 and the resulting stimulation of NK cells, might play an important, sometimes unacknowledged, adjunct role in many current gene therapy anti-cancer trials.

Current clinical trials are supplementing the innate inflammatory response to plasmid DNA by delivering genes encoding Th1 cytokines such as IL-2, IL-12 and IFN-α. Several companies are testing this strategy on patients with melanoma, renal cell carcinoma, prostate cancer or head and neck cancer (Table 3). Valentis (Burlingame, CA, USA) is also testing direct intratumor injection of melanomas with plasmids encoding IL-2 and the superantigen *Staphylococcus* enterotoxin B (SEB) formulated in DOTIM–cholesterol. Veterinary trials in domestic dogs with melanomas showed that intratumoral injections of SEB and canine IL-2 genes induced partial or complete remission in seven of eleven dogs surviving at least 12 weeks after the initial treatment[100]. Coley Pharmaceutical Group is testing an indirect cytokine approach in non-Hodgkin's lymphoma by immunizing patients with proprietary CpG oligonucleotide sequences. These trials seek to counter the heterogeneity and poor immunogenicity of many tumors by inducing a strong, localized Th1 response. Ideally, boosting of intratumoral immunity will also generate T cells that attack metastatic sites.

## Conclusions

Since the relatively recent demonstrations of bacterial DNA adjuvanticity and the immune response to injected plasmid DNA, many imaginative approaches to DNA vaccination have been tested. Much of what we currently know is based on empirical observations, and the mechanisms behind these varied approaches are not fully understood. It seems improbable that there will be one 'best way' to administer DNA vaccines. Instead, different combinations of formulation, route of administration, co-stimulatory genes and boosting regimen will probably be required for

**Table 3. Recent clinical trials of DNA immunotherapies for cancer**

| Principal gene(s) | Formulation | Institute | Sponsor | Phase |
|---|---|---|---|---|
| **B cell lymphoma** | | | | |
| Anti-idiotype (Vaxid) | Naked DNA | SU | Vical[a] | I/II |
| **Breast cancer** | | | | |
| B7-1 | NA | UPA Med | None | I |
| **Head and neck cancer** | | | | |
| IL-2 | DOTMA/Chol | JHU Med | Valentis[b]/Roche[c] | IIb |
| IL-2 | DOTMA/Chol | LMU | Valentis/Roche | I |
| HLA-B7 (Allovectin-7) | DMRIE/DOPE | UCMC | Vical | II |
| HLA-B7 (Allovectin-7) | DMRIE/DOPE | AZCC | Vical | II |
| IL-2 | DOTMA/Chol | Multi | None | I |
| IL-12 | PINC™ | DFCI | Valentis/Roche | I/II |
| IFN-γ | NA | UPA/UKY | Valentis/Roche | IIa |
| IL-12 + IFN-γ | PINC™ | UPA | Valentis/Roche | II |
| Gp-100 | DMRIE/DOPE | NCI | Vical | I/II |
| **Malignant angioendothelioma** | | | | |
| IFN-γ | PINC™ | UMI | Valentis | IIa |
| **Melanoma** | | | | |
| IL-2 + SEB | DOTIM/Chol | UCHSC | Valentis | IIa |
| HLA-B7 (Allovectin-7) | DMRIE/DOPE | Multi | Vical | II |
| HLA-B7 + Dacarbazine | DMRIE/DOPE | Multi | Vical | III |
| Gp-100 | NA | NIH | NCI-CTEP | II |
| MART-1 | NA | UAB | None | I |
| Gp-100 + GM-CSF | Gold particles | UWI | PowderJect[d] | I |
| **Non-Hodgkin's lymphoma** | | | | |
| CpG 7909 | CpG oligo | UIO | Coley Pharmaceuticals[e] | I/II |
| **Prostate cancer** | | | | |
| IL-2 (Leuvectin) | DMRIE/DOPE | UCLA | Vical | II |
| PMSA | Naked DNA | AFBR | None | II |
| **Renal cell carcinoma** | | | | |
| IL-2 (Leuvectin) | DMRIE/DOPE | UCLA | Vical | II |

[a]San Diego, CA, USA; [b]Burlingame, CA, USA; [c]Basel, Switzerland; [d]Oxford, UK; [e]Wellesley, MA, USA.
Abbreviations: NA, no available information; AFBR, American Foundation for Biological Research (Bethesda, MD, USA); AZCC, University of Arizona Cancer Center (Tucson, AZ, USA); Chol, cholesterol; CpG 7909, oligonucleotide containing a proprietary unmethylated CpG sequence; DFCI, Dana-Farber Cancer Institute (Harvard Medical School, Boston, MA, USA); DOPE, dioleoylphosphatidylethanolamine; DOTIM, 1-[2-(9-(*Z*)-octadecenoyloxy)ethyl]-2-(8-(*Z*)-heptadecenyl)-3-(hydroxyethyl)imidazolinium chloride; DOTMA, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium chloride; DMRIE, *N*-(1-(2,3-dimyristyloxypropyl)-*N*,*N*-dimethyl-(2-hydroxyethyl) ammonium bromide; GM-CSF, granulocyte-macrophage-colony-stimulating factor; gp-100, glycoprotein-100; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; JHU Med, Johns Hopkins University Medical School (Baltimore, MD, USA); LMU, Ludwig Maximilians University (Munich, Germany); Mart-1, melanoma-specific melanoma antigen recognized by T cells 1; Multi, multiple sites; NCI, National Cancer Institute (Bethesda, MD, USA); NCI-CTEP, National Cancer Institute-Cancer Therapy Evaluation Program (Bethesda, MD, USA); NIH, National Institutes of Health (Bethesda, MD, USA); PINC, protective interactive non-condensing delivery system; PMSA, prostate-specific membrane antigen; SEB, *Staphylococcus* enterotoxin B; SU, Stanford University (Palo Alto, CA, USA); UAB, University of Alabama (Birmingham, AL, USA); UCHSC, University of Colorado Health Science Center (Denver, CO, USA); UCLA, University of California at Los Angeles (Los Angeles, CA, USA); UCMC, University of Cincinnati Medical Center (Cincinnati, OH, USA); UIO, University of Iowa (Iowa City, IA, USA); UKY, University of Kentucky (Lexington, KY, USA); UMI, University of Michigan (Ann Arbor, MI, USA); UPA, University of Pennsylvania (Philadelphia, PI, USA); UPA Med, University of Pennsylvania Medical School (Philadelphia, PI, USA); UWI, University of Wisconsin (Madison, WI, USA).

each pathogen or tumor. It is already clear that not all of the lessons learned from animal models can apply to human vaccines. Therefore, new paradigms to safely and rapidly move DNA vaccines into clinical trials are required. The initial clinical trial results should provide guidance for further refinements and successes.

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