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# DNA Vaccines: Vector Design, Delivery, and Antigen Presentation

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**Abstract** Inoculations with antigen-expressing plasmid DNAs (DNA vaccines) in the production of protective immune responses. Since the initial development of DNA vaccines more than 5 years ago, major strides have been made in the design of efficient vaccine vectors and in the process of vaccine delivery. However, many questions remain regarding the mechanism of cellular transfection and in the development of immune responses. This review addresses functional aspects of DNA vaccines, including vector design and delivery, as well as cellular transfection and antigen presentation. J. Cell. Biochem. Suppls. 30/31:304–311, 1998. 

**Key words:** DNA vaccines; gene therapy; vectors; immune response; antigen presentation

The direct inoculation of antigen-expressing DNA (DNA vaccines) represents a powerful new approach to raising immune responses. The antigens are synthesized in transfected cells and obey the trafficking, modification, and antigen presentation rules of eukaryotic cells. Very low levels of antigen (typically nanogram levels) induce both antibody and cytolytic T-cell responses. This facile method of immunization supports the testing of the immunogenicity of novel recombinant molecules and the screening of DNA libraries. Uses include prophylaxis against infectious diseases, the control of tumors, and the manipulation of allergies and autoimmune disease [reviewed in Donnelly et al., 1997]. DNA-based immunizations readily raise polyclonal and monoclonal antibodies, T cell help and cytolytic T cells [reviewed in Donnelly et al., 1997]. The use of DNA for immunization holds high promise for basic studies on immune responses.

As with many other groundbreaking discoveries, the initial observation that led to the creation of DNA vaccines was an accident. During the late 1980s, with hopes of curing muscular dystrophy by gene therapy, Jon Wolff and Phil Felgner began investigating new methods to introduce expression plasmids into muscle. Using a variety of lipid and cationic vehicles to deliver pDNA into muscle by needle injection, they were surprised to observe that the negative control, pDNA alone (saline DNA), was actually far superior at transfecting cells in vivo than any other method they were testing [Wolff et al., 1990]. Stephan Johnston and colleagues demonstrated an altogether new technique for in vivo delivery of pDNA. They used a particle bombardment device, otherwise known as a gene gun, to literally shoot pDNA-coated gold beads into the epidermis of mice [Tang et al., 1992]. Working independently, Harriet Robinson and colleagues used inoculations of a pDNA encoding a replication incompetent influenza hemagglutinin-expressing retrovirus to successfully protect chickens against a lethal challenge of influenza virus [Robinson et al., 1993]. On the basis of earlier studies, in which they observed that as few as $10^5$ retroviral particles were sufficient to raise protective immune responses, Robinson realized that despite a very low transfection efficiency ($\sim 10^{-6}$–$10^{-8}$), inoculations of 100 µg of pDNA ($\sim 10^{13}$ plasmids) could still produce sufficient transfection events to mount a protective immune response. Their study was the first to use an antigen-expressing plasmid as a DNA vaccine.

This review focuses on the basic issues related to the molecular and cellular biology of DNA vaccines. The basic features of plasmid vectors are described and the latest enhancements to vaccine vector design are discussed.

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This paper also explores different methods of pDNA inoculation and focuses on the various cells transfected by each method. Finally, several models are discussed that may explain how antigen expression and processing contributes to the developing immune response.

## PLASMID DESIGN

The design of DNA vaccine vectors mimics the design of in vitro expression vectors. Each plasmid contains the elements that ensure high antigen expression, including a strong promoter, a multicloning site for insertion of an antigen gene, and a mammalian polyadenylation signal for efficient transcription termination. In addition, a bacterial origin of replication and a bacterial antibiotic resistance gene allow for plasmid replication and selection during bacterial culture (Fig. 1A).

Early research on DNA vaccines focused on the design of optimum antigen-expressing vectors. The most important findings related to the use of highly active promoters with broad tissue tropism. By far, the highest level of gene expression was observed with the cytomegalovirus (CMV) immediate early gene promoter. The promoter was further optimized by including the first intron (intron A) of the immediate early gene [Chapman et al., 1991]. Other promoters such as Simian virus 40 (SV40) and the Rous sarcoma virus (RSV) LTR have also been used successfully [Lee et al., 1997]. The BGH and SV40 polyadenylation signals have been used most commonly. In a comparison of various polyadenylation signals, one study showed slightly higher levels of mRNA and protein expression with plasmids containing a BGH poly A signal [Montgomery et al., 1993].

The most recent research on vector design focused on the addition of features that could enhance or customize the immune response (Fig. 1B). For instance, in attempts to express two genes simultaneously, people have used dicistronic vectors or multiple gene-expressing plasmids. In the dicistronic vectors, an internal ribosome entry site (IRES) was incorporated within the plasmid proximal to the second gene [Clarke et al., 1997]. For multiple gene-expressing vectors, individual promoter, gene, and poly A signal units were linearly arranged within the same plasmid [Iwasaki et al., 1997a]. To enhance proteasome-dependent protein degradation, and consequently MHC class I presentation of antigen, a ubiquination signal was incorporated proximal to the gene insertion site [Rodriguez, et al., 1997; Wu and Kipps, 1997]. To express a secreted protein, leader sequences have been placed distal to the promoter element [Haddad et al., 1997]. To restrict gene expression to a limited number of tissues, cell specific promoters have been installed [Xiang et al., 1997]. To provide cytokine enhancement of the immune response, researchers have used either cytokine/antigen fusion genes or have incorporated cytokine genes into multigene-expressing vectors [Iwasaki et al., 1997a]. Others have enhanced immune responses by placing immunostimulatory CpG DNA sequences

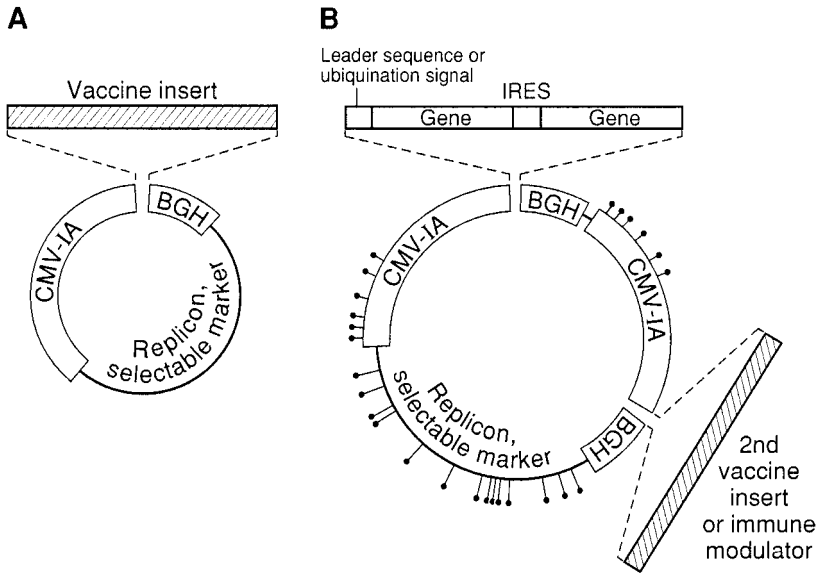


**Fig. 1.** Schematic representation of vectors for DNA immunization. **A:** Basic plasmid design. CMV-IA, CMV promoter containing intron A; BGH, bovine growth hormone polyadenylation signal. **B:** Additional features for vectors used in DNA immunization. IRES, internal ribosome entry site; CMV-IA, CMV promoter containing intron-A; BGH, bovine growth hormone polyadenylation signal; black lollipops, CpG immunostimulatory sequences.

into the vector backbone [Sato et al., 1996]. Finally, to broaden the antigenic capacity of DNA vaccines, vectors have been designed to incorporate cDNAs libraries from particular pathogens. Inoculation with a pool of such vectors, expression library immunization, has successfully protected mice against pathogen challenge even when the antigens that conferred protection were unknown [Barry et al., 1995].

## METHODS OF DNA DELIVERY

Several methods of pDNA delivery have been used by investigators to transfect cells. Two of the most well-studied methods of pDNA delivery—saline DNA injections and gene gun DNA inoculations—are discussed in this review. Other methods to deliver pDNA have been used, including liposome-encapsulated DNA [Kuklin et al., 1997], cochleate DNA complexes [Gould-Fogerite and Mannino, 1996], and attenuated *Shigella* as a transport vehicle [Sizemore et al., 1995]. Thus far, limited evidence is available to demonstrate unequivocally the efficacy with which these methods of DNA delivery can generate protective immune responses.

### Injections of DNA in Saline

This method entails the use of pDNA diluted to a desired concentration in saline. The saline DNA solution can then be administered by syringe to a variety of injection sites including, intramuscular (i.m.), intradermal (i.d.), intravenous (i.v.) [Zhu et al., 1993], and intrafollicular (thyroid) [Sikes et al., 1994]. Additionally, epidermal delivery has been accomplished by scarification [Raz et al., 1994]. To enhance uptake and distribution of DNA to cells, some investigators co-inject a mild anesthetic or a sucrose solution [Davis et al., 1993]. The precise mechanism by which cells take up pDNA is unknown. It has been speculated that nonspecific processes such as pinocytosis may be responsible; however, a role for a pDNA receptor has not been excluded. For many antigens, an inoculation of 50–100 μg of pDNA with or without a boost will result in a strong immune response. However, as little as 1 μg of pDNA given i.m. or i.d. can raise a measurable immune response [Feltquate et al., 1997].

### Gene Gun

A particle-bombardment device, otherwise known as a gene gun, uses a regulated burst of helium gas to propel pDNA-coated gold beads (~1–3 μm in diameter) into the freshly shaven skin of an animal [Tang et al., Johnston, 1992]. Plamid DNA-coated gold beads penetrate cells of the epidermis and consequently deposit pDNA intracellularly. Thus, a gene gun inoculation is a direct means of transfecting cells. Because of this, gene gun inoculations appear to be more efficient than other DNA delivery methods since direct delivery avoids the dilution of pDNA within extracellular fluid. Each "shot" carries ~0.25 μg of pDNA. Several "shots" can be given to adjacent regions of skin to increase the delivered dose. Typically, 0.5–2.0 μg of pDNA with or without a boost generates a strong immune response. However, as little as a single inoculation of 0.4 ng of pDNA has been shown to generate a measurable immune response [Robinson et al., 1995].

Besides offering multiple options for DNA immunization, the different methods of DNA inoculation can influence the type of immune response. One component of most immune responses is the T-helper (Th) cell. Th cells function by providing "help" in the form of cytokines to B cells and cytolytic T cells. Th cells exist in two forms—Th1 and Th2—and they serve to support distinct types of immune responses. Th1 cells stimulate the development of cytolytic T cells and assist B cells to make IgG2a antibody, while Th2 cells signal B cells to produce IgG1 antibody. A pathogenic relationship has been demonstrated for certain disease processes and for each of these particular types of immune responses. Thus, methods that can subvert or prevent the development of one or the other type of immune response may be important. To date, most reports have shown that saline DNA immunizations stimulate Th1 immune responses, whereas gene gun DNA immunizations produce Th2 responses (Table I) [Feltquate et al., 1997]. However, several factors can affect this general pattern. Whether an antigen is secreted, membrane-bound, or intracellularly located seems to influence the subsequent immune response [Lewis et al., 1997]. When given as a saline DNA immunization, antigens that are cell associated tend to produce Th1 immune responses, while secreted antigens do not elicit one specific type of response (Table I). For instance, an i.m. saline DNA immunization with the membrane-bound

influenza hemagglutinin produces a Th1 response. However, a similar immunization with a secreted form of the same antigen generates a Th2 response. Exactly how a cell-associated form of an antigen and a secreted form of the same antigen can produce different types of immune responses is unclear. After gene gun DNA immunization, most antigens tested have raised Th2 responses (Table I). Other factors that can affect Th cell differentiation include the type of antigen [Leitner et al., 1997] and co-inoculation of cytokines or other immunostimulants [Gurunathan et al., 1997].

A current area of active investigation involves the delivery of pDNA to mucosal tissues. Many pathogens enter the body through mucosal tissues lining such structures as the nasal passages, lungs, gastrointestinal tract, and reproductive tract. An important component to the protective barrier offered by the mucosal lining is the antibody subtype known as IgA. IgA antibody is typically derived from immune responses that have originated in mucosal tissues. Thus, a major focus of general vaccine research is to design vaccines that can be administered to mucosal tissues and elicit mucosal immunity (IgA antibody). With respect to DNA vaccines, very few studies have produced protective immune responses when pDNA was administered to mucosal tissues. The most productive methods for pDNA delivery thus far have come from liposome-encapsulated DNA complexes [Chen et al., 1997] or during saline DNA inoculations in which cholera toxin was coadministered [Kuklin et al., 1997]. More improved methods of DNA delivery are needed as well as a better understanding of which transfected cells are required for the development of an effective immune response.

**TABLE I. Effect of Method of Inoculation and Cellular Location of Antigen on the Type of Immune Response Elicited by DNA Immunization**

| Method | Intracellular | Membrane-bound | Secreted |
|---|---|---|---|
| Saline DNA | Th1[a] | Th1 | Mixed[b] |
| Gene gun | Th2[c] | Th2 | Th2 |

[a]Th1 indicates a predominantly IgG2a antibody response or interferon-γ (IFN-γ) production by restimulated T cells.
[b]Mixed indicates no specific type of response (Th1 and Th2).
[c]Th2 indicates a predominantly IgG1 antibody response or interleukin-4 (IL-4) production by restimulated T cells.

## CELLULAR TRANSFECTION AND ANTIGEN EXPRESSION

Depending on the route and method of DNA inoculation, a variety of cells are transfected with pDNA. The total quantity of antigen produced by cells has been measured to range on the order of picograms to nanograms per day, while the kinetics of antigen expression varies with the cell type and site of inoculation. The following is a list of cell types known to be transfected during DNA immunization.

### Myocytes

After i.m. saline DNA inoculation, the most commonly transfected cell is the myocyte. Approximately 1–3% of myocytes within a muscle bundle are transfected and express antigen after a single saline DNA injection [Wolff et al., 1992]. As measured by immunohistochemistry, antigen expression by muscle cells is long-lived. Luciferase expression was observed to persist for at least 19 months after an inoculation with a luciferase-expressing pDNA, while pDNA has been detected in muscle for greater than 1 year [Wolff et al., 1992]. However, a portion of antigen-producing muscle cells may be destroyed by antigen-specific cytotoxic T lymphocytes (CTL) within weeks as the immune response develops.

### Keratinocytes

During both gene gun and i.d. saline DNA immunization, keratinocytes are the most commonly transfected cell [Raz et al., 1994]. Approximately 1–5% of keratinocytes within an inoculation site are transfected and express antigen after DNA inoculation. Antigen expression peaks at 24 h and is essentially absent after several days as a result of the natural sloughing of skin [Raz et al., 1994].

### Macrophages and Dendritic Cells

Recently, it has become evident that tissue macrophages and dendritic cells (DC) are transfected during DNA inoculations by most methods of DNA delivery. The use of polymerase chain reaction (PCR) demonstrated that macrophages residing in inoculated tissues harbor pDNA. Macrophages from lymph nodes and spleen have also been observed to contain pDNA [Chattergoon et al., 1998]. Cells from these tissues acquire pDNA by either of two nonmutu-

ally exclusive processes. As little as 20 min after a DNA injection, pDNA can be found in lymph nodes, suggesting that pDNA can leak out of tissues and travel to draining lymph nodes where macrophages and dendritic cells may be transfected [Kuklin et al., 1997]. Additionally, 24 h after a gene gun inoculation of fluorescein-painted skin, labeled Langerhan DCs containing a gold bead were found in draining lymph nodes, suggesting that recently transfected cells can migrate to draining lymph nodes [Condon et al., 1996]. Currently, it is uncertain how long the macrophages and dendritic cells express antigen and whether these cells are long-lived or are removed by the developing immune response.

### Other Cells

Other types of epithelial cells are also transfected during saline DNA inoculations. These cells constitute a very small subset of the total number of cells transfected following i.m. and i.d. saline DNA immunization. However, after an intravenous or intrafollicular saline DNA inoculation, vascular endothelial and thyroid follicular cells are the predominant cells transfected, respectively [Sikes et al., 1994; Zhu et al., 1993].

An important area of future research will focus on the actual methods by which cells can take up pDNA. Antisense research has demonstrated that short oligonucleotides can easily be taken up by many cell types, using a variety of biological processes. However, the much larger pDNA molecules apparently require a more complex mechanism of uptake, as evidenced by the much lower transfection efficiency and relatively restricted cell specificity of pDNA uptake. Formal evidence for a "pDNA receptor" is currently limited to a few studies that demonstrated that macrophage scavenger receptor bearing cells within the liver specifically bound i.v. injected pDNA [Kawabata et al., 1995]. A more sophisticated understanding of the mechanism of pDNA uptake should contribute to more improved pDNA design, and perhaps enhanced pDNA uptake efficiency. Such improvements may not only augment the immunogenicity of DNA vaccines but may contribute to research on gene therapy as well.

## ANTIGEN PRESENTATION

Professional antigen-presenting cells (APCs), including macrophages and dendritic cells, are essential for the production of most immune responses. Central to APC function is the processing and presentation of immunogenic peptides from antigenic proteins. APCs acquire protein antigens either through the endogenous production of antigen or by acquiring it exogenously. The protein antigen is processed into short peptides and placed into small grooves on major histocompatibility complex (MHC) molecules, a T-cell recognition complex found on the surface of cells. Antigen-specific T cells that recognize the peptide–MHC complexes on APCs are activated and perform a variety of functions one of which is to help B cells produce antigen-specific antibody.

Considering that most cells producing antigen are nonprofessional APCs (myocytes, keratinocytes), a great deal of study has focused on which cells actually serve as APCs during DNA immunization. Several lines of evidence suggest that nonprofessional APC, such as muscle cells, do not act as APCs during DNA immunization. Using bone marrow chimeric mice, several groups have demonstrated unequivocally that bone marrow-derived cells, which include professional APCs, function as APCs during both gene gun and saline DNA inoculations [Corr et al., 1996; Doe et al., 1996; Iwasaki et al., 1997b].

To explain how APCs receive antigen for presentation, two models have been proposed [Pardoll and Beckerleg, 1995]. In the first model, directly transfected APCs are considered the primary cells responsible for driving the immune response (Fig. 2A). These cells can process and present antigen through an endogenous processing pathway. Transfected nonprofessional APCs (keratinocytes or muscle cells) would serve a minimal role in contributing to the immune response. In the second model, the majority of transfected cells are considered to act as "factories," producing antigen for APCs to acquire, process, and present to the immune system (Fig. 2B). Directly transfected APC would constitute a small fraction of the total number of APCs involved in the developing immune response. The bulk of the immune response would be proportional to antigen production by cells.

### Directly Transfected APCs

Evidence supporting this model demonstrates that directly transfected APCs are capable of producing an immune response and that antigen production by nonprofessional APCs may

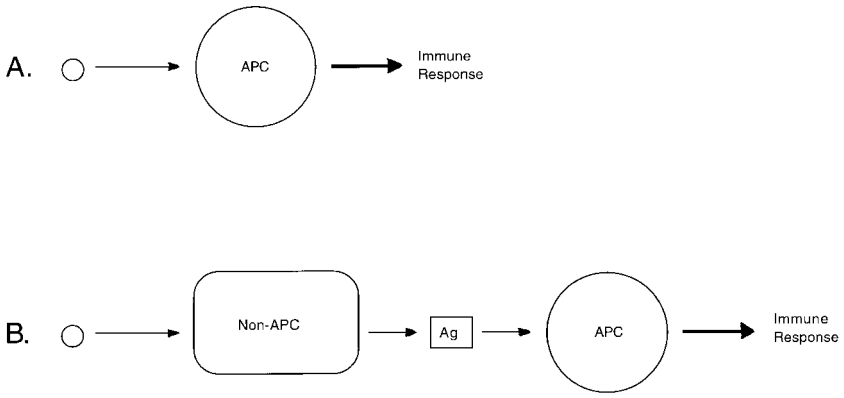


**Fig. 2.** Model depicting modes of antigen acquisition for antigen presentation during DNA immunization. **A:** Direct transfection. Plasmid DNA directly transfects APC, which then stimulates an immune response. **B:** "Factory" model. Plasmid DNA transfects non-APC (i.e. skeletal muscle cell, keratinocyte). Antigen (Ag) produced by non-APC is acquired by APC, which then stimulates an immune response.

not contribute significantly to the magnitude of the immune response. For instance, a vaccine vector containing an APC-specific promoter (MHC class II promoter) was capable of raising an immune response [Xiang et al., 1997]. The magnitude of the immune response was diminished when compared with controls that received a vector using a CMV promoter. It was unclear whether the diminished response was attributable to a net reduction in the number of APCs or because the MHC class II promoter had a much lower activity than the CMV promoter. At a minimum, however, Xiang and colleagues demonstrated that antigen production by only APC was sufficient to raise an immune response. Thus, transfection and antigen production by non-APC "factory" cells was unnecessary for the development of an immune response. Similarly, another group demonstrated the transfer of DC-containing antigen-expressing pDNA was sufficient to generate a measurable immune response in mice [Casares et al., 1997]. The addition of a ubiquination signal to an antigen gene can eliminate the ability of the antigen to stimulate an antibody response [Rodriguez et al., 1997]. Since the antigen can still generate a very good CTL response, it was concluded that the rapid degradation of the antigen limited the range of cells that could have "seen" the antigen to include only those cells that were producing the antigen [Rodriguez et al., 1997]. Plasmid DNA has been found in APCs in lymph nodes and spleen [Chattergoon et al., 1998] and in draining lymph nodes as quickly as 20 min after an i.m. saline DNA immunization [Kuklin et al., 1997]. Taking advantage of this finding, Torres et al. [1997] used timed muscle ablations to demonstrate that antigen production by muscle cells was not necessary for the generation of an immune response. Removal of a muscle bundle seconds after it was injected with pDNA did not prevent the generation of an immune response equal in magnitude to nonablated control mice. Thus, sufficient pDNA might have been leaving the injection site to transfect APC located elsewhere.

### Factory Model

Most evidence supporting this model demonstrate that antigen production by non-APCs is important for maximizing or enhancing the immune response. For instance, the study using an APC-specific promoter referred to above [Xiang et al., 1997] can be interpreted to support the factory model. One possible explanation was that an insufficient amount of antigen was produced because only a very few number of transfected cells were capable of producing antigen. Second, in contrast to the results of muscle ablation studies described above, when skin was removed soon after gene gun DNA inoculation, the immune response was abolished [Torres et al., 1997]. The longer skin was kept in place after DNA inoculation, the greater the immune response. Thus, for gene gun skin inoculations, the immune response was in direct proportion to the production of antigen by keratinocytes. Finally, non-APC-produced antigen was capable of generating an immune response. Injection of an antigen-producing stably transfected myocyte cell line into the muscle of naive mice resulted in the generation of an equivalent CTL response as mice receiving an intramuscular DNA immunization [Ulmer et al., 1996]. One possible conclusion from this study was that the role of APC in DNA immunization could be considered secondary to the skeletal muscle cell's job as a "factory" for antigen production.

On the basis of these studies, production of the optimum immune response appears to rely on a combination of both models. Although direct transfection of APCs may be sufficient to elicit an immune response, it seems plausible that the maximum immune response will be produced by the synergism of both pathways. The development of an immune response using different methods and routes of DNA inoculation may depend unequally on one model compared with the other. More research into the early phases of the immune response, including aspects of antigen acquisition and processing, will be needed to help understand the contribution each model may have in the immune response.

Rapid advances in vaccine vector design and a better understanding of the cellular processes driving the immune response have contributed to the current success of DNA vaccines. However, many questions pertaining to the cellular and biochemical mechanisms of DNA vaccines remain. The key events surrounding cellular uptake of pDNA and the relationship between antigen expression and presentation remain to be elucidated. Future improvements in DNA vaccine delivery and transfection efficiency may translate into major breakthroughs for the related field of gene therapy.

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