Annu. Rev. Immunol. 2000. 18:927–974

DNA VACCINES: Immunology, Application, and Optimization*

Sanjay Gurunathan[1], Dennis M. Klinman[2], and Robert A. Seder[1]

[1]*Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892 (Corresponding author);*

[2]*Section of Retroviral Immunology, Center for Biologics Evaluation and Research, US Food and Drug Administration, Bethesda, Maryland 20892; e-mail: seder@nih.gov*

Key Words CpG sequences, plasmid DNA

■ **Abstract** The development and widespread use of vaccines against infectious agents have been a great triumph of medical science. One reason for the success of currently available vaccines is that they are capable of inducing long-lived antibody responses, which are the principal agents of immune protection against most viruses and bacteria. Despite these successes, vaccination against intracellular organisms that require cell-mediated immunity, such as the agents of tuberculosis, malaria, leishmaniasis, and human immunodeficiency virus infection, are either not available or not uniformly effective. Owing to the substantial morbidity and mortality associated with these diseases worldwide, an understanding of the mechanisms involved in generating long-lived cellular immune responses has tremendous practical importance. For these reasons, a new form of vaccination, using DNA that contains the gene for the antigen of interest, is under intensive investigation, because it can engender both humoral and cellular immune responses. This review focuses on the mechanisms by which DNA vaccines elicit immune responses. In addition, a list of potential applications in a variety of preclinical models is provided.

INTRODUCTION

The concept of vaccination was demonstrated over 200 years ago when Jenner showed that prior exposure to cowpox could prevent infection by smallpox. Over the last century, the development and widespread use of vaccines against a variety of infectious agents have been a great triumph of medical science. Despite these successes, vaccines for many pathogens throughout the world, including human immunodeficiency virus (HIV) and the agents of malaria and tuberculosis, are

either ineffective or unavailable. One of the impediments to successful vaccination against the aforementioned infectious agents is that they likely require a cellular immune response for protection. In this regard, although all currently licensed vaccines are efficient at inducing antibody responses, only vaccines derived from live attenuated organisms induce cellular immunity efficiently. It should be noted, however, that widespread use of live attenuated vaccines might be precluded by practical constraints such as manufacturing and safety concerns. Thus, the demonstration over the last decade that plasmid DNA vaccines can induce both humoral and cellular immune responses in a variety of murine and primate disease models has engendered considerable excitement in the vaccine community.

The historical basis for DNA vaccines rests on the observation that direct in vitro and in vivo gene transfer of recombinant DNA by a variety of techniques resulted in expression of protein. These approaches included retroviral gene transfer, using formulations of DNA with liposomes or proteoliposomes (1–3), calcium phosphate-coprecipitated DNA (4), and polylysine-glycoprotein carrier complex (5). In the seminal study by Wolff et al of ''plasmid or naked'' DNA vaccination in vivo, it was shown that direct intramuscular inoculation of plasmid DNA encoding several different reporter genes could induce protein expression within the muscle cells (6). This study provided a strong basis for the notion that purified/recombinant nucleic acids (''naked DNA'') can be delivered in vivo and can direct protein expression. These observations were further extended in a study by Tang et al (7), who demonstrated that mice injected with plasmid DNA encoding hGH could elicit antigen-specific antibody responses. Subsequently, demonstrations by Ulmer et al (8) and Robinson et al (9) that DNA vaccines could protect mice or chickens, respectively, from influenza infection provided a remarkable example of how DNA vaccination could mediate protective immunity. The mouse study further documented that both antibody and $CD8^+$ cytotoxic T-lymphocyte (CTL) responses were elicited (8), consistent with DNA vaccines stimulating both humoral and cellular immunity.

DNA vaccination might provide several important advantages over current vaccines (Table 1). (*a*) DNA vaccines mimic the effects of live attenuated vaccines in their ability to induce major histocompatibility complex (MHC) class I-restricted $CD8^+$ T-cell responses, which may be advantageous compared with conventional protein-based vaccines, while mitigating some of the safety concerns associated with live vaccines. (*b*) DNA vaccines can be manufactured in a relatively cost-effective manner and stored with relative ease, eliminating the need for a ''cold chain'' (the series of refrigerators required to maintain the stability of a vaccine during its distribution). In light of these potential advantages, this review focuses on the mechanisms by which DNA vaccines induce immune responses. In addition, we have provided a table of diseases for which DNA vaccines are effective in animal models. For additional information on DNA vaccination with an emphasis on viral infections, we refer to the recent review

TABLE 1 Comparative analysis of various vaccine formulations

		DNA vaccine	Live attenuated	Killed/protein subunit
Immune response				
Humoral	B cells	+ + +	+ + +	+ + +
Cellular	$CD4^+$	+ + + Th1[a]	+/− Th1	+/− Th1
	$CD8^+$	+ +	+ + +	−
Antigen presentation		MHC class I & II	MHC class I & II	MHC class II
Memory	Humoral	+ + +	+ + +	+ + +
	Cellular	+ +	+ + +	+/−
Manufacturing				
	Ease of development and production	+ + + +	+	+ +
	Cost	+ + +	+	+
	Transport/Storage	+ + +	+	+ + +
Safety		+ + +[b]	+ +[c]	+ + + +

[a]Th2 responses can be induced by gene gun immunization in mice.
[b]Data available only from Phase 1 trials.
[c]Live/attenuated vaccines may be precluded for use in immunocompromised patients and certain infections such as HIV.

by Robinson & Pertmer (10). Finally, a comprehensive web site on DNA established by Whalen (10a) can be found at www.genweb.com/dnavax.html.

REQUIREMENTS FOR A DNA VACCINE VECTOR

There are several factors that influence the type of immune response induced by DNA vaccination. This section outlines the two basic elements of a DNA vaccine that influence the transcription and modulation of the immune responses. A more comprehensive discussion of how the plasmid DNA can be optimized for a specific type of immune response is presented in a later section.

Expression Plasmid Backbone

DNA vaccines consist of the foreign gene of interest cloned into a bacterial plasmid (Figure 1). The plasmid DNA is engineered for optimal expression in eukaroytic cells. Requisites include (*a*) an origin of replication allowing for growth in bacteria (the *E. coli:*Co1EI origin of replication in PUC plasmids is most commonly used for this purpose, because it provides large copy numbers in bacteria with high yields on purification); (*b*) a bacterial antibiotic resistance gene (this allows for plasmid selection during bacterial culture; the ampicillin resistance gene, the most common resistance gene used for studies in mice, is precluded for

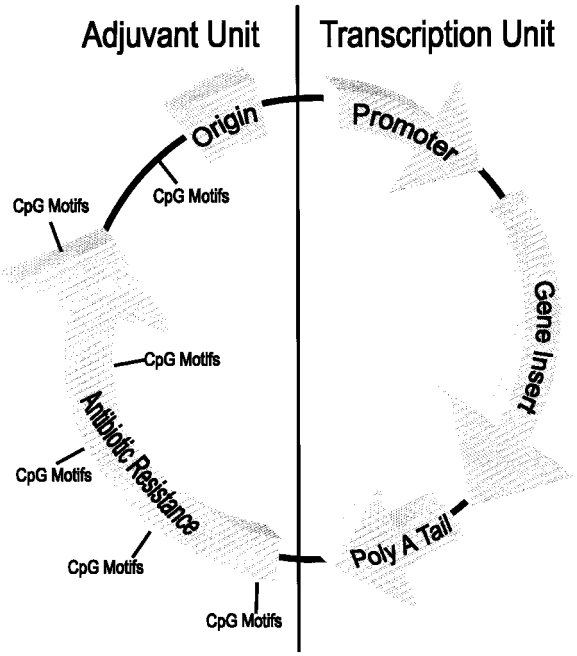


Figure 1 Schematic for the basic requirements of a plasmid DNA vector. The essential features for a plasmid DNA vector include a transcriptional unit, which consists of a viral promoter (i.e. cytomegalovirus), an insert containing the antigen, and transcription/termination sequences (Poly A). The other essential components include a bacterial origin of replication and antibiotic resistance gene, allowing for growth and selection in bacteria. The adjuvant properties of a plasmid vector are highly influenced by the number of CpG motifs within the plasmid backbone.

use in humans, and kanamycin is often used); (*c*) a strong promoter for optimal expression in mammalian cells (for this, virally derived promoters such as from cytomegalovirus (CMV) or simian virus 40 provide the greatest gene expression); and (*d*) stabilization of mRNA transcripts, achieved by incorporation of polyadenylation sequences such as bovine growth hormone (BGH) or simian virus 40.

Contribution of Immunostimulatory Cytidine-Phosphate-Guanosine Motifs

In addition to the requirements outlined above, DNA vaccines also contain specific nucleotide sequences that play an important role in the immunogenicity of these vaccines. Yamamoto et al were the first to report that synthetic oligodeoxynucleotides (ODNs) with sequences patterned after those found in bacterial DNA could activate natural killer cells to secrete interferon (IFN)-γ (11). They hypothesized that palindromic sequences present in the synthetic ODNs were

responsible for this stimulation. More recently, it was shown that a specific sequence motif present in bacterial DNA elicited innate immune responses characterized by the production of interleukin (IL)-6, IL-12, tumor necrosis factor (TNF)-α, IFN-γ, and IFN-α (12–14). This motif consists of an unmethylated cytidine-phosphate-guanosine (CpG) dinucleotide with appropriate flanking regions. In mice, the optimal flanking region is composed of two 5' purines and two 3' pyrimidines (14, 15). Such motifs are 20-fold more common in microbial than mammalian DNA, owing to differences in frequency of use and the methylation pattern of CpG dinucleotides in prokaryotes vs eukaryotes (16, 17). CpG motifs directly activate B cells to proliferate or secrete antibody (15). In addition, they directly induce professional antigen-presenting cells [APCs; i.e. macrophages and dendritic cells (DCs)] to secrete cytokines (12, 18, 19). Natural killer (NK) cells are indirectly activated by CpG motifs through cytokines induced by APCs (20). Finally, T cells are also stimulated directly or indirectly by CpG motifs, depending on their baseline activation state (21). Because CpG motifs have such a prominent role in enhancing the immune response after DNA vaccination, a more detailed summary of their role is highlighted below in the section discussing approaches to vaccine optimization.

IMMUNOLOGY OF DNA VACCINATION

An important first step in the rational design of a vaccine is to understand the immune correlates of protection. For most viral and bacterial infections, primary protection is mediated by a humoral immune response (production of antibodies). For intracellular infections such as *Mycobacterium tuberculosis, Leishmania major,* and other parasites, protection is mediated by cellular immunity. Moreover, for some diseases [e.g. human immunodeficiency virus (HIV) infection, herpes, and malaria], both humoral and cellular responses are likely to be required. The cellular immune response comprises primarily $CD4^+$ and $CD8^+$ T cells. These cells recognize foreign antigens that have been processed and presented by APCs in the context of MHC class II or class I molecules, respectively. Exogenous antigens provided by killed/inactivated pathogens, recombinant protein, or protein derived from live vaccines are taken up by APCs by phagocytosis or endocytosis and presented by MHC class II molecules to stimulate $CD4^+$ T cells, which can help generate effective antibody responses. In contrast, MHC class I molecules associate with antigens synthesized within the cytoplasm of the cell (with rare exceptions) and are generally elicited by live or DNA vaccines. From an immunologic standpoint, based on the broad range of effector cells generated and the memory responses they induce, live attenuated vaccines represent the vaccines of choice for those diseases requiring both humoral and cellular responses (Table 1). From a practical and safety standpoint, however, live or live attenuated vaccines raise several issues that can preclude their widespread use. In this regard, DNA vaccines—which resemble live attenuated vaccines in their ability to induce

both humoral and cellular responses—may prove to be useful alternatives. In the next section, the mechanism by which DNA vaccines induce specific types of immunity is discussed.

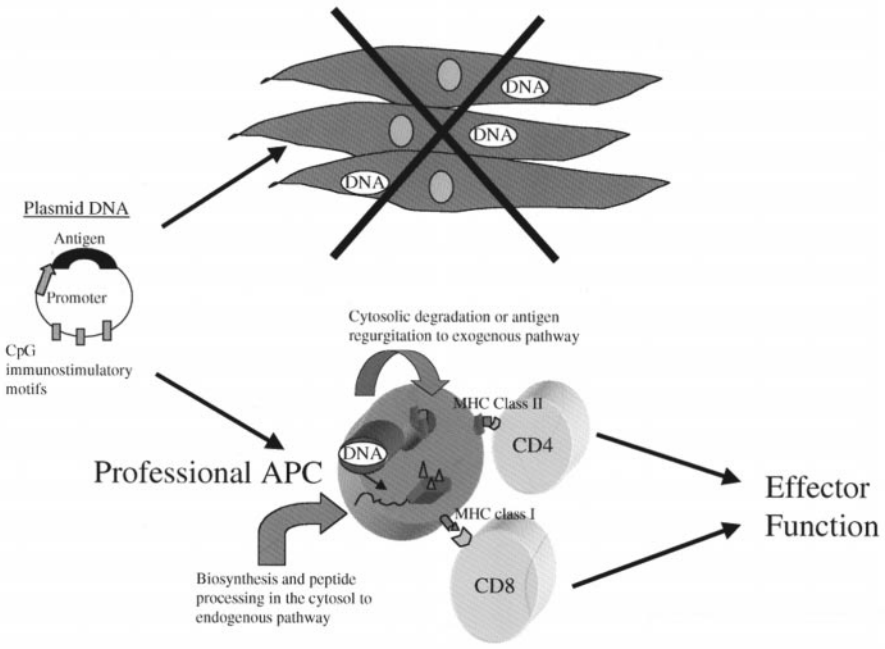
Mechanism of Antigen Presentation

One intriguing aspect of DNA vaccination involves the mechanism by which the antigen encoded by the foreign gene introduced into the bacterial plasmid is processed and presented to the immune system. Studies demonstrate that the quantity of antigen produced in vivo after DNA inoculation is in the picogram to nanogram range. Given the relatively small amounts of protein synthesized by DNA vaccination, the most likely explanation for the efficient induction of a broad-based and sustained immune response is the immune-enhancing properties of the DNA itself (i.e. CpG motifs) and/or the type of APC transfected. There are at least three mechanisms by which the antigen encoded by plasmid DNA is processed and presented to elicit an immune response: (*a*) direct priming by somatic cells (myocytes, keratinocytes, or any MHC class II-negative cells); (*b*) direct transfection of professional APCs (i.e. DCs); and (*c*) cross-priming in which plasmid DNA transfects a somatic cell and/or professional APC and the secreted protein is taken up by other professional APCs and presented to T cells. These three mechanisms are highlighted in Figure 2.

Direct Transfection of Professional Antigen-Presenting Cells—Bone Marrow-Derived Cells Directly Mediate Cellular Immune Responses after DNA Vaccination Several elegant studies with bone marrow-chimeric mice have conclusively demonstrated that bone marrow-derived APCs play a key role in the induction of the immune response after DNA vaccination. In these studies, parent into F1 bone marrow-reconstituted mice created a mismatch between the haplotypes of somatic cells and bone marrow-derived cells. The immune response generated on subsequent DNA immunization was found to be restricted to the haplotype of reconstituted bone marrow, providing conclusive evidence that bone marrow-derived cells were responsible for priming immune responses after DNA vaccination (22–24).

Dendritic Cells Are the Principal Cells Initiating the Immune Response after DNA Vaccination The above findings were further extended to evaluate the cellular mechanisms responsible for the activation of T cells after DNA immunization. In particular, studies were aimed at defining the specific type of APCs regulating the immune response after DNA vaccination. The first study to address this question showed that isolated DCs but not B cells or keratinocytes from DNA-vaccinated mice were able to efficiently present antigen to T cells in vitro (25). Moreover, in the same study it was estimated that only a small proportion of the DCs (0.4%) was transfected with plasmid DNA (25). Similar results were obtained in two additional studies in which the injection of DNA led to direct

A. Direct Transfection of Bone-Marrow Derived APCs



B. Direct Transfection of Somatic Cells

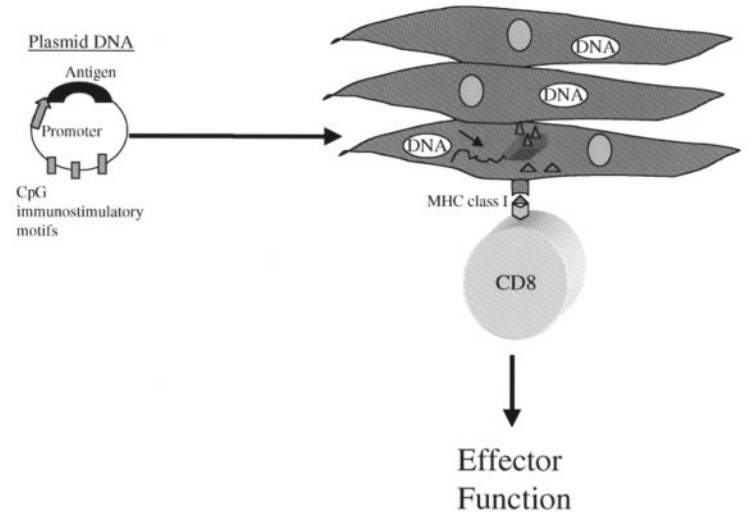


Figure 2 Legend under Figure 2c.

C. Cross Priming

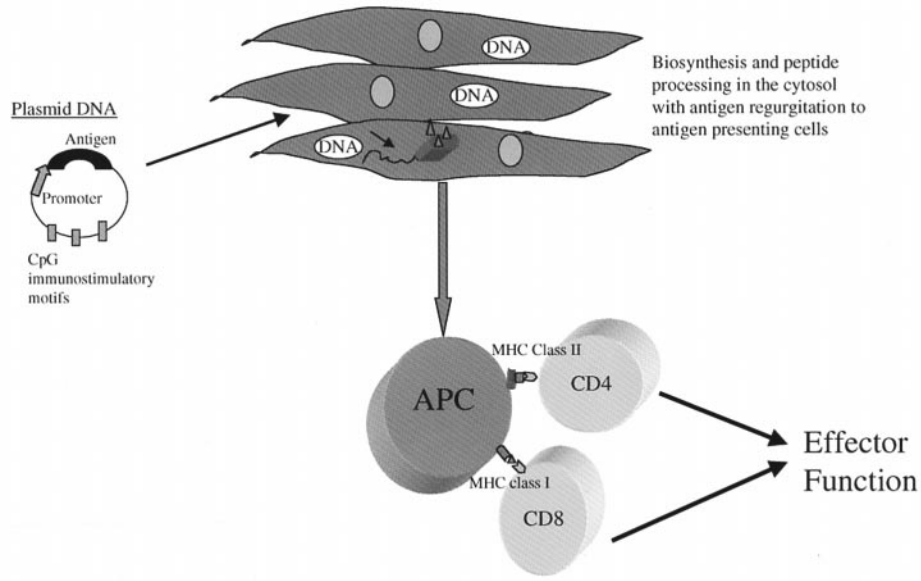


Figure 2 Mechanisms of antigen presentation after DNA immunization. A. Bone marrow-derived antigen-presenting cells (APCs)s mediate immune responses after DNA vaccination. Injection of plasmid DNA leads to direct transfection of a small number of DCs that present antigen to T cells. B. Direct priming of immune responses by somatic cells (myocytes, keratinocytes, or any major histocompatibility complex class-II–negative cells). This result could occur after injection of plasmid DNA into muscle or skin, leading to protein production and presentation to T cells by the somatic cells themselves. Alternatively, C. Protein production by transfected somatic cells may be taken up by professional APCs, leading to T-cell activation (cross-priming).

transfection of small numbers of DCs (26, 27). It is notable that in both of these studies there was general activation and migration of large numbers of DCs that were not transfected. Finally, direct in vivo visualization of antigen-expressing DCs from draining lymph nodes after gene gun vaccination was demonstrated in a separate study in which gold particles and protein expression from a reporter gene could be co-localized within a cell that had morphologic indices consistent with a DC (28). Taken together, the preponderance of data clearly demonstrates that DCs play a key role in induction of the immune response after DNA vaccination. Furthermore, these data suggest that the predominant contribution to priming immune responses after DNA vaccination involves a small number of directly transfected DCs. Additionally, as noted above, the question arises whether the enhancement in the number of migrating DCs not directly transfected with DNA, seen in many studies, could also present antigen via additional mechanisms such as cross-priming (see below).

Direct Priming of Somatic Cells—Skin vs Muscle The initial seminal study by Wolff et al (6) demonstrating the success of ''plasmid or naked'' DNA vaccination in vivo involved the direct intramuscular inoculation of plasmid DNA, leading to expression of protein within the transfected cell. The other important study by Ulmer et al (8), showing that direct intramuscular inoculation of plasmid DNA induced a strong $CD8^+$ CTL to influenza nucleoprotein, provided the first evidence that cellular responses could be induced in vivo and have a potentially important protective role. These and several additional studies suggested that muscle cells were critically involved in the initiation of immune responses after DNA vaccination. One conceptual difficulty with this premise is that, although muscle cells express MHC class I molecules, they do not express other cell surface molecules (i.e. CD80 and CD86) that are critical in optimizing T-cell priming. Therefore, they are not likely to be as efficient at presenting antigen as are DCs. This difficulty raised a question about the exact role that muscle cells play in the induction of cellular immune responses after intramuscular DNA vaccination. To address whether expression of antigen by myocytes was sufficient to induce protective immunity in vivo, it was shown that transfer of stably transfected myoblasts expressing an influenza nucleoprotein protected mice from infectious challenge (29). Although these data suggested that expression of viral protein by muscle cells in vivo is sufficient for CTL-mediated protection, the question of whether CTLs were induced directly by myocytes expressing protein directly or by transfer of protein from myocytes to professional APCs (cross-priming) remained open.

Experiments were undertaken to directly test whether muscle cells alone are sufficient to prime immune responses. In one study, using bone marrow chimeras to examine the contribution of bone marrow- and non-bone marrow–derived cells to CTL priming, it was shown that antigen-specific CTL responses could be induced by non-bone marrow–derived (muscle) cells only when mice were vaccinated with DNA encoding the antigen and CD86 (30). By contrast, in a separate study with a plasmid DNA encoding a different antigen, it was shown that plasmids encoding CD86, IL-12, or granulocyte/macrophage colony-stimulating factor DNA failed to induce muscle cells to prime for CTL responses (31). Taken together, although these studies both show that muscle cells alone are not efficient at priming immune responses, one study does suggest that muscle cells expressing CD86 are sufficient to induce a response. Finally, the finding that removing the muscle immediately (within 10 min) after immunization does not alter the subsequent immune response (32) provides additional evidence that injected plasmid DNA is likely to gain access to the lymphatic or circulatory system, thus obviating the need for transfection of muscle cells at the site of injection.

For other somatic cells, it has been shown that keratinocytes and Langerhans cells constitute the major cell types transfected by plasmid DNA after injection into the skin (33, 34). In contrast to the data mentioned above regarding removing muscle, immediate removal of skin after DNA vaccination prevented development of immune responses (32). Moreover, in a separate study, it was shown that

transplantation of vaccinated skin <12 h postvaccination could elicit an immune response in naive animals (35). By contrast, little or no immune response could be initiated when the period of transplantation exceeded 24 h. These data suggest that cells that migrated from the epidermis within 24 h of immunization induced the primary immune response after DNA vaccination. Finally, it was shown that the magnitude of the primary immune response increased when the vaccination site was left intact (35). Taken together, these data suggest that antigen-expressing nonmigratory cells such as keratinocytes may continue to produce antigen to augment the immune response (27, 35).

Cross-Priming As discussed above, secreted or exogenous proteins undergo endocytosis or phagocytosis to enter the MHC class II pathway of antigen processing to stimulate $CD4^+$ T cells. Endogenously produced proteins/peptides (e.g. viral antigens) are presented to the immune system through an MHC class I-dependent pathway to stimulate naive $CD8^+$ T cells. Although peptides derived from exogenous sources are generally excluded from presentation on MHC class I molecules, there are now several examples showing that this can occur in vivo (36–40). Moreover, the concept of cross-priming, in which triggering of $CD8^+$ T-cell responses can occur without de novo antigen synthesis within the APCs, provides an additional mechanism by which DNA immunization can enhance immune responses. During cross-priming, antigen or peptides (both MHC class I and II) generated by somatic cells (myocytes or keratinocytes) can be taken up by professional APCs to prime T-cell responses. The demonstration that transfer of myoblasts expressing an influenza nucleoprotein into F1 hybrid mice induced CTL responses restricted by the MHC haplotype of the recipient mice provided the first evidence that transfer of antigen from myocytes to professional APCs can occur in vivo in the absence of direct transfection of bone marrow–derived cells (29, 41). In addition, cross-priming can occur when professional APCs process secreted peptides or proteins from somatic cells and/or other APCs by phagocytosis of either apoptotic or necrotic bodies (42, 43). This is supported by a study showing that cross-priming of DCs occurred when keratinocytes expressing antigen were exposed to irradiation in vitro, leading to cell death (27).

In summary, the overwhelming evidence suggests that bone marrow-derived APCs, but not somatic cells, directly induce immune responses after DNA vaccination; however, because somatic cells such as myocytes or keratinocytes constitute the predominant cells transfected after DNA inoculation via muscle or skin injection, respectively, these cells may serve as a reservoir for antigen. Thus, somatic cells can be important in the induction of immune responses via cross-priming and may play a role in augmenting and/or maintaining the response.

Cellular Immunity

CD4+ T Helper Cell Responses $CD4^+$ T cells play a central role in immune homeostasis. There are at least three major functions that $CD4^+$ T cells can mediate. First, activated $CD4^+$ T cells have a critical role in promoting B-cell survival

and antibody production through CD40L-CD40 interactions (44). Second, $CD4^+$ T cells, through production of IL-2 and/or through CD40L-CD40 costimulation, provide helper function to $CD8^+$ T cells (45–47). Finally, $CD4^+$ T cells secrete a myriad of cytokines that have profound immunoregulatory effects in many disease states. In this regard, it has been demonstrated that activated $CD4^+$ T cells can be segregated into two distinct subsets based on their production of certain cytokines (48). For example, T-helper-1 (Th1) cells exclusively produce IFN-γ, whereas $CD4^+$ T cells that exclusively produce IL-4, IL-5, and IL-13 are designated as T-helper-2 (Th2) cells. Although several factors have been shown to influence the differentiation of Th1- and Th2-type cells, the cytokine milieu present at the time of initial T-cell priming appears to be the most important (48, 49). Thus, the presence of IL-12 facilitates differentiation toward a Th1 phenotype, whereas the presence of IL-4 allows for Th2 differentiation.

Because CpG motifs present in bacterial DNA can trigger the immune system to induce a variety of proinflammatory cytokines including IL-12, it would follow that the generation of Th1 responses may be a general property of DNA vaccines. Indeed, DNA vaccination has been successfully applied to several animal models of infection in which induction of a Th1 response correlates with protection (e.g. tuberculosis and leishmaniasis). DNA vaccination has also proven to be successful in a mouse model of respiratory syncytial virus infection, in which it is likely that antibodies correlate with protection. It is important that, in this infection, killed/inactivated vaccines induced a Th2-type response, which was associated with unfavorable pathology and outcome (50). This is a striking example in which DNA vaccination (by preferentially inducing a Th1 response) has a definite advantage over a formalin-inactivated respiratory syncytial virus vaccine by changing the qualitative immune response (51). Additional evidence that DNA vaccination favors a Th1 response stems from the observation that the predominant immunoglobulin (Ig) isotype detected after DNA vaccination is IgG2a (52). Of note, however, is that, under certain circumstances, DNA vaccines can also induce Th2 responses. Perhaps the best example of this involves using the gene gun method of immunization. Pertmer et al (53) first demonstrated increased IL-4 production in mice repetitively immunized by gene gun, while production of IFN-γ concomitantly decreased. As a further correlate, it was shown that the predominant Ig isotype generated after repetitive gene gun immunization was IgG1, whereas the predominant Ig isotype generated after intramuscular immunization was IgG2a (53). These observations were extended by the work of Feltquate et al (54), who substantiated the finding that different predominant T helper-type cytokines were generated by gene gun versus intramuscular DNA immunization. Preferential Th2 responses occurred whether DNA plasmids on gold beads ''shot'' into the skin or into surgically exposed muscle. Taken together, these data suggest that the use of the gene gun has a powerful influence on the induction of Th2 response regardless of the route of immunization. A potential explanation for why Th2-type responses are induced by gene gun is that the gun delivers plasmid DNA directly into cells, thus bypassing surface interaction of

CpG motifs, present in the plasmid backbone, with the APCs to mediate the release of proinflammatory Th1-type cytokines. Moreover, because DNA vaccines target DCs, it is possible that different methods of immunization could target different subsets of DCs that have been shown to preferentially bias helper T cell responses (55–59). Finally, there is some evidence that the nature of the antigen used (secreted vs intracellular) can preferentially bias T-helper responses (60, 61). It should be noted, however, that these studies used antibody subtypes rather than direct measurement of cytokine production as a surrogate for T-helper responses.

The potential of DNA vaccines to strongly influence $CD4^+$ T-helper cell responses has several practical implications. For infectious diseases, the ability of DNA vaccines to preferentially generate Th1 responses may be particularly useful for preventing intracellular infections requiring Th1 immunity or for modulating ongoing immune responses to optimize intracellular killing. First, in terms of influencing an ongoing response, it was shown that CpG ODN treatment could strikingly enhance IFN-γ and diminish IL-4 production in BALB/c mice that were already infected with *Leishmania major*, suggesting that Th1-type responses could be induced in the course of inducing ongoing Th2 response (62). Additionally, a Th1 response generated by DNA immunization may prevent or limit an ongoing Th2 response, for example, in allergic or asthmatic diseases. This was demonstrated in a study by Raz et al (63), who showed that a Th2 response (reflected by antigen-specific production of IL-4 and IgE antibody) generated by vaccination with β-galactosidase (β-gal) protein plus alum immunization could be altered (decreased IL-5 and IgE production) when these mice were boosted with plasmid DNA encoding β-gal. These data raise the broader question of whether immunotherapy with DNA vaccines affects already differentiated Th2 cells at a single-cell level or influences naive and/or activated but uncommitted $CD4^+$ T cells toward Th1 cytokine production at the population level. In addition to its ability to influence an established Th2 response, in a rat model of allergic hyperresponsiveness, it was demonstrated that injection of plasmid DNA encoding a house dust mite allergen prevented the induction of IgE and reduced airway hyperreactivity (64). In that study, the suppression of allergic responses could be transferred by CD4-depleted T cells. These findings raise the possibility that $CD8^+$ T cells can suppress IgE production and confirm the ability of DNA vaccines to induce both MHC class I- and class II-restricted responses (64). Finally, in experimental models of autoimmune disease, it appears that type I cytokine production (IL-12 or IFN-γ) correlates with disease progression. Therefore, it might be expected that DNA vaccination would not be useful for preventing or limiting ongoing autoimmune diseases associated with Th1 responses (65), yet it was demonstrated that vaccination of mice with DNA encoding a gene for a pathogenic T-cell receptor (Vβ8.2) for experimental autoimmune encephalitis (EAE) actually protected mice. Protection was associated with a reduction in the Th1 response and increase in the Th2 response (66). The mechanism for this novel observation remains to be elucidated.

Cytotoxic T-Lymphocyte Responses As noted above, one of the major advantages of DNA vaccines is the ability to generate antigens endogenously, making them accessible to $CD8^+$ T cells via an MHC class I pathway (8). Although CD8 responses are also generated by live vaccines, they are difficult to induce with conventional protein-based vaccines. Moreover, owing to the potential safety concerns about certain live viral vaccinations, the induction of $CD8^+$ CTL responses after DNA vaccination may represent a principal advantage of this type of vaccine approach. In addition, because plasmid DNA encoding an antigen can be easily modified, this method of vaccination allows for optimization of both the qualitative and quantitative aspects of CTL responses (see section on vaccine optimization below).

While it is clear that DNA vaccination is an effective method of inducing $CD8^+$ T-cell responses, there are at least two critical issues concerning the ability of this vaccine approach to mimic the responses of those achieved with live viral vaccines. The first point relates to the magnitude of the CTL response, whereas the second relates to the generation of CTL responses against dominant and subdominant epitopes. In three separate studies of DNA vaccination against lymphocytic choriomeningitis virus (LCMV) infection, mice inoculated with DNA encoding an LCMV protein generated no detectable CTL responses before infectious challenge (67–69). DNA-vaccinated mice, however, were protected from challenge with LCMV. Furthermore, in one of these studies, it was shown that mice inoculated with live LCMV had CTL activity that was immediately detectable ex vivo (69). Taken together, these data show that, in the LCMV mouse model, although live viral infection but not DNA vaccination induced a detectable frequency of effector CTLs immediately ex vivo, DNA vaccination did induce low numbers of precursor CTLs that expanded in vivo after infectious challenge sufficient for protection. In contrast to the LCMV model, the frequency of CTL precursors from cells of mice that were vaccinated with plasmid DNA encoding a Sendai virus nucleoprotein were comparable to those elicited by live Sendai virus infection in a previous report (70). Similarly, in a separate study, it was shown that CTLs generated from mice vaccinated with plasmid DNA encoding influenza nucleoprotein were comparable to those derived from mice that were infected with influenza virus (71). Thus, depending on the antigen and viral model system used, DNA vaccination can elicit CTL responses that are similar to live viral infection after short-term in vitro culture. Perhaps the critical issue of whether DNA vaccination is similar to live viral infection will be resolved by comparing the effector CTL responses immediately ex vivo without any further in vitro culturing. Current techniques, using MHC class I-specific tetramers and intracellular cytokine staining, should clarify this question. These issues are relevant to the optimization of vaccines for infections such as HIV infection or malaria, in which a high precursor frequency of effector CTLs at the time of infection may be required to limit dissemination of infection.

Although the magnitude of CTLs induced by DNA vaccination may be sufficient for protection after infectious challenge, an additional consideration is

whether DNA vaccines can elicit the same breadth of response as that induced by natural infection. In this regard, although the number of epitopes available in a primary CTL response is relatively large after viral infection, the effector CTL responses selected by the host are often limited to a few dominant epitopes. Additionally, responses to subdominant epitopes may be important in mediating an effector role in the absence of CTL responses to a dominant epitope. For instance, two separate studies showed that DNA vaccines encoding an influenza or Sendai virus nucleoprotein were able to elicit CTL responses against both dominant and subdominant epitopes (70, 71). These data suggest that DNA vaccines can elicit broad memory responses to multiple epitopes. In this aspect, DNA vaccines resemble live viral vaccines by inducing a broad precursor CTL frequency and memory.

Humoral Responses

Immunization with plasmid DNA can induce a strong antibody response to a variety of proteins in animal species, including mice, non-human primates, and, most recently, human subjects. Moreover, the humoral response generated by DNA vaccination has been shown to be protective in several animal models in vivo. Because conventional protein vaccines also induce protective antibody responses, it is useful to not only review the mechanism by which DNA vaccines induce antibody responses but also highlight potential differences and determine whether DNA vaccination offers any advantages compared with other types of vaccination.

Dose Response and Kinetics of Antibody Response Induced by DNA Vaccination With regard to the quantitative aspect of antibody production after DNA vaccination, it was shown in two separate studies with DNA-encoding influenza hemagglutinin antigen that the antibody responses peaked and reached a plateau between 4 and 12 weeks after a single DNA immunization in mice (72, 73). Furthermore, antibody production is increased in a dose-responsive manner with either a single injection or multiple injections of DNA by various routes of immunization (72, 73). Although dosage and frequency of immunizations may affect the kinetics and magnitude of the response, it is interesting that single or multiple injections with an optimal dose of DNA did not significantly affect the amount of antibody produced once a plateau had been reached (72, 73). Finally, although the duration of the antibody response can be long lived [significant serum levels were present up to 1.5 years postvaccination (34, 72)], this duration is highly variable and depends on the model system and vaccine used.

Comparison of Antibody Responses between DNA Vaccination and Protein or Live Infection: Effects on Avidity, Magnitude, Isotype, and Induction of Neutralizing Antibody As noted above, peak antibody responses after DNA vaccination occur 4–12 weeks postvaccination. Most studies comparing antibody production after DNA, protein, and live virus immunization use this time range.

In comparing humoral responses in mice vaccinated with DNA H1 hemagglutinin and mice immunized with a sublethal viral challenge with H1N1 influenza, the amount of antibody produced was substantially greater and peaked more rapidly in the sublethally infected mice than in the DNA-vaccinated mice (74). Similarly, in a separate study comparing the antibody response to DNA encoding the hemagglutinin (HA) antigen and live influenza infection, the antibody titers in mice vaccinated with live influenza were higher than in DNA-vaccinated mice, although this result was seen with only certain antibody isotypes (72). In comparing the antibody response elicited by vaccination with DNA and protein, it was shown that antibody titers and avidity were significantly lower in mice vaccinated with DNA encoding a malarial surface protein than in those vaccinated with the protein alone (75). By contrast, in one study directly comparing the kinetics of antibody response after vaccination with DNA-encoding ovalbumin (OVA) and OVA protein, there did not appear to be a difference in total OVA-specific antibody production when DNA was administered intradermally at 2 or 4 weeks postvaccination (76). In this study, antibody induced by DNA had a higher avidity than that induced by protein.

The antibody subtypes induced by DNA vaccination include IgG, IgM, and IgA. Moreover, as noted in the previous section, DNA vaccination generally enhances Th1 cytokine production. Because cytokines such as IL-4 and IFN-γ can direct IgG1, and IgG2a production, respectively, it follows that the subclass of antibodies generated by pDNA vaccination will be biased toward IgG2a production. While this appears to be a general property of DNA vaccination in mice, it has been shown that DNA encoding secreted antigen generated higher levels of IgG1 than did membrane-bound antigen (60). Moreover, as noted above, the route of DNA vaccination (gene gun) can also preferentially bias toward IgG1 production (54).

Finally, the ability of DNA vaccines to generate neutralizing antibodies suggests that antigen expressed in vivo after DNA vaccination can assume a native configuration. In this regard, the ability of plasmid DNA encoding influenza HA to generate neutralizing antibody suggests that HA was present in its native form, because the epitopes of HA that are recognized by these antibodies are formed by noncontiguous regions within HA. Thus, DNA vaccines may generate antibody responses that more closely resemble those seen after natural infection and provide a potential advantage over conventional protein vaccines. Since some recombinant proteins may lack linear determinants or conformational epitopes required for efficient generation of neutralizing antibodies. Data to support this were shown for mice immunized with a DNA vaccine encoding HIV gp120, in which sera contained antibodies reactive to linear epitopes within the V3 region of gp120 whereas sera from mice immunized with recombinant gp120 contained much lower levels of V3-specific antibodies (77). Similar results were observed with a rabbit model of papilloma virus (CRPV) infection. In this model, immunization with plasmid DNA encoding a major capsid protein L1 induced neutralizing antibody. In that study, adsorption experiments with native L1 or

denatured LI protein suggested that vaccination with plasmid DNA encoding LI elicited conformationally specific neutralizing antibody (78).

Memory Immunity

The hallmark of any successful vaccine is the ability to induce long-term memory. Current vaccines—whether live attenuated or protein subunit—are successful at generating durable humoral responses. For diseases requiring cellular immunity such as parasitic, mycobacterial, and certain viral infections, however, it is not yet clear how memory responses are generated and maintained after vaccination. Regarding humoral immunity, it has been shown that mice vaccinated with DNA encoding an HA antigen had levels of anti-HA antibodies comparable with or greater than those from convalescent sera of previously infected mice that persisted over 1 year (34, 72). In other studies, however, plasmid DNA encoding a nucleoprotein of the LCMV virus administered by gene gun (69) or intramuscularly (67) either failed to give appreciable antibody responses before challenge or the responses had waned by 4 months postimmunization. Taken together, these results indicate that, while DNA vaccination can be effective at inducing long-term antibody responses, this effect may depend on the type of antigen used in the vaccine.

In terms of cellular immunity, it was recently shown that the frequency of antigen-specific $CD4^+$ T cells as measured by proliferation remained elevated for $\leq$40 weeks postvaccination. Of interest, antigen was detectable only for 2 weeks postvaccination in DCs in the draining lymph nodes but for $\leq$12 weeks in keratinocytes (27). Moreover, a functional assay performed in vivo appeared to demonstrate no source of antigen present in the spleen or lymph nodes 20 days postvaccination. Taken together, these data showed that antigen-specific $CD4^+$ T cells are activated in the draining lymph nodes and migrate to the spleen, where they can persist for up to 40 weeks in the absence of detectable antigen (27). More definitive evidence showing that DNA can induce long-lived Th1 effector responses in vivo involved a mouse model of *L. major* infection. This study demonstrated that vaccination with plasmid DNA encoding a specific leishmanial antigen is more effective than vaccination with leishmanial protein plus IL-12 protein in maintaining antigen-specific Th1 cells capable of controlling *L. major* infection (79). These data provided evidence that DNA vaccination can induce long-term Th1 responses and suggested that DNA vaccination may be more effective than vaccination with protein plus adjuvant (i.e. IL-12). Reasons for the enhanced efficacy of DNA vaccination over protein and adjuvant may include low levels of persistent antigen and/or IL-12 induced by the CpG in the plasmid DNA.

Induction of Long-Term Cytotoxic–T-Lymphocyte Responses after DNA Vaccination Although few studies have assessed the induction of $CD8^+$ T-cell responses for prolonged periods after DNA vaccination, there is a report showing

that CTL responses could be observed ≤68 weeks after intradermal injection of DNA encoding a nucleoprotein from influenza virus (34). In a separate study, DNA-primed CTL responses to hepatitis B virus envelope proteins could be detected for ≤4 months post-DNA injection (80). It should be noted that these responses were detected only after cells were re-cultured in vitro for several days and then tested. Thus, the relative effectiveness of DNA vaccination for generating fresh, memory effector CTL responses remains to be determined. One other potentially important finding relating to DNA vaccination and induction of memory $CD8^+$ T-cell responses is that CpG motifs are potent stimulators of type-1 interferons. It was originally reported by Tough et al that IFN-α enhances the proliferation of $CD8^+$ T cells expressing a surface marker, consistent with a memory phenotype (81). More recent work showed that CpG DNA appeared to stimulate T cells by inducing type-1 interferons from APCs (82). Although these studies are important in establishing a role for IFN-α in regulating activation of $CD8^+$ T cells, a functional in vivo role for these cells remains to be elucidated.

Mechanisms by Which DNA Vaccinations Induce Sustained Humoral and Cellular Immune Responses There is evidence that long-lived antigen-specific proliferative responses that are induced by DNA vaccination are maintained in the absence of detectable antigen (27). In contrast, one of the original studies on DNA vaccination by Wolff et al showed that intramuscular inoculation of plasmid DNA encoding several different reporter genes resulted in protein expression for >1 year (83). These data raise several possibilities as to how DNA vaccines induce long-term responses: (*a*)antigen is continuously present at low levels sufficient for antigen presentation but below the limit of detection as assessed by polymerase chain reaction or currently available functional assays. Alternatively, plasmid DNA may not be detectable, but synthesized antigen could persist in vivo (i.e. follicular DCs), providing a reservoir to maintain the immune response; (*b*) plasmid DNA as well as antigen are completely gone, and responses are antigen independent (27); and/or (*c*) memory cells generated by DNA vaccines differ qualitatively from those achieved by other forms of vaccination such as protein plus adjuvant.

APPROACHES TO VACCINE OPTIMIZATION

Because different diseases have specific requirements for protective immunity, a rational approach to vaccine optimization would reflect these distinct requirements. Thus, one of the principal advantages of DNA vaccination is the ease with which plasmid DNA can be manipulated to alter the quantitative and qualitative aspects of the immune response. In this section, we discuss the factors that affect the efficiency of DNA vaccines and highlight how DNA vaccines can be influenced or tailored to generate the desired immune response.

Vector Optimization

Role of Gene Regulatory Elements and Multicistronic Vectors One of the most important considerations in optimizing a DNA vaccine is the appropriate choice of a vector. The basic requirements for a plasmid vector are described above. It is generally believed that the level of gene expression in vivo obtained after DNA vaccination correlates with the immune response generated. Therefore, several laboratories sought to improve gene expression and immune responses after plasmid DNA vaccination. These approaches included optimizing gene regulatory elements within the plasmid backbone (e.g. promoter-enhancer complex or transcription termination signals) or modifying the plasmid backbone itself to enhance gene expression. As noted above, a requisite for a DNA vaccine vector is a promoter that stimulates a high level of gene expression within mammalian cells. Virally derived promoters have generally provided the greatest gene expression in vivo, whereas eukaryotic promoters are weaker (84, 85). The CMV immediate early enhancer-promoter produced the highest transgene expression in various tissues when compared with other promoters (84, 85). Furthermore, because optimal expression of certain mammalian genes depends on splicing of the mRNA transcript, inclusion of the first intron (intron A)of the immediate early gene from CMV in the promoter-enhancer complex further enhanced expression (86). To study the effects of manipulating transcriptional termination elements on gene expression, several different kinds of termination sequences have been studied. In one study, replacing the BGH transcriptional termination element with a transcriptonal terminator derived from the rabbit β-globin gene improved gene expression (87). Several other modifications that enhance gene expression have been examined. To express two or multiple genes in the same cell, dicistronic or multicistronic vectors with internal ribosome entry sites were studied. These vectors could be particularly useful in constructing multivalent vaccines from two or more different antigens from the same or different pathogens (88).

Effects of Manipulating Heterologous Genes on the Immune Response Optimizing codon usage for eukaryotic cells can also enhance expression of antigens. Codon bias has been observed in several species, and the use of selective codons in a particular gene correlates with efficiency of gene expression (89). This correlation was shown by using a plasmid expressing listeriolysin O, in which codons frequently used in murine genes were substituted for the native codons for the encoded antigen. This substitution led to enhanced CTL and protective immunity (90). Similar results were noted in mice, by using the HIV-1 gp120 sequence (91) or gp160 sequence (92).

A plasmid may also be engineered so that the encoded protein is either secreted or localized to the interior of the cell. Several studies show that the type and magnitude of the immune response depend on whether an antigen is secreted, bound on the surface of the cell, or retained within the cell. For example, secreted proteins induced higher IgG titers than the same antigen localized either on the

cell membrane or within the cell (60, 93–95). It is unclear from these studies how DNA immunization induces antibody production against intracellular, noncytopathic proteins, because B cells require free or membrane-bound linear determinants or conformational epitopes to initiate the process of clonal expansion for efficient antibody production. These concepts suggest that a nonsecreted intracellular antigen would not elicit antibody production (27). The evidence that the nature of the antigen used (secreted vs intracellular) can preferentially bias T-helper responses is less clear. In two separate studies, it was demonstrated that secreted antigens induced a higher IgG1:IgG2a ratio (suggesting a Th2 bias) than did antigens that remained cell associated (membrane anchored or cytosolic); however, these studies analyzed antibody subtypes rather than directly measuring cytokines and thus provide only a surrogate for T-helper responses (60, 93, 95). In a separate study, plasmid DNA expressing either secreted or intracellular antigen induced comparable levels of antigen-specific IFN-γ on in vitro stimulation (94). Taken together, these data suggest that cellular localization of the antigen after DNA immunization may play a role in modulating immune responses, although this role may depend on the nature of the antigen and model system used (95).

Optimizing Cytotoxic–T-Lymphocyte Responses

Enhancing Delivery into the Major Histocompatibility Complex Class I Pathway CTL responses can be enhanced by engineering the antigen to target specific cellular compartments. An example for this engineering is the use of N-terminal ubiquitination signals, which target the protein to proteosomes, leading to rapid cytoplasmic degradation and presentation via the MHC class-I pathway. In this regard, it was demonstrated that a DNA vaccine encoding β-gal that was fused with ubiquitin was more effective at inducing CTL responses than was a plasmid encoding β-gal alone. The latter construct was also less efficient at inducing antibody responses, suggesting that the transfected gene product was rapidly degraded intracellularly and that processing precluded the release of native polypeptides or proteins for efficient antibody production (96). These results are in agreement with studies in other model systems targeting HIV Nef (97) and LCMV nucleoprotein (98, 99).

Another approach is to design vectors that use the E3 leader sequence from adenovirus, which facilitates transport of antigens directly into the endoplasmic reticulum for binding to MHC class-I molecules, bypassing the need for the TAP transporter. The addition of the E3 leader sequence appeared to improve CTL responses for certain antigens (100, 101) but did not improve CTL in other model systems (100). These data suggest that endoplasmic reticulum-targeting of T-cell epitope DNA vaccines may not enhance the immune response for all antigens.

Epitope-Specific Responses: Minigenes and Multiple Epitopes Another interesting approach for improving the ability of DNA vaccines to generate cell-

mediated responses is to engineer vaccines that elicit epitope-specific CTL responses. Several groups have successfully used minimal-epitope vaccines to induce CTL responses (100–107). Furthermore, it was demonstrated that these minimal-epitope vaccines could function in isolation and when linked to other epitopes in a ''string-of-beads'' vaccine. This approach may be advantageous, because a combination of antigenic epitopes can generate a broader immune response than a DNA vaccine encoding for a single antigen. Moreover, this approach may be effective in developing a single vaccine against multiple pathogens. In this regard, epitopes from several different pathogens could be combined in a single plasmid DNA vaccine, providing an advantage over a conventional DNA vaccine strategy with a plasmid-encoding antigen(s) against a single pathogen. In a study by Thomson et al, mice vaccinated with a DNA plasmid, encoding multiple contiguous minimal-CTL epitopes derived from five separate viruses and a parasite epitope derived from malarial protein, generated MHC class I–restricted CTL responses to each of these epitopes. Furthermore, these CTLs were protective after infectious viral challenge (104). In a separate study, a novel vector containing a polyepitope construct from HIV and *Plasmodium falciparum* was also effective in generating CTL responses in mice (103).

Inclusion of a helper epitope can also enhance CTL activity after DNA vaccination (108). In a study designed to ascertain whether CTL responses generated by DNA vaccines are dependent on MHC class-II/CD4 help, CTL responses generated against a minimal epitope class-I–restricted OVA peptide were compared with those of a similar construct with the adjacent MHC class-II–restricted epitope. Very low or negligible CTL responses were observed in mice vaccinated with a minimal-epitope MHC class-I–restricted DNA construct. In contrast, mice vaccinated with either a full-length ovalbumin construct or a DNA construct with both MHC class-I and class-II epitopes induced a robust CTL response (108). These observations are in contrast to several studies in which minimal-epitope DNA vaccines generated robust CTL responses. Potential explanations for these differences include the following: (*a*)the polyepitope vaccines could lead to the assembly of neoepitopes that served to generate MHC class-II help; (*b*) CpG sequences can potently activate DCs in a nonspecific manner (27) and prime $CD8^+$ T cells in the absence of CD4 help; and (*c*) CpG motifs induce IFN-α, a cytokine shown to be important in expansion of $CD8^+$ T cells (82).

Role of Cytosine-Phosphate-Guanosine Motifs

Over the past decade, portrayals of DNA as immunologically inert have been challenged. New data indicate that bacterial DNA can trigger and instruct the immune system to respond to danger and plays an important role in host defense. This role includes B-cell activation resulting in antibody production, stimulation of cytokine-producing cells, and activation of the innate immune system. The subsequent identification of CpG motifs present in bacterial DNA as potent immu-

nostimulatory molecules has spurred tremendous interest in the development of immune-based therapies and of a new generation of experimental vaccines.

Immunostimulatory Properties of Cytosine-Phosphate-Guanosine DNA As noted above, it was recently shown that a specific sequence motif present in bacterial DNA elicits an innate immune response characterized by the production of IL-6, IL-12, TNF-α, and IFN-γ. Several lines of evidence suggest that CpG motifs in plasmid vectors contribute to the immunogenicity of DNA vaccines. First, vectors lacking protein-encoding inserts induce cytokine production in vitro in a manner indistinguishable from bacterial DNA (109). Second, when the cytosine of the CpG dinucleotides present in plasmid vectors is selectively methylated with Sss-I CpG methylase, the vaccine's ability to stimulate cytokine production in vitro and antibody or CTL production in vivo is concomitantly reduced (14, 109). Third, coadministering ODN that contains CpG motifs with an antigenic protein boosts the antibody and cellular response similar to that achieved by DNA vaccination with a plasmid encoding the same antigen (110–112). Indeed, coadministering vector alone (without the antigen-encoding insert) also improves the immune response elicited by DNA vaccines. Presumably, CpG motifs present in the vector act as adjuvants in a fashion similar to CpG ODNs. This observation raises the interesting possibility that higher doses of a DNA vaccine or the coadministration of multiple antigen-encoding plasmids might synergistically boost the immune response to each element of a multicomponent vaccine.

Perhaps the strongest evidence the CpG motifs contribute to the immunogenicity of DNA vaccines was provided by Sato et al, who substituted a CpG-containing *ampR* gene for a *kanR*-selectable marker in a β-gal–encoding plasmid. They found that the reengineered plasmid elicited a higher IgG antibody response, more CTLs, and greater IFN-γ production than did the original vector (14). The same effect was observed when additional CpG motifs were introduced into the plasmid backbone of the *kanR*-containing vector, a result subsequently confirmed in several other vectors by other laboratories (52, 109, 113, 114). As noted above, this effect is most apparent when low doses of DNA vaccine are administered, presumably because, at high dose, endogenous CpG motifs in plasmid vectors perform the same function. Thus, additional CpG motifs may decrease the amount of vaccine required to induce an immune response rather than increase the absolute magnitude of that response. Indeed, CpG motifs appear to be limited in their ability to augment antibody and cytokine production in vivo such that too many CpG motifs may actually reduce immunogenicity (114). For example, introducing 16 additional CpG motifs into the plasmid backbone improved the humoral immune response by the DNA vaccines, whereas introducing 50 such motifs was detrimental. The above studies were performed in mice, the animals in which the effects of CpG ODNs were first described. Of interest, the 6-base-pair motif that induces optimal stimulation in mice is less effective when tested on cells of primate origin (human, monkey, or chimpanzee). Thus, efforts to improve the efficacy of DNA vaccines intended for human use would require identification of

those sequence motifs that are optimally immunostimulatory in humans. Toward this end, Liang et al (115) identified several ODNs that induced proliferation and Ig secretion of human B cells. They did not, however, systematically examine the size or sequence of the CpG motif that provided optimal immune activation. Ballas et al (116) reported that an AACGTT motif embedded in an ODN at least 15 base pairs in length stimulated the proliferation of human NK cells ; however, it is unclear whether this motif is optimally immunostimulatory. In this context, recent evidence suggests that at least two different human cell types respond to ODN stimulation and that different CpG motifs are required to stimulate these distinct cell populations. These findings suggest that it may be possible to tailor the type of immune response elicited by a DNA vaccine by selectively engineering one, the other, or both types of stimulatory motifs into a vector.

Immonosuppressive DNA Motifs Whereas CpG-containing bacterial DNA causes immune stimulation in vivo and in vitro, coadministration of mammalian DNA can block such activation. This suppression may account for the inability of mammalian DNA, which contains CpG motifs (albeit at much lower frequency than bacterial DNA), to stimulate the immune system. Several laboratories have shown that a subset of nonstimulatory ODNs can suppress the immune activation induced by ODNs that contain CpG motifs. Hacker et al (117) showed that an excess of non-CpG ODNs could inhibit the uptake of fluorescein-isothiocyanate–labeled CpG ODNs. This inhibition abrogated the ability of CpG ODNs to induce immune stimulation, interfering with cytokine production and stress kinase activation (117). Recent work by Krieg et al (114) confirmed that the immunostimulatory activity of CpG ODNs could be blocked by certain non-CpG motifs. They showed that eliminating suppressive motifs (tandem repeats of GpC) from the plasmid backbone of a DNA vaccine improved immunogenicity up to threefold. These observations demonstrate the complexity of the interaction between DNA sequence motifs and the immune system.

An important feature of CpG motifs is their ability to stimulate multiple types of immune cells. They improve antigen-presenting function by monocytes, macrophages, and DCs, induce proliferation of B cells, and boost antibody production by antigen-activated lymphocytes. Efforts are under way to identify the sequence motifs that are optimally active in humans, to determine whether different motifs can be used to regulate discrete elements of the immune system, and to establish where in the plasmid these immunostimulatory sequences can be introduced to greatest benefit. Presumably, this will include the elimination of suppressive motifs present in the plasmid backbone. These efforts are likely to yield vectors with significantly improved immunostimulatory capacities for clinical use.

Role of Cytokines and Costimulatory DNA Adjuvants

Because cytokines or costimulatory cell surface molecules play a crucial role in generation of the effector T-cell subsets and in determining the magnitude of the response, several groups have used plasmid DNA encoding various cytokine or

costimulatory molecules to enhance or bias the immune response generated by DNA vaccination. The studies with cytokine-encoding DNA and their effects on humoral and cellular immunity are summarized in Table 2 (211–244).

TABLE 2 Cytokine and costimulatory DNA adjuvants

Cytokine	Antibody	Cellular response	CTL	References
IL-1	↑IgG	↑proliferation	↑CTL	211-213
	↑IgG2a	↑IFN-γ		
IL-2	↑IgG	↑proliferation	↑CTL	212-219
	↑IgG2a	↑IFN-γ		
	↑IgG1[a]			
IL-4	↑IgG	↑proliferation		156, 212, 213,
	↑IgG1	↓DTH		216, 218, 219
		↑IL-4		
IL-5	↑IgG	± proliferation		213
IL-6				220
IL-7	↑IgG2a	↑IFN-γ		217
	↑IgG1			
IL-8		↑Neutrophils		221
IL-10	↑G	↓DTH		213, 219,
	↓IgG2a	↓proliferation		222-224
IL-12	↑Ig2a	↑DTH	↑CTL	31, 79, 156, 212,
	↑ or ↓ IgG1[a]	↑proliferation		213, 217-219,
	↑ or ↓ IgG[a]	↑IFN-γ		225-229
IL-15	?↑IgG[a]	± ↑proliferation	↑CTL	213
IL-18	↑IgG	↑proliferation	↑CTL	213
TNF	↑IgG	↑proliferation	↑CTL	93, 213
GM-CSF	↑IgG	↑proliferation	↑CTL	31, 93, 156, 211,
	↑IgG2a and	↑IFN-γ		212, 216, 218,
	IgG1[a]	↑IL-4		219, 230-233
TGF-β	↑IgG1	↓DTH		157, 234
		↓proliferation		
		↓cytokines		
IFN-γ	↑IgG2a	↑ or ↓proliferation	↑CTL	93, 157, 212,
	↑ or ↓IgG[a]	↑IFN-γ, ↓IL-5		218, 233, 235
IFN-α				236, 237
B7-1 (CD80)			↑CTL[a]	31, 238-241
B7-2 (CD86)		↑DTH	↑CTL[a]	31, 238-241
		↑Proliferation		

(*continued*)

TABLE 2 (continued) Cytokine and costimulatory DNA adjuvants

Cytokine	Antibody	Cellular response	CTL	References
CD40L	↑IgG2a ↑IgG, ↑IgG1[a]	↑IFN-γ	↑CTL	226, 242
ICAM-1 (CD54)		↑proliferation ↑IFN-γ ↑β-chemokines	↑CTL	243
LFA-3		↑proliferation ↑IFN-γ ↑β-chemokines	↑CTL	243
L-selectin	↑IgG ↑IgG2a>IgG1	↑proliferation		244
CTLA4	↑IgG ↑IgG1>IgG2a	↑proliferation		244

[a]Changes in antibody or cellular responses are not in agreement between studies.

Alternative Boost

Although DNA vaccination alone can elicit potent humoral and cellular responses to many antigens, it appears that for certain antigens (e.g. HIV envelope proteins and malarial proteins), the immune response generated by DNA vaccination may be suboptimal for protection. In such instances, alternative booster regimens have been shown to be helpful. The most common of these booster regimens have used either recombinant protein or poxviruses. Thus, for HIV, because multiple DNA vaccinations elicit only modest and transient titers of neutralizing antibody (118–124), there have been many studies evaluating the effects of peptide (125) or protein boosting after DNA vaccination (119, 126–128). In two separate studies that used rhesus macaques, it was shown that antibody production could be substantially increased in monkeys vaccinated with DNA encoding an HIV-1 envelope protein followed by a protein boost (127, 128). In one of these reports, monkeys were protected after an infectious challenge (127). In a separate study, rabbits primed with various HIV-1 *env*-expressing plasmids had a rapid increase in the titer of antibody after a protein boost; however, the avidity and neutralizing activity rose more slowly. In contrast to HIV, high titers of antibody with good avidity and persistence were induced after DNA vaccination encoding an influenza virus HA glycoprotein without any protein boost (119). Taken together, these data underscore a potential difference between HIV and other viral proteins in requiring a protein boost after DNA vaccination to optimize both the qualitative and quantitative aspects of the humoral response.

As noted above, although protein boosting enhanced the antibody response after DNA vaccination, there is little evidence that it affects the cellular immune response. Because cellular immunity might be required for protection against diseases such as HIV infection and malaria, there have been several studies attempting to increase cellular responses after DNA vaccination by using recombinant poxviruses. In several such studies, boosting with recombinant poxvirus substantially enhanced CTL and/or IFN-γ responses in mice primed with DNA encoding either a malarial (129–131) or HIV envelope protein (132, 133). It should be noted that, whereas antibody production was also increased after DNA and poxvirus boosting in mice, by using vectors encoding malarial proteins (130), antibody production was not enhanced and eventually declined in rhesus macaques vaccinated with DNA and boosted with fowl pox-encoding HIV proteins (132). To conclude, in both malaria- and HIV-infected rodent and nonhuman primate models, DNA vaccination followed by poxvirus boosting gave consistent and striking increases in cellular immunity. One caveat that may be important with regard to vaccination against diseases requiring both humoral and cellular immunity (i.e. HIV infection) is whether this type of boosting also limits antibody responses.

Modes of Administration

Route and Dosage A variety of routes of DNA injection, including intramuscular, intradermal, intravenous, intraperitoneal (134), epidermal delivery by scarification (34), oral (135–138), intranasal (134, 139–144), vaginal (145, 146), and, more recently, noninvasive vaccination to the skin (147) have been studied. The most common immunization routes studied have been intramuscular and, to a lesser extent, subcutaneous or intradermal. DNA is administered in a variety of diluents including distilled water, saline, and sucrose. For intramuscular injections, although some investigators have used agents such as cardiotoxin, bupivacaine, or hypertonic solutions (148, 149) to pretreat the muscle tissue to improve responses, additional studies suggest little benefit. Whereas the optimal dose depends on the particular antigen and model system used, typically, 10 to 100 μg of plasmid DNA is required to elicit responses when administered intramuscularly or subcutaneously. By contrast, immunization of DNA by gene gun often requires 0.1–1 μg of plasmid DNA to induce antibody or CTL responses. Thus, in terms of the amount of DNA used, immunization of plasmid DNA with a gene gun is the most efficient mode of delivery (134); however, as noted above, DNA immunization via gene gun can qualitatively alter the type of immune response that is generated. Although doses of 25 to 100 μg per injection (intramuscularly) are usually sufficient in mice, higher doses appear to be required in primates or humans. In a study of human volunteers given a DNA vaccine encoding a malarial antigen, doses of plasmid DNA in the 500- to 2500-μg range gave enhanced CTL responses (150). Whereas a single vaccination with DNA can induce both an antibody and CTL response in several model systems, both cellular

and humoral immune responses are increased by successive boosting (one or two additional immunizations). This requirement for multiple immunizations is well documented for the induction of humoral responses to HIV envelope proteins (118–124). It should be noted, however, that, in one study, antibody responses to HIV-1 gp120 were actually enhanced in rhesus macaques when the number of DNA vaccinations (as delivered by gene gun) was reduced but the interval between immunizations increased, suggesting the importance of a rest period between immunizations (128). Similar results were noted after DNA vaccination (given either by gene gun or intramuscularly) that expressed the circumsporozoite protein from *Plasmodium berghei* in mice (151).

Mucosal Immunization Induction of mucosal immunity by DNA immunization after immunization by several different mucosal routes has been studied. These include application of plasmid DNA intranasally (134, 139–144), intratracheally (134, 152, 153), by aerosol (154, 155), by genital-tract immunization (145, 146), and by oral administration (135–138). In addition, in several studies, plasmid DNA was combined with various immunity-enhancing regimens such as cholera toxin (140, 141), plasmid-encoding cytokines (156), liposomes (139, 152, 154, 155), or other adjuvants (142, 143).

There has been great interest in generating specific types of immune responses after mucosal immunization. In autoimmune models of disease, oral administration of protein can lead to immune tolerance. By contrast, for viral infections such as HIV and herpes simplex virus, the generation of potent antibody and/or cellular responses may be critical in mediating protection. Owing to the importance of the mucosal immune response for these diseases, studies were undertaken to compare the immune responses elicited by mucosal immunization with those achieved after systemic immunization. First, for antibody production, although many studies showed that serum IgG responses after mucosal immunization were comparable with those elicited after systemic immunization with the same plasmid constructs (142, 143, 146), other groups have demonstrated that mucosal immunization did not lead to an efficient induction of serum IgG responses (134, 140, 141). Of note is that mucosal immunization was superior to systemic immunization at inducing and sustaining mucosal IgA responses in all studies in which data examining this effect were available (142, 143, 146). Whereas mucosal DNA vaccination was advantageous in generating mucosal IgA responses, it was demonstrated in a murine model of herpes simplex virus infection that, despite the presence of virus-specific IgA at the time of challenge, virus could persist and replicate at the mucosal site of challenge (157). These results suggest either a failure of these immunization regimens to induce an adequate IgA response or the requirement for additional immune mechanisms to control viral replication at the mucosal site.

For cellular immunity, the ability of DNA vaccines given mucosally or systemically to induce local mucosal T-cell responses has not been directly demonstrated; however, cellular responses have been studied from spleen cells of mice

after vaccination via systemic or mucosal routes. In two of these studies, delayed-type hypersensitivity (DTH) responses and specific cytolytic activity from spleen cells was comparable between the two routes (142, 143). To conclude, determination of whether mucosal or systemic vaccination with DNA affects the cellular response at specific mucosal sites will likely be important for designing vaccines against such pathogens as HSV and HIV.

A potentially exciting means of mucosal DNA delivery is the use of microparticles. Plasmid DNA trapped in these biodegradable microparticles, composed of polymers such as polylactice-coglycolides or chitosan, can be administered orally and has been shown to induce both mucosal and systemic immune responses (135, 138). The ability of polylactice-coglycolide–entrapped DNA vaccines to induce protective immune responses to rotavirus challenge after oral administration was demonstrated in two separate studies (136, 137). In addition to uses for infectious pathogens, oral administration of DNA vaccines has also been shown to be useful in treating allergic diseases. Recently, an oral DNA vaccine containing the gene for the main peanut allergen (*Arah2*) protected mice against peanut-induced anaphylaxis. This protection was correlated with a reduction of IgE (a surrogate for a Th2 response), providing further evidence that DNA vaccination by its preferences to stimulate Th1 responses may have broad clinical applications (138). Finally, delivery of plasmid DNA orally with attenuated enteric bacteria such as *Salmonella* or *Shigella* spp. is an active area of investigation (see below).

Carrier-Mediated Approaches to Optimizing DNA Vaccines It appears that a majority of the DNA injected intramuscularly is degraded by extracellular deoxyribonucleases (158, 159). It follows that protecting plasmid DNA from extracellular degradation by introducing it directly into target cells should optimize DNA uptake. Several methods of carrier-mediated DNA transfection have been successful.

Gene gun Gene gun technology uses a gas-driven biolistic bombardment device that propels gold particles coated with plasmid DNA directly into the skin (7, 33, 134, 160). These gold particles are propelled directly into the cytosol of target cells, resulting in transgene expression levels higher than those obtained by comparable doses of ''naked DNA.'' This mode of immunization induces protective immunity in several animal models of disease.

Liposomes Liposomes are bilayered membranes consisting of amphipathic molecules (polar and nonpolar portions) such as phospholipids, forming unilayered or multilayered (lamellar) vesicles. Unilamellar vesicles have a single bilayer membrane surrounding an aqueous core and are characterized by either being small or large unilamellar vesicles, whereas multilayered vesicles have several lipid bilayers separated by a thin aqueous phase. Because liposomes can be prepared with significant structural versatility based on vesicle surface charge, size,

lipid content, and coentrapment of adjuvants, they offer considerable flexibility toward vaccine optimization. The full scope of the use of liposomes to increase the effect of DNA vaccines is currently an active area of investigation. Intramuscular injection of plasmid DNA (hepatitis B surface antigen) entrapped in liposomes elicited 100-fold increased antibody titers and increased levels of both IFN-γ and IL-4 when compared with those in animals injected with ''naked DNA'' (161). A similar result on antibody augmentation was seen when DNA/liposome complexes were administered intranasally (139).

Cochleates Cochleates are rigid calcium-induced spiral bilayers of anionic phosolipids. They have a unique structure that is different from that of liposomes. They are relatively stable after lyophilization or in harsh environments. It is believed that, on contact with target cell membrane, a fusion event occurs between the membrane and the outer layer of the cochleate leading to delivery of the contents of the cochleate into the cytosol. It has been reported that DNA/cochleate formulations were able to induce strong CTL and antibody responses after parenteral or oral administration (162–164).

Microparticle encapsulation Another potentially exciting means of DNA delivery is the use of biodegradable polymeric microparticles. Plasmid DNA trapped in these polymers (e.g. polylactice-coglycolides or chitosan) can be given systemically or to mucosal surfaces (orally or via the respiratory tract). The ability of polylactice-coglycolide–entrapped DNA vaccines to induce asystemic and mucosal immune responses after oral or intraperitoneal administration has been demonstrated (see above).

Attenuated organisms Delivery of DNA can also be accomplished by attenuated intracellular bacteria. Intracellular bacteria, carrying the DNA, undergo phagocytosis by APCs, delivering plasmid DNA into the host cell phagosome or cytosol. The released DNA is then transcribed, resulting in expression of encoded antigens. Attentuated strains of invasive bacteria *Shigella flexneri* (165, 166), *Salmonella typhimurium* (167, 168), and *Listeria monocytogenes* (169) have been used for the delivery of plasmid DNA. For *S. typhimurium*, the bacteria are lysed within the phagosome, releasing plasmid DNA from this compartment into the cytoplasm via an unknown mechanism. Vaccination of mice with attenuated *S. typhimurium* transformed with plasmid DNA encoding lysteriolysin induced specific antibody as well as T-cell responses (167). Moreover, in a separate study, fluorescent DCs were demonstrated after oral administration of *S. typhimurium* harboring plasmid DNA encoding green fluorescent protein. These data provided evidence that this delivery system could target relevant immune cells, leading to efficient induction of an immune response (168). For *Shigella* infection, after phagocytosis and lysis within host cells, antigenic material is released directly into the cytoplasm. Immunization by using attenuated *S. flexneri* transformed with a bacterial plasmids encoding β-gal led to induction of a strong antigen-specific

humoral and cellular response (166). In a separate study, it was shown that mice vaccinated with attenuated *Shigella* vaccine harboring measles virus genes induced a vigorous antigen-specific response (170). Finally, delivery of eukaryotic expression vectors in murine macrophage cell lines by attenuated suicide *L. monocytogenes* has also been reported (169). Whereas immunization with naked DNA has not been reported to lead to genomic integration with a significant frequency (see below), delivery of DNA by *L. monocytogenes* has resulted in chromosomal integration in vitro (169, 171), raising safety concerns with this technology.

Alphaviruses are arthropod-borne togaviruses with a positive-polarity and single-stranded RNA genome that can replicate in a large number of animal hosts. Development of a variety of expression strategies has made it possible to deliver foreign genes in vivo by using alphaviruses (reviewed in 172). During infection, viral RNA replication is initiated by translation of viral nonstructural replicase proteins directly from the viral genome. During replication, both full-length genomic RNA and RNA initiated from an internal viral subgenomic promoter are synthesized. These subgenomic RNA transcripts are produced in excess relative to the genomic RNA and serve as mRNA for viral structural proteins. Thus, the natural viral life cycle permits striking amplification of mRNA. It has been shown that substitution of a heterologous gene for a viral structural gene results in high-level expression of the heterologous gene. Recently, the development of a layered plasmid DNA-based expression system by using alphaviruses has been described (173–176). The mode of heterologous gene expression from alphavirus-derived expression vectors differs from that of conventional DNA vaccine plasmids in that transcription of heterologous genes is achieved in multiple steps. The first step involves the generation of viral genomic RNA that functions as a template for mRNA synthesis. Second, taking advantage of the virus life cycle, amplification of mRNA is achieved to drive the synthesis of antigen-encoding sequences. As the virus encodes machinery required for RNA replication and amplification in the host cell cytoplasm, high levels of protein production can be obtained, thus circumventing many problems associated with nuclear gene expression (such as limitation of transcription factors, RNA transport, etc). This method of gene delivery provides an exciting advance in the field of DNA vaccines, because these vectors can express heterologous proteins at higher levels than can conventional DNA vaccines (177).

Somatic transgene immunization The concept of expressing T-cell epitopes in Ig has been demonstrated in foreign genes inserted into one or more of the complementary determining regions in the Ig heavy-chain molecule (antigenized antibodies), leading to induction of an immune response against the heterologous epitopes. A DNA-based approach as an alternative to the above has recently been described. In two separate studies, it was demonstrated that antigenized antibody-DNA constructs containing either a B-cell epitope or a B- and T-cell epitope engineered to different complement-determining regions led to the production of an antibody response directed against both epitopes (178, 179). Unlike conven-

tional DNA vaccines, immunization with these constructs led to an efficient detection of both transgene expression in vivo and transgene product in the serum.

APPLICATION

For details on models for specific applications of DNA vaccines, see Tables 3A–3D, which are produced in their entirety at the Annual Reviews world-wide-web site (www.AnnualReviews.org). These tables provide data on models for allergic diseases (Table 3A), autoimmune diseases (Table 3B), infectious diseases (Table 3C), and tumors (Table 3D).

SAFETY

A number of safety concerns have been raised about the use of DNA vaccines. These include the possibility that such vaccines may (*a*)integrate into the host genome, thereby increasing the risk of malignancy (by activating oncogenes or inactivating tumor suppressor genes); (*b*) induce responses against transfected cells, thereby triggering the development of autoimmune disease; (*c*) induce tolerance rather than immunity; and/or (*d*) stimulate the production of cytokines that alter the host's ability to respond to other vaccines and resist infection (180).

Plasmids can persist at the site of injection for many months. They can also be found far from the original site of injection (including the gonads), perhaps carried by transfected lymphocytes or macrophages. Long-term persistence may be especially common for plasmids that encode self-antigens, because these do not induce an immune response against the cell they transfect. To date, there is no clear evidence that plasmids integrate, yet neither has this possibility been eliminated. Efforts to prove that high-molecular-weight (genomic) DNA does not contain plasmids (proof that integration has not taken place) have failed, in part because of contamination of the high-molecular-weight fraction by plasmid concatamers combined with the enormous sensitivity of the polymerase chain reaction. To overcome this problem, investigators digested genomic DNA with a restriction enzyme that is specific for a single site within the plasmid. By repeatedly digesting and isolating high-molecular-weight DNA, most but not all of the plasmid can be eliminated (181). Whether the few remaining copies of plasmids represent integration events remains to be determined.

Concern that DNA vaccines might promote the development of autoimmune diseases arises from the immunostimulatory activity of CpG motifs in the plasmid backbone. It has been known for many years that bacterial DNA can induce the production of anti–double-stranded-DNA autoantibodies in normal mice and accelerate the development of autoimmune disease in lupus-prone animals (182–184). The CpG motifs present in bacterial DNA and DNA vaccines stimulate the production of IL-6 and block the apoptotic death of activated lymphocytes, both

functions that predispose to the development of systemic lupus erythematosus by facilitating persistent B-cell activation (185–190).

These findings led several groups to investigate whether systemic autoimmune disease was induced or accelerated by the CpG motifs (191). With sensitive spot enzyme-linked immuno spot (ELIspot) assays, the absolute number of B cells secreting autoantibodies was studied in normal mice repeatedly immunized with a DNA vaccine. Shortly after vaccination, the number of IgG anti-DNA–secreting cells rose by two- to threefold (192). This was accompanied by a 35%–60% increase in serum IgG anti-DNA antibody titer. This modest rise in autoantibody level did not, however, result in the development of disease in normal mice or accelerate disease in lupus-prone animals (191–194). Thus, although the theoretical possibility remains that a subset of DNA vaccines (particularly those encoding determinants cross-reactive with self) may induce or accelerate autoimmune disease, findings to date suggest that the level of autoantibody production elicited by DNA vaccines is insufficient to induce such an outcome.

The situation is somewhat more complex for organ-specific autoimmune disease, whose induction is promoted by strong type I immune responses. In an IL-12–dependent model of experimental allergic encephalomyelitis, animals treated with CpG motifs and then challenged with myelin basic protein developed autoreactive Th1 effector cells that caused experimental allergic encephalomyelitis (65). In a molecular mimicry model, CpG motifs acted as potent immunoactivators, inducing autoimmune myocarditis when coinjected with *Chlamydia*-derived antigen (195). These findings indicate that CpG motifs may trigger deleterious autoimmune reactions under certain circumstances. Balancing these safety concerns is the observation that toxicity has not been reported among normal animals treated with therapeutic doses of DNA vaccines or CpG ODNs. In addition, hundreds of human volunteers have been exposed to plasmid DNA vaccines without serious adverse consequences.

Most vaccines intended for human use are administered to infants and children. Owing to the immaturity of their immune systems, newborns exposed to foreign antigens are at risk for developing tolerance rather than immunity (196). A number of factors influence the development of neonatal tolerance, including the nature, concentration, and mode of antigen presentation to the immune system as well as the age of the host (197–199). Because the protein encoded by a DNA vaccine is produced endogenously and expressed in the context of self-MHC, the potential exists for the neonatal immune system to recognize it as ''self,'' resulting in tolerance rather than immunity. Consistent with such a possibility, a DNA vaccine encoding the circumsporozoite protein of malaria was found to induce tolerance rather than immunity in newborn mice (200). Neonatal animals treated with this vaccine were unable to generate T- or B-cell responses when challenged with pCSP as adults, thereby remaining at increased risk from infection despite immunization (200, 201). In this system, the induction of tolerance was critically dependent on the age at which the vaccine was administered. Tolerance was observed only when vaccine was administered to mice <8 days of age; however,

decreased protection was also observed in geriatric mice (>2 years of age), raising concern that DNA vaccines might be less immunogenic in the elderly as well as in the very young (202). Efforts are under way to improve the overall immunogenicity of DNA vaccines by coadministering plasmids encoding cytokines or costimulatory molecules. Recent results suggest that these approaches can improve immunization of neonates and the elderly (199, 203–209).

Safety concerns also arise from the use of CpG motifs or cytokine-encoding plasmids as adjuvants to improve the in vivo response elicited by DNA vaccines. An important component of immune homeostasis is through a balance in the production of Th1 cytokines (which promote cell-mediated immunity) and Th2 cytokines (which facilitate humoral immune responses or counterregulate Th1 responses). These two classes of cytokine-producing cells form a dynamic and mutually inhibitory network, because Th1 cytokines can block the maturation of Th2-type cells and vice versa. The overproduction of one type of cytokine can disrupt immune homeostasis, thereby altering the host's response to other vaccines, susceptibility to infection, and predisposition to develop autoimmune disease. Although the use of cytokine-encoding plasmids is growing in popularity, relatively little information is available on their long-term safety. Although no serious side effects have been reported after the administration of cytokine-encoding plasmids in animals, it is unclear whether systematic efforts to detect such events were undertaken. Studies indicate that cytokine-encoding plasmids given in conjunction with antigen do alter the cytokine milieu (ratio of Th1-:Th2-secreting cells) and ultimately bias the immune response. In contrast, cytokine DNA given alone did not appear to alter immune reactivity against unrelated antigens and did not lead to the development of autoimmunity (203, 210). Indeed, no change in the frequency of Th1 or Th2 cytokine-secreting precursors was detected in mice treated multiple times with IFN-γ-, IL-4-, or granulocyte/macrophage colony-stimulating factor–encoding plasmids. It thus appears that the cytokine released by transfected cells primarily affects local rather than systemic immunity, leaving serum cytokine levels generally unchanged.

CONCLUSIONS

DNA vaccines moved very rapidly from laboratory phenomena into clinical trials. This transition was sustained by our ability to harness the tools of molecular biology to design antigen-encoding plasmids capable of inducing immune responses against pathogens for which no conventional vaccine was available, yet enthusiasm for this new technology must be tempered by an appreciation of its potential risks. The long-term sequelae of DNA vaccination have received little attention despite the capacity of these plasmids to persist in vivo for months or years. As multicomponent DNA vaccines and DNA vaccines encoding both cytokines and antigens become more common, the possibility for detrimental side effects will increase. DNA vaccines have the potential to be administered to mil-

lions of children and/or adults. Thus, adverse events occurring even at low frequency (<1 /1000) could affect many thousands of otherwise healthy individuals. Adequate preclinical studies coupled with large-scale human trials will still be needed to establish the risk of this new vaccine approach. To aid in this effort, the Food and Drug Administration has published "*Points to Consider*" a document that provides valuable suggestions for the evaluation of the safety, potency, and immunogenicity of candidate DNA vaccines (210a).

ACKNOWLEDGMENTS

We thank Brenda Rae Marshall for editing the manuscript.

Visit the Annual Reviews home page at www.AnnualReviews.org.

LITERATURE CITED

1. Nicolau C, Le Pape A, Soriano P, Fargette F, Juhel MF. 1983. In vivo expression of rat insulin after intravenous administration of the liposome-entrapped gene for rat insulin I. *Proc. Natl. Acad. Sci. USA* 80:1068–72
2. Kaneda Y, Iwai K, Uchida T. 1989. Increased expression of DNA cointroduced with nuclear protein in adult rat liver. *Science* 243:375–78
3. Mannino RJ, Gould-Fogerite S. 1988. Liposome mediated gene transfer. *Biotechniques* 6:682–90
4. Benvenisty N, Reshef L. 1986. Direct introduction of genes into rats and expression of the genes. *Proc. Natl. Acad. Sci. USA* 83:9551–55
5. Wu GY, Wu CH. 1988. Receptor-mediated gene delivery and expression in vivo. *J. Biol. Chem.* 263:14621–24
6. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL. 1990. Direct gene transfer into mouse muscle in vivo. *Science* 247:1465–68
7. Tang DC, De Vit M, Johnston SA. 1992. Genetic immunization is a simple method for eliciting an immune response. *Nature* 356:152–54
8. Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, Gromkowski SH, Deck RR, De Witt DM, Friedman A, Hawe LA, Leander KR, Martinez D, Perry HC, Shiver JW, Montgomery DC, Liu MA. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 259:1745–49
9. Robinson HL, Hunt LA, Webster RG. 1993. Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. *Vaccine* 11:957–60
10. Robinson HL, Pertmer TM. 1999. DNA vaccines for viral infection: basic studies and applications. *Adv. Virus Res.* In press

10a. Whalen R. http://www.genweb.com/dnavax/dnavax.html

11. Yamamoto S, Yamamoto T, Shimada S, Kuramoto E, Yano O, Kataoka T, Tokunaga T. 1992. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. *Microbiol. Immunol.* 36:983–97
12. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. 1996. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6,

interleukin 12, and interferon γ. *Proc. Natl. Acad. Sci. USA* 93:2879–83
13. Halpern MD, Kurlander RJ, Pisetsky DS. 1996. Bacterial DNA induces murine interferon-γ production by stimulation of interleukin-12 and tumor necrosis factor-α. *Cell Immunol.* 167:72–78
14. Sato Y, Roman M, Tighe J, Lee D, Corr M, Nguyen MD, Silverman GJ, Lotz M, Carson DA, Raz E. 1996. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* 273:352–54
15. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546–49
16. Cardon LR, Burge C, Clayton DA, Karlin S. 1994. Pervasive CpG suppression in animal mitochondrial genomes. *Proc. Natl. Acad. Sci. USA* 91:3799–803
17. Razin A, Friedman J. 1981. DNA methylation and its possible biological roles. *Prog. Nucl. Acid Res. Mol. Biol.* 25:33–52
18. Stacey KJ, Sweet MJ, Hume DA. 1996. Macrophages ingest and are activated by bacterial DNA. *J. Immunol.* 157:2116–22
19. Jakob T, Walker PS, Krieg AM, Udey MC, Vogel JC. 1998. Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J. Immunol.* 161:3042–49
20. Cowdery JS, Chace JH, Yi AK, Krieg AM. 1996. Bacterial DNA induces NK cells to produce IFN-γ in vivo and increases the toxicity of lipopolysaccharides. *J. Immunol.* 156:4570–75
21. Bendigs S, Salzer U, Lipford GB, Wagner H, Heeg K. 1999. CpG-oligodeoxynucleotides co-stimulate primary T cells in the absence of antigen-presenting cells. *Eur. J. Immunol.* 29:1209–18
22. Corr M, Lee DJ, Carson DA, Tighe H. 1996. Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J. Exp. Med.* 184:1555–60
23. Iwasaki A, Torres CA, Ohashi PS, Robinson HL, Barber BH. 1997. The dominant role of bone marrow-derived cells in CTL induction following plasmid DNA immunization at different sites. *J. Immunol.* 159:11–14
24. Doe B, Selby M, Barnett S, Baenziger J, Walker CM. 1996. Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells. *Proc. Natl. Acad. Sci. USA* 93:8578–83
25. Casares S, Inaba K, Brumeanu TD, Steinman RM, Bona CA. 1997. Antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope. *J. Exp. Med.* 186:1481–86
26. Porgador A, Irvine KR, Iwasaki A, Barber BH, Restifo NP, Germain RN. 1998. Predominant role for directly transfected dendritic cells in antigen presentation to $CD8^+$ T cells after gene gun immunization. *J. Exp. Med.* 188:1075–82
27. Akbari O, Panjwani N, Garcia S, Tascon R, Lowrie D, Stockinger B. 1999. DNA vaccination: transfection and activation of dendritic cells as key events for immunity. *J. Exp. Med.* 189:169–78
28. Condon C, Watkins SC, Celluzzi CM, Thompson K, Falo LD Jr. 1996. DNA-based immunization by in vivo transfection of dendritic cells. *Nat. Med.* 2:1122–28
29. Ulmer JB, Deck RR, Dewitt CM, Donnhly JI, Liu MA. 1996. Generation of MHC class I-restricted cytotoxic T lymphocytes by expression of a viral protein in muscle cells: antigen presentation by non-muscle cells. *Immunology* 89:59–67
30. Agadjanyan MG, Kim JJ, Trivedi N, Wilson DM, Monzavi-Karbassi B, Morrison LD, Nottingham LK, Dentchev T,

Tsai A, Dang K, Chalian AA, Maldonado MA, Williams WV, Weiner DB. 1999. CD86 (B7–2) can function to drive MHC-restricted antigen-specific CTL responses in vivo. *J. Immunol.* 162: 3417–27

31. Iwasaki A, Stiernholm BJ, Chan AK, Berinstein NL, Barber BH. 1997. Enhanced CTL responses mediated by plasmid DNA immunogens encoding costimulatory molecules and cytokines. *J. Immunol.* 158:4591–601
32. Torres CA, Iwasaki A, Barber BH, Robinson HL. 1997. Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. *J. Immunol.* 158:4529–32
33. Yang NS, Burkholder J, Roberts B, Martinell B, McCabe D. 1990. In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. *Proc. Natl. Acad. Sci. USA* 87:9568–72
34. Raz E, Carson DA, Parker SE, Parr TB, Abai AM, Aichinger G, Gromkowski SH, Singh M, Lew D, Yankauckas MA, Baird SM, Rhodes GH. 1994. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc. Natl. Acad. Sci. USA* 91:9519–23
35. Klinman DM, Sechler JM, Conover J, Gu M, Rosenberg AS. 1998. Contribution of cells at the site of DNA vaccination to the generation of antigen-specific immunity and memory. *J. Immunol.* 160:2388–92
36. Wraith DC, Vessey AE, Askonas BA. 1987. Purified influenza virus nucleoprotein protects mice from lethal infection. *J. Gen. Virol.* 68:433–40
37. Staerz UD, Karasuyama H, Garner AM. 1987. Cytotoxic T lymphocytes against a soluble protein. *Nature* 329:449–51
38. Harding CV, Song R. 1994. Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules. *J. Immunol.* 153:4925–33
39. Falo LD Jr, Kovacsovics-Akowski M, Thompson K, Rock KL. 1995. Targeting antigen into the phagocytic pathway in vivo induces protective tumour immunity. *Nat. Med.* 1:649–53
40. Udono H, Srivastava PK. 1993. Heat shock protein 70-associated peptides elicit specific cancer immunity. *J. Exp. Med.* 178:1391–96
41. Fu TM, Ulmer JB, Caulfield MJ, Deck RR, Friedman A, Wang S, Liu X, Donnelly JJ, Liu MA. 1997. Priming of cytotoxic T lymphocytes by DNA vaccines: requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. *Mol. Med.* 3:362–71
42. Albert ML, Sauter B, Bhardwaj N. 1998. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 392:86–89
43. Albert ML, Pearce SF, Francisco LM, Sauter B, Roy P, Silverstein RL, Bhardwaj N. 1998. Immature dendritic cells phagocytose apoptotic cells via $\alpha V\beta 5$ and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* 188:1359–68
44. Banchereau J, Bazan F, Blanchard D, Briere F, Galizzi LP, van Kooten C, Liu YJ, Rousset F, Saeland S. 1994. The CD40 antigen and its ligand. *Annu. Rev. Immunol.* 12:881–922
45. Ridge JP, Di Rosa F, Matzinger P. 1998. A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. *Nature* 393:474–78
46. Bennett SRM, Carbone FR, Karamalis F, Flavell RA, Miller J, Heath WR. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393:478–80
47. Schoenberger SP, Toes REM, van der Voort EIH, Offringa R, Melief CJM. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393:480–83
48. Seder RA, Paul WE. 1994. Acquisition

of lymphokine-producing phenotype by $CD4^+$ T cells. *Annu. Rev. Immunol.* 12:635–73

49. O'Garra A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 8:275–83
50. Waris ME, Tsou C, Erdman DD, Zaki SR, Anderson LJ. 1996. Respiratory synctial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. *J. Virol.* 70:2852–60
51. Li X, Sambhara S, Li CX, Ewasyshyn M, Parrington M, Caterini J, James O, Cates G, Du RP, Klein M. 1998. Protection against respiratory syncytial virus infection by DNA immunization. *J. Exp. Med.* 188:681–88
52. Roman M, Martin-Orozco E, Goodman JS, Nguyen MD, Sato Y, Ronaghy A, Kornbluth RS, Richman DD, Carson DA, Raz E. 1997. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* 3:849–54
53. Pertmer TM, Roberts TR, Haynes JR. 1996. Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector DNA delivery. *J. Virol.* 70:6119–25
54. Feltquate DM, Heaney S, Webster RG, Robinson HL. 1997. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J. Immunol.* 158:2278–84
55. Suss G, Shortman K. 1996. A subclass of dendritic cells kills CD4 T cells via Fas/Fas ligand-induced apoptosis. *J. Exp. Med.* 183:1789–96
56. Pulendran B, Smith JL, Caspary G, Brasel K, Pettit D, Maraskovsky E, Maliszewski CR. 1999. Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc. Natl. Acad. Sci. USA* 96:1036–41
57. Smith AL, de St Groth BF. 1999. Antigen-pulsed $CD8\alpha^+$ dendritic cells generate an immune response after subcutaneous injection without homing to the draining lymph node. *J. Exp. Med.* 189:593–98
58. Rissoan MC, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal Malefyt R, Liu YJ. 1999. Reciprocal control of T helper cell and dendritic cell differentiation. *Science* 283:1183–86
59. Maldonado-Lopez R, De Smedt T, Michel P, Godfroid J, Pajak B, Heirman C, Thielemans K, Leo O, Urbain J, Moser M. 1999. $CD8\alpha^+$ and $CD8\alpha^-$ subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J. Exp. Med.* 189:587–92
60. Boyle JS, Koniaras C, Lew AM. 1997. Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. *Int. Immunol.* 9:1897–906
61. Haddad D, Liljeqvist S, Stahl S, Andersson I, Perlmann P, Berzins K, Ahlborg N. 1997. Comparative study of DNA-based immunization vectors: effect of secretion signals on the antibody responses in mice. *FEMS Immunol. Med. Microbiol.* 18:193–202
62. Zimmermann S, Egeter O, Hausmann S, Lipford GB, Rocken M, Wagner H, Heeg K. 1998. CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J. Immunol.* 160:3627–30
63. Raz E, Tighe H, Sato Y, Corr M, Dudler JA, Roman M, Swain SL, Spiegelberg HL, Carson DA. 1996. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody for-

mation by plasmid DNA immunization. *Proc. Natl. Acad. Sci. USA* 93:5141–45
64. Hsu CH, Chua KY, Tao MH, Lai YL, Wu HD, Huang SK, Hsieh KH. 1996. Immunoprophylaxis of allergen-induced immunoglobulin E synthesis and airway hyperresponsiveness in vivo by genetic immunization. *Nat. Med.* 2:540–44
65. Segal BM, Klinman DM, Shevach EM. 1997. Microbial products induce autoimmune disease by an IL-12-dependent pathway. *J. Immunol.* 158:5087–90
66. Waisman A, Ruiz PJ, Hirschberg DL, Gelman A, Oksenberg JR, Brocke S, Mor F, Cohen IR, Steinman L. 1996. Suppressive vaccination with DNA encoding a variable region gene of the T-cell receptor prevents autoimmune encephalomyelitis and activates Th2 immunity. *Nat. Med.* 2:899–905
67. Martins LP, Lau LL, Asano MS, Ahmed R. 1995. DNA vaccination against persistent viral infection. *J. Virol.* 69:2574–82
68. Yokoyama M, Zhang J, Whitton JL. 1995. DNA immunization confers protection against lethal lymphocytic choriomeningitis virus infection. *J. Virol.* 69:2684–88
69. Zarozinski CC, Fynan EF, Selin LK, Robinson HL, Welsh RM. 1995. Protective CTL-dependent immunity and enhanced immunopathology in mice immunized by particle bombardment with DNA encoding an internal virion protein. *J. Immunol.* 154:4010–17
70. Chen Y, Webster RG, Woodland DL. 1998. Induction of $CD8^+$ T cell responses to dominant and subdominant epitopes and protective immunity to Sendai virus infection by DNA vaccination. *J. Immunol.* 160:2425–32
71. Fu TM, Friedman A, Ulmer JB, Liu MA, Donnelly JJ. 1997. Protective cellular immunity: cytotoxic T-lymphocyte responses against dominant and recessive epitopes of influenza virus nucleoprotein induced by DNA immunization. *J. Virol.* 71:2715–21
72. Deck RR, DeWitt CM, Donnelly JJ, Liu MA, Ulmer JB. 1997. Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. *Vaccine* 15:71–78
73. Robinson HL, Boyle CA, Feltquate DM, Morin MJ, Santoro JC, Webster RG. 1997. DNA immunization for influenza virus: studies using hemagglutinin- and nucleoprotein-expressing DNAs. *J. Infect. Dis.* 176:S50–55 (Suppl)
74. Boyle CM, Morin M, Webster RG, Robinson HL. 1996. Role of different lymphoid tissues in the initiation and maintenance of DNA-raised antibody responses to the influenza virus H1 glycoprotein. *J. Virol.* 70:9074–78
75. Kang Y, Calvo PA, Daly TM, Long CA. 1998. Comparison of humoral immune responses elicited by DNA and protein vaccines based on merozoite surface protein-1 from *Plasmodium yoelii*, a rodent malaria parasite. *J. Immunol.* 161:4211–19
76. Boyle JS, Silva A, Brady JL, Lew AM. 1997. DNA immunization: induction of higher avidity antibody and effect of route on T-cell cytotoxicity. *Proc. Natl. Acad. Sci. USA* 94:14626–31
77. Peet NM, McKeating JA, Ramos B, Klonisch T, De Souza JB, Delves PJ, Lund T. 1997. Comparison of nucleic acid and protein immunization for induction of antibodies specific for HIV-1 gp120. *Clin. Exp. Immunol.* 109:226–32
78. Donnelly JJ, Martinez D, Jansen KU, Ellis RW, Montgomery DL, Liu MA. 1996. Protection against papillomavirus with a polynucleotide vaccine. *J. Infect. Dis.* 173:314–20
79. Gurunathan S, Prussin C, Sacks DL, Seder RA. 1998. Vaccine requirements for sustained cellular immunity to an intracellular parasitic infection. *Nat. Med.* 4:1409–15
80. Davis HL, Schirmbeck R, Reimann J,

Whalen RG. 1995. DNA-mediated immunization in mice induces a potent MHC class I-restricted cytotoxic T lymphocyte response to the hepatitis B envelope protein. *Hum. Gene Ther.* 6:1447–56
81. Tough DF, Borrow P, Sprent J. 1996. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science* 272:1947–50
82. Sun S, Zhang X, Tough DF, Sprent J. 1998. Type I interferon-mediated stimulation of T cells by CpG DNA. *J. Exp. Med.* 188:2335–42
83. Wolff JA, Ludtke JJ, Acsadi G, Williams P, Jani A. 1992. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. Mol. Genet.* 1:363–69
84. Manthorpe M, Cornefert-Jensen F, Hartikka J, Felgner J, Rundell A, Margalith M, Dwarki V. 1993. Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice. *Hum. Gene Ther.* 4:419–31
85. Cheng L, Ziegelhoffer PR, Yang NS. 1993. In vivo promoter activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment. *Proc. Natl. Acad. Sci. USA* 90:4455–59
86. Chapman BS, Thayer RM, Vincent KA, Haigwood NL. 1991. Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. *Nucl. Acids Res.* 19:3979–86
87. Norman JA, Hobart P, Manthorpe M, Felgner P, Wheeler C. 1997. Development of improved vectors for DNA-based immunization and other gene therapy applications. *Vaccine* 15:801–3
88. Wild J, Gruner B, Metzger K, Kuhrober A, Pudollek HP, Hauser H, Schirmbeck R, Reimann J. 1998. Polyvalent vaccination against hepatitis B surface and core antigen using a dicistronic expression plasmid. *Vaccine* 16:353–60
89. Lewin B, ed. 1994. *Genes V.* Oxford Univ. Press/Cell. 5th ed.
90. Uchijima M, Yoshida A, Nagata T, Koide Y. 1998. Optimization of codon usage of plasmid DNA vaccine is required for the effective MHC class I-restricted T cell responses against an intracellular bacterium. *J. Immunol.* 161:5594–99
91. Andre S, Seed B, Eberle J, Schraut W, Bultmann A, Haas J. 1998. Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *J. Virol.* 72:1497–503
92. Vinner L, Nielsen HV, Bryder K, Corbet S, Nielsen C, Fomsgaard A. 1999. Gene gun DNA vaccination with Rev-independent synthetic HIV-1 gp160 envelope gene using mammalian codons. *Vaccine* 17:2166–75
93. Lewis PJ, Cox GJ, van Drunen Littel-van den Hurk S, Babiuk LA. 1997. Polynucleotide vaccines in animals: enhancing and modulating responses. *Vaccine* 15:861–64
94. Inchauspe G, Vitvitski L, Major ME, Jung G, Spengler U, Maisonnas M, Trepo C. 1997. Plasmid DNA expressing a secreted or a nonsecreted form of hepatitis C virus nucleocapsid: comparative studies of antibody and T-helper responses following genetic immunization. *DNA Cell Biol.* 16:185–95
95. Rice J, King CA, Spellerberg MB, Fairweather N, Stevenson FK. 1999. Manipulation of pathogen-derived genes to influence antigen presentation via DNA vaccines. *Vaccine* 17:3030–38
96. Wu Y, Kipps TJ. 1997. Deoxyribonucleic acid vaccines encoding antigens with rapid proteasome-dependent degradation are highly efficient inducers of cytolytic T lymphocytes. *J. Immunol.* 159:6037–43
97. Tobery TW, Siliciano RF. 1997. Targeting of HIV-1 antigens for rapid intracellular degradation enhances cytotoxic T lymphocyte (CTL) recognition and the

induction of de novo CTL responses in vivo after immunization. *J. Exp. Med.* 185:909–20

98. Rodriguez F, Zhang J, Whitton JL. 1997. DNA immunization: ubiquitination of a viral protein enhances cytotoxic T-lymphocyte induction and antiviral protection but abrogates antibody induction. *J. Virol.* 71:8497–503
99. Whitton JL, Rodriguez F, Zhang J, Hassett DE. 1999. DNA immunization: mechanistic studies. *Vaccine* 17:1612–19
100. Ciernik IF, Berzofsky JA, Carbone DP. 1996. Induction of cytotoxic T-lymphocytes and antitumor immunity with DNA vaccines expressing single T-cell epitopes. *J. Immunol.* 156:2369–75
101. Iwasaki A, Dela Cruz CS, Young AR, Barber BH. 1999. Epitope-specific cytotoxic T lymphocyte induction by minigene DNA immunization. *Vaccine* 17:2081–88
102. Yu Z, Karem KL, Kanangat S, Manickan E, Rouse BT. 1998. Protection by minigenes: a novel approach of DNA vaccines. *Vaccine* 16:1660–67
103. Hanke T, Schneider J, Gilbert SC, Hill AV, McMichael A. 1998. DNA multi-CTL epitope vaccines for HIV and *Plasmodium falciparum:* immunogenicity in mice. *Vaccine* 16:426–35
104. Thomson SA, Sherritt MA, Medveczky J, Elliott SL, Moss DJ, Fernando JG, Brown LE, Suhrbier A. 1998. Delivery of multiple CD8 cytotoxic T cell epitopes by DNA vaccination. *J. Immunol.* 160:1717–23
105. Suhrbier A. 1997. Multi-epitope DNA vaccines. *Immunol. Cell Biol.* 75:402–8
106. Wang R, Doolan DL, Charoenvit Y, Hedstrom RC, Gardner NJ, Hobart P, Tine J, Sedegah M, Fallarme V, Sacci JB Jr, Kaur M, Klinman DM, Hoffman SL, Weiss WR. 1998. Simultaneous induction of multiple antigen-specific cytotoxic T lymphocytes in nonhuman primates by immunization with a mixture of four *Plasmodium falciparum* DNA plasmids. *Infect. Immun.* 66:4193–202
107. Shi YP, Hasnain SE, Sacci JB, Holloway BP, Fujioka H, Kumar N, Wohlhueter R, Hoffman SL, Collins WE, Lal AA. 1999. Immunogenicity and in vitro protective efficacy of a recombinant multistage *Plasmodium falciparum* candidate vaccine. *Proc. Natl. Acad. Sci. USA* 96:1615–20
108. Maecker HT, Umetsu DT, DeKruyff RH, Levy S. 1998. Cytotoxic T cell responses to DNA vaccination: dependence on antigen presentation via class II MHC. *J. Immunol.* 161:6532–36
109. Klinman DM, Yamshchikov G, Ishigatsubo Y. 1997. Contribution of CpG motifs to the immunogenicity of DNA vaccines. *J. Immunol.* 158:3635–39
110. Brazolot Millan CL, Weeratna R, Krieg AM, Siegrist CA, Davis HL. 1998. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc. Natl. Acad. Sci. USA* 95:15553–58
111. Davis HL, Weeranta R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM. 1998. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J. Immunol.* 160:870–76
112. Klinman DM. 1998. Therapeutic applications of CpG-containing oligodeoxynucleotides. *Antisense Nucl. Acid Drug Dev.* 8:181–84
113. Klinman DM, Barnhart KM, Conover J. 1999. CpG motifs as immune adjuvants. *Vaccine* 17:19–25
114. Krieg AM, Wu T, Weeratna R, Efler SM, Love-Homan L, Yang L, Yi AK, Short D, Davis HL. 1998. Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. *Proc. Natl. Acad. Sci. USA* 95:12631–36
115. Liang H, Nishioka Y, Reich CF, Pisetsky DS, Lipsky PE. 1996. Activation of human B cells by phosphorothioate oli-

godeoxynucleotides. *J. Clin. Invest.* 98: 1119–29

116. Ballas ZK, Rasmussen WL, Krieg AM. 1996. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* 157:1840–45
117. Hacker H, Mischak H, Miethke T, Liptay S, Schmid R, Sparwasser T, Heeg K, Lipford GB, Wagner H. 1998. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* 17:6230–40
118. Richmond JF, Mustafa F, Lu S, Santoro JC, Weng J, O'Connell M, Fenyo EM, Hurwitz JL, Montefiori DC, Robinson HL. 1997. Screening of HIV-1 Env glycoproteins for the ability to raise neutralizing antibody using DNA immunization and recombinant vaccinia virus boosting. *Virology* 230:265–74
119. Richmond JF, Lu S, Santoro JC, Weng J, Hu SL, Montefiori DC, Robinson HL. 1998. Studies of the neutralizing activity and avidity of anti-human immunodeficiency virus type 1 Env antibody elicited by DNA priming and protein boosting. *J. Virol.* 72:9092–100
120. Mustafa F, Richmond JF, Fernandez-Larsson R, Lu S, Fredriksson R, Fenyo EM, O'Connell M, Johnson E, Weng J, Santoro JC, Robinson HL. 1997. HIV-1 Env glycoproteins from two series of primary isolates: replication phenotype and immunogenicity. *Virology* 229:269–78
121. Fuller DH, Murphey-Corb M, Clements J, Barnett S, Haynes JR. 1996. Induction of immunodeficiency virus-specific immune responses in rhesus monkeys following gene gun-mediated DNA vaccination. *J. Med. Primatol.* 25:236–41
122. Fuller DH, Haynes JR. 1994. A qualitative progression in HIV type 1 glycoprotein 120-specific cytotoxic cellular and humoral immune responses in mice receiving a DNA-based glycoprotein 120 vaccine. *AIDS Res. Hum. Retrovir.* 10:1433–41
123. Lu S, Wyatt R, Richmond JF, Mustafa F, Wang S, Weng J, Montefiori DC, Sodroski J, Robinson HL. 1998. Immunogenicity of DNA vaccines expressing human immunodeficiency virus type 1 envelope glycoprotein with and without deletions in the V1/2 and V3 regions. *AIDS Res. Hum. Retrovir.* 14:151–55
124. Lu S, Manson K, Wyand M, Robinson HL. 1997. SIV DNA vaccine trial in macaques: post-challenge necropsy in vaccine and control groups. *Vaccine* 15:920–23
125. Okuda K, Xin KO, Tsuji T, Bukawa H, Tanaka S, Koff WC, Tani K, Honma K, Kawamoto S, Hamajima K, Fukushima J. 1997. DNA vaccination followed by macromolecular multicomponent peptide vaccination against HIV-1 induces strong antigen-specific immunity. *Vaccine* 15:1049–56
126. Barnett SW, Rajasekar S, Legg H, Doe B, Fuller DH, Haynes JR, Walker CM, Steimer KS. 1997. Vaccination with HIV-1 gp120 DNA induces immune responses that are boosted by a recombinant gp120 protein subunit. *Vaccine* 15:869–73
127. Letvin NL, Montefiori DC, Yasutomi Y, Perry HC, Davies ME, Lekutis C, Alroy M, Freed DC, Lord CI, Handt LK, Liu MA, Shiver JW. 1997. Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. *Proc. Natl. Acad. Sci. USA* 94:9378–83
128. Fuller DH, Corb MM, Barnett S, Steimer K, Haynes JR. 1997. Enhancement of immunodeficiency virus-specific immune responses in DNA-immunized rhesus macaques. *Vaccine* 15:924–26
129. Hanke T, Blanchard TJ, Schneider J, Hannan CM, Becker M, Gilbert SC, Hill AV, Smith GL, McMichael A. 1998. Enhancement of MHC class I-restricted peptide-specific T cell induction by a

DNA prime/MVA boost vaccination regime. *Vaccine* 16:439–45

130. Sedegah M, Jones TR, Kaur M, Hedstrom R, Hobart P, Tine JA, Hoffman SL. 1998. Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine. *Proc. Natl. Acad. Sci. USA* 95:7648–53
131. Schneider J, Gilbert SC, Blanchard TJ, Hanke T, Robson KJ, Hannan CM, Becker M, Sinden R, Smith GL, Hill AV. 1998. Enhanced immunogenicity for $CD8^+$ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nat. Med.* 4:397–402
132. Kent SJ, Zhao A, Best SJ, Chandler JD, Boyle DB, Ramshaw IA. 1998. Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. *J. Virol.* 72:10180–88
133. Robinson HL, Montefiori DC, Johnson RP, Manson KH, Kalish ML, Lifson JD, Rizvi TA, Lu S, Hu SL, Mazzara GP, Panicali DL, Herndon JG, Glickman R, Candido MA, Lydy SL, Wyand MS, McClure HM. 1999. Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. *Nat. Med.* 5:526–34
134. Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL. 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. USA* 90:11478–82
135. Jones DH, Corris S, McDonald S, Clegg JC, Farrar GH. 1997. Poly(DL-lactide-co-glycolide)-encapsulated plasmid DNA elicits systemic and mucosal antibody responses to encoded protein after oral administration. *Vaccine* 15:814–17
136. Chen SC, Jones DH, Fynan EF, Farrar GH, Clegg JC, Greenberg HB, Herrmann JE. 1998. Protective immunity induced by oral immunization with a rotavirus DNA vaccine encapsulated in microparticles. *J. Virol.* 72:5757–61
137. Herrmann JE, Chen SC, Jones DH, Tinsley-Bown A, Fynan EF, Greenberg HB, Farrar GH. 1999. Immune responses and protection obtained by oral immunization with rotavirus VP4 and VP7 DNA vaccines encapsulated in microparticles. *Virology* 259:148–53
138. Roy K, Mao HQ, Huang SK, Leong KW. 1999. Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat. Med.* 5:387–91
139. Klavinskis LS, Gao L, Barnfield C, Lehner T, Parker S. 1997. Mucosal immunization with DNA-liposome complexes. *Vaccine* 15:818–20
140. Ban EM, van Ginkel FW, Simecka JW, Kiyono H, Robinson HL, McGhee JR. 1997. Mucosal immunization with DNA encoding influenza hemagglutinin. *Vaccine* 15:811–13
141. Kuklin N, Daheshia M, Karem K, Manickan E, Rouse BT. 1997. Induction of mucosal immunity against herpes simplex virus by plasmid DNA immunization. *J. Virol.* 71:3138–45
142. Sasaki S, Hamajima K, Fukushima J, Ihata A, Ishii N, Gorai I, Hirahara F, Mohri H, Okuda K. 1998. Comparison of intranasal and intramuscular immunization against human immunodeficiency virus type 1 with a DNA-monophosphoryl lipid A adjuvant vaccine. *Infect. Immun.* 66:823–26
143. Sasaki S, Sumino K, Hamajima K, Fukushima J, Ishii N, Kawamoto S, Mohri H, Kensil CR, Okuda K. 1998. Induction of systemic and mucosal immune responses to human immunodeficiency virus type 1 by a DNA vaccine formulated with QS-21 saponin adjuvant via intramuscular and intranasal routes. *J. Virol.* 72:4931–39
144. Sasaki S, Fukushima J, Hamajima K,

Ishii N, Tsuji T, Xin KQ, Mohri H, Okuda K. 1998. Adjuvant effect of Ubenimex on a DNA vaccine for HIV-1. *Clin. Exp. Immunol.* 111:30–35

145. Wang B, Dang K, Agadjanyan MG, Srikantan V, Li F, Ugen KE, Boyer J, Merva M, Williams WV, Weiner DB. 1997. Mucosal immunization with a DNA vaccine induces immune responses against HIV-1 at a mucosal site. *Vaccine* 15:821–25
146. Livingston JB, Lu S, Robinson H, Anderson DJ. 1998. Immunization of the female genital tract with a DNA-based vaccine. *Infect. Immun.* 66:322–29
147. Shi Z, Curiel DT, Tang DC. 1999. DNA-based non-invasive vaccination onto the skin. *Vaccine* 17:2136–41
148. Davis HL, Jasmin BJ. 1993. Direct gene transfer into mouse diaphragm. *FEBS Lett.* 333:146–50
149. Davis HL, Michel ML, Mancini M, Schleef M, Whalen RG. 1994. Direct gene transfer in skeletal muscle: plasmid DNA-based immunization against the hepatitis B virus surface antigen. *Vaccine* 12:1503–9
150. Wang R, Doolan DL, Le TP, Hedstrom RC, Coonan KM, Charoenvit Y, Jones TR, Hobart P, Margalith M, Ng J, Weiss WR, Sedegah M, de Taisne C, Norman JA, Hoffman SL. 1998. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 282:476–80
151. Leitner WW, Seguin MC, Ballou WR, Seitz JP, Schultz AM, Sheehy MJ, Lyon JA. 1997. Immune responses induced by intramuscular or gene gun injection of protective deoxyribonucleic acid vaccines that express the circumsporozoite protein from *Plasmodium berghei* malaria parasites. *J. Immunol.* 159:6112–19
152. Tsan MF, White JE, Shepard B. 1995. Lung-specific direct in vivo gene transfer with recombinant plasmid DNA. *Am. J. Physiol.* 268:L1052–56
153. Meyer KB, Thompson MM, Levy MY, Barron LG, Szoka FC Jr. 1995. Intratracheal gene delivery to the mouse airway: characterization of plasmid DNA expression and pharmacokinetics. *Gene Ther.* 2:450–60
154. Stribling R, Brunette E, Liggitt D, Gaensler K, Debs R. 1992. Aerosol gene delivery in vivo. *Proc. Natl. Acad. Sci. USA* 89:11277–81
155. McDonald RJ, Liggitt HD, Roche L, Nguyen HT, Pearlman R, Raabe OG, Bussey LB, Gorman CM. 1998. Aerosol delivery of lipid:DNA complexes to lungs of rhesus monkeys. *Pharm. Res.* 15:671–79
156. Okada E, Sasaki S, Ishii N, Aoki I, Yasuda T, Nishioka K, Fukushima J, Miyazaki J, Wahren B, Okuda K. 1997. Intranasal immunization of a DNA vaccine with IL-12- and granulocyte-macrophage colony-stimulating factor (GM-CSF)–expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens. *J. Immunol.* 159:3638–47
157. Kuklin NA, Daheshia M, Chun S, Rouse BT. 1998. Immunomodulation by mucosal gene transfer using TGF-β DNA. *J. Clin. Invest.* 102:438–44
158. Kawabata K, Takakura Y, Hashida M. 1995. The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. *Pharm. Res.* 12:825–30
159. Lew D, Parker S, Latimer T, Abai A, Kuwahara-Rundell A, Doh S, Yang Z-Y, Gromkowski S, Nabel G, Manthrope M, Norman J. 1995. Cancer gene therapy using plasmid DNA: pharmacokinectic study of DNA following injection in mice. *Hum. Gene Ther.* 6:553–64
160. Williams RS, Johnston SA, Riedy M, De Vit MJ, McElligott SG, Sanford JC. 1991. Introduction of foreign genes into

tissues of living mice by DNA-coated microprojectiles. *Proc. Natl. Acad. Sci. USA* 88:2726–30

161. Gregoriadis G, Saffie R, de Souza JB. 1997. Liposome-mediated DNA vaccination. *FEBS Lett.* 402:107–10
162. Gould-Fogerite S, Kheiri MT, Zang F, Wang Z, Scolpino AJ, Feketeova E, Canki M, Mannino RJ. 1998. Targetting immune response induction with cochleate and liposomal-based vaccines. *Adv. Drug Del. Rev.* 32:273–87
163. Mannino RJ, Gould-Fogerite S. 1997. Antigen cochleate preparations for oral and systemic vaccination. In *New Generation Vaccines*, eds. ML Levine, GC Woodrow, JB Kaper, GS Cobon. New York: Marcel Dekker. 2nd ed.
164. Gould-Fogerite S, Mannino RJ. 1996. Mucosal and systemic immunization using cochleate and liposome vaccines. *J. Liposome Res.* 2:357–79
165. Sizemore DR, Branstrom AA, Sadoff JC. 1995. Attenuated Shigella as a DNA delivery vehicle for DNA-mediated immunization. *Science* 270:299–302
166. Sizemore DR, Branstrom AA, Sadoff JC. 1997. Attenuated bacteria as a DNA delivery vehicle for DNA-mediated immunization. *Vaccine* 15:804–7
167. Darji A, Guzman CA, Gerstel B, Wachholz P, Timmis KN, Wehland J, Chakraborty T, Weiss S. 1997. Oral somatic transgene vaccination using attenuated *S. typhimurium. Cell* 91:765–75
168. Paglia P, Medina E, Arioli I, Guzman CA, Colombo MP. 1998. Gene transfer in dendritic cells, induced by oral DNA vaccination with *Salmonella typhimurium*, results in protective immunity against a murine fibrosarcoma. *Blood* 92:3172–76
169. Dietrich G, Bubert A, Gentschev I, Sokolovic Z, Simm A, Catic A, Kaufmann SH, Hess J, Szalay AA, Goebel W. 1998. Delivery of antigen-encoding plasmid DNA into the cytosol of macrophages by attenuated suicide *Listeria monocytogenes. Nat. Biotechnol.* 16:181–85
170. Fennelly GJ, Khan SA, Abadi MA, Wild TF, Bloom BR. 1999. Mucosal DNA vaccine immunization against measles with a highly attenuated *Shigella flexneri* vector. *J. Immunol.* 162:1603–10
171. Courvalin P, Goussard S, Grillot-Courvalin C. 1995. Gene transfer from bacteria to mammalian cells. *C.R. Acad. Sci. III* 318:1207–12
172. Tubulekas I, Berglund P, Fleeton M, Liljestrom P. 1997. Alphavirus expression vectors and their use as recombinant vaccines: a minireview. *Gene* 190:191–95
173. Driver DA, Latham EM, Polo JM, Belli BA, Banks TA, Chada S, Brumm D, Chang SMW, Mento SJ, Jolly DJ, Dubensky TW. 1995. Layered amplification of gene expression with a DNA gene delivery system. *Ann. NY Acad. Sci.* 772:261–64
174. Dubensky TW Jr, Driver DA, Polo JM, Belli BA, Latham EM, Ibanez CE, Chada S, Brumm D, Banks TA, Mento SJ, Jolly DJ, Chang SM. 1996. Sindbis virus DNA-based expression vectors: utility for in vitro and in vivo gene transfer. *J. Virol.* 70:508–19
175. Herweijer H, Latendresse JS, Williams P, Zhang G, Danko I, Schlesinger S, Wolff JA. 1995. A plasmid-based self-amplifying Sindbis virus vector. *Hum. Gene Ther.* 6:1161–67
176. Berglund P, Smerdou C, Fleeton MN, Tubulekas I, Liljestrom P. 1998. Enhancing immune responses using suicidal DNA vaccines. *Nat. Biotechnol.* 16:562–65
177. Hariharan MJ, Driver DA, Townsend K, Brumm D, Polo JM, Belli BA, Catton DJ, Hsu D, Mittelstaedt D, McCormack JE, Karavodin L, Dubensky TW Jr, Chang SM, Banks TA. 1998. DNA immunization against herpes simplex virus: enhanced efficacy using a Sindbis virus-based vector. *J. Virol.* 72:950–58
178. Gerloni M, Baliou WR, Billetta R,

Zanetti M. 1997. Immunity to *Plasmodium falciparum* malaria sporozoites by somatic transgene immunization. *Nat. Biotechnol.* 15:876–81

179. Xiong S, Gerloni M, Zanetti M. 1997. Engineering vaccines with heterologous B and T cell epitopes using immunoglobulin genes. *Nat. Biotechnol.* 15:882–86
180. Klinman DM, Takeno M, Ichino M, Gu M, Yamshchikov G, Mor G, Conover J. 1997. DNA vaccines: safety and efficacy issues. *Springer Semin. Immunopathol.* 19:245–56
181. Martin T, Parker SE, Hedstrom R, Le T, Hoffman SL, Norman J, Hobart P, Lew D. 1999. Plasmid DNA malaria vaccine: the potential for genomic integration after intramuscular injection. *Hum. Gene Ther.* 10:759–68
182. Gilkeson GS, Ruiz P, Howell D, Lefkowith JB, Pisetsky DS. 1993. Induction of immune-mediated glomerulonephritis in normal mice immunized with bacterial DNA. *Clin. Immunol. Immunopathol.* 68:283–92
183. Gilkeson GS, Pippen AM, Pisetsky DS. 1995. Induction of cross-reactive anti-dsDNA antibodies in preautoimmune NZB/NZW mice by immunization with bacterial DNA. *J. Clin. Invest.* 95:1398–402
184. Steinberg AD, Krieg AM, Gourley MF, Klinman DM. 1990. Theoretical and experimental approaches to generalized autoimmunity. *Immunol. Rev.* 118:129–63
185. Klinman DM. 1990. Polyclonal B cell activation in lupus-prone mice precedes and predicts the development of autoimmune disease. *J. Clin. Invest.* 86: 1249–54
186. Klinman DM, Steinberg AD. 1987. Systemic autoimmune disease arises from polyclonal B cell activation. *J. Exp. Med.* 165:1755–60
187. Linker-Israeli M, Deans RJ, Wallace DJ, Prehn J, Ozeri-Chen T, Klinenberg JR. 1991. Elevated levels of endogenous IL-6 in systemic lupus erythematosus: a putative role in pathogenesis. *J. Immunol.* 147:117–23
188. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 356: 314–17
189. Krieg AM. 1995. CpG DNA: a pathogenic factor in systemic lupus erythematosus? *J. Clin. Immunol.* 15:284–92
190. Yi AK, Hornbeck P, Lafrenz DE, Krieg AM. 1996. CpG DNA rescue of murine B lymphoma cells from anti-IgM-induced growth arrest and programmed cell death is associated with increased expression of c-myc and bcl-xL. *J. Immunol.* 157:4918–25
191. Katsumi A, Emi N, Abe A, Hasegawa Y, Ito M, Saito H. 1994. Humoral and cellular immunity to an encoded protein induced by direct DNA injection. *Hum.Gene Ther.* 5:1335–39
192. Mor G, Singla M, Steinberg AD, Hoffman SL, Okuda K, Klinman DM. 1997. Do DNA vaccines induce autoimmune disease? *Hum. Gene Ther.* 8:293–300
193. Xiang ZQ, Spitalnik SL, Cheng J, Erikson J, Wojczyk B, Ertl HC. 1995. Immune responses to nucleic acid vaccines to rabies virus. *Virology* 209:569–79
194. Gilkeson GS, Conover J, Halpern M, Pisetsky DS, Feagin A, Klinman DM. 1998. Effects of bacterial DNA on cytokine production by (NZB/NZW)F1 mice. *J. Immunol.* 161:3890–95
195. Bachmaier K, Neu N, de la Maza LM, Pal S, Hessel A, Penninger JM. 1999. Chlamydia infections and heart disease linked through antigenic mimicry. *Science* 283:1335–39
196. Silverstein AM, Segal S. 1975. The ontogeny of antigen-specific T cells. *J. Exp. Med.* 142:802–4

197. Marodon G, Rocha B. 1994. Activation and 'deletion' of self-reactive mature and immature T cells during ontogeny of Mls-1a mice: implications for neonatal tolerance induction. *Int. Immunol.* 6:1899–904
198. Silverstein AM. 1977. Ontogeny of the immune response: a perspective. In *Development of Host Defenses,* eds. MD Cooper, DH Dayton, pp. 1–10. New York: Raven
199. Sarzotti M, Robbins DS, Hoffman PM. 1996. Induction of protective CTL responses in newborn mice by a murine retrovirus. *Science* 271:1726–28
200. Mor G, Yamshchikov G, Sedegah M, Takeno M, Wang R, Houghten RA, Hoffman S, Klinman DM. 1996. Induction of neonatal tolerance by plasmid DNA vaccination of mice. *J. Clin. Invest.* 98:2700–5
201. Ichino M, Mor G, Conover J, Weiss WR, Takeno M, Ishii KJ, Klinman DM. 1999. Factors associated with the development of neonatal tolerance after the administration of a plasmid DNA vaccine. *J. Immunol.* 162:3814–18
202. Klinman DM, Conover J, Bloom ET, Weiss W. 1998. Immunogenicity and efficacy of DNA vaccination in aged mice. *J. Gerontol.* 53:B281–86
203. Ishii KJ, Weiss W, Klinman DM. 1999. Prevention of neonatal tolerance by plasmid encoding GM-CSF. *Vaccine.* In press
204. Sarzotti M, Dean TA, Remington MP, Ly CD, Furth PA, Robbins DS. 1997. Induction of cytotoxic T cell responses in newborn mice by DNA immunization. *Vaccine* 15:795–97
205. Wang Y, Xiang Z, Pasquini S, Ertl HC. 1997. Immune response to neonatal genetic immunization. *Virology* 228: 278–84
206. Prince AM, Whalen R, Brotman B. 1997. Successful nucleic acid based immunization of newborn chimpanzees against hepatitis B virus. *Vaccine* 15:916–19
207. Davis HL, Brazolot Millan CL. 1997. DNA-based immunization against hepatitis B virus. *Springer Semin. Immunopathol.* 19:195–209
208. Bot A, Bot S, Bona C. 1998. Enhanced protection against influenza virus of mice immunized as newborns with a mixture of plasmids expressing hemagglutinin and nucleoprotein. *Vaccine* 16:1675–82
209. Manickan E, Yu Z, Rouse BT. 1997. DNA immunization of neonates induces immunity despite the presence of maternal antibody. *J. Clin. Invest.* 100:2371–75
210. Ishii KJ, Weiss WR, Ichino D, Verthelyi D, Klinman DM. 1999. Activity and safety of DNA plasmids encoding IL-4 and IFN-γ. *Gene Ther.* 6:237–44
210a. U.S. Food and Drug Administration. *Points to Consider.* http://www.fda.gov/cber/points.html
211. Hakim I, Levy S, Levy R. 1996. A nine-amino acid peptide from IL-1β augments antitumor immune responses induced by protein and DNA vaccines. *J. Immunol.* 157:5503–11
212. Maecker HT, Umetsu DT, DeKruyff RH, Levy S. 1997. DNA vaccination with cytokine fusion constructs biases the immune response to ovalbumin. *Vaccine* 15:1687–96
213. Kim JJ, Trivedi NN, Nottingham LK, Morrison L, Tsai A, Hu Y, Mahalingam S, Dang K, Ahn L, Doyle NK, Wilson DM, Chattergoon MA, Chalian AA, Boyer JD, Agadjanyan MG, Weiner DB. 1998. Modulation of amplitude and direction of in vivo immune responses by co-administration of cytokine gene expression cassettes with DNA immunogens. *Eur. J. Immunol.* 28:1089–103
214. Raz E, Watanabe A, Baird SM, Eisenberg RA, Parr TB, Lotz M, Kipps TJ, Carson DA. 1993. Systemic immunological effects of cytokine genes injected into skeletal muscle. *Proc. Natl. Acad. Sci. USA* 90:4523–27

215. Chow YH, Huang WL, Chi WK, Chu YD, Tao MH. 1997. Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin-2. *J. Virol.* 71:169–78
216. Geissler M, Gesien A, Tokushige K, Wands JR. 1997. Enhancement of cellular and humoral immune responses to hepatitis C virus core protein using DNA-based vaccines augmented with cytokine-expressing plasmids. *J. Immunol.* 158:1231–37
217. Prayaga SK, Ford MJ, Haynes JR. 1997. Manipulation of HIV-1 gp120-specific immune responses elicited via gene gun-based DNA immunization. *Vaccine* 15:1349–52
218. Chow YH, Chiang BL, Lee YL, Chi WK, Lin WC, Chen YT, Tao MH. 1998. Development of Th1 and Th2 populations and the nature of immune responses to hepatitis B virus DNA vaccines can be modulated by codelivery of various cytokine genes. *J. Immunol.* 160:1320–29
219. Kim JJ, Simbiri KA, Sin JI, Dang K, Oh J, Dentchev T, Lee D, Nottingham LK, Chalian AA, McCallus D, Ciccarelli E, Agadjanyan MG, Weiner DB. 1999. Cytokine molecular adjuvants modulate immune responses induced by DNA vaccine constructs for HIV-1 and SIV. *J. Interferon Cytokine Res.* 19:77–84
220. Larsen DL, Dybdahl-Sissoko N, McGregor MW, Drape R, Neumann V, Swain WF, Lunn DP, Olsen CW. 1998. Coadministration of DNA encoding interleukin-6 and hemagglutinin confers protection from influenza virus challenge in mice. *J. Virol.* 72:1704–8
221. Hengge UR, Chan EF, Foster RA, Walker PS, Vogel JC. 1995. Cytokine gene expression in epidermis with biological effects following injection of naked DNA. *Nat. Genet.* 10:161–66
222. Rogy MA, Auffenberg T, Espat NJ, Philip R, Remick D, Wollenberg GK, Copeland EM III, Moldawer LL. 1995. Human tumor necrosis factor receptor (p55) and interleukin 10 gene transfer in the mouse reduces mortality to lethal endotoxemia and also attenuates local inflammatory responses. *J. Exp. Med.* 181:2289–93
223. Daheshia M, Kuklin N, Kanangat S, Manickan E, Rouse BT. 1997. Suppression of ongoing ocular inflammatory disease by topical administration of plasmid DNA encoding IL-10. *J. Immunol.* 159:1945–52
224. Manickan E, Daheshia M, Kuklin N, Chun S, Rouse BT. 1998. Modulation of virus-induced delayed-type hypersensitivity by plasmid DNA encoding the cytokine interleukin-10. *Immunology* 94:129–34
225. Kim JJ, Ayyavoo V, Bagarazzi ML, Chattergoon MA, Dang K, Wang B, Boyer JD, Weiner DB. 1997. In vivo engineering of a cellular immune response by coadministration of IL-12 expression vector with a DNA immunogen. *J. Immunol.* 158:816–26
226. Gurunathan S, Irvine KR, Wu CY, Cohen JI, Thomas E, Prussin C, Restifo NP, Seder RA. 1998. CD40 ligand/trimer DNA enhances both humoral and cellular immune responses and induces protective immunity to infectious and tumor challenge. *J. Immunol.* 161:4563–71
227. Tsuji T, Hamajima K, Fukushima J, Xin KQ, Ishii N, Aoki I, Ishigatsubo Y, Tani K, Kawamoto S, Nitta Y, Miyazaki J, Koff WC, Okubo T, Okuda K. 1997. Enhancement of cell-mediated immunity against HIV-1 induced by coinoculation of plasmid-encoded HIV-1 antigen with plasmid expressing IL-12. *J. Immunol.* 158:4008–13
228. Rakhmilevich AL, Turner J, Ford MJ, McCabe D, Sun WH, Sondel PM, Grota K, Yang NS. 1996. Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established pri-

mary and metastatic murine tumors. *Proc. Natl. Acad. Sci. USA* 93: 6291–96

229. Kim JJ, Ayyavoo V, Bagarazzi ML, Chattergoon M, Boyer JD, Wang B, Weiner DB. 1997. Development of a multicomponent candidate vaccine for HIV-1. *Vaccine* 15:879–83
230. Svanholm C, Lowenadler B, Wigzell H. 1997. Amplification of T-cell and antibody responses in DNA-based immunization with HIV-1 Nef by co-injection with a GM-CSF expression vector. *Scand. J. Immunol.* 46:298–303
231. Weiss WR, Ishii KJ, Hedstrom RC, Sedegah M, Ichino M, Barnhart K, Klinman DM, Hoffman SL. 1998. A plasmid encoding murine granulocyte-macrophage colony-stimulating factor increases protection conferred by a malaria DNA vaccine. *J. Immunol.* 161:2325–32
232. Lee AH, Suh YS, Sung YC. 1999. DNA inoculations with HIV-1 recombinant genomes that express cytokine genes enhance HIV-1 specific immune responses. *Vaccine* 17:473–79
233. Xiang Z, Ertl HC. 1995. Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunity* 2:129–35
234. Song XY, Gu M, Jin WW, Klinman DM, Wahl SM. 1998. Plasmid DNA encoding transforming growth factor-β1 suppresses chronic disease in a streptococcal cell wall-induced arthritis model. *J. Clin. Invest.* 101:2615–21
235. Lim YS, Kang BY, Kim EJ, Kim SH, Hwang SY, Kim TS. 1998. Potentiation of antigen specific, Th1 immune responses by multiple DNA vaccination with ovalbumin/interferon-γ hybrid construct. *Immunology* 94:135–41
236. Yeow WS, Lawson CM, Beilharz MW. 1998. Antiviral activities of individual murine IFN-α subtypes in vivo: intramuscular injection of IFN expression constructs reduces cytomegalovirus replication. *J. Immunol.* 160:2932–39
237. Horton HM, Anderson D, Hernandez P, Barnhart KM, Norman JA, Parker SE. 1999. A gene therapy for cancer using intramuscular injection of plasmid DNA encoding interferon α. *Proc. Natl. Acad. Sci. USA* 96:1553–58
238. Tsuji T, Hamajima K, Ishii N, Aoki I, Fukushima J, Xin KQ, Kawamoto S, Sasaki S, Matsunaga K, Ishigatsubo Y, Tani K, Okubo T, Okuda K. 1997. Immunomodulatory effects of a plasmid expressing B7–2 on human immunodeficiency virus-1-specific cell-mediated immunity induced by a plasmid encoding the viral antigen. *Eur. J. Immunol.* 27:782–87
239. Kim JJ, Bagarazzi ML, Trivedi N, Hu Y, Kazahaya K, Wilson DM, Ciccarelli R, Chattergoon MA, Dang K, Mahalingam S, Chalian AA, Agadjanyan MG, Boyer JD, Wang B, Weiner DB. 1997. Engineering of in vivo immune responses to DNA immunization via codelivery of costimulatory molecule genes. *Nat. Biotechnol.* 15:641–46
240. Corr M, Tighe H, Lee D, Dudler J, Trieu M, Brinson DC, Carson DA. 1997. Costimulation provided by DNA immunization enhances antitumor immunity. *J. Immunol.* 159:4999–5004
241. Horspool JH, Perrin PJ, Woodcock JB, Cox JH, King CL, June CH, Harlan DM, St. Louis DC, Lee KP. 1998. Nucleic acid vaccine-induced immune responses require CD28 costimulation and are regulated by CTLA4. *J. Immunol.* 160:2706–14
242. Mendoza RB, Cantwell MJ, Kipps TJ. 1997. Immunostimulatory effects of a plasmid expressing CD40 ligand (CD154) on gene immunization. *J. Immunol.* 159:5777–81
243. Kim JJ, Tsai A, Nottingham LK, Morrison L, Cunning DM, Oh J, Lee DJ, Dang

K, Dentchev T, Chalian AA, Agadjanyan MG, Weiner DB. 1999. Intracellular adhesion molecule-1 modulates β-chemokines and directly costimulates T cells in vivo. *J. Clin. Invest.* 103:869–77

244. Boyle JS, Brady JL, Lew AM. 1998. Enhanced responses to a DNA vaccine encoding a fusion antigen that is directed to sites of immune induction. *Nature* 392:408–11

Annual Review of Immunology
Volume 18, 2000

CONTENTS

Discovering the Role of the Major Histocompatibility Complex in the Immune Response, *Hugh O. McDevitt* 1
Receptor Selection in B and T Lymphocytes, *David Nemazee* 19
Molecular Basis of Celiac Disease, *Ludvig M. Sollid* 53
Population Biology of Lymphocytes: The Flight for Survival, *Antonio A. Freitas, Benedita Rocha* 83
Nonclassical Class II MHC Molecules, *Christopher Alfonso, Lars Karlsson* 113
Negative Regulation of Cytokine Signaling Pathways, *Hideo Yasukawa, Atsuo Sasaki, Akihiko Yoshimura* 143
T Cell Activation and the Cytoskeleton, *Oreste Acuto, Doreen Cantrell* 165
The Specific Regulation of Immune Responses by CD8+ T Cells Restricted by the MHC Class Ib Molecule Qa-1, *Hong Jiang, Leonard Chess* 185
The Biology of Chemokines and their Receptors, *Devora Rossi, Albert Zlotnik* 217
Dendritic Cells in Cancer Immunotherapy, *Lawrence Fong, Edgar G. Engleman* 245
CD8 T Cell Effector Mechanisms in Resistance to Infection, *John T. Harty, Amy R. Tvinnereim, Douglas W. White* 275
Glucocoricoids in T Cell Development and Function, *Jonathan D. Ashwell, Frank W. M. Lu, Melanie S. Vacchio* 309
Molecular Genetics of Allergic Diseases, *Santa Jeremy Ono* 347
Immunology at the Maternal-Fetal Interface: Lessons for T Cell Tolerance and Suppression, *A. L. Mellor, D. H. Munn* 367
Regulation of B. Lymphocyte Responses to Foreign and Self-Antigens by the CD19/CD21 Complex, *Douglas T. Fearon, Michael C. Carroll, Michael C. Carroll* 393
Regulatory T Cells in Autoimmunity, *Ethan M. Shevach* 423
Signal and Transcription in T Helper Development, *Kenneth M. Murphy, Wenjun Ouyang, J. David Farrar, Jianfei Yang, Sheila Ranganath, Helene Asnagli, Maryam Afkarian, Theresa L. Murphy* 451
The RAG Proteins and V (D) J Recombination: Complexes, Ends, and Transposition, *Sebastian D. Fugmann, Alfred Ian Lee, Penny E. Shockett, Isabelle J. Villey, David G. Schatz* 495
The Role of the Thymus in Immune Reconstitution in Aging, Bone Marrow Transplantation, and HIV-1 Infection, *Barton F. Haynes, M. Louise Markert, Gregory D. Sempowski, Dhavalkumar D. Patel, Laura P. Hale* 529
Accessing Complexity: The Dynamics of Virus-Specific T Cell Responses, *Peter C. Doherty, Jan P. Christensen* 561
The Role of Chemokine Receptors in Primary, Effector, and Memory Immune Responses, *Federica Sallusto, Charles R. Mackay, Antonio Lanzavecchia* 593
Phosphorylation Meets Ubiquiination: The Control of NF-Kappa-B Activity, *Michael Karin, Yinon Ben-Neriah* 621
Reservoirs for HIV-1: Mechanisms for Viral Persistence in the Presence of Antiviral Immune Responses and Antiretroviral Therapy, *Theodore Pierson, Justin McArthur, Robert F. Siliciano* 665

Regulation of Antibody Responses via Antibodies, Complement, and Fc Receptors, *Birgitta Heyman* 709
Multiple Roles for theMajor Histocompatibility Complex Class I--Related Receptor, FcRn, *Victor Ghetie, E. Sally Ward* 739
Immunobiology of Dendritic Cells, *Jacques Banchereau, Francine Briere, Christophe Caux, Jean Davoust, Serge Lebecque, Yong-Jun Liu, Bali Pulendran, Karolina Palucka* 767
An Address System in the Vasculature of Normal Tissues and Tumors, *E. Ruoslahti, D. Rajotte* 813
Genomic Views of the Immune System, *Louis M. Staudt, Patrick O. Brown* 829
Viral Subversion of the Immune System, *Domenico Tortorella, Benjamin E. Gewurz, Margo H. Furman, Danny J. Schust, Hidde L. Ploegh* 861
DNA Vaccines: Immunology, Application, and Optimization, *Sanjay Gurunathan, Dennis M. Klinman, Robert A. Seder* 927
Gamma Delta Cells: A Right Time and a Right Place for a Conserved Third Way of Protection, *Adrian C. Hayday* 975