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BRIEF REVIEWS

DNA Vaccines: Progress and Challenges[1]

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In the years following the publication of the initial in vivo demonstration of the ability of plasmid DNA to generate protective immune responses, DNA vaccines have entered into a variety of human clinical trials for vaccines against various infectious diseases and for therapies against cancer, and are in development for therapies against autoimmune diseases and allergy. They also have become a widely used laboratory tool for a variety of applications ranging from proteomics to understanding Ag presentation and cross-priming. Despite their rapid and widespread development and the commonplace usage of the term "DNA vaccines," however, the disappointing potency of the DNA vaccines in humans underscores the challenges encountered in the efforts to translate efficacy in preclinical models into clinical realities. This review will provide a brief background of DNA vaccines including the insights gained about the varied immunological mechanisms that play a role in their ability to generate immune responses. The Journal of Immunology, *2005, 175: 633–639.*

Deoxyribonucleic acid vaccines are the simplest embodiment of vaccines that, rather than consisting of the Ag itself, provide genes encoding the Ag. The development of DNA vaccines grew from efforts to generate MHC class I-restricted CTL responses by capitalizing on the understanding of different intracellular Ag-processing pathways. It had become understood that proteins synthesized in somatic cells could generate peptides that would associate with MHC class I molecules for presentation to $CD8^+$ lymphocytes with their subsequent activation. Thus, because the focus of vaccine development expanded to include cellular responses as well as Abs, means were sought to introduce proteins into the MHC class I-processing pathway.

Felgner and colleagues (1) initially showed that unformulated plasmid DNA (derived from bacteria), encoding a marker protein and using a promoter capable of functioning in mammalian cells, could be taken up by muscle cells in mice following direct i.m. injection with resultant synthesis of the encoded protein. The low amount of protein produced, the apparent lack of transfection of professional APCs by this route, and the absence of any replicative step made it surprising that i.m. immuniza-

tion of mice with plasmid DNA encoding a viral protein could generate $CD8^+$ CTL, as well as Abs (2). These CTL were potent enough to protect mice from subsequent lethal challenge with a heterosubtypic strain of influenza, i.e., a strain that was not only of a different subtype from the strain from which the gene had been cloned, but that had arisen 34 years later. Quite rapidly, a number of laboratories demonstrated the robustness of the technology using off-the-shelf vectors encoding a variety of Ags to induce either immune responses or even protection in a host of disease models (reviewed in Ref. 3).

Mechanisms of action

The initial observations led to a series of studies intended to determine how such vaccines could work (Fig. 1). These studies covered three general areas: the source of Ag presentation, the immunological properties of the DNA itself, and the role of cytokines in eliciting the immune responses.

Early studies with reporter genes showed that the method of delivery of the DNA affected the range of cell types that were transfected. Bombardment of the epidermis with plasmid coated onto gold microbeads tended to directly transfect epidermal keratinocytes and also Langerhans cells, which were shown to migrate rapidly to regional lymph nodes (Ref. 4; reviewed in Ref. 5). In this case, the source of Ag presentation and costimulatory molecules appeared straightforward, because professional APCs were transfected directly. Intramuscular injection of plasmid predominantly led to transfection of myocytes. Direct uptake of plasmid by professional APCs after i.m. injection was much more difficult to demonstrate directly and appeared to be much less frequent (6). Nonetheless, bone marrow-derived APCs were shown in studies of parental$\rightarrow F_1$ bone marrow chimeric mice to be absolutely required for the induction of MHC class 1-restricted CTLs after i.m. DNA vaccination (7–9). Furthermore, transplantation of myoblasts stably transfected with a gene encoding influenza nucleoprotein likewise gave rise to MHC class 1-restricted CTL, and in bone marrow chimera studies, the restriction element also was shown to be the MHC of the bone marrow-derived APCs and not that of the myoblasts themselves (7).

The exact mechanism by which transplanted myoblasts provide cross-priming of $CD8^+$ T cells remains to be determined.

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FIGURE 1. Gene immunization. The viral gene of interest is converted to DNA, which is inserted in a bacterial plasmid. The DNA plasmids carrying one or several genes or several different plasmids each carrying one or several genes can be administered i.m., in the skin or at the mucosa. The same gene(s) can be introduced in a viral or bacterial vector and used either as the only vaccine or as a boosting component to the first DNA vaccination. The plasmid enters the cell nucleus, where the gene initiates transcription, followed by protein production in the cytoplasm. Secreted proteins induce cytokines, T help, and Abs that will react with and eliminate virus. APCs present peptides in context of the MHC of the vaccinated individual and activate cytokines and killer cells, which in turn will lyse virus-infected cells. DNA itself or cytokines in the immune cascade activate NK cells. In the therapeutic treatment of HIV, NK cells may, although poorly, lyse cells presenting HIV foreign proteins. In prophylactic vaccination, naïve B and T cells are primed by proteins and by APC presenting peptides, respectively. In therapeutic vaccination, the Ags may provide both priming of new responses, in cases where there has been no priming as in cancer, or a boost of memory responses, i.e., in persons with a chronic infectious disease.
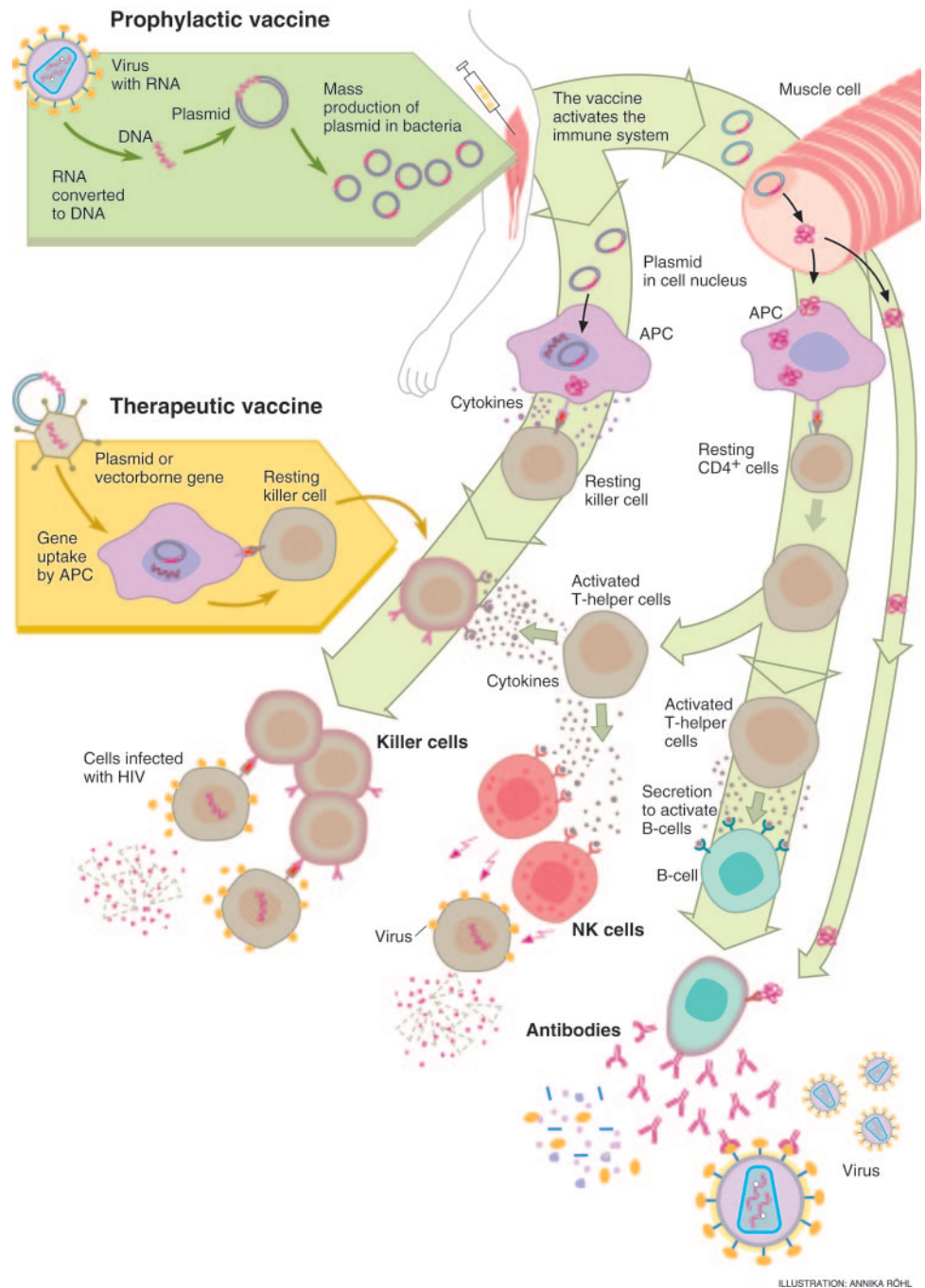
Uptake of apoptotic cells by professional APCs, transfer of processed peptide (alone or with heat-shock proteins), and transfer of protein all are feasible, and all may occur. EBV or HIV DNA present in apoptotic bodies can be transferred into APCs and viral epitopes effectively presented by MHC class I molecules (10). Proteins and peptides complexed with heat-shock proteins may be re-presented by APCs (11). Fusion of Ags with heat shock protein or herpes simplex VP22 to facilitate internalization of secreted proteins by APCs has been reported to increase the effectiveness of i.m. DNA vaccines, although this has been controversial (reviewed in Ref. 12). Influenza nucleoprotein is capable of forming virus-like particles and of being released from transfected cells with high efficiency; the literature contains multiple reports of MHC class I processing and presentation in vivo of exogenous Ags including virus-like particles (13–15). Thus, i.m. DNA immunization has the potential to result in conventional priming as well as cross-priming that may occur both at the protein and at the nucleic acid levels. Recent efforts to improve the performance of DNA vaccines (see below) are focused on improving uptake and expression of plasmid in professional APCs to take advantage of "orthodox" MHC class 1-restricted priming pathways.

Other studies focused on the immunological properties of the plasmid DNA itself. Bacterial DNA containing unmethylated CpG sequences was found to act as a polyclonal activator of B cells in vitro and as an adjuvant in vivo. Further studies showed that oligonucleotides containing unmethylated CpG sequences activated target cells through TLR9 (reviewed in Ref.

16). However, to demonstrate an adjuvant effect for protein Ags in vivo requires microgram doses of the CpG oligo, prepared with a synthetic backbone that is resistant to endonuclease degradation. Even milligram doses of plasmid may not provide a dose of CpG equivalent to that found in the synthetic CpG adjuvant. Furthermore, in one study, plasmid DNA vaccines were found to be immunogenic in both normal and TLR9$^{-/-}$ mice (17). Nonetheless, increasing the total amount of plasmid administered i.m. by mixing coding and noncoding plasmids clearly increased the subsequent immune responses in a range of animal species (3). Absent an effect on TLR9, this finding may indicate an effect of increasing quantities of plasmid on the persistence, processing, or uptake of DNA by a mechanism that is not yet defined.

Cytokines or chemokines delivered simultaneously with DNA vaccines as plasmids or proteins have proven useful for studying their roles in immune responses (18). IL-12 DNA administered together with HIV DNA plasmids has been shown to enhance Th1 immunity and to decrease the Th2 response. Concomitant administration of the cytokines IL-2 and GM-CSF augmented both B and T cell responses (19). GM-CSF has been used successfully in several studies to amplify the primary responses to HIV DNA (19, 20). Letvin and colleagues showed that monkeys immunized with DNA encoding both the HIV Ags and a form of IL-2 increased cellular immune responses and resistance to disease following challenge with a pathogenic simian-human immunodeficiency virus. Administration of recombinant exogenous IL-2 has given more mixed results (21–23). Because detailed studies of pharmacokinetics and biodistribution of the recombinant cytokines and cytokine gene products have not usually been done in concert with the studies of immune responses, it has proven difficult to translate these results across species and into different experimental models. More research is needed in this area.

Second-generation DNA vaccines

Early in the development of DNA vaccines, it became clear that maximizing the expression of the encoded Ag was critical to the induction of potent immune responses. Strong constitutive promoters, such as CMVintA, were and are generally favored over regulated or endogenous eukaryotic promoters (24). Synthetic genes are likewise generally favored over endogenous viral or bacterial sequences to allow removal of negative regulatory sequences (e.g., inhibitory elements in HIV, late genes in HPV) and adapt codon usage to more closely reflect that of eukaryotes (25). Finally, high plasmid doses, up to multiple milligrams, now are being used in animal models and clinical trials (26).

Protein modifications that facilitated cell surface expression or secretion (e.g., addition of secretion signal sequences) generally were associated with increased immunogenicity, whereas Ags retained in the cell, such as HCV E1, generally tended to be weaker immunogens (24, 27–30). Proteins that could be shed from cells as virus-like particles, for example, influenza nucleoprotein, human papillomavirus L1, and HIV p55 gag also were found to be strong immunogens in laboratory animals when used in DNA vaccines despite not being targeted to a classical secretory pathway (Ref. 31; reviewed in Ref. 3). Targeting the expressed protein to specific intracellular compartments, e.g., by ubiquitination or fusion to lysosomal-associated membrane protein, could increase presentation by MHC class I but also could result in ablation of Ab or CD4$^+$ T cell responses after

i.m. injection of plasmid (32–34). In contrast, when the DNA was given by gene gun, both of these modifications reportedly increased all categories of immune responses to proteins and minigenes, underscoring the differential effects of targeting DNA to transfect different cell types (reviewed in Ref. 12).

Structural modifications also have been used to increase the diversity of epitopes presented by a single DNA vaccine. Initially, some expression vectors were constructed using minigenes expressing a single minimal peptide epitope or multiple short peptides joined together in a "string of beads" approach. These composite sequences tended to create potentially dominant novel epitopes and, although immunogenic in mice, have been less immunogenic in human clinical trials (35). Where larger polypeptides were coexpressed with short peptides, responses to the longer protein tended to dominate, suggesting that presentation of longer or more native polypeptides may be more efficient. Recent designs favor the use of larger sequence elements, with blocks of sequence rearranged or mutated to eliminate biological activities (30, 36). These Ag designs will be reaching clinical trials in the near future. Combinations of full-length genes, and also large gene segments, mixed together on separate plasmids are being explored preclinically and clinically in HIV, HCV, and malaria (37, 38).

Formulations and targeting

Part of the initial appeal of naked DNA vaccines resulted from their ability to induce immune responses without any special formulation. However, biodistribution studies showed that the number of plasmid DNA molecules surviving to transfect target cells after i.m. injection was only a small fraction of the total DNA injected. The quest for higher immune responses led to a proliferation of different approaches for formulating DNA vaccines to protect the DNA from degradation and improve transfection efficiency. After many years of work, the field can be sorted into several general categories: transfection-facilitating lipid complexes, microparticulates, and classical adjuvants (39). Lipid complexes can include varying combinations of cationic lipids and cholesterol (40). Microparticulates include DNA adsorbed to or entrapped in biodegradable microparticles such as poly-lactide-co-glycolide or chitosan, or complexed with non-ionic block copolymers or polycations such as polyethyleneimine (39, 41, 42). Among the classical adjuvants, aluminum phosphate is noteworthy for its effectiveness and simplicity of preparation (43). Microparticulates appear to improve delivery of DNA to APCs by facilitating trafficking to local lymphoid tissue via the afferent lymph and facilitating uptake by dendritic cells (44–46). Alum phosphate does not bind DNA, and in fact, cationic alum formulations that do bind DNA generally are not immunogenic. Alum phosphate is thought to act by recruiting APCs to the site of the i.m. injection, where a proportion of muscle cells would be expressing the Ag encoded by the DNA vaccine. Future potential to improve formulations may be facilitated by redesign of the plasmid itself. Minimal expression elements consisting of linear DNA comprising a promoter and gene, blocked at both ends with synthetic hairpin oligonucleotides to prevent degradation, were shown to be as potent as closed circle plasmids (47). Incorporation of a synthetic element has the potential to greatly facilitate the addition of different ligands and targeting moieties.

Tissue damage or irritation leading to regeneration of myocytes may be important in enhancing immune responses to

DNA vaccines. Early studies suggested that agents that caused muscle necrosis, such as cardiotoxin or bupivicaine, increased immune responses to DNA vaccines administered while the muscle was regenerating (48, 49). This was thought to be due to increased protein expression in regenerating myocytes, but recruitment of APCs by inflammatory responses also may play some role. Later, hydrostatic damage caused by injection of relatively larger volumes of fluid was implicated as a mechanism for the relatively high immunogenicity of plasmid DNA vaccines when given i.m. in mice compared with larger animals (45). The polymer and adjuvant formulations currently under evaluation also may work in part through a local inflammatory component. Most recently, electroporation, which has the potential both to force DNA into cells and to create damage to adjacent muscle cells, has emerged as the most potent method for delivering DNA i.m. (50). However, electroporation also has been found to result in increased levels of integration of plasmid into the genome of host cells (51).

An alternative site of administration of plasmid DNA and design of plasmid targets B cells as APCs. Intravenous or intrasplenic injection of plasmid expressing an Ag fused to the Ig H chain and controlled by a B cell-specific promoter can efficiently transfect B cells, which then can serve as APCs to both $CD4^+$ and $CD8^+$ T cells (52). Injection of plasmid-transfected B cells can elicit $CD4^+$ and $CD8^+$ T cell responses even in $RelB^{-/-}$ mice lacking bone marrow-derived dendritic cells. Immunization with transfected B cells can provide protective immunity in an influenza virus challenge model in mice.

Epidermal immunization by gene gun tends to target epidermal Langerhans cells, potentially favoring direct presentation to $CD4^+$ and $CD8^+$ T cells over cross-priming (5, 53), whereas jet injection was used for direct targeting of mucosal cells in humans (54). The gene gun serves as a useful platform to study the effects of protein trafficking within and among APCs on immune responses. Prolonging the life span of transfected APCs with concurrent administration of antiapoptotic factors such as RNA inhibitors of Bak or Bax can substantially increase $CD8^+$ T cell responses (55). Fusion of Ags to C3d (56) or CTLA4 (57) can increase Ab responses to DNA vaccines delivered by this method.

Mixed modality vaccines

During the evolution of DNA vaccines, it became apparent that, although DNA alone could sometimes yield Ab responses comparable with unadjuvanted protein immunogens, e.g., influenza HA, for sheer magnitude of Ab titers DNA alone could not equal a potent protein plus adjuvant. Therefore, various approaches were tested that sought to take advantage of combining the ability of DNA to prime Ab responses with the ability of recombinant proteins to boost them. DNA-protein prime-boost regimens have been studied extensively in HIV (58), providing partial protection from simian-human immunodeficiency virus challenge (59) and also have been studied in anthrax (60), tuberculosis (61), and in transmission-blocking vaccines for both vivax and falciparium malaria (62).

Both malaria and also HIV have been used to test immunization regimens comprising a DNA prime and a viral vector boost. Malaria is acquired at a young age, and for complete protection, it likewise may be necessary to immunize at a very early age. It was shown experimentally that 7-day-old mice with maternal Abs could acquire $CD8^+$ related protective immunity with a circumsporozoite protein DNA vaccine together with GM-CSF, followed by boosting with the same Ag in a poxvirus vector at 1 mo of age (63). In contrast, a malaria DNA vaccine prime with a modified vaccinia Ankara boost encoding sporozoite and liver stage epitopes was weakly immunogenic in healthy, malaria-naive, U.K. adults, and was insufficient to protect against malaria challenge by infected mosquitoes (64). The subjects had a statistically significant (3-day) delay in developing parasitemia upon heterologous challenge with a chloroquine-sensitive strain of malaria, 3D7, but all became infected (65).

DNA primes with viral vector boosts also may enhance protective responses to HIV env, although in most such studies the anti-env responses induced by this approach did not include neutralizing Abs (66). Studies of CTL precursor frequencies and circulating tetramer-positive and IFN-γ-producing $CD8^+$ CTL in response to DNA vaccines also showed that, although DNA alone could generate a primed $CD8^+$ T cell population, viral vectors were more effective at inducing expanded populations of circulating effector CTL. Again, the logic of combining the two modalities has led to the exploitation of DNA vaccine priming followed by boosting with various viral and other gene delivery vectors to expand the effector $CD8^+$ T cell populations (67). In human clinical trials, immune responses to DNA followed by modified vaccinia Ankara appeared similar to viral vector immunization alone (35). Where multiple immunizations with the viral vector alone were included in the trials, this approach could be equally as potent as the DNA prime-boost. However, multiple immunizations with the same viral vector can results in diminishing responses to subsequent immunizations as immunity to the viral vector develops. Therefore, DNA prime-vector boost approaches still may prove useful.

Therapeutic DNA vaccines

By therapeutic immunization, it is possible to get an immediate feedback on which immunogens may have a clinical impact. The assumption is that similar types of immune responses as in prophylaxis will be needed, but this is far from proven. Recruitment of $CD4^+$ helper cell activity appears to be the most important task in immunotherapy of HIV/SIV infection, because memory $CD4^+$ cells are the ones primarily infected and deleted during the course of the infection. The effects of depleting $CD8^+$ cytotoxic cells in retrovirus infection were demonstrated in primates with SIV infection, where the ablation of the virus-induced $CD8^+$ T cell response led to a further increased viral load (68). In cancer, both MHC class 1-restricted $CD8^+$ T cell responses against epitope peptides expressed on tumor cells and Ab responses against Ig expressed on B cell lymphomas have been demonstrated.

A number of studies suggest a potential for clinical benefit from therapeutic vaccination in HIV. During antiretroviral treatment, acutely SIV-infected macaques were immunized with a vaccinia construct NYVAC encoding SIV Gag, Pol, and Env proteins. Vaccination elicited anti-SIV specific $CD4^+$ T cell responses in animals with a low viral load. Vaccine-induced $CD8^+$ T cell responses were elicited only in vaccinated animals receiving antiretroviral treatment. After structured therapy interruption, animals in the vaccinated group had transient viremia that was quickly suppressed. Rhesus macaques with SIV-mac251 infection were treated with antiretrovirals and

vaccinated with or without IL-2 with a poxvirus vector expressing the SIVmac structural and regulatory genes. Following antiretroviral treatment interruption, the viral set point was significantly lower in vaccinated than in control macaques (69). Topical DNA-based immunization in macaques was designed to express most of the regulatory and structural genes in dendritic cells. Immunological, virological, and clinical benefit for SIV-infected macaques on highly active antiretroviral therapy including hydroxyurea, enhanced viral control following treatment interruptions (70). These results point to a role for therapeutic immunization in protecting against viral rebound upon withdrawal of antiretroviral treatment.

Therapeutic clinical studies have been performed with DNA vaccines carrying gp160 or with regulatory genes. In dose escalation studies, *env* and *rev* genes as well as regulatory genes appeared safe in HIV-1-infected individuals (71, 72). It was possible to show the induction of substantial Ag-specific CD4$^+$ Th cells by DNA vaccination with regulatory genes *rev*, *tat*, and *nef* in patients and decreased viral loads with DNA representing the *env* and *pol* genes (73). New CD8$^+$ cells were reactive with HIV-infected cells (74). It is thus possible to induce relevant T cell reactivities in infected patients, despite their immunosuppressed state. Such responses also could be obtained with highly active antiretroviral therapy. However, the duration of immune responses were short, and various adjuvants to genetic immunization appear necessary. Lu et al. (75) treated HIV-infected patients with their own dendritic cells, cultured and matured ex vivo, pulsed with the patients' own inactivated viral strain and then given back to the patient. In half of these individuals, virological control was obtained for several months without antiviral chemotherapy. This lends hope to similar procedures using more easily available DNA constructs representing the subtype of the patient.

The ability to screen Ags rapidly, design specific types of expression constructs, and combine both in vivo and ex vivo approaches has made immunotherapy of cancer a worthwhile field for the study of both DNA and RNA vaccines. Patient-specific DNA vaccines for therapy of B cell lymphomas and multiple myelomas based on single-chain Fv's derived from individual patients' cancers were shown to be effective in animal models and are being studied in clinical trials (reviewed in Ref. 76).

Results with dendritic cells modified ex vivo exemplify what could be accomplished with DNA vaccines provided APCs could be targeted efficiently. Dendritic cells pulsed ex vivo with mRNA extracted from human tumors presented tumor Ags, and induced CD8$^+$ T cell responses when used as immunogens (reviewed in Ref. 77). This approach induced relatively weak CD4$^+$ T cell responses, although these could be improved by reducing levels of invariant chain expression. Human subjects demonstrated tumor-specific CD8$^+$ T cell ELISPOT responses against prostate-specific Ag and telomerase, respectively, after immunization with DCs pulsed with prostate-specific Ag or renal cell carcinoma mRNA. Some clinical studies report modulations of disease associated with the demonstration of tumor-specific immune responses. Larger studies conducted in earlier-stage cancer patients will determine whether these immune responses confer substantial clinical benefit.

The future

After some 15 years of experimentation, DNA vaccines have become well established as a research tool in animal models.

However, DNA vaccines so far have shown low immunogenicity when tested alone in human clinical trials. A significant effort has been put forward to identify methods of enhancing the immune response to plasmid DNA to enable its general use as a method of immunization in humans. So far, the improvements that have been seen are incremental, but this work is both continuing and making progress. The knowledge that is being gained in the pursuit of more effective DNA vaccines also is enriching the development of "conventional" vaccine approaches, and this understanding may well facilitate the invention of effective new vaccines for cancer and infectious diseases.

Acknowledgments

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