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DNA Vaccines: A Ray of Hope*

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ABSTRACT: Vaccines represent the most commonly employed immunologic intervention in medicine today. DNA vaccination or genetic immunization is a rapidly developing technology that offers new approaches for the prevention of disease. This method of vaccination provides a stable and long-lived source of the protein vaccine, and it is a simple, robust, and effective means of eliciting both antibody- and cell-mediated immune responses. Furthermore, DNA vaccines have a number of potential advantages such as they can address several diseases in one vaccine, they are cheap and easy to produce and have no special cold storage requirement because they are extremely stable. It has proven to be a generally applicable technology in various preclinical animal models of infectious and noninfectious diseases, and several DNA vaccines have now entered phase I/II, human clinical trials. There are several hurdles that need to be overcome on the road to the use of DNA vaccines widely. These include the technical challenges of improving delivery and/or potency so that low doses of DNA can achieve the efficacy of conventional vaccines.

KEY WORDS: vaccine, disease, immunization, antibodies, cytotoxic T lymphocytes, B lymphocytes.

I. INTRODUCTION

Vaccines have probably prevented more disease than any other modern medical intervention. Nucleic acids or in other words 'naked DNA' is rightly being considered as the "third generation of vaccines". From the study of model systems in animals, there seems little doubt that DNA vaccines are a distinct possibility for the future of infectious disease prevention in human populations. DNA-based immunization also offers an extremely powerful tool for molecular immunologists to study the immune system and to develop new vaccines and other immunotherapeutic approaches. Vaccines made of DNA are being developed as a form of gene therapy that uses the patients own cellular machinery to make foreign proteins that stimulate an immune response. Vaccines have been in use for over 100 years. First-generation and the oldest vaccines were developed by Louis Pasteur and his successors by using attenuated and killed forms of microorganisms, the second was

the use of defined natural or recombinant components of whole organisms. Both these formulations contain protein or proteinaceous substances. Even if polysaccharides or small organic molecules were used, they were coupled to carrier proteins. Protein-based immunization thus is at the basis of the well-established public health measure of vaccination (Dixon, 1995).

In 1990, Wolff et al. demonstrated direct gene transfer into mouse muscle *in vivo* with reporter constructs (Wolff et al., 1990). Transfection with DNA *in vivo* was also reported by Williams et al. (1991) using the biolistic approach. Using a number of reporter genes, these investigators have demonstrated that injection of either purified RNA or DNA could result in the expression of the appropriate enzyme activity within the skeletal muscle. When 100 μg of purified DNA consisting of the reporter gene linked to a Rous Sarcoma Virus Long Terminal Repeat was injected, episomal plasmid DNA could be detected by Southern blot 30 days later, and enzyme activity persisted for at least 60 days after injection (Wolff et al., 1990). The stability of this episomal form of DNA is presumably due to the low proliferative state of myocytes *in vivo*.

Injection of plasmid DNA as a vaccine was first demonstrated to be effective using influenza as a model, where it was shown that DNA encoding nucleoprotein (NP) induced cytotoxic T lymphocytes (CTLs) and cross-strain protection in mice (Ulmer et al., 1993). The effectiveness of DNA vaccines against viruses, parasites, and cancer cells has been demonstrated in numerous animal models. Numerous studies have already shown that immunization of experimental animals with plasmid DNA encoding antigens from a wide spectrum of bacteria, viruses, protozoa, and cancers leads to protective humoral and cell-mediated immunity. This type of immunization has also modulated the effects of autoimmune and allergic diseases and provided some hope for the control of cancer.

This new approach comes as an aid for the prevention of infectious diseases for which the conventional vaccines have failed. The ease with which DNA may be manipulated means that vaccines can be custom designed to meet many needs.

II. METHODOLOGY AND ADVANTAGES

A. Properties of Vectors

DNA-mediated immunization involves the direct introduction of a plasmid DNA encoding an antigenic protein that is then expressed within cells of the organism (Ulmer et al., 1993; Wang et al., 1993a; Davis et al., 1993; Fynan et al., 1993). The *in vivo* expression of that protein using expression systems leads to the induction of antigen-specific immune responses. Because the genes transferred by the plasmids require the host cellular machinery to be expressed, DNA-based immunization most resembles a virus infection.

Plasmid DNA constructs used for gene vaccination are similar to those used for delivery of reporter or therapeutic genes. Basically, it has three major units (1) a plasmid backbone that delivers adjuvant, mitogenic activity and convenient cloning sites for the insertion of genes of interest, (2) a transcription unit comprising a strong viral promoter/enhancer sequences, antigen cDNA and polyadenylation addition se-

quences, which together direct protein synthesis (Davis, 1997), (3) an antibiotic-resistance gene to confer antibiotic-selected growth in *E. coli*. These selection markers ensure that only plasmid-containing bacteria will propogate during culture.

The most frequently used regulatory elements for DNA vaccines are those known to mediate high levels of gene expression under mammalian cell culture conditions. These include the human cytomegalovirus immediate/early promoter (pCMVIE) (Boshart et al., 1985), the Rous sarcoma virus (RSV) LTR (Gorman et al., 1982), and the SV40 early promoter (Moreau et al., 1981) used in conjunction with the SV40 or bovine growth hormone 3′-untranslated region (BGH-3′ UTR) transcript termination/polyadenylation sequences (Pfarr et al., 1986). Most vaccination vectors also contain an intron, because expression of many mammalian genes may be dependent on, or may be increased by, the inclusion of an intron. The best plasmid for eliciting immune responses utilizes the CMVIE promoter and intron A (Chapman et al., 1991) with the BGH 3′-UTR. The plasmid is grown in bacteria, purified (free of contaminants mainly endotoxin), dissolved in saline solution, and then simply injected into the host. When a DNA vaccine plasmid is taken up by host cells, the DNA enters the nucleus where it is transcribed into RNA, the mRNA is transported into the cytoplasm and it is translated into protein (Figure 1). Due to the lack of an origin of replication that is functional in eukaryotic cells, these plasmids neither replicate in the mammalian host nor integrate within the chromosomal DNA of the animal.

The technology has its real and potential advantages over conventional approaches to vaccination. The DNA vaccines do not require cultivation of dangerous infectious agents. There is no risk of an attenuated vaccine strain mutating back to a virulent form. Any variant of a protein can be tested through application of simple recombinant DNA methods. It is possible to modify the sequence of an antigenic protein or to add heterologous epitopes by simple manipulations performed on the plasmid DNA. This can be followed by direct assessment of the changed immunogenicity of the protein after direct injection of the plasmid DNA. Using this methodology it is easier to understand the structure-function relationships between the protein and the immune response to the antigen. When compared with conventional vaccines, the DNA is very stable and is resistant to extremes of temperature, thus storage, transport, and distribution of DNA-based vaccines would be facilitated. DNA vaccines may be safer than certain live virus vaccines, specially in immunocompromized patients. They may be constructed in a way so that genes from several different pathogens are included on the same plasmid, thus potentially decreasing the number of vaccinations required for children. If DNA vaccines come into widespread use for public health applications, vaccines for many diseases could be produced rapidly because in the end the product is simply a DNA plasmid. If new infectious diseases appear in the future, as they surely will, perhaps these new tools will be used to combat them more effectively.

DNA vaccines elicit antibodies and CD4+ T cell responses in animals, but at the immunological level their major advantage has been their capacity to induce antigen-specific CD8+ T cell responses, including CTLs, which is a major mechanism of protection against intracellular pathogens.

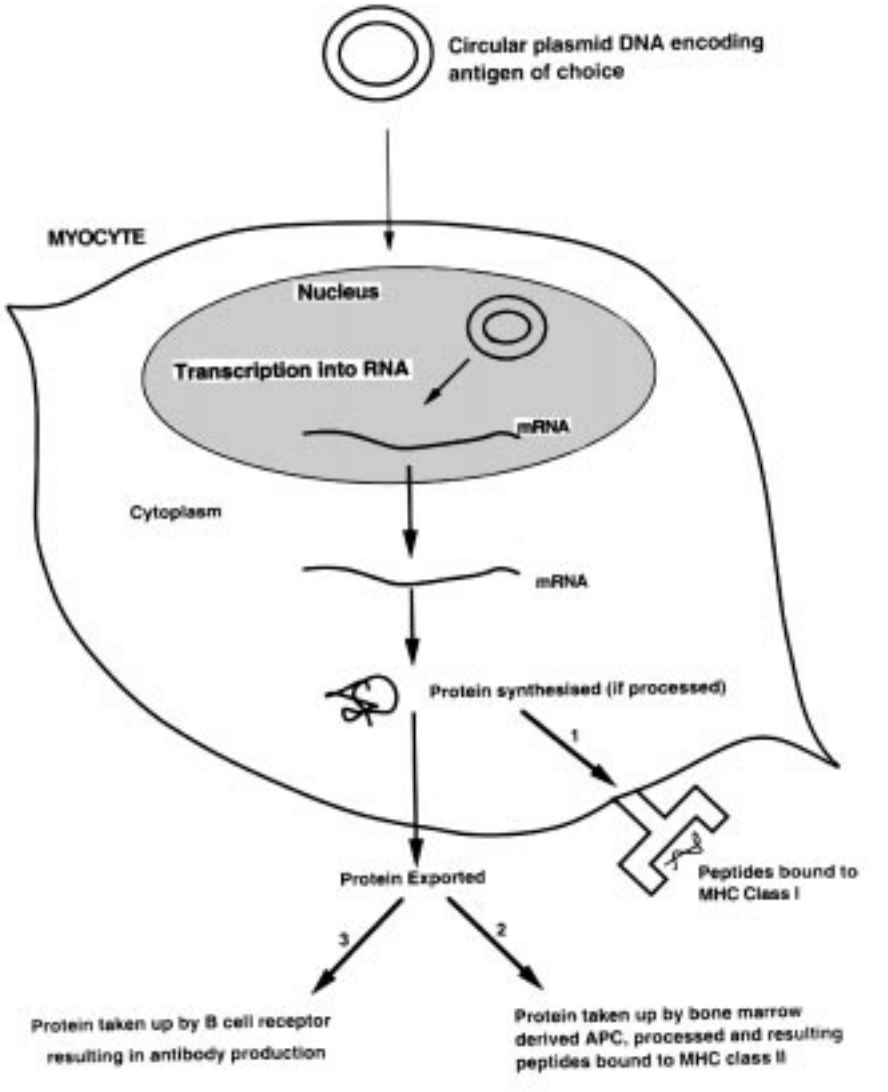

FIGURE 1. Schematic representation of steps required for immune response to DNA-encoded antigens. Circular plasmid DNA enters the myocyte and is transported into the nucleus. The DNA is transcribed into RNA, and the resulting RNA is translocated to the cytoplasm and translated, producing the protein antigen. The antigen is then presented to the immune system by a number of mechanisms. (1) The antigen is processed intracellularly and the resulting peptides are presented on the cell surface bound to major histocompatibility (MHC) class I molecules, where they serve in selection of cytotoxic T lymphocytes. (2) Protein antigen is transferred to bone marrow-derived antigen-presenting cell (APC), degraded and its peptides are presented on the cell surface bound to MHC class II molecules, where they stimulate T-helper cells. (3) The exported protein is taken up by B cells, where it can serve in selection of B cells.

B. DNA Vaccine Delivery

Direct intramuscular injection of plasmid DNA has been relatively widely used to induce immune responses. It has been found that simple saline solutions appear to be quite reasonable carriers often resulting in transfection of between 1 to 5% of myofibrils in the vicinity of the injection site in the case of intramuscular administration. DNA preparations that are typically used to transfect cells *in vitro*, such as calcium phosphate precipitates or liposomal preparations, do not appear to enhance the efficiency of *in vivo* transfer (Wolff et al., 1990; Manthorpe et al., 1993). It has been suggested that muscle that was made edematous by intramuscular injection of hypertonic 25% sucrose solution 20 min prior to administration of DNA in mice gave increased reporter gene expression (Davis et al., 1993), but such an approach may not be feasible for use in humans as it would be in laboratory animals.

It has been reported in few cases that coinjection of toxic agents intended to cause muscle necrosis and repair either prior to or concurrently with injection of DNA can increase gene transfer and expression. These include local anesthetics such as bupivicaine and myotoxins such as cardiotoxin (Davis et al., 1993; Vitadello et al., 1994; Wang et al., 1993a). The improved uptake of DNA in regenerating muscles composed of immature fibers also suggests that newborn mammalian muscle may also take up DNA more effectively than adult muscle.

Although the greatest experience is with injection into skeletal muscle, other tissues have also been shown to express gene products after DNA injection, including cardiac muscle, liver, and dermis (Raz et al., 1994). The technology in wide use for intradermal DNA injections is the gene gun. The gene gun takes advantage of the ability of ballistically accelerated microscopic gold particles to penetrate cell membranes without killing the cell. By mixing these gold particles with purified DNA in the presence of a polycation such as spermidine, the nucleic acid becomes coated onto the gold particles. These DNA-coated gold particles are loaded into the gene gun and the end is abutted to a shaved area of skin. Discharge of the gene gun results in penetration of the dermis between 0.1 to 5 mm, depending on the chosen projectile force. This form of DNA injection has been shown to transduce cells in both the dermis and epidermis.

The introduction of DNA can be accomplished by simple intramuscular or interdermal injections using needles, as well as by propelling DNA-coated gold particles to various tissues, preferentially the dermis (Fynan et al., 1993). While intramuscular injection of DNA appeared to generate the best response, intravenous, intranasal, intradermal, and subcutaneous immunizations also induced significant protection. When ballistic inoculation of DNA-coated gold particles was evaluated, equivalent levels of protection were achieved using 2 to 3 logs lower total DNA dose (Fynan et al., 1993).

In a recent study different routes were used to deliver the HBsAg-encoding plasmid DNA or the recombinant HbsAg particles: different doses of expression plasmid DNA (10 μg or 100 μg per mouse) or of recombinant HBsAg lipoprotein particles were injected into different muscles, into subcutaneous tissue (at the base of the tail), into the peritoneal cavity, or intravenously (into the tail vein). The results show that the intramuscular and subcutaneous but not the intravenous and intraperitoneal injection of 'naked DNA' efficiently and reliably primes

cellular and humoral immune responses. In contrast, recombinant HBsAg particles injected by all four routes (without adjuvants) efficiently primed specific humoral and CTL responses. These data demonstrate that the choice of routes to deliver 'naked' plasmid DNA for obtaining efficaceous immunogenicity of the expressed antigen is restricted (Bohm et al., 1998).

Three different vaccination sites were compared for efficiency of immunization with naked DNA (Forg et al., 1998). Using the bacterial lac Z gene as a model, all three sites of the mouse (skeletal muscle, dermis of the abdominal skin or of the ear pinna) could express the gene product beta-galactosidase but varied in the expression time with muscle tissue showing the longest expression. Expression time, however, did not correlate with immune response intensity. The ear pinna was the most effective and muscle the least effective priming site for specific humoral and cytotoxic T-cell-mediated immune responses. The pinna was also found to be a priviledged site for induction of antitumor responses and for genetic immunization (Forg et al., 1998).

DNA vaccines appear to act as their own adjuvant owing to the presence of immunostimulatory DNA sequences that have in common a cytosine preceding a guanosine (CpG) motif (Klinman et al., 1996; Klinman et al., 1997; Sato et al., 1996; Krieg et al., 1995). These are present at a higher frequency (1/16) bases) in bacterial DNA than in vertebrate DNA (1/50 to 1/60 bases). The frequency of methylated cytosines in CpG base pairs from eukaryotic DNA is 70 to 90%, whereas only 5% of the cytosines in CpG base pairs from prokaryotic DNA are methylated.

It has been shown recently that bacterial DNA induces the production of cytokines involved in both cell-mediated and humoral immune responses (Klinman et al., 1996). The CpG DNA can enhance the specific immunity in mice immunized with recombinant hepatitis B surface antigen. The possible mechanisms for the enhancing effects are the ability of CpG oligonucleotides to induce costimulatory molecule expression *in vitro* and *in vivo* and to drive B cell isotype shifting *in vitro* (Davis et al., 1998). A recent study has shown that the immunogenicity of a DNA vaccine was significantly reduced by methylating it's CpG motifs and was significantly increased by co-administering exogenous CpG-containing DNA (Klinman et al., 1997).

The effect of immunostimulatory CpG DNA motifs was examined on a DNA vaccine for Dengue type 2 in a recent study. The results showed that CpG motifs present in pUC19 significantly improved the antibody response to a suboptimal dose of 3.1 μg of the DNA vaccine and a higher percentage of mice survived the challenge. These studies illustrate that immunostimulatory CpG DNA motifs can be used to lower the minimum dose required to produce an antibody response (Porter et al., 1998). Recently, it has been reported that when mice were systematically primed with influenza virus mixed with CpG oligonucleotide, the virus-specific antibody titres were about seven times higher than in mice immunized without CpG. These studies further show that stimulatory CpG oligonucleotides are promising new immune enhancers for vaccination application (Moldoveanu et al., 1998).

It has been shown in several instances that DNA-mediated immunization to a

single antigen will provide protection against infection by a pathogen (Ulmer et al., 1993).

C. Effect of Co-Stimulatory Molecules

Following intramuscular inoculation of plasmid DNA, antigen is expressed at significant levels (Wolff et al., 1990). Muscle cells express or can be induced to express adhesion molecules, cytokines, and MHC class I and II molecules, but they do not seem to express the costimulatory molecules required for efficient antigen presentation (Goebels et al., 1992; Hohfield et al., 1994; Michaelis et al., 1993). The CD80 (B7-1) and CD86 (B7-2) molecules interact with the CD28/CTLA4 molecules on T cells, providing an important second signal in addition to ligation of the T-cell receptor to the MHC peptide complex (Linsley et al., 1990). It has been shown recently that CD80 or CD86 coimmunization with HIV-1 envelope or gag/pol DNA vaccine cassettes had a minimal effect on the level of antigen-specific humoral responses; however, coinjection with CD86 resulted in a significant increase in the level of antigen-specific T-helper cell proliferation (Kim et al., 1997). In another study, it has been reported that co-administration of CD80 and CD86 expression cassettes along with HIV-I immunogen resulted in a dramatic increase in MHC class I-restricted and CD8+ T-cell-dependent CTL responses in both mice and chimpanzees (Kim et al., 1998a; Kim et al., 1998b). Interaction of CD40 with its ligand (pCD40L) can induce CD40 bearing antigen-presenting cells to express immune stimulatory accessory molecules that facilitate immune recognition. It was observed that coinjection of pCD40L in BALB/c mice enhanced the antibody response to beta-galactosidase induced by intramuscular or intradermal injection of a plasmid DNA vector encoding beta-galactosidase (placZ). The CTL response specific for P815 cells transfected with placZ were also enhanced by coinjection of pCD40L. This study indicates that pCD40L can serve as a genetic adjuvant capable of augmenting humoral and cellular immune responses to antigens encoded by plasmid DNA expression vectors (Mendoza et al., 1997).

Mice vaccinated with a DNA plasmid encoding a polyepitope or polytope protein that contained multiple contiguous minimal murine CTL epitopes made MHC-restricted CTL responses to each of the epitopes and protective CTL were demonstrated in recombinant vaccinia virus, influenza virus, and tumor challenge models. These responses could be enhanced by co-delivering a gene for granulocyte-macrophage CSF and appeared to be induced in the absence of CD4+ T-cell-mediated help (Thomson et al., 1998).

The use of cytokines as immunological adjuvants can enhance various immune responses when administered during the immune response to a particular antigen. When interleukin (IL-2) is administered as multiple injections after the antigen, it enhances the protection against challenge with the infectious agent (Anderson et al., 1987, Weinberg et al., 1988). In a recent study, it has been shown that coexpression of IL-2 and the HBV envelope protein within the same plasmid vector results in at least a 100-fold increase in its ability to induce humoral and cellular immune responses to HBsAg (Chow et al., 1997).

III. USE OF DNA VACCINES IN VARIOUS DISEASE MODELS

The DNA vaccine has been tried in a number of viral diseases such as hepatitis B, C, rabies, herpes, AIDS, bacterial such as tuberculosis and parasitic diseases such as malaria. In addition to these, it has also been used in certain types of cancers.

A. Viral Diseases

1. Hepatitis B

The most studied DNA vaccine so far is the vaccine against hepatitis B. The hepatitis B virus (HBV) is a noncytopathic enveloped virus that causes acute and chronic necroinflammatory liver disease and hepatocellular carcinoma. The first vaccine against HBV to be applied to humans involved injection of HBsAg particles that had been purified from the plasma of chronic carriers (Maupas et al., 1976). Currently used vaccines are recombinant protein vaccines derived from HBsAg-producing yeast cells (Valenzuela et al., 1982) or Chinese hamster ovary cells that contain the S or the S plus pre-S2 genes, respectively (Michel et al., 1984). Despite their high efficacy, subunit HBV vaccines are not used widely in developing areas of the world where they are most needed, owing to the high cost of production.

Since the discovery of this new technology, a number of studies have been carried out using hepatitis B surface antigen gene as a vaccine. The first study showed that direct intramuscular injection of a plasmid vector encoding the HBsAg gave rise to secretion of the viral surface protein into the circulation, which led to the production of antibodies (Davis et al., 1993). In a later study it was shown that intramuscular injection of plasmid DNA expression vectors encoding the three envelope proteins of hepatitis B virus induced humoral responses in mice specific to several antigenic determinants of the viral envelope (Michel et al., 1995). A single injection of DNA was able to induce high levels of antibodies to HbsAg in mice that were sustained for at least 6 months. The first antibodies appeared within 1 to 2 weeks after injection of DNA and included antibodies of the IgM isotype. Over the next few weeks, an IgM to IgG class switch occurred, indicating helper T-lymphocyte activity (Michel et al., 1995).

The intramuscular injection of mice with the expression vectors containing all or part of the hepatitis B virus gene induces a strong humoral response that is sustained for up to 74 weeks without boost. Although the antibody levels do not diminish significantly over time, they can be further increased severalfold by boosting with a second injection of DNA or an injection of recombinant HBsAg protein (Davis et al., 1996a). Genetic vaccination of two chimpanzees against HBV was carried out, because the chimpanzee is the only animal model for HBV infection. The animal injected with 2 mg DNA attained >100 milliinternational units/ml (mIU) of anti-HBs antibody after one injection and 14,000 mIU after four injections. A smaller dose (400 μg) induced lower and transient titers, but a strong anamnestic response occurred 1 year later (Davis et al., 1996b).

The effect of DNA-mediated immunization for immunotherapy of chronic HBV carriers using the HBsAg transgenic mouse as a model was also examined.

It has been shown that the immune response induced after a single intramuscular injection of DNA resulted in complete clearance of circulating HbsAg and in the long-term control of transgene expression in hepatocytes (Mancini et al., 1996). The nature of immune responses to hepatitis B virus DNA vaccines can be modulated by co-administration of various cytokine genes. While GM-CSF, IL-2, IL-4, and IL-12 increased both IgM and IgG antibody titers, IFN-γ was unique in that it increased IgM but suppressed IgG antibody titers (Chow et al., 1998). These results demonstrate that application of a cytokine gene in a DNA vaccine formulation can influence the differentiation of Th cells as well as the nature of an immune response and thus may provide a strategy to improve its prophylactic and therapeutic efficacy.

Recently, a polyvalent vaccine against hepatitis B surface and core antigens has been constructed using a dicistronic expression plasmid. Intramuscular injection of this plasmid DNA into mice elicited polyvalent humoral and cytotoxic T lymphocyte responses to HBsAg and HBcAg (Wild et al., 1998).

Recently, intradermal and intramuscular routes of delivery of a DNA vaccine against the hepatitis B virus in Aotus monkeys has been compared. Monkeys were immunized with 400 μg of a DNA vaccine that encoded HBsAg. The primary humoral response induced by intramuscular delivery of the DNA vaccine was very poor. On the other hand, intradermal delivery of the DNA vaccine in saline induced a potent antibody response that was further augmented six-fold by the addition of *E. coli* DNA. Therefore, for immunization of primates with DNA vaccines, the intradermal route appears to be better (Gramzinski et al., 1998). All the studies described have shown strong antibody and CTL responses but still no data are available on protection from infection after DNA immunization.

2. Hepatitis C

Exposure to hepatitis C virus is associated with a high prevalence of persistent viral infection and the development of chronic liver disease and hepatocellular carcinoma. Recovery from acute infection may depend on the generation of broad-based cellular immune responses to viral structural and nonstructural proteins.

It has been shown in a number of studies that injection of a DNA construct for the hepatitis C virus core protein generates core-specific antibody responses, lymphoproliferative responses, and cytotoxic T-lymphocyte activity (Major et al., 1995; Lagging et al., 1995; Tokushige et al., 1996; Inchauspe et al., 1997). Plasmids expressing different domains of the hepatitis C virus envelope E2 glycoprotein were injected in BALB/c mice intramuscularly or intraepidermally via a gene gun, and induced humoral immune responses were evaluated. The intraepidermal injections resulted in higher seroconversion rates and antibody titers than did the intramuscular injections. This study demonstrated that different routes of injection of HCV E2 plasmids can result in quantitatively and qualitatively different humoral immune responses (Nakano et al., 1997).

For enhancing the immunogenicity of nonsecreted viral structural proteins at both B and T cell levels, several chimeric HBV-HCV constructs were prepared that were designed to express and secrete the HCV core protein along with various regions of

the hepatitis B envelope protein. These chimeric proteins were capable of generating CD4+ inflammatory T cell and CD8+ CTL activity against the HBV as well as HCV components of the fusion proteins. The proliferative activity of T cells as well as the humoral immune responses to HCV core protein were substantially enhanced by some chimeric fusion protein when compared with the HCV core protein alone (Geissler et al., 1998). Recently, it was observed that the HCV nonstructural proteins NS3, NS4, and NS5 are particularly good immunogens and produced cellular immune responses when adminstered in BALB/c mice as a DNA construct (Encke et al., 1998).

Inoculation of Buffalo rats with plasmids carrying the hepatitis C virus envelope genes induced both antibody and lymphoproliferative responses.

These responses were greatly enhanced by codelivery of the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene (Lee et al., 1998a, Lee et al., 1998b). In another study using hepatitis C virus structural proteins (nucleocapsid and envelope E2) for immunization of mice, a predominant Th1 response was induced with the production of IgG2a antibodies and CTLs against HCV determinants (Inchauspe et al., 1998).

3. Influenza

In addition to hepatitis B and C, the DNA vaccine approach has been tried in a number of other viral infections. The intramuscular injection of a DNA expression vector encoding a conserved, internal protein of influenza A resulted in the generation of significant protective immunity against subsequent viral challenge. Primary cytotoxic T lymphocytes and NP-specific antibodies were produced. The protection was against a virulent strain of influenza A that was heterologous to the strain from which the DNA was cloned (Ulmer et al., 1993).

4. Rabies

In case of rabies virus, it has been shown that when a plasmid vector containing the full-length rabies virus glycoprotein (G protein) under the control of SV40 or cytomegalovirus promoter is inoculated into mice, a specific B- and T-cell-mediated immune response is generated, resulting in protection against challenge with a virulent strain of the virus (Xiang et al., 1995). Monkeys inoculated with DNA encoding the glycoprotein of rabies virus survived the virus challenge (Lodmell et al., 1998), demonstrating DNA immunization-mediated protective immunity in non-human primates against lethal challenge with a human viral pathogen of the central nervous system.

5. Herpes

Immunization with naked plasmid DNA encoding a major glycoprotein of herpes simplex virus glycoprotein B provided protection against viral challenge. On the basis of adoptive transfer studies and measurements of T cell functions *in vitro*, the protection appeared to be mediated by CD4+ T cells (Manickan et al., 1995).

6. Myocarditis

Coxsackievirus B3 (CVB3), a member of the picornavirus group, along with other

enteroviruses, appears to be associated with about 50% of acute myocarditis cases and approximately 25% of dilated cardiomyopathy cases in humans (Kandolf et al., 1989). It was shown recently that plasmids encoding the structural proteins of CVB3 when injected intramuscularly in mice, resulted in only low levels of virus-specific antibodies. However, DNA vaccination with the major structural protein VP1 protected about three-fourths of the mice from a lethal challenge with CVB3 (Henke et al., 1998).

7. AIDS

The potential utility of plasmid DNA as a component of an AIDS vaccine is currently an area of active investigation. While the mechanisms for protection are not fully understood, the growing consensus is that a strong CTL response as well as a neutralizing antibody response will be necessary to prevent infection with HIV and its progression to disease. It has been shown that DNA vaccines can generate HIV-specific and SIV-specific CTLs, Th cells, and Abs in mice and non-human primates (Wang et al., 1993a; Wang et al., 1993b; Shiver et al., 1995; Wang et al., 1995; Shiver et al., 1996; Yasutomi et al., 1996; Lekutis et al., 1997).

Recently, the immunogenicity and efficacy of an HIV-1 DNA vaccine encoding env, rev, and gag/pol in a chimpanzee model system has been analyzed. The immunized animals developed specific cellular and humoral immune responses, and when challenged with a heterologous strain of HIV-1 were found to be protected from infection (Boyer et al., 1997). The efficiency of genetic immunization with hepatitis B virus envelope-based vectors fused with DNA encoding the V3 loop of HIV was tested in mice and rhesus macaques, and this administration was found to induce strong humoral and cytotoxic responses to antigenic determinants of both viruses (Borgne et al., 1998).

As discussed previously, cytokines play a very crucial role in modulating the immune response. In a study involving application of cytokines to modulate vaccine-elicited immune responses, it has been shown that the immune responses elicited by an HIV-1 gp 120 plasmid DNA vaccine can be augmented or suppressed by administration of plasmid-encoding cytokines. These findings stress the need to first prime the immune system with a specific antigen followed by amplification of the response with cytokines (Barouch et al., 1998).

The immunomodulatory effects of an IL-2 expresison plasmid on an HIV-1 DNA vaccine were studied using intranasal administration of the combination. When the vaccine and expression plasmid were incorporated into cationic liposomes and administered to mice, the HIV-1-specific delayed-type hypersensitivity response and cytotoxic T lymphocyte activity were significantly increased. These results demonstrate that the IL-2 expression plasmid strongly enhances the HIV-1-specific immune response via activation of Th-1 cells (Xin et al., 1998). In a related study the adjuvant effect of low-viscosity carboxymethylcellulose sodium salt (CMES-L) on immune response induced by DNA vaccination of HIV-1 was examined. BALB/c mice were immunized with HIV-DNA vaccine formulated with CMCS-L via the intranasal (i.n.) and i.m. routes. The results showed that i.n. immunization of HIV-DNA vaccine formulated with CMCS-L enhanced an HIV-specific mucosal antibody and cell-mediated immune response (Hamajima et al., 1998). The adjuvant effect of another agent saponin QS-21 for augmentation of the systemic and mu-

cosal immune responses to HIV-1 has also been studied in a murine model (Sasaki et al., 1998). QS-21 was found to act as a mucosal adjuvant in DNA vaccination, acting through stimulation of the Th-1 subset (Sasaki et al., 1998). Similarly, the use of mannan-coated *N*-*t*-butyl-*N*′-tetradecyl-3-tetradecylamino-propionamidine (diCI4-amidine) as an adjuvant for a DNA vaccine encoding glycoprotein 160 of HIV-1 enhanced the antigen-specific immune responses. The results of both antigen-specific immunoglobulin isotype analysis and cytokine measurement showed that the immunogenic DNA incorporated into mannan-coated diC14-amidine elicits Th1-biased immune responses (Sasaki et al., 1997; Toda et al., 1997).

The use of liposomes on the immunomodulation of an HIV-1-specific DNA vaccine candidate (pCMV160/REV) has been studied. By immunizing with pCMV160/REV and cationic liposomes through various routes (intramuscular, intraperitoneal, subcutaneous, intradermal, and intranasal), higher levels of both antibody production and delayed-type hypersensitivity (DTH) were induced than by using the DNA vaccine alone. The HIV-1-specific cytotoxic T lymphocyte activity was stronger on immunization with the DNA vaccine and cationic liposome combination (Ishii et al., 1997).

The gp-120-derived V3 loop of HIV-1 is involved in co-receptor interaction, thereby affecting cell tropism, and also contains an epitope for antibody neutralization. This makes it an attractive vaccine candidate. In a recent study to improve the immunogenicity of V3 in DNA vaccines, a plasmid expressing MNV3 as a fusion protein with the highly immunogenic middle (preS2+S) surface antigen of hepatitis B virus (HBsAg) was constructed. DNA vaccination with the chimeric MNV3/HBsAg plasmid elicited humoral responses against both viruses within 3 to 6 weeks, which peaked at 6 to 12 weeks and remained stable for at least 25 weeks. In addition, specific CTL responses were induced in all mice against both MNV3 and HBsAg already within the first 3 weeks and lasting up to 11 weeks. Thus, HBsAg acts as a 'genetic vaccine adjuvant' augmenting and accelerating the cellular and humoral immune response against the inserted MN V3 loop (Fomsgaard et al., 1998).

The induction and regulation of immune responses following the co-delivery of proinflammatory cytokines (IL-1 alpha, TNF-alpha, and TNF-beta), Th1 cytokine (IL-2, IL-12, IL-15, and IL-18), and Th2 cytokine (IL-4, IL-5, and IL-10) with an HIV1 DNA immunogen was examined. An enhancement of antigen-specific humoral response with the co-delivery of Th2 cytokine genes IL-4, IL-5, and IL-10, as well as those of IL-2 and IL-18 was observed. A dramatic increase in antigen-specific T helper cell proliferation was seen with IL2 and TNF alpha gene co-injections and a significant enhancement of the cytotoxic response with co-administration of TNF-alpha and IL-15 genes was also observed. These increases in CTL response were both MHC class I restricted and CD8+ T cell dependent (Kim et al., 1998). In an interesting study it has been reported that optimized codon usage of an injected DNA sequence considerably increases both humoral and cellular immune responses (Andre et al., 1998). A synthetic HIV type 1 gp 120 (syngp 120) sequence in which most wild-type codons were replaced with codons from highly expressed human genes was generated. In BALB/c mice, DNA immunization with syngp 120 resulted in significantly increased antibody titers and CTL reactivity, suggesting a direct correlation between ex-

pression levels and the immune response. Thus, synthetic genes with optimized codon usage represent a novel strategy to increase the efficacy and safety of DNA vaccination (Andre et al., 1998).

As our closest genetic relative, the chimpanzee provides the most important model for preclinical safety and immunogenecity studies. Using plasmid DNA vaccines expressing various genes of HIV to immunize adult, pregnant, and infant chimpanzees, it has been shown that these vaccines are safe and well tolerated in all of these groups (Bagarazzi et al., 1998). The same vaccines have induced both humoral and cellular immunity in each instance.

Recently, the efficacy of combining immunization with HIV-1 DNA and HIV-1 recombinant proteins to obtain protection from chimeric simian/human immunodefficiency virus (SHIV) was determined (Putkonen et al., 1998). Four cynomolgous monkeys received four gene gun immunizations intraepidermally of plasmid DNA encoding HIV-1 lai env (gp 160), gag, tat, nef, and rev proteins. Ten μg of DNA was used per immunization. The animals were boosted twice intramuscularly with 50 μg of recombinant proteins. The antibody responses were amplified following the administration of the recombinant subunit boosts. One month after the final subunit immunization, the vaccinated animals together with four control animals were challenged intravenously with infectious doses of SHIV that expresses the env, tat, and rev genes of HIV-1 and gag and nef from SIV. The consecutive HIV-1 DNA and recombinant protein immunizations induced B and T cell responses but not protection against SHIV replication or reduction of the viral load (Putkonen et al., 1998). In all the studies, DNA-based vaccines have been shown to induce antibody responses and in some cases cellular responses in animal models. However, still evidence supporting the role of these in conferring immunity to infection is lacking.

B. Bacterial Diseases

In addition to viral diseases, the DNA vaccination approach has been tried for bacterial diseases as well. The only vaccine for tuberculosis Bacillus Calmette-Guerin (BCG) is a live bacterial vaccine that is far from ideal for protecting against tuberculosis. When mice were injected with plasmid DNA encoding a single mycobacterial antigen (65-kDa heat shock protein), specific cellular and humoral responses to the protein were detected and mice became immune to subsequent challenge with *Mycobacterium tuberculosis.* The protection level was equivalent to that obtained by vaccinating with live BCG, whereas immunizing with the protein was ineffective (Tascon et al., 1996). In a related study, immunization with DNA constructs encoding one of the secreted components of *M. tuberculosis*, antigen 85, induced substantial humoral and cell-mediated immune responses and protection against challenge with live *M. tuberculosis* and *M. bovis bacille* Calmette-Guerin (Huygen et al., 1996).

Therefore, DNA vaccines for bacterial diseases appear feasible notwithstanding the potential concerns regarding bacterial protein expression in eukaryotic cells.

C. Parasitic Diseases

Malaria is a parasitic disease for which no successful vaccine has been developed so far, despite considerable efforts to

develop subunit vaccines that offer protective immunity. Immunization with irradiated sporozoites protects animals and humans against malaria, and the circum-sporozoite protein is a target of this protective immunity. It has been shown that intramuscular injection of BALB/c with plasmid DNA encoding the *Plasmodium yoelii* circumsporozoite protein (PyCSP) induced higher levels of antibodies and cytotoxic T lymphocytes against the *P. yoelii* circum-sporozoite protein than did immunization with irradiated sporozoites. Mice immunized with this vaccine had an 86% reduction in liver-stage parasite burden after challenge with infectious doses of sporozoites (Sedegah et al., 1994). A later study examined the nature and localization of the antibody and cytokine-secreting cells activated by immunization with this plasmid construct (Mor et al., 1995). The initial humoral response was localized to the draining lymph nodes and was characterized by the production of IgG1 anti-PyCSP Abs and the Th2 cytokine IL-4. In contrast, the secondary response was dominated by IFN-γ production (a Th1 cytokine) and the secretion of IgG2a anti-PyCSP Abs in the spleen (Mor et al., 1995).

Recombinant vaccinia viruses expressing antigens derived from pathogens have often been used to induce cellular immune responses that may be protective. The modified virus Ankara strain of vaccinia was attenuated by multiple serial passages and has lost 31 kb of DNA including host range genes and genes encoding cytokine receptors (Sutter and Moss, 1992). In a recent study, the prime-boost immunization strategy with a combination of various recombinant vaccinia virus strain and plasmid DNA has been investigated. Using plasmid DNA encoding *P. berghei* antigens thrombospondin-related adhesive protein and the circumsporzoite protein as priming agents and boosting with recombinant modified virus Ankara (MVA), complete protection against sporozoite challenge was observed in both BALB/c and C57 BL/6 mice. The specific order of immunization, DNA priming followed by MVA boosting, was essential for protection (Schneider et al., 1998). The sequential immunization with *P. yoelii* CSP DNA vaccine, recombinant vaccinia, and synthetic peptide *P. yoelii* CSP vaccine was also tested in another study. The results indicated that priming with *P. yoelii* CSP DNA and boosting with recombinant vaccinia expresssing PyCSP were associated with greater immunogenicity and protective immunity than priming and boosting with PyCSP DNA alone (Sedegah et al., 1998).

In a recent study using *P. yoelii* as a model for malaria vaccine development, it has been shown that a DNA plasmid encoding the *P. yoelii* circumsporozoite protein can protect mice against sporozoite infection. Co-administration of a new plasmid PyCSP1012 with a plasmid encoding murine granulocyte-macrophage colony stimulating factor (GM-CSF) was shown to increase protection against malaria. While PyCSP 1012 alone protected 28% of mice, the protection increased to 58% when a GM-CSF encoding plasmid was added (Weiss et al., 1998). Immunization of rhesus monkeys with a mixture of DNA plasmids encoding four *P. falciparum* proteins induced detectable antigen-specific cytotoxic T lymphocytes after *in vitro* restimulation of peripheral blood mononuclear cells. These studies provide the foundation for multigene immunization for the protection against some diseases (Wang et al., 1998a).

The immunogenicity and efficacy of two DNA plasmids expressing different amounts of *P. berghei* circumsporozoite protein were

evaluated by immunizing mice intramuscularly or epidermally and by varying the number of immunizations (one to three doses) and interval between immunizations. Expanding the interval between immunizations gave the strongest effect, increasing efficacy and boosting antibody (Leitner et al., 1997). Immunization of mice with DNA vaccines encoding the full-length form and C and N termini of *P. yoelii* merozoite surface protein 1 provided partial protection against sporozoite challenge and resulted in boosting of antibody titers after challenge (Becker et al., 1998).

It has been shown that, when the C terminus (PyC2) of *Plasmodium yoelii* merozoite surface protein-1 (MSP-1) was expressed with glutathione *S*-transferase (GST) as a fusion protein (GST-PyC2), it elicited Ab-mediated protective immune responses in BALB/c mice. In continuation of this study the humoral responses to a DNA vaccine (V3) encoding GST-PyC2 have been examined (Kang et al., 1998). When BALB/c mice were immunized with V3 plasmid, anti-PyC2 antibodies were successfully induced. In contrast to protein immunization, there was no significant increase in the avidity of antibodies during the course of DNA immunization. These data suggest that there may be little or no affinity maturation of specific antibody during DNA immunization in this system (Kang et al., 1998).

Recently, in a very interesting study on the use of malaria DNA vaccine in humans, it was shown that volunteers developed antigen-specific, genetically restricted, CD8(+) T-cell-dependent CTL responses. This study provides a foundation for further human testing of this potentially revolutionary vaccine technology (Wang et al., 1998b).

D. Cancers

Expression of DNA encoding fragments of tumor-specific proteins as neo-antigens or surrogate antigen works in breaking immunological tolerance and leads to the generation of tumor-specific immune responses. For nonvirally induced tumors, plasmids may prove to be an efficient way to build subunit and multisubunit vaccines based on the genetic changes that occur in carcinogenesis.

Idiotypic antigens are clearly defined tumor-associated protein antigens, that can induce protective immunity against lymphoma. Intramuscular idiotypic DNA vaccination in a mouse model induces low levels of antiidiotype antibody in serum. Levels can be increased dramatically by co-injection of DNA plasmids encoding either IL-2 or GM-CSF and specific proliferative antiidiotypic T cells are induced (Stevenson et al., 1995).

Idiotypic determinants of the immunoglobulin expressed on the surface of B-cell lymphomas are tumor-specific antigens, that can be targeted by immunotherapy. Immunizations with DNA constructs encoding the idiotype (Id) of a murine B-cell lymphoma induced specific anti-Id antibody responses and protected mice against tumor challenge. The use of DNA encoding an Id/GM-CSF (idiotype/granulocyte-macrophage colony-stimulating factor) fusion protein improved vaccine efficacy. These studies indicate that DNA may be a simple and efficacious means of inducing immune responses against a weak, otherwise unrecognized tumor antigen, provided additional stimuli are included with the DNA (Syrengelas et al., 1996).

DNA immunization has been used to block tumor growth in a model system in

which a defined 9 amino acid epitope from the nucleoprotein of influenza virus was used as a surrogate tumor-associated antigen (Iwasaki et al., 1998). A mastocytoma cell line of DBA/2 origin (P815) was transfected with a plasmid encoding the minimal H-2Kd-restricted NP (147–155) cytotoxic T-lymphocyte (CTL) epitope, pCMV/Npep, to generate the cell line designated P815-Npep. Mice primed and boosted once with a plasmid encoding the full-length NP gene, pCMV/NP, but not with the mini gene pCMV/Npep developed a strong NP (147-155) specific CTL response within 2 weeks after the boost. When challenged with P815-Npep cells, pCMV/NP-immunized DBA/2 mice were protected from tumor challenge, whereas control mice immunized with the vector backbone rapidly developed lethal tumor. These data indicate that intramuscular plasmid DNA immunization can be used to mobilize an effective CD8+ CTL-mediated antitumor response (Iwasaki et al., 1998).

Recently, it has been suggested that liposome-mediated transfection of tumor cells with an episomal, high copy number plasmid expressing both IL-12 subunits is a promising approach to cancer vaccination, a strategy that could be implemented *ex vivo* in treating malignancies such as metastatic ovarian cancer (Hoshino et al., 1998).

IV. HUMAN TRIALS WITH DNA VACCINES

A DNA-based vaccine containing HIV-1 env and rev genes was tested for safety and host immune response in 15 asymptomatic HIV-infected patients who were not using antiviral drugs. These successive groups received three doses of the vaccine (30, 100, or 300 μg) at 10-week intervals. Antibody against gp 120 increased in individual patients in the 100- and 300-μg groups. Some increases were also noted in CTL activity against gp160-bearing targets and in lymphocyte proliferative activity (MacGregor et al., 1998). These findings demonstrate the safety and potential immunogenicity of an HIV-directed DNA-based vaccine.

In another study conducted on smaller number of patients, it was shown that cellular cytotoxic responses were induced by DNA vaccination in HIV-1-infected patients (Calarota et al., 1998). The patients were immunized with DNA constructs encoding the nef, rev, or tat regulatory genes of HIV-1. This vaccination induced detectable memory cells in all patients and specific cytotoxicity in some patients. The CTLs that developed were MHC-class I restricted and mainly of CD8+ origin (Calarota et al., 1998).

In an interesting study, it has been shown recently that malaria-naive volunteers who were vaccinated with plasmid DNA encoding a *Plasmodium falciparum* protein developed antigen-specific, genetically restricted, CD8+ T-cell-dependent cytotoxic T lymphocytes (Wang et al., 1998). Responses were directed against all 10 peptides tested and were restricted by six human lymphocyte antigen (HLA) class I alleles.

The enhancement of humoral immune responses specific to HIV-1 in infected patients after therapeutic inoculation with nucleic acid vaccine expressing HIV-1 envelope glycoprotein has been reported in a recent study (Ugen et al., 1998). The serological analysis on the human study group vaccinated with the 100-μg dose of the DNA

plasmid vaccine indicated a boosting of humoral immune responses in the individuals after vaccination. This boosting effect was noted against recombinant gp120 and a V3 loop peptide from gp120. In addition, these results also indicated that the vaccine preparation was well tolerated in the individuals and no significant side effects were noted (Ugen et al., 1998). Recently, a DNA-based vaccine containing HIV-1 env and rev genes was tested for safety and host immune responses in 15 HIV-infected asymptomatic patients receiving no antiviral therapy (Boyer et al., 1999). Successive groups of patients received three doses of vaccine at 30, 100, or 300 μg at a 10-week interval in a dose-escalation trial. Enhanced specific lymphocyte proliferative activity against HIV-1 env was observed in multiple patients. The majority of patients in the highest dose group exhibited an increase in immune parameters. These studies support further investigation of this technology for the production of antigen-specific immune responses in humans (Boyer et al., 1999).

V. CONCLUDING REMARKS AND FUTURE DIRECTIONS

DNA vaccination has initiated a new era of vaccine research as it offers an extremely powerful tool to develop new vaccines and other immunotherapeutic approaches. Unlike conventional protein vaccines, plasmid DNA vaccination leads to antigen processing and loading onto both MHC class I and class II molecules and, in this respect, more closely resembles a viral infection. Therefore, the potential of DNA-mediated immunization to partially mimic viral infection promises the efficacy of live attenuated vaccines without the risk of inadvertent infection. The characteristic immune response that is induced by gene vaccination with naked plasmid DNA has multiple potential applications in the field of infectious and parasitic diseases, allergy, and cancer. The reason for the lower antibody response induced by gene vaccination is the minute amount of gene product produced *in vivo*. Improved gene expression combined with improved immunostimulatory (ISS) sequences inserted into the plasmid DNA backbone or use of ISS-containing oligonucleotides as adjuvant are proven to enhance antibody responses. These measures may expand the application of this emerging technology to develop preventive vaccines for infectious diseases where high levels of neutralizing antibodies are required. Ultimately, although a number of human trials are underway, only results from further experimentation will help to determine the likelihood of success of this technology in humans.

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