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DNA Vaccine

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ABSTRACT

The DNA vaccine has proven to be one of the most promising applications in the field of gene therapy. Due to its unique ability to readily induce humoral as well as cellular immune responses, it attracted great interest when the concept was first confirmed in the early 1990s. After thousands of articles related to the DNA vaccine were published, scientists began to realize that although the DNA vaccine is very effective in small animal models, its effectiveness in recent clinical trails is rather disappointing. Therefore, current effort has been shifted to understanding the different performance of the DNA vaccine in mouse and large animal models and on how to transfer the success of the DNA vaccine in small animals to large animals and humans.   © 2005, Elsevier Inc.

I. INTRODUCTION

With the exception of clean water, vaccines have been the most effective modalities in reducing human mortality from infectious diseases. With an effective vaccine, the World Health Organization (WHO) was able to declare the complete eradication of smallpox around the world in the 1970s. Similarly, the world is now almost free of polio, with the exception of only six countries (CDC). Effective vaccines have been successfully developed for some pathogens. However, lack of effective vaccines for others [e.g., the Bacilli-Calette-Guerin (BCG)-based vaccine for tuberculosis] and the emergence of new pathogens (e.g., severe acute respiratory syndrome [SARS] virus) warrant continuous effort in vaccine development. In addition, the recent bioterror threat makes it more urgent to develop new or alternative vaccines for those pathogens that can be potentially used for bioterror purposes.

Vaccines are traditionally prepared with either live attenuated or killed bacteria or viruses. In many cases, these approaches have proven to be successful. Vaccines based on live attenuated or killed pathogens are usually very potent and can induce all aspects of immune responses. However, serious safety concerns may preclude the use of these approaches in the development of vaccines for some pathogens such as HIV (Baba *et al.*, 1999). New generation vaccines, such as recombinant protein-based vaccines, synthetic peptide-based vaccines, lipid-based vaccines/antigens, and vaccines based on polysaccharides, are thought to be potentially safer than traditional vaccines. Unfortunately, these new generation vaccines are often very poorly immunogenic, partially because the components having adjuvant activity were discarded in the purification or synthesis process. Some less or well defined structures of pathogens, such as bacterial cell wall components, unmethylated DNA, or double-stranded

(ds) RNA, may activate the host defense system by acting as potent "danger" signals. Elimination of these components leads to poor immunogenicity. The reduced or lack of ability for some new generation vaccines to induce a cell-mediated immune (CMI) response, especially a cytotoxic T lymphocyte response (CTL), is another limitation. For example, a recombinant protein-based vaccine elicits mainly humoral immune response (i.e., IgG and IgE production) if administered without proper adjuvants. The polysaccharide vaccine, without conjugation to an appropriate carrier protein, generates only a T cell independent (TI) response by the production of IgM (Lesinski and Westerink, 2001). Importantly, it is currently believed that CMI, especially the CTL response, is as critical as neutralizing antibodies for the effective control of intracellular pathogens such as HIV, hepatitis, tuberculosis, and malaria. In case of tumor vaccines, the ability to induce a tumor-specific CTL response is thought to be indispensable for them to be effective. Therefore, the discovery in the early 1990s that a vaccine based on plasmid DNA can induce both humoral and cellular immune responses produced great excitement in the vaccine and immunology community.

Many different terms, such as genetic vaccine, polynucleotide vaccine, and nucleic acid vaccine, have been used to name the DNA vaccine. In 1994 the WHO chose the term nucleic acid vaccine, subtermed into the DNA vaccine and RNA vaccine. The DNA vaccine was based on the finding that administration of recombinant plasmid DNA into an animal resulted in the expression of a foreign protein encoded by the plasmid (Wolff et al., 1990). Soon after this initial finding, Tang et al. (1992) for the first time demonstrated the elicitation of an immune response against a foreign protein by introducing a plasmid encoding the interesting antigen protein directly into mouse skin with a gene gun. Then, almost simultaneously, Ulmer et al. (1993) and Fynan et al. (1993) showed that immunization with plasmid DNA could protect mice against a lethal influenza challenge. Subsequently, thousands of papers have been published demonstrating that the DNA vaccine is potentially effective for a wide variety of diseases, including infectious diseases, cancers, autoimmune diseases, and allergic diseases.

II. COMPOSITION OF DNA VACCINE

To generate a DNA vaccine, the interesting antigen-encoding gene is inserted into a bacterial plasmid under the control of an appropriate eukaryotic promoter (e.g., the CMV promoter from cytomegalovirus in most cases). Due to the difference in codon usage preference between bacteria and eukaryotic cells, the antigen gene is often modified by point mutation to improve the efficiency

of gene expression. Purified and detoxified plasmid DNA from bacteria is then administered into the host animal. From those plasmids that were picked up by appropriate cells and made their way into nuclei, the host cell will use its own gene transcription and protein expression machines to produce the interesting antigen. The host regards the expressed antigen as foreign and will then mount an immune response against it.

       In addition, appropriate numbers of unmethylated CpG motifs with the right flanking sequences are usually engineered into the backbone of the plasmid. As explained later, the CpG motif is immunostimulatory and induces the production of Th1 type cytokines (INF-γ, TNF-α, IL-12, etc) and the upregulation of costimulatory molecules such as CD80 and CD86 on antigen-presenting cells (APCs) (Akira *et al.*, 2001; Shimada *et al.*, 1985; Tokunaga *et al.*, 1984; Yamamoto *et al.*, 1988).

III. ADVANTAGES AND DISADVANTAGES OF DNA VACCINE

In addition to its ability to elicit both humoral and cellular immune responses, the DNA vaccine is thought to be potentially safer than the traditional vaccine. It is relatively more stable and potentially more cost-effective for manufacture and storage. Also, multiple antigens may be combined into one plasmid to target multiple pathogens or multiple components of a single pathogen. In addition, the unmethylated CpG motifs on bacterial plasmid DNA in the context of flanking sequences have proven to be immunostimulatory by acting as a pathogen associated molecular pattern (PAMP) molecule and interacting with the Toll-like receptor 9 (TLR9) (Akira *et al.*, 2001). However, the DNA vaccine does have limitations. Specifically, the DNA vaccine tends to be relatively poorly immunogenic, often requiring a large dose to be effective. Since its discovery in the early 1990s, the DNA vaccine has mainly been administered by either intramuscular (im) injection of naked plasmid DNA or by gene gun-mediated administration (i.e., ballistic penetration of pDNA adsorbed on gold beads into skin). Intramuscular injection has proven to be very effective in small animal models. However, the effectiveness of the DNA vaccine in non-human primates and humans in recent studies has not been encouraging, especially in eliciting an antibody response (Calarota *et al.*, 2001; Conry *et al.*, 2002; Klencke *et al.*, 2002; Le *et al.*, 2000; Mincheff *et al.*, 2000; Rosenberg *et al.*, 2003; Tacket *et al.*, 1999; Tagawa *et al.*, 2003; Timmerman *et al.*, 2002; Wang *et al.*, 1998; Weber *et al.*, 2001). Gene gun-mediated administration resulted in a better immune response than an intramuscular injection in clinical trials (Roy *et al.*, 2000). However, the administration of gold beads into humans might be problematic in long term.

IV. MECHANISMS OF IMMUNE INDUCTION FROM DNA VACCINE

As stated by Hilleman (1998) a vaccinologist's knowledge about immunology should be essential and simple. In general, immune responses include antibody production (humoral immune response), CTL response, and cytokine-mediated type 1 (Th1) and type 2 (Th2) T helper responses. In addition, APCs and B lymphocytes (known as detector cells), CD8$^+$ toxic T cells and B lymphocytes (known as effectors), and CD4$^+$ T cells (known as facilitators) determine the type of immune response.

It is now known that in order to successfully induce a primary immune response, professional APCs are required. This is because only the professional APCs can provide both the first signal and the secondary signal required for successful antigen presentation. Almost all cells (can) express MHC class I molecules, but only the professional APCs express or can be induced to express the secondary signal molecules such as CD80 and CD86. An interaction between a peptide epitope-loaded MHC molecule and the T cell receptor (TCR) without the appropriate secondary signal will lead to anergy. Dendritic cells (DCs) are the most potent antigen-presenting cells (Banchereau and Steinman, 1998). Immature DCs, such as Langerhan's cells (LCs), are extremely well equipped for antigen capture, and the capture of antigens induces maturation and mobilization of DCs (Banchereau and Steinman, 1998). In light of these findings, a successful antigen must make its way to APC, especially to DC to initiate a primary immune response.

The outcome of an immune response is determined partially by how the antigen is presented by the APCs to the T and B cells, exogenously or endogenously. For a live vaccine such as the live viral vaccine, the virus is still infectious to the host cells, including APCs. The relevant antigen is produced by the host cells. Therefore, the antigen is generated inside the cells (endogenously) and is processed by the proteosome apparatus into small peptides, which are then transferred into the endoplasmic reticulum where they can bind to the newly synthesized MHC class I molecules (Harding and Song, 1994). The MHC class I molecule with peptide epitope on its groove is mobilized onto the cell surface where it may be recognized by antigen-specific CD8$^+$ T cells with the appropriate TCR. This endogenous presentation results in a CTL immune response. The fact that a DNA vaccine may elicit a CTL immune response is also due to the endogenous presentation of the encoding antigen. However, for the nonlive vaccine, such as the protein (subunit)-based vaccine, the antigen is taken up by APCs in the intercellular spaces by endocytosis. In this pathway, the antigen is processed inside the endosome and lysosome. Some of the endosomal proteolytic degradation products will occupy the epitope groove of the class II MHC molecule and be transported to the extracellular surface of the APCs.

This class II MHC molecule, together with the epitope, will be available for recognition by CD4$^+$ T cells. The CD4$^+$ T cells may develop into either Th1 type or Th2 type cells. The Th1 cell and its related cytokines [interleukin-2 (IL-2), interferon-γ (IFN-γ), etc.] help initiate CMI, whereas the Th2 helper and its related cytokines (IL-4, IL-10, etc.) direct a humoral immune response (Castellino *et al.*, 1997).

As mentioned earlier, for the DNA vaccine, plasmid DNA has primarily been administered by either im injection or via the gene gun into skin. It was found that gene expression from the administered plasmid was predominantly in the myoblasts after im injection and in the keratinocytes and fibroblasts after gene gun-mediated administration. These patterns of expression raised questions on how the expressed antigen is presented to T cells. Although myoblasts and keratinocytes express MHC class I molecules, they do not express other secondary signal molecules (e.g., CD 80 and CD 86). Therefore, theoretically, presentation of expressed antigen by these nonprofessional cells is more likely to tolerize than stimulate T cells. In addition, experiments carried out by Iwasaki *et al.* (1997) and Corr *et al.* (1996) clearly ruled out the possibility that the somatic cells (transfected with plasmid expressing the antigen only) directly presented antigens to the T cells. In the study by Corr *et al.* (1996), parent→F1 bone marrow chimeras, in which H-2^{bxd} recipient mice received bone marrow that expressed only H-2^b or H-2^d MHC molecules, were injected (im) with naked plasmid DNA encoding the nucleoprotein from the A/PR/8/34 influenza strain, which has epitopes for both H-2D^b and H-2K^d on a single antigen. The resulting CTL responses were restricted to the MHC haplotype of the bone marrow alone and not to the other haplotype expressed by the myocytes of the recipient (Corr *et al.*, 1996). These results showed that dendritic cells and other APCs that are differentiated from bone marrow cells, instead of somatic cells, were responsible for antigen presentation. This may explain why intradermal gene gun injection often induced better immune responses than im injection. The gene gun may target DCs in the viable skin epidermis more effectively and directly, resulting in a very potent immune response. Moreover, significantly less DNA is required to elicit an immune response by the gene gun compared to im needle injection. Within the skin, there is a high population of immature DCs or LCs. In contrast, much fewer DCs exist in the muscle.

Currently, there are three proposed mechanisms of antigen presentation by the DNA vaccine: (1) direct transfection of professional APCs (i.e., DCs) and the presentation of expressed antigen by the professional APCs; (2) direct priming by modified somatic cells (myocytes or keratinocytes); and (3) cross-priming in which plasmid DNA transfects a somatic cell and/or professional APC and the proteins secreted from the transfected cells are taken up by

other professional APCs and presented to T cells (Gurunathan *et al.*, 2000; Liu, 2003; Takashima and Morita, 1999).

The first mechanism of the direct transfection of professional APCs and the presentation of the self-expressed antigen by the APCs is easily understandable and has been well documented. It was reported that as few as 500 transfected mouse DCs are sufficient to successfully elicit an immune response (Takashima and Morita, 1999). Many experiments have also demonstrated the existence of cross-priming. In cross-priming, the antigen may be expressed by somatic cells transfected with the plasmid DNA. The antigen protein or peptide will then be picked up by professional APCs and presented to T cells. As aforementioned, the secreted or exogenous protein undergoes endocytosis or phagocytosis to enter the MHC class II antigen processing pathway to stimulate $CD4^+$ T cells. Endogenously produced proteins are processed by the proteosome apparatus and are presented through the MHC class I pathway to stimulate naive $CD8^+$ T cells. Although peptides derived from exogenous sources generally cannot be presented on MHC class I molecules, there are now many examples showing that this does occur if proper adjuvants or delivery systems are used (Falo *et al.*, 1995; Raychaudhuri and Rock, 1998). During cross-priming, the antigen or peptide (both MHC class I and II) generated by somatic cells (myocytes or keratinocytes) can be taken up by professional APCs to prime the T-cell response. A study carried out by Ulmer *et al.* (1996) clearly demonstrated that cross-priming from myocytes to APC happens. In their study, influenza NP-expressing myoblasts $(H-2^k)$ were injected intraperitoneally into F1 hybrid mice $(H-2^{dxk})$ (Ulmer *et al.*, 1996). It was found that NP CTL restricted by the MHC haplotype of both parental strains was induced. $H-2^d$-restricted epitopes must have somehow found their way to move from the myoblasts to the $H-2^d$-expressing APCs. It is, of course, assumed that the very unlikely case of direct transfer of the NP expression plasmid from the myoblast to APC did not happen.

As to direct priming by transfected somatic cells, this mechanism is theoretically very unlikely. However, Agadjanyan *et al.* (1999) pointed out that when mice were vaccinated with DNA encoding both antigen and CD86, transfected muscle cells can prime an antigen-specific CTL response (Agadjanyan *et al.*, 1999). In this case, the myoblast expresses both HIV-1 envelope protein and CD86. It is interesting to find that CD86 alone can provide the necessary secondary signal. Another study showed that the transfected fibroblasts are able to induce an antigen-specific MHC class I-restricted response if they are physically relocated to secondary lymphoid tissue (Kundig *et al.*, 1995). In lymphoid tissues, a secondary signal may be provided by other cells to the fibroblasts. Thus, nonprofessional cells may be able to prime an immune response when the appropriate secondary signal is provided.

V. THE IMMUNOSTIMULATORY ACTIVITY OF CpG MOTIF

As mentioned earlier, one of the advantages of bacterial plasmid DNA vaccine is that it has a built-in powerful adjuvant, the unmethylated CpG motif. Bacterial DNA was unknowingly used experimentally as a component of an adjuvant over 60 years when Freund used the whole mycobacterial extract as a major constituent in his adjuvant formula based on empirical results (Freund, 1951). It has been shown that the mycobacterial DNA in the Freund's adjuvant contributed to the adjuvant effect (Shimada et al., 1985; Tokunaga et al., 1984; Yamamoto et al., 1988). Yamamoto et al. (1988) found that a purified nucleic acid fraction from the BCG vaccine has a limited antitumor activity that appears to be mediated through its ability to activate NK cells and to induce the production of interferon. Treatment of this fraction with DNase substantially reduced this activity (Tokunaga et al., 1999). This confirmed the immunostimulatory property of mycobacterial DNA. It is now known that the unmethylated CpG motif with its flanking sequences, even in the form of oligonucleotide, is strongly immunostimulatory (Klinman et al., 1999; Krieg et al., 1995).

The unmethylated CpG motif has been found to be a ligand for TLR9 (Hemmi et al., 2000). TLR9 belongs to a group of TLRs. TLRs were identified as major recognition receptors for a pathogen-associated molecular patterns (PAMP) such as lipopolysaccharides (LPS), peptidoglycan, lipoteichoic acid, and CpG-containing oligonucleotides (CpG ODN) (Hemmi et al., 2000; Poltorak et al., 1998). The term Toll was originally referred to as a cell surface receptor governing dorsal/ventral orientation in early *Drosophila* larvae (Stein et al., 1991). It was later found to also play a crucial role in antifungal defense, together with other antimicrobial peptides (Lemaitre et al., 1996). In the 1990s, the first mammalian protein structurally related to *Drosophila* Toll was identified and is now called the human Toll-like receptor (Medzhitov et al., 1997). To date, 10 human and 9 murine transmembrane proteins have been shown to belong to the mammalian TLR family (Akira et al., 2001; Zarember and Godowski, 2002). Very recently, a TLR11 responding specifically to uropathogenic bacteria was discovered in mice (Zhang et al., 2004).

Toll and TLR family proteins are characterized by the presence of an extracellular domain with leucine-rich repeats and an intracytoplasmic region containing a Toll/interleukin-1 receptor homology (TIR) domain. Individual mammalian TLR appears to recognize distinct microbial components. For example, LPS (Poltorak et al., 1998), bacterial lipoproteins (sBLP) (Aliprantis et al., 1999) and yeast (Underhill et al., 1999), flagellin (Hayashi et al., 2001), dsRNA (Alexopoulou et al., 2001), small antiviral compounds (Hemmi et al., 2000), and bacterial DNA (CpG-DNA) (Hemmi et al., 2000) engage TLR4, TLR2, TLR5, TLR3, TLR7, and TLR9, respectively.

Different TLRs can exert distinct, but overlapping, sets of biological effects, and increasing evidence indicates that this can be attributed to both common and unique aspects of the signaling mechanisms. All functional studies characterize the TLR signal via the TLR/IL-1 receptor pathway because the presence of the TIR domain can interact with the adaptor protein MyD88. The adaptor molecule MyD88 is recruited to the receptor complex, followed by engagement of the IL-1R-associated kinases (IRAK). IRAK1 and IRAK4 are serine-threonine kinases involved in the phosphorylation and activation of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (Medzhitov *et al.*, 1998; Muzio *et al.*, 1998; Wesche *et al.*, 1997). In contrast, IRAK-M lacks kinase activity and regulates TLR signaling negatively by preventing the dissociation of phosphorylated IRAK1 and IRAK4 from MyD88. Phosphorylated TRAF6 leads to the activation of downstream kinases, such as the stress kinase JNK1/2 and the IκB kinase (IKK) complex (Baud *et al.*, 1999). This event frees nuclear factor-κB (NF-κB) from IκB and allows its nuclear translocation and the subsequent transcriptional activation of many proinflammatory genes, which encode cytokines, chemokines, adhesion molecules, and immune receptors. All of these molecules are involved in engaging and controlling the innate immune response and in orchestrating the transition to an adaptive immune response (Medzhitov, 2001).

TLR signaling stimulates the maturation of DCs, which migrate to the lymph nodes where they stimulate T cells by the presentation of MHC complexes. Antigen presentation alone can stimulate pathogen-specific T-cell clones, but is not sufficient to trigger efficient T-cell expansion. Clonal T-cell expansion requires an additional signal delivered by costimulatory molecules such as CD80/86. TLR signaling functions to trigger adaptive immunity by enhancing the expression of not only MHC molecules, but also of these costimulatory molecules in DCs (Kaisho and Akira, 2001).

Stimulation by bacterial CpG motifs generally leads to the production of Th1-type cytokines and to the upregulation of costimulatory molecules on lymphocytes and APCs. The interaction of CpG motifs with TLR9 was further confirmed by the fact that in TLR9 knockout mice, all CpG DNA-induced effects, including cytokine production, B-cell proliferation, and DC maturation, were completely abolished (Hemmi *et al.*, 2000). In fact, the immunostimulatory effect of CpG motif is so strong that CpG motifs containing oligonucleotides are now being used as a vaccine adjuvant. In tumor vaccine development, the CpG motif is an adjuvant of special interest, as generally, the CpG motif can skew the immune response to be Th1 biased, which favors the induction of CTL for tumor killing. However, caution needs to be applied in the repeated administration of CpG motifs. Heikenwalder *et al.* (2004) reported that repeated CpG oligodeoxynucleotide administration led to lymphoid follicle destruction and immunosuppression (Heikenwalder *et al.*, 2004). They reported that daily intraperitoneal

injection of 60 μg CpG-ODN dramatically alters the morphology and functionality of mouse lymphoid organs. By day 7, lymphoid follicles were poorly defined; follicular dendritic cells and germinal center B lymphocytes were suppressed. Accordingly, CpG-ODN treatment for more than 7 days strongly reduced the primary humoral immune response and immunoglobulin class switching. By day 20, mice developed multifocal liver necrosis and hemorrhagic ascites. Of course, it is very unlikely for anybody to develop a vaccine that contains so many CpG motifs and to administer it daily for up to 7–20 days intraperitoneally. The finding is mentioned here simply to point out that high doses of CpG motifs could be potentially toxic.

VI. ROUTE OF ADMINISTRATION

The DNA vaccine was originally administered by an intramuscular injection or via a gene gun to the skin. These two have continued to be the main means/routes of administration. Meanwhile, many other routes have been tried. These include subcutaneous injection, intradermal injection, noninvasive topical application onto the skin (Tang *et al.*, 1997), intranasal and intravaginal applications (Livingston *et al.*, 1998; Park *et al.*, 2003), and vaccination via oral mucosa or gastrointestinal (GI) mucosa (oral). Depending on the route, different immune responses have been elicited. For example, due to the fact that most of the pathogens enter the host through either the mucosal surface or the skin, an immune response that can neutralize or kill the pathogens on the mucosal surface prior to their entrance is therefore desired. Also, due to the common mucosal system, dosing of vaccine from one mucosal site (e.g., nasal) may lead to a mucosal response on another mucosal site (e.g., vaginal) (McGhee *et al.*, 1992). To achieve a mucosal immune response, the DNA vaccine has been applied on the nasal, oral, GI, or vaginal mucosal surface with specially designed devices or delivery systems. The following paragraphs provide a few examples for administration of the DNA vaccine by mucosal routes.

        The oral buccal mucosa is covered by a network of DCs analogous to LCs in the skin. LCs represent the major APCs in human buccal mucosa epithelium. In addition, a high density of T cells and mucosal-associated lymphoid tissue (MALT) are present in the buccal mucosa. Therefore, immunization via the buccal mucosa may represent an attractive mucosal immunization approach. In fact, the efficacy of oral buccal mucosal DNA immunization has been confirmed by many using transepithelial needle injection, jet injection, or gene gun. For example, Lundholm *et al.* (1999) found that jet injection of plasmid DNA into the oral cheek of mice induced a very strong IgA mucosal response specific to the encoding HIV-1 proteins (gp160, p24, and TAT) (Lundholm *et al.*, 1999). To avoid the inconvenience posed by those means of

DNA administration, Cui and Mumper (2002a) developed a buccal mucoadhesive film based on polymers Noveon and Eudragit S-100. When plasmid DNA (encoding β-galactosidase protein as a model antigen)-loaded film was applied to rabbit buccal mucosa, a serum-specific IgG level comparable to that induced by a subcutaneous injection of aluminum hydroxide (Alum) adjuvanted β-galactosidase protein was induced (Cui and Mumper, 2002a). In addition, strong serum IgA and splenocyte proliferative responses were induced. Although data are promising, application of vaccine on oral buccal mucosa has the limitation that tolerance instead of immunity might be very often induced. The buccal mucosa is in contact with many foreign antigens daily. However, rarely is an immune response induced.

Oral administration of vaccines is desired from both an immunological aspect and a patient compliance point of view. Oral administration of vaccines is convenient for patients and may make large population immunization more feasible. Moreover, oral administration of vaccines has been shown to induce both systemic and mucosal immune responses (Russell-Jones, 2000). The intestinal mucosa is rich in DCs and other MALT. However, the polio vaccine is still the only marketed vaccine administered orally, perhaps illustrating the difficulty in developing an effective oral vaccine. The extensive enzymatic systems and harsh physical and chemical environments in the GI tract make it difficult to develop an active oral vaccine. Therefore, a DNA vaccine for oral administration (at experimental level) is often encapsulated into particles. Encapsulation of DNA inside microparticles may provide protection to DNA.

Jones et al. (1997) reported the first oral DNA vaccine by encapsulating a plasmid-expressing insect luciferase protein into PLGA microparticles and administering it to mice by oral gavage (Jones et al., 1997). The authors observed good serum IgG, IgA, and IgM antibody responses and, most importantly, a significant level of mucosal IgA in saliva and stool samples. In contrast, unencapsulated DNA gave undetectable responses.

Soon after, Herrmann's group used this method to orally immunize mice with more relevant antigens, such as the capsid proteins of rotavirus, VP4, VP6, and VP7 (Chen et al., 1997, 1998; Herrmann et al., 1996, 1999). Using rotavirus VP6 DNA vaccine-encapsulated PLGA microparticles, Chen et al. (1998) reported that one dose of vaccine given to BALB/c mice elicited both rotavirus-specific serum antibodies and intestinal IgA. Moreover, after challenge with homologous rotaviruses, virus shedding was reduced significantly compared to control mice, which were immunized with PLGA microparticles encapsulated with VP6-free plasmid (Chen et al., 1998). Similar results were observed when the VP6 gene was replaced with VP4 and/or VP7 (Herrmann et al., 1999). These studies represented the first demonstration of protection against an infectious agent after oral administration of a DNA vaccine.

VII. IMMUNOLOGY OF THE IMMUNE RESPONSES FROM DNA VACCINE

Many studies have demonstrated the preclinical efficacy of the DNA vaccine in disease models of infectious disease, cancer, allergy, and autoimmune disease. Interested readers may refer to the supplemental tables tabulated by Gurunathan et al. (2000) for a comprehensive list of allergy, autoimmune disease, bacterial infection, and tumor models for which DNA vaccines have been attempted. Many more have been added since 2000. CTL, antibody, and different types of T-cell helper responses have been generated depending on the disease, antigen, animal, and route of administration.

A. Humoral response

Immunization with plasmid DNA can induce antibody responses in a variety of proteins in animal species, particularly in mouse. Moreover, the humoral response generated by DNA vaccination has been shown to be protective in several animal models *in vivo*. However, as detailed later, the antibody response in humans from the DNA vaccine has not been encouraging. The antibody response from the DNA vaccine is weak at the beginning then peaks and reaches a plateau between 1 and 3 months after a single DNA immunization in mice (Deck et al., 1997). Furthermore, within a certain dose range, antibody production is generally increased in a dose-responsive manner with either a single injection or multiple injections of DNA by various routes of immunization (Deck et al., 1997). The resulting antibody response can be long lived (e.g., significant serum levels were present up to 1.5 years after vaccination) (Deck et al., 1997; Raz et al., 1994). Unfortunately, when antibody responses from DNA, protein, and live virus vaccines were compared, the response from the DNA vaccine is generally weaker than that from protein-based vaccine and that from the liver virus vaccine. For example, in a study comparing the antibody responses to DNA encoding the hemagglutinin (HA) antigen and live influenza infection, the antibody titer in mice vaccinated with live influenza was significantly higher than that in DNA-vaccinated mice (Deck et al., 1997). In comparing the antibody responses elicited by vaccination with DNA encoding a malarial surface protein and the protein itself, both antibody titers and avidity were significantly lower in mice vaccinated with DNA than in those vaccinated with the protein (Kang et al., 1998). In contrast, in one study directly comparing the kinetics of an 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 at 2 or 4 weeks postvaccination when DNA was administered intradermally (Boyle et al., 1997). In this study, antibody induced by DNA had a higher avidity than that induced by protein. It

is worthwhile to point out that many parameters can affect the results from these comparisons. First of all, it is difficult to choose the doses of DNA and protein to compare. Therefore, one has to compare the highest antibody response from the DNA vaccine with the highest antibody response from other vaccines. Second, it is well known that when a protein is injected alone, usually no or very weak immune responses will be induced. Thus, an adjuvant such as Alum is often used. In the case of the DNA vaccine, intramuscular injection of "naked" DNA alone can lead to an antibody response. Third, the adjuvant or delivery system makes a difference in terms of the resulting responses. For example, previous studies found that the antibody response from the DNA vaccine was weaker than that from a protein-based vaccine adjuvanted with Alum when the DNA vaccine is administered as a "naked" unformulated plasmid. However, when the plasmid is formulated with nanoparticles as the carrier, the resulting antibody response was then stronger than that from the same protein-based vaccine (Cui and Mumper, 2002b). Finally, the route of administration has a significant effect. As mentioned earlier, when plasmid DNA was applied to rabbit buccal mucosa via the mucoadhensive films we developed, a strong antibody response was observed. However, when the protein antigen encoded by the plasmid was applied to rabbits in the same route with the same device, no detectable antibody response was induced (Cui and Mumper, 2002a). Therefore, caution should be applied when carrying out these comparisons. However, in general, especially in clinical trials, the antibody response from the DNA vaccine is not encouraging.

DNA vaccination induces the production of many subtypes of antibodies, including IgG, IgM, and IgA. Moreover, in most cases, antibodies generated by DNA vaccines are skewed toward IgG2a due to the fact that the CpG motifs on plasmid DNA stimulate production of the Th1 cytokine. However, exceptions do exist because DNA vaccination with a gene gun on skin was preferentially bias toward IgG1 production (Feltquate *et al.*, 1997). One reason to explain this is that when the DNA vaccine is administrated via the gene gun on skin, the DNA dose is much lower than in the case of intramuscular injection. Therefore, there may not be a sufficient amount of CpG motifs being administered. This has yet to be confirmed.

Finally, regarding the memory humoral response, it has been shown that mice vaccinated with DNA encoding an influenza viral HA antigen had levels of anti-HA antibodies comparable to or greater than those from convalescent sera of previously infected mice that persisted over 1 year (Martins *et al.*, 1995; Torres *et al.*, 1997). However, in other studies, plasmid DNA encoding a nucleoprotein of the LCMV virus administered intramuscularly failed to give appreciable antibody responses before viral challenge (Deck *et al.*, 1997). Thus, depending on the type of antigen used, DNA vaccination may be effective at inducing a long-term antibody response in some animal species.

B. Cellular immune response

As mentioned earlier, one of the advantages of the DNA vaccine is that it can induce cellular immune responses, including a CTL response, while it does not have the safety concern posed by other replicable vaccines, such as live virus. Both the CD4$^+$ T-cell response and the CD8$^+$ T-cell response (i.e., CTL) from DNA vaccination are discussed here.

Functionally, CD4$^+$ T cells may be divided into Th1 and Th2. Some researchers proposed a Th0 cell population, which has the function of both Th1 and Th2 cells. However, others believe that Th0 cells may just be the physical mixture of these two. Th1 cells produce IFN-γ exclusively, whereas Th2 cells produce IL-4, IL-5, and IL-13 exclusively. Also, the presence of Th1 cytokines facilitates the differentiation of CD4$^+$ T cells toward a Th1 phenotype and prevents the development of Th2, whereas the presence of Th2 cytokines allows for Th2 differentiation and prevents the differentiation of Th1 T cells. CD4$^+$ T cells can mediate at least three major functions. (1) Activated CD4$^+$ T cells promote B-cell survival and antibody production (Banchereau *et al.*, 1994). (2) CD4$^+$ T cells, through production of IL-2 and/or through CD40L-CD40 costimulation, provide helper function to CD8$^+$ T cells (Bennett *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998). (3) CD4$^+$ T cells secrete a variety of cytokines that regulate the resulting immune response as mentioned previously.

Because the CpG motif in bacterial DNA induces the production of a variety of proinflammatory cytokines, including IL-12, TNF-α, and INF-γ, it is understandable that the DNA vaccine generally skews the response toward Th1. Scientists have taken advantage of this property of the DNA vaccine to develop vaccines for tumor, which needs a strong Th1, especially CTL response to kill. Also, the DNA vaccine has been used to treat allergy, which is associated with a high-level production of the IgE antibody and Th2 cytokines. For example, Roy *et al.* (1999) developed a chitosan-encapsulated DNA vaccine for peanut allergy. Mice receiving chitosan-encapsulated DNA containing a dominant peanut allergen gene (pCMVArah2) produced secretory IgA and serum IgG2a (Roy *et al.*, 1999). Compared to nonimmunized mice or mice treated with "naked" DNA, mice immunized with chitosan-encapsulated DNA showed a substantial reduction in allergen-induced anaphylaxis associated with reduced levels of IgE, plasma histamine, and vascular leakage (Roy *et al.*, 1999).

The DNA vaccine is able to generate antigens endogenously, making them accessible to CD8$^+$ T cells via an MHC class I pathway. Numerous publications have demonstrated the induction of CTL response by DNA vaccines. Although the CTL response can also be induced by a live vaccine, it is difficult to induce with a protein-based vaccine. Of course, with a proper delivery system such as particles, a protein-based vaccine occasionally induces a CTL response (Falo *et al.*, 1995). Depending on the disease models used, the

magnitude of the CTL response from the DNA vaccine is, in most cases, comparable to that from a live viral vaccine. Also, the DNA vaccine can induce a CTL response against both dominant and subdominant epitopes. This may be useful for the development of a DNA vaccine for tumor immunotherapy. In their development process, tumor cells often become tolerated to the CTL response against the dominant epitopes of tumor-specific or tumor-associated antigens. Therefore, successful induction of CTL responses to the subdominant epitopes on these antigens should be helpful for tumor killing.

As to the memory cellular immune response, it has been shown that the frequency of antigen-specific CD4$^+$ T cells measured by proliferation remained elevated for 40 weeks postvaccination. Also, Raz et al. (1994) reported that a single intradermal needle injection of 0.3–15 μg of naked plasmid DNA induced an anti-influenza nucleoprotein-specific antibody and CTL that persisted for at least 68–70 weeks after vaccination (Raz et al., 1994). In a separate study, Davis et al. (1995) reported that a CTL response from the DNA vaccine to hepatitis B virus envelope proteins could be detected 4 months postvaccination. Also, Chen et al. (1999) showed that CTL activity specific for both dominant and subdominant epitopes on the Sendai virus nucleoprotein gene could be recalled readily 1 year after DNA vaccination and that the frequencies of CTL precursors specific for both of these epitopes were relatively high (Chen et al., 1999). Chattergoon et al. (2004) reported that coadministration of an IL-12 encoding plasmid pIL-12 with plasmid encoding HIV gp160 and influenza A hemagglutinin not only significantly enhanced the resulting CTL response, but also led to a more persistent CTL response.

In summary, depending on the route, antigen, species, and so on, a DNA vaccine may induce a very comprehensive and potent immune response.

VIII. CLINICAL TRIALS OF DNA VACCINES

The success of the DNA vaccine in a variety of small animal models propelled it into a number of human clinical trials for diseases or pathogens, including HIV (Boyer et al., 2000; MacGregor et al., 1998, 2000; Ugen et al., 1998; Weber et al., 2001), malaria (Epstein et al., 2004; Le et al., 2000; McConkey et al., 2003; Moorthy et al., 2003; Wang et al., 1998, 2004a), hepatitis B (Roy et al., 2000; Tacket et al., 1999), human papillomavirus (HPV) 16-associated anal dysplasia (Klencke et al., 2002), and many other cancers (Mincheff et al., 2000; Rosenberg et al., 2003; Tagawa et al., 2003; Timmerman et al., 2002). The selection of disease models for human clinical trials clearly showed that researchers were taking advantage of the ability of the DNA vaccine to induce cellular immune responses. As mentioned earlier, for a vaccine to be effective against those

Table 11.1. A Summary of Recent Human Trials with DNA Vaccines

Antigen	Pathogen or disease	Subjects	Route	Safety	Immune response	Clinical response	Reference
Env, Rev	HIV	15 asympto-matic HIV patients	im	Safe	Ab, CTL, T proliferative, IFN-γ, β-chemokine release	N/A	Boyle et al. (1997); MacGregor et al. (1998, 2000, 2002); Ugen et al. (1998)
gp120, gp160	HIV	4 asympto-matic HIV patients	im	Safe	No Ab response, Cellular response not reported	N/A	Weber et al. (2001)
Nef, Rev, Tat	HIV	9 asymptomatic patients	im	Safe	CTL	N/A	Calarota et al. (2001)
PfCSP	Malaria	20 healthy people	im	Few mild reaction	No Ab, CTL positive	N/A	Le et al. (2000); Wang et al. (1998)
PfCSP then RTS/AS02A	Malaria	24 healthy	im	Safe, well tolerated	Ab, CTL, Th	N/A	Epstein et al. (2004); Wang et al. (2004a)
TRAP and MVA	Malaria	63 people	im id	Safe	CTL, IFN-γ, Th1	Partial protection	McConkey et al. (2003); Moorthy et al. (2003)
HBsAg	HBV	7 healthy subjects	Powder-Ject	Well tolerated	No response	N/A	Tacket et al. (1999)

HBsAg	HBV	12 healthy subjects	Powder-Ject	Safe, well tolerated	Ab in 12/12 subjects, CTL, Th	N/A	Roy et al. (2000)
HPV 16 E7	HPV	12 anal dysplasia patients	im	Well tolerated	10/12 CTL	3/12 partial histological response	Klencke et al. (2002)
PSMA with CD86	Prostate cancer	26 prostate cancer patients	im	Safe	Partially shown signs of immunization	Cannot conclude	Mincheff et al. (2000)
CEA, HBsAg	Colorectal carcinoma	17 patients	im	Grade I injection toxicity	0/17 anti-CEA, 4/17 proliferative response to CEA, 6/8 anti-HBsAg	No	Conry et al. (2002)
Id linked to MsIg	Lymphoma	12 patients	im id	Good	6/12 humoral and T-cell responses	No	Timmerman et al. (2002)
gp100	Melanoma	22 patients	im id	Good	No response	No	Rosenberg et al. (2003)
Tyrosinase	Melanoma	26 stage IV patients	Intra-nodal infusion	Well tolerated	11/26 tetramer response	No but longer survival time	Tagawa et al. (2003)

diseases or pathogens, it is believed that a strong specific CTL response, as well as antibody response, is required. In general, the DNA vaccine was found to be safe in humans. It was well tolerated. Only very weak and minor local side effects were observed in a small portion of the participants. Depending on different routes of administration, humoral and cellular immune responses can be generated. However, the immune responses, especially antibody response, have not been encouraging in most of the trials. The following findings are from some reported DNA vaccine clinical trials. Table 11.1 provides a brief summary of some clinical trial data.

A. HIV vaccine

The first human trial of the DNA vaccine for the treatment of HIV infection was reported by MacGregor et al. (1998). In their study, a DNA-based vaccine encoding HIV-1 env and rev genes was tested for safety and host immune response in 15 asymptomatic HIV-infected patients. Successive groups received three doses of vaccine intramuscularly (30, 100, or 300 μg) at 10-week intervals in a dose-escalation trial. The vaccine induced no local or systemic reactions, and no laboratory abnormalities were detected. Specifically, no patient developed anti-DNA antibody or showed muscle enzyme elevations. No consistent change occurred in CD4 or CD8 lymphocyte counts or in plasma HIV concentration. Antibody against gp120 increased in individual patients in the 100- and 300-μg groups. Some increases were noted in CTL activity against gp160-bearing targets and in lymphocyte proliferative activity. For the 100-μg treatment, ELISA showed that binding of antisera from the subjects to gp120 was increased significantly after vaccination. When the binding of pre- and postvaccination antisera against a V3 loop peptide derived from gp130MN was measured, an enhancement of binding (at 1:4500 dilution) of 47.6% 8-week postvaccination was observed. These data demonstrated that the DNA vaccine boosted the antibody response in humans. In case of cellular immune responses, some changes were noted in CTL activity against gp160-bearing targets. Enhanced specific lymphocyte proliferative activity against the HIV-1 envelope was observed in multiple patients. The majority of patients who exhibited an increase in any immune parameters were within the 300-μg dose group, the group with the highest dose. In at least one of multiple assays, the six subjects who received the 300-μg dose had DNA vaccine-induced antigen-specific lymphocyte proliferative responses and antigen-specific production of both IFN-γ and β-chemokine. Furthermore, four of five subjects in the 300-μg dose group responded to both rev and env components of the vaccine. The responses did not persist within inoculated individuals and scored in different individuals at different times in the trial (Boyer et al., 2000; MacGregor et al., 1998, 2000, 2002; Ugen et al., 1998). Taken together, these studies show that the safety

profile for the DNA vaccine is excellent. Both humoral and cellular immune responses, including CTL, are inducible in humans. Also, the vaccine can stimulate multiple immune responses in vaccine-naive subjects when multiple antigens were encoded in the plasmid DNA.

In addition to the work by Weiner and colleagues, Weber et al. (2001) reported a phase I study of a HIV-1 gp160 DNA vaccine. Asymptomatic HIV-1-infected subjects with CD4$^+$ lymphocyte counts >500/μl were injected intramuscularly four times with 400 μg of HIV-1-modified gp160 env and rev coding DNA vaccine at 0, 4, 10, and 28 weeks. The DNA vaccine was safe and did not induce anti-DNA autoimmune antibodies. Vaccination had no long-term effects on the CD4$^+$/CD8$^+$ lymphocyte counts, plasma HIV-1 RNA concentrations, or disease progression. However, anti-gp120 and anti-gp160 antibody titers did not change significantly over a time period of 28 weeks and did not increase in response to vaccination compared to the baseline value. In other words, this DNA vaccination did not cause any significant antibody response (Weber et al., 2001). Calarota et al. (2001), however, reported that vaccination with a gene combination raised a broad HIV-specific CTL response (Calarota et al., 2001). In their trial, the efficacy of a combination of DNA plasmids encoding the nef, rev, and tat HIV-1 regulatory genes in inducing cellular immune responses was analyzed in asymptomatic HIV-1-infected patients. Patients initially selected for having low or no detectable immune responses to Nef, Rev, or Tat antigens developed MHC class I-restricted cytolytic activities as well as enhanced bystander effects. The most remarkable change observed after immunization with the gene combination was an increase in CTL precursors to target T cells infected with the whole HIV-1 genome (Calarota et al., 2001). An in vitro assessment of the expression of single and combined gene products showed that this was consistent with the induction of CTL responses in vivo.

B. Malaria vaccine

Malaria is an increasingly uncontrolled public health problem; 1–3 million people die annually from *Plasmodium falciparum* infection. A preventative vaccine is likely to be among the most effective means for its control. In 1998, Wang and colleagues reported the induction of antigen-specific CTL responses in humans by a malaria DNA vaccine. In their study, 20 healthy adult volunteers were enrolled in a phase I safety and tolerability clinical study of a DNA vaccine encoding a malaria antigen. Volunteers received three intramuscular injections of one of four different dosages (20, 100, 500, and 2500 μg) of the *P. falciparum* circumsporozoite protein (PfCSP) encoding plasmid DNA at monthly intervals and were followed for up to 12 months. Local reactogenicity and systemic symptoms were few and mild. There were no severe or serious

adverse events, clinically significant biochemical or hematologic changes, or detectable anti-DNA antibodies. The volunteers developed antigen-specific, genetically restricted, CD8^{+} T-cell-dependent CTLs. Responses were directed against all 10 peptides tested and were restricted by six human lymphocyte antigen (HLA) class I alleles. This was the first demonstration in healthy naïve humans of the induction of CD8^{+} CTLs by a DNA vaccine, including CTLs, that were restricted by multiple HLA alleles in the same individual. However, very disappointingly, despite the induction of excellent CTL responses, the DNA vaccination failed to induce detectable antigen-specific antibodies in any of the volunteers (Le et al., 2000; Wang et al., 1998). More recently, this group reported that patients who received the PfCSP DNA vaccine followed by a recombinant protein vaccine have antibody and CD8^{+} and CD4^{+} T-cell responses, suggesting that this heterologous prime-boosting approach might be viable (Epstein et al., 2004; Wang et al., 2004a). Hill and colleagues at the University of Oxford approached this by alternative dosing with a DNA vaccine and recombinant modified vaccinia virus Ankara (MVA) (McConkey et al., 2003; Moorthy et al., 2003). They showed that a heterologous prime-boost vaccination regime of DNA either intramuscularly or epidermally, followed by intradermal recombinant MVA, induces high frequencies of IFN-γ-secreting, antigen-specific T-cell responses in humans to a preerythrocytic malaria antigen, thrombospondin-related adhesion protein (TRAP). These responses are 5- to 10-fold higher than the T-cell responses induced by the DNA vaccine or recombinant MVA vaccine alone and produce partial protection manifest as delayed parasitemia after sporozoite challenge with a different strain of P. falciparum. DNA ME-TRAP and MVA ME-TRAP are safe and immunogenic for effector and memory T-cell induction. MVA ME-TRAP, with or without prior DNA ME-TRAP immunization, was more immunogenic and more cross-reactive in malaria-exposed individuals than in malaria-naive individuals. Both CD4^{+} and CD8^{+} T cells were induced by these vaccines (McConkey et al., 2003; Moorthy et al., 2003).

C. Hepatitis B vaccine

Tacket et al. (1999) reported a HBV DNA vaccine human trial in 1998. The study was designed to determine the safety and immunogenicity of a DNA vaccine consisting of a plasmid-encoding hepatitis B surface antigen (HBsAg) delivered by the PowderJect XR1 gene delivery system into human skin. Seven healthy adult volunteers received two immunizations on days 0 and 56. The vaccine was well tolerated. However, only one out of six seronegative volunteers developed high titers of persistent anti-HBsAg Ab after a single immunization (Tacket et al., 1999). They reasoned that the lack of immune response might be due to the extremely low DNA dose (0.25 μg) used.

A similar trial carried out by the PowderJect Vaccines Inc. produced quite different and encouraging results. Roy et al. (2000) reported an induction of antigen-specific $CD8^+$ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine into the skin. The needle-free PowderJect system was used to deliver gold particles coated with DNA directly into cells of the skin of 12 healthy, hepatitis-naive human volunteers. Three groups of four volunteers received three administrations of DNA encoding the surface antigen of HBV at one of the three dose levels (1, 2, or 4 μg). The vaccine was safe and well tolerated, causing only transient and mild to moderate responses at the site of administration. All the volunteers developed protective antibody responses of at least 10 mIU/ml. In volunteers who were positive for the HLA class I A2 allele, the vaccine also induced antigen-specific $CD8^+$ cells that bound HLA-A2/ HBsAg (335–343) tetramers, secreted IFN-γ, and lysed target T cells presenting a HBsAg CTL epitope (Roy et al., 2000). These results demonstrated that the DNA vaccine may induce protective antibody titers in humans, depending on how the DNA vaccine is administrated.

D. HPV 16-associated anal dysplasia

Klencke and colleagues (2002) at UCSF tested the PLGA microsphere-encapsulated DNA vaccine developed by Zycos, Inc. (Lexington, MA) in treating HPV 16-associated anal dysplasia. High-grade dysplasia induced by high-risk types of human papillomavirus (HPV) precedes invasive cancer in anal squamous epithelium just as it does in the cervix. In the trial, each subject was treated with four im injections of 50–400 μg of ZYC101 at 3-week intervals. The plasmid DNA in the ZYC101 encodes for multiple HLA-A2-restricted epitopes derived from the HPV-16 E7 protein, one of two HPV oncoproteins (E6 and E7) consistently expressed in neoplastic cells. Twelve eligible subjects with HPV-16 anal infection and a HLA-A2 haplotype were enrolled in the study. ZYC101 was well tolerated in all subjects at all dose levels tested. Three subjects experienced partial histological responses, including 1 of 3 subjects receiving the 200-μg dose and 2 subjects at the 400-μg dose level. Using a direct Elispot, 10 of 12 subjects demonstrated an increased immune response to the peptide epitopes encoded within ZYC101; each continued to show elevated immune responses 6 months after the initiation of therapy.

E. DNA vaccine for cancer immunotherapy

Because it requires a CTL response to kill tumor cells, the ability to induce a cellular immune response by the DNA vaccine makes it attractive in developing cancer vaccines. Advancement in biochemical and genetic techniques in the

last decade has facilitated the discovery of a great number of tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs). In most animal studies, the tumor DNA vaccine has proven to be very effective, although the tumors were grafted artificially in almost all the cases. These successful preclinical data propelled some tumor DNA vaccines into human trials.

In 2000, Mincheff and co-workers published their results from a trial of naked DNA and adenoviral immunizations for immunotherapy of prostate cancer. The prostate-specific membrane antigen (PSMA) was used as the tumor antigen. Immunizations included extracellular human PSMA DNA as well as human CD86 DNA into separate expression vectors (PSMA and CD86 plasmids) and into a combined PSMA/CD86 plasmid. In addition, the PSMA gene was inserted into a replication-deficient adenoviral expression vector. Twenty-six patients with prostate cancer were entered into a phase I/II toxicity-dose escalation study. Immunizations were performed intradermally at weekly intervals. Doses of DNA between 100 and 800 μg and of recombinant virus at 5×10^8 PFUs per application were used. They found no immediate or long-term side effects following immunizations. All patients who received the initial inoculation with the viral vector followed by PSMA plasmid boosts showed signs of immunization, as evidenced by the development of a delayed-type hypersensitivity reaction after the PSMA plasmid injection. In contrast, of the patients who received a PSMA plasmid and CD86 plasmid, only 50% showed signs of successful immunization. Of the patients who received PSMA plasmid and soluble granulocyte–macrophage colony-stimulating factor (GM-CSF), 67% were immunized. However, all patients who received the PSMA/CD86 plasmid and soluble GM-CSF became immunized. Patients who did not immunize during the first round were later successfully immunized after a boost with the viral vector. The heterogeneity of the medical status and the presence of concomitant hormone therapy in many patients did not permit unequivocal interpretation of data with respect to the effectiveness of the therapy. However, several responders, as evidenced by a change in the local disease, distant metastases, and PSA levels, were identified.

In 2002, Conry et al. reported safety and immunogenicity results from a dose-escalation clinical trial of a dual expression plasmid encoding a carcinoembryonic antigen (CEA) and HBsAg in 17 patients with metastatic colorectal carcinoma (Conry et al., 2002). CEA was selected as a prototypic tumor-associated self-antigen, and the HBsAg cDNA was included as a positive control for an immune response to the DNA vaccine without relying on breaking tolerance to a self-antigen. Groups of 3 patients received escalating single im doses of the DNA vaccine at 0.1, 0.3, and 1.0 mg. Subsequent groups of 3 patients received three repetitive 0.3- or 1.0-mg doses at 3-week intervals. A final group of 2 patients received three repetitive 2.0-mg doses at 3-week intervals. Toxicity was limited to transient grade 1 injection site tenderness, fatigue, and creatine

kinase elevations, each affecting a minority of patients in a nondose-related manner. Repetitive dosing of the DNA vaccine induced HBsAg antibodies in 6 out of 8 patients, with protective antibody levels achieved in 4 of these patients. Although 4 of 17 patients developed lymphoproliferative responses to CEA after vaccination, CEA-specific antibody responses were not observed in any subject, echoing the results from other human clinical trials mentioned earlier. Also, this study shows that the antibody response is dependent on the antigen used. HBsAg is known to be very antigenic. This is probably why anti-HBsAg Ab was detected in all patients while anti-CEA Ab was not detected in any individual. No objective clinical responses to a DNA vaccine were observed among this population of patients with widely metastatic colorectal carcinoma.

Levy and colleagues reported results from a DNA vaccine encoding a chimeric idiotype in patients with B-cell lymphoma (Timmerman *et al.*, 2002). B-cell lymphomas express tumor-specific immunoglobulin, the variable regions of which [idiotype (Id)] can serve as a target for active immunotherapy. The safety and immunogenicity of naked DNA Id vaccine in 12 patients with follicular B-cell lymphoma were investigated. The DNA encoded a chimeric immunoglobulin molecule containing variable heavy and light chain immunoglobulin sequences derived from each patient's tumor, linked to the IgG2a and κ mouse immunoglobulin (MsIg) heavy and light chain constant region chains, respectively. Patients in remission after chemotherapy received three monthly im injections of the DNA in three dose-escalation cohorts of 4 patients each (200, 600, and 1800 μg). After vaccination, 7 of 12 patients mounted either humoral ($n = 4$) or T-cell-proliferative ($n = 4$) responses to the MsIg component of the vaccine. In one patient, a T-cell response specific to autologous Id was also measurable. A second series of vaccinations were then administered using a needle-free injection device (Biojector) to deliver 1800 μg both im and intradermally (id); 9 of 12 patients had humoral ($n = 6$) and/or T-cell ($n = 4$) responses to MsIg. Six of 12 patients exhibited humoral and/or T-cell anti-Id responses; however, these were cross-reactive with Id proteins from other patient's tumors. Subsequently, a third series of vaccinations were carried out using 500 μg of human granulocyte–macrophage colony-stimulating factor DNA mixed with 1800 μg of Id DNA. The proportion of patients responding to MsIg remained essentially unchanged (8 of 12), although humoral or T-cell responses were boosted in some cases. Throughout the study, no significant side effects or toxicities were observed.

The result from Rosenberg *et al.* (2003) in NCI on a DNA vaccine encoding gp100 melanoma–melanocyte antigen in patients with metastatic melanoma reported in 2003 was very disappointing. In their study, 22 patients with metastatic melanoma were randomized to receive plasmid DNA either intradermally ($n = 10$) or intramuscularly ($n = 12$). One patient (4.5%)

exhibited a partial response of several subcentimeter cutaneous nodules. All other patients had progressive disease. Of 13 patients with cells available before and after immunization, no patient exhibited evidence of the development of an anti-gp100 cell response using *in vitro* boost assays. They were unable to demonstrate significant immunologic or clinical responses to plasmid DNA encoding the "self" nonmutated gp100 tumor antigen.

The study carried out by Tagawa *et al.* on melanoma DNA vaccine encoding tyrosinase epitopes was more encouraging (Tagawa *et al.*, 2003). Groups of eight stage IV melanoma patients each received 200, 400, or 800 μg of DNA intranodally by pump over 96 h every 14 days for four cycles. Blood was collected for immunologic assays and to measure plasmid in serum prior to treatment and 4 and 8 weeks later. Scans and X-rays were performed at baseline and after 8 weeks. It was found that the treatment was well tolerated, with only five patients demonstrating grade 1–2 toxicity. Vaccination by 96-h infusions of plasmid into a groin lymph node resulted in only one episode of catheter leakage in 107 cannulations. Detection of plasmid in serum was rare and transient in two patients. Immune responses by a peptide-tetramer assay to tyrosinase amino acids 207–216 were detected in 11 of 26 patients. Although clinical responses were not seen, survival of the heavily pretreated patients on this trial was unexpectedly long, with 16 of 26 patients alive at a median follow-up of 12 months.

The failure of DNA vaccine human trials for tumor agrees well with the reported tumor immunotherapy human trials with other vaccines. Possible reasons include the crippled immune system of cancer patients, especially after their chemotherapy and/or radiotherapy. In addition, tumors have developed a great number of escaping mechanisms, such as the loss of MHC I molecules, which makes the tumor cells unresponsive to CTL killing.

In summary, many human clinical trials have been reported in the past several years on DNA vaccine against quite a few disease models. Although they have all shown that the DNA vaccine is safe or causes very minor side effects, the resulted immunological responses have not been encouraging with no or low antibody response detected in most of the cases, often no clinical response, and somewhat weak cellular response in some trials. Therefore, research on how to transfer the success of DNA vaccine in small animals into large animals and humans is urgently needed.

IX. SAFETY ISSUES

Although the DNA vaccine was thought to be safer than the traditional live (viral) vaccine and phase I clinical trials reported no serious side effects from the DNA vaccine, there still are several concerns, including the integration of

plasmid DNA into the host genome, induction of autoimmune response, induction of tolerance instead of immunity, and the effect of the CpG motif on the overall long-term immune response. Organizations such as FDA, WHO, and the European Union have already had their guidelines on the regulation of the DNA vaccine. The documents listed the manufacturing, preclinical, and human clinical issues relevant to the development of the DNA vaccine and described potential safety concerns that vaccine developers should address prior to the initiation of clinical trials. Several of these issues mentioned earlier are discussed briefly.

A. Plasmid integration

Similar to gene therapy, there is widespread concern that plasmid DNA might integrate into the host genome and increase the chance of malignant transformation, genomic instability, or cell growth dysregulation. However, for many years, the integration of plasmid DNA in host genome was not reported, making it difficult to reach a regulatory consensus concerning the magnitude to the problem (Gurunathan *et al.*, 2000). It is well known that it is not technically easy to remove free plasmid DNA from the host genomic DNA. Wang *et al.* (2004b) clearly showed the integration of plasmid DNA into mouse genome, especially after intramuscular DNA electroporation (Wang *et al.*, 2004b). For needle-injected mice, only about 17 copies of plasmid DNA were detected in 1 μg of genomic DNA. This agrees well with previous reports. It was indicated early that 3–30 copies of a DNA vaccine plasmid were associated with host genomic DNA 2 months after intramuscular needle injection (Ledwith *et al.*, 2000; Martin *et al.*, 1999). The calculated mutation rate from 3 to 30 copies per genome is 3000 times lower than the spontaneous mutation rate of 10^{-5} per cell. However, because the plasmid DNA copies in genome DNA were detected by polymerase chain reaction (PCR), PCR may not be able to detect all the integrations such as those short fragments. In addition, the effect from the integration of long stretches of plasmid DNA containing strong regulatory sequences might be more significant than that from the small spontaneous point mutations.

This integration rate, however, was sharply increased to about 980 copies/μg genomic DNA in the case of electroporated DNA (Wang *et al.*, 2004b). Electroporation increased the plasmid tissue level by approximately 34-fold. Using a quantitative gel purification assay for integration, electroporation was found to markedly increase the level of plasmid associated with genomic DNA. To confirm the integration and to identify the insertion sites, Wang *et al.* (2004b) developed a new assay referred to as repeat-anchored integration capture (RAIC) PCR, which is capable of detecting rare integration events in a complex mixture *in vivo*. Using this assay, they identified four

independent integration events. Sequencing of the insertion sites suggested a random integration process, but with short segments of homology between the vector breakpoint and the insertion site in three of the four cases. This highlights the dilemma scientists are facing. Ways to improve the efficiency of the DNA vaccine such as electroporation might at the same time increase the risk (integration) of the DNA vaccine. Therefore, it is necessary to continue monitoring the integration rate of the DNA administration approaches prior to entrance into a human clinical trial.

B. CpG effect and autoimmune response

As noted earlier, the CpG motif in the plasmid DNA biases the immune response toward Th1. This could be deleterious when a humoral response is required and therefore increases the susceptibility of the host to infections that need Th2 responses. In addition, the Th1-biased response in long term might lead to the development of Th1-mediated organ-specific autoimmune diseases. Segal et al. (1997) reported that in a murine model, the CpG motif, by enhancing the production of IL-12, promoted the development of experimental allergic encephalomyelitis, a Th1-dependent organ-specific autoimmune disease. Klinman et al. (1999) reported that when repeatedly administering (ip) CpG ODN two to four times a month, the animals remained healthy and developed neither macroscopic nor microscopic evidence of tissue damage or inflammation. In contrast, Heikenwalder et al. (2004) reported that repeated CpG ODN administration led to lymphoid follicle destruction and immunosuppression. These conflicting data clearly show that more thorough studies need to be carried out to investigate the long-term effect of the CpG motif.

C. Tolerance

In 1997, Mor and colleagues reported that the DNA vaccine encoding the circumsporozoite protein of the malaria induces tolerance rather than immunity when administered to 2- to 5-day-old mice, although it induced a strong protective immune response against live sporozoite challenge in adult BALB/c mice (Mor et al., 1996). Neonatally tolerized animals were unable to mount antibody, cytokine, or cytotoxic responses when rechallenged with the DNA vaccine in vitro or in vivo. Tolerance was specific for immunogenic epitopes expressed by the vaccine-encoded, endogenously produced antigen. This confirmed that in neonatals, an endogenously presented antigen from the DNA vaccine might be viewed by the neonatal as self. However, further studies pointed out that whether tolerance or immunity will develop is dependent on many parameters, such as dose, recipient's age, and the nature of antigen (Ichino et al., 1999). For example, for influenza, rabies, and HBV, no tolerance was

observed, even in neonatals. Finally, a decreased response was also observed in aged mice (over 2 years old), suggesting that the DNA vaccine might be not effective in elderly people (Bender *et al.*, 1998). This is probably due to the generally weaker immune system in elderly people.

X. CONCLUSION

Since its first discovery in 1992, great advances have been made in the DNA vaccine field. The DNA vaccine has proven to be very successful for many diseases in small animal models. However, recent clinical trials have shown that it is far from being effective in humans. Research on how to transfer the success of DNA vaccines in small animal to human is thus needed.

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