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# **DNA Vaccines for Prophylactic or Therapeutic Immunization Against Hepatitis B Virus**

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### **Abstract**

DNA vaccines, with which the antigen is synthesized *in vivo* after direct introduction of its encoding sequences, offer a unique method of immunization that may overcome many of the deficits of traditional antigen-based vaccines. By virtue of the sustained *in vivo* antigen synthesis and the comprised stimulatory CpG motifs, plasmid DNA vaccines appear to induce strong and long-lasting humoral (antibodies) and cell-mediated (T-help, other cytokine functions and cytotoxic T-cells) immune responses. In animal models, DNA vaccines against hepatitis B virus (HBV) give humoral and cell-mediated immunity superior to that of the current traditional antigen-based vaccines, indicating the possibility of a more effective prophylactic vaccine against HBV. Furthermore, DNA vaccines can overcome tolerance to and expression of HBV proteins in a transgenic mouse model of the HBV chronic carrier, opening up the possibility of an effective therapeutic DNA vaccine to treat chronic carriers of HBV.

**Key Words:** DNA vaccine, hepatitis B, immunotherapy, antibody, cytotoxic T-lymphocyte.

### **What Is a DNA Vaccine?**

Traditional vaccines against infectious diseases involve administration of some form of the pathogen itself. This can be either the whole pathogen in a killed (e.g., by treatment with formaldehyde or heat) or attenuated (i.e., rendered nonpathogenic through growth under abnormal conditions or genetic alteration) form. Alternatively, the vaccine may comprise a subunit of the pathogen, such as the envelope protein used in the current vaccines against the hepatitis B virus (HBV). Such subunit vaccines may be purified (e.g., from the plasma of HBV chronically infected individuals) or produced artificially in cell lines as a recombinant protein (e.g., the current HBV vaccine from yeast cells).

In contrast to the traditional antigen-based vaccines, DNA vaccines involve introduction of plasmid DNA encoding the antigen. A plasmid is a double-stranded, closed circular form of DNA that can be produced easily in bacteria and then purified by chromatography. Upon injection into the body, some of the DNA enters cells of the body, where it enters the nucleus and, without integration into the genome, uses the cell's machinery to direct synthesis of the encoded antigenic protein. Since the body makes the antigen itself, it is in a sense its own vaccine factory.

The gene incorporated into the DNA vaccine can encode one or more complete proteins, or alternatively, only a portion of a protein that contains B- and/or T-cell epitopes. Since one can easily choose which genes or parts of genes to include, it is possible to exclude any gene related to virulence or toxicity. In addition to antigen-encoding sequences, DNA vaccines also contain genetic elements that permit or facilitate growth of the plasmid in bacteria (e.g., a bacterial origin of replication to allow amplification of large quantities of plasmid DNA in bacteria, and a prokaryotic selectable marker gene such as an antibiotic resistance gene to ensure that only bacteria containing the plasmid can survive and divide in culture). In addition, DNA vaccines permit expression of the antigen in the body (e.g., promoter and enhancer sequences to drive transcription and a transcription termination element to ensure appropriate termination of the expressed mRNA and polyadenylation).

### **Why Do We Need New and Better Vaccines?**

#### **What Is the Goal of Vaccination?**

The goal of vaccination is to induce immune responses against components of the pathogen, so that infection or disease will be prevented or attenuated if the real pathogen is encountered. Two general types of immunity may be induced in response to an antigen, namely humoral immunity (the production of antibodies) and cell-mediated immunity (CMI), which involves a number of different cellular responses.

Antigen-specific antibodies are produced by B-cells that recognize and bind circulating, soluble antigen via surface antibody receptors. This process alone results in secretion of T-independent antibody isotypes (IgM and IgG3). However, for induction of the longer-lasting, high affinity T-dependent antibodies (e.g., IgG1, IgG2a), B-cells must be further stimulated by cytokines secreted from activated T-helper ( $T_H$ ) cells (see below). Some activated B-cells become plasma cells that provide long-term memory.

CMI is the result of activated T-cells that recognize short segments of antigen (peptides) presented on cell surfaces via molecules encoded by the major histocompatibility complex of genes (MHC). Class I MHC presentation occurs on any nucleated cell in which there is endogenous synthesis of an antigen (e.g., infected cells). Some T-cells (usually CD8+) are activated by such MHC-class I antigen-presenting cells to differentiate into cytotoxic T-lymphocytes (CTL), although the influence of cytokines secreted by  $T_h$  cells is required for them to be fully potent. CTL can act on infected cells presenting antigen by class I MHC by both cytolytic mechanisms (e.g., secretion of perforin, which puts holes into the cell membrane) and non-cytolytic mechanisms (e.g., secretion of cytokines that prevent virus replication).

Other T-cells (usually CD4+) are activated to become  $T_h$  cells by recognizing antigen presented on class II MHC molecules that are found exclusively on professional antigen-presenting cells (APCs) such as macrophages and dendritic cells.  $T_h$  cells secrete cytokines and are of two basic types.  $T_h1$  cytokines (e.g., IL-2, IFN- $\gamma$ , IL-12) stimulate both CTL and B-cells, while  $T_h2$  cytokines (e.g., IL-4, IL-5, IL-10) act predominantly on B-cells.  $T_h1$  cytokines can also have direct effects on viral replication or assembly (1, 2).

Ideally, a vaccine will safely induce both antibodies and CMI. While humoral immunity alone is sufficient for prophylaxis against some diseases (e.g., HBV), CTL are still desirable and are absolutely essential for many diseases. In particular, treatment of chronic infections such as HBV requires CMI.

### **What Are the Shortcomings of Current Antigen-Based Vaccines?**

Vaccines containing a live pathogen, either an attenuated (e.g., hepatitis A vaccine) or related but less virulent (e.g., vaccinia for smallpox) version of the virulent strain, are usually highly effective at inducing a full range of immune responses by virtue of the intracellular synthesis of antigen with the controlled "infection." The major disadvantage of these vaccines is the possibility of reversion to a virulent form, especially in immunocompromised individuals (e.g., neonates, HIV-infected or malnourished individuals). In addition, not all pathogens may be attenuated (3).

On the other hand, whole-killed or subunit vaccines do not induce intracellular synthesis of antigen, and because of this there is poor or absent presentation of antigen on class I MHC, and thus poor induction of a CTL response. CMI may be further compromised when the vaccine is administered with an adjuvant. Aluminum hydroxide (alum), which is the only adjuvant currently licensed for human use, has a strong  $T_h2$  bias that effectively abrogates CTL responses (4). Subunit vaccines pose no risk of inadvertent infection but tend to have poor immunogenicity without adjuvants. On the other hand, whole-killed vaccines may be virulent with incomplete inactivation or have poor immunogenicity due to the inactivation procedure (e.g., denaturation of epitopes by heat or formaldehyde). In addition, not all pathogens can be grown in tissue culture, making it impossible to produce a whole-killed vaccine from them (3).

Thus, current antigen-based vaccines have several important shortcomings. Although such

vaccines have proven highly effective against many diseases, there still remain numerous diseases for which it has not been possible to produce effective and safe vaccines (3, 5). Also, even for diseases for which effective vaccines exist, it may be desirable to develop less expensive, heat-stable, multivalent, single-dose vaccines for use in less-developed countries. DNA vaccines have the potential to overcome many of the limitations of antigen-containing vaccines.

### **What Are the Advantages of DNA Vaccines?**

DNA vaccines, by virtue of the *in vivo* synthesis of antigen, induce both humoral and cell-mediated immunity, and this without the risk of inadvertent infection. Thus, they offer the efficacy of a live, attenuated vaccine with the safety of a subunit vaccine. They also offer, at least in animal models, the possibility of long-lasting immunity without boost (6). This is probably due, at least in part, to the prolonged antigen synthesis. Finally, by virtue of immunostimulatory sequences within their backbone (i.e., CpG motifs, discussed below), they are their own adjuvant and generally induce the highly desirable  $T_H1$ -type responses that include CTL and cytokines that can control viral replication.

Other advantages of DNA vaccines may be important for vaccine delivery to less-developed areas of the world. These include the low cost of production, the relative ease of manufacturing (with a single process for all DNA vaccines), and the heat-stability of DNA, obviating the need for a “cold chain.” Also, it should be possible to make multivalent vaccines against several pathogens by cloning genes encoding different antigens into a single vector (for co-linear expression) or by mixing different plasmids together.

### **How Do DNA- and Protein-Based Vaccines Compare?**

Disease-specific, DNA-based immunization has now been demonstrated in animal models against numerous viral, bacterial and parasitic human and veterinary diseases (7, 8). In almost all cases, strong antibody and CTL responses have been noted, and, where it has been possible to test, protection against live pathogen challenge. Our work has concentrated on the development of DNA vaccines against HBV (9), and the remainder of this review will provide an overview of preclinical evaluation of DNA vaccines and protein vaccines with DNA adjuvants for prophylactic or therapeutic immunization against HBV.

The surface coat of HBV is encoded by a single open reading frame divided into three domains (pre-S1, pre-S2 and S) by three separate ATG start codons. As such, three different polypeptides may be made: the small or major surface protein (S), the middle protein (pre-S2 + S) and the large protein (pre-S1 + pre-S2 + S). The HBV envelope contains mostly S protein, with lesser amounts of the larger forms. The B- and T-cell epitopes of the envelope are known collectively as hepatitis B surface antigen (HBsAg) (10).

The current prophylactic vaccine against HBV contains recombinant S protein with alum as adjuvant. When injected into mice, it induces antibodies against HBsAg (anti-HBs) but usually not

CTL, owing to the T-helper type 2 ( $T_H2$ ) bias of the alum. On its own, recombinant HBsAg induces lower levels of anti-HBs and weak CTL (4). In comparison, a DNA vaccine encoding the S gene of HBV, when injected intramuscularly (IM) in mice, induces high titers of anti-HBs and strong CTL responses. Even after a single injection of DNA, high antibody titers are maintained for essentially the life of the mouse (6, 11, 12). HBV DNA vaccines have also been shown to be effective in chimpanzees (13, 14).

In very young mice, protein vaccines are less effective than in adult mice, owing to the immaturity of their immune system. However, DNA vaccines, which can continue to produce antigen until the immune system is mature enough to respond to it, induce strong humoral and cell-mediated responses even in mice as young as one day old. In such young mice, there is no response with a protein-alum vaccine (15).

### **What Makes DNA Vaccines So Effective?**

There are probably three reasons why DNA vaccines are so effective: (i) the *in vivo* synthesis of antigen, which likely occurs in both professional APCs and non-APCs (e.g., skin fibroblasts and muscle cells), leads to appropriate presentation of antigenic peptides in the context of class I and II MHC molecules; (ii) the prolonged synthesis of antigen, which may be “self-boosting”; and (iii) the presence of immunostimulatory CpG motifs in the DNA backbone.

### **Antigen Presentation**

Although direct transfection of APCs is not absolutely essential (16), only professional APCs are able to present the antigen that induces CMI (16–19). Also, it is likely that direct transfection of some APCs (e.g., dendritic cells) does occur. Immediate removal of the target muscle after IM injection of DNA does not diminish CTL responses (20), indicating that plasmid leaves immediately, perhaps via the lymphatic system to regional lymph nodes where it can directly transfect APCs. Removal of the skin target site during the first few days does abrogate the immune response (20), indicating that transfected APCs in the skin do not track immediately to lymphatic tissue. Delivery of DNA vaccines to the skin has been shown to directly transfect APCs (i.e., Langerhans' cells) (21). While APCs are essential for initiating immune responses, it is probable that antigen released from other transfected cells (e.g., muscle) can help boost antibody production by B-cells.

### **Longevity of Antigen Expression**

In theory, antigen expression from plasmid DNA can continue for a long period, and in a post-mitotic cell such as the muscle fiber, indefinitely. However, in fact, antigen-expressing muscle cells are destroyed around 10–20 days after DNA injection by an immune-mediated mechanism, since it does not occur in mice with severe combined immunodeficiency (SCID) (6). It is likely that antigen-specific CTL destroy antigen-expressing cells by virtue of their presentation of antigenic peptides on MHC class I molecules.

### **Immunostimulatory CpG Motifs**

CpG motifs are unmethylated cytosine-guanine dinucleotides commonly found in bacterial but not vertebrate DNA; they cause potent activation of the innate immune system. It appears that the mammalian immune system has evolved the capability to recognize and respond to such CpG motifs as an early immune activation to help fight off bacterial infection. Bacterial DNA or oligonucleotides containing CpG motifs (CpG ODN) cause B-cell proliferation and immunoglobulin secretion, monocyte cytokine secretion, and activation of natural killer (NK) cell lytic activity and increased cytokine secretion (especially  $T_h1$  cytokines such as IFN- $\gamma$ , TNF- $\alpha$  and IL-12) (22, 23). CpG ODN can also act to augment antigen-specific responses owing to: (i) a synergy of CpG effects on B-cells and B-cell activation through antigen-receptors, (ii) direct effects on APCs (i.e., dendritic cells), such as up-regulation of co-stimulatory molecules, which can improve T-cell activation, and (iii) induction of cytokines that in turn augment both antibody and CTL responses. Since plasmids contain many CpG motifs, DNA vaccines can act as their own adjuvant, and indeed it has been demonstrated that DNA vaccines are dependent on the presence of such motifs to be effective (24–26).

We have also evaluated CpG ODN with recombinant HBsAg in mice and show it to be a potent adjuvant for induction of  $T_h1$ -type responses, including CTL and predominantly IgG2a antibodies (4). The  $T_h1$  response dominates over the  $T_h2$  response of alum when the two adjuvants are administered together. Furthermore, CpG ODN can redirect a previously established (with alum)  $T_h2$  response to a  $T_h1$  response, even in very young mice (15).

### **What Is the Need for a DNA Vaccine Against Hepatitis B?**

Hepatitis B poses a serious worldwide problem (27). Most infected adults recover completely, sometimes without even realizing that they were infected, and only a small proportion (< 1%) may die from fulminant hepatitis. A more serious problem is that of chronic infection, with 5–15% of infected adults and 90–95% of infected newborns failing to clear infection. There are an estimated 300 million individuals chronically infected with HBV today; and in addition to serving as reservoirs of HBV, they run a greatly increased risk of developing cirrhosis and hepatocellular carcinoma (10).

There are highly effective prophylactic vaccines available for HBV; however, these usually require three doses over 6 months, and that is unacceptably long for persons at risk of exposure to HBV (e.g., health care professionals). Even after three doses, up to 15% of individuals (depending on age and immune status) do not respond adequately, and in at least some cases, this is genetically determined (28–30). Based on animal studies, DNA vaccines may be able to induce immunity more quickly and in a greater proportion of the population. In particular, DNA vaccines may be more effective for immunization of newborns, especially those born to chronic carrier mothers. Furthermore, the protein vaccine is relatively costly to manufacture, making it prohibitively expensive for delivery to some of the poorer countries. DNA vaccines are inexpensive to manufacture, and delivery to less-developed areas of the world is facilitated by the fact that they are heat stable and do not require refrigeration.

There is currently no effective treatment for chronic HBV infection. Chronic carriers of HBV who eventually overcome infection, either spontaneously or as a result of interferon therapy, acquire a broad range of anti-HBV immune responses similar to those developed by persons recovering from acute infection. These include HBV-specific CTL and T<sub>h</sub>1 cytokines, which may be important for non-cytopathic control of viral replication. Successful immunotherapy for chronic HBV infection will likely involve induction of such T<sub>h</sub>1-like immune responses. DNA vaccines may be one approach that can induce such immune responses. Of particular interest is the fact that DNA vaccines are especially effective in inducing CTL. Although not essential for protective immunity against HBV, CTL may be important for avoiding or overcoming the chronic carrier state. Indeed, many previously infected individuals, even years after clinical and serological recovery, have traces of HBV in their blood and HBV-specific CTL that express activation markers indicative of recent contact with antigen (31). These results suggest that sterilizing immunity may not occur after HBV infection and that chronic activation of CTL is responsible for keeping the virus under control.

Transgenic mice that constitutively express HBsAg in the liver are a model of the neonatally infected “healthy” chronic HBV carrier, in that they are tolerant to the high levels of circulating antigen and do not produce any anti-HBs antibodies. DNA-based immunization of such HBsAg-transgenic mice by IM injection of HBsAg-expressing DNA results in a rapid appearance of anti-HBs antibodies and a concomitant loss of circulating antigen, which is largely due to down-regulation of transgene expression in the liver (32). This appears to be T-cell dependent (both CD4+ and CD8+) and B-cell independent, based on results after passive transfer of cells from normal mice immunized with HBsAg-expressing DNA. The T-cell effect is predominantly non-cytolytic, since there is no elevation of liver enzymes in the serum, nor is there any histological evidence of liver necrosis at the time that circulating HBsAg is rapidly decreasing. It is probably due to a cytokine-mediated effect, since in a similar transgenic model it was shown that passive transfer of HBsAg-specific CD8+ CTL caused a transient down-regulation of HBV transgene expression which was mediated in a predominantly non-cytolytic fashion by IFN- $\gamma$  and TNF- $\alpha$  (1, 2, 33, 34).

### **What Are the Safety and Regulatory Issues?**

Several issues are being carefully considered as DNA vaccines go into clinical trials.

### **Possibility of Tolerance to Foreign Antigen**

One of the initial worries was that continuous expression of the antigenic protein (in particular in muscle, which is post-mitotic) might induce immunological tolerance. This was based on the finding that luciferase reporter gene expression could continue for nearly two years in muscles of mice (35). However, luciferase appears to be an exceptional protein that is very poorly immunogenic, at least in mice. After IM injection of plasmid DNA encoding an antigenic protein such as HBsAg, expression continues for only a few weeks, owing to immune-mediated destruction of the antigen-expressing cells (36). In any event, tolerance has never been demonstrated for any of the many animal models of DNA vaccines developed to date.

### **Possibility of an Integrative Event**

Another safety concern is the possibility that the DNA may integrate into the host's chromosomal DNA and cause an insertional mutagenic event by activation of a proto-oncogene or by inactivation of a tumor suppresser gene. The likelihood of an insertional mutagenic event is low, since: (i) most injected DNA is rapidly degraded in the extracellular space; (ii) the plasmid DNA vectors are designed to remain episomal; (iii) most non-integrated DNA would soon be lost during subsequent cell division; and (iv) integration is not possible in mature muscle fibers as they are permanently post-mitotic. The incidence of an insertional mutagenic event is estimated to be  $10^3$  times lower than the rate of spontaneous mutagenesis (37).

### **Possibility of Immune Response to DNA and Induction of Autoimmune Disease**

Plasmid DNA can induce the production of antibodies against single- but not double-stranded DNA; however, anti-ssDNA antibodies are not pathogenic, and the fear that they could contribute to undesired autoimmune reactions against the host's DNA has not materialized, even in lupus-prone animal models (38–40).

### **Other Unwanted Immunostimulatory Effects**

The adjuvant effect of CpG motifs appears to be a desirable and necessary event for the induction of strong immune responses with DNA vaccines. Nevertheless, there may be situations where a strong  $T_H1$  stimulatory effect that includes IL-12 induction is undesirable, such as in persons prone to autoimmune disease.

### **How Do DNA Vaccines Work in Humans?**

Encouraged by the extremely good responses obtained in animal models, researchers have carried out several clinical trials using DNA vaccines (41–44). However, results have been disappointing, indicating the need to further improve DNA vaccines. Increased efficacy could result from improvements in efficiency of transfection, in expression of antigen, or in the induction of immune responses against the antigen.

### **Efficiency of Transfection**

Pure plasmid DNA does not transfect with a high efficiency many cell types other than mature skeletal muscle fibers. The use of delivery systems, especially targeted delivery systems, may help overcome this. In particular, it may be desirable to target DNA vaccines to APCs, since it is now clear that antigen must be presented by professional APCs. However, any delivery system will most likely increase the cost and complexity of vaccine manufacturing.

### **Increased Expression of Antigen**

Changes in vector design may allow increased expression of antigen by having stronger promoters or increased stability of the mRNA through the addition of introns.

### **Enhanced Immune Responses to Expressed Antigen**

Various strategies to enhance immune responses following DNA-based immunization have

been reported. These include the addition of CpG immunostimulatory motifs (24–26) and the use of other non-DNA adjuvants (45). It is also possible to improve immune responses through the co-expression of cytokines (46, 47) or co-stimulatory molecules (47, 48).

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