

DNA Vaccines for Emerging Infectious Diseases: What If?

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A novel and powerful method for vaccine research, colloquially known as DNA vaccines, involves the deliberate introduction into tissues of a DNA plasmid carrying an antigen-coding gene that transfects cells *in vivo* and results in an immune response. DNA vaccines have several distinct advantages, which include ease of manipulation, use of a generic technology, simplicity of manufacture, and chemical and biological stability. In addition, DNA vaccines are a great leveler among researchers around the world because they provide unprecedented ease of experimentation. To facilitate diffusion of information, an Internet site has been established called THE DNA VACCINE WEB (URL:<http://www.genweb.com/dnavax/dnavax.html>). In this review, a brief survey is undertaken of the experimental models and preclinical work on DNA vaccines to contribute to a greater awareness of the possibilities for emerging infectious diseases.

"But there is no single problem that is more pressing than our fast-deteriorating relations with the microbial world" wrote Barbara Culliton (1), the editor-in-chief of *Nature Medicine*, at the end of 1995. This stark statement concluded her comments on, among other threats, the reemergence of cholera and plague, the growing number of Lyme disease cases, and humanity's occasional but frenetic duels with Ebola virus. What if one day an Ebola-infected traveler makes it to the boarding lounge and embarks on an airplane? As pointed out by David Heymann, director of the World Health Organization's new Division of Emerging Diseases, the virus would spread to far corners of the world (2) with dramatic consequences. This scenario has already been played out with human immunodeficiency virus (HIV). Vaccines have traditionally been used as weapons against health threats. In the case of HIV infection, the basis for one has not yet been clearly delineated. For scientific, commercial, and practical reasons vaccines cannot solve all the problems posed by emerging infectious organisms. However, novel and powerful methods for vaccine research, and possibly for vaccines themselves, hold some promise in our efforts to curb emerging disease threats. These methods involve the deliberate introduction of a DNA plasmid carrying a protein-coding gene that transfects cells *in vivo* (albeit at a low efficiency) and expresses an antigen causing an immune response (3). This procedure, known as a DNA vaccine, is perhaps better described as

DNA-mediated or DNA-based immunization, with the understanding that the objective is not to raise an immune response to the DNA itself.

This method is conceptually sound and experimentally straightforward; however, its most novel aspect is that it works at all! It was not expected that pure plasmid DNA could be taken up by cells, after parenteral introduction in a simple saline solution (4), to levels allowing expression of enough protein to induce an immune response. A more radical method of introducing DNA involves the bombardment of DNA-coated gold particles. When applied to the skin, these particles produce good immune responses with much less DNA than is required by other routes, such as intramuscular or intradermal needle injection (5). More esoteric still is the application of pure DNA solution (as nose drops) to the nasal membranes, which has been reported to work (5) but is perhaps too inefficient for further consideration.

DNA vaccines have distinct advantages: They can be manufactured far more easily than vaccines composed of an inactivated pathogen, subcellular fraction, or recombinant protein. Since almost all plasmids can be manufactured in essentially the same way, substantial economies of scale can be achieved. DNA is very stable and resists temperature extremes; consequently, the storage, transport, and distribution of DNA-based vaccines are more practical and less expensive. In addition to the commercial, there are vaccine research and development considerations. It is now possible to change the sequence of an antigenic protein, or to add heterologous epitopes, by simply introducing mutations to the plasmid DNA. The immunogenicity of the modified protein can be directly

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assessed after injecting the plasmid DNA. This simple method could increase considerably our understanding of the immune response to antigens.

In addition, both in vaccine research and in actual use, DNA-mediated immunization is the great leveler among researchers around the world. It is easy to use because once the protein coding sequences are cloned into a suitable expression vector, the direct introduction of the plasmid vector (into mice for example) allows experimental assessment of the immune response and its consequences, without further experimental steps such as the preparation of a recombinant protein as antigen.

Because it is so straightforward and requires only simple molecular biologic techniques, the method should be practical in many laboratories around the world. It would be unrealistic to deny that certain diseases are not of great interest to large pharmaceutical firms. Moreover, even when triumphs over disease are achieved, as in the case of hepatitis B virus (HBV) infection (6), marketing strategies can exclude the vaccine from regions where it would do the most good. DNA-mediated immunization can be used in countries that cannot implement more complicated and expensive strategies.

What if DNA vaccine research could be carried out worldwide by a generic technology, where human creativity adds substantial value to the work? This short review outlines the possibilities offered by DNA-mediated immunization.¹ I will review experimental models and preclinical work on DNA vaccines and discuss new developments that are based on the idea that DNA delivery can be used to induce immune response to proteins.

Animal Models of DNA Vaccines

Various experimental models of DNA vaccination have been reported (Table 1). Most of the pathogens studied have been viruses, which is consistent with the method used: since the genes transferred by the plasmids require the host cellular machinery to be expressed, DNA-based immunization most resembles a virus infection. However, genes from other microorganisms have also been used with success. The types of polypeptides expressed are often the envelope proteins of viruses, but various proteins have been used. Indeed, it is not obvious what aspects of a protein produce an effective immune response by this un-

usual method of antigen delivery.

The immune responses obtained are clearly broad-based when they have been well characterized. In several models, antibodies are reproducibly induced, and the antibodies ultimately are of the immunoglobulin G (IgG) type, indicating a T-dependent class switch. In the case of immunization for HBV surface antigen, the fine specificity of the humoral response in mice mimics, to a certain extent, that observed during infection in humans (12). T-cell proliferation and cytokine secretion have been studied in several models, and the cytokine profile indicates a Th1 type response, characterized by the secretion of interleukin-2 and γ -interferon. The immune responses can be remarkably long lasting; however, the duration of the immune response does not appear to have any deleterious effects on the animals because they are protected against challenge long after immunization (41). Also, in some cases, as in the DNA-mediated immunization against HBV surface antigen, antibody levels reach a plateau at titers of about 10^4 and remain stable for at least 18 months. If the mice are boosted with a second DNA injection at 7 months, a further 10-fold increase in titer can be obtained (14).

Cytotoxic T lymphocytes (CTL) are invariably induced to class I epitopes of the proteins encoded by the transferred genes. These CTL responses can be quite strong (10), and DNA-mediated immunization can circumvent haplotype-linked nonresponsiveness (13). Cytotoxic immune responses are thought to be important in clearing viral infections because this type of T-cell response allows the immune system to recognize virally infected cells and destroy them, thus removing the virus.

What is surprising about the induction of CTL with DNA-mediated immunization is that either professional antigen-presenting cells have been successfully transfected, or the free protein can be processed more effectively with CTL induction than with a classical immunization protocol. A large body of immunologic evidence suggests that only professional antigen-presenting cells can prime T lymphocytes in the first stages of the immune response. Thus, the transfection of muscle fibers, for example, would not normally be expected to lead to such efficient immune responses. Solution of this enigma should provide insight into some fundamental immune processes. In the meantime, DNA-based immunization is an excellent and

¹ An Internet site, THE DNA VACCINE WEB (URL <http://www.genweb.com/Dnavax/dnavax.html>), provides journal references, abstracts, experimental protocols, available plasmids, and other information about DNA vaccines.)

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Table 1. Animal models of DNA vaccines

Pathogen	Antigen	Animal species	Reference
Bovine herpesvirus	Glycoprotein	Cattle, mouse	(7)
Hepatitis B virus	Capsid protein (core antigen)	Mouse	(48)
Hepatitis B virus	Envelope protein (surface antigen)	Mouse Rabbit Rat Chimpanzee*	(3, 8-14, 53)
Hepatitis C virus	Core/nucleocapsid	Mouse	(12, 15)
Herpes simplex virus	Glycoprotein B Glycoprotein D ICP27	Mouse [*]	(16-18)
Human immuno-deficiency virus-1	Envelope glycoprotein gp160 Noninfectious particles	Mouse Nonhuman primates	(19-22)
House dust mite	Allergen	Rat*	(52)
Influenza virus	Hemagglutinin Matrix protein Nucleoprotein	Chicken* Ferrets* Mouse* Nonhuman primates*	(23-29)
<i>Leishmania major</i>	Major surface glycoprotein	Mouse*	(50)
Lymphocytic choriomeningitis virus	Glycoprotein Nucleoprotein	Mouse ^{*@}	(30-32)
<i>Mycobacterium tuberculosis</i>	<i>M. leprae</i> hsp65	Mouse	(33)
<i>Mycoplasma pulmonis</i>	<i>M. pulmonis</i> DNA <i>M. pulmonis</i> DNA expression library	Mouse*	(34, 35)
Papillomavirus	Major capsid protein L1	Rabbit*	(51)
<i>Plasmodium yoelii</i>	Circumsporozoite protein	Mouse	(36-38)
Rabies virus	Glycoprotein	Mouse*	(39-41)
Simian immuno-deficiency virus	Env, gag	Monkey	(49)
<i>Schistosoma japonicum</i>	Paramyosin (Sj97)	Mouse	(42)

The symbols | and * refer to reports of partial or complete protection, respectively, to challenge by the infectious agent. In one case, enhanced immunopathology (@) after intracranial viral challenge has been reported (32).

Updated versions of this table can be found on THE DNA VACCINE WEB site (URL <http://www.genweb.com/Dnavax/dnavax.html>) where links are provided to the Medline abstracts of most references. See also a compendium of recent articles on DNA vaccines in a special issue of the Annals of the New York Academy of Sciences, Volume 772 (New York Academy of Sciences Conference: "DNA Vaccines: A Novel Approach to Vaccination," Arlington, VA, April 7-9, 1995). The Medline abstracts of the articles in this issue can be found on THE DNA VACCINE WEB.

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simple tool to achieve CTL responses for which efficient methods have been needed for years.

One of the first uses of DNA-mediated immunization was to induce such cytotoxic cellular immunity to a conserved protein of influenza A virus to determine if overcoming its seasonal variation was possible. Ulmer et al. (26), obtained cytotoxic T lymphocytes directed against the conserved influenza nucleoprotein, which protected mice against the disease, even when they were challenged with a heterologous virus carrying a different hemagglutinin but the same nucleoprotein sequence.

The crucial point, however, is the ability of DNA-based immunization to protect animals from infection upon challenge, and this has been demonstrated in several model systems, particularly with influenza and rabies viruses, as well as *Mycoplasma pulmonis* and *Plasmodium yoelii*. Donnelly et al. (29) have shown that a mixture of plasmids can be used to induce antibody to the influenza hemagglutinin surface protein and cytotoxic immunity to the viral nucleoprotein and matrix protein. This DNA vaccine protected ferrets and African green monkeys against viral challenge by using an antigenically distinct human influenza virus more effectively than the contemporary commercial vaccine. The influenza model has now been the subject of much preclinical work, and human trials are in progress.

One human trial uses plasmid vectors expressing HIV-1 genes delivered to HIV-seropositive persons by intramuscular injection. This protocol uses Marcaine to facilitate DNA uptake, although the mechanism of this effect has not been clearly delineated (20). More clinical trials will likely be initiated since plasmid DNA is now considered an innocuous substance compared with other genetic vectors used in therapy.

Further Questions on Methods

Most studies listed in Table 1 have used intramuscular needle injection to deliver DNA; however, intradermal particle bombardment works very well (provided that one has access to biostatic apparatus). Intradermal particle bombardment may ultimately work better than intramuscular injection in primates, including humans, since extensive connective tissue in muscle may impede DNA diffusion and transfection in larger mammals that do not lead sedentary lives in cages.

In most of our own experimental work (8-12), we have used a protocol that induces muscle regeneration and increases the number of transfected

muscle fibers (43), presumably because of improved diffusion of the DNA and better transfection efficiency of immature fibers. The actual protocol is probably not suitable for routine prophylactic vaccination in humans, although it could be considered for therapeutic immunization. Nonetheless, this model system shows that muscle fibers can take up DNA better under some circumstances, and further understanding of this process may improve formulations for DNA uptake in muscle. Such improvements will almost certainly be a prerequisite for intramuscular DNA vaccination in humans. The improved uptake of DNA in regenerating muscles composed of immature fibers also suggests that newborn mammalian muscle may also take up DNA more effectively than adult muscle. Since improving childhood vaccination programs is a goal, this characteristic may be an important consideration in the use of DNA vaccines, if they can be used for children.

The regeneration protocol is particularly useful for scientists who are beginning research with DNA-mediated immunization. The use of available control plasmids to inject DNA for validating the procedure is also an important consideration. The various steps that can be taken to achieve reproducible immunization with plasmid DNA transfer have been discussed (44). Careful attention to the details of the intramuscular injection protocol (43,45) can make a substantial difference in the outcome.

Vaccine Development and the Limits of the Protective Immune Response

A successful vaccine must confer protective response to the recipient, and therefore, the limits of the immune response must be known. This knowledge can be purely empirical, as in the case of the first polio vaccines for which the precise protective epitopes were not known. Such knowledge is not required when using a killed or attenuated viral preparation. In contrast, for recombinant vaccines a single protein should induce an immune response that will provide sterilizing immunity; this is also true for DNA vaccines and represents a major conceptual limitation in the use of this approach for vaccination. Although mixtures of individual proteins or their genes can be envisioned, in the case of recombinant protein vaccines this would be prohibitively complicated and expensive. For DNA vaccines this is far easier to imagine since the injected material is always DNA, and no matter what genes are carried by the plasmid, the production process is the same.

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The only human vaccine that uses a recombinant protein as its basis is that against HBV, which has been used for nearly 10 years. Such a high-tech vaccine was possible because empty viral particles, from the plasma of persons chronically infected with HBV, could be purified and used to induce humoral immunity against the so-called surface antigen that would protect against infection. The plasma-derived particles are still used as a vaccine in many countries, in part because of the cost of the recombinant product (6). The envelope proteins of viruses are always good targets for inducing protective immunity.

However, this rational clearly breaks down in the case of a virus, such as HIV, which mutates so rapidly, apparently in response to immune pressure. The fundamental knowledge required to determine what would comprise a potential HIV vaccine is still lacking, and therefore, it is unlikely that a vaccine can be developed until further basic research provides more insight. Thus HBV and HIV illustrate two extremes in vaccine development: with HBV a simple antibody response to a single antigen neutralizes the virus, whereas with HIV some form of cytotoxic immune response is probably necessary. DNA-mediated immunization has a role to play in further research.

Expression Library Immunization

An attempt has been made to use DNA-mediated immunization to develop a systematic method for producing a vaccine. Barry et al. (34) took advantage of the very small amounts of DNA required with the particle bombardment method (5,46). Since a single nanogram of DNA coated on the gold particles can induce an immune response, one microgram can potentially introduce a thousand different genes. On this basis, a library of gene fragments was prepared from *Mycoplasma pulmonis* by cloning the genomic DNA into a plasmid expression vector. Since this organism has a relatively small genome (about 10^6 base pairs), enough of the total DNA protein-coding sequences might be expressed to induce immunity to the pathogen. Since only a small part of the genetic complement of the organism is expressed and expression is mostly from only a fragment of genes and not entire functional proteins, pathogenic effects would be avoided, while all the advantages of broad-based immunity produced by a DNA vaccine would be present.

Protection against *M. pulmonis* has been achieved after immunization with different expression libraries (34). The next steps could consist of

using the method to screen for the gene or genes responsible for protective immunity. Seen in this light, this approach solves the problems of having a more general way to determine the limits of protection, which is vital in establishing what the protein or DNA composition of a vaccine would be. The search for a single gene, or a small number of genes, should be facilitated by the use of the DNA-based immunization method. However, if many gene products are required to confer protection, defining the correct mixture may not be straightforward.

This discussion raises another consideration. From several points of view (at least regulatory and manufacturing), a vaccine must contain defined components. Therefore, the expression library immunization approach cannot be used with simple mixtures of uncharacterized gene fragments. Although in principle it is possible, would anyone be prepared to have a human vaccine composed of an undefined mixture of HIV or Ebola virus gene fragments that seemed to confer protection in animal models? Once again, the simple method means that further creative research may rapidly provide insight into vaccine design.

What If an Ebola Outbreak Threatens the World?

Let us take the most provocative scenario to illustrate what can perhaps be done, in principle and in practice, in the face of a rampantly infectious viral disease. When preventive measures against an agent like Ebola virus are needed on an emergency basis, speed is imperative. The filoviruses Ebola and Marburg are extremely pathogenic, causing a fulminating febrile hemorrhagic disease; they grow fast, kill most cells, and the infected person bleeds to death, usually within 48 hours of infection.

These viruses are enveloped filamentous particles with a nonsegmented negative-strand RNA. The genes of both members of the filovirus genus, the Marburg virus group and the Ebola group, have already been cloned and sequenced. There does not, as yet, seem to be any great variation among the proteins sequences for a given virus; however, Ebola and Marburg are not highly related serologically. The filoviruses are similar to paramyxoviruses, such as the respiratory syncytial virus.

This basic molecular information is enough for testing the ability of one or more of the genes to induce an antibody and CTL response that might help protect against disease. One would choose genes whose proteins are not responsible for the

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pathology of the virus, but this should be relatively easy, since the growth potential of the virus largely accounts for its extreme toxicity. One option is the gene encoding the glycoprotein, a viral membrane protein requiring glycosylation for its native structure. Such glycosylation might be important for the antigenicity of the protein if it is to mimic the filamentous envelope of the virus. Here, the fact that DNA vaccination involves synthesis of the protein in the cells of the mammalian recipient of the DNA vaccine means that the appropriate glycosylation pattern can be produced.

If Ebola or Marburg, or a related filovirus, escaped a restricted ecologic niche, the following scenario might unfold. If the emerged virus is found (by serologic testing) to be one of the existing, characterized ones, existing cloned genes could be used. Otherwise, a virus isolate would need to be obtained, and the genes would need to be cloned. Cloning can be accomplished easily since related genes are available for probes in molecular cloning experiments.

Since one strategy for prophylactic vaccination is to induce a humoral response to the outer coat of the virus and block entry to the cells, cloning would allow an attempt to quickly counter the virus infection or at least to slow down its spread in the organism or within the population. A full-length envelope glycoprotein gene could be isolated from the cloned virus genome (perhaps by polymerase chain reaction) and cloned directly into a suitable DNA vaccine plasmid vector. A GMP production run could be carried out, perhaps within a week, and DNA vaccine could begin to be distributed to areas most critically at risk.

The above scenario assumes that DNA vaccines will be accepted for use in humans, which seems likely for high-risk situations, and that the basic method for preparing GMP-grade plasmid DNA is available, which is currently the case. Since August 1995, a collaboration between Qiagen (Hilden, Germany) and Pharos (Seraing, Belgium) has offered full-GMP production of plasmid DNA to companies and the scientific community in general. The molecular method needed to rapidly go from a virus isolate to the vaccinating plasmid vector should represent only a relatively minor bottleneck to the development of an urgently needed vaccine, at least compared with any other approach used before. It is not surprising that Bernard Dixon, writing in *Bio/Technology* (47), has called DNA vaccines "the third vaccine revolution."

This review poses questions about DNA vaccines and suggests that the answers lie in new methods of research and development. If DNA-mediated immunization were used in all countries that have expertise in molecular biology, novel vaccines would be developed. Ultimately, a major goal of the DNA vaccine approach for public health might well be to bring vaccine development within the reach of researchers working on infectious disease problems in which there is no great commercial interest. If DNA vaccines come into widespread use for public health applications, vaccines for many diseases could be produced rapidly since, in the end, the product is simply a DNA plasmid.

What if such a method were used for human vaccination? The cost of production and delivery of vaccines would be reduced, thus allowing vaccines to reach areas of the world somewhat deprived of preventive public health measures, particularly the recent biotechnologic methods. If new infectious diseases appear in the future, as they surely will, perhaps these new tools will be used to combat them more effectively.

Today's research method can be tomorrow's vaccine. DNA vaccines will be within the means of many more populations and countries since full GMP production technology will be both simpler than technology for other products and far more available to research scientists. A little more than 2 years stood between the first published description of a DNA vaccine and the beginning of the first clinical trial, which indicates that the necessary infrastructure for producing DNA vaccines was rapidly put in place. But more importantly, this short time span bodes well for the ability of public health agencies all over the world to bring scientific research to bear on diseases relevant to their own situation and to disease prevention.

Dr. Whalen is director of research, French National Center for Scientific Research. His previous work at the Pasteur Institute in Paris, France, concerned gene expression in muscle tissue, which serendipitously led to the study of DNA-mediated immunization that used the hepatitis B surface antigen as a model. Dr. Whalen collaborated closely with Drs. Heather Davis, Loeb Institute for Medical Research and the University of Ottawa, Ottawa, Canada, and Marie-Louise Michel and Maryline Mancini, both of the Pasteur Institute.

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