Nominations from FDA's Center for Biologic Evaluation and Research

FDA INITIATIVE TO STUDY THE SAFETY OF DNA-BASED PRODUCTS (CBER)

INTRODUCTION

DNA-based products are the fastest growing segment of CBER's product portfolio, numbering >200 as of January 1, 1998. These products include: 1) DNA vaccines designed to cure cancer and to prevent infectious diseases (including AIDS, tuberculosis and malaria), 2) synthetic oligonucleotides (oligos) that inhibit the development of allergies, 3) antisense molecules that correct abnormalities in gene function, and 4) gene therapy products for a broad range of indications, including the correction of in-born errors of metabolism, protection of host cells from chemotherapy, and stimulation of protective immune responses.

DNA-based products are manufactured in a variety of forms, including plasmids (injected intramuscularly, encapsulated in liposomes, or coated onto gold microspheres), synthetic oligos (alone or conjugated to protein antigens) and viral vectors (e.g., retrovirus-, vaccinia- and adenovirus-based). Uptake of DNA-based products by host cells leads to the expression of the self or foreign protein encoded by the DNA. DNA plasmids have a number of advantages over other biological products, including ease and expense of manufacture and stability. Although large numbers of DNA-based products are under development or in clinical trials, none have yet been licensed by the FDA

Early studies of DNA-based products involved critically ill patients in whom long-term safety was a secondary issue. More recently, DNA vaccines and synthetic oligos have been developed for use in relatively healthy children and adults, making long-term safety a dominant concern. Unfortunately, conventional pre-clinical toxicological studies are poorly suited to evaluate the life-long toxicity of DNA-based products. The FDA has limited authority to require long-term studies to assess the non-acute safety risks arising from the use of biologics, although there is a critical need for such studies, as described below. Moreover, the bulk of the gene therapy and DNA vaccine community consists of academic sponsors and small biotechnology firms that lack the resources to perform long term large-scale toxicity studies for each product. Of equal concern, the FDA cannot direct sponsors to share and/or publish their results, making it difficult to reach a regulatory consensus on these safety issues. For these reasons, the FDA has a compelling need to perform in-house studies examining the long-term safety of DNA-based products.

SPECIFIC SAFETY ISSUES

ISSUE 1: PERSISTENCE AND INTEGRATION

DNA-based products are capable of persisting long-term *in vivo* and/or integrating into the host's genome. The former could result in prolonged, unregulated, ectopic production of self or foreign proteins, while the latter could result in mutation, the induction of genetic instability, translocations, gene activation and/or gene inactivation.

There is evidence that DNA delivered via retroviral vectors can integrate into the host cell's genome and behave as insertional mutagens. Similarly, plasmid DNA vaccines enter host cells where they can persist long term and integrate. A variety of pathogenic consequences could result from such integration events, including cell transformation leading to tumor formation. Given the intended use of DNA-based products (such as vaccines) in children, the life-long risk to the recipient posed by such integration events is of considerable concern.

ISSUE 2: GONADAL DISTRIBUTION

Studies have shown that many DNA-based products can reach gonadal tissue (even when administered to presumably sequestered sites). For example, DNA vaccines delivered intramuscularly or intradermally reach the gonads where they persist for days to months. This raises the possibility of reproductive toxicity, dysgenesis of germ calls, and the risk of transmission of altered genetic material to subsequent human generations.

ISSUE 3: ECTOPIC PROTEIN PRODUCTION AND ABNORMAL IMMUNE ACTIVATION

Both viral vectors and DNA vaccines carry genes that stimulate host cells to secrete self and foreign proteins. The production of such proteins can persist long term, is not physiologically regulated, and cannot be terminated should deleterious side effects arise. In addition, immunomodulatory sequences are present in most DNA-based products that stimulate strong inflammatory responses. Not surprisingly, immune deviation and organ-specific autoimmunity have been observed in animals treated with DNA-based products. These findings underscore concern that DNA-based therapy could promote the development of autoimmune disease and jeopardize immune homeostesis.

CONCLUSIONS

Preclinical studies suggest that DNA-based products may yield breakthroughs in the treatment of cancer, the prevention of infectious diseases, and the correction of inborn errors of metabolism. Yet only limited efforts have been directed towards identifying and quantifying the long-term risks posed by these biologic products. The FDA has the unique responsibility of evaluating the risks and benefits of this class of biologic and determining whether clinical trials should proceed. With this responsibility in mind, CBER scientists have developed research proposals to address these critical safety concerns. Our projects involve state-of-the art single-cell assays and PCR technology to detect and tract minute quantities of DNA, and animal models designed to monitor immune function and tumor development. Support is required to: i) evaluate the distribution and duration of DNA-based products *in vivo*, ii) monitor their rate of integration and their mutagenicity, iii) set limits on the frequency of adverse events resulting from the distribution and uptake of DNA to somatic and germline cells, and iv) determine whether ectopic expression of DNA-encoded proteins adversely affects the integrity of the immune system or other critical host functions. These studies will establish both the short- and long-term safety profile of DNA-based products, the level of risk associated with exposure to these agents, and provide insight into the manifestations of these risks including the key attendant biologic processes that are associated with toxicity.

DNA Based Therapies

Introduction

DNA-based therapies are currently being developed for the treatment of a wide range of human diseases. Examples include plasmid DNA encoding one or more antigenic proteins for vaccines against viral and bacterial pathogens, triplex forming synthetic oligonucleotides to modulate gene expression, antisense oligonucleotides and ribozymes to selectively inactivate mRNA's, and viral vectors for gene therapy. Although three therapies show significant promise, by their very nature, all pose risk of interacting with the host genome or disrupting normal cellular processes in unexpected and unpredictable ways with potentially adverse consequences. Therefore, it is essential to identify hazards and potential risks associated with therapies prior to widespread clinical application. The potential for DNA-based therapies to persist makes evaluation of long-term safety a top priority. A major target population for many of these therapies (i.e., DNA vaccines) will be healthy young children who may harbor DNA constructs over a significant portion of their life span. At the present time, FDA has only limited authority to require evaluation of non-acute long-term safety from sponsors. Moreover, the majority of manufacturers of DNA-based products are small biotechnology companies and academic sponsors that lack resources to perform long-term large-scale studies for each of their products. Therefore, FDA is launching an initiative to study the long-term safety of DNA-based products. As part of that initiative, they are interested in conducting long-term animal studies on DNA based products. The object of this document is to provide a general outline of how such studies might be conducted and a framework within which to consider how NIEHS/NTP might best participate in this effort.

Background

Synthetic Oligonucleotides

Oligonucleotides based on the natural occurring phosphodiester linkage are subject to extensive nuclease degradation *in vivo* and are not considered useful as potential therapies. As a result, oligonucleotides currently being developed for clinical applications are based on modifications of the phosphate ester linkage which render them less sensitive to nuclease digestion; these include oligonucleotides based on phosphotriester, phosphoramidate, phosphorothioate, methylphosphonate, and oligonucleotides synthesized from ✂-anometric nucleotide monomers. In general, synthetic oligonucleotides bind efficiently to the targeted RNA/DNA sequences and are more resistant to nuclease cleavage than phosphodiester-based nucleotides. However, oligonucleotides containing modified linkages are ultimately degraded within a time frame of hours to days and therefore clinical use will require repeated administration of these substances. The product of oligonucleotide cleavage by nucleases will be modified nucleotide monomers that are potential substrates for normal cellular polymereses. Therefore, there is a potential risks that incorporation of the modified nucleotides into DNA could lead to mutations. There is also the possibility that oligonucleotides could produce unanticipated responses by acting as decoys that bind to

proteins involved in normal cellular regulation and compete for binding to the natural target sequence.

DNA Vaccines

Plasmid DNA vaccines are constructed from bacterial plasmids and may carry one or more genes that code for specific protein antigens in addition to elements necessary for selection and replication in bacteria and eukaryotic sequences necessary for gene expression. The use of plasmid DNA for vaccines is based on the observation that plasmids injected directly into skeletal or cardiac muscle persist extra-chromosomally in muscle cells and express their encoded genes. Plasmids are also taken up by cells in the skin after intradermal injection; however, muscle and skin appear to be unique in this respect since it has not been possible to demonstrate similar uptake of plasmid DNA by other tissues. The persistence of plasmid vaccines and the continued expression of protein antigen, as well as the potential to integrate into the host genome resulting in insertional mutagenesis, are potential risks associated with the use of plasmid DNA for vaccines.

Viral Vectors

Viral vectors offer the possibility of transferring genes into nearly any cell type or tissue with the potential for long term stable transgene expression. Vectors based on retroviruses and adenoviruses have been the most widely used. Retroviral vectors efficiently integrate a DNA copy of their genome directly into the host genome ensuring long-term stability and transmission of the transgene to cell progeny. Since all viral genes have been partially or completely deleted during construction of the vector, only an intact transgene is integrated. Vectors based on the Maloney Murine Leukemia Virus have been widely used, but the inability of these vectors to transduce non-dividing cells has prompted the development of vectors based on other retroviruses such as lentiviruses (HIV-1) which are capable of transducing terminally differentiated as well as proliferating cells. Since retroviral integration into host cell DNA occurs at apparently random genomic locations, insertional mutagenesis is a potential risk associated clinical use of retroviral vectors.

Vectors based on adenoviruses and adeno-associated viruses effectively transduce all types of dividing and non-dividing cells, which is considered their major attribute. They are also considered safe; interic-coated capsules containing adenovirus have been widely used m oral vaccines, and more than 90% of the adult human population has been infected by adeno-associated virus. Vectors based on adeno-associated virus integrate at a preferred site on human chromosome 19 provided certain viral genes are present in the vector. In the absence of the viral genes, integration may occur at random genomic locations, or the DNA may remain extra-chromosomal. Adenoviral vector DNA rarely integrates into the host genome but instead remains in the nucleus as a non-replicating extra-chromosomal element. Since the vector is not passed on to daughter cells or may be lost, repeated administration may be necessary for long-term gene therapy. Vectors based on both adeno- and adeno-associated virus contain intact viral genes. Low level expression of viral genes may effect normal cellular processes or target the transduced cells for immune destruction. The presence of viral genes also increases the risk of generating replication competent virus through recombination with wild type virus.

Insertional mutagenesis associated with integration of vector DNA is also a potential long-term risk.

Long Term Safety Issues

Although significant development of DNA-based products will continue, the current generation of constructs contains fundamental features that may change only slightly in the future. Therefore, it is important to begin establishing a database in experimental animals against which future generations of constructs, as well as human data, may be compared. The FDA has identified three major safety issues associated with prolonged exposure to the current generation of DNA based products that should be evaluated in long-term animal studies.

1.) The intracellular persistence of DNA-based products and the possibility and consequences of integration into the host cell gnome.

2.) The possibility that gonadal tissue will be exposed, the consequences of long-term persistence in the gonads and potential for germline integration and transmission.

3.) The consequences of long-term exposure to self or foreign proteins, whose production may not, in general, be subject to normal physiological controls.

General Outline of Animal Studies

To address these issues the FDA has proposed three general studies:

1.) Evaluate the influence of routes of administration and formulation on the distribution and persistence of DNA plasmids and synthetic oligonucleotides.

2.) Evaluate the potential for DNA used therapeutically to alter cytokine production and the associated immune milieu.

3.) Evaluate the safety and immunogenicity of DNA vaccines in pregnant women and newborns.

Rationale and Experimental Outline

A number of the potential adverse effects of long-term exposure to DNA-based products may involve infrequent events, e.g., plasmid DNA integration. Therefore, the basic approach is to maximize the probability of detecting an adverse response by administering the test agent at high doses. If there is no response under a worst case scenario, then a rather low level of risk would be suggested. If a positive response is observed, then the conditions that led to that response could be evaluated in more detail to determine under what conditions this response might signal potential risks of similar events in humans. The general experimental procedure would be as follows:

1.) Administer the test article by the clinically relevant routes under conditions that maximize

uptake of the vector. The route of administration used will depend on the specific product under evaluation. Typically, synthetic oligonucleotides are administered by intramuscular or intravenous injection, DNA vaccines are administered by intramuscular or intradermal injection, and viral vectors are administered by *ex vivo* exposure, producer cell injection, or direct injection of a vector solution into the target tissues or organs.

2.) Verify uptake and function of the test article in target tissues within a short time (hours or days) after administration. For oligonucleotides, this might also include evaluation of the metabolism, disposition, and pharmacokinetics if this information is not available.

Verifying uptake and function requires demonstrating the presence and function of the test article in the target issue. For an antisense synthetic oligonucleotides targeted to a specific mRNA, this would require demonstrating reduced levels of the specific mRNA and gene product in cells of the target tissue. For a Plasmid DNA vaccine, this would mean demonstrating expression of the encoded protein antigen in cells of the target tissue and determining whether the plasmid was integrated or extra-chromosomal. Similarly, for viral vectors, it would be necessary to confirm expression of the therapeutic transgene in cells of the target tissue and determine whether the vector DNA has been integrated.

3.) In longer-term studies, monitor for toxic and/or carcinogenic response, persistence and function of the test article in selected tissues, and other end points such as immune response parameters, at periodic time points.

For plasmid vaccines and viral vectors, this will involve monitoring expression of the encoded protein(s) and determining whether integration has occurred. Immunomodulatory oligonucleotides, plasmid DNA vaccines, and all vectors have the potential to alter normal immune homeostasis and, therefore, the impact of long-term exposure on a number of immune response parameters, e.g. cytokine production, must be evaluated at regular intervals. To determine a toxic or carcinogenic response, it will be necessary to examine selected tissues for the presence of pathology associated with exposure to the test agent. A tumor response observed during, or at the end, of the study may require determining whether viral or plasmid integration into the host cell genome was a factor. In general, this will entail examining tumor cell DNA for inserts, and if present, determining whether the tumors are clonal for the insertion site.

Animal Models

Mice have been used extensively for investigating DNA-based therapies. This animal model offers the advantages of being a well characterized immune system and the use of relatively small quantifies of test agent is an advantage. The unreliability of immunological reagents for rats and the need to use much greater quantities of test agent make rats a less desirable choice for long-term studies.

Therapies such as antisense and triplex forming oligonucleotides designed to target a specific mRNA or gene would best be evaluated in an animal model of the disease or condition that the product was designed to treat. Studies of these therapies, in the appropriate animal model, would provide information about the consequences of long-term **i**nhibition of expression of a specific

gene or exposure to the specific agent. The results of such studies would be most relevant to the human population suffering from the specific disease or condition.

For other DNA-based therapies such as immunomodulatory oligonucleotides or DNA vaccines intended for use in the general populations, long-term studies address more generic issues such as persistence, disruption of immune homeostasis, or insertional mutagenesis. These are often associated with the general class of therapy, e.g. DNA vaccines, rather than the response to a specific member of the class. It is this latter type of study that is being addressed and for these types of studies a more general animal model would be acceptable.

Because of the extensive historical control database, the $B6C3F_1$ mouse is well suited for use in long-term studies, especially studies that will examine long-term persistence of DNA-based products and for studies used to examine associated immune response parameters. BALB/c mice have been used extensively in immnunological studies and have also been used in a number of long-term studies and therefore would be useful in studies intended primarily to evaluate the impact of treatment on immune function.

Another potentially useful model for which there is a historical control date base is the p53 deficient (+/-) mouse. Loss or inactivation of the remaining wild type allele at the p53 locus as a result of integration of plasmid DNA, proviral DNA from a retroviral vector, or adenoviral DNA would lead to the development of a tumors within a relatively short time period. Therefore, this model has the potential of providing an estimate of the frequency of integration at a specific genomic locus. In addition, the p53(+/-) deficient mouse represents a model of humans carrying a comparable germ line alteration who may be potential candidates for gene therapy. For example, this provides the possibility of examining whether p53 deficiency, and/or the presence of an inactive, altered p53 allele would predispose these mice to greater risk from exposure to plasmid DNA vaccines or oligonucleotide therapies than "normal" wild type B6 mice.

CBER RECOMMENDED RESEARCH PROJECTS THAT EXAMINE THE SAFETY OF DNA-BASED THERAPEUTIC AGENTS

A. Potential of DNA Used Therapeutically to Alter Cytokine Production and the Immune Milieu.

Project Background:

Serious safety concerns arise from the use of DNA plasmids (encoding cytokines or foreign proteins) to alter the production of immunoregulatory cytokines in vivo. It has been established that DNA vaccines, like the bacterial DNA from which they are derived, contain unmethylated CpG dinucleotides that stimulate B and T cell proliferation (Nature 374:546, 1995). Recent work indicates that these CpG motifs alter the pattern of cytokines produced following DNA vaccination, resulting in the selective activation of IL-6, IL-12, IFNg, TNFa, and IL-18 secreting cells. Based on these findings, vaccine developers are incorporating additional CpG motifs into their DNA vaccines, hoping to strengthen and/or modify the type of immune response induced. To achieve a similar effect, DNA vaccines are being co-administering with DNA plasmids encoding immunostimulatory cytokines (including IL-12, IFNg and GM-CSF).

Normal immune homeostasis is maintained by balancing the production of Th1 cytokines (which promote cell-mediated immunity) and Th2 cytokines (which facilitate humoral immune responses). These two classes of cytokine form a dynamic and mutually inhibitory network, since Th1 cytokines can block the maturation of Th2-type cells, and vice-versa. The over-production of one class of cytokines can result in long-term disruption of immune homeostasis, with serious consequences. For example, animals with reduced Th1 cytokine production have increased susceptibility to infection by pathogens such as *Candida albicans* and *Leishmania major*, whereas reduced Th2 cytokine production increases susceptibility to parasites such as *Trichuris muris*. Moreover, altering the cytokine milieu may significantly reduce the immune response elicited by conventional attenuated/killed vaccines. Finally, changes in cytokine balance can predispose to the development of autoimmune disease.

The issue is whether CpG-induced production of the Th1-associated cytokines IFNg and IL-12, reinforced by repeated DNA vaccination, reduce the host's ability to mount a protective Th2 cytokine responses or increases its susceptibility to autoimmune disease. Concomitant concerns are whether detrimental effects will result from the administration of cytokine-encoding plasmids (which stimulate long-term cytokine production), or synthetic oligonucleotides (used as immune adjuvants and to stimulate a protective "innate" immune response).

We propose to monitor the impact of repeatedly administering CpG-containing DNA, and plasmids encoding Th1 and Th2 cytokines, on the immune system's capacity to maintain homeostasis and combat infection.

Description of Project:

This project will examine whether cytokine-encoding plasmids, DNA vaccines and synthetic oligos expressing CpG motifs disrupt cytokine homeostasis. Recent studies suggest that these agents can alter the cytokine profile of mice in such a way as to increase susceptibility to experimental allergic encephalomyelitis (EAE), an autoimmune model of multiple sclerosis. This finding demonstrates that increased Th1 cytokine production can deleteriously affect the host's immune system. We propose to analyze whether repeated vaccination with CpG-containing DNA motifs and cytokine-encoding DNA plasmids alters i) the balance between Th1 and Th2 cytokine secreting cells present *in vivo* (using ELIspot assays developed at CBER), ii) the maturation of Th0 cells into Th1 and Th2 cells, iii) susceptibility to subsequent infection, and iv) predisposition to the development of organ-specific autoimmune disease. Since newborns and young children are major recipients of human vaccines, we also plan to examine the effect of CpG motifs and cytokine-encoding plasmids on maturation of the neonatal immune system (this is detailed in a subsequent protocol).

To achieve these ends, we suggest monitoring the activation of Th1 and Th2 cytokine secreting cells following treatment of adult mice with each category of immunomodulatory DNA (using ELIspot assays (Current protocols in Immunology, 6:19, 1994). The effect of multiple DNA vaccinations, or administration of cytokine-encoding plasmids, on the Th1: Th2 balance should be addressed in long-term studies of adult mice. Animals should also be studied to determine i) their susceptibility to infectious agents (C. *albicans* for Th1 responses, T. *muris* for Th2 responses), ii) their susceptibility to autoimmune disease (using the EAE model), and iii) their

ability to respond to conventional protein based vaccines (using tetanus toxoid as a model). Similar techniques could be used to monitor the effect of vaccination on the development of the neonatal immune system.

B. Influence of Administration Route and Formulation on the Distribution and Persistence of DNA Plasmids and Synthetic Oligonucleotides.

Project Background:

Preclinical studies suggest that DNA vaccines may be effective in the treatment of cancer and in the prevention of infectious diseases. Additional evidence suggests that immunostimulatory fragments of DNA (so called "CpG motifs") may be useful as immune adjuvants or as "universal vaccines" capable of improving the immune system's capacity to prevent infection by a broad array of pathogens. Finally, DNA vectors are being commonly used for human gene therapy. The vectors are based elements of AAV, adenoviruses, and lentiviruses. INDs involving these agents are a rapidly growing component of CBER's review portfolio.

As discussed during a recent meeting of the Vaccines Advisory Committee and the NIH Recombinant DNA Advisory Committee, CBER reviewers have obtained evidence that DNA-based products are widely disseminated *in vivo*, that they persist long-term, and that they may integrate into the genome of host cells. This constellation of findings raises the possibility of DNA-based products causing mutations, genetic instability, translocations, gene-activation and/or gene inactivation. Depending on the site where these events occur, pathogenic consequences including tumor formation could result.

An associated concern is the ability of DNA vaccines to reach gonadal tissue (even when administered intramuscularly or intradermally). Rodent studies suggested that plasmids can persist for months in the gonads, introducing the possibility of reproductive toxicity, dysgenesis of germ cells, and the risk of transmission of altered genetic material to subsequent human generations. Very little is currently known about the type of cells in the gonads transfected by the plasmid DNA, and whether the plasmid poses a long-term threat to the germ line.

Obtaining accurate long-term safety data concerning DNA vaccines and gene therapy vectors is of considerable importance, since these agents are now being considered for use in relatively healthy children and adults. The same is true of synthetic CpG oligos, which may be used as immune adjuvants with conventional vaccines or to improve immune function in general. Unfortunately, conventional preclinical toxicological studies are poorly suited to evaluate the life-long toxicity posed by these DNA-based products. The FDA's authority to require long-term studies to assess the non-acute safety risks arising from the use of DNA vaccines is limited, and we cannot direct sponsors to share and/or publish their results, making it difficult to reach a regulatory consensus on these important safety issues.

Description of Project:

To optimize the likelihood of detecting plasmid DNA, synthetic oligos or gene therapy vectors requires that: i) high doses of DNA be administered repeatedly, ii) DNA be delivered in a vehicle that maximizes cellular uptake, iii) the effect of the DNA (i.e., immune activation or protein production) be demonstrated, providing evidence of gene transcription/translation and verifying the relevance of the model selected) and iv) the host does not develop an immune response against cells expressing the encoded protein (such a response might eliminate cells carrying

integrated copies of the plasmid, reducing the sensitivity of detecting such events).
We envision the testing of several DNA plasmids (encoding both self and foreign proteins), CpG-containing synthetic oligos, and viral-based gene therapy vectors to analyze these issues. These agents should be injected intramuscularly (in some cases formulated with liposomes) and DNA persistence, distribution and expression studied using sensitive PCR assays capable of detecting 1 - 10 copies of the relevant DNA per 150,000 cell nuclei (see project describing PCR-based method for quantitative analysis of DNA biodistribution). As a worse case scenario (depending on the availability of resources) these agents could also be injected intravenously.

We propose that studies examine the persistence and distribution of these DNA-based agents over time. This will require sacrificing animals and obtaining tissue from multiple sites, including the gonads, thymus, spleen, lymph nodes, bone marrow, heart, kidney, blood, skin, and muscle, for analysis by PCR. DNA from any site found PCR positive >2 months post plasmid injection should be size fractionated on agarose gels to separate unintegrated plasmid from genomic DNA. Integration of the plasmid into the host cell genome can be confirmed by sequencing (appropriate primers for each type of DNA are available). The frequency of such integration events can then be determined. If plasmid or oligo is found in the gonads of male mice, sperm samples should be collected periodically to determine whether reproductive cells carry the plasmid. Alternatively, *in situ* hybridization may be performed to determine whether the plasmid is limited to non-germline cells.

These studies will help establish the long-term safety profile of DNA-based products, the level of risk associated with exposure to these agents, and will provide insight into the manifestations of these risks, including the key attendant biologic processes associated with toxicity.

C. Safety and Immunogenicity of DNA Vaccines in Pregnant Women and Newborns.

Project Background:

A revolutionary approach to vaccine development involves the use of antigen-encoding DNA plasmids to induce cellular and humoral responses against pathogenic viruses, parasites, and bacteria. DNA vaccines are composed of an antigen-encoding gene whose expression is regulated by a strong mammalian promoter incorporated into a plasmid backbone of bacterial DNA. The gene is transcribed and translated by host cells, which then express the encoded protein in the context of self-MHC, resulting in a strong immune response.

Determining whether DNA vaccines can be safely and effectively administered to pregnant women and newborns is of considerable public health importance. Most American vaccines are administered to newborns and young children, and certain DNA vaccines (such as those against malaria) must target pregnant women (who are at highest risk of the pathologic consequences of malaria infection).

A DNA vaccine encoding the circumsporozoite protein (CSP) of malaria is being developed that protects adult mice from infection in preclinical studies. Yet the immunogenicity and efficacy of this vaccine has never been examined in pregnancy, and the vaccine was found to induce tolerance rather than immunity in newborns. Sponsors of DNA vaccine trials have been reticent to initiate studies in high-risk populations, due to the safety and immunogenicity problems posed by such groups. We propose to examine the effect of pyCSP vaccination in pregnant BALB/c mice and their offspring. These studies will establish whether DNA vaccination results in protective immunity or causes deleterious side effects (such as tolerance or immune deviation).

Pregnancy can profoundly alter the immune response to a vaccine. Specifically, Th1-type immune responses are broadly suppressed in pregnant women (this reduces the risk of a maternal response against paternal antigens present on the fetus). Indeed, agents shown to enhance Th1 responses during pregnancy can substantially increase the rate of spontaneous abortion. DNA vaccination during pregnancy might therefore provoke one of two deleterious outcomes: successful immunization could induce a strong Th1 response that compromises the pregnancy, or the absence of Th1 reactivity could prevent successful immunization. Balancing these potential negative outcomes is the possibility that vaccination during pregnancy could protect mothers from infection. Maternal vaccination could also be of benefit to her offspring, since immunity against sporozoite infection is passed via breast milk.

Additional safety and immunogenicity issues are raised by the vaccination of newborns. Due to immaturity of their immune system, newborns exposed to foreign antigens are at risk of developing tolerance rather than immunity. This risk is increased by DNA vaccination, since the encoded antigen is produced endogenously and expressed in the context of self-MHC. As noted above, that tolerance rather than immunity can result when neonates are vaccinated with pyCSP. These findings emphasize the importance of conducting thorough animal studies of DNA vaccines before exposing mothers or newborns to potentially serious side effects.

Description of Project:

We suggest a series of studies to evaluate the safety, persistence, immunogenicity and efficacy of the DNA vaccine for malaria. Female BALB/c mice should be vaccinated before, at various times during, and after pregnancy. Some of their offspring should also be treated with pyCSP (the plasmid encoding the circumsporozoite protein of malaria) at 1-5 days of age. Spleen and lymph node cells from vaccinated and control animals should be studied by ELIspot and ELISA assays (with and without culture in the presence of recombinant purified CSP and synthetic peptides) to monitor the nature, duration, and magnitude of the resultant antigen specific cytokine and antibody responses. Animals could then be challenged with live sporozoites to monitor vaccine efficacy.

We also suggest that the safety of DNA vaccines in mothers and their offspring be determined. Such studies could examine: i) whether the immunization alters the ability of either mothers or pups to mount normal immune responses to malarial or non-malaria proteins, ii) whether a persistent alteration in the ratio of Th1: Th2 cytokine secreting cells results from pyCSP vaccination (indicative of immune deviation), and iii) the tissue distribution and persistence of plasmid in mothers and their offspring, including whether neonatal tolerance leads to long-term plasmid persistence.

To accomplish these goals, we suggest the vaccination of adult female BALB/c mice IM with 50 *u*g of pyCSP before, at various times during, and after pregnancy. Litter sizes of newborn mice from control and vaccinated mothers should then be monitored; subgroups of offspring could then immunized with pyCSP at 1-5 days or 8 weeks of age (when adult immune responses are elicited). We also suggest that some mice be re-vaccinated up to three times at 6 week- intervals (to maximize the induction of protective immunity). Spleen and lymph node cells from these mice should be studied using CTL, ELIspot, and ELISA assays (with and without *in vitro* culture in the presence of recombinant purified CSP and peptides) to determine the nature, duration, and magnitude of the resultant antigen specific cytokine and antibody responses. Other animals could be challenged with 100 ID_{50} of live sporozoites to examine vaccine efficacy. Based on the results of these studies, animals could then be co-injected with 50 *u*g of plasmid encoding murine GM-CSF, IL-12, IFNg or IL-4 and these studies repeated.

We also suggested studies that examine the effect of vaccination on the ability of mothers and their offspring to mount normal Th1 and Th2 responses to the CSP protein, and to non-malarial proteins (including conventional antigens such as ovalbumin bound to heat-killed Brucella abortis or emulsified in Freunds adjuvant to induce strong Th1 and Th2 responses). Antigen-specific *in vitro* re-stimulation assays coupled with cytokine-specific ELIspot assays will evaluate whether pyCSP vaccination (with and without cytokine-encoding plasmids) induces short or long-term immune deviation (animals will be followed for up to one year). Results will be confirmed by analyzing the subclass of IgG antibodies induced by these antigens.

Other studies could examine the offspring of vaccinated and control mothers immunized with pyCSP at 1-5 days of age to determine whether tolerance develops. These animals can then be challenged with live sporozoites to examine their susceptibility to infection. Both newborn and

older animals should be sacrificed and their tissues analyzed by PCR to determine whether the pyCSP plasmid crosses the placenta and/or persists long-term *in vivo*.

D. Development of a PCR-based Method for Quantitative Analysis of Vector Biodistribution and Persistence in Safety Assessment of DNA-based Gene Therapies.

Project Background:

CBER has received close to two hundred applications to conduct gene therapy trials using recombinant DNA vectors. An important issue in assessing the risks and benefits of gene therapy is that of biodistribution and persistence of the vector. Before gene therapy trials are performed in humans, preclinical animal studies are used to examine vector dissemination in various tissues and the rates of disappearance of vector. Both biodistribution and persistence are important considerations. For example, detection of vector in gonadal tissue would raise concerns over the possibility of germ cell alteration. Subsequent absence of vector detection in gonadal tissue would suggest that either that germ cells were unaffected or that any possible germ cell alteration was transitory. Thus studies of biodistribution and persistence can inform decisions such as whether or not to recommend that patients practice birth control after treatment with DNA-based products. The probability of long-term toxicities can also be assessed if kinetics of vector persistence and clearance are understood. Preclinical animal studies of vector distribution and persistence in tissues rely on PCR due to its inherent sensitivity. Surprisingly, many preclinical gene therapy studies have shown unexpected persistence of vector DNAs in a variety of tissues including gonads. Unfortunately, most PCR assays used by gene therapy trial sponsors were inadequately controlled for PCR sensitivity and efficiency in the different tissues surveyed. These observations resulted in a FDA presentation entitled "Risks of Gonadal Distribution and Inadvertent Germline Integration of Patients Receiving Direct Administration of Gene Therapy Vectors" at the December 15, 1997 meeting of the NIH Recombinant DNA Advisory Committee. Lack of quantitative PCR data prevented accurate assessment of the public health risk entailed by gene therapy. It was also noted that, due to the lack of standardized quantitative approaches, it was difficult to compare PCR studies using similar vectors but conducted by different laboratories.

Many factors could affect biodistribution and integration of vector DNA including route of administration, formulation, vector type, and even vector sequence. In particular, gene therapy vectors such as AAV or lentivirus vectors, which can and do integrate, need careful assessment by quantitative, accurate PCR methods. Development of a reliable, reproducible, and widely applicable method to quantify vector after administration in a variety of tissues would tremendously enhance gene therapy risk assessment.

The intent of this project is to construct a single internal standard that can be used to quantify a variety of gene therapy vectors. The techniques for construction and use of a single internal standard molecule that can be used to measure many different target sequences have been established and demonstrated by FDA scientists. Figure 1 illustrates the structure and use of such an internal standard designed to measure expression of 20 different oncogenes. This approach was also used for quantitative clinical measurements of human herpesvirus burden in post transplant lymphoproliferative disorder. The use of internal standards allows for absolute quantification of vector copies. In addition, internal controls can demonstrate that the PCR reaction is equally efficient in the different samples being compared. It is known that DNA from different sources can differentially affect PCR reaction kinetics. Without the use of internal PCR standards, it is difficult to assess this inhibitory effect in a quantitative manner. In preclinical studies using different tissues, comparisons between these tissues is not optimal unless adequate controls for PCR reaction efficiency are used.

Description of Project:

The internal standard for PCR should be constructed so that it can be used to perform quantitative measurements of vector sequence in both preclinical animal studies and in patient samples after gene therapy vector administration. This single nucleic acid standard will take the form of a plasmid that can be used to generate an internal standard containing PCR primers for a variety of vectors including plasmids, AAV, adenovirus, and the newly emerging class of lentivirus-based vectors. The DNA sequences used to generate PCR primers should be chosen from available DNA sequence databases that originate from segments of each vector type which do not change after insertion of the therapeutic gene. For example, a sequence derived from plasmid backbone would be useful to quantify a large variety of plasmid-based vectors. The standard plasmid should be designed so that other primer sets can be incorporated as new vector classes arise. The internal standard should also contain PCR primers from cellular genes so that the amount of vector can be normalized to the number of cells in the tissue sample. The process of construction of such an internal PCR standard could be as follows. After selection of appropriate PCR primer sequences, an insert containing all the desired primers placed in tandem array is synthesized by overlap extension PCR. The PCR sequences for a variety of vectors and genes are thus linearly juxtaposed on a single stretch of synthetic DNA. This piece is cloned into an appropriate plasmid and propagated to generate as much standard molecule as may be needed. This approach can be used to quantify both RNA and DNA arising from vector sequences.

Following construction, this internal standard molecule can be used to validate sensitivity and precision of quantitative detection of gene therapy vector sequences in spiked tissue form rodents. The internal standard vector can also be made available for preclinical investigations of vector distribution and persistence and for monitoring of patients after vector administration.