Chapter 15

DNA vaccines: Mechanisms and aspects of relevance for biosafety

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1. Introduction

DNA vaccines represent a new approach to protect against infectious disease, and hence may improve human and animal welfare, reduce antibiotic usage and reduce the spread of pathogens. Edible and injectable DNA vaccines hold prospects for rapid immunization against a variety of diseases that are difficult to eradicate with traditional vaccines and antibiotics. Other potential uses of DNA vaccines include treatment of cancer, autoimmune diseases and allergies. DNA vaccines have several attractive benefits: low cost, ease of production and improved quality control, heat stability, identical production processes for different vaccines, and the possibility of producing multivalent vaccines (Kwang 2000). On the other hand, there is a limited scientific understanding of the mechanisms underlying uptake, persistence and degradation of DNA vaccines following their injection into humans or animals. The main areas of uncertainty are related to the immunological impact, tissue distribution and persistence after injection and whether the DNA vaccine can leak into the environment.

In this chapter, we summarize the uses, mechanisms, immunological parameters, and environmental issues associated with the introduction of DNA vaccines, and identify areas where more research is needed.

2. The use of DNA vaccines

A DNA vaccine consists of a bacterial plasmid with a strong viral promoter, the gene of interest (a gene encoding the immunostimulatory protein), and a polyadenylation/transcriptional termination sequence. The plasmid is grown in bacteria, purified, dissolved in a saline solution, and then administrated by direct intramuscular injection of naked DNA (in ng and μg amounts) to activate protein expression in vivo and to ultimately induce an immune response and disease protection.

The advances in the field of DNA vaccines in recent years have been profound. In 2003, the US Centers for Disease Control (CDC) expedited delivery of an experimental veterinary DNA vaccine developed by the CDC and manufactured by Aldevron (Fargo, ND) (Bouchie 2003). The target for vaccination was the wild Californian condor and the purpose was to protect this endangered species from becoming infected with the West Nile virus. In Canada, an Infectious Hematopoietic Necrosis Virus (IHNV) DNA vaccine (Apex-IHN®) developed by Aqua Health Ltd. (Canada), an affiliate of Novartis, was cleared for marketing by the Canadian Food Inspection Agency on 15 July 2005 (Novartis media release 19 July 2005). Currently, a number of experimental human DNA vaccines have entered phase 1 clinical trials. However, the biosafety aspects have not yet been thoroughly investigated, and it may be expected that these aspects will become increasingly important when the vaccines enter the regulatory approval process prior to commercial use of DNA vaccines.

3. The immune system and immune responses by DNA vaccination

Both mammalian and fish defence systems include, roughly defined, leucocytes, and their products most often localized in lymphoid organs, such as the thymus, spleen and kidney. In addition, several other tissues harbour defence cells and proteins (e.g. liver, skin, intestines, and gills). The immune system contains both adaptive and innate defence mechanisms that eradicate pathogens in a concerted manner. Within minutes after infection, the innate defence is activated, whereas two to three weeks are required to eradicate the pathogens by mechanisms of the adaptive defence (Fig. 15.1).

The innate defence mechanisms involve 1) cell-derived defence factors (e.g. defensive peptides, complement components, reactive oxygen radicals, interferons and receptors), and 2) leucocytes such as monocytes, macrophages, dendritic cells, scavenger endothelial cells, and granulocytes (Fig. 15.2). The expression, amount and activities of the cell-derived defence factors may increase upon activation of innate defence. Almost all organs and tissues in humans and animals contain cells and components of innate defence.

The adaptive defence system, specific for an infectious agent, concerns the immune response that involves: 1) cells and specifically recognizing molecules mediating eradication of e.g. virus infected cells, and 2) the production of reactive antibodies that bind to antigenic determinants against pathogens and foreign substances. These processes mainly involve antigen presenting cells (APC; i.e. macrophages and dendritic cells) that are a partner in both innate and adaptive arms, T- and B cells (Fig. 15.2). The adaptive machinery of defence also creates memory cells that, upon reactivation, induce rapid immune responses and is the rationale for vaccinology.

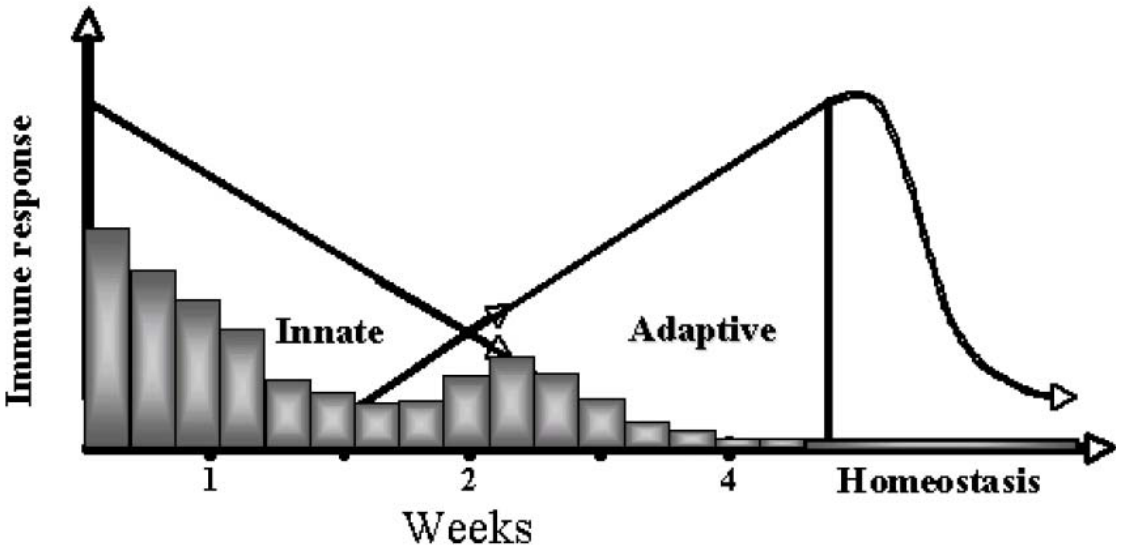

Figure 15.1. The innate immune response is immediate in nature and involves cellular and soluble (humoral) antimicrobial factors and is contributing to the eradication of pathogens. In the case of surviving pathogens (e.g. for 7–14 days), adaptive immune defence mechanisms may bring about final destruction of pathogens whereby homeostasis reoccurs. Bars show pathogen load in the host.

3.1 Persistence and uptake of DNA vaccines after injection in animals

It has been reported that immediately after injection of plasmid DNA intravenously most of the DNA is rapidly degraded (Hashida et al. 1996). The products from the degradation are either used as nutrients or excreted in the urine. Interstitial (extracellular) and cellular nucleases have been reported to be responsible for DNA degradation in mice (Hashida et al. 1996). In preliminary experiments, almost 100% of plasmid DNA is degraded in salmon blood within one hour. The degradation is most probably due to nuclease (DNAse) activity, since known nuclease inhibitors block degradation. Despite rapid breakdown in blood, a minor fraction of intact DNA vaccine remains in the muscle tissue at the injection site, together with fractions of blood-transported intact DNA in organs such as the kidney and liver (Tonheim et al. 2007).

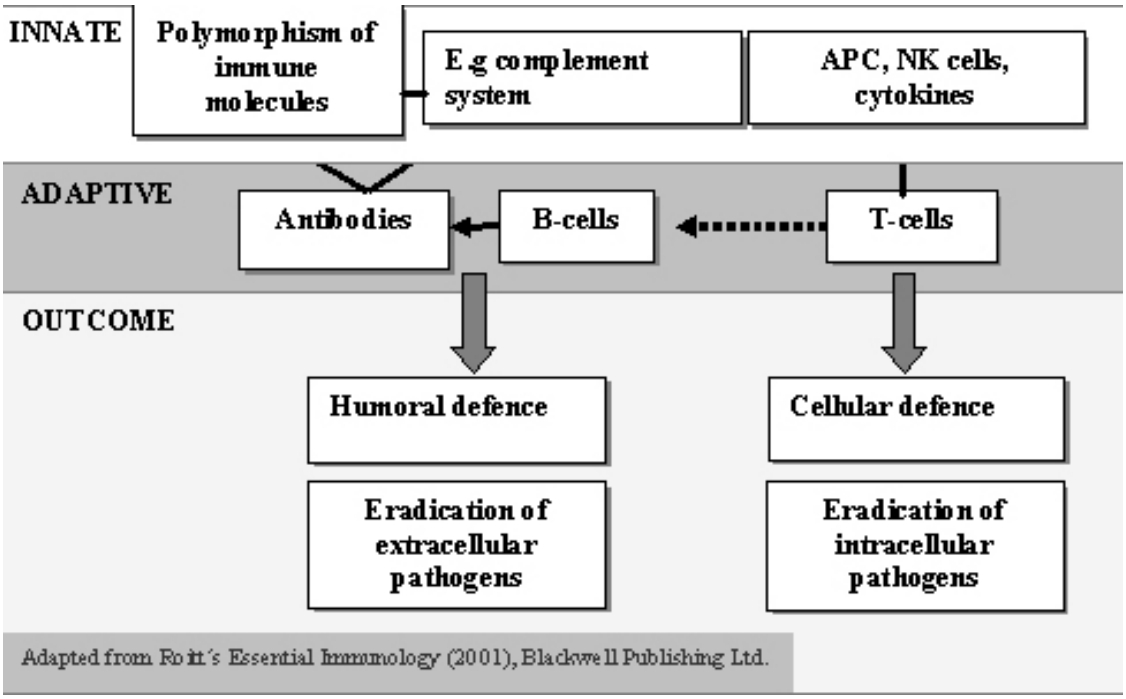

Figure 15.2. The concerted action of the innate and the adaptive immune defences.

The outcome of an immune response depends on the infectious agent, host cell activation, and the cytokine (signalling molecules) profile generated by the leucocytes. In general, humoral immunity (generation of antibodies) will resolve infection caused by extracellular pathogens (e.g. many bacteria) whereas cellular activation may cause eradication of intracellular pathogens by actions of e.g. enzymes, oxide radicals and membranolytic substances. Of importance is the interplay between innate, adaptive, humoral and cellular defences. For instance, both complement and antibodies may facilitate antiviral effects and activated cells (cellular defence) may degrade extracellular bacteria. To increase the efficacy of eradication of pathogens many defence molecules show hyper-variability, in which single nucleotide polymorphism (in genome) induces mutation in the amino acid sequences of proteins. Some of the mutations may cause increased affinity to pathogenic structures, thereby causing higher probability of pathogen scavenging. DNA vaccination causes activation of innate, adaptive, humoral and cellular defences. It is highly acknowledged that the DNA vaccine induced activation of the cellular defence is utmost important in fighting viral pathogens.

A prerequisite for expression of the immunostimulatory protein is that the DNA vaccine (purified plasmid DNA) is taken up by the host cells, transferred to the cytosol and eventually transported to the nucleus before any expression occurs. Several passive and active mechanisms have been described concerning receptor binding and/or uptake of DNA. The uptake processes are described as uptake by endocytosis (phagocytosis (cell eating) and pinocytosis (cell drinking)).

Pinocytosis is utilized essentially by all cell types and occurs by multiple pathways, i.e. clathrin-mediated endocytosis, caveolin-mediated endocytosis, chlatrin- and caveolin-independent endocytosis, and macropinocytosis (Belting et al. 2005). Macrophages, granulocytes and dendritic cells carry out phagocytosis (e.g. of dead cells, bacteria, large molecular complexes), and they are found in many parts of humans and animals. In particular, macrophages, residing in close connection to the bloodstream, are highly phagocytic, thus functioning as an important element in the reticuloendothelial system – together with scavenger endothelial cells. These scavenger cells are responsible for the highest uptake and degradation of plasmid DNA (Kawabata et al. 1995; Takagi et al. 1998). The liver is the main scavenger organ in mammalian species, whereas the kidney and heart have this function in fish.

The endocytic pathway normally confers total degradation of DNA – especially if the DNA is transported to the end-point terminal (lysosomes). However, tiny amounts of DNA may escape the endocytic compartments and degradation. This DNA may be trafficked through the nuclear membrane into the nucleus where transcription occurs.

Transport vehicles (carriers) have been commonly used to increase the efficacy of transgene production and are used in conjunction with DNA vaccines. Such vectors include polyplexes (positive charged cationic polymers, such as poly–L/D-lysine), lipoplexes (cationic lipids: Liposomes) and molecular conjugates (cell receptor ligands conjugated to DNA) (Medina-Kauwe et al. 2005). Their main advantages are that they confer DNA condensation, inhibit DNAses and facilitate endosomal escape of DNA by endosomal membrane association. In spite of these advantages, low-level transfection, relative to viral delivery of DNA/viral infection, often occurs since there are many obstacles to overcome to mediate efficient gene transfer. In conclusion, intracellular trafficking including endosomal escape and cytosolic processes mediating nuclear import of DNA vaccines are issues that warrant further research.

The nuclear membrane filter excludes intact plasmid DNA larger than *c.*40 kDa and the DNA is thus retained in cytosol (Median-Kauwe et al. 2005). Active nuclear transfer mechanisms must be present to facilitate transgene expression mediated by large DNA fragments or intact DNA. Such processes are ‘catalysed’ by nuclear importins or nuclear localization signals (NLS) that are proteins and peptides respectively, that help large molecules to reach the nucleus (Medina-Kauwe et al. 2005). Further, there are reports that describe transgene production, for instance both after intravenous, intraperitoneal and intramuscular injection of naked luciferase-coding plasmid DNA in rainbow trout (e.g. firefly luciferase (enzyme)) in distant organs such as the kidney and spleen (Romøren et al. 2004). More experiments addressing the tissue distribution versus transgene production are needed to elucidate molecular mechanisms of DNA persistence and stability of the expression of the immunostimulatory gene product.

3.2 Immune responses to DNA vaccination

After DNA vaccination, two main immune responses evolve in a time-dependent manner, the first being an immediate response generated by innate defence mechanisms and the second being a late specific response with production of specific antibodies and activation of a cytotoxic response (cytolysis of cells expressing the transgene on their cell membrane) (Fig. 15.1). It has, for instance, been reported that the immediate response following DNA vaccination in fish with a

rhabdo virus (VHSV) DNA vaccine also confers protection against other viral diseases and is thus not pathogen-specific (Lorenzen et al. 2002). Furthermore, genes important in both cellular and humoral defence have been reported to be significantly up-regulated one to three days after DNA vaccination using a VHS-G plasmid construct, whereas the number of differentially expressed genes at days seven and twenty-one have decreased considerably (Byon et al. 2005). This is also in line with the suggestion that pDNA containing the G-protein confers a strong effect on the immune system at early time-points. The long-term effect of pDNA on the immune system is, however, not known.

3.2.1 Innate immune response to DNA vaccines

Bacterial DNA, invertebrate DNA and DNA from some viruses differ structurally from vertebrate DNA because they contain increased frequencies of CpG dinucleotides (Bird 1986). It has been reported that toll-like receptor 9 (TLR9) recognizes such CpG motifs (Hemmi et al. 2000). TLR9 binding of DNA and subsequent intracellular activation induces production of cytokines and type I interferons that augment the host fighting viral infections. It is suggested that CpG containing DNA vaccines (plasmids produced in bacteria) confer an immediate and efficient anti-viral effect – as observed by Lorenzen et al. (2002).

3.2.2 Adaptive immune response to DNA vaccines

After DNA vaccination, host cells may produce the antigen of interest and these antigens may be endocytosed by antigen presenting cells (APC). Peptides of the endocytosed antigen will be presented on MHC class II molecules to T cells leading to production of antibodies by plasma cells. Administration of DNA vaccines has proven to be an effective means for generation of humoral immune responses (antibody production) specific for the encoded antigen(s) (Russell et al. 1998; Fernandez-Alonso et al. 2001; Nusbaum et al. 2002; Verri et al. 2003). A combination DNA vaccine, consisting of multiple discrete plasmids encoding several different antigens of a pathogen, may be employed to induce a broader spectrum of immune responses. This would be effective for vaccination against viruses that undergo antigenic variations (Lee et al. 1996; Wang & Nicholson 1996; Kibenge et al. 2001). Although there may be high vaccine efficacy (increased survival), the potency (amount of specific antibodies generated) may be relatively low compared with traditional vaccines. To obtain increased potency of the DNA vaccines, one may apply higher doses, a prime boosting regime or co-administration of plasmids encoding cytokines or co-stimulatory molecules.

3.2.3 Cytotoxic T cell responses and DNA vaccines

Both viral infection and DNA immunization induce intracellular expression of antigens that may be presented on MHC class I molecules (Dijkstra et al. 2001) which, in turn, activate TCR/CD8+ T-lymphocytes to lyse the 'infected' cells. After DNA vaccination, CTL responses and subsequent cell lysis may eliminate 1–5% of the muscle cells that have been transfected and express, for example, viral antigens on their surfaces. An intramuscular injection of any solution will cause tissue damage, wound repair and tissue remodelling. However, the destruction of 1–5% of muscle cells after DNA vaccination would be unlikely to have clinically significant effect on the performance of the injected muscle. The damaged muscle cells will be replaced by the migration and fusion of satellite cells within existing myotubes as part of normal cellular turnover. It is suggested that the magnitude of cellular turnover caused by DNA vaccination is not higher than by viral and bacterial infections (Donnelly et al. 1997).

4. The need for research on the effects of DNA vaccination

Before distributing any genetically modified DNA constructs (e.g. DNA vaccines) into a new location/ecosystem, important questions and knowledge gaps concerning environmental and

health effects need to be addressed. A number of hypothetical effects, both beneficial and harmful, have only modest scientific support. There are three main issues that need to be resolved:

- Knowledge gaps related to the biology of uptake, the tissue and organ distribution and persistence of the DNA vaccine in the host organism.
- Knowledge gaps with regard to potential unintended physiological effects on the host organisms, including unwanted immune response.
- Knowledge gaps arising from unintended release of the DNA vaccine into the open environment as a result of the expected human error in large-scale vaccination processes or from the vaccinated host organism itself.

4.1 Knowledge gaps related to uptake, distribution and persistence of the DNA vaccine
Preliminary experiments have revealed that organs and tissues rich in leucocytes have accumulated intact DNA (plasmid DNA) for more than one month in salmon after intraperitoneal (ip) and intramuscular (im) injections (Myhr & Dalmo 2005). For instance, in sea bream, intact plasmids were found at the injection site two months after intramuscular injection (Verri et al. 2003). Similar findings have been described for rainbow trout (Anderson et al. 1996). Further, it has been shown that not only muscle cells but also cells in tissues very distal to the injection site (muscle) have expressed the transgene after plasmid injection (Romøren et al. 2004). Moreover, it has been shown that glass catfish have been expressing a transgene as long as two years after injection (Dijkstra et al. 2001). These reports illustrate that plasmid DNA can persist in fish for long time periods after the initial injection. Undoubtedly, there is an urgent need to analyse the longevity of DNA vaccines with respect to immunological parameters, the risk of gene transfer to the host's genome or intestinal bacteria, or other exposed organisms after release of plasmid DNA (excreted into the gastrointestinal tract of the vaccinated host).

4.2 Knowledge gaps with regard to potential unintended immune response
Concerning unintended long-term effects of plasmid DNA on the immune system, no experiments have been conducted to address this issue in any animal species so far although modern tools for gene expression analysis are available. It seems that the immediate short-term elevation of the expression of certain immune genes is normalized within three weeks after DNA vaccination, as reported for Japanese flounder (Byon et al. 2005; 2006). To our knowledge, no microarray analysis on samples obtained from DNA vaccinated mammalian species has been performed.

4.3 Potential effects of unintended environmental release of DNA vaccines
If the transgenes are released into the environment after vaccination, DNA products could be distributed unintentionally over vast areas, and have potentially mediating effects in a range of organisms after horizontal gene transfer. DNA is more resistant to immediate breakdown in the ecosystems, both terrestrial and aquatic ecosystems (Heinemann & Roughan 2000) and after uptake from the gastrointestinal tract micro-organisms, than previously assumed. There are, however, few published studies investigating the stability, horizontal transfer and uptake of released DNA constructs in terrestrial and aquatic systems, including in fish and mammals.

5. Legal implications of the usage of DNA vaccines

At present, scientific uncertainty concerning the risks of introducing DNA vaccines creates disagreements about which legal frameworks should be applied for risk assessments in approval procedures. For instance, scientists and policymakers in Norway and in the EU disagree about how to regulate DNA vaccines (Foss & Rogne 2003). Central to this discussion are the regulatory definitions of 'medicinal products' and 'genetic modification' and which regulatory system to involve. In the United States, the US Food and Drug Administration has asserted that genetic

constructs distributed to animals fall under the legal definition of a drug substance. This corresponds with regulations in Europe; the European Agency of Medicinal Products authorizes pharmaceuticals based on modern biotechnology through a centralized procedure. However, as a part of the European procedure, national GMO authorities are involved in evaluating environmental risks of both the medicinal products and the animals receiving them. For instance, the Norwegian Directorate for Nature Management has stated that a DNA-vaccinated animal is to be considered as genetically modified (GM) for as long as the added DNA is present in the animals This may have implications for the need for labelling and traceability. Accordingly, the current limited scientific understanding of the fate of DNA vaccines, such as host distribution and persistence, has clear policy implications.

6. Conclusions

DNA vaccines hold promises for protection against a range of diseases caused by viruses and intracellular bacteria, for which there at present are no efficient vaccines based on either live, attenuated viruses or vaccines containing recombinant viral antigens. However, the present lack of biological understanding of the health and environmental effects of distribution of DNA vaccines creates a challenge with regard to their perceived safety and regulatory basis. Targeted studies of specific knowledge gaps, as identified here, must therefore be incorporated into the vaccine research and development agenda; encouraging broad and long-term thinking.

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