REVIEW

Arch. Immunol. Ther. Exp., 2007, **55**, 387–398
PL ISSN 0004-069X

DOI 10.1007/s00005-007-0044-4

DNA vaccines: are they still just a powerful tool for the future?

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Received: 2007.06.25, **Accepted:** 2007.10.29, **Published online first:** 2007.12.03

Abstract

Vaccination is historically one of the most successful strategies for the prevention of infectious diseases. For safety reasons, modern vaccinology tends toward the usage of inactivated or attenuated microorganisms and uses predominantly subunit vaccines. The antigens need to be clearly defined, pure, stable, appropriately composed, and properly presented to the immune system of the host. Differing ratios of various proportions between specific $CD4^+$ and $CD8^+$ T cell responses are essential for conferring the required protection in the case of individual vaccines. To stimulate both $CD4^+$ and $CD8^+$ T cells, the antigens must be processed and presented to both antigen-presentation pathways, MHC I and MHC II. Protein antigens delivered by vaccination are processed as extracellular antigens. However, extracellularly delivered antigen can be directed towards intracellular presentation pathways in conjugation with molecules involved in antigen cross-presentation, e.g. heat shock proteins, or by genomic-DNA vaccination. In this overview, current knowledge of the host immune response to DNA vaccines is summarized in the introduction. The subsequent sections discuss techniques for enhancing DNA vaccine efficacy, such as DNA delivery to specific tissues, delivery of DNA to the cell cytoplasm or nucleus, and enhancement of the immune response using molecular adjuvants. Finally, the prospects of DNA vaccination and ongoing clinical trials with various DNA vaccines are discussed.

Key words: DNA vaccine, plasmid, delivery systems, CTL, MHC I, MHC II.

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INTRODUCTION

Immunization with plasmid DNA (DNA-based vaccination) is a relatively novel technique for the efficacious stimulation of a specific cellular and humoral immune response to protein antigens. DNA vaccines deliver transgenes, which code for antigens, directly into the cells of immunized host organisms. Such antigens are expressed in a similar way as antigens during viral infection [35]. Antigens are processed identically as proteins synthesized in cytoplasm [95, 96] and peptide fragments are presented to the immune system on cell-surface MHC I molecules. If the DNA vaccine-coded proteins are secreted from the cell, they could be processed by MHC II and could elicit a specific antibody response [47, 107].

DNA-based immunization is a new and attractive strategy in the prophylaxis and treatment of infections caused by extracellular and intracellular pathogens. The method of application, dose, boosting schemes, and species used are factors that influence the strength and nature of the elicited immune response.

THE ADVANTAGES OF DNA VACCINATION

Vaccination with plasmid DNA may offer several important advantages over traditional vaccines, e.g. the relative stability of DNA, the specificity of the antigen produced, and the possibility of guiding the type of elicited specific immune response [63]. One of the major benefits of DNA vaccines is that host cells express a vaccine-coded antigen and thus the antigen presents epitopes which may resemble native viral epitopes more closely than is the case with other vaccination approaches. Intracellularly processed epitopes are presented to the host immune system in a way similar to that of a natural viral infection (MHC I presentation followed by $CD8^+$ T cell responses), but without the risk associated with the administration of infectious agents [39]. DNA vaccines encoding several glycoproteins, i.e. multivalent vaccines, can be delivered to the host in a single dose. A few micrograms to milligrams of plasmid DNA are sufficient for inducing a vigorous immune response. DNA vaccines can also be manufactured in a relatively cost-effective manner and stored with relative ease [40].

The temperature-stable storage of DNA in a lyophilized form is feasible and more practical for transport and distribution as well [36]. Another important advantage of DNA vaccines is their therapeutic potential in ongoing chronic viral infections. DNA vaccines are very promising tools for an effective induction of a protective immune response against viral infections (HBV, HCV, HIV) and parasitic infections (malaria, leishmaniasis) [26, 40, 63].

THE CONSTRUCTION OF DNA VACCINES

DNA vaccines are principally derived from bacterial plasmids [96]. Conventional plasmid DNA vaccines consist of two different parts: 1) a eukaryotic cistron coding for the target antigen and consisting of a strong promoter/enhancer, cDNA coding for the target antigen(s) (full-length or truncated), and a polyadenylation/termination signal, and 2) sequences necessary for the manipulation-construction and amplification of plasmid DNA in a prokaryotic host (*E. coli*) and consisting of the origin of replication (usually from *E. coli*), a multiple cloning site, and an antibiotic-resistant gene used as the selection marker during bacterial amplification [8, 34, 36, 95].

The majority of eukaryotic promoters used are derived from the human cytomegalovirus (HCMV) or the Rous-sarcoma virus (RSV). HCMV and RSV were found to give the highest levels of antigen expression after intramuscular (i.m.) DNA injection [36]. Other promoters tested are tissue-specific promoters, e.g. the myocyte-specific desmin promoter [57], the actin promoter, major creatine kinase promoter, alphaglobin promoter, chicken beta-actin promoter, and adenovirus promoter. These have been tested primarily for i.m. DNA application.

Efficacious expression of protein from DNA vaccines is dependent on the presence of DNA vaccine in the nucleus. The effective targeting of plasmid DNA to the cell nucleus is dependent on the presence of DNA sequences recognized by proteins or peptides called nuclear localization signals (NLSs) [9], which form a DNA-NLS complex recognized by cytoplasmic proteins called importins which dock DNA-NLS complexes to the nuclear membrane receptor/transporter (nuclear pore complex) as a nuclear import substrate [14]. Nuclear DNA transport is typical for viral DNA. The most effectively recognized DNA sequence is localized within the SV40 early promoter enhancer region of Simian virus DNA. This DNA sequence was shown to be effective in the nuclear transport of plasmid DNA [21]. Other DNA sequences have been identified and tested, but their recognition through cellular NLSs followed by the transport of plasmid to the nucleus is generally poor. An alternative approach is based on the chemical linkage of plasmid DNA with an NLS. A variety of NLSs were tested (large SV40 T-antigen, M9 sequence of heterogeneous nuclear ribonucleoprotein, adenovirus 3 fiber protein residues), but they generally obtained unsatisfactory results in *in vivo* experiments [14]. This was most likely linked to the size-exclusion limit for active nuclear transport of such artificial DNA-NLS complexes. Studies of the viral DNA nuclear transport mechanism are promising because some viral DNA, although more than ten times larger than standard vaccination DNA plasmids, are effectively transported to the nucleus [14].

PolyA-termination signals, which provide stabilization of mRNA transcripts, are commonly taken from bovine growth hormone or from SV40 [34, 35].

THE MECHANISM OF ANTIGEN PRESENTATION FOLLOWING DNA VACCINATION

DNA vaccines stimulate both exogenous (MHC class II-restricted) and endogenous (MHC class I-restricted) antigen-presentation pathways. MHC I-restricted cytotoxic T lymphocytes (CTL) may be induced after DNA vaccination by: a) directly transfected somatic cells (myocytes, keratinocytes, or any MHC II-negative cells) which present expressed antigen on MHC I, b) directly transfected professional antigen-presenting cells (APCs) such as dendritic cells (DCs) which, besides MHC I presentation of the expressed antigen, effectively stimulate naive T cells via a variety of co-stimulatory molecules and cytokines, or c) by cross-priming when protein expressed by DNA-transfected somatic cells is taken up by the surrounding professional APCs and presented to T cells [24, 97]. The cross-priming phenomenon, discovered by Bevan in 1976, is at present used as a general explanation for the re-presentation of exogenously derived cell-associated antigens on both MHC I and MHC II molecules [12, 44].

When the DNA vaccine reaches the cell nucleus, transcription is initiated and subsequent translation of the coded protein takes place on cytosolic ribosomes. The proteasome complex processes the expressed protein and released peptides are transported to the endoplasmic reticulum through the membrane transporter complex TAP-1 and TAP-2. Inside the endoplasmic reticulum, the peptides are bound to MHC I molecules [48, 92], which subsequently migrate to the cytoplasmic membrane and present the peptides to the surrounding $CD8^+$ T cells [49].

Professional APCs, such as macrophages, B cells, and DCs, play a central role in the regulation of the immune response to any vaccine. In contrast to somatic cells, APCs can present the antigen to both MHC I and MHC II molecules and thus stimulate T-helper cells ($CD4^+$), which control other T cell or B cell responses [63, 108]. DCs are much more effective than other APCs because of their unique ability to prime naive T cells. Thus DCs are most likely the key cells initiating the immune response to the DNA vaccine [40].

Intradermal (i.d.) and gene-gun application would

directly target the plasmids to Langerhan's cells, which represent immature DCs located in the stratum spinosum of the epidermis. DCs can migrate to lymphatic organs (the spleen and the lymph nodes) where they activate antigen-specific T lymphocytes. Apart from high levels of MHC I and MHC II, DCs express the stimulatory molecules B7.1 and B7.2 (CD80 and CD86, respectively) that are required for immune response initiation [22].

If non-APCs such as myocytes take up the DNA vaccine (following i.m. injection), the expressed antigen is delivered to T cells by cross-presenting DCs [35, 39, 49, 99]. Cross-presentation and cross-priming are responsible for inducing the immune response to apoptotic and/or necrotic bodies [40], which may also be associated with DNA vaccination.

Thus DNA immunization results in the induction of both humoral (antibody) and cellular (T cell) immune responses. The major advantage of DNA vaccines is their ability to generate a strong cellular immunity with preference for MHC I-restricted $CD8^+$ cytotoxic T cells and MHC II-restricted T helper type 1 (Th1) cell responses [8]. On the other hand, DNA vaccines can induce Th2 responses and results from a number of studies indicate that DNA vaccination can be effective in inducing long-term antibody responses [89, 90]. This effect may depend on the type of antigen coded by the vaccine [40].

Effective DNA vaccination should generate a long--term memory immune responses. There are several possible ways for DNA vaccines to induce a long-term response: a) antigen is continuously expressed at a low level sufficient for antigen presentation and b) plasmid DNA as well as antigen are completely gone and the response is antigen independent. Memory cells generated by DNA vaccines probably differ qualitatively from those achieved by other forms of vaccination, such as protein plus adjuvant [1, 40]. Memory $CD8^+$ T cell and B cell populations are the most relevant for vaccine development.

The most critical factors for current DNA vaccines are: 1) the efficacy with which the DNA vector reaches the target cells' nuclei (transfection efficacy) and 2) the amount of actual protein synthesized in DNA vaccine--transfected cells. It has been estimated that injection of microgram doses of DNA plasmid results in the production of only nanograms of protein [40]. It is difficult to quantify the number of plasmids that enter the cell nucleus and the number of plasmids that are degraded before they enter the nucleus. It is estimated that more than 90% of the DNA plasmids never reach the cytoplasm and only about 0.1% of cytoplasmatically localized DNA plasmids enter the nucleus, where gene expression is initiated [3]. Identifying and overcoming each hurdle along the DNA vaccine entry pathway (low uptake across the plasma membrane, inadequate release of DNA molecules within the cell's cytoplasm resulting in reduced DNA stability, and a lack of nuclear targeting) can improve DNA vaccine efficacy [68].

THE ENHANCEMENT OF EFFICACY OF DNA VACCINATION

DNA vaccination was described in 1990 when Wolff et al. [114] demonstrated induced gene expression after direct i.m. injection of naked plasmid DNA into experimental mice. Naked DNA was then injected into various tissues with the aim of comparing the intensity of proteosynthesis and the host immune response. Besides i.m. injection the most frequently tested injection routes were i.d., intravenous, subcutaneous, epidermal, intraepidermal, intraperitoneal, injection into lymphatic follicles, and injection into the thyroid gland [19, 40, 72, 113]. Different routes of administration lead to markedly different levels of protein expression as well as different levels of intensity and quality (Th1, Th2, antibody) of the immune response.

The skin was found to be one of the best sites for immunization due to the ease of skin injection and the high concentration of DCs (in the skin Langerhans cells), macrophages, and lymphocytes, which are necessary for the induction of the immune response [85]. In contrast, muscle is generally not equipped with DCs and elicitation of the immune response probably relies on cross-presentation, the effectiveness of which is limited by the dose of available antigen [44]. Thus methods which increase antigen expression by increased uptake of the DNA by muscle cells could dramatically improve the applicability of i.m. DNA vaccination.

The enhancement of the antigen expression from DNA vaccine delivered to the cell by specific delivery systems and DNA targeting

DNA delivery methods can be classified into two general types: 1) mechanical and electrical strategies for introducing naked DNA into cells, including microinjection, particle bombardment, and the use of electroporation, and 2) DNA delivery systems which can be classified into biological viral DNA delivery systems and chemical non-viral delivery systems (DNA-binding polymers and liposomes).

Physical methods for increasing naked DNA delivery

DNA vaccination into skin and muscle. Wang et al. [110] tested the effect of i.m. injection followed by electroporation on plasmid uptake in mice. The electroporation increased muscle cell plasmid uptake by approximately 6- to 34-fold.

Direct i.d. or i.m. DNA injection vaccination very often results in the induction of a Th1 response characterized by interferon (IFN)-γ synthesis and predominately IgG2a antibodies in mice [6, 24]. The doses applied by needle injection are normally in the range of about 10–100 μg of naked plasmid DNA. In the case of gene-gun DNA application, only 0.1–1 μg of plasmid DNA is sufficient to induce antibody or CTL responses. The gene-gun or biolistic system uses compressed heli-

um to propel micrometer-sized colloidal gold particles coated with precipitated plasmid DNA (DNA-coated microparticles) directly into the epidermal cells. In mice, intraepidermal gene-gun DNA inoculation generates a prominent Th2 response with interleukin (IL)-4 production and an excess of the IgG1 isotype [26, 36, 40]. The approximately $\log_{10}$ of the DNA dose may explain the dominance of the Th2 response because low plasmid doses result in a low CpG motif moiety, which is important for a Th1 response elicitation [62, 87].

Needle-free jet injection (Biojector) is another DNA delivery approach which has been investigated extensively as a method of i.d. immunization of laboratory animals, such as mice, pigs, rabbits, dogs, and monkeys [17, 76]. Gramzinski et al. [38] analyzed the effect of various routes for immunizing with a DNA vaccine encoding hepatitis B surface antigen (HBsAg). Experiments confirmed that needle-free injection of a DNA vaccine (biojection of DNA) induces a greater immune response to HBsAg antigen than i.d. or i.m. injection using the classic needle-syringe approach [38]. Similar results were obtained in the earlier experiments of Baizer et al. [4]. The needle-free injection of DNA has been tested in several human clinical trials as well [27], e.g. a Gag-Pol candidate HIV DNA vaccine [103].

The delivery of DNA vaccines to the liver. A high level of DNA vaccine expression in liver cells was achieved by the rapid injection of naked plasmid DNA in relatively large volumes via the tail vein, the portal vein, the hepatic vein, and the bile duct in mice and rats [61, 112]. This liver-specific approach has been designated "hydrodynamic delivery" and it is increasingly being used as a research tool for elucidating the mechanisms of gene expression and the role of genes and their cognate proteins in the pathogenesis of diseases in laboratory animal models [45, 46, 101]. The procedure has also been shown to be effective in large animals such as dogs and non-human primates [96, 116]. The hydrodynamic approach is proving to be a very useful research tool not only for gene expression studies, but also more recently for the delivery of small interfering RNA [46, 59, 71]. The application of DNA vaccine to the liver is associated with enormous protein expression followed by a strong antibody- and cell-mediated immune response. When the same dose of DNA was administered by hydrodynamic application or by i.m. or i.d. injection, the antigen-specific antibody levels induced by the hydrodynamic application were approximately 40 times higher (Raska et al., unpublished result).

DNA vaccine minimization to minicircle DNA. Minicircles are small circular DNA molecules that are derived from parental DNA plasmids by specific recombination. Antibiotic resistance genes, selection markers, and bacterial origins of replication are fully removed by the specific recombination [13, 80]. Minicircles contain only the gene of interest, making them promising tools for DNA vaccination and gene therapy. Chen et al. [15] have shown that minicircles can express high (45- and 560-fold more) and persistent levels of target protein in mouse liver compared with their parent plasmids. This could be attributed to a higher transfection efficacy and the low to minimal content of CpG in minicircle DNA. Avoidance of bacterial DNA also increases the safety of DNA application because no antibiotic-resistant genes can be passed to pathogenic bacteria present in host tissues.

Non-physical methods for enhancing DNA delivery to target cells: chemically modified DNA vaccines

Non-viral carrier systems: DNA/polyplexes. DNA carriers tested for DNA vaccination are various molecules which complex with DNA by 1) electrostatic forces between negatively charged DNA molecules and a positively charged carrier (or cationic ions on a negatively charged carrier), 2) analogous to the natural DNA-protein interaction, or 3) artificial covalent linkage between DNA and a carrier [81]. The DNA/carrier complexes protect DNA from serum DNases, increase transmission of DNA through the cytoplasmic membrane of target cells, allow targeting to specific tissues, and some of them induce the escape of DNA entrapped in endosomes by promoting endosomal disruption (weak bases such as chloroquine, the proton-sponge effect of many polymers) [69]. Non-viral DNA vaccine delivery systems are based on 1) electrostatic complexation of DNA with cationic polymers (poly-L-lysine, protamine sulfate, polyethyleneimine, chitosan, polyethylene glycol, poly-(D,L-lactide-co-glycolide)), complexes commonly termed DNA/polyplexes, 2) electrostatic complexation and condensation of DNA with artificial cationic lipids or lipopolyamines (DC-chol, DOTMA, DOTAP, DOSPA, DOGS) which are mixed together with zwitterionic helper lipids responsible for the fusion of complexes with the target cell membrane (Chol, DOPE, DPPC), complexes commonly termed DNA/lipoplexes or DNA/lipopolyplexes, 3) complexation of DNA with artificial anionic lipids (DMPG) through electrostatic interaction mediated by Na^+ and K^+ ions which are supplemented with zwitterionic helper lipids (DPPC), complexes commonly termed fluid DNA/liposomes, 4) association of DNA with proteins or peptides (histones, peptide tyrosine-lysine-alanine-$(lysine)_8$-tryptophan-lysine, Fab fragments of anti-DNA antibodies, cationic viral proteins μ and Vp1) which in combination with cationic polymers or lipids facilitate nuclear targeting, and 5) complexation of DNA with dendrimers with a very low degree of polydispersity (PAMAM), complexes commonly termed DNA/dendrimers (Table 1) [8, 68, 69, 78, 84, 86, 104].

Cationic liposomes and polymers are accepted as effective vectors for gene delivery with low immunogenicity, unlike viral vectors [84]. Liposomes are often used for systemic (intravenous, i.m., i.d.) or topical (nasal, oral) DNA administration [31, 43, 50, 69, 74, 84]. The final DNA/lipopolyplex structures, DNA concentration, ratio of cationic moiety to DNA, and supplements such as condensing agents, endosmolytic agents,

Table 1. Composition of the most frequently used non-viral carriers

Lipids	Abbreviation	Structure
Cationic lipids	DC-chol	3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol
	DOTMA	N-[2,3-(dioleyloxy)propyl]-N,N,N-trimethylammonium chloride
	DOTAP	1,2-bis(oleoyloxy)-3-(trimethylammonio)propane
	DOSPA	2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate
	DOGS	Dioctadecylamidoglycylspermine
Zwitterionic lipids	Chol	Cholesterol
	DOPE	Dioleoylphosphatidylethanolamine
	DPPC	Dipalmitoylphosphatidylcholine
Anionic lipids	DMPG	1,2-dimyristoyl-sn-glycero-3-[phosphor-rac-(1-glycerol)]
Dendrimers	PAMAM	Polyamidoamine

or nuclear targeting molecules are the most critical factors in the transfection efficacy of each formulation [36, 50, 86].

The development of modified DNA/lipopolyplexes suitable for intravenous application and targeting to tissues and organs such as heart, lung, liver, spleen, or kidney is desirable and necessary. The current focus is on decreasing the toxicity of complexes and increasing transfection efficacy and tissue specificity. To target specific tissue, the carriers are modified by linkage to various ligands allowing DNA complexes to be recognized by specific tissue and cell populations.

Targeting to the liver is possible by linking liposomes or other polymers (polylysine, PEI, polyamidoamine dendrimers) with a sugar motive recognized by the liver-specific asialoglycoprotein receptor, which is expressed on hepatocytes. Such ligands are composed of trimeric terminal galactose [2, 70, 117]. Lipid-based DNA/complexes are a promising approach in systemic application because of the minimal toxicity of particular lipids (DPPC) in contrast to cationic polymers [84]. On the other hand, lipid-based DNA complexes are larger in size and are thus limited in their penetrability into liver-specific compartments (Disse spaces), which are the port for hepatocyte targeting [11, 33, 42, 56]. Therefore an effort has been made to decrease the size of final complexes by protamine sulfate or poly-L-lysine, which increased the transfection efficacy in some *in vitro* experiments by up to 30 times [2, 78].

Targeting the DNA/complexes to DCs, mainly by interaction with the mannose receptor on the DC surface, is immunologically very promising. A specific ligand, mannose, was linked to a variety of polymers and lipids used for DNA/polymer preparation which were tested *in vitro* or *in vivo* according to their DC specificity and transfectability. Unfortunately, the high transfection efficacy commonly achieved in *in vitro* experiments is still dramatically diminished under *in vivo* conditions [23, 74].

Viral carrier systems. One highly efficacious delivery system for DNA vaccines, or, more precisely, genetic vaccines, is based on recombinant viral vectors derived either from attenuated viruses used for preventive vaccination (vaccinia, poliovirus, hepatitis B virus, measles virus) or from viruses such as human adenovirus (HAdV), adeno-associated virus (AAV), alphavirus, vesicular stomatitis virus (VSV), or poxviruses other than vaccinia [41]. In contrast to plasmid DNA vaccines, virally vectored genetic vaccines induce a specific immune response not only against the expressed transgene, but also against the viral capsid and/or envelope and this response is often effective even after the first immunization. Therefore, repeated immunization, often necessary for elicitation of a satisfactory immune response against the transgene product, could be inefficient. Virally vectored genetic vaccination is generally performed as the second immunization after DNA priming, i.e. a heterologous vaccination approach [10]. The choice of transgene, viral vector, dose, route of application, prime/boost regimen, and number of immunizations are the most important factors influencing appropriate antigen-specific humoral and $CD4^+$ and $CD8^+$ cellular responses. Furthermore, delivery efficacy of viral vectors could be dramatically hampered by preexisting immunity to the capsid or envelope proteins in a population. Only a few viral vectors (alphavirus, VSV, and some serotypes of HAdV or AAV) are considered to be unrecognized by preexisting immunity of vaccines. Finally, similarly to DNA plasmid, a protective immune response elicited in experimental animals by virally vectored genetic immunization may not necessarily be observed in subsequent human clinical trials. This could be attributed to the above-mentioned preexisting immunity, the restricted range of viral hosts, restricted viral tissue tropism, or to many other, not yet well-described factors.

Poxvirus-derived vectors are some of the most frequently tested. They are safe and genetically stable double-stranded (ds) DNA vectors whose entire life cycle occurs in the cytoplasm of somatic cells. They do not enter the nucleus and therefore do not integrate into the host genome. Poxviruses are capable of carrying large amounts of foreign genetic material. Two vaccinia-derived vectors, the New York Vaccinia Virus

(NYVAC) and Modified Vaccinia Virus Ankara (MVA), have been tested in human stage I and II clinical trials for the prevention of infections such as HIV infection, malaria, tuberculosis [41, 60, 93]. Edible bait vaccine, Raboral VR-G, is licensed worldwide for the prevention of rabies in animals. Vectors derived from avian poxviruses such as canarypox virus (ALVAC) and fowlpox virus (FWPV-9) are expected not to be recognized by preexisting immunity in humans. Poxvirus-vectored transgenes induce good specific humoral and $CD4^+$ and $CD8^+$ cellular responses [10, 18, 30].

Recombinant adenoviruses are extensively tested for both vaccination and gene therapy. They contain dsDNA in a rigid icosahedral non-enveloped nucleocapsid. The wild-type of adenovirus does not integrate into the genome. Nevertheless, HAdVs are oncogenic for animals and thus the safety of HAdV therapy is still an open question [111]. Replication-incompetent recombinant HAdVs are able to carry transgenes of up to 8 kb. Adenoviruses are used because of effective cellular uptake and transgene expression. Wild and recombinant adenoviruses induce intense inflammatory responses followed by an induction of serotype-specific immunity, which could hamper the effectiveness of a subsequent vaccination with the same serotype [79]. Among the about 40 known HAdV serotypes, HAdV-5 is the most explored for recombinant vaccine application. HAdV-5 has one of the weakest pathogenicities associated with mild upper respiratory disease and fever in humans. Recombinant HAdVs were tested in animal models as a potential vaccine for HIV, malaria, SARS, and Ebola. Heterologous DNA-prime/recombinant adeno-boost immunization induced both humoral and $CD4^+$ and $CD8^+$ cell responses in experimental animals. A weak response was detected in human volunteers. Because the prevalence of HAdV-5 is relatively high in the population, altered surface proteins or chimeric HAdV-5 carrying the surface proteins of another serotype are developed for avoiding the hampering effect of preexisting immunity [5].

AAV are members of the *Parvoviridae* family of single-stranded (ss) DNA non-enveloped viruses of the genus *Dependovirus* [10]. Recombinant AAVs are non-replicative but persist within the cells as non-episomal, mainly circular DNA. The integration frequency determined in rodent and rabbit muscle tissue is less than approximately 10^{-7}, which is two orders lower than spontaneous mutations in human genes. Recombinant AAVs could package 5 kb of ssDNA, including ITR (transgene <4.5 kb) [58]. Within the cell, the ssDNA genome is transcriptionally active after conversion to a dsDNA template, which takes *in vivo* a few weeks, a necessary delay before the induction of an immune response. Therefore, dsAAVs were developed. The maximum capacity for transgene insertion is 1.2 kb [109]. In mice and rhesus macaques, a single i.m. injection of serotype 2 AAV expressing HIV antigens induced robust specific antibody and $CD8^+$ cell responses [115]. A significantly lower response in humans could be attributed to many factors, including preexisting immunity, because the presence of specific neutralizing antibodies ranges from 35–80% according to age group and geographic location. Specific antibodies recognize other serotypes, but their neutralizing activity seems to be less prominent, for example AAV-1 with an affinity for muscle and hepatocytes, AAV-6 for airway epithelium, AAV-7 for muscle, and AAV-8 for hepatocytes. Beside vaccination, AAV vectors are one of the most frequently tested vectors for gene replacement therapy [7].

Alphavirus vectors were earlier used for *in vitro* recombinant protein expression. Their wild precursors belong to the *Togavirus* family of positive ssRNA enveloped viruses which replicate entirely in cytoplasm. A high level of transgene expression from this viral promoter is achieved due to the self-replicating nature of viral RNA and the efficient inhibition of translation of host mRNA by the viral replicase. Because of limited prevalence, the preexisting immunity to the vector seems to be minimal. In experimental animals, immunization with vectors based on Sindbis, Semliki Forest, and Venezuelan equine encephalitis viruses induced a cellular and humoral immune response to the transgene. In the case of HIV-1 antigens the immune response in non-human primates was able to significantly reduce the viral load after challenge [10, 51].

The VSV belongs to the *Rhabdoviridae* family of enveloped negative-sense ssRNA viruses. VSV-derived vectors are able to generate viral particles that express foreign transmembrane protein on the membrane surface. Because VSV proteins do not down-regulate the interferon response of an infected cell, VSV could exhibit promising adjuvant activity. Although wild-type VSV could induce neuropathy after intranasal application to mice, recombinant VSVs were proved to be safe in non-human primates after intranasal and i.m. application. Immunization of non-human primates with VSV expressing HIV and SIV antigens demonstrated efficacy in subsequent challenge with the highly pathogenic SHIV89.6P virus. Thus the potential neural toxicity of VSV needs to be finally solved before entering into the clinical trials [25].

A few additional viral vectors were designed for genetic vaccination. One viral vector of future vaccines will be probably based on the attenuated measles virus, an enveloped negative-sense ssRNA virus. At present, genetically stable recombinant vaccine strains are available for cloning up to three transgens (Edmonston, Schwartz). In animal experiments a recombinant measles vector expressing HIV-1 envelope antigen induced neutralizing antibodies and envelope-specific $CD4^+$ and $CD8^+$ cell responses after a single dose [67]. Although about 90% of the population experienced a measles infection or vaccination, it is estimated that 10 years after the measles experience the immune response does not preclude successful immunization with recombinant measles vaccines. Furthermore, it is expected that within 10 to 20 years measles will be eradicated [10].

DNA/polyplexes for targeting DNA vaccine to mucosal surfaces. Mucosal immunity establishes the first line of the defense against pathogens which attack the body via mucosal surfaces [8]. Induction of the mucosal immune response, either cellular or humoral, requires local mucosal application of antigen or an antigen-coding DNA vaccine. Although some mucosal response is detectable after a systemic DNA vaccination, i.m. injection and gene-gun delivery of plasmid DNA have a limited ability to induce mucosal immune responses [99]. Therefore, various mucosal DNA application routes have been tested to achieve sufficient antigen production on mucosal surfaces (intranasal and intratracheal application, inhalation of DNA vaccine in aerosol form, application of DNA on external genital mucosa, and oral administration) [32, 40, 65]. Complexing DNA with various polymers enhances DNA uptake on mucosal surfaces. DNA/polyplexes adhered to the mucosal cells either through specific receptors or through electrostatic interaction between a negatively charged mucosal cell surface and positively charged DNA/polyplexes. When DNA/lipoplexes were delivered orally or intranasally, they induced a significant mucosal immune response, including secretory IgA responses [82, 94].

Enhancement of the immune response by mechanisms affecting immune response: molecular adjuvants in mono- and bi-cistronic DNA vaccines, CpGs

Molecular adjuvants based on cytokine or co-stimulatory molecules

An alternative means of enhancing the efficiency of DNA vaccines is the use of genetic adjuvants. Genetic adjuvants are most often genes coding for cytokines, chemokines, or co-stimulatory molecules. The cDNA can be either administered in separate DNA plasmids (monocistronic DNA vaccine) or the cDNA is cloned into parental DNA vaccine plasmid under separate promoters or under promoters shared with antigen-coding DNA sequence separated by an internal ribosome entry site element (bicistronic DNA plasmid) [29].

Such molecules supply T cells or DCs with the second, antigen-independent stimulatory signal. Several co-stimulatory molecules have been tested for enhancement of a) APC activation (CpG, CD40L, MIP-1α), b) CTL response (IL-1, IL-2, IL-12, IL-15, IL-18, GM-CSF, IFN-γ, CD40L, ICAM-1, LFA-3), c) Th1-type antibody production: IgG2a in mice (IL-1, IL-2, IL-7, IL-12, GM-CSF, IFN-γ, CD40L) or Th2-type antibody production: IgG1 in mice (IL-4, IL-7, IL-10, TGF-β), and d) cellular response associated with IFN-γ induction (IL-1, IL-2, IL-7, IL-12, GM-CSF, IFN-γ, CD-40L, ICAM-1, LFA-3) [3, 16, 18, 35, 40, 64, 83, 102]. Two additional co-stimulatory molecules of the tumor necrosis factor receptor superfamily expressed on activated T cells are antiapoptotic, i.e. 4-1BB (CD137), expressed on $CD8^+$ T cells, and its $CD4^+$ T cell analogue OX40 (CD134), which protect activated T cells from death and thus enhance the antigen-specific T cell response. Stimulating these receptors may be useful in DNA vaccine development [77].

The specificity of molecular adjuvants used to elicit mucosal immunity

During mucosal DNA vaccination, specific parameters are critical for the use of molecular adjuvants. The proinflammatory cytokines IL-1α, IL-12, and IL-18 were tested for antibody and mucosal CTL responses. IL-15 has the potential to increase antigen-specific CTL activity and is thus particularly interesting due to its potential role in regulating the homeostasis of memory T cells [64]. IL-5 and IL-6 were shown to be able to markedly increase IgA reactivity to co-expressed heterologous antigen. Monocyte chemoattractant protein-1 was effective in increasing mucosal IgA secretion and CTL responses [105]. RANTES, lymphotactin, MIP-1β, MIP-2, human neutrophil peptides (HNP-1, HNP-2, HNP-3), IFN-γ, and IFN-β are important candidates for use as adjuvants [28, 100].

Modification of the immune response by fusion of antigen with signal peptides

Another approach used for modification of the immune responses to DNA vaccines includes the addition of heterologous gene fragments encoding localization or secretory signals or fusion of the antigen-coding cDNA with the sequence coding for ligands which drive the antigen to sites appropriate for immune induction. For example, a number of studies have shown that higher titers of antigen-specific IgG (IgG1 in mice) were elicited when the antigen was secreted rather than localized on the cell membrane or within the cell [40, 66]. The cellular localization of heterologous antigen may play a role in modulating the immune response, although the role may depend upon the nature of the antigen and/or the model system used. Another strategy consists of fusing the antigen-encoding gene with the ubiquitin-encoding gene, thereby accelerating cytoplasmic degradation of the antigen by targeting it to proteasomes and improving class I antigen presentation [35]. Enhancement of the immune response could additionally be reached by fusion of antigen with the hsp70-binding viral J-domain during the construction of DNA vaccines. This molecule stabilizes fusion antigen by binding to the hsp70 protein, which in addition serves as an instruction molecule to induce an increase in the $CD8^+$ T lymphocyte and B lymphocyte response [91]. Further, DNA vaccine potency may be improved through fusion of the antigen-coding DNA with the endosomal/lysosomal sorting signal sequence (derived from lysosome-associated membrane protein type 1; LAMP-1), which directs the expressed antigen towards MHC class II molecules. Thus the $CD4^+$ T cell response could be significantly enhanced [53].

The above-mentioned use of the bicistronic vector is

the most common practice for heterologous production of the binary protein complex, but these methods are primarily in the research stage [52]. Bicistronic vectors could be useful in constructing multivalent vaccines derived from two, and possibly more, different antigens originating from one or more microorganisms.

CpG motifs

An element common to the majority of plasmids are the cytosine-phosphate-guanosine dinucleotides (flanked by two 5´ purines and two 3´pyrimidines recognized in mice) [34], called CpG motifs, which are unmethylated when the plasmids are amplified in a bacterial host. Hypomethylated or unmethylated CpG nucleotides are specific to bacterial DNA, but are very rare in eukaryotic DNA. CpGs play an important role as an immunomodulatory component of DNA vaccines [20]. They are recognized by the Toll-like receptor (TLR)9 localized in the cytoplasm [106]. CpGs are potent stimulators of B cell proliferation and antibody secretion. CpG induce APCs (macrophages and DCs) to secrete Th1-type cytokines IL-12 and IFN-α/β, which activate natural killer cells and T cells ($CD8^+$) [40, 54]. Rankin et al. showed that CpG motifs or sequences which are effective in mice are ineffective in humans [88]. This highlights the species diversity within TLR substrate specificity. The amount of CpGs in plasmid backbones could be changed by simple recombinant technology. Thus the addition of CpGs to DNA plasmids could theoretically decrease the DNA vaccine dose used for a single immunization. However, from murine experiments it is clear that too many CpG motifs can actually reduce immunogenicity [40].

SAFETY

The major safety concerns here are: 1) the integration of the plasmid DNA into the host genome, thereby increasing the risk of malignancy by activating protooncogenes or inactivating oncosuppressors, 2) transfer of antibiotic-resistant genes to surrounding bacteria, 3) the induction of an immune response to transfected cells, resulting in the development of an autoimmune disease, 4) the stimulation of cytokine responses that alter the host immune homeostasis, and 5) the induction of tolerance rather than immunity [24, 37, 55, 73].

At the present time there is still no evidence for plasmid DNA integration into the host genome although traces of plasmid DNA are detectable in host cells up to one year after DNA vaccination. Current methods used for the detection of plasmid integration into genomic DNA are not sufficiently sensitive or specific and this question remains open [40].

The spread of antibiotic resistance is another important question. DNA plasmid could be detected far from the original site of injection (in the case of i.m. or i.d. application). The responsible carriers were transfected lymphocytes and macrophages. Such cells, of course, could spread antibiotic resistance genes to bacteria. There is, however, no direct experimental evidence to date for this phenomenon [40]. Both plasmid integration and antibiotic resistance transfer could be effectively minimized by minicircle DNA technology, as mentioned above [15].

Autoimmune response a) against DNA plasmid, mediated by anti-DNA antibodies or b) against transfected cells, mediated by a type I immune response was experimentally modeled in mice [40]. DNA vaccination was able to moderately increase the DNA-specific serum antibody titers for a limited period of time, but clinical signs of autoimmunity were not confirmed. CpG motifs, on the other hand, may induce a truly harmful autoimmune response when injected together with autoantigens such as the myelin basic protein (inducing encephalomyelitis) or a *Chlamidia*-derived antigen (inducing myocarditis) in experimental models. The above effects were not found in healthy animals treated with therapeutic DNA vaccine doses and no sign of toxicity was found in human volunteers exposed to DNA vaccines during clinical trials [40, 41, 64].

The use of cytokine genes in modern multicistronic DNA vaccines could theoretically disrupt the immune homeostasis and increase susceptibility to other infections. It could also be associated with exacerbation of autoimmune or allergic diseases. Experimental observations have confirmed that the cytokines are released locally and that serum cytokine levels are unchanged [40].

Induction of tolerance rather than immunity after DNA vaccination is a problem for both extreme age groups. Very young animals (mice under 8 days of age) and very old ones (mice older than 2 years) respond weakly to DNA vaccination. In newborn mice, DNA vaccine encoding circumsporozoite protein of the malaria plasmodium induced tolerance which was long lasting [75]. Thus DNA vaccination schedules for both extreme age groups would need to be separately tested, possibly modified by use of cytokines or addition of costimulatory molecules.

CLINICAL TRIALS

The majority of ongoing human DNA vaccination trials are focused on assessing vaccine safety and immunogenicity [63, 98]. As estimated from the US database at www.clinicaltrials.gov as of June 2007, approximately one hundred DNA vaccination clinical trials have been registered (irrespective of their trial phase). A large proportion of DNA vaccines use viral vectors as delivery systems. The DNA vaccine Gendicine, produced by SiBiono Genetech, uses an adenovirus vector for delivery of DNA encoding the p53 suppressor. It is registered by the Chinese FDA for treatment of head and neck squamous cell carcinoma [78]. Another adenovirus-vectored p53-encoding DNA vac-

cine, Advexin, produced by Introgene Therapeutis, is being tested in phase III clinical trials for the same indications in the US.

If we focus on plasmid (naked) DNA vaccines, approximately 40 clinical trials had been registered by www.clinicaltrials.gov as of May 2007. Ten have attained phase II. The majority of DNA vaccines are focused on HIV-1 infection. Other infectious diseases include hepatitis B, malaria, Ebola hemorrhagic fever, West Nile virus infection, and avian flu. The other trials deal with various cancers (hepatocellular carcinoma, renal cell carcinoma, pancreatic cancer, chronic lymphocytic leukemia, breast cancer, ovarian cancer, prostate cancer, bladder cancer, synovial sarcoma, leiomyosarcoma, lung cancer, and melanoma).

All ongoing clinical trials have confirmed minimal toxicity and good tolerance to DNA vaccines. However, the still poor immune response to the majority of clinically tested DNA vaccines is a great challenge for further optimization using novel antigen, delivery systems, and prime-boost-based schedules.

CONCLUSIONS

DNA vaccines remain a promising approach for inducing both humoral and cellular immune responses. One of the more interesting aspects of DNA vaccination is the mechanism of inducing antibody- and/or cell--mediated immune responses. This is associated either with antigen presentation on vaccine transfected cells or with cross-presentation of the antigen by DCs, which are the only known cells capable of inducing both $CD8^+$ and $CD4^+$ T cell response. DNA vaccines are effective inducers of host immune protection against viral, bacterial, fungal, and parasitic infections and may be suitable for cancer therapy. Multicistronic DNA vaccines that, for example, coexpress cytokines may be able to modulate any autoimmunity or allergic reactions. One basic obstacle to applying DNA vaccines in human medicine is their still relatively poor immunogenicity, linked to low transfection efficacy and low antigen production. Thus the development of effective delivery systems is the main research challenge in this area of immunology.

Acknowledgment: This work was supported by grant MSM 6198959223 (Ministry of Education, Youth, and Sport, Czech Republic).

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