Recent advances towards the clinical application of DNA vaccines

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ABSTRACT

DNA vaccination is an attractive method for therapeutic vaccination against intracellular pathogens and cancer. This review provides an introduction into the DNA vaccination field and discusses the pre-clinical successes and most interesting clinical achievements thus far. Furthermore, general attributes, mechanism of action and safety of DNA vaccination will be discussed. Since clinical results with DNA vaccination so far show room for improvement, possibilities to improve the delivery and immunogenicity of DNA vaccines are reviewed. In the coming years, these new developments should show whether DNA vaccination is able to induce clinically relevant responses in patients.

KEYWORDS

DNA vaccination, cancer vaccination, infection diseases

WHAT IS DNA VACCINATION?

DNA vaccination, or genetic vaccination, is the common name for vaccination methods that induce immunity by transfecting host cells with DNA that encodes an antigen, rather than by injecting antigens in the form of protein or peptide. Once transfected, cells of the host start producing the protein encoded by the DNA leading to an immune response against this protein along similar lines as responses that occur against conventional vaccines. Nonetheless DNA vaccines have clear advantages that we will address in the next paragraphs.

Currently, no DNA vaccines are registered for use in humans. Although some DNA vaccines are registered for veterinary use (e.g. a prophylactic West Nile virus DNA vaccine for horses[1,2] and a therapeutic DNA vaccine for melanoma in dogs[3,4]) and plenty of reports show efficacy of DNA vaccination in non-human primates, evidence for efficacy in humans is scarce. However, there is an ongoing research effort aimed at putting the DNA vaccination platform to human use. Based on these studies the advantageous attributes of DNA vaccines have become clear.

GENERAL ATTRIBUTES OF DNA VACCINES AND ADVANTAGES OVER ALTERNATIVE VACCINATION PLATFORMS

In contrast to the complicated processes needed for vaccines such as attenuated viruses or subunit protein vaccines, plasmid DNA (pDNA) is easy to design and construct. Moreover, it is cheap and also relatively easy to manufacture. Furthermore, pDNA is fairly stable at room temperature[5] again in contrast to attenuated viral vaccines, whose storage and global delivery are complicated by the need to keep the vaccines cold. Another attractive feature of DNA vaccines is that, since the antigen is made in situ, it will inherently get post-translationally modified in a similar way as during infection with the cognate pathogen. Another important attribute of DNA vaccination is that the protection induced by DNA vaccines tends to skew towards cellular immunity, which is believed to be crucial for successful vaccination against intracellular pathogens (e.g. viruses) and cancers.[6,7]

Apart from these minor advantageous attributes, one feature of DNA vaccines stands out in comparison with vaccines based on vector systems such as modified vaccinia Ankara (MVA) and adenoviruses. This concerns

so-called 'vector-specific immunity': the phenomenon that pre-existing immunity against the vector decreases the efficacy of the vaccine. For example, in a study using an MVA-based vector, patients with pre-existing immunity against smallpox responded significantly less to vaccination than individuals without pre-existing immunity against the vector.[8] It should be noted that a bias was present in this study though, as older patients were overrepresented in the group with pre-existing vector-specific immunity (smallpox immunisation was terminated in 1980) and ageing is associated with decreasing immune responses. However, in a study using an adenoviral-based HIV vaccine an attenuating effect of pre-existing adenoviral immunity was observed as well.[9] A variant to this phenomenon is the situation in which, during consecutive boosts, the immunity directed against irrelevant components of a vaccine hampers that against the relevant antigen, resulting in loss of efficacy.[10]

Besides loss of efficacy, vector-specific immunity may result in more serious side effects as illustrated in the STEP trial. This clinical trial used a modified adenovirus (strain Ad5) encoding HIV antigens[11] and included individuals with both high and low titres of pre-existing antibodies directed against Ad5. The study was stopped for futility (i.e. it was obvious that vaccine efficacy would not be demonstrable). However, after extended follow-up, vaccinees with pre-existing anti-Ad5 antibodies showed higher infection rates with HIV within 18 months after vaccination than those in the placebo group.[12] In individuals with low anti-Ad titres, no difference in HIV infection rate was observed. It has to be noted that despite substantial efforts, no causal link has been established between Ad5 seropositivity and HIV infection (PLoS One. 2012; 7(4): e33969). Nevertheless, these results highlight the need to increase our understanding of the role of anti-vector immunity in adenoviral- and MVA-based vaccination.

Contrary to adenoviral- and MVA-based vaccines, DNA vaccines consist simply of naked pDNA. Sometimes it is formulated with a synthetic carrier, but it never contains other antigens. Vaccination only leads to the production of those proteins that are specifically desired for the immune response. Hence, vector-specific immune responses do not arise. This facilitates regimens based on multiple consecutive boosts. Based on the fact that the majority of the current human vaccines require two or more administrations, it is unlikely that a single administration will suffice for novel vaccines against difficult targets such as HIV, mycobacteria or cancer. For such targets, a scenario in which multiple boosts are required during a lifetime for optimal protection seems more realistic. Even more so if protection depends on cellular immunity, as in the process of ageing cellular immunity wanes considerably.[13] Currently, only DNA, RNA, subunit and (long) peptide

vaccines provide optimal 'boostability' in this respect since these platforms do not expose the vaccinee to potentially immunogenic moieties other than the antigen of choice.

MECHANISM

More than 20 years after its introduction, the mechanism of B- and T-cell induction after DNA vaccination is still only partly understood. Initial reports suggested a mechanism where the injected DNA transfected professional antigen presenting cells (pAPCs) present at the injection site, leading to their maturation, migration to the draining lymph node (DLN) and subsequent priming of T and B cells.[14] However, this notion was contradicted by reports showing that DNA vaccines encoding antigens driven by myocyte-specific promoters were equally immunogenic as those driven by ubiquitous promoters.[15] Since myocytes can neither migrate to the DLN nor prime T cells (they do not express co-stimulatory molecules) it quickly became clear that pAPCs can ingest the antigen produced by myocytes and then prime the ensuing immune response. Surprisingly, apart from priming B and CD4+ T cells as part of a humoral response, these pAPCs also primed a strong cellular response consisting of CD8+ T cells.

Why is this surprising? Exogenous antigen ingested by pAPCs localises to endocytic vesicles that fuse with the lysosome, leading to degradation of the contents. The antigenic fragments then get loaded onto MHC-II molecules to form an MHC-II–peptide complex that is targeted back to the cell membrane in order to stimulate cognate CD4+ T cells in the DLN (which in turn provide help for B-cell priming). The fact that these vesicles are known not to contain MHC-I molecules and hence do not generate MHC-I–peptide complexes for CD8+ T cell stimulation made this 'priming pathway' hard to swallow as the pathway responsible for CD8+ T-cell priming. However, in recent years various molecular mechanisms have been described that explain how antigens ingested by pAPCs are presented on MHC-I, leading to CD8 T cell stimulation. These mechanisms are referred to as 'cross-presentation'. The mechanism in which pAPCs get transfected themselves (the standard pathway for generation of MHC-I–peptide complexes in any cell) instead of ingesting the antigen, is referred to as 'direct presentation'. Currently, it is clear that both pathways contribute to T-cell priming by DNA vaccines (See *figure 1* for a schematic diagram of antigen expression and presentation upon DNA vaccination).

In DNA vaccination the amount of antigen produced is very limited due to the low transfection efficiency of host cells by the pDNA. This limitation makes the quantity of antigen that is expressed by the transfected tissue the

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Figure 1. *Schematic diagram of antigen expression and presentation upon DNA vaccination. DNA is taken up by cells via endocytosis or via direct cytosolic uptake. After endosomal escape, cytosolic trafficking and nuclear entry, the pDNA can be transcribed into mRNA, followed by intracellular translation of the antigen. For T-lymphocyte activation, antigens have to be presented in the context of MHC class I or MHC class II molecules in the presence of co-stimulatory molecules (such as CD80/86 and CD40). Since professional antigen presenting cells (pAPCs) are the only cell type that have both classes of MHC, can express co-stimulatory molecules and can migrate to the lymphatic system, their role in antigen presentation and T-lymphocyte activation is crucial. Via extracellular release by non-pAPCs or cell death, antigens can enter the MHC II pathway. Antigens produced by direct transfection of pAPCs are presented by MHC I. In addition, antigen intracellularly produced by non-pAPCs can enter the MHC class I pathway in pAPCs by a process called cross-presentation. (Reprinted from reference 58, with permission from Informa Healthcare)*

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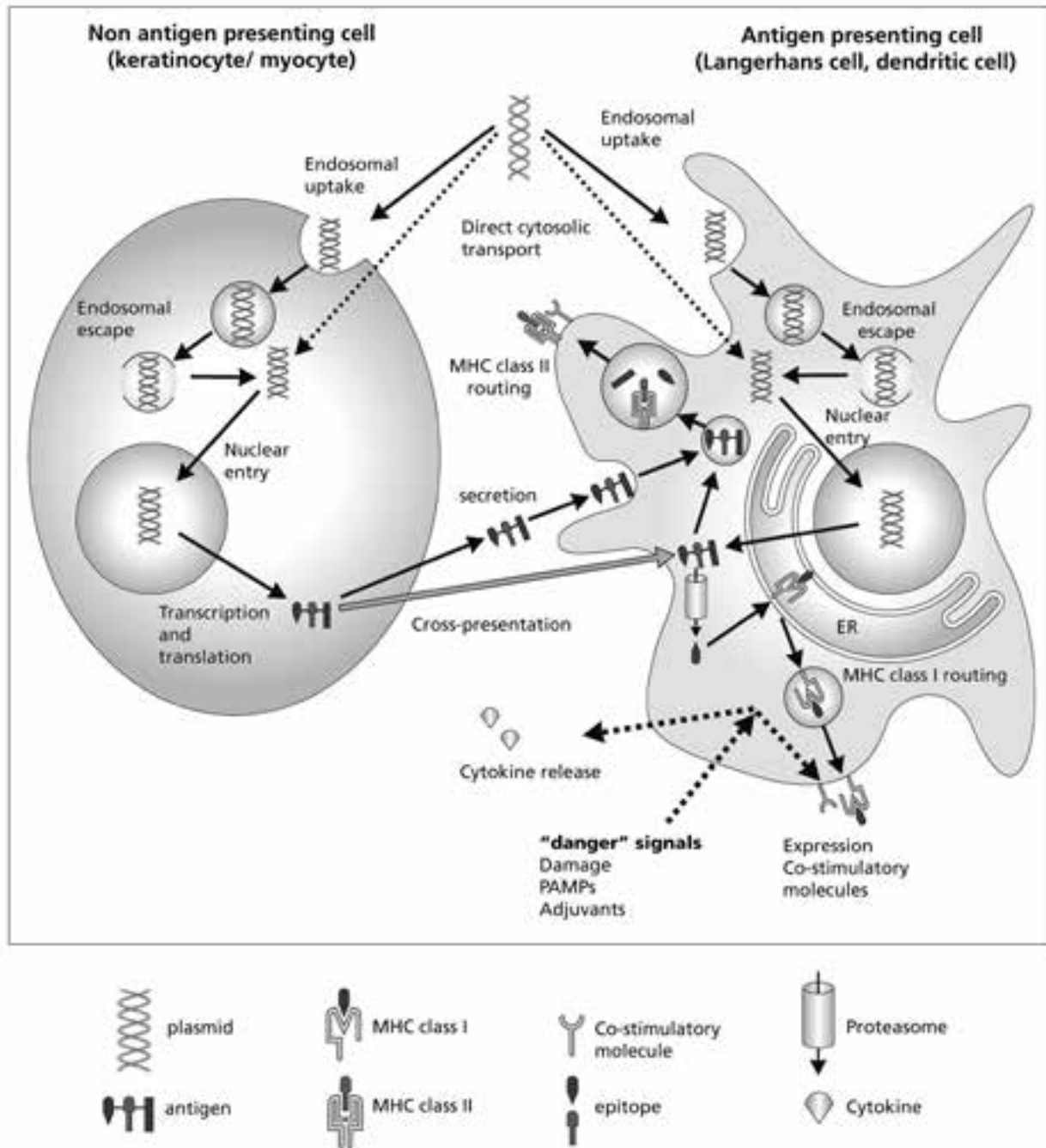
Achilles heel of the method. Therefore, it is crucial that the little antigen that is produced is maximally available for antigen presentation by pAPCs. As efficient presentation along each route requires different antigenic properties, it is very important to know whether priming occurs optimally via direct or cross presentation. At the moment this is unclear. Neither do we know what circumstances favour each pathway.

Besides the availability of antigen, both cross and direct presentations require maturation signals for the pAPCs in order to present the antigen. During infection with a pathogen these signals are provided by non-self 'molecular patterns' on the pathogen that are perceived by receptors on the pAPCs. These 'pathogen-associated molecular patterns' (PAMPs) ligate 'pattern-recognition receptors' (PRRs) on pAPCs leading to their maturation and migration to the DLN. Examples of common PAMPs are double-stranded RNA, components of the cell wall of gram-negative bacteria (LPS) and DNA sequences containing unmethylated cytosine-guanine DNA sequences ('CpG' sequences). These molecules are all scarce or absent in the host and abundant in viruses and bacteria. Hence they have evolved to provide the first 'danger signal' to be perceived by the host immune system upon infection. This signal appears to be indispensable for mounting a B- or T-immune response.

In vaccines that do not contain live attenuated pathogens, this 'danger signal' is provided by so-called 'adjuvants'. These are substances that are added to the formulation in order to trigger the PRRs of pAPCs, thereby providing the danger signal leading to their maturation and migration to the DLN. Contrary to this, DNA vaccines have long been thought to have adjuvancy by their own merit. Due to its bacterial origin, *pDNA* is abundant in unmethylated CpG sequences, which ligate a PRR on the pAPC that is named 'Toll-like receptor 9' (TLR9).

Surprisingly, DNA vaccination studies in TLR9 deficient (-/-) and proficient (+/+) mice have not pointed out a clear role for TLR9 and CpG motifs. Babiuk *et al.*[16] found that TLR9+/+ and TLR9-/-mice mounted immune responses of similar potency. Most reports show that activation via TLR9 is not necessary for the generation of immune responses by DNA vaccines,[17,18] but does increase their potency to some extent. An explanation that accounts for this is that although CpG-mediated activation of DCs plays a role, other danger signals are generated during transfection of host tissue, making the TLR9 signal less critical. For example, in keratinocytes the presence of pDNA in the cytoplasm can be detected by other molecular sensors such as DAI (DNA-dependent activator of IFN-regulatory factors) and AIM-2 (absent in melanoma-2),[19] resulting in the activation of a cell stress signalling complex named the inflammasome and leading to the production of immunogenic cytokines such as IL-1 and IL-18.

INTERESTING TRIAL RESULTS

A number of human clinical trials of DNA vaccines have been performed or are ongoing for infectious diseases, as well as cancer and autoimmune diseases (reviewed in references 20 and 21). We will briefly summarise some of the results below.

After an initial series of disappointing clinical trials in the early 1990s, the first moderately successful DNA vaccination result in humans was obtained with a malaria-specific DNA vaccine.[22] This trial demonstrated the emergence of vaccine specific T cells in the peripheral blood of 11 out of 20 malaria-naive volunteers after three intramuscular pDNA injections. The study did not assess clinical benefit. Similarly, gene gun administration of HBV DNA was able to induce antibodies in 12 out of 16 patients who had not responded to the licensed (recombinant protein based) vaccine.[23] Although protection against HBV was not assessed, the induced antibody titres were considered protective based on data obtained with conventional HBV vaccines.

Various other trials have provided proof of principle for the capacity of DNA vaccines to induce humoral and cellular immune responses in humans.[23-26] However, the immune responses measured were not as robust as anticipated from the preclinical studies in any of these trials. For example, HIV-infected patients with high viral counts mounted a modest T-cell response against HIV Nef after DNA vaccination with a DNA vaccine encoding several HIV antigens,[27] without any effect on viral counts.

In an effort to combine the qualities of DNA vaccines with those of adenoviral- or MVA-based vaccines, so-called 'heterologous prime-boost' regimens have been developed. In these regimens the strong but broad (partly vector-specific) immunity induced by MVA- or adenoviral-based vaccines gets focused on the relevant antigens by DNA vaccination, which is very specific but less potent. Usually, in these regimens the DNA vaccine comes first as a 'prime', followed by the vector-based vaccine as a 'boost'. Particularly for HIV, where T cells may be the key to protection, many such trials have been performed and are ongoing, utilising boosts with modified adenoviruses[28-30] or MVA.[8,31-36]

DNA priming followed by MVA boosting has been studied clinically[37-39] for malaria vaccination with moderate success. One study that included a live malarial challenge following immunisation demonstrated that a DNA prime encoding the ME-TRAP antigen followed by an (ME-TRAP recombinant) MVA boost resulted in partial protection from challenge with live parasite.[37]

Currently, worldwide 27 clinical trials involving DNA vaccination are ongoing (*table 1*, based on www.clinicaltrials.gov). Notably, 15 of the 27 clinical trials are directed against tumour antigens, of which five against virus (HPV) derived tumour antigens. This illustrates that over the

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Table 1. *Number of trials worldwide involving DNA vaccination that are actively recruiting patients*

Disease	Number of trials currently open
HIV	8
CIN / Cervical cancer	4
Hepatitis B	1
Hepatitis C	1
HPV-related head/neck cancer	1
Influenza	1
Leukaemia	1
Lymphoma	2
Breast cancer	1
Prostate cancer	3
NET of skin (Merkel cell carcinoma)	1
Ovarian cancer	1
Pancreatic cancer	1
Allergy for Japanese Red Cedar	1

CIN = cervical intraepithelial neoplasia; HIV = human immunodeficiency virus.

last 20 years the focus of DNA vaccine development has shifted to tumour antigens, probably due to better funding opportunities in that field. In the Netherlands no clinical DNA vaccination studies are currently recruiting. However, in the first half of 2013 a phase I clinical trial will open at the Netherlands Cancer Institute/Antoni van Leeuwenhoek hospital for HPV-related cancer patients.

SAFETY IN PATIENTS

In general, DNA vaccines are considered safe for both patient and environment. Studies have reported good tolerability of DNA vaccines in humans and local reactivity at the injection site is the most commonly reported side effect in clinical trials thus far.[26,40-42] Nonetheless, a major concern of using DNA vaccines in the clinic is the potential risk that genetic information of the plasmid is integrated in the host genome of somatic cells. Genomic integration can occur during random or homologous recombinations and might lead to the activation of oncogenes or the inactivation of tumour suppressor genes, potentially resulting in neoplastic transformation. This remote risk is worth considering when DNA vaccines are applied for therapeutic vaccination in young patients.

To provide a context in the assessment of this risk, it is generally accepted to compare the integration rate of pDNA with the spontaneous mutation frequency of autologous genes. Although this spontaneous mutation rate varies largely between genes and individuals (based on external factors such as smoking or UV exposure) 2×10^{-6} spontaneous gene-inactivating mutations per

gene is generally accepted as the standard value. This number is adapted from a study by Cole *et al.* in which the mutation frequency of three genes in circulating cells was determined in several hundred volunteers.[43]

To our knowledge, genomic integration of DNA vaccines in humans has never been studied in a similar setting, probably because of the difficulty to obtain a biopsy from the administration site. Nevertheless, several studies have analysed the integration of pDNA in animal models such as mice, rabbits and guinea pigs.[44-49] Although genomic integration could be confirmed in these animal studies, integration rates were always several-fold lower than the spontaneous integration rate. Furthermore, the probability that a random integration occurs at a growth-regulatory gene (thus initiating oncogenesis) is even lower, since many integration events will be innocuous. Multiplying the low levels of integration observed with the low probability of interfering with growth-regulatory genes results in an extremely low risk of oncogenesis. Using mRNA instead of plasmid could theoretically annihilate the risk of genomic integration.[50] Nevertheless, the high production costs and lower stability of mRNA constitute a limitation for the broad application of RNA vaccination.

Vertical transmission of vaccine-derived pDNA into germline cells is another potential risk of DNA vaccines. However, this only occurs when pDNA is injected directly into the gonads[51] and not when DNA vaccines are administered in other tissues. This means that vertical transmission is not a relevant risk when DNA vaccines are administered via the common intramuscular and intradermal delivery routes.

DELIVERY OF DNA VACCINES

As discussed above, the clinical responses upon DNA vaccination thus far are rather disappointing. To overcome this, the DNA vaccination field is putting a lot of focus on optimisation of the delivery methods, carrier molecules and genetic optimisation of the construct used. Two administration routes are commonly used for the administration of DNA vaccines: intramuscular (IM) and intradermal (ID). Upon IM administration, the encoded antigen will primarily be produced in myocytes that can potentially transfer their antigen to pAPCs for cross presentation. This administration route will result in the highest levels of antigen expression but may not be the most immunogenic, since the frequency of pAPCs in muscle tissue is rather low. Although ID delivery of DNA vaccines does not lead to the amount of protein production that is obtained upon IM injection, it is potentially much more immunogenic, since the skin is the natural port d'entrée of pathogens and full with pAPCs ready to take up and present antigens.

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In the past decade a large number of technical devices have been developed for IM and ID delivery of DNA vaccines. The 'gene gun', also referred to as biolistic particle delivery system, is a commonly used tool. This so-called 'particle-mediated epidermal delivery' (PMED) method requires the pDNA to be coated onto cold particles in order to be shot into the skin.[52] In a similar fashion DNA can been shot in surgically exposed muscle tissue.

Furthermore, electroporation (EP) is used as a strategy to increase the transfection of DNA vaccines upon IM or ID administration. EP uses short electrical pulses to destabilise cell membranes. Under optimal conditions, this will lead to the formation of transient pores, which allows the entrance of macromolecules such as DNA into the cell. It is thought that electro-permeabilisation is followed by electrophoretic displacement of the negatively charged DNA molecule into the cytoplasm of the cell. Several research groups and companies are developing EP-based devices for the delivery of DNA vaccines and some of these devices have already been tested in the clinic.[26,42] Jet injection, ultrasound and micro needles are other mechanical delivery methods that are currently under development for the delivery of DNA vaccines.[53-55]

Our group has developed a technique called DNA tattooing for the intradermal administration of DNA vaccines.[56] We have shown that this strategy is highly immunogenic in mice and non-human primates.[57] The potency of DNA tattooing is probably mediated by the abundance of danger signals that are generated in the damaged skin upon mechanical disruption by the tattoo needles. Clinical trials that are currently running should prove whether DNA tattooing is also immunogenic in patients.

In addition to technical delivery devices, naked pDNA is often formulated into a synthetic carrier molecule/nanoparticle composed of lipids or polymers, in order to increase pDNA stability and cellular uptake (reviewed in reference 58).

EFFORTS TO IMPROVE DNA VACCINES

Another common way to improve DNA vaccines is to increase the immunogenicity of the encoded antigen. Roughly two methods can be distinguished, 1) the addition of genetic adjuvants and 2) the modification of the gene encoding the antigen itself. A genetic adjuvant is a protein with adjuvant properties that is encoded by the pDNA together with the antigen and hence co-expressed with the antigen, bolstering the immune response towards this antigen. Examples are GM-CSF[59-61] HGMB1[62] and IL-15.[61] Most often these adjuvants are encoded for by a separate plasmid that is admixed with the DNA vaccine. A more sophisticated way is to combine the genetic adjuvant and the antigen in one plasmid (i.e. in a bicistronic cassette). This last method ensures that any transfected cells express both the antigen and the adjuvant.

Many different modification strategies have been suggested in order to optimise the immunogenicity of the antigen itself. We shall briefly discuss three common approaches, i.e. codon optimisation,[63] addition of signal sequences[64,65] and genetic fusion to an entire protein or protein domain, referred to as a carrier protein. Codon optimisation means that the gene encoding the antigen is rewritten for optimal transcription and translation in the species that the vaccine is meant for. Within the redundancy of the genetic code the optimal tRNA for any amino acid varies from species to species. Especially when native prokaryotic or viral genes are used in DNA vaccines, codon optimisation can considerably augment its transcription in the eukaryotic cells of the vaccinated host.[66]

The addition of signal sequences can target the antigen to different subcellular compartments, thereby improving the immunogenicity of DNA vaccines.[64,65,67] Moreover, by the addition of motifs with affinity for receptors on pAPCs that are involved in antigen uptake, antigens may be targeted

Table 2. *Selected examples of carrier proteins known to improve the immunogenicity of HPV16 E7 or E6 encoding DNA vaccines*

Carrier protein	Antigen	Proposed mode of action	Reference
Mycobacterium tuberculosis HSP-70	E7	Provision of CD4+ T-cell help, increased antigen uptake by DC	Chen et al., 2000
Heat shock protein 60	E6, E7	Increased antigen uptake by DC	Huang et al., 2007
Calreticulin	E6, E7	Targeting of antigen into the antigen presentation pathway	Cheng et al., 2001; Peng et al., 2004
Extracellular domain of Flt3 ligand	E7	Altered subcellular localisation/ increased antigen uptake by DC	Hung et al., 2001b
HSV VP22	E7	Antigen spreading, improved antigen stability	Michel et al., 2002
E. coli β-glucoronidase	E7	Enhanced stability/ altered subcellular localisation	Smahel et al., 2004
Pseudomonas aeruginosa exotoxin A (domain II)	E7	Enhanced cross presentation	Hung et al., 2001a
Invariant chain with PADRE epitope insertion	E6	Provision of CD4+ T cell help	Wu et al., 2011
IP-10	E7	Enhanced antigen presentation, chemoattraction	Kang et al., 2011
TTFC	E6, E7	Provision of CD4+ help, increased antigen stability	Oosterhuis et al., 2011; Stevenson et al., 2004

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to pAPCs with the intention to make their presentation more efficient.[68]

Fusion of the antigen to a carrier protein is another trick that is often employed in the design of DNA vaccines. To illustrate this, *table 2* summarises popular carrier proteins used for fusions with HPV-16 E6 and E7. Although many different mechanisms have been postulated to explain the positive effect of such fusions on the immunogenicity of an antigen, we propose three mechanisms to be most important in this respect. Firstly, genetic fusions often affect the half-life of the antigen.[69-71] We have shown that antigen half-life is a critical determinant of DNA vaccine immunogenicity.[72] Allegedly this is because antigens that accumulate in the transfected cell are more efficiently cross-presented. Secondly, these carriers are often foreign proteins that are likely to contain CD4+ helper epitopes. Since DNA vaccine-induced CD8+ T-cell responses are strictly dependent on CD4+ T-cell help (as illustrated by the fact that MHC class II deficient mice do not mount detectable T-cell responses upon DNA vaccination[73]) at least part of the potentiating effect of any foreign carrier protein can be explained by the addition of CD4+ T-cell help. Thirdly, a carrier protein can affect the subcellular localisation of the antigen. Many of the commonly used carrier proteins (such as calreticulin and invariant chain) are likely to impact on the subcellular localisation of the antigen and hence may act via this mechanism. In this regard, localisation of E6 and E7 to the endoplasmic reticulum (ER) of a cell can increase the T-cell responses measured in peripheral blood by an unknown mechanism.[74,75]

We have recently developed so-called modular DNA vaccines[74] and demonstrate that the addition of ER localisation/retention signals combined with a set of minimal CD4+ T-cell epitopes can tremendously improve the immunogenicity of HPV16 E6 and E7 encoding DNA vaccines. The key advantage of this approach is that besides the antigen only minimal additional sequences are added, thereby preventing off-target immune responses. In conclusion it can be said that many different strategies can be applied to improve the immunogenicity of antigens encoded in DNA vaccines. As the molecular mechanisms are being unravelled, the opportunities to rationally improve DNA vaccines become manifold.

CONCLUSIONS

As discussed, DNA vaccines form an interesting platform for therapeutic vaccination against viral infections and cancers. Since mice to men translation appears to be extremely complex in DNA vaccination, future widespread clinical application depends on the successful development of new delivery techniques, adjuvants and the genetic optimisation of the plasmids used. Hopefully these improvements will eventually lead to DNA vaccine products that are immunogenic enough to be applied as a standalone modality in the clinic.

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