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REVIEW ARTICLE

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DNA vaccines

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Abstract Immunization by genes encoding immunogens, rather than with the immunogen itself, has opened up new possibilities for vaccine research and development and offers chances for new applications and indications for future vaccines. The underlying mechanisms of antigen processing, immune presentation and regulation of immune responses raise high expectations for new and more effective prophylactic or therapeutic vaccines, particularly for vaccines against chronic or persistent infectious diseases and tumors. Our current knowledge and experience of DNA vaccination is summarized and critically reviewed with particular attention to basic immunological mechanisms, the construction of plasmids, screening for protective immunogens to be encoded by these plasmids, modes of application, pharmacokinetics, safety and immunotoxicological aspects. DNA vaccines have the potential to accelerate the research phase of new vaccines and to improve the chances of success, since finding new immunogens with the desired properties is at least technically less demanding than for conventional vaccines. However, on the way to innovative vaccine products, several hurdles have to be overcome. The efficacy of DNA vaccines in humans appears to be much less than indicated by early studies in mice. Open questions remain concerning the persistence and distribution of inoculated plasmid DNA in vivo, its potential to express antigens inappropriately, or the potentially deleterious ability to insert genes into the host cell's genome. Furthermore, the possibility of inducing immunotolerance or autoimmune diseases also needs to be investigated more thoroughly, in order to arrive at a well-founded consensus, which justifies the widespread application of DNA vaccines in a healthy population.

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Introduction

DNA vaccination utilizes direct inoculations of DNA expression vectors, such as plasmids encoding for a specific antigen under the control of a eukaryotic promotor, to stimulate the in vivo synthesis of immunogenic proteins and immune responses. In contrast to conventional inactivated antiviral or tumor vaccines, endogenously synthesized immunogens possess a natural conformation and undergo posttranslational modifications and immune recognition pathways which, in principle, do not differ from those of the natural antigens.

Gene therapy has paved the way for DNA vaccines. As part of their work to establish a gene therapy approach for muscular dystrophy Wolff et al. (1990) demonstrated that expression plasmids could effectively be transferred into muscle cells by injecting them as a simple saline solution. Tang et al. (1992), already using the "gene gun" as a new delivery technique, propagated genetic immunization as a simple method for eliciting an immune response. Just one year later, the first reports on immunoprotective DNA vaccines in mice were published, using the influenza virus model and genes encoding the viral nucleoprotein (Ulmer et al. 1993) or hemagglutinin (Fynan et al. 1993; Robinson et al. 1993). At about the same time other groups had started studying the immune responses in mice after injection of genes derived from the human immunodeficiency virus (Wang et al. 1993) or hepatitis B virus (Davis et al. 1993) and successfully raised immune responses against hepatitis B virus surface antigen in transgenic mice, which were immunotolerant against conventional vaccine antigens (Mancini et al. 1993). Thus, very rapidly, convincing evidence for a reliable concept had been produced and has encouraged research groups all over the world to built upon the foundation laid. Since then various vaccine research approaches have also been tested by using DNA vaccines. Due to the fact that DNA vaccines produce their target antigens in vivo within the host cell, which is the ideal way of stimulating cytotoxic T-cells, vaccine research on chronic and persistent infectious diseases,

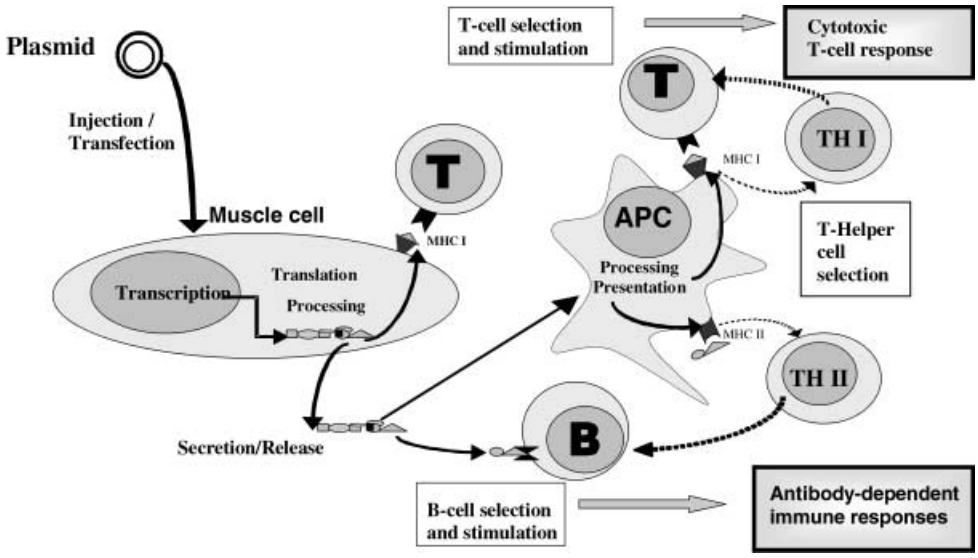

Fig. 1 Immune mechanisms induced by inoculation with naked DNA. The example shows a myocyte which is transfected by the inoculated DNA, produces the antigen encoded by the plasmid, and exposes processed peptides of the antigen together with MHC-I molecules to stimulate a T-cell response after cross-linking with the T-cell receptor. Antigen released from the cell stimulates B-cells by binding to their immunoglobulin-like receptors and induce a humoral immune response. Alternatively, released antigen – but also expression plasmids – may be taken up by antigen-presenting cells (*APC*). These are able to present their antigens to the immune system via MHC-I and MHC-II pathways, thus stimulating and regulating both arms of the immune system

parasite infestations, and tumors has been intensified, as new hope has arisen that these can be more effectively treated. In addition, DNA vaccines offer many possibilities of specifically directing and regulating immune responses, which stimulates considerations about their use to overcome immunotolerances, allergies and autoimmune diseases.

Various methods of improving the plasmids, uptake and application of DNA, and antigen expression have been evaluated and have improved our understanding of what happens after the DNA is inoculated. Quite soon, the approach had been supported by sufficient information and brought to a degree of maturity to qualify for first studies in humans, the first of which were published in 1998 (Calarota et al. 1998; MacGregor et al. 1998; Ugen et al. 1998; Wang et al. 1998).

This exciting new technology developed extremely rapidly and the number of new publications has grown exponentially. This review does not attempt to give an extensive account of the work published in this field; it intends to provide a reasonably short summary of the major aspects of this technology with a critical view on its future application in humans. Earlier reviews, partly focusing more on mechanistic and on preclinical model studies, are recommended for obtaining a deeper insight into the field, but also for getting other people's opinions on those aspects, which are open to hypotheses and speculations (see, e.g., Donnelly et al. 1998; Feltquate 1998; Lai and Bennett 1998; Leitner et al. 1999; Whitton et al. 1999).

Stimulation of immune responses by DNA plasmids

When an expression plasmid preparation is injected into a body tissue, e.g., a muscle bundle, plasmids are taken up by the myocytes – and to a lesser extent by other cells – and the encoded information is transcribed and translated into proteins. The DNA uptake by muscle cells does not appear to be very efficient (Wolff et al. 1992), as only a very low percentage of cells at the injection site express the introduced genes. Newly synthesized protein is processed through the proteasome complex and small peptides of the same are bound to major histocompatibility complex type I (MHC-I) molecules. These molecules now migrate to the cytoplasmic membrane and present these peptides to CD8-bearing T-lymphocytes (see Fig. 1). Binding of $CD8^+$ T-cells results in the selection and stimulation of peptide-specific cytotoxic T-cells (CTL). Appearance of the same peptides on the surface of cells would then trigger a lethal CTL attack against those cells.

For the stimulation of B-cell receptors and antibody responses, the antigens must be released from the producing cells in order to get into contact with B-cells circulating in the blood stream. It is not known whether myocytes actively secrete the antigens, but necrotic or apoptotic cell death, as well as CTL-lysis of cells presenting the antigens, would also release antigens into the environment and expose these to B-cells.

A simplified model, as described above, postulates that myocytes would directly act as antigen-presenting cells. This certainly does not provide the complete picture or may even describe an irrelevant mechanism. Based upon our current knowledge, the matter appears to be more complex. Foreign antigens expressed by myocytes can definitely induce immune responses, as demonstrated by Ulmer et al. (1996), who injected an anti-

gen-producing, stably transfected myocyte cell line and elicited immune responses against the antigen. On the other hand, if the muscle bundle in which plasmids have been inoculated is surgically removed as soon as possible (within a few minutes) after injection, no negative effect on the stimulation of CTL and antibody responses has been observed in mice (Torres et al. 1997). Thus, the plasmids were distributed away from the inoculation site very rapidly and must have stimulated immune responses via other cells. Most likely plasmids were rapidly transported away with tissue fluids; alternatively they can also be taken up by phagocytotic, migrating cells, which then evade the muscle. Based upon existing limited investigations, plasmid DNA can obviously transfect a wide range of cell types and appears to be readily taken up by cells of the reticuloendothelial system, including macrophages and dendritic cells (Condon et al. 1996; Parker et al. 1999; Lunsford et al. 2000).

Professional antigen-presenting cells (APC), such as macrophages and dendritic cells (DC) most likely play a central role in the immune regulation upon inoculation of DNA vaccines. In addition to antigen processing and presentation by the MHC-I pathway, APC can also present antigens via MHC-II molecules to stimulate T-helper cells, which regulate B-cell responses (see Fig. 1). APC are either transfected directly by the plasmids or antigen released from any other transfected cell, serving as an antigen-producing factory, is taken up by phagocytosis. In particular, intradermal and gene gun inoculations would directly target the plasmids to Langerhans cells, which are a specific form of DC, located in the stratum spinosum of the epidermis.

Immune responses with DNA vaccines compared with conventional vaccines

Conventional vaccines either consist of whole, inactivated microorganisms or purified components thereof ("inactivated vaccines") or of attenuated, but still infectious microorganisms ("live vaccines"). Whereas inactivated vaccines have excellent safety records, but usually require immunopotentiating adjuvants and booster injections to stimulate sufficiently protective immune responses, most live vaccines can achieve a similar or more durable protection after only single applications, as they imitate a natural infection. The disadvantage of live vaccines, however, is that their mode of action is based upon a narrow and delicate balance between replication competence and lack of pathogenicity, between (over-) attenuation and (lack of) efficacy. Live vaccines are more potent stimulators of CTL responses and are particularly successful against viral diseases, as the relevant antigens and targets for the immune effector mechanisms are located in or on cells, which are in many cases not accessible by humoral antibodies.

DNA vaccines apparently combine the optimal characteristics of both categories of conventional vaccines. They are not infectious, but they produce their antigens in vivo, which is an important prerequisite for stimulating strong cytotoxic T-cells and optimal immune responses. Concerning antigen processing and presentation to the immune system, the active intracellular synthesis of antigens makes a fundamental difference compared with exogenous antigens.

Intracellularly synthesized proteins are regularly processed into peptides by the proteasome complex. The peptides are transported to the endoplasmatic reticulum by specialized transporter proteins and assembled into a complex consisting of MHC-I molecules, β2-microglobulin, and the peptide. Antigen presentation by MHC-I molecules – which is present on almost any cell type – triggers a T-cell response of the Th-I type, which is predominantly regulated by interleukin 2 (IL-2), interferon gamma (IFN-γ), and tumor necrosis factor beta (TNF-β) and associated with a higher level of IgG2a antibodies. As a main effector mechanism, the MHC-I-mediated immune stimulation selects and stimulates cytotoxic T cells (CTL), which are able to migrate through tissues and recognize and destroy cell that expose the specific antigen/peptide together with MHC-I.

Extracellular antigens are processed differently. Specific and professional antigen-presenting cells (APC) take up the antigen via unspecific pinocytosis or by specific, (immunoglobulin Fc-fragment or complement-) receptor-mediated endocytosis, which restricts the antigens to intercellular vesicles. Proteolysis within these vesicles normally results in peptides, which are presented via MHC-II molecules. MHC-II-peptide complexes stimulate specific T-helper cell subsets, which provide help to B-cell responses. These are predominantly regulated by interleukins 4, 5, 10, 13 and characterized mainly by antibodies of the IgG-1 subtype.

It should be noted that alternative pathways exist, by which exogenous antigens can also be linked to MHC-I molecules and thus stimulate CTL responses. These require that either the antigen is released into the cytosol to get access to the proteasome or that the antigen fragments are bound to unoccupied or recycled MHC-I molecules inside the vacuolar compartments or bind to MHC-I after being secreted from the cell.

Figure 2 summarizes the major characteristics of antigen presentation, immune regulation and responses against extracellular and intracellularly synthesized antigens as described above. For more detailed information, an article by Sztein and Mitchell (1997), who expertly summarized this complex and only partially understood field, is recommended.

Inactivated vaccines are more active against extracellular antigens, but less effective against cellular or intracellular antigens. Both live vaccines and DNA vaccines are quite obviously better suited to fighting intracellular infections, in particular chronic viral diseases and tumors. At least in theory, DNA vaccines also provide several tools to influence or modify the type of immune response generated, e.g. by co-expressing specific cytokines, by selectively raising T-cell responses against protective, but normally suppressed antigens, or by targeting the plasmids to certain cells by choosing cell-specific promotors. This

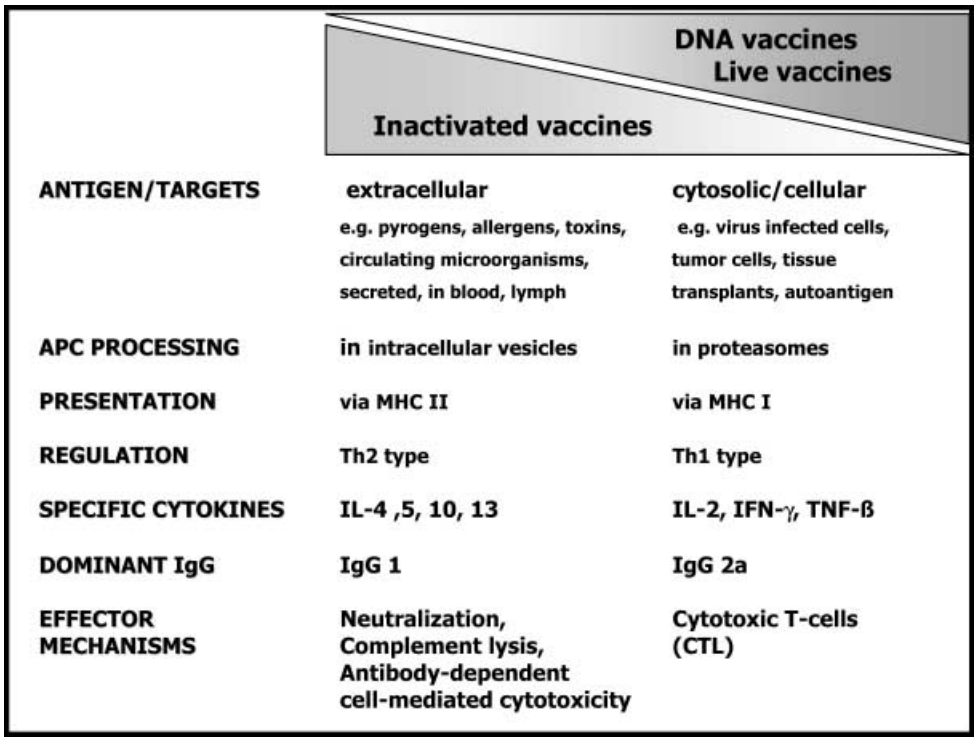

Fig. 2 Antigen processing and immune regulation mechanisms induced by intracellularly synthesized and by exogenous antigens and their relationship to conventional and DNA vaccines. Inactivated vaccines predominantly induce Th2 type antibody responses, whereas intracellularly produced antigens primarily induce a Th1 type of immune response. Depending on the antigen and the method of delivery, DNA vaccines can induce cytotoxic T-cells, but may also stimulate Th2 type antibody responses

opens up entirely new possibilities for using vaccines also to break tolerances, to treat autoimmune diseases, or to modify imbalanced immune responses.

Plasmids for the application of naked DNA

Expression plasmids to be used for DNA vaccination consist of five basic and essential elements: a bacterial origin of replication, a strong mammalian cell promotor/enhancer, a multicloning site for insertion of foreign antigens, a mammalian polyadenylation/termination signal, and an antibiotic resistance gene for the selection during bacterial culture. Most of the frequently used promotors are derived from the human cytomegalovirus (immediate early) gene or from other viruses. Other promotors, e.g. the myocyte-specific desmin promotor (Kwissa et al. 2000), may be used to target the antigen to a specific cell type by limiting expression to these cells. PolyA-termination signals are commonly taken from bovine growth hormone or from certain viruses. Ampicillin, neomycin, or kanamycin are most often used for plasmid selection. Another common element of most plasmids are GpC dinucleotides. These unmethylated nucleotides in particular base contexts are specific for bacteria, but not for eukaryotic genes. They are potent direct stimulators of B-cells and also indirectly activate natural killer cells and T-cells. It is likely that their mode of action is similar to that of Freund's adjuvant (Krieg 2001; Rothenfusser et al. 2001).

Bicistronic vectors containing two genes under the control of a single promotor, as well as multiple gene-expressing vectors with individual promotors and termination signals for each inserted gene, are in use. Various cytokines and co-stimulatory molecules of the MHC/T-cell receptor stimulation complex were expressed together with specific antigens in order to enhance or steer the immune response into a specific direction. Interestingly, ubiquitin, a molecule that targets proteins to the proteasome, has been co-expressed by plasmids in order to rapidly degrade antigens before they can be released into the extracellular compartment to stimulate B-cells. This resulted in CTL responses, but the antibody response was reduced or lost (Rodriguez et al. 1997). These examples show that almost unlimited options exist to modify and optimize plasmids and antigen inserts for any specific purpose (Lewis et al. 1997; Feltquate 1998; Leitner et al. 1999; Whitton et al. 1999). Whereas some of these options are generally for technical purposes only, others could influence and modify the quality of the resulting immune responses. Once established and confirmed as a reliable model, many of these plasmid modifications would enable us to gain a better understanding of the complex immune regulation for a number of interesting immunogens and, in the longer term, to specifically design improved or new vaccines.

Vaccine formulation and mode of application

The majority of experimental DNA vaccines are simply applied as a physiological saline solution. No specific formulations are needed to render the plasmids applicable for their intended use, thus any laboratory is able to study prototype DNA vaccines without worrying about adequate adjuvants, stabilizers, and acceptable buffers. Saline/plasmid preparations are typically injected intra-

muscularly or intradermally. For research purposes, application by skin scarification, intravenous injections, and injections into lymph follicles or into thyroidal tissues have been studied. The doses applied are normally in the range of about 100 µg plasmid DNA per application, but sometimes, and particularly in other species than mice, even more than 1,000 µg has been used.

Frequent reimmunization schemes and rather high concentrations of plasmid DNA indicate that dose response and efficacy are still below a desirable and practical level. Since regenerating tissues show a higher degree of antigen expression, attempts have been made to induce muscular tissue necrosis by co-injections of local anaesthetics, hypo-osmotic sucrose solutions, and snake venom-derived cardiotoxin. Due to local reactions as described by Fomsgaard et al. (1998), these approaches are most likely not acceptable for commonly applied vaccines. Likewise, attempts to improve plasmid uptake by electrophoration (Kadowaki et al. 2000; Widera et al. 2000) may be interpreted as an indicator of insufficient activity of many injected DNA vaccines studied.

Particle-mediated "gene gun" applications may present an interesting alternative, as apparently much lower doses of plasmid DNA are required. The gene gun uses compressed helium to propel micrometer-sized, colloidal gold particles coated with plasmids into the epidermis. Due to the limited adsorption capacity of the gold particles, less than 1 µg of plasmid DNA is typically delivered per individual shot. Applications to multiple sites per vaccination and repeated boosting schemes are commonly used to deliver immunogenic doses. Compared to intramuscular injections, gene gun applications seem to be a more efficient method of raising immune response for two reasons: firstly, the transfection efficiency may be higher, as the plasmids can be directly inoculated into cells and, secondly, improved immune responses could result from the fact that a higher proportion of the plasmids is brought into contact with professional APC (Langerhans cells) or transfects keratinocytes (Raz et al. 1994), which have the ability to produce immunopotentiating cytokines (Nickoloff and Turka 1993).

Many further application modes and formulations have been and are being investigated, most of these aiming at mucosal immune responses. Examples are liposome formulations, formulation into bioadhesive polymers or biodegradable nanoparticles, aerosol applications, inoculations together with cholera toxin, and plasmids packed into replication-deficient microbial carriers, such as enteric bacteria or viruses with a tropism to mucosal cells. The extent and intensity to which these studies have been conducted does not enable us to draw generally applicable conclusions, except that route of application, dose, boosting schemes, and the species studied are factors that influence the strength and nature of the resulting immune response (McCluskie et al. 1999). The finding that naked expression plasmids for hepatitis B surface antigen, applied to the skin in aqueous solution, are taken up by hair follicles and resulted in quite acceptable cellular and humoral immune responses (Fan et al. 1999) may illustrate that basically any mode of application may be possible, but may not necessarily be ideal for a standardized application of vaccine products, requiring a high degree of reliability when used under highly variable practical conditions.

Identification and characterization of protective immunogens

The development of new and successful vaccines is mainly restricted by technical and scientific limitations concerning the identification and adequate presentation of protective antigens. DNA vaccination appears to be the ideal tool to overcome these difficulties. Whereas traditional vaccine research was greatly limited by the technical resources required to produce the desired antigens in sufficient quantities, DNA immunization offers a much simpler approach for screening large numbers of antigens and for detecting CTL-inducing antigens. Even an expression library, consisting of several thousand different plasmids and representing the entire genome of a microorganism, may be used successfully for immunization studies. If immunity is detected, smaller fractions may subsequently be tested in order to identify the protective individual plasmids and antigens (Barry et al. 1995). This "blind" expression library immunization approach still has some restrictions, since interference by many irrelevant proteins may occur, but these can be overcome by cloning cDNA or open reading frames into the expression library (Johnston and Barry 1997).

When an immunogenic or even protective plasmid/antigen has been identified, it is rarely effective enough to qualify right away for a vaccine. Improvements can be achieved by modifications of the plasmid framework, but the antigen's configuration and expression characteristics may also need major improvements. It may be presented as a cytosolic, intracellular antigen or as a secreted, extracellular variant. It may be more effective, if expressed together with a particular ligand or with or without its membrane anchor sequence. And even the optimized, single antigen may not be sufficiently active to stimulate protective responses under practical conditions, so that antigen combinations, either within the same plasmid or by mixing different expression plasmids will be needed.

The most important part of any successful vaccine research and development, however, is the availability of adequate model systems to monitor the vaccine preparation's protective effects. Mouse studies have quite frequently turned out to be not representative of what is to be expected in humans, which is at least in part due to genetic restrictions of the antigen presentation and immune response to certain antigens. For example, dominant CTL responses to the matrix protein of influenza virus are restricted to HLA-A2 allele in humans, which is lacking in mice (Ulmer and Liu 1996). On the other hand, largely unknown pharmacokinetic parameters (e.g. tissue distribution and degradation before coming into contact with immune cells) of a vaccine plasmid or anti-

gen certainly differ significantly between mice and larger species.

Immunogenicity and efficacy in preclinical models and clinical trials

Immunogenicity and in several cases also protective responses after DNA vaccination have been demonstrated in numerous animal models for viral, bacterial, and parasitic infections and have been summarized and reviewed by Lai and Bennet (1998), Alarcon et al. (1999) and Tuteja (1999). Some studies were also done in monkeys or primates (Boyer et al. 1997; Davis 1998; Gramzinski et al.1998) and in farm animals, representing vaccine target species (summarized by Beard and Mason 1998). In general these studies were quite encouraging but also illustrate that, despite the repeated application of high doses of plasmid DNA, immunogenicity was often rather low and that intradermal inoculations – preferably particle-mediated intracellular applications – may be more immunogenic than intramuscular injections. Examples of vaccination studies comparing DNA vaccines with existing vaccines and first clinical trials in humans will be briefly summarized below in order to draw some preliminary conclusions on the efficacy of DNA vaccines.

Polack et al. (2000) compared vaccination by an attenuated live measles vaccine with DNA vaccines encoding the measles virus H (hemagglutinin) and/or F (fusion) protein. Rhesus monkeys vaccinated with either 500 µg plasmid by intradermal injection or 8 µg of plasmid via gene gun delivery showed only 1–10% of the serological responses seen within the classical vaccine, however, eight of the 14 DNA-vaccinated monkeys developed protective antibody titres. A second vaccination was given, followed by a challenge infection 7 months later. All six gene-gun-vaccinated monkeys and two out of three in each group of the intradermally injected animals remained healthy; one animal, each injected with either H, F, or H+F plasmids, developed a mild disease. The attenuated live vaccine was given only once. It produced CTL responses similar to those seen in DNA-vaccinated monkeys, but higher neutralizing antibodies and resulted in complete protection.

Ashok and Rangarajan (2001) immunized mice with two doses of 100 µg plasmids encoding the E (envelope), prME (precursor-matrix/envelope), NS1 (non-structural), or the prME plus NS1 antigens of the Japanese encephalitis virus. Compared to a conventional, inactivated vaccine, antibody responses were undetectable, borderline, or very low. Whereas all mice in the conventional vaccine group survived an intracerebral challenge infection, only about half of the mice in each of the four DNA vaccine groups survived the challenge infection.

Several clinical studies of DNA vaccines in humans have already been conducted. These were predominantly designed as safety studies in small groups of volunteers and the immune parameters measured allow only limited conclusions on efficacy. The first human trials tested human immunodeficiency virus (HIV) Type 1 DNA vaccines containing either the viral *nef*, *rev*, or *tat* regulatory genes (Calarota et al. 1998) or a combination of envelope and rev genes (MacGregor et al. 1998; Boyer et al. 2000). Both studies showed that three or four intramuscular injections of 100 or 300 µg plasmid DNA were required to induce or to increase CTL responses in asymptomatic HIV-infected individuals.

Using the hepatitis B virus surface antigen as target and a gene-gun delivery device, Tacket et al. (1999) did not induce a primary immune response through two vaccinations with 0.25 µg plasmid DNA. In a subsequent trial three vaccinations at higher doses (1, 2, or 4 µg) were tested by using higher plasmid concentrations and multiple site applications (Roy et al. 2001). Compared with the conventional, recombinant antigen vaccine, which normally results in a seroconversion rate of 50–60% after the priming dose, the seroconversion rate after the first DNA vaccination was 17% and peak antibody levels were low. The highest dose appeared to result in a better seroconversion than lower doses. After the third vaccination, all 12 individuals in the trial had antibody levels of 10 mIU/ml, which for conventional vaccines is considered as being protective.

Wang et al. (1998) and Le et al. (2000) published different aspects of a clinical trial of a DNA vaccine against the *Plasmodium falciparum* circumsporozoite (CSP) protein. Twenty individuals received three intramuscular injections of 20, 100, 500, or 2,500 µg DNA. No detectable antibodies were induced in any of the volunteers. (Compared to that, earlier mouse studies resulted in good antibody responses, but also showed, that titers were three times higher after intradermal than after intramuscular injection.) CTL-responses were seen in two out of five individuals in the two lower dose groups, in three out of five in the 500 µg dose group, and in four out of five in the 2,500 µg dose group. Ten different CSP peptides, representing six types of human lymphocyte antigen (HLA) class I presentation restrictions, were used to test for CTL responses and revealed multiple HLA restrictions even within the same individual. Aggravated by allelic variations of CTL epitopes in *Plasmodium falciparum* in nature, this genetic restriction of CTL response is considered to be the major obstacle to malaria vaccine development.

Clinical trials with DNA vaccines for tumor indications, such as B-cell lymphomas or malignant melanomas, are currently in preparation or have already started (Stevenson et al. 1999; Walsh et al. 2000). Due to the low number of subjects in phase I trials, the heterogeneity of their medical status, and due to concomitant treatments and therapies, definite results on the efficacy can hardly be expected from those first trials. On the other hand, anti-tumor indications may be the first in which DNA vaccines show clinical benefits, because directly measurable therapeutic effects may be seen. A completed clinical study reported by Mincheff et al. (2000) appears to support these expectations: changes of local tumors and metastases were seen in prostate cancer patients after vaccination with plasmids or replication-deficient viral vectors expressing prostate-specific membrane antigen. The clinical observations apparently justified commenc-

Table 1 Specific safety aspects to be considered for DNA vaccines. For more detail see text and refer to the Guidelines for DNA vaccines (European Agency for the Evaluation of Medicinal Products 1999: Note for guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products. CPMP/BWP/3088/99 draft; http://www.emea.eu.int/pdfs/human/bwp/308899en.pdf and US Food and Drug Administration 1996: Points to consider on plasmid DNA vaccines for preventive infectious disease indications. Docket No. 96N-0400; http://www.fda.gov/cber/gdlns/plasmid.pdf)

Genetic construct	Regulatory control signals Antibiotic resistance markers Unintended, alternative reading frames Chromosomal integration/ insertion mutagenesis Expression of cytokines, co-stimulatory molecules Replication-deficiency of bacterial/viral vector systems
Application mode	Local tolerance Facilitators (toxins, local anaesthetics, adjuvants) Hypo-osmotic formulations Application devices
Pharmacokinetics	Tissue distribution Persistence of introduced sequences Duration of expression
Pharmacodynamics	TH1/TH2 response imbalances Interactive dysfunctions mediated by cytokines and co-stimulatory molecules
Immunotoxicology	Cellular autoimmune reactions Anti-DNA and anti-nuclear antibodies Immunotolerance

ing a phase II trial to specifically evaluate the effectiveness.

General safety considerations

Vaccines must have a safety profile, which takes into consideration that they are normally administered to thousands or even millions of healthy individuals with highly variable predispositions, including individuals with impaired or immature immune responses, such as the elderly or babies and children. As a consequence, novel vaccines deviating from well-established systems with known safety records must be investigated very thoroughly and broadly for their potential to induce undesirable side-effects. For DNA vaccines, particular attention must be given to potential risk factors inherent to the genetic construct, the formulation, mode of application, pharmacokinetics, pharmacodynamic characteristics of the vaccine, and to specific immunotoxicological risk factors (Table 1). The methodological repertoire to assess the safety profile of a DNA vaccine will by no means be restricted to standard toxicology tests, but will have to apply an additional, wide spectrum of molecular genetic, biochemical, histochemical, biological, and immunological methods.

A complete characterization of the plasmid/vector system used must be provided as a base for a risk evaluation with particular emphasis on tumorigenicity due to insertion mutagenesis and undesired pharmacodynamic and immunotoxicological effects. Plasmids containing retroviral LTRs (long terminal repeats), oncogenes, sequences with homologies to the human genome, sequences with cell growth functions, and unintended reading frames, should be avoided in any case in DNA vaccines.

In the context of gene therapy, the issue of insertion mutagenesis and resulting tumor formation has been studied intensively. Uncertainties remain, as plasmids may persist for a long time in various types of cells, but based on model studies in vitro and in vivo, a critical mutational event is expected to occur several orders of magnitude below the spontaneous mutation rate (Kurth 1995; Martin et al. 1999). There appears to be a consensus that the risk is almost negligible and for the moment it is at least acceptable to conduct initial clinical trials and safety studies. However, we must be aware that a single negative event can rapidly change this perception.

The local tolerance of a DNA vaccine may not be as good, as it is frequently stated with regard to plasmids, applied in saline. High doses and co-administered adjuvants and “facilitators”, such as local anaesthetics, hypo-osmotic solutions, or toxins, may have a significant influence on the tolerability of a DNA vaccine. Furthermore, frequent or multiple-site applications may be safe and acceptable from a regulatory point of view – although they may not necessarily be tolerated and widely accepted by the recipients.

Pharmacokinetic studies will be absolutely necessary to monitor the tissue distribution of any particular expression vector, as well as the duration of its persistence and the duration of antigen expression. Existing studies indicate that plasmids applied intramuscularly primarily transfect local tissues, lymphoid organs, and highly vascularized tissues. “Worst-case” studies with intravenous applications resulted in a more widespread dissemination, during which plasmids were initially found in all examined tissues except in the gonads and brain (Parker et al. 1999; Lunsford et al. 2000). Plasmid DNA injected intramuscularly in mice was shown to persist for more than a year and expression of a luciferase reporter molecule was detected for at least 19 months, which is a considerable part of the life-span of a mouse (Wolff et al. 1992). Gene-gun-delivered plasmids are considered as being less persistent, since plasmids are mainly transfecting terminally differentiated keratinocytes and are lost after several days due to natural sloughing of the skin (Raz et al. 1994); however, dendritic cells of the skin (Langerhans cells) are also readily transfected and may transport the plasmids to any other organ (Condon et al. 1996; Torres et al. 1997).

Before extended applications in human clinical trials are considered, the pharmacodynamic characteristics of the vaccine should be investigated. Furthermore, repeated application toxicology studies and immunotoxicology evaluations are to be performed. These studies must be designed to give adequate information on toxicological and undesired systemic side-effects under the same conditions as those envisaged for normal use, but should also include overdosing and frequent vaccination in rapid succession. Specific studies may be required to address potential effects in specific risk groups and under predisposing conditions for immune disorders. Immunologically triggered side-effects after DNA vaccination have been postulated due to the specific mode of action and have been observed in a few studies. These aspects of DNA vaccines will be discussed below in a separate chapter.

Immunotolerance and autoimmune responses

Proteins expressed endogenously and presented together with self-MHC molecules may under certain conditions be recognized as self-antigen, resulting in the induction of immunotolerance rather than immunity. This phenomenon has been observed, when 2–5-day-old mice were vaccinated with a *Plasmodium falciparum* CSP-encoding DNA vaccine (Mor et al. 1996; Klinman et al. 1997). Whereas 2–6-month-old mice responded to vaccination and boosting, neonatal mice of different HLA types did not show an anamnestic response and remained unresponsive when revaccinated after 2 or 6 months. The tolerance induced by early vaccination was antigen-specific, as it could not be induced by control plasmids and as mice challenged with exogenous CSP antigen responded only to a different set of epitopes, but were not tolerized. Similarly, immune responses were significantly reduced in aged mice and the protection rate upon challenge infection in aged mice was reduced to only one half of that observed in young mice. Tolerance induction may be a specific characteristic of that particular vaccine studied, as other DNA vaccines studied by the same group and elsewhere did not show neonatal tolerance; however, the example shows, that tolerance induction must be carefully studied if a vaccine is intended also for use in very young or aged individuals and in immune-compromised persons.

Another area of concern is the likelihood of induction or acceleration of autoimmune diseases. Efficient plasmid expression would inevitably lead to an immune attack against those cells presenting the encoded, foreign antigens. Florid myositis reactions and destruction of transfected muscle fibres after repeated vaccination with DNA vaccines cells have in fact been described by Davis et al. (1997) and Whitton et al. (1999), whereas others did not find anti-myocyte antibodies or myositis (Mor et al. 1997). As clinical safety studies regularly measured muscle enzymes, but did not mention critical elevations, these effects may either only be related to certain plasmid constructs or they could be mild and transient and not really be relevant if restricted to muscle cells. Similar effects in other cell types, however, could be more critical and emphasize the necessity of a careful evaluation of a DNA vaccine's distribution, persistence, and expression in various cell types.

Bacterial DNA plasmids – as opposed to mammalian DNA – can stimulate anti-DNA antibodies. Autoantibodies against ubiquitous antigens, such as nucleoproteins and DNA, can induce specific diseases (e.g., lupus erythemathodes). In severe cases immune complexes accumulate to considerable amounts in the kidney glomerula, causing severe dysfunctions. Studies addressing this aspect were performed in specific mouse strains, which develop lupus erythematodes symptoms at an advanced age. When four subsequent DNA vaccinations were given to these "lupus-prone" mice, the development of immune complex deposition and glomerulonephritis was not aggravated or accelerated. Repeated DNA vaccination of normal mice stimulated a moderate increase of anti-DNA autoantibody levels, which remained 40-fold below those levels found in mice having acute lupus symptoms (Mor et al. 1997). Along with clinical trial reports mentioning no relevant elevations of antibodies against nucleoproteins or DNA, these studies indicate that, due to the weak immunogenicity of plasmids far below disease-inducing levels, the risk of antinuclear autoantibodies may be less critical than expected.

A look back and into the future

DNA vaccines are occasionally also referred to as "third generation vaccines", a term which raises very high expectations and suggests that a variety of applications and products are to be expected quite soon. We are well advised, to remind ourselves that, based upon mouse model studies, similar terms were also used in the recent past for monoclonal antibody immunotherapy and for anti-idiotype vaccines. These certainly did not fulfill those high expectations. It can be reasonable expected that DNA vaccines will not share the same fate, as their active principle is not founded on just a single epitope. However, limited efficacy, as it has been observed quite frequently with DNA vaccines, reminds us that there is a wide gap between successful vaccines in mice and successful vaccines for humans.

Quite obviously, DNA vaccines need to be improved in order to qualify as a vaccine product. In particular, the DNA uptake and expression by the target cells need to be enhanced. For safety reasons it would also be desirable to develop vector systems that are highly specific for certain target cells. Nevertheless, DNA vaccines are an excellent opportunity to extend the very small technical and scientifically largely unknown base on which current vaccines have been developed. DNA vaccines offer quite reasonable chances to successfully develop several new indications and applications for vaccines. Progress towards applications for complex, persistent infections, for parasite vaccines, or for currently still rather hypothetical indications, such as vaccines against aller-

gies or autoimmune diseases, will certainly be slow. Tumor vaccines, however, could be the first practical applications for DNA vaccines. With some optimism one can envisage that the first of these may be licensed within the coming 5–10 years, as partial therapeutic effects would already justify their use and since safety requirements for these vaccines may be much lower than for traditional, prophylactic vaccines.

While DNA vaccines as products may still have a rather unpredictable future, as a research tool they can be considered as representing a revolution for the entire field of vaccinology. The technology enables us to identify and screen vaccine target antigens in a much more direct, faster, and more efficient way and has motivated scientists all over the world to resume application-oriented vaccine research. Currently, DNA vaccines are still methods or tools, which – like almost any real innovation of the past – simply need more time than just a decade to mature into useful applications. Finding an effective and protective new vaccine antigen takes time and has always been preceded by many unsuccessful approaches. And even when a research product has qualified for product development, the following development period will – also for DNA vaccines – not be much shorter than the average 10 years that it takes to develop a vaccine product, because efficacy, safety, and quality have to be newly developed for each individual vaccine. Developing a new vaccine technology to maturity is certainly no simpler task than developing the technology to make instant coffee, which according to Rosen (1976) took 22 years from patent application to a commercial product!

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