

Review

Preclinical and Clinical Safety Studies on DNA Vaccines

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ABSTRACT

DNA vaccines are based on the transfer of genetic material, encoding an antigen, to the cells of the vaccine recipient. Despite high expectations of DNA vaccines as a result of promising preclinical data their clinical utility remains unproven. However, much data is gathered in preclinical and clinical studies about the safety of DNA vaccines. Here we review current knowledge about the safety of DNA vaccines. Safety concerns of DNA vaccines relate to genetic, immunologic, toxic, and environmental effects. In this review we provide an overview of findings related to the safety of DNA vaccines, obtained so far. We conclude that the potential risks of DNA vaccines are minimal. However, their safety issues may differ case-by-case, and they should be treated accordingly.

INTRODUCTION

Vaccines are one of the most effective means to prevent infectious diseases, and generally have a very good safety profile. Vaccines may be conventional or genetically engineered vaccines, and either live or inactivated. Adverse events after vaccination are often a consequence of the vaccine's nature. Thus attenuated pathogens in live vaccines may still be too virulent, or they may revert back to virulence as recently occurred with live attenuated poliovirus vaccine strain.¹ Inactivated vaccines are considered safer than live vaccines, however, inactivation can be incomplete, e.g., the Cutter accident,² or poses inadvertent consequences. For example, a formalin-inactivated and aluminium-adjuvanted respiratory syncytial virus (RSV) vaccine caused enhanced disease upon subsequent natural RSV infection.³

For DNA vaccination, genetic material (usually a plasmid) encoding an antigen is transferred to cells of a vaccine recipient. These cells express the antigen and present it to the recipient's immune system. This method of vaccination has many potential advantages. No unsafe infectious agents are involved. DNA vaccines can induce both humoral and cytotoxic T cell responses, even without replication. They can even induce immunity in newborn individuals that have high levels of maternal antibodies, which bind and neutralize conventional vaccines. There is a potential for encoding multiple immunogenic epitopes with the purpose of raising protection against several diseases by a single vaccine. Compared with many conventional vaccines, DNA vaccines are relatively stable. Furthermore, DNA vaccines may be developed quickly and easily once a gene has been identified. They therefore have great potential in controlling emerging pathogens (such as SARS or pandemic influenza), or bioterrorist attacks.

Despite initial success in preclinical studies, to date, the efficacy of DNA vaccines in clinical trials has been disappointing and it is uncertain whether the high expectations associated with DNA vaccines will be fulfilled. Recent studies focus on the improvement of DNA vaccine efficacy.⁴ In the meantime, preclinical and clinical studies have yielded much safety data. This review provides an overview of DNA vaccine safety data and investigates how regulatory authorities have addressed the safety aspects.

PRECLINICAL OBSERVATIONS ON POTENTIAL RISKS

A number of safety concerns have been identified considering the application of DNA vaccines for human use⁵⁻⁹ as reflected in guidance documents that exist for DNA vaccines.¹⁰⁻¹² These concerns pertain to genetic, immunological, toxicological, and environmental effects (Table 1). In this section an overview is given of safety data from preclinical studies, i.e., usually in small laboratory animals.

General safety. Parker et al¹³ studied the safety of repeated intramuscular injection of a plasmid DNA malaria vaccine at doses of 1, 10 and 100 µg in mice and doses of 150 and 450 µg in rabbits. Hanke et al.¹⁴ studied intramuscular injection of mice with two 50 µg doses of plasmid DNA. Tuomela et al.¹⁵ studied the general safety of DNA vaccination in rats who received a single dose of 200 µg plasmid DNA intravenous, intramuscular or intradermal. In all administration methods and at all doses applied, DNA vaccines were well-tolerated and nontoxic.

Biodistribution and persistence. The biodistribution and persistence of a plasmid is dependent on the route of administration and delivery method. Shortly after intramuscular injection, plasmid DNA was detected in many organs remote from the site of injection in mice.^{13,16,17} In rat, plasmid DNA was detected in the lymph nodes.¹⁵ Several weeks after injection, plasmid DNA could only be detected at the site of injection where it persisted for the time of the study.¹³⁻¹⁷ Occasionally, plasmid was detected in gonads, but it dissipated rapidly.^{13,17} The level of plasmid DNA at the injection site was below 100 copies/ µg DNA after initial injection with 100–200 µg DNA.^{13,15,17} Kim et al¹⁸ have shown that 30 minutes after injection 33% of the initial concentration was present. Ninety minutes after injection less than 1% remained. The amount of plasmid DNA in the organs remote from the injection site was 2–3 orders of magnitude lower than at the injection site.¹⁸

After intravenous administration in mice and rat, plasmid DNA was initially distributed at a relatively low amount to all tissues examined except the gonads and brain, in which no plasmid DNA was detected. However, plasmid DNA was rapidly cleared.^{13,15} Less than 1% of the initial concentration was detected in blood at 30 minutes post-administration in mice, and no plasmid was detected 60 minutes post-administration.¹⁸

Bureau et al¹⁹ studied radiolabeled plasmid DNA to study its biodistribution after intramuscular injection; between 5 minutes and 3 hours after injection more than 90% of the plasmid had been cleared. A small part of the injected DNA seemed to be relatively protected from DNase I and persisted, probably because it is involved in the transfection process.

Long-term persistence might facilitate the integration of plasmid DNA into the host's genome. Furthermore, long-term expression could cause long-term skewing of the immune system influencing subsequent immunizations and infections. One study found that DNA delivered into mouse muscle was stably expressed for 19 months, even though no integration could be detected.²⁰ Recently, Armengol et al.²¹ reported that DNA injected in mouse muscle persisted for up to two years and was expressed at a low, but significant level.

Integration into the host genome. Integration of plasmid DNA into the recipient's genome appears the major point of attention of DNA vaccination. Integration may occur randomly or by homologous recombination. Integration could lead to activation of oncogenes, inactivation of tumor suppressor genes, or, when integrated into the chromosomal DNA of germ line cells, to vertical transmission. Suicide vectors have been developed that induce apoptotic cell death of transfected cells.²²⁻²⁶ Such suicide vectors may be important to alleviate the concerns of potential integration and cell transformation.

Table 1 **Safety aspects of DNA vaccines**

Genetic effects

Long-term persistence

Integration into the host genome thereby increasing the risk of mutagenesis and oncogenesis

Germline integration and vertical transmission

Immune-mediated effects

Induction of auto-immunity by breaking tolerance to self-antigen or induction of anti-DNA antibodies

Induction of immunological tolerance

Altered immune responsiveness to other vaccines and infection

Toxicity and immunotoxicity

Environmental effects

Environmental spread

Horizontal transmission inside or outside the vaccinated host after recombination Vertical transmission

Integration was first examined with plasmid DNA vaccines encoding the influenza NP gene,²⁷ and later with plasmids encoding the influenza HA or M gene, as well as the HIV *gag* gene.²⁸ After vaccination of mice and guinea pigs with plasmid DNA, high molecular weight DNA was isolated and purified from non-integrated plasmid using pulsed-field gel electrophoresis, followed by detection of the plasmid with PCR. The sensitivity of the PCR was approximately 1 plasmid copy per µg DNA, representing approximately 150,000 nuclei.²⁸ Essentially all detectable plasmid DNA in treated muscle tissue was extrachromosomal. Thus, random integration might have occurred, but at frequencies of <1–8 copies in 150,000 nuclei.²⁸ This would be at least three orders of magnitude below the spontaneous mutation rate of gene-inactivating mutations, making the authors to conclude that the risk of mutation due to plasmid integration following intramuscular inoculation is negligible.²⁸

After vaccination of mice, the amount of plasmid DNA expressing the *Plasmodium falciparum* circumsporozoite protein (*PfCSP*) that persisted in muscle tissue for 30 to 60 days post-injection was about 10 fg per µg of genomic DNA (in the range of 1,500 copies per 150,000 nuclei). PCR assays after agarose gel purification indicated that, 3-30 copies of plasmid DNA remained associated per 150,000 genomes,²⁹ which is in line with the data above.²⁸

Injection of a plasmid DNA vaccine containing the human papilloma virus type 16 E7 gene, whose protein product is known to increase integration in vitro,³⁰ did not result in detectable integration in mice as studied by PCR on gel-purified genomic DNA.¹⁷ Biojector delivery, compared with needle injection, greatly increased the uptake of plasmid by host cells, but did not result in a detectable increase in integration frequency. Aluminium phosphate adjuvant had no effect on the tissue distribution and integration frequency.¹⁷ Studies in rat^{31,32} or fish³³ have also not been able to demonstrate any integration event.

In contrast to the studies mentioned above, Wang et al,³⁴ using a newly developed PCR assay, identified four independent integration events upon plasmid injection followed by electroporation in vivo. This PCR uses a vector-specific primer and a genomic primer based on repetitive DNA. The PCR detects covalent junction of plasmid-to-genomic DNA sequences after repeated rounds of gel purification to remove free plasmid DNA. Electroporation markedly increased plasmid tissue levels and its association with genomic DNA; after gel-purification approximately 980 copies of plasmid DNA were

found to be associated with 1 µg of high molecular weight genomic DNA, whereas for the muscle DNA samples from non electroporated mice, only 17 copies/µg DNA were found. These results suggest that electroporation increased plasmid integration frequency.³⁴

The results of Wang et al.³⁴ demonstrate that integration events—at a very low frequency—cannot be neglected. When new technologies improve the efficiency of DNA delivery an increase in integration events can be expected. Therefore, for each new plasmid DNA to be used as a clinical vaccine integration should be considered.

Vertical transmission. Evidently, when plasmid is transmitted to the gonads, germ line chromosomal integration and germ line transmission could occur. Plasmid DNA may be detected in gonads shortly upon injection,^{13,17} but integration of plasmid DNA into chromosomal germ line DNA has not been observed so far.¹⁷ Since the level of plasmid DNA in the gonads was rapidly cleared the risk on germline transmission is expected to be minimal.

Induction of auto-immunity. There is concern that DNA vaccines might induce auto-immunity. The immunostimulatory activity of unmethylated CpG motifs in the plasmid backbone can lead to the formation of anti-DNA antibodies, which might accelerate the development of auto-immune diseases.^{6,7,35} The introduction of other immunomodulatory molecules such as cytokines, may also result in the induction of auto-immune responses to these molecules. Also the attachment of peptides, for example a nuclear localization signal, to the DNA vectors might induce auto-immunity. Another mechanism by which auto-antibodies might arise is through destruction of injected muscle cells as a result of DNA vaccination. However, in this respect, it is unlikely that DNA vaccines would pose any greater risk than conventional vaccines.⁶

To examine whether the CpG motifs in DNA vaccines are capable of stimulating systemic auto-immune disease, studies were performed with normal BALB/c mice and B/W mice. B/W mice develop lupus erythematoses symptoms at an advanced age, which is associated with the overproduction of IgG anti-DNA antibodies. BALB/C and B/W mice were immunized and boosted several times intramuscularly with DNA vaccines. In normal mice, boosting resulted in an increase of the number of spleen cells producing IgG anti-DNA by up to 3-fold as estimated with ELISpot assay. Also a small increase in serum IgG anti-DNA antibody levels was observed. The magnitude of the rise was quite modest when compared to the spontaneous production of auto-antibodies in B/W mice, and did not result in the development of disease in normal mice. The onset of disease was not accelerated in B/W mice nor was an increase in B cells producing anti-DNA antibodies observed compared to untreated animals.^{36,37}

Also in other studies no auto-immune mediated pathology,^{13,38} or development of systemic auto-immune responses³⁹ were observed in normal animals after DNA vaccination. No anti-DNA antibodies were observed in sera after DNA vaccination of mice,^{13,39,40} rat,¹⁵ rabbits,¹³ fish,³³ and non-human primates.⁴¹

Certain transgenes may increase the risk of anti-DNA antibodies. Transient secondary antibody responses against DNA were found in 7 out of 11 rabbits that received genes encoding the viral nucleic acid-binding proteins HIV-1 reverse transcriptase (RT), HIV-1 nef, and truncated hepatitis C virus (HCV) core. In this study the immune response against DNA was probably stimulated by protein-nucleic acid complexes.⁴²

In conclusion, although DNA vaccines can induce the formation of anti-DNA antibodies, no evidence has been found that DNA vaccines do induce systemic autoimmune diseases, neither in healthy animals, nor in animals that are at-risk for the development of autoimmune diseases.

Organ-specific auto-immune diseases are characterized by the selective activation of Th1-type immune responses. Therefore Th1 cytokine production induced by DNA vaccination might increase an animal's susceptibility to organ-specific auto-immunity.³⁶ Segal et al.⁴³ indeed have shown that CpG motifs can convert resting auto-reactive T cells into effector cells through induction of IL-12. In a molecular mimicry model, CpG motifs acted as potent immunoadaptors inducing auto-immune myocarditis when coinjected with *Chlamydia*-derived antigen.⁴⁴ Thus, theoretically, DNA vaccination may trigger deleterious auto-immune reactions under certain circumstances. However, toxicity has not been observed in normal animals treated with therapeutic doses of DNA vaccines or CpG oligodeoxynucleotides.^{7,45}

A point of attention is whether cytokine-encoding plasmids may induce auto-antibodies against these cytokines. This question appears unexamined.

Induction of immunological tolerance. Evidently, vaccination is usually applied to induce immunity. However there appears to be a fine line between the induction of immunity and the induction of tolerance. Most vaccines intended for human use are administered to infants and children. Because of the immaturity of their immune system, vaccinated newborns may develop tolerance rather than immunity.⁴⁶ Vaccination of 2–6-month-old mice with a DNA vaccine encoding the circumsporozoite protein (pCSP) of malaria resulted in immunity. However, neonatal mice of different MHC types and younger than seven days of age did not develop a serum antibody response,^{47,48} and remained unresponsive when revaccinated as adults.⁴⁸ The tolerance induced by early vaccination was antigen-specific, as it was not induced by DNA plasmids encoding other malaria proteins,³⁶ or by DNA vaccines against other infectious diseases.^{49,50} However, the examples above show that safety testing of each new DNA vaccine that will be used in children or newborns is extremely important.

Modulation of immune response. When plasmids encode cytokines, they might affect the host's immune capacity and therewith lead to long-term safety risks. Coadministration of immunostimulatory molecules, either as protein or gene, can result in their release into the circulation, potentially causing undesirable, systemic, effects. Indeed, in some studies such effects are described. IL-12-encoding plasmid enhanced susceptibility to a challenge with feline infectious peritonitis virus (FIPV).⁵¹ Infection of mice that were genetically resistant to infection by ectromelia virus (a mouse pox virus) with an IL-4-expressing ectromelia virus caused high mortality rates. Also infection of immunized, resistant mice with this IL-4-expressing ectromelia recombinant virus caused substantial mortality, probably caused by suppression of cytotoxic T-lymphocyte cytolytic activity and inhibition of memory responses.⁵² Ishii et al.⁵³ showed that plasmids encoding IFN-γ or IL-4 boosted immunity against a coadministered vaccine without unwanted side effects.

Toxicity and immunotoxicity. An unanticipated safety problem has been reported recently after DNA vaccination against tuberculosis in mice. In an immunotherapeutic vaccination study with mice that were infected with *Mycobacterium tuberculosis*, vaccination with a DNA vaccine encoding the 65-kDa heat shock protein of *Mycobacterium leprae* caused pulmonary necrosis.⁵⁴ Similar severe reactions were seen in mice given a DNA vaccine encoding the Ag85 antigen of *M. tuberculosis*.⁵⁴ The basis of this reaction is not precisely known, but could be due to a potent CD8⁺ CTL response. The reaction does not seem to be restricted to DNA vaccines since exacerbated lung pathology has also been observed in immunotherapeutic studies

with mycobacterium antigens.^{55,56} Furthermore, in mice that were given BCG vaccine as immunotherapeutic, pyrogenic responses, suggesting lung damage, were observed.⁵⁷ However, this indicates that *M. tuberculosis* DNA vaccines should be used with caution in individuals who may have already been exposed to *M. tuberculosis*.⁵⁵

Toxic effects might further be exerted by the biological function of the expressed proteins, but so far this question appears largely unexamined. Interestingly, a plasmid DNA vaccine expressing fragment C gene of type A botulinum neurotoxin, appeared nontoxic to the injected animals and provided protection against lethal type A botulinum neurotoxin challenges.⁵⁸ Evidently, such a vaccine minimizes human safety issues associated with the production of vaccine containing hazardous botulinum. Similarly, immunization of mice with plasmid encoding fragment C protein, the nontoxic C-terminal domain of tetanus toxin, protected mice against lethal challenge with tetanus toxin.⁵⁹

ENVIRONMENTAL EFFECTS

Environmental risks of DNA vaccines received little attention so far in literature, probably because the risks are considered very low. Possible environmental risks of DNA vaccines that can be anticipated are: (1) environmental spread of recombinant plasmid vectors by shedding or by consumption of vaccinated animals, (2) recombination with viruses, bacteria, or parasites inside the vaccinated host, and consequently the generation and spread of genetically modified organisms (GMOs), (3) recombination with viruses, bacteria, or parasites outside the vaccinated host after shedding, (4) integration in the genome of germ line cells.

Injected plasmids might spread accidentally, e.g., by the transfer of plasmids to other species via excreted body fluids, or when the immunized animal or human dies. Little is known about the shedding of plasmid DNA after application of a DNA vaccine to animals or humans. Only Comerota et al⁶⁰ reported data on plasmid DNA shedding. No plasmid DNA was detectable in urine of human subjects after intramuscular administration of a naked plasmid DNA in a phase 1 clinical trial. Once shedded into the environment DNA will be degraded.^{61,62} Therefore, release of plasmid DNA into the environment is not likely to have any direct impact on other organisms. Even if bacteria or other organisms take up the plasmid, and/or recombination occurs between their genomic DNA and the plasmid DNA, the resulting recombinant organism will only overgrow the population and pose an ecological risk if the gene(s) present on the plasmid provide a certain survival advantage to the organism. This might for example occur when the recombinant organism obtains an altered tissue or species tropism, altered virulence, or when antibiotic resistance or metabolic genes provide a survival advantage in particular niches.

Plasmid DNA can also be spread by consumption of vaccinated animals. The fate of ingested DNA was examined in several studies and it was found that food-ingested DNA could be traced to several organs.^{16,63,64} However, it is not likely that the ingestion of plasmid DNA by consumption of vaccinated animals poses any greater risk than the consumption of "natural" DNA.

Recombination with natural viruses, bacteria or parasites inside the vaccinated person or animal is partly determined by the degree of homology between plasmid DNA and naturally occurring micro-organisms. However, chances on such recombination events and subsequent shedding and (long-term) transmission will probably be extremely low, especially when DNA vaccines are administered

intramuscularly. Evidently, such risks do also exist for conventional live or inactivated vaccines, which also contain nucleic acids. Since, in contrast to live vaccines, DNA vaccines do not replicate in mammalian cells, the risk of recombination with resident micro-organisms will likely even be much lower for DNA vaccines.

A number of bacterial species are naturally competent and able to take up naked DNA. However, in most bacteria special uptake sequences are required for this process. These uptake sequences have often been identified,⁶⁵ and DNA vaccines should be free of such sequences. *Helicobacter pylori*, a pathogen of the stomach, does not encode a specific sequence for uptake of naked DNA, and transfer of naked DNA to this ubiquitous human pathogen should be investigated. *H. pylori* is the first naturally transformable gram-negative species shown to lack such a transformation-targeting system.⁶⁶

In conclusion, DNA vaccines do not seem to pose any risks for the environment, although this assumption has not been rigorously tested.

CLINICAL OBSERVATIONS IN HUMANS

DNA vaccines have entered the clinic for initial safety and immunogenicity testing in humans for various infectious diseases, like human immunodeficiency virus (HIV) infections, malaria, tuberculosis, influenza virus infections, hepatitis B and C and cytomegalovirus (CMV) infections. Current phase 1 and 2 trials are also studying DNA vaccines as potential immunotherapeutics for various cancers, including colon cancer, human follicular lymphoma and cutaneous T-cell lymphoma.⁶⁷ All DNA vaccines used so far were well tolerated with no local or systemic serious adverse effects.⁶⁸⁻⁷¹ No changes occurred in vital signs, complete blood counts, serum creatinine, other blood chemical parameters, or urinalysis.^{68,72,73} No significant increase in antinuclear antibodies and anti-DNA antibodies were detected.^{68,72-74} Serum liver chemical values increased in some subjects, but returned to normal during the study.⁶⁸ Even at doses as high as 2500 µg per injection no serious adverse effects, no antinuclear or anti-DNA antibodies, and no abnormalities in blood chemistry or urinalysis were observed.⁷² Toxicity in one study was limited to transient grade 1 injection site tenderness, fatigue, and creatine kinase elevations indicative of some muscle damage.⁷⁵ Unfortunately, clinical studies in humans have shown lower efficacy than what had been expected based on animal studies.

An hepatitis B DNA vaccine has been clinically tested by coating the DNA onto gold beads, which were then propelled into the epidermis with a "gene gun". This vaccine was well tolerated at all pressures. However, in a first phase 1 trial the vaccine did not induce primary immune responses possibly due to the low dose of DNA (0.25 µg) used.⁷⁴ In a second trial, designed as a dose escalation study with administration of DNA concentrations from 1 µg to 4 µg in total, the vaccine elicited both humoral and cellular immune responses.⁷³ In a trial with human subjects that did not respond to conventional hepatitis B vaccines the DNA vaccine elicited antibody responses in 12 out of 16 subjects.⁷¹

DNA vaccination strategies against HIV focus on the induction of HIV-specific CTL responses in order to eliminate infected cells. Calarota et al⁷⁶ describe a clinical trial in which they use a DNA vaccine with the regulatory HIV-1 genes *nef*, *rev* and *tat*. HIV-1 specific responses were elicited in 8 out of 9 patients. However, no decrease in viral load was observed. CTL responses in asymptomatic HIV patients were also observed in a clinical trial with a plasmid encoding the env and rev proteins.^{68,77,78} The same vaccine was also

tested in a study with seronegative volunteers where a transient CTL response was observed.⁷⁹ Transient T-cell responses were also reported by Mwau et al.⁷⁰ after vaccination with a DNA vaccine with the *gag* gene and more than 40 small DNA sequences encoding antigenic regions of other HIV proteins.

In a phase 1 safety and tolerability study of a malaria DNA vaccine (*Plasmodium falciparum* circumsporozoite protein (*PfCSP*) DNA plasmid), 20 volunteers received 3 intramuscular injections of one of 4 different dosages (20–2500 µg). Despite the induction of CTL responses,⁸⁰ this DNA vaccination failed to induce detectable antigen-specific antibodies in any participant.⁷² In an attempt to elicit both antibody and T cell responses, a *PfCSP* recombinant protein vaccine (a partially protective *PfCSP* vaccine) was administered together with adjuvant to volunteers that were previously immunized with the *PfCSP* DNA vaccine and to naive volunteers. This sequential immunization with DNA and recombinant protein (also called heterologous prime-boost regime), was well-tolerated, safe, and led to antibody, and CD4⁺ and CD8⁺ T cell responses, which were enhanced compared to the vaccines consisting of DNA or recombinant protein only.⁸¹ Also in a phase 1 trial of a priming immunization with a DNA vaccine and a boosting immunization with a modified vaccinia virus Ankara (MVA) poxvirus vaccine, higher T cell responses were reported than obtained by either vaccine alone in malaria-naive individuals.⁸²

REGULATORY ASPECTS OF DNA VACCINES

The most important regulatory documents that refer to DNA vaccines are listed in (Table 2). For comparison relevant regulations from both Europe (EU) and the USA are shown. The last column shows globally recognized regulatory documents, especially from the WHO and the International Conference on Harmonization (ICH) of technical requirements for the registration of pharmaceuticals for human use. WHO documents are not binding, but are meant to guide national regulatory authorities and manufacturers on the characterization, manufacture, preclinical safety evaluation, and clinical development of medicinal products by outlining international regulatory expectations. As such the documents are also meant to facilitate international harmonization. The WHO documents are written by expert groups consisting of experts from research institutes, industry, academia, and regulatory authorities. ICH documents are written to aid harmonization on the requirements for investigations on medicinal products in Europe, the USA and Japan. Representatives from the industry and regulatory authorities draft these documents. Once an ICH document has become final, the Committee for Human Medicinal Products (CHMP); previously named Committee for Proprietary Medicinal Products (CPMP) adopts such a document for the European Union. Therefore the ICH documents can be seen as a regulatory basis for medicinal products in the European Union. The legal framework for the regulatory requirements in Europe is laid down in Directives issued by the European Commission or Council and/or Parliament. These Directives are implemented in National laws. Based on the Directives, more detailed guidance is issued by the European Medicines Agency (EMA)/CHMP in the form of Notes for Guidance (NfG) and Points to Consider (PtC) documents. These documents are no regulations, but provide guidance on the data required for applications for marketing authorization. For the most these documents are also applicable for clinical trial applications. Only the most relevant Directives and guidance documents are listed in Table 2. Other more general guidance documents may also be applicable to some extent.

According to Commission Directive 2003/63/EC (medicinal products for human use),⁸³ DNA vaccines are considered gene therapy medicinal products. This directive recognizes that conventional requirements for safety evaluation may not always be appropriate. This understanding that DNA vaccines differ from conventional medicinal products is reflected in the relevant guidelines, where it is emphasized that these type of products need a case-by-case approach for evaluation and often require studies with tailor-made design. The most important regulatory guidance document within the EU for DNA vaccines is the Note for Guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products.¹⁰ Other relevant documents are the Note for Guidance on preclinical pharmacological and toxicological testing of vaccines⁸⁴ and the Guideline on Preclinical Safety Evaluation of Biotechnology-Derived Products.⁸⁵ For gene therapy medicinal products also GMO legislation applies,^{86–88} however, the GMO legislation could also be interpreted in such a way that DNA vaccines do not fall under the scope of this legislation. This is further discussed below.

Specific considerations as expressed in the CHMP Note for Guidance document¹⁰ that apply to DNA vaccines are the possibility of integration of the construct in the genomic DNA and the distribution and extent of expression of the vector gene. DNA integration should be studied and the potential consequences of such an integration event should be considered. It should be studied to which tissues the DNA vaccine is distributed and where it is expressed and for how long. In this respect distribution to gametes needs special attention, as genomic manipulation of human germ line cells, intentional or unintentional, is prohibited. In addition, the possibility of immunopathological effects should be given thorough consideration, e.g., the formation of antinuclear antibodies, the induction of auto-immunity due to molecular mimicry, or the formation of insoluble immune complexes that might precipitate in target organs such as the kidney or the liver. Higher doses of the vaccine should also be investigated in toxicology studies. Rapid and vast expression of the antigen might aggravate adverse side effects or trigger an extensive immune response when expression levels are high or when the vaccine is readministered. On the other hand, prolonged expression of the antigen might induce tolerance. Usually, standard genotoxicity studies are not appropriate for DNA vaccines. However, the type of complexing material might be a concern necessitating such testing.

A specific concern associated with DNA vaccines is the integration of vaccine DNA in the genomic DNA causing insertional mutagenesis, especially when the insertion takes place in or near an oncogene. When integration in genomic DNA occurs, tumorigenicity may be a concern that could make additional testing necessary. Also long-term expression of growth factors or immunosuppressive molecules could be reason to study tumorigenicity.

The relevant guidance documents issued in the USA¹¹ and by the WHO¹² (see Table 2) provide essentially similar guidance on the preclinical aspects of DNA vaccines as the CHMP Note for guidance. Both USA and WHO guidance documents express the need for a flexible approach and a scientific rationale for the design of the studies. In the USA the Points to Consider on plasmid DNA vaccines for preventive infectious diseases¹¹ is now being updated in the Draft Guidance for Industry Considerations for plasmid DNA vaccines for infectious disease indications.⁸⁹ The gained insight in the safety of DNA vaccines, obtained through preclinical and clinical studies is reflected in this adapted document.

Naked DNA vaccines are products for gene transfer and can as such be considered to be inside the scope of legislature for GMOs.

Table 2 **Regulatory documents for DNA vaccines**

	EU	USA	World-Wide (Not Binding)
Legislation	EU Directives 2001/83/EC and 2003/63/EC (medicinal products for human use) and 2001/20/EC (clinical trials)	USA Code for Federal Regulations Parts 50, 56, 58, 210, 211, 312, 600, 601 and 610	
Main guidance documents	NfG on the quality, preclinical and clinical aspects of gene transfer medicinal products (CPMP/BWP/3088/99)	PfC on plasmid DNA vaccines for preventive infectious disease indications	WHO Guidelines for assuring the quality of DNA vaccines (WHO Technical Report Series N. 878, 1998; Annex 3)
Other relevant guidance documents	NfG on preclinical pharmacological and toxicological testing of vaccines (CPMP/SWP/465/95); ICH Topic S6, Guideline on Preclinical Safety Evaluation of Biotechnology-Derived Products (CPMP/ICH/302/95);	PfC in the production and testing of new drugs and biologicals produced by recombinant DNA technology (4/85); Supplement to PfC in the production and testing of new drugs and biologicals produced by recombinant DNA technology Nucleic acid characterisation and genetic stability (4/92); PfC in human somatic cell therapy and gene therapy (8/91).	WHO guidelines on nonclinical evaluation of vaccines (WHO/BS/03 1969 revised, DRAFT, November 2003); ICH Topic S6, Guideline on Preclinical Safety Evaluation of Biotechnology-Derived Products (CPMP/ICH/302/95)

NfG, notes for guidance; PfC, points to consider; ICH, International Conference on Harmonization.

Inside the European Union, the use of GMOs is subject to 2 Council Directives. Contained use is regulated by Council Directive 90/219/EC,⁸⁸ as amended by Council Directive 98/81/EC.⁸⁶ Deliberate release into the environment is regulated by Directive 2001/18/EC.⁸⁷ Commission Decision 2002/623/EC⁹⁰ establishes guidance notes supplementing Annex II of directive 2001/18/EC. Directive 2001/18/EC leaves some room for interpretation on the issue of whether naked DNA is considered to be inside the scope of this directive.

In the EU, the national regulations are relatively diverse. The United Kingdom, Italy, and France consider treatment of a patient by gene therapy as contained use of a GMO. However, the United Kingdom authorities view the vector as being contained by the patient, while Italian authorities insist on containment of the treated patient. In several other countries (e.g., Denmark, The Netherlands, Sweden) clinical trials with products for gene therapy are considered to constitute deliberate release of a GMO into the environment. In The Netherlands, this currently also applies to plasmid DNA vectors, and consequently a trial is subject to an elaborate public procedure in which the Ministry for the Environment is the competent authority and which includes two rounds of consultation of the public. Recently, the Netherlands Commission on Genetic Modification (COGEM) advised the Ministry for the Environment to simplify the procedure for those DNA vectors that do not contain sequences that can lead to replication of the DNA, or sequences that can become part of viruses or bacteria, such as packaging signals or bacterial uptake sequences. COGEM advised to confine with a duty to report for those DNA vectors that are replication incompetent and in addition do not contain sequences that can enhance recombination with viruses.

Concerns of national competent authorities regarding environmental risks of plasmid DNA vectors are sparsely and briefly described. The Dutch competent authority requests a description of regulatory and structural sequences in the vector, a description of the (lack of knowledge of) functions in the donor organism of the

different sequences, and of the possibility of sequences (of viral origin) in the vector interacting with autonomously replicating microorganisms. While such interaction is theoretically possible, it is questionable if the result will be significant as long as the vector DNA does not contain any means for replicating itself. The United Kingdom competent authorities have a comprehensive on-line compendium of guidance provided by the Advisory Committee on Genetic Modification (ACGM).⁹¹ Apart from specific control measures for work with naked oncogenic DNA by laboratory workers, the compendium does not identify any risks of naked DNA to the environment. For the centralized licensing procedure through the EMEA, EC Directive 2003/63⁸³ requires an environmental risk assessment to be made for both GMO and nonGMO medicinal products. However, for DNA vaccines, the only Note for Guidance available (DNA vaccines non-amplifiable in eukaryotic cells for veterinary use)⁹² does not mention any environmental issues at all. A similar situation is present in the USA. The Points to Consider on plasmid DNA vaccines for preventive infectious disease indications¹¹ of the FDA deals with quality, safety and clinical issues, but does not, however, address the environment. Altogether this gives the impression that environmental risks of DNA vaccines are not considered grave and the issue is not a priority for national authorities.

DISCUSSION

The field of DNA vaccination is rapidly moving forward. Many researchers are doing their utmost to improve the rational design of DNA vaccines, to enhance their uptake by host cells, to improve the presentation of their encoded antigens, or their immunogenicity. Driving forces behind this scientific development are, beyond doubt, the potential to develop vaccines more rationally (compared with the old-fashioned trial-and-error approach), and more quickly, which is extremely important when vaccines are needed against newly emerging pathogens, or against bioterrorist's attacks. Safety data accumulated so far indicate that DNA vaccines have a good safety profile in

preclinical and clinical phase I studies. DNA vaccines may be intrinsically more safe than conventional vaccines, because they are non-life, non-replicating, and non-spreading. They also appear well-tolerated, i.e., nontoxic. This does not imply that there are no safety concerns. One of them is integration of plasmid DNA into the host genome. An integration event has been demonstrated upon intramuscular injection followed by electroporation, but at a frequency below that of spontaneous gene-inactivating mutations.³⁴ Integration of plasmid DNA into host genomic DNA will be very rare, but we may foresee that the frequency of integration may increase (somewhat) when techniques assure more efficient uptake and nuclear localization of plasmid DNA to enhance their immunogenicity. The question, unanswered at the moment, is whether a low frequency of plasmid integration has any biological significance at all.

Although the risks of DNA vaccines may be very small, we should try to minimize them, because vaccines may be administered very widely. Therefore factors favoring integration should be identified and avoided as far as possible and reasonable. In general, all elements favoring uptake, integration, transposition, replication, enhancement (of oncogenes), or selection (resistance genes) should not be used, or only with extreme care. For example, genes encoding bacteriophage integrases and retroviral long terminal repeats should not be included in plasmid DNA vaccines.^{93,94} Although remote, the consequences of plasmid integration can be minimized by assuring that plasmids do not contain promoter and enhancer sequences that have the potential to alter the expression of cellular oncogenes.⁹⁵ These latter sequences may be used in gene therapy protocols, but should not be incorporated in DNA vaccines. Homology between plasmid and host DNA is likely a factor determining integration efficiency. Expression of host genes, for example coding for immunologically relevant molecules such as cytokines, could also enhance the risk of integration and should thus be used with caution.

Safety may also be enhanced, for example, by using suicide vectors that induce apoptotic cell death of transfected cells,²²⁻²⁶ which is important to alleviate the concerns of potential integration and cell transformation. Further development of RNA vaccines, though more difficult to produce, may be important, because they lack the potential of DNA integration into the genome. The potential, but remote, risks of insertional transformation and oncogenesis should be tested thoroughly in the preclinical phase in *in vitro* models and in models of oncogenesis that use transgenic or conventional knockout mice. Characterization of insertional events and their consequences in cell lines *in vitro* may indicate different risk categories for plasmid vectors. Finally, expression of host molecules may also promote the development of auto-immunity. This aspect, especially the development of auto-antibodies directed against cytokines, deserves attention.

Environmental risks of DNA vaccines received little attention. EC Directive 2003/63⁸³ requires an environmental risk assessment for both GMO and non-GMO medicinal products. An environmental risk assessment analysis is described for the recombinant live oral cholera vaccine, CVD 103-HgR.⁹⁶ The strain was derived from *V. cholerae* Classical Inaba strain 569B by deletion of 95% of both chromosomal copies of the *ctxA* gene, which encodes the toxic A subunit of the cholera toxin, and by insertion of a mercury resistance marker. It is the first, and currently only, GMO registered as a vaccine for human use. The environmental risk assessment included genetic structure and stability, its (in) ability to transfer toxin or other virulence determinants to other micro-organisms, its (in) ability to stably convert to a toxinogenic phenotype, and the absence of genetic traits, which may confer some competitive advantage under natural

environmental conditions. In a similar way an environmental risk assessment should be made for DNA vaccines. We consider that the environmental risks for DNA vaccines are very small. As discussed in this report, the chance of integration for DNA vaccines is very low. Integration into germline cells has even never been observed. Similarly, we believe that the chance of recombination with other organisms or uptake by bacteria is very small. Even if recombinant organisms arise it is not very likely that they will overgrow the population and have any ecological impact. Nevertheless, the expected low environmental risk of DNA vaccines is mainly based on assumptions and this aspect deserves more attention. An environmental risk assessment for DNA vaccines should include shedding data, information about sequence homology with other organisms, information about presence of sequences implicated in DNA dissemination (uptake, conjugation, transposition, mobilization and bacterial transformation), information about genes that could be favorable for other organisms, and integration studies. Furthermore, to get more insight in the fate of plasmid DNA in the environment, it would be worthwhile to study the stability of plasmid DNA, its uptake by bacteria (e.g., by *H. pylori*), and recombination with viruses.

Use of plasmid DNA vaccines is in some countries strictly regulated, therewith applying rules for deliberate release for gene therapy protocols. Given the small risks associated with the use of plasmid vaccines, the application of such rules is questionable as advised by the COGEM.

In conclusion, DNA vaccines promise to become a flexible and easy way to design and produce vaccines against important public health threats, especially when their immunogenicity will be enhanced. DNA vaccines are considered to be safe and this is supported by data from literature. Additional safety issues may originate from their encoded sequences or added substances, and these should be assessed on a case-by-case base.

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