Biosafety of DNA vaccines: New generation of DNA vectors and current knowledge on the fate of plasmids after injection.

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Abstract

DNA vaccination has been widely studied to develop new, alternative, efficient and safe vaccines for humans and animals. Many efforts have been made to increase the immunising potential of these vaccines and three veterinary vaccines are now available on the market. Much work is also being dedicated to develop effective DNA vaccines for humans. However, this new vaccination technique raises issues concerning biosafety due to the nature of the vector, i.e. a DNA molecule that contains sequences of prokaryotic origin (e.g. genes for antibiotic resistance). This review describes the development of the new generation of DNA vectors that are partially or completely devoid of elements of prokaryotic origin and outlines the results of studies on the fate of plasmids after their injection *in vivo*.

I. From discovery to industrial application

In 1990, Wolff *et al.* showed that the injection of a DNA plasmid in mouse muscle resulted in a significant expression of the protein encoded by the plasmid [1]. Starting with this discovery, various antigens encoded by plasmids have been successfully used to induce the production of antibodies [2, 3] and cytotoxic T lymphocytes [4], thereby demonstrating the potential of this strategy for DNA vaccination and gene therapy. Progress in this field has resulted in the development and the marketing of three veterinary DNA vaccines since 2005. Two of them are authorised for use in the United States: one targets the West Nile virus infection in horses and the other targets canine malignant melanoma. The third vaccine, authorised for use in Canada on salmon, is directed against the infectious hematopoietic necrosis virus.

II. First- and new generation of DNA vectors for DNA vaccination

II.1 First-generation of plasmids bearing prokaryotic elements

DNA vaccines are basically composed of plasmids encoding the vaccine antigens and saline solutions. The plasmids contain an antibiotic resistance gene controlled by a prokaryotic promoter and a prokaryotic origin of replication that allow for selection and replication of plasmids in transformed bacteria. The plasmid transcription unit is usually composed of a strong and ubiquitous viral promoter that confers optimal expression of the gene of interest in eukaryotic cells, as well as a termination-polyadenylation sequence usually derived from the simian virus 40 (SV40) genome or from the bovine growth hormone gene [5, 6]. The prokaryotic sequences of conventional DNA vaccines have occasionally been shown to induce negative effects *in vitro* and *in vivo*. For example, the expression of the neomycin resistance gene has been reported to hinder the expression of the gene of interest in mammalian cells [7]. Likewise, ampicillin gene expression has been shown to reduce transgene expression *in vivo* compared to kanamycin gene expression [8]. Moreover, unmethylated CpG sequences that are present in certain plasmids are recognised by the Toll-like receptor 9 (TLR9), which leads to an adjuvant effect through the activation of the innate immune system [9]. However, studies have shown that TLR9 or CpG are not necessarily essential for the induction of innate immunity after the injection of a DNA vaccine [10, 11]. In fact, CpG can play a role independently of TLR9 in primary B cells [12] and TANK-binding kinase-1 signalling pathways can be activated by dsDNA *in vitro* [13, 14] and *in vivo*

[15]. dsDNA can thus mediate adjuvanticity and, because it induces T-cell responses, dsDNA is essential for the immunogenicity of DNA vaccines [16].

Stimulation of the immune system may be beneficial and expected when plasmid DNA is intended for vaccination purposes, but not for gene therapy.

II.2 New generation of DNA vectors partially or totally devoid of prokaryotic elements

To minimise all the adverse effects described, plasmids partially or totally devoid of prokaryotic elements have been developed. Selection of bacteria transformed by the plasmids during the production process was initially based on antibiotic resistance and has now been replaced by antibiotic-free selection systems. In these selection systems, the prokaryotic origin of replication has been maintained. For example, selection mechanism can be based on an RNA/RNA antisens interaction involving the naturally occurring RNA I derived from the origin of replication of the plasmid [17] or on operator-repressor titration [18, 19]. The latter system has been implemented in a DNA vaccine [20], tested in many clinical trials and has been shown to be safe in a good laboratory practice toxicology study [21].

In addition, other vectors that are totally devoid of prokaryotic elements, such as plasmids or linear dumbbell-shaped expression cassettes, have also been developed. In 1997, Darquet *et al.* [22] developed a plasmid that does not contain any prokaryotic elements by producing supercoiled recombinant circular DNA molecules called minicircles. These molecules do not contain either the prokaryote origin of replication or antibiotic resistance genes, both having been eliminated during site-specific recombination. Following recombination, minicircles are purified from miniplasmids, which are circular DNA molecules containing prokaryotic elements, and from parental plasmids that may still be present. Various types of site-specific recombination techniques have been used to improve the yields of minicircles. The first type of site-specific recombination technique uses bacteriophage λ integrase to mediate recombination between the *attP* and *attB* sites. The yield of recombined plasmids is low, corresponding to roughly 60% of starting material [22, 23]. The second recombination technique uses Cre recombinase [24]. The yield in minicircles for a 7 kbp parental plasmid is roughly 200 µg/l culture, while 20 kbp parental plasmid yields only 40 µg/l culture. Purification of minicircles is done using caesium chloride density gradients. The density gradient separates minicircles from both miniplasmids and non-recombined

plasmids. However, undesirable dimeric, concatemeric and relaxed minicircles are frequently copurified along with the targeted supercoiled minicircles. The third technique utilises phage φ31 integrase to catalyse recombination and produce minicircles; the resulting product is then purified on commercially available affinity columns. This technique does not require either digestion with enzymes or purification on a caesium chloride gradient. The I-SceI site and the expression of I-SceI endonuclease gene by the plasmid linearises non-recombined plasmids and miniplasmids, which are then eliminated by exonucleases. The yield of minicircles is nine times higher than with the original protocol, or 1.8 mg/l culture with a purity of 97%. However, concatemers and miniplasmids are still present. A fourth technique was developed in 2008 by Mayrhofer *et al.* [25]. This technique is called recombination-based plasmid separation technology (RBPS technology). It consists of using ParA resolvase to mediate recombination and affinity chromatography based on protein-DNA interactions for purification. Using this technique, recombination efficiencies of 99.57% can be attained. Unlike other techniques, neither multimers nor concatemers are copurified and the final purified product contains 98.8% minicircles, 1% miniplasmids and 0.2% parental plasmids.

Although minicircle DNA molecules have low immunogenicity since most of the unmethylated CpG motifs have been eliminated, they may nonetheless be interesting for use in vaccines. The first advantage is their small size that may improve the dissemination of DNA and their entry into the nucleus. Moreover, Molnar *et al.* have shown that plasmid size has an inverse relationship with the level of transgene expression [26]. In the case of minicircles, it has been demonstrated that transfection and expression levels are higher compared to conventional plasmids [22, 23, 27, 28]. The second advantage is the absence of antibiotic resistance genes. The third advantage is the reported reduction in the risk of plasmid transfer to local bacteria owing to the absence of the prokaryotic origin of replication.

The other type of vector completely free of prokaryotic elements, linear dumbbell-shaped expression cassettes, is constructed using one of two methods described thus far: linear dsDNA molecules generated *in vitro* either by polymerase chain reaction (PCR) amplification or by endonuclease processing from plasmids. In the case of PCR amplification from plasmids, the PCR product is end-protected by ligation with hairpin oligodeoxynucleotides (ODN). Hirata *et al.* have demonstrated that, after electroporation, PCR fragments show better expression compared to the plasmids carrying the same gene [29]. Moreover, PCR fragments can induce protection against influenza A challenge in mice [30]. The endonuclease processing technique has been used by the Mologen AG pharmaceutical company, which has developed a minimalistic immunogenic defined gene expression vector (MIDGE). Plasmids,

which contain the expression cassette of interest, are digested by restriction endonuclease like *Eco3I* I [31], the ends ligated with hairpin ODN and DNA is purified by anionic exchange column chromatography. Hairpin ODNs can be used to bind molecules such as peptides, proteins or sugars to DNA. For example, nuclear localisation signal (NLS) peptides can be added to direct the DNA to the nucleus [32]. MIDGE vectors and MIDGE-NLS vectors successfully support expression *in vitro* and *in vivo* [31, 33, 34], to support immune responses *in vivo* [31-33], protecting cats or mice against challenges with feline immunodeficiency virus [35] or Leishmania major [34], respectively, and to enhance protective anti-tumor immunity [36].

III. The different routes of administration

Before describing the fate of plasmids after injection, it appears appropriate to recall briefly the different routes of administration thereof. There are two main routes of administration for DNA vaccines: intramuscular injection and intradermal injection. Subcutaneous injection, application to mucosal surfaces, intravenous and intranodal injections are other possible routes of administration, but they have been used much less frequently. Plasmid DNA can be directly injected in an aqueous solution — in which case it is called naked DNA — , complexed with liposomes [37] or packaged in viral [38, 39] or bacterial [40] vectors. In this review, we will focus on the routes of administration for delivering naked DNA.

The most widely employed delivery method is intramuscular injection. Naked DNA in saline is injected directly into skeletal muscle tissue using a hypodermic needle. It has been demonstrated that the induced immune response is primarily a T_H1 cell-mediated immune response [41]. From 95 to 99% of intramuscularly injected plasmids, which are found in the interfibrillar space, are degraded in the muscle tissue within 90 min post-administration [42]. To counter this low transfection rate, injection of naked DNA can be assisted by electroporation [43]. Electroporation consists in creating an electric field by applying an electric pulse between electrodes placed in the muscle. This technique increases the intensity of the humoral immune response, although the cell-mediated immune response remains predominant [41, 44, 45]. Another technique that increases the intensity of the humoral immune response by intramuscular administration is a high-pressure liquid injection using a Biojector^{TM} [46].

Naked plasmid DNA administered by intradermal injection is often injected with a hypodermic needle. However, other delivery methods are also used, such as the gene gun that propels DNA adsorbed on gold beads [47], Biojector^{TM} [48, 49], topical application of naked

DNA [50], or tattooing [51]. These different methods primarily induce humoral immunity characterised by a T_H2 response and a pronounced production of IgG1 antibodies [52-57]. However, intradermal plasmid DNA injection methods cannot be qualified as inducing a predominantly humoral immune response. Electroporation following intradermal injection of naked DNA and intradermal injection of MIDGE-NLS may induce a predominant cell-mediated immune response [33, 34, 58].

Other routes of administration involve subcutaneous injection and mucosal surface delivery. In the case of subcutaneous methods, naked DNA can be injected by a needle [59] or by high-pressure gas [60]. In the case of mucosal delivery, naked DNA can be deposited in the nasal [61] or buccal [61, 62] cavity or delivered by topical application on, for example, the eye [63] or vaginal mucosa [64]. Unlike intramuscular and cutaneous (intradermal and subcutaneous) administration, mucosal surface delivery induces systemic and mucosal immunity [61, 63].

IV. Fate of plasmid DNA after injection

IV.1.Plasmid DNA uptake mechanisms following intramuscular injection

Induction of an immune response after immunisation by naked plasmid DNA is possible if the gene of interest coded by the plasmid DNA is expressed in the nucleus of the host cell. Before the gene of interest can be expressed, injected plasmid DNA must overcome three obstacles.

The first obstacle involves crossing the plasma membrane. DNA is a polyanion and thus cannot interact with cell plasma membranes which are negatively charged. However, Wolff *et al.* indirectly showed that naked DNA can enter cells after injection in mouse muscle tissue [65]. Several studies have been conducted to explain this result. Studies on the role of disrupted plasma membranes or on transient membrane pores induced during injection have been inconclusive compared to experiments that have demonstrated active cellular mechanisms of DNA uptake by using low temperature conditions to inhibit energy production [66, 67]. There are two main active uptake mechanisms: fluid-phase endocytosis, i.e. not mediated by a receptor, and adsorptive endocytosis. DNA uptake is inhibited in excesses of non-coding DNA or dextran sulphate, illustrating the importance of the negative charge of DNA in the uptake mechanism [66, 68]. These results reject the hypothesis of uptake by fluid-phase endocytosis. A large number of membrane receptors may be involved in oligonucleotide capture by cells. However, none of them have been shown to be necessary and sufficient for DNA uptake. Moreover, given the concentration of membrane receptors on the cell surface, it is highly likely that plasmid DNA interacts with several receptors

simultaneously. Not all cell types take up plasmid DNA in the same way. This suggests that uptake mechanisms depend on certain sequences that may be specific to cell type [69, 70].

The second obstacle involves crossing the cytoplasm. The cytoplasm is made up of a network of microfilaments, microtubules and a variety of subcellular organelles that float in the cytosol. The cytoskeleton generates mechanical resistance in the cell and transports organelles and large complexes. The structure of the cytoskeleton, the presence of organelles and the high concentration of proteins limit the diffusion of large macromolecules, such as plasmid DNA [71]. Thus, it is thought that the cytoplasm hinders plasmid transport to the nucleus. However, plasmid DNA has a mechanism to circumvent this impediment. Studies using microinjection or electroporation delivery methods have shown that plasmid DNA associates with proteins that resemble dyneins whereby the microtubule network helps transport plasmid DNA to the nucleus [72, 73]. Plasmid DNA can also cross the cytoplasm another way. Studies have demonstrated that labelled plasmid DNA co-localises with endosomal markers [74, 75], confirming plasmid DNA uptake by endocytosis. When endosomes engulf a plasmid, plasmid DNA is protected from degradation for most of its journey to the nucleus. While little is known on how naked DNA is released from the endolysosome, studies with complexed DNA have shown that a few plasmid DNA molecules are released into the cytoplasm, while the rest are degraded [76]. To curtail its metabolic instability in the cytoplasm, the DNA molecule must be escorted up to the nuclear membrane. A study on the behaviour of different plasmids in different cells showed a half-life of about 90 min after micro-injection in the cytoplasm and only 0.1% of plasmid DNA that was micro-injected actually entered the nucleus. The characterisation of the mechanisms involved in this rapid elimination showed that DNA is digested by DNase enzymes in the cytoplasm [77].

The third obstacle involves crossing the nuclear membrane. There are three possible ways for plasmid DNA to enter the nucleus. DNA can be imported into the nucleus through nuclear pores by simple diffusion, be incorporated during disassembly of the nuclear membrane during mitosis or enter via nuclear pores by facilitated diffusion. The nuclear membrane possesses nuclear pore complexes (NPC) that play an important role in protein transport to and from the cytoplasm and the nucleoplasm. DNA fragments smaller than 250 bp are capable of diffusing passively into the nucleus via the NPCs while fragments longer than 250 bp cannot [78]. Several research groups have demonstrated the importance of nuclear membrane disassembly in the transfection efficiency for DNA larger than 250 bp. However, cellular division has a relatively weak effect on expression. There are three possible explanations for this weak effect. First, the plasmid may be degraded by the time mitosis occurs. Second,

plasmids may be excluded from re-forming nuclei. Third, plasmids may be differentially partitioned to each daughter cell [79]. Plasmid DNA may finally enter the nucleus in two ways: either by taking advantage of nuclear membrane disassembly in dividing cells or by crossing an intact membrane by facilitated diffusion via NPCs [79]. In the latter case, it has been suggested that DNA molecules are associated with polypeptides, such as transcription factors, that contain a nuclear localisation signal (NLS) [80]. Transcription factors not only have an NLS sequence that interacts with nuclear import receptors, but also possess amino acid sequences that recognise the promoter or enhancer sequences of the plasmid DNA. Dean *et al.* showed that a plasmid containing early enhancer-promoter SV40 sequences interact with transcription factors via a 72 bp early enhancer SV40 sequence, termed DNA nuclear targeting sequence (DTS), and that these transcription factors transfer the plasmid to the nucleus [81, 82]. Inclusion of DTS elements into minicircle DNA causes a 30-fold increase in efficiency in cells and a 6-fold increase in mouse lungs [27]. DTS elements can be used to enhance levels of gene transfer in a cell-specific manner in non-dividing cells, however, the presence of DTS elements does not influence levels of expression in some tissues [83].

IV.2.Cellular distribution of plasmid DNA after intramuscular injection

Striated muscle tissue is made up of muscle cells, called myocytes, satellite cells that induce the regeneration of muscle cells and connective tissue. Myocytes are large multinucleated cells (syncytia). They contain around a hundred nuclei that are located under the plasma membrane and their cytoplasm contains contractile proteins (myofilaments). Myocytes and satellite cells are enveloped in a basal lamina and a layer of connective tissue, the endomysium [84]. It has been demonstrated that 5 minutes after intramuscular injection with rhodamine-labelled DNA, the DNA was localised in and between myocytes [85]. Myocytes containing the plasmid DNA express the gene of interest [85].

The connective tissue spans three levels of organisation and includes the epimysium that lines the whole muscle, the perimysium that forms a sheath around bundles of myocytes and the endomysium that surrounds each myocyte [86]. Several research groups have shown that macrophages and dendritic cells are always present at all three organisational levels of connective tissue [87-89]. Plasmid DNA that has been administrated intramuscularly and traced with DNA labels or with green fluorescent protein (GFP) expression coded by the plasmid DNA is not only found in myocytes, but also in antigen-presenting cells, such as macrophages and B cells [75, 85, 90, 91]. However, no plasmid DNA-coded proteins have

been reported in dendritic cells, although transplantation experiments in mice suggest that the transfer of antigens from muscle cells to the major histocompatibility complex (MHC) class I molecules of antigen-presenting cells — and therefore also dendritic cells — can occur [92]. Myeloid $CD11c^+$ (dendritic) cells that have been transfected during intramuscular immunisation appear to make up 2% of all myeloid cells present in mouse muscle [75].

IV.3.Tissue distribution of plasmid DNA after intramuscular injection

Differences in distribution and persistence of a plasmid can be attributed to several factors such as the route of administration, the number of injections, the quantity of plasmid DNA and the animal species under study [93]. Given the variability of these factors, it is difficult to compare the results from different studies in detail. However, it is possible to draw some general conclusions. Whatever the route of administration, the site of injection is the area where the plasmid remains the most concentrated and persists the longest. However, all organs under observation have shown evidence of plasmid DNA as of the first minutes following injection, but with lower quantities of plasmids and for shorter periods [46, 93-104].

In particular, it has been shown that more than 90% of injected plasmids were found in mouse thigh muscles 5 minutes after intramuscular injection of less than 20 µg of plasmid DNA coding for the hAAT protein and that, throughout the duration of the experiment, less than 10% of plasmid DNA was present in the observed organs at any given point in time [68]. Persistence of the plasmid at the point of injection varies with the animal model and the identity and amount of plasmid DNA injected. However, in most studies, the determination of plasmid DNA persistence times has been limited by the duration of the study. It has been shown that plasmid DNA persists more than 2 years in mice [105], at least 28 days in rats [94], 54 days in sheep [100], 70 days in goldfish [106], 90 days in rainbow trout (Kurath, 2006 and [103]), 10 weeks in turkeys [93] and in one out of three pigs after 4 weeks [101]. Plasmid concentrations are maximal after several minutes, for example 12 min in pigs [101], then decrease to trace amounts after several hours. In fact, more than 98% of plasmids are eliminated after 9 h at the injection site in mice [68]. After several minutes, plasmid DNA is also present at low quantities in other organs for short periods of time. The organs tested after intramuscular injection include all vascularised organs [97], but most studies test for plasmid presence in blood, liver, spleen, kidneys, lungs, draining lymph nodes, the opposite muscle and gonads. Blood-brain barriers and blood-gonad barriers do not seem to act on plasmid

DNA since gonads and the brain show evidence of plasmid DNA in several studies after intramuscular injection [96, 97, 107-109]. The mechanism involved in the dissemination of plasmid DNA throughout the body has not been completely determined. However, the presence of plasmids in immune cells [85, 91, 95], in lymph [85, 96, 100, 110] and blood [95] may contribute to their dissemination.

Plasmid DNA is generally detected by PCR, Southern blot or by quantitative PCR. These methods do not differentiate functional plasmid DNA, which expresses the protein of interest, from degraded plasmids. One study showed that it was possible to rescue plasmid DNA up to 6 months post-injection via intramuscular administration of 100 µg of plasmids in mice [111]. In rainbow trout, DNA is detected for at least 90 days in muscle tissue, whereas the expression of the protein of interest is greatest at 14 days in muscle, kidneys and thymus glands and can no longer be detected after 28 days [103]. However, studies in mice and turkeys have shown that the plasmid expresses the protein of interest in muscle tissue as long as it persists [93, 105]. Antigen expression does not only depend on the state of the plasmid, but also on the animal model employed, the identity of the antigen, the viral promoter, and the delivery method [93, 112-114].

IV.4.Clearance of plasmid DNA after injection

After intramuscular injection, plasmid DNA is either transfected into cells or it remains free in the body. According to the route it takes in the host, plasmid DNA will be eliminated differently. It is difficult to differentiate between the fate of transfected plasmid DNA and the fate of free plasmid DNA. Antigen clearance can be studied to determine the fate of transfected plasmids and the fate of free plasmid DNA can be determined by studying the general circulation.

Little is known about the host factors that limit long-term plasmid DNA expression. Elimination of myocytes expressing the antigen appears to depend on the immunogenicity of the antigen and the presence of a functional immune system [115, 116]. One study showed that the innate immune system, mediated by macrophages and natural killer (NK) cells may be involved in the elimination of antigen expression [117]. Payette *et al.* showed that the decrease in HB antigen expression after intramuscular injection is mediated by mechanisms that induce the major histocompatibility complex II (MHC II) and antibodies [118]. However, because it induces a high humoral immune response, the HB antigen model is not appropriate for studying the role of cell-mediated immune responses during antigen expression. The role

of cell-mediated immune responses in antigen clearance has been clarified by Letvin *et al.* [115, 119]. After establishing a correlation between the decrease in antigen expression and cell-mediated immune response [115], they determined that $CD4^+$ cells, but not NK cells, macrophages or $CD8^+$ cells, are largely responsible for this decrease via the Fas/FasL mechanism and via their MHC II molecules. Plasmid DNA may then be degraded by apoptosis or, in the case of necrotic cells, by DNases present in the serum such as, for example, murine DNAse1I3 that is secreted by macrophages [120, 121].

Due to variability in quantities of plasmid isomers, variability in plasmid size and variability in the animal models used, the different studies cannot be directly compared in detail. However, the clearance of free plasmid DNA may be induced by different factors. The presence of endonucleases in mouse muscle mediated the degradation of 95 to 99% of naked plasmid DNA within 90 min of intramuscular injection, which most likely occurred in the extracellular space [42]. After intramuscular injection in pigs, plasmid DNA bioavailability — i.e. the quantity of plasmid DNA that reaches the general blood circulation — is low, around 10% [101]. The highest concentrations of plasmid DNA are detected in the blood around 15 min post-injection and they rapidly decrease thereafter [101, 122]. Blood circulation appears to play a role in the dissemination of plasmid DNA to tissues other than muscle. Moreover, one study showed that higher quantities of plasmid DNA are found in blood than in organs [96]. Some plasmids in the bloodstream may be degraded by nucleases present in blood. The degradation rate of supercoiled plasmids found in mouse blood, after intramuscular injection, is 20.9% after 10 min, 34% after 1 h, 86.8% after 1 day and 97.8% after 1 week [96]. Moreover, studies showed that free plasmid DNA present in the blood may be internalised and degraded by liver non-parenchymal cells after intravenous injection [123, 124]. Some plasmid DNA, intramuscularly injected in mice, is eliminated in urine and faeces. Urine collected 10 minutes post-injection already showed a strong signal that continued to increase up to the end of the 1 week study. At 10 minutes post-injection, faeces only showed a weak signal, while after 1 h, the signal became much stronger [96]. However, no studies to detect the presence of encoded proteins in urine and in faeces have been conducted to date.

IV.5. Potential risk of plasmid DNA genomic integration

The main safety concern for DNA vaccination is that partial or complete plasmid genome integration could result in insertional mutagenesis and possibly in the activation of oncogenes or inactivation of tumor suppressor genes [125]. Few studies have been undertaken on this subject until recently and most have concluded that the rate of plasmid genomic integration is

negligible [46, 108, 126-130], because plasmid integration could not be unambiguously detected. Furthermore, the strategies used in these studies could not clearly distinguish between integrated plasmids and non-integrated residual genome-associated plasmids. Thus, the plasmid sequences detected in these studies were assumed to be host genome-integrated copies; based on this assumption, integration appears to occur at rates that are at least three orders of magnitude below the spontaneous mutation rate in mammalian genomes [131]. To confirm plasmid integration, Wang *et al.* [132] designed an elegant and efficient method to detect and identify insertion sites after plasmid delivery. To detect rare integration events, their approach is based on PCR amplification of a sequence found between a repeated genome sequence and a known plasmid sequence. Furthermore, to increase the probability of potential integration, they used the more potent electroporation delivery technology. They thereby effectively detected four independent plasmid integration events in the mouse genome and this frequency of events is lower than the rate of spontaneous mutation in mammalian genomes [131].

VI. Conclusion:

DNA vaccination is an elegant and simple concept. Many efforts continue to be made to better understand the mechanisms behind DNA vaccination and to find new strategies to improve vaccination efficiency. Progress in this field has resulted in three veterinary vaccines that have been authorised for use. However, biosafety of DNA vaccination remains a major issue. Concerns specific to the DNA vaccination concept stem from the use of injected DNA molecules, which can potentially integrate the host genome. Host gene expression can be affected if integration occurs in host genes or if genes of prokaryotic origin, such as those that confer resistance to antibiotics, integrate into the host genome. One way to limit these risks is to develop and use plasmids or other DNA molecules that lack the genes for antibiotic resistance and the prokaryotic elements needed for their replication in bacteria. As described above, these kinds of vectors already exist and their vaccine efficacies are currently being tested in several large animal models.

There is a pressing need to understand how integration events occur. For instance, integration events may depend on the presence of specific nucleic acid sequences or on the concentration of plasmids in a specific tissue type or near the host genome. To date, only one study showing

random integration events has been published [132]. Understanding the mechanisms involved in integration would be useful for developing strategies to limit these integration events. Another goal in the future is to develop a technique that can detect, or at least increase the probability of detecting, integration events, which are described as rare. The above-described method (see IV.5) for detecting actual integration events has limitations. In fact, under these technical conditions, the absence of amplification is not sufficient proof that integration has not occurred. The drawback of this PCR technique that detects integration events is that the distance between the plasmid sequences targeted by the primers is high. Therefore, if the integrated plasmid fragment does not contain one of the primer sequences, it will not be detected. One way to enhance the probability of detecting integration events is to reduce the distance between the plasmid-targeted primers by using new techniques such as high-throughput sequencing with primers targeting sequences distributed throughout the plasmid. The Food and Drug Administration recommends evaluating whether the DNA plasmid has integrated the genome of the vaccinated animals when the plasmids persist at levels exceeding 30,000 copies per µg of host DNA [133]. The European Medicines Agency (EMA) recommends using the most sensitive available methods for this purpose [134]. The development of new and more efficient methods to assess plasmid integration into host genomes strives to satisfy these recommendations.

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