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

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40 **Abstract**

41

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

51

## 52 **I. From discovery to industrial application**

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

63

64

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

### 66 **II.1 First-generation of plasmids bearing prokaryotic elements**

67

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

86 [15]. dsDNA can thus mediate adjuvanticity and, because it induces T-cell responses, dsDNA  
87 is essential for the immunogenicity of DNA vaccines [16].  
88 Stimulation of the immune system may be beneficial and expected when plasmid DNA is  
89 intended for vaccination purposes, but not for gene therapy.

90  
91

## 92 **II.2 New generation of DNA vectors partially or totally devoid of prokaryotic** 93 **elements**

94  
95 To minimise all the adverse effects described, plasmids partially or totally devoid of  
96 prokaryotic elements have been developed. Selection of bacteria transformed by the plasmids  
97 during the production process was initially based on antibiotic resistance and has now been  
98 replaced by antibiotic-free selection systems. In these selection systems, the prokaryotic  
99 origin of replication has been maintained. For example, selection mechanism can be based on  
100 an RNA/RNA antisense interaction involving the naturally occurring RNA I derived from the  
101 origin of replication of the plasmid [17] or on operator-repressor titration [18, 19]. The latter  
102 system has been implemented in a DNA vaccine [20], tested in many clinical trials and has  
103 been shown to be safe in a good laboratory practice toxicology study [21].

104 In addition, other vectors that are totally devoid of prokaryotic elements, such as  
105 plasmids or linear dumbbell-shaped expression cassettes, have also been developed. In 1997,  
106 Darquet *et al.* [22] developed a plasmid that does not contain any prokaryotic elements by  
107 producing supercoiled recombinant circular DNA molecules called minicircles. These  
108 molecules do not contain either the prokaryote origin of replication or antibiotic resistance  
109 genes, both having been eliminated during site-specific recombination. Following  
110 recombination, minicircles are purified from miniplasmids, which are circular DNA  
111 molecules containing prokaryotic elements, and from parental plasmids that may still be  
112 present. Various types of site-specific recombination techniques have been used to improve  
113 the yields of minicircles. The first type of site-specific recombination technique uses  
114 bacteriophage  $\lambda$  integrase to mediate recombination between the *attP* and *attB* sites. The yield  
115 of recombined plasmids is low, corresponding to roughly 60% of starting material [22, 23].  
116 The second recombination technique uses Cre recombinase [24]. The yield in minicircles for a  
117 7 kbp parental plasmid is roughly 200  $\mu\text{g/l}$  culture, while 20 kbp parental plasmid yields only  
118 40  $\mu\text{g/l}$  culture. Purification of minicircles is done using caesium chloride density gradients.  
119 The density gradient separates minicircles from both miniplasmids and non-recombined

120 plasmids. However, undesirable dimeric, concatemeric and relaxed minicircles are frequently  
121 copurified along with the targeted supercoiled minicircles. The third technique utilises phage  
122  $\phi$ 31 integrase to catalyse recombination and produce minicircles; the resulting product is then  
123 purified on commercially available affinity columns. This technique does not require either  
124 digestion with enzymes or purification on a caesium chloride gradient. The I-SceI site and the  
125 expression of I-SceI endonuclease gene by the plasmid linearises non-recombined plasmids  
126 and miniplasmids, which are then eliminated by exonucleases. The yield of minicircles is nine  
127 times higher than with the original protocol, or 1.8 mg/l culture with a purity of 97%.  
128 However, concatemers and miniplasmids are still present. A fourth technique was developed  
129 in 2008 by Mayrhofer *et al.* [25]. This technique is called recombination-based plasmid  
130 separation technology (RBPS technology). It consists of using ParA resolvase to mediate  
131 recombination and affinity chromatography based on protein-DNA interactions for  
132 purification. Using this technique, recombination efficiencies of 99.57% can be attained.  
133 Unlike other techniques, neither multimers nor concatemers are copurified and the final  
134 purified product contains 98.8% minicircles, 1% miniplasmids and 0.2% parental plasmids.  
135 Although minicircle DNA molecules have low immunogenicity since most of the  
136 unmethylated CpG motifs have been eliminated, they may nonetheless be interesting for use  
137 in vaccines. The first advantage is their small size that may improve the dissemination of  
138 DNA and their entry into the nucleus. Moreover, Molnar *et al.* have shown that plasmid size  
139 has an inverse relationship with the level of transgene expression [26]. In the case of  
140 minicircles, it has been demonstrated that transfection and expression levels are higher  
141 compared to conventional plasmids [22, 23, 27, 28]. The second advantage is the absence of  
142 antibiotic resistance genes. The third advantage is the reported reduction in the risk of plasmid  
143 transfer to local bacteria owing to the absence of the prokaryotic origin of replication.  
144 The other type of vector completely free of prokaryotic elements, linear dumbbell-shaped  
145 expression cassettes, is constructed using one of two methods described thus far: linear  
146 dsDNA molecules generated *in vitro* either by polymerase chain reaction (PCR) amplification  
147 or by endonuclease processing from plasmids. In the case of PCR amplification from  
148 plasmids, the PCR product is end-protected by ligation with hairpin oligodeoxynucleotides  
149 (ODN). Hirata *et al.* have demonstrated that, after electroporation, PCR fragments show better  
150 expression compared to the plasmids carrying the same gene [29]. Moreover, PCR fragments  
151 can induce protection against influenza A challenge in mice [30]. The endonuclease  
152 processing technique has been used by the Mologen AG pharmaceutical company, which has  
153 developed a minimalistic immunogenic defined gene expression vector (MIDGE). Plasmids,

154 which contain the expression cassette of interest, are digested by restriction endonuclease like  
155 *Eco3I* I [31], the ends ligated with hairpin ODN and DNA is purified by anionic exchange  
156 column chromatography. Hairpin ODNs can be used to bind molecules such as peptides,  
157 proteins or sugars to DNA. For example, nuclear localisation signal (NLS) peptides can be  
158 added to direct the DNA to the nucleus [32]. MIDGE vectors and MIDGE-NLS vectors  
159 successfully support expression *in vitro* and *in vivo* [31, 33, 34], to support immune responses  
160 *in vivo* [31-33], protecting cats or mice against challenges with feline immunodeficiency virus  
161 [35] or *Leishmania major* [34], respectively, and to enhance protective anti-tumor immunity  
162 [36].

163

### 164 **III. The different routes of administration**

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

173 The most widely employed delivery method is intramuscular injection. Naked DNA in saline  
174 is injected directly into skeletal muscle tissue using a hypodermic needle. It has been  
175 demonstrated that the induced immune response is primarily a  $T_{H1}$  cell-mediated immune  
176 response [41]. From 95 to 99% of intramuscularly injected plasmids, which are found in the  
177 interfibrillar space, are degraded in the muscle tissue within 90 min post-administration [42].  
178 To counter this low transfection rate, injection of naked DNA can be assisted by  
179 electroporation [43]. Electroporation consists in creating an electric field by applying an  
180 electric pulse between electrodes placed in the muscle. This technique increases the intensity  
181 of the humoral immune response, although the cell-mediated immune response remains  
182 predominant [41, 44, 45]. Another technique that increases the intensity of the humoral  
183 immune response by intramuscular administration is a high-pressure liquid injection using a  
184 Biojector<sup>TM</sup> [46].

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

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

194 Other routes of administration involve subcutaneous injection and mucosal surface delivery.  
195 In the case of subcutaneous methods, naked DNA can be injected by a needle [59] or by high-  
196 pressure gas [60]. In the case of mucosal delivery, naked DNA can be deposited in the nasal  
197 [61] or buccal [61, 62] cavity or delivered by topical application on, for example, the eye [63]  
198 or vaginal mucosa [64]. Unlike intramuscular and cutaneous (intradermal and subcutaneous)  
199 administration, mucosal surface delivery induces systemic and mucosal immunity [61, 63].

200

#### 201 **IV. Fate of plasmid DNA after injection**

##### 202 **IV.1. Plasmid DNA uptake mechanisms following intramuscular injection**

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

206 The first obstacle involves crossing the plasma membrane. DNA is a polyanion and thus  
207 cannot interact with cell plasma membranes which are negatively charged. However, Wolff *et*  
208 *al.* indirectly showed that naked DNA can enter cells after injection in mouse muscle tissue  
209 [65]. Several studies have been conducted to explain this result. Studies on the role of  
210 disrupted plasma membranes or on transient membrane pores induced during injection have  
211 been inconclusive compared to experiments that have demonstrated active cellular  
212 mechanisms of DNA uptake by using low temperature conditions to inhibit energy production  
213 [66, 67]. There are two main active uptake mechanisms: fluid-phase endocytosis, i.e. not  
214 mediated by a receptor, and adsorptive endocytosis. DNA uptake is inhibited in excesses of  
215 non-coding DNA or dextran sulphate, illustrating the importance of the negative charge of  
216 DNA in the uptake mechanism [66, 68]. These results reject the hypothesis of uptake by fluid-  
217 phase endocytosis. A large number of membrane receptors may be involved in  
218 oligonucleotide capture by cells. However, none of them have been shown to be necessary  
219 and sufficient for DNA uptake. Moreover, given the concentration of membrane receptors on  
220 the cell surface, it is highly likely that plasmid DNA interacts with several receptors



221 simultaneously. Not all cell types take up plasmid DNA in the same way. This suggests that  
222 uptake mechanisms depend on certain sequences that may be specific to cell type [69, 70].  
223 The second obstacle involves crossing the cytoplasm. The cytoplasm is made up of a network  
224 of microfilaments, microtubules and a variety of subcellular organelles that float in the  
225 cytosol. The cytoskeleton generates mechanical resistance in the cell and transports organelles  
226 and large complexes. The structure of the cytoskeleton, the presence of organelles and the  
227 high concentration of proteins limit the diffusion of large macromolecules, such as plasmid  
228 DNA [71]. Thus, it is thought that the cytoplasm hinders plasmid transport to the nucleus.  
229 However, plasmid DNA has a mechanism to circumvent this impediment. Studies using  
230 microinjection or electroporation delivery methods have shown that plasmid DNA associates  
231 with proteins that resemble dyneins whereby the microtubule network helps transport plasmid  
232 DNA to the nucleus [72, 73]. Plasmid DNA can also cross the cytoplasm another way. Studies  
233 have demonstrated that labelled plasmid DNA co-localises with endosomal markers [74, 75],  
234 confirming plasmid DNA uptake by endocytosis. When endosomes engulf a plasmid, plasmid  
235 DNA is protected from degradation for most of its journey to the nucleus. While little is  
236 known on how naked DNA is released from the endolysosome, studies with complexed DNA  
237 have shown that a few plasmid DNA molecules are released into the cytoplasm, while the rest  
238 are degraded [76]. To curtail its metabolic instability in the cytoplasm, the DNA molecule  
239 must be escorted up to the nuclear membrane. A study on the behaviour of different plasmids  
240 in different cells showed a half-life of about 90 min after micro-injection in the cytoplasm and  
241 only 0.1% of plasmid DNA that was micro-injected actually entered the nucleus. The  
242 characterisation of the mechanisms involved in this rapid elimination showed that DNA is  
243 digested by DNase enzymes in the cytoplasm [77].

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

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

270

271

#### 272 **IV.2. Cellular distribution of plasmid DNA after intramuscular injection**

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

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

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

294

295

### 296 **IV.3. Tissue distribution of plasmid DNA after intramuscular injection**

297 Differences in distribution and persistence of a plasmid can be attributed to several factors  
298 such as the route of administration, the number of injections, the quantity of plasmid DNA  
299 and the animal species under study [93]. Given the variability of these factors, it is difficult to  
300 compare the results from different studies in detail. However, it is possible to draw some  
301 general conclusions. Whatever the route of administration, the site of injection is the area  
302 where the plasmid remains the most concentrated and persists the longest. However, all  
303 organs under observation have shown evidence of plasmid DNA as of the first minutes  
304 following injection, but with lower quantities of plasmids and for shorter periods [46, 93-  
305 104].

306 In particular, it has been shown that more than 90% of injected plasmids were found in mouse  
307 thigh muscles 5 minutes after intramuscular injection of less than 20 µg of plasmid DNA  
308 coding for the hAAT protein and that, throughout the duration of the experiment, less than  
309 10% of plasmid DNA was present in the observed organs at any given point in time [68].  
310 Persistence of the plasmid at the point of injection varies with the animal model and the  
311 identity and amount of plasmid DNA injected. However, in most studies, the determination of  
312 plasmid DNA persistence times has been limited by the duration of the study. It has been  
313 shown that plasmid DNA persists more than 2 years in mice [105], at least 28 days in rats  
314 [94], 54 days in sheep [100], 70 days in goldfish [106], 90 days in rainbow trout (Kurath,  
315 2006 and [103]), 10 weeks in turkeys [93] and in one out of three pigs after 4 weeks [101].  
316 Plasmid concentrations are maximal after several minutes, for example 12 min in pigs [101],  
317 then decrease to trace amounts after several hours. In fact, more than 98% of plasmids are  
318 eliminated after 9 h at the injection site in mice [68]. After several minutes, plasmid DNA is  
319 also present at low quantities in other organs for short periods of time. The organs tested after  
320 intramuscular injection include all vascularised organs [97], but most studies test for plasmid  
321 presence in blood, liver, spleen, kidneys, lungs, draining lymph nodes, the opposite muscle  
322 and gonads. Blood-brain barriers and blood-gonad barriers do not seem to act on plasmid

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

328 Plasmid DNA is generally detected by PCR, Southern blot or by quantitative PCR. These  
329 methods do not differentiate functional plasmid DNA, which expresses the protein of interest,  
330 from degraded plasmids. One study showed that it was possible to rescue plasmid DNA up to  
331 6 months post-injection via intramuscular administration of 100 µg of plasmids in mice [111].  
332 In rainbow trout, DNA is detected for at least 90 days in muscle tissue, whereas the  
333 expression of the protein of interest is greatest at 14 days in muscle, kidneys and thymus  
334 glands and can no longer be detected after 28 days [103]. However, studies in mice and  
335 turkeys have shown that the plasmid expresses the protein of interest in muscle tissue as long  
336 as it persists [93, 105]. Antigen expression does not only depend on the state of the plasmid,  
337 but also on the animal model employed, the identity of the antigen, the viral promoter, and the  
338 delivery method [93, 112-114].

339  
340

#### 341 **IV.4. Clearance of plasmid DNA after injection**

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

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

357 of cell-mediated immune responses in antigen clearance has been clarified by Letvin *et al.*  
358 [115, 119]. After establishing a correlation between the decrease in antigen expression and  
359 cell-mediated immune response [115], they determined that CD4<sup>+</sup> cells, but not NK cells,  
360 macrophages or CD8<sup>+</sup> cells, are largely responsible for this decrease via the Fas/FasL  
361 mechanism and via their MHC II molecules. Plasmid DNA may then be degraded by  
362 apoptosis or, in the case of necrotic cells, by DNases present in the serum such as, for  
363 example, murine DNase1I3 that is secreted by macrophages [120, 121].

364 Due to variability in quantities of plasmid isomers, variability in plasmid size and variability  
365 in the animal models used, the different studies cannot be directly compared in detail.  
366 However, the clearance of free plasmid DNA may be induced by different factors. The  
367 presence of endonucleases in mouse muscle mediated the degradation of 95 to 99% of naked  
368 plasmid DNA within 90 min of intramuscular injection, which most likely occurred in the  
369 extracellular space [42]. After intramuscular injection in pigs, plasmid DNA bioavailability —  
370 i.e. the quantity of plasmid DNA that reaches the general blood circulation — is low, around  
371 10% [101]. The highest concentrations of plasmid DNA are detected in the blood around 15  
372 min post-injection and they rapidly decrease thereafter [101, 122]. Blood circulation appears  
373 to play a role in the dissemination of plasmid DNA to tissues other than muscle. Moreover,  
374 one study showed that higher quantities of plasmid DNA are found in blood than in organs  
375 [96]. Some plasmids in the bloodstream may be degraded by nucleases present in blood. The  
376 degradation rate of supercoiled plasmids found in mouse blood, after intramuscular injection,  
377 is 20.9% after 10 min, 34% after 1 h, 86.8% after 1 day and 97.8% after 1 week [96].  
378 Moreover, studies showed that free plasmid DNA present in the blood may be internalised  
379 and degraded by liver non-parenchymal cells after intravenous injection [123, 124]. Some  
380 plasmid DNA, intramuscularly injected in mice, is eliminated in urine and faeces. Urine  
381 collected 10 minutes post-injection already showed a strong signal that continued to increase  
382 up to the end of the 1 week study. At 10 minutes post-injection, faeces only showed a weak  
383 signal, while after 1 h, the signal became much stronger [96]. However, no studies to detect  
384 the presence of encoded proteins in urine and in faeces have been conducted to date.

385

#### 386 **IV.5. Potential risk of plasmid DNA genomic integration**

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

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

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## 409 **VI. Conclusion:**

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

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

425 random integration events has been published [132]. Understanding the mechanisms involved  
426 in integration would be useful for developing strategies to limit these integration events.  
427 Another goal in the future is to develop a technique that can detect, or at least increase the  
428 probability of detecting, integration events, which are described as rare. The above-described  
429 method (see IV.5) for detecting actual integration events has limitations. In fact, under these  
430 technical conditions, the absence of amplification is not sufficient proof that integration has  
431 not occurred. The drawback of this PCR technique that detects integration events is that the  
432 distance between the plasmid sequences targeted by the primers is high. Therefore, if the  
433 integrated plasmid fragment does not contain one of the primer sequences, it will not be  
434 detected. One way to enhance the probability of detecting integration events is to reduce the  
435 distance between the plasmid-targeted primers by using new techniques such as high-  
436 throughput sequencing with primers targeting sequences distributed throughout the plasmid.  
437 The Food and Drug Administration recommends evaluating whether the DNA plasmid has  
438 integrated the genome of the vaccinated animals when the plasmids persist at levels exceeding  
439 30,000 copies per  $\mu\text{g}$  of host DNA [133]. The European Medicines Agency (EMA)  
440 recommends using the most sensitive available methods for this purpose [134]. The  
441 development of new and more efficient methods to assess plasmid integration into host  
442 genomes strives to satisfy these recommendations.

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## 448 **References**

449

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