

# Plasmid DNA Vaccines: Tissue Distribution and Effects of DNA Sequence, Adjuvants and Delivery Method on Integration into Host DNA

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## Abstract

A variety of factors could affect the frequency of integration of plasmid DNA vaccines into host cellular DNA, including DNA sequences within the plasmid, the expressed gene product (antigen), the formulation, delivery method, route of administration, and the type of cells exposed to the plasmid. In this report, we examined the tissue distribution and potential integration of plasmid DNA vaccines following intramuscular administration in mice and guinea pigs. We compared needle versus Biojector (needleless jet) delivery, examined the effect of aluminum phosphate adjuvants, compared the results of different plasmid DNA vaccines, and tested a gene (the human papilloma virus E7 gene) whose protein product is known to increase integration frequency in vitro. Six weeks following intramuscular injection, the vast majority of the plasmid was detected in the muscle and skin near the injection site; lower levels of plasmid were also detected in the draining lymph nodes. At early time points (1–7 days) after injection, a low level of systemic

exposure could be detected. Occasionally, plasmid was detected in gonads, but it dissipated rapidly and was extrachromosomal – indicating a low risk of germline transmission. Aluminum phosphate adjuvant had no effect on the tissue distribution and did not result in a detectable increase in integration frequency. Biojector delivery, compared with needle injection, greatly increased the uptake of plasmid (particularly in skin at the injection site), but did not result in a detectable increase in integration frequency. Finally, injection of a plasmid DNA vaccine containing the human papilloma virus type 16 E7 gene, known to increase integration in vitro, did not result in detectable integration in mice. These results suggest that the risk of integration following intramuscular injection of plasmid DNA is low under a variety of experimental conditions.

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## Introduction

The primary safety concern for DNA vaccines is their potential to integrate into host cellular DNA [1–5]. We developed a sensitive assay for the detection of integration in vivo, which involves separation of high-molecular-

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weight (HMW) genomic DNA from extrachromosomal plasmid by gel electrophoresis, followed by detection of potentially integrated plasmid in the gel-purified genomic DNA by polymerase chain reaction (PCR). In the accompanying manuscript [6], we described the assay in detail and demonstrated that intramuscular injection of three different plasmid DNA vaccines in mice did not result in a significant level of detectable integration in the injected muscle.

When considering the risk of integration, the tissue distribution of the plasmid should be examined, since the type of cells exposed to the plasmid could affect the potential for integration. For example, the potential for integration should be greater in cells that are actively proliferating. In addition, exposure of the gonads to the plasmid, and potential integration into germline DNA, should be examined to determine the risk of germline transmission.

A variety of other factors could potentially affect the frequency of integration into cellular DNA. First, the DNA sequence of the plasmid could affect integration. It is known that short segments of DNA, sometimes of unpredictable sequence, can affect rates of integration or recombination. Such sequences include the VDJ recombination signal sequence and related sequences [7, 8], *chi*-like elements and minisatellites [9–13], *Alu* segments [14–16], a recombinase recognition signal found in hepatitis B and mammalian genomes [17], and topoisomerase II recognition sites [17, 18]. Second, the protein product (antigen) encoded by the plasmid DNA vaccine could conceivably affect integration. For example, expression of human papilloma virus (HPV) type 16 E6 and E7 proteins increase rates of plasmid integration *in vitro* by 3- to 30-fold, presumably by interacting with cell-cycle-regulatory proteins [19]. Third, the formulation use of adjuvants, delivery method, and route of administration could each affect either the cellular uptake of plasmid or the type of cells exposed to plasmid, and thus could potentially affect the integration frequency.

In the present study, using several different plasmid DNA vaccines for influenza and HIV genes, we examined the tissue distribution of plasmid DNA vaccines following intramuscular administration to mice or guinea pigs. In addition, we investigated the effect of aluminum phosphate adjuvant on tissue distribution and integration frequency, compared needle versus Biojector (needleless jet) delivery, and tested a gene (HPV16 E7) whose product is known to increase integration frequency *in vitro*.

## Methods

### Plasmids

Plasmid constructs were made in variations of the V1Jn vector [20, 21]. In V1Jns, a 13-bp linker containing an *Sfi*I restriction site was inserted into a *Kpn*I site within the bovine growth hormone (BGH) terminator (the polyadenylation signal was not affected). In V1Jp, a 34-bp linker containing two *Sfi*I sites (separated by a *Pac*I site) was inserted in the same location.

Six different plasmid DNA vaccines were tested: VIJp-HA, a 6,614-bp plasmid containing the hemagglutinin (HA) gene of influenza A/Georgia; V1Jp-M1, a 5,674-bp plasmid containing the matrix (M1) gene of influenza A/Beijing; V1Jns-tPA-gag, a 6,375-bp plasmid containing the HIV gag gene with a 5'-secretory signal from tissue plasminogen activator; V1Jns-GAG2, a second plasmid construct containing the HIV gag gene; V1Jns-HPV16-E7wt, a 5,162-bp plasmid containing the wild-type HPV16 E7 gene, and V1Jns-HPV16-E7mutant, a 5,162-bp plasmid containing a mutant, inactivated E7 gene. Plasmids were purified and characterized as described previously [6], with the exception that the E7 plasmids were purified by CsCl banding rather than by high-pressure liquid chromatography.

### Animal Treatment and Tissue Collection

Balb/c mice were injected in *both* quadriceps with 50  $\mu$ l of either plasmid or vehicle (saline or phosphate-buffered saline). The doses of plasmid (per injection) used in mice were 100  $\mu$ g per quadriceps for the V1Jp-HA and V1Jp-M1 studies, 150  $\mu$ g per quadriceps for the V1Jns-HPV16-E7 plasmids, 160  $\mu$ g per quadriceps for V1Jns-tPA-gag alone and 135  $\mu$ g per quadriceps for V1Jns-tPA-gag mixed with 700  $\mu$ g/ml aluminum (as aluminum phosphate). For short-term tissue distribution studies (1–7 days' duration), the mice received a single dose of plasmid and were necropsied at the time point indicated in Results. For longer-term studies, mice received either a single injection or two injections (spaced 3 weeks apart) and were necropsied 6 weeks after the last injection, as indicated in Results.

Biojector delivery studies, including a comparison with needle injection, were carried out in guinea pigs. The Biojector is a needleless injection device that utilizes pressurized CO<sub>2</sub> to propel a narrow stream of liquid through the skin during injection. Both the needle and Biojector injections were target for intramuscular delivery to the hamstrings, using dye studies to optimize Biojector conditions. A #2-tip was used for the Biojector, which was held perpendicular to the muscle during injection. The guinea pigs were injected in *both* hamstrings with 200  $\mu$ l of either plasmid or vehicle (saline). The doses of plasmid (per injection) used in guinea pigs were 1.1 mg per hamstrings for V1Jns-tPA-gag, and approximately 1.4 mg per hamstrings for V1Jns-GAG2. Guinea pigs were necropsied 6 weeks after injection.

At necropsy, animals were anesthetized using isoflurane and bled via the vena cava into EDTA-containing tubes. The blood was stored at  $-70^{\circ}$ . To assess the tissue distribution of the plasmid, multiple tissues were usually removed, including blood, brain, thymus, heart, lung, liver, spleen, mesenteric lymph nodes, kidneys, ovaries or testes, inguinal nodes, skin at the injection site, and the injected muscle (quadriceps in mice, hamstrings in guinea pigs). The muscle was removed last to avoid contamination of other tissues. For tissue distribution studies within the 1st week after injection, the injection site tissues (muscle, skin, inguinal node) were not taken in order to avoid contamination of distal sites. Tissues were rinsed in phosphate-buff-

ered saline, placed in cryotubes, frozen in liquid nitrogen, placed in storage boxes, sealed in plastic bags, and stored at  $-70^{\circ}$  until DNA isolation.

#### DNA Isolation and Conventional PCR Analysis

DNA isolation, gel purification and conventional PCR analysis are described in detail in the accompanying manuscript [6]. Briefly, total DNA was isolated from tissue samples (generally a pool of like tissue taken from 5 cohort animals) using digestion with RNase (and  $\alpha$ -amylase for liver) followed by proteinase K; extraction with phenol:chloroform and chloroform, and precipitation with isopropanol. Total DNA samples were assayed for the presence of plasmid by either conventional or TaqMan PCR. Stringent procedures were implemented to prevent contamination throughout the various procedures.

For conventional PCR, DNA samples were usually assayed for three segments of the relevant plasmid, including the CMV-III segment of the cytomegalovirus promoter, the BGH-II segment of the BGH terminator, and one or more segments of the antigen gene (the HA-I or HA-III segments of HA gene, the M-II segment of the matrix gene, the GAG-I segment of V1Jns-tPA-gag, or the GAG-II segment of V1Jns-GAG2). For the HPV16 E7 study, only the CMV and BGH segments were assayed. For gel-purified samples where material was limited, only 1 or 2 segments were usually assayed. The oligonucleotide primers for the GAG-II segment are listed in table 1. All other primers were described in the accompanying paper [6].

Sample preparation and PCR analysis was carried out as described [6]. The sensitivity of the conventional PCR reaction was routinely 1 copy of plasmid/ $\mu$ g DNA. The level of plasmid was estimated by comparison of the PCR product intensity with that of a reconstruction positive control (including standards from 1 to 16 copies/ $\mu$ g DNA). For samples with higher plasmid levels, the samples

were diluted into control mouse DNA such that their PCR product intensities fell within those of the standard curve (1–16 copies/ $\mu$ g DNA).

#### TaqMan PCR

In the some studies, DNA samples were also assayed by 'TaqMan' real-time quantitative PCR. TaqMan was sensitive to approximately 1 copy/ $\mu$ g DNA, and was quantitative between 0 and  $10^6$  copies/ $\mu$ g DNA. Samples were assayed for 1–3 segments of the plasmid, including the CMV segment from the cytomegalovirus promoter (CMV), the BGH segment from the bovine growth hormone terminator (BGH), and the GAG segment from V1Jns-tPA-gag. The oligonucleotide primers, the fluorescent TaqMan probe (with FAM and TAMRA dyes on each end), and the expected product size for each segment are listed in table 2.

Prior to TaqMan PCR, DNA samples were heat-denatured at  $94^{\circ}$  for about 15 min. TaqMan PCR reactions were carried out in a final volume of 50  $\mu$ l containing a final concentration of  $1 \times$  Master Mix (from Perkin-Elmer), 200 nM probe, 180 nM of each primer, and 0.5  $\mu$ g of sample DNA. Amplification and real-time fluorescence detection were performed using the ABI Prism 7700 Sequence Detector. In each TaqMan PCR experiment, a positive control titration curve was assayed along with the test samples. Generally, the titration curve consisted of 0, 10,  $10^2$ ,  $10^3$ ,  $10^4$ ,  $10^5$  and  $10^6$  copies of plasmid/ $\mu$ g DNA. The 0 samples from the titration curve served as a negative control to detect contamination. In addition, H<sub>2</sub>O (no DNA)-negative control reactions were included. Samples were generally assayed in quadruplicate. For data analysis, the baseline was generally determined between cycles 3 and 15. A standard curve was generated by the instrument using the positive controls, plotting Ct – the cycle number where the fluorescence crossed a threshold value – versus the log of plasmid copies/ $\mu$ g DNA. The threshold was set such that the standard curve would have the maximum possible correlation coefficient, which was generally between 0.990 and 1.0. The level of plasmid in samples was determined by comparison of samples Ct values with the standard curve.

#### Integration Assay

The integration is described in detail in the accompanying manuscript [6]. A variety of gel purification procedures were used. Restriction enzyme digestion was *not* used, and therefore the gel-purified (recovered) DNA could be considered representative of the whole.

**Table 1.** Conventional PCR primers

| Segment | Oligonucleotide primers  | Product size, bp |
|---------|--|------------------|
| GAG-II  | 5'-CTG AAT GCC TGG GTG AAG GTG GTG<br>5'-TCA GGC CCA GGA TGA TCC ACC TCT | 358              |

**Table 2.** TaqMan primers and probes

| Segment | Oligonucleotide primers/probes              | Name   | Product size, bp |
|---------|---|--------|------------------|
| BGH     | 5'-ATG CGG TGG GCT CTA TGG                  | BGH-F2 | 72               |
|         | 5'-TTC TTT CTG GCC CAG GAG G                | BGH-R1 |                  |
|         | fam-CCA GGT GCT GAA GAA TTG ACC CGG T-tamra | BGH-Pr |                  |
| CMV     | 5'-TGA ACC GTC AGA TCG CCT G                | CMV-F  | 68               |
|         | 5'-TCG GTC CCG GTG TCT TCT AT               | CMV-R  |                  |
|         | fam-ACG CCA TCC ACG CTG TTT TGA CCT-tamra   | CMV-Pr |                  |
| GAG     | 5'-CAA GAT TGT GAG GAT GTA CTC C            | GAG-F  | 86               |
|         | 5'-ACC TGT CCA CAT AGT CCC TGA              | GAG-R  |                  |
|         | fam-ATC CTG GAC ATC AGG CAG GGC-tamra       | GAG-Pr |                  |

**Table 3.** Plasmid levels at distal sites after intramuscular injection in mice: approximate copies/ $\mu$ g DNA

| Tissue <sup>a</sup>  | Plasmid level, copies/ $\mu$ g DNA <sup>c</sup> |        |        |                      |                   |        |                      |                         |         |   |         |
|----------------------|---|--------|--------|----------------------|-------------------|--------|----------------------|-------------------------|---------|---|---------|
|                      | 2.0 mg/ml V1Jp-HA                               |        |        |                      | 2.0 mg/ml V1Jp-M1 |        |                      | 3.2 mg/ml V1Jns-tPA-gag |         | 2.7 mg/ml V1Jns-tPA-gag + AlPO <sub>4</sub> |         |
|                      | 1 day   | 3 days | 7 days | 6 weeks <sup>b</sup> | 1 day             | 7 days | 6 weeks <sup>b</sup> | 2 days                  | 6 weeks | 2 days                                      | 6 weeks |
| Peripheral blood (F) | Pos   | Pos    | 0      | 0                    | 8->16             | 1-4    | 0                    | 1-8                     | 0       | 1-8   | n.d.    |
| Peripheral blood (M) | Pos   | Pos    | 0      | 0                    | 8->16             | 1-2    | 0                    | 1-8                     | 0       | 1->16                                       | n.d.    |
| Thymus               | Pos   | Pos    | 0      | 0                    | 1-2               | 0      | 0                    | 0                       | 0       | 0   | n.d.    |
| Spleen               | Pos   | Pos    | 0      | 0                    | 1-4               | 1-4    | 0                    | 1-2                     | 0       | 1-2   | n.d.    |
| Ovaries              | Pos   | 0      | 0      | 0                    | 20-80             | 4-20   | 0                    | 8-40                    | 0       | 1-8   | n.d.    |
| Testes               | 0   | 0      | 0      | 0                    | 1-2               | 1      | 0                    | 0                       | 0       | 0   | n.d.    |
| Brain                | Pos   | Pos    | 0      | 0                    | 1-2               | 1-4    | 0                    | 1-2                     | 0       | 1-4   | n.d.    |
| Heart                | Pos   | Pos    | ~ 1    | n.d.                 | 1                 | 0      | n.d.                 | 1-4                     | 0       | 0   | n.d.    |
| Lung                 | Pos   | Pos    | ~ 1    | n.d.                 | 8->16             | 1-8    | n.d.                 | 1                       | 0       | 0   | n.d.    |
| Liver                | Pos   | Pos    | ~ 1    | n.d.                 | 8->16             | 1-2    | n.d.                 | >16                     | 0       | 1-4   | n.d.    |
| Kidney               | Pos   | Pos    | ~ 1    | n.d.                 | 8-16              | 1-4    | n.d.                 | 1-8                     | 0       | 1-4   | n.d.    |
| Mesenteric node      | Pos   | Pos    | 0      | n.d.                 | 8->16             | 4-16   | n.d.                 | 1-8                     | 0       | 8->16                                       | n.d.    |

F = Female; M = male; n.d. = not determined.

<sup>a</sup> Tissues were taken at various times after injection (last injection). Mice were injected in each quadriceps with 50  $\mu$ l of either 2.0 mg/ml V1Jp-HA, 2.0 mg/ml V1Jp-M1, 3.2 mg/ml V1Jns-tPA-gag or 2.7 mg/ml V1Jns-tPA-gag + 700  $\mu$ g/ml Al (as AlPO<sub>4</sub>). DNA was isolated from a pool of like tissue from cohort animals (same treatment group/sex/time point). Each of the tissues shown, except ovaries, was examined in male animals; blood was examined in both males and females. At 6 weeks, both male and female pools were assayed for each tissue examined, and in each case both were found to be negative (data not shown separately).

<sup>b</sup> Mice received 2 doses of vaccine (3 weeks apart) and were necropsied 6 weeks after a second dose (9 weeks total).

<sup>c</sup> Plasmid level was estimated by conventional PCR. Values > 16 copies/ $\mu$ g DNA were estimated by dilution analysis. The levels of plasmid in the V1Jp-HA study are shown as positive (Pos) or negative (0), however most positive samples were between 1 and 16 copies/ $\mu$ g DNA (at 7 days, the positive samples near the limit of detection and therefore had ~ 1 copy/ $\mu$ g DNA).

Three conventional agarose gel procedures were used: the TAE procedure, which utilizes 0.5% agarose (FMC SeaKem Gold) gels in Tris-acetate-EDTA (TAE) buffer, electrophoresed at 40 V for 22.5 h; the TBE procedure, which utilizes 1% agarose (FMC SeaKem Gold) gels in Tris-borate-EDTA (TBE) buffer, electrophoresed at 50 V for 45 h (V1Jp-HA study) or 70 V for 32 h (V1Jp-M1 and V1Jns-tPA-gag studies), and the *Gibco*TBE procedure, which utilizes 1.2% agarose (Gibco/BRL) gels in TBE buffer, electrophoresed at 80 V for 22 h.

Genomic DNA was also purified by pulsed-field gel electrophoresis using the BioRad CHEF (Clamped Homogeneous Electric Field) Mapper System. For the CHEF procedure, the electrophoresis parameters were determined by the instrument using an autoalgorithm for separation of 10–50 kb DNA. DNA was recovered from gel slices by electroelution (ee). The gel-purified, HMW genomic DNA was assayed by conventional PCR for potentially integrated plasmid.

## Results

### *Tissue Distribution Studies in Mice*

The tissue distribution following intramuscular injection in mice was examined for three different plasmid DNA vaccines: V1Jp-HA, containing the influenza HA gene; V1Jp-M1, containing the influenza matrix gene, and

V1Jns-tPA-gag, containing the HIV gag gene. V1Jns-tPA-gag was tested both in the absence and presence of aluminum phosphate adjuvant. As shown in table 3, at early time points (1–7 days) after a single intramuscular injection, a low level of systemic exposure was detected for all treatment groups. The level of plasmid at these distal sites was low, generally below 16 copies/ $\mu$ g DNA, and dissipates to undetectable levels by 6 weeks postdose. There was no significant difference between the various plasmids, or between plasmid with and without aluminum phosphate adjuvant.

### *Analysis of Gonad DNA for Integrated Plasmid*

Because of concern over germline transmission, mouse testis and ovary samples that were positive for the presence of plasmid were assayed for integrated plasmid. HMW genomic DNA was purified by agarose gel electrophoresis to remove free plasmid, and the gel-purified DNA was then reassayed by PCR to detect potentially integrated plasmid. Both the testis and ovary samples were negative for integrated plasmid after gel purification (table 4).

**Table 4.** Plasmid detected in gonads following intramuscular injection is extrachromosomal

| Tissue <sup>a</sup> | Plasmid treatment       | Time point, days | Plasmid level (pre-gel) copies/ $\mu$ g DNA <sup>b</sup> | Rounds of gel purification (steps) <sup>c</sup> | Plasmid level (post-gel) copies/ $\mu$ g DNA |
|---------------------|-------------------------|------------------|--|---|--|
| Testes              | 2.0 mg/ml V1Jp-M1       | 1                | 1–2  | 2 (TAE, TBE, ee)                                | 0  |
| Ovaries             | 2.0 mg/ml V1Jp-M1       | 1                | 20–80  | 2 (TAE, TBE, ee)                                | 0  |
|                     | 3.2 mg/ml V1Jns-tPA-gag | 2                | 8–40   | 2 (TAE, TBE, ee)                                | 0  |

<sup>a</sup> Mice were injected in each quadriceps with 50  $\mu$ l of the indicated plasmid, and tissues were taken at indicated times.

<sup>b</sup> Plasmid level before and after gel purification was determined by conventional PCR. Values > 16 copies/ $\mu$ g DNA were estimated by dilution analysis.

<sup>c</sup> Gel purification procedures are abbreviated as described in Methods. ‘ee’ refers to electroelution and to a stopping point in the multiround gel purifications.

**Table 5.** Effect of aluminum phosphate adjuvant on integration of V1Jns-tPA-gag plasmid after intramuscular injection in mice

| Tissue <sup>a</sup>   | Delivery <sup>b</sup>       | Sex | Plasmid level (pre-gel) copies/ $\mu$ g DNA <sup>c</sup> | Rounds of gel purification (steps) <sup>d</sup> | Plasmid level (post-gel) copies/ $\mu$ g DNA |
|-----------------------|-----------------------------|-----|--|---|--|
| Quadriceps            | Plasmid                     | F   | 8,010  | n.d.  | n.d.   |
|                       | Plasmid                     | M   | 24,900   | 6 (TAE, TBE, TAE, TBE, ee, GibcoTBE, ee)        | 1–4  |
|                       | Plasmid + AlPO <sub>4</sub> | F   | 13,200   | 4 (TAE, TBE, TAE, GibcoTBE, ee)                 | 0  |
|                       | Plasmid + AlPO <sub>4</sub> | M   | 13,800   | 4 (TAE, TBE, TAE, GibcoTBE, ee)                 | 0–1  |
| Skin (injection site) | Plasmid                     | F   | 120  | n.d.  | n.d.   |
|                       | Plasmid                     | M   | 1,270  | 4 (TAE, TBE, TAE, GibcoTBE, ee)                 | 0  |
|                       | Plasmid + AlPO <sub>4</sub> | F   | 370  | 2 (TAE, TBE, ee)                                | 0  |
|                       | Plasmid + AlPO <sub>4</sub> | M   | 5,030  | 4 (TAE, TBE, TAE, GibcoTBE, ee)                 | 0  |
| Inguinal node         | Plasmid                     | F   | 0  | n.d.  | n.d.   |
|                       | Plasmid                     | M   | 1–8  | 2 (TAE, ee, TBE, ee)                            | 0  |
|                       | Plasmid + AlPO <sub>4</sub> | F   | 0  | n.d.  | n.d.   |
|                       | Plasmid + AlPO <sub>4</sub> | M   | 0  | n.d.  | n.d.   |

n.d. = Not determined.

<sup>a</sup> Tissues were taken approximately 6 weeks after dosing. Ovaries, testes, blood, brain, thymus, spleen, heart, lung, liver, kidney and mesenteric nodes were negative for the presence of plasmid for all samples (with or without AlPO<sub>4</sub>, male or female) and are therefore not shown.

<sup>b</sup> Mice were injected with 50  $\mu$ l per quadriceps of either 3.2 mg/ml V1Jns-tPA-gag plasmid alone or 2.7 mg/ml plasmid + 700  $\mu$ g/ml Al (as AlPO<sub>4</sub>).

<sup>c</sup> Plasmid level in quadriceps and skin samples before gel purification was determined by TaqMan PCR. Plasmid level in all other tissues including inguinal nodes, and in all gel-purified samples, was determined by conventional PCR.

<sup>d</sup> Gel purification procedures are abbreviated as described in Methods. ‘ee’ refers to electroelution and to a stopping point in the multiround gel purifications.

#### *Effect of Aluminum Phosphate Adjuvant*

The V1Jns-tPA-gag plasmid was analyzed in the absence and presence of aluminum phosphate adjuvant. As noted above, aluminum phosphate had no effect on the tissue distribution of the plasmid (table 3). As shown in

table 5, there was also no significant effect on plasmid uptake in either the injected quadriceps, skin at the injection site, or the draining lymph nodes. Note the high but variable uptake of plasmid in skin with intramuscular injection.

**Table 6.** Effect of Biojector delivery on tissue distribution and integration of V1Jns-tPA-gag plasmid in guinea pigs

| Tissue <sup>a</sup>   | Delivery <sup>b</sup> | Sex | Plasmid level (pre-gel) copies/ $\mu$ g DNA <sup>c</sup> | Rounds of gel purification (steps) <sup>d</sup>              | Plasmid level (post-gel) copies/ $\mu$ g DNA |
|-----------------------|-----------------------|-----|--|--|--|
| Hamstrings            | Needle                | F   | 2–16   | n.d.   | n.d.   |
|                       | Needle                | M   | 2,800  | n.d.   | n.d.   |
|                       | Biojector             | F   | 120  | n.d.   | n.d.   |
|                       | Biojector             | M   | 2,700  | 5 (TAE, TBE, TAE, GibcoTBE, ee, CHEF, ee)                    | 1–8  |
| Skin (injection site) | Needle                | F   | 1,800  | n.d.   | n.d.   |
|                       | Needle                | M   | 68,000   | n.d.   | n.d.   |
|                       | Biojector             | F   | 72,000   | n.d.   | n.d.   |
|                       | Biojector             | M   | 430,000  | 7 (TAE, TBE, TAE, GibcoTBE, ee, CHEF, ee, TAE, GibcoTBE, ee) | 2–8  |
| Inguinal node         | Needle                | F   | 0  | n.d.   | n.d.   |
|                       | Needle                | M   | 0  | n.d.   | n.d.   |
|                       | Biojector             | F   | 1–8  | n.d.   | n.d.   |
|                       | Biojector             | M   | 1–16   | 2 (TAE, TBE, ee)   | 0  |
| Peripheral blood      | Needle                | F   | 0  | n.d.   | n.d.   |
|                       | Needle                | M   | 0  | n.d.   | n.d.   |
|                       | Biojector             | F   | 0  | n.d.   | n.d.   |
|                       | Biojector             | M   | 1–4  | 1 (TAE, ee)  | 0  |
| Brain                 | Needle                | F   | 0  | n.d.   | n.d.   |
|                       | Needle                | M   | 0  | n.d.   | n.d.   |
|                       | Biojector             | F   | 0  | n.d.   | n.d.   |
|                       | Biojector             | M   | ~ 1  | 1 (TAE, ee)  | 0  |
| Thymus                | Needle                | F   | 0  | n.d.   | n.d.   |
|                       | Needle                | M   | 0  | n.d.   | n.d.   |
|                       | Biojector             | F   | 0  | n.d.   | n.d.   |
|                       | Biojector             | M   | 1–2  | 1 (TAE, ee)  | 0  |

n.d. = Not determined.

<sup>a</sup> Tissues taken approximately 6 weeks after injection. Ovaries, testes, spleen, heart, lung, liver, kidney and mesenteric nodes were negative for the presence of plasmid for all samples (Biojector or needle, males or female) and are therefore not shown.

<sup>b</sup> Guinea pigs were injected with 200  $\mu$ l per hamstring of 5.5 mg/ml V1Jns-tPA-gag plasmid, either by needle or Biojector (needleless jet) delivery.

<sup>c</sup> Plasmid level before and after gel purification was estimated by conventional PCR. Dilution analysis was used for samples with >16 copies/ $\mu$ g DNA.

<sup>d</sup> Gel purification procedures are abbreviated as described in Methods. 'ee' refers to electroelution and to a stopping point in the multiround gel purifications.

Following gel purification to remove free plasmid, there was little to no detectable plasmid remaining in any of the treated samples, indicating that the plasmid remained extrachromosomal. This was true both in the absence and presence of the adjuvant (table 5).

Residual plasmid that remains with HMW genomic DNA following gel purification could represent integrated plasmid. However, it is also possible that the residual plasmid is extrachromosomal. Purification procedures almost never yield 100% pure material, and 1 copy ( $<1 \times$

$10^{-17}$  g) of plasmid/ $\mu$ g genomic DNA represents an impurity level of <100 billionth by weight. The residual free plasmid could be 'trapped' in the genomic DNA, or it could be large linear concatemers of similar size to genomic DNA such that it comigrates with genomic DNA during electrophoresis. Even if the residual plasmid in the gel-purified genomic DNA did represent integrated plasmid, it was calculated that 1 copy of integrated plasmid/ $\mu$ g of genomic DNA (representing 150,000 diploid cells) would be at least three orders of magnitude below the fre-

**Table 7.** Effect of Biojector delivery on uptake and integration of V1Jns-GAG2 plasmid in guinea pigs

| Animal No. <sup>a</sup>      | Plasmid level, copies/ $\mu$ g DNA <sup>c</sup> |                    |               |                       |
|------------------------------|---|--------------------|---------------|-----------------------|
|                              | hamstrings <sup>b</sup>                         |                    | skin          |                       |
|                              | needle  | Biojector          | needle        | Biojector             |
| 1                            | 21  | 74,933             | 30            | 36,200                |
| 2                            | 11  | 1,377              | 1,787         | 512,667               |
| 3                            | 25  | 35                 | 12            | 135                   |
| 4                            | 21  | 1,141              | 250           | 458,667               |
| 5                            | 18  | 1,090              | 55            | 1,343                 |
| 6                            | 11  | 20                 | 641           | 54,700                |
| 7                            | 19  | 215                | 128           | 9,560                 |
| 8                            | 21  | 57                 | 72            | 125,333               |
| 9                            | 14  | 212                | 19            | 96,700                |
| 10                           | 18  | 28                 | 30            | 354                   |
| Average $\pm$ SD             | 18 $\pm$ 5                                      | 7,900 $\pm$ 23,600 | 300 $\pm$ 560 | 130,000 $\pm$ 193,000 |
| Pool (pre-gel) <sup>d</sup>  | 12  | 5,800              | 290           | 165,000               |
| Pool (post-gel) <sup>e</sup> | 0   | 1–8                | 0             | 1–2                   |

<sup>a</sup> Male guinea pigs were injected with 200  $\mu$ l per hamstring of 7.13 mg/ml V1Jns-GAG2 plasmid, either by needle or Biojector (needleless jet).

<sup>b</sup> Tissues were taken approximately 6 weeks after injection. Only the hamstrings, skin at the injection site, and inguinal nodes were examined. Treated inguinal node samples were sporadically positive, with 0–2 copies/ $\mu$ g DNA, for both needle and Biojector delivery (data not shown).

<sup>c</sup> Plasmid level was determined by TaqMan PCR. Results represent the average of 3 plasmid segments for each sample. The average and standard deviation of the 10 individual samples is given.

<sup>d</sup> DNA from cohort animals was pooled. Plasmid levels in the pool were determined by TaqMan PCR.

<sup>e</sup> Pooled samples were subjected to 4 rounds of gel purification (TAE, TBE, TAE, GibcoTBE, ee), except for the Biojector hamstring sample which had 6 rounds (TAE, TBE, TAE, GibcoTBE, ee, CHEF, CHEF, ee). Gel purification procedures are abbreviated as described in Methods. 'ee' refers to electroelution and to a stopping point in the multiround gel purifications. Plasmid level after gel purification was determined by conventional PCR.

quency of spontaneous gene-inactivating mutations [5, 6].

#### *Comparison of Biojector with Needle Delivery*

Biojector studies were carried out in guinea pigs because mice are too small for the device. Biojector delivery was optimized for intramuscular injection during trial experiments with a dye solution. In the first study, guinea pigs were injected once in each hamstring with V1Jns-tPA-gag plasmid. Six weeks after injection, the tissue distribution and potential integration of the plasmid were investigated. DNA was isolated from a pool of like tissue taken from cohort animals (same treatment group/sex). As shown in table 6, compared with needle, Biojector delivery led to a greater uptake of the plasmid in skin, and

also caused slightly more dispersion of the plasmid to distal sites. However, Biojector delivery did not lead to significant levels of integrated plasmid detectable after gel purification (table 6).

In the second Biojector study, male guinea pigs were injected with V1Jns-GAG2 plasmid. Tissue DNA was isolated from individual animals to permit a better assessment of plasmid uptake. Although there was considerable animal-to-animal variability, Biojector delivery led to significantly higher levels of plasmid uptake in hamstrings and skin at the injection site, as compared with needle delivery (table 7). For integration assays, the cohort DNA was pooled and subjected to gel purification. Again, gel purification removed essentially all of the plasmid, indicating that the plasmid was extrachromosomal.

**Table 8.** Integration studies in mice using V1Jns-HPV16 E7 plasmids: comparison of wild-type versus mutant E7 genes

| Tissue <sup>a</sup>   | E7 gene <sup>b</sup> | Plasmid level (pre-gel) copies/ $\mu$ g DNA <sup>c</sup> | Rounds of gel purification (steps) <sup>d</sup> | Plasmid level (post-gel) copies/ $\mu$ g DNA |
|-----------------------|----------------------|--|---|--|
| Quadriceps            | Wild-type            | 100–800  | 5 (TAE, TBE, TAE, TBE, ee, CHEF, ee)            | <1   |
|                       | Mutant               | 100–800  | 5 (TAE, TBE, TAE, TBE, ee, CHEF, ee)            | 1–8  |
| Skin (injection site) | Wild-type            | 10–40  | 2 (TAE, TBE, ee)                                | 0  |
|                       | Mutant               | 10–20  | 2 (TAE, TBE, ee)                                | 0  |
| Inguinal node         | Wild-type            | $\leq 1$   | 2 (TAE, TBE, ee)                                | 0  |
|                       | Mutant               | $\leq 1$   | 2 (TAE, TBE, ee)                                | 0  |

<sup>a</sup> Tissues were taken approximately 6 weeks after dosing. Analysis was limited to the tissues shown.

<sup>b</sup> Mice were injected with 50  $\mu$ l per quadriceps of 3.0 mg/ml V1Jns-HPV E7 plasmid, containing either a wild-type (active) or mutated (inactivated) E7 gene from HPV type 16.

<sup>c</sup> Plasmid level was estimated using conventional PCR, using dilution analysis for samples with >16 copies/ $\mu$ g DNA.

<sup>d</sup> Gel purification procedures are abbreviated as described in Methods. 'ee' refers to electroelution and to a stopping point in the multiround gel purifications.

#### *Comparison of Wild-Type versus Mutant E7 Genes*

Since the HPV16 E7 gene has been shown to increase integration frequency *in vitro*, we compared a plasmid expression vector containing the wild-type (active) E7 gene with a similar plasmid containing a mutant (inactivated) E7 gene as a control. If the E7 gene had a significant effect on integration *in vivo*, then the level of plasmid associated with genomic DNA after gel purification should be greater in the wild-type E7 versus mutant E7 treatment groups. As shown in table 8, neither treatment group exhibited significant levels of integrated plasmid, and the wild-type E7 gene had no effect on the detectable level of integration.

#### **Discussion**

Since the type of cells exposed to the plasmid could affect integration frequency, analysis of tissue distribution should be an integral part of any integration study. After intramuscular injection, the vast majority of the plasmid remains near the injection site. At early time points (1–7 days after injection), a low level of systemic exposure can be detected. However, the plasmid is present at these distal sites at low levels (usually less than 16 copies/ $\mu$ g DNA), and it dissipates rapidly such that it is usually undetectable by 6 weeks. Plasmid was occasionally detected in ovaries and testes; however, it is unlikely

that the plasmid was within germ cells, and we demonstrated that the plasmid was extrachromosomal, suggesting that there is little risk of germline transmission.

A second factor that could affect integration frequency is the sequence of the plasmid itself, since specific nucleotide sequences as short as 7 bp are known to affect integration or recombination. Between the present study, the accompanying paper [6] and our original exploratory study [5], we have tested 7 different plasmid DNA vaccines – all involving a derivative of the V1J vector. The genes tested include the influenza nucleoprotein [5], HA, and matrix genes, the HIV-1 gag gene (with and without a tPA secretory signal), and the wild-type and mutant E7 genes of HPV16. We have not detected any significant differences in the tissue distribution or integration frequency between these different plasmids, suggesting there are no *cis*-acting elements in these plasmids that enhance integration to the detectable range.

A third factor that could affect integration is the expressed gene itself. The HPV16 E7 gene is known to stimulate integration *in vitro* by up to 30-fold. However, we detected no effect of E7 in the present study. It is likely that the rapid cell proliferation observed *in vitro* is necessary to observe the effects of the E7 gene product, which is thought to act by interfering with cell cycle-regulatory proteins [19].

The formulation is a fourth factor that could affect integration frequency. In the present study, we found that

aluminum phosphate adjuvant had no effect on plasmid tissue distribution or integration frequency. The adjuvant has been found to enhance the immune responses elicited by the vaccine; apparently this is due to direct adjuvant effect rather than an enhancement of plasmid uptake.

Finally, a fifth factor that could affect integration is the delivery method and route of administration. We found that Biojector delivery could enhance the uptake of plasmid in the muscle and skin at the injection site, as well as lead to slightly greater dispersion of the plasmid to distal sites. However, the Biojector did not lead to significant levels of integrated plasmid, even in samples that had >100,000 copies/ $\mu$ g DNA of free plasmid.

In conclusion, the results of the present study and the accompanying paper [6] suggest that the risk of plasmid integration following intramuscular injection is exceedingly small under a variety of experimental conditions, including needle or Biojector delivery, the presence or

absence of aluminum phosphate adjuvant, and 7 different plasmid DNA vaccines – including one whose gene product is known to enhance plasmid integration *in vitro*. While these results add support to the safety of plasmid DNA vaccines, the database of preclinical safety information for DNA vaccines is still quite limited, and it is advisable to investigate tissue distribution and potential integration whenever there is a significant change in DNA vaccination protocol.

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