

Investigation of DNA Integration into Reproductive Organs Following Intramuscular Injection of DNA in Mice

Fatemeh Vahedi*¹, Najmeh Nazari², and Shirin Arbabi²,
Yasser Peymanfar¹

Abstract

Background: DNA immunization with plasmid DNA encoding bacterial, viral, parasitic, and tumor antigens has been reported to trigger protective immunity. The use of plasmid DNA vaccinations against many diseases has produced promising results in animal and human clinical trials; however, safety concerns about the use of DNA vaccines exist, such as the possibility of integration into the host genome, and elicitation of adverse immune responses.

Methods: In this study, we examined the potential integration and bio-distribution of pcDNA3.1+PA, a new vaccine candidate with GenBank accession # EF550208, encoding the PA63 gene, in reproductive organs of mice; ovaries and uterus in female, and testis in male. Animals of both sexes were injected intramuscularly with pcDNA3.1+PA. Host genome integration and tissue distribution were examined using PCR and RT-PCR two times monthly for six months.

Results: RT-PCR confirmed that pcDNA3.1+PA was not integrated into the host genome and did not enter reproductive organs.

Conclusions: This finding has important implications for the use of pcDNA3.1+PA plasmid as a vaccine and opens new perspectives in the DNA vaccine area.

Keywords: DNA, Intramuscular injection, Integration, Mice, Reproductive organs

Introduction

DNA vaccines represent an attractive approach to immunization with regard to their specific properties, which include stability, and simplicity of preparation (1). Although hundreds of clinical trials in this area have reported success, in fact, few DNA vaccines have been marketed (2-3).

Probably the issue of most concern to investigators is the possible integration of injected DNA into chromosomes (4). Such DNA integration could range from no effect to cancer through alteration of normal DNA; therefore, DNA vaccines have been vigorously examined for evidence of even minimal integration of the injected DNA into chromosomes (5-7).

Determination of tissue distribution of the injected DNA vaccines is essential to identify tissues exposed to exogenous DNA. Potential adverse consequences due to integration into the host genome should be considered (8). Organs containing germline cells are emphasized in consideration of the possible transfer of foreign DNA to future generations (4, 8). Polymerase chain reaction (PCR) is a suitable technique to detect the presence of foreign DNA in purified tissues (9-10).

We previously showed that intramuscular administration of plasmid pcDNA3.1+PA, Genbank accession # EF550208, encoding the PA63 protective antigen gene, resulted in a

1: Razi Vaccine & Serum Research Institute, Mashhad, Iran

2: Biochemistry Department, Payam-e-Noor University, Mashhad, Iran

*Corresponding author: Fatemeh Vahedi; Tel: +98 511-8431780; Fax: +98 511-8420430; E-mail: vahedif@yahoo.com

Received: May 21, 2012; Accepted: Aug 6, 2012

protective immune response. The response profile was representative of mixed Th1 and Th2 responses, skewed to a Th1 response.

As mentioned previously, characterization of plasmid bio-distribution and genome integration are critical for the design of new delivery strategies and biosafety in DNA vaccine research.

In this study, we examined the potential integration and bio-distribution of pcDNA3.1+PA in the reproductive organs of mice; ovaries and uterus in female, and testis in male. Host genome integration and tissue distribution were examined over six months using PCR and RT-PCR.

Materials and Methods

The construction of plasmid pcDNA3.1, containing the gene for protective antigen PA63 (pcDNA3.1+PA, GenBank accession # EF550208), has been previously described (11). Maxi-preparation of pcDNA3.1+PA was performed using the PhoenIX™ maxi-prep kit, as described by the manufacturer (QBiogene, Inc., CA).

Twenty-eight female and 28 male BALB/c mice, 6-8 weeks of age, were obtained from the Razi Vaccine and Serum Research Institute (RVSRI), Iran. Mice were injected with 100 µg of plasmid in PBS at a final volume of 100 µl/mouse (w/v). Experimental mice received pcDNA3.1+PA and eight control mice received pcDNA3.1. The plasmid was administered intramuscularly (I.M.) into the right quadriceps muscle at three separate sites. At two-week intervals following administration one male and one female mouse were euthanized and samples of uterus and ovary from females, and testis from males, were obtained and stored at -70°C. Samples were treated using the TriPure kit (Roche), and total RNA and DNA were isolated according to the manufacturer's protocols. Subsequently, RNA was extracted with chloroform and precipitated with isopropyl alcohol. The DNA was isolated by ethanol precipitation of the interphase and phenol phase. The precipitated DNA was washed with 0.1 M sodium citrate in absolute ethanol followed by 75% ethanol. Extracted RNA and DNA were dissolved in nuclease-free water (Invitrogen).

Total cellular RNA (10 µg/ml) was reverse-transcribed using oligo(dT) primers and reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Contaminating plasmid DNA was removed by treatment with amplification-grade DNase I (Invitrogen). The cDNA (2 µg) was amplified by one starting cycle at 95 °C for 5 min, followed by 35 cycles at 94°C for 1 min, 65°C for 1 min, 72°C for 2 min, and final extension at 72 °C for 10 min, using the primer pairs 5'ACAAGTAAGCTTACCATGGTTCCAGACCGTGAC3' and 5'CTCGAGCTTCAATTACCTTATCCT3', resulting in a 1721-base pair (bp) cDNA fragment encoding PA in positive samples, and the primer pairs 5' CCTTCCTGGGCATGGAGTCCTG3' and 5' GGAGCAATGATCTTGATCTTCG 3', resulting in a 220-bp cDNA fragment encoding α-actin.

Total DNA was extracted from all samples using the TriPure reagent according to the manufacturer's instructions. Rescued plasmids were analyzed by PCR using PA primers as described above.

Results

pcDNA3.1+PA plasmid was purified and analyzed by spectrophotometry and agarose gel electrophoresis. In addition, restriction digestion followed by agarose gel electrophoresis verified the integrity of the plasmid. The vaccine was administered and tissues were harvested as scheduled.

Tissue samples from reproductive organs were collected for detection of plasmid DNA and PA message bi-weekly for 6 months. Three days after inoculation, RT-PCR analysis demonstrated the presence of PA transcripts and injected plasmid as DNA in muscles near the injection sites. These samples were considered to be positive controls during our study.

All samples were subjected to TriPure reagent and their DNA and RNA isolated. Amplification of a 220-bp band of α-actin cDNA confirmed isolation of RNA from the collected reproductive tissues and efficiency of the RT-PCR procedure (Fig. 1). A 1,720-bp PA fragment was amplified from DNA extracted from muscle (Fig. 2); however, efforts to

amplify PA cDNA by RT-PCR from RNAs extracted from collected reproductive tissues were unsuccessful (Fig. 3).

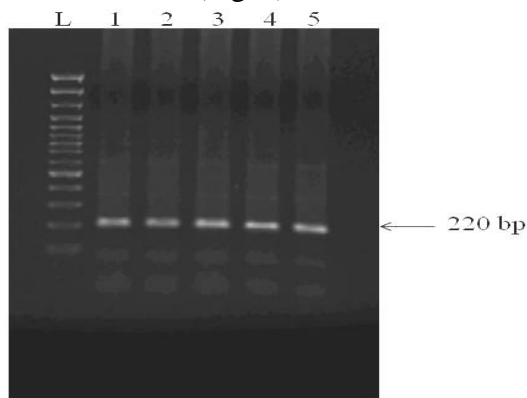


Fig. 1. Agarose gel electrophoresis following RT-PCR with actin primers. RNA was isolated from collected reproductive tissues of injected mice. Lane L: GeneRuler™ 100 bp DNA Ladder (Fermentas). Lane 1: Ovary, lane 2: Testis, lane 3: Uterus, lane 4: Muscle.

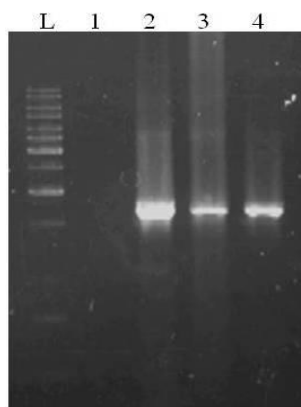


Fig. 2. Agarose gel electrophoresis following PCR using PA primers and DNA extracted from muscles of injected mice. Lane L: GeneRuler™ 100 bp DNA Ladder (Fermentas). Lane 1: Muscle, lane 2: Muscle, lane 3: 1721-bp positive control, lane 4: Negative control.

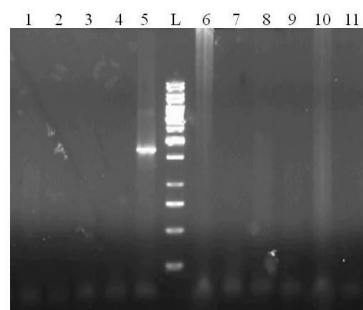


Fig. 3. Agarose gel electrophoresis following RT-PCR with PA primers. RNA was extracted from collected reproductive tissues of injected mice. Lane L: GeneRuler™ 1 kb DNA Ladder (Fermentas). Lanes 1-4 and 6-11: RNAs extracted from reproductive tissues of injected mice, lane 5: 1721-bp positive control.

Discussion

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), formulation, delivery method, route of administration, and type of cells exposed to the plasmid (6).

In this report, we examined the tissue distribution and potential integration of plasmid DNA vaccines following intramuscular administration in mice.

Plasmid bio-distribution, persistence, and integration studies were initially recommended to address questions regarding patient safety. An important question is whether subjects in DNA vaccine trials are at heightened risk from long-term expression of the encoded antigen, either at the site of injection or an ectopic site, and/or plasmid integration. Theoretical concerns regarding DNA integration include the risk of tumorigenesis if insertion reduces the activity of a tumor suppressor or increases the activity of an oncogene. In addition, DNA integration may result in chromosomal instability through the induction of chromosomal breaks or rearrangements (8, 13).

A typical bio-distribution and persistence study assesses the presence of plasmid collected from a panel of tissues at multiple time points ranging from a few days to several months post-administration. The panel of tissues typically includes blood, heart, brain, liver, kidney, bone marrow, ovaries, testes, lung, draining lymph nodes, spleen, and muscle at the site of administration and subcutis at the injection site. Plasmid levels are calculated using a quantitative real time polymerase chain reaction assay (Q-PCR) validated for sensitivity, specificity, and the absence of inhibitors (14).

Studies examining plasmid bio-distribution and persistence indicate that DNA vaccines prepared from a common plasmid vector, but encoding different antigens, behave similarly. Conventional intramuscular, subcutaneous, intradermal, and particle-mediated delivery of DNA plasmids rarely result in long-term persistence of vector DNA at ectopic sites; however, tissue at or near the site of administration frequently contains thousands of copies of plasmid per microgram of host DNA for periods exceeding 60 days. Studies assessing the nature of this DNA indicate that the vast majority is not integrated (14).

A typical integration study will assess all tissues containing the persisting DNA plasmid. The FDA recommends that at least four independent DNA samples be analyzed. Each sample may include DNA pooled from several different donors. Q-PCR is generally used to detect and quantify the amount of plasmid DNA present in each genomic DNA preparation (14).

The frequency of integration into the cellular genome could be affected by several factors, such as the plasmid sequence, the presence of chi-like elements (6), and *Alu* segments (15); however, the integration of bacterial plasmid DNA is not so simple. The mammalian genome appears to possess a mechanism to protect its integrity. In addition, the results provided by Ledwith *et al.*, using different

plasmid constructs, suggest the risk of plasmid DNA vaccine integration following intramuscular inoculations is negligible (4); therefore, the use of plasmid DNA in gene therapy may be safer than vector systems.

Our results suggest that this vaccine is safe for clinical use and the use of a plasmid containing the PA gene is reliable for gene therapy purposes, as well as vaccination in a clinical setting by intramuscular route.

Acknowledgement

The authors express their thanks to all staff in Research department of Razi vaccine & serum research institute, Mashhad, Iran for their support. Hereby, the authors confirm there is no conflict of interest.

References

1. Srinivasan R, Wolchok JD. Tumor antigens for cancer immunotherapy: therapeutic potential of xenogeneic DNA vaccines. *J Transl Med.* 2004 Apr 16;2(1):12.
2. Dhama K, Mahendran M, Gupta PK, Rai A. DNA vaccines and their applications in veterinary practice: current perspectives. *Vet Res Commun.* 2008 Jun;32(5):341-56.
3. Kutzler MA, Weiner DB. DNA vaccines: ready for prime time? *Nat Rev Genet.* 2008 Oct;9(10):776-88.
4. Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG, 2nd, *et al.* Plasmid DNA vaccines: assay for integration into host genomic DNA. *Dev Biol (Basel).* 2000;104:33-43.
5. Webster RG, Robinson HL. DNA vaccines: a review of developments. *BioDrugs.* 1997 Oct;8(4):273-92.
6. Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG, 2nd, *et al.* Plasmid DNA vaccines: investigation of integration into host cellular DNA following intramuscular injection in mice. *Intervirology.* 2000;43(4-6):258-72.
7. Shaw DR, Strong TV. DNA vaccines for cancer. *Front Biosci.* 2006;11:1189-98.
8. Manam S, Ledwith BJ, Barnum AB, Troilo PJ, Pauley CJ, Harper LB, *et al.* Plasmid DNA vaccines: tissue distribution and effects of DNA sequence, adjuvants and delivery method on integration into host DNA. *Intervirology.* 2000;43(4-6):273-81.
9. Orsag P, Kvardova V, Raska M, Miller AD, Ledvina M, Turanek J. Quantitative real-time PCR study on persistence of pDNA vaccine pVax-Hsp60 TM814 in beef muscles. *Genet Vaccines Ther.* 2008;6:11.
10. Wolff JA, Ludtke JJ, Acsadi G, Williams P, Jani A. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum Mol Genet.* 1992 Sep;1(6):363-9.
11. Vahedi F, Taiebi Meibody N, Kianizadeh M, Mahmoudi M. Construction of a Eukaryotic Plasmid Encoding *Bacillus anthracis* Protective Antigen, a Candidate for DNA Vaccine. *Iran J Immunol.* 2005;3:134-40.
12. Vahedi F, Kyd J, Mozeni Jula G, Afzalaghaeei M, Kianizadeh M, Mahmoudi M. A DNA vaccine candidate for *B. anthracis* immunization, pcDNA3.1+PA plasmid, induce Th1/Th2 mixed responses and protection in mice. *World Journal of Microbiology and Biotechnology* 2008;24:2171-8.
13. Wang Z, Troilo PJ, Wang X, Griffiths TG, Pacchione SJ, Barnum AB, *et al.* Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. *Gene Ther.* 2004 Apr;11(8):711-21.
14. Guidance for Industry: Considerations for Plasmid DNA Vaccines for Infectious Disease Indications [database on the Internet] 2007.
15. van de Lagemaat LN, Gagnier L, Medstrand P, Mager DL. Genomic deletions and precise removal of transposable elements mediated by short identical DNA segments in primates. *Genome Res.* 2005 Sep;15(9):1243-9.