Original Article

DNA-Based Vaccine Is More Efficient than Non-Pathogenic Live Vaccine for Prevention of HPV16 E7-Overexpressing Cancers

Amin Daemi [1,2], Sahar Hosseinzadeh [1,2], Azam Bolhassani [2]*, Elnaz Agi [2]

1 Molecular Immunology and Vaccine Research Laboratory, Pasteur Institute of Iran, Tehran, Iran
2 Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran

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Abstract:

Introduction: Vaccinology provides promising approaches for the control of various infectious diseases. Among different strategies, DNA vaccines offer attractive research opportunities for development of vaccines for induction of antigen-specific immunity owing to their stability, simplicity of delivery, safety and cost effectiveness. However, there is a need to increase their potency by the use of adjuvants such as glycoprotein 96 and electroporation delivery. On the other hand, the attenuated or non-pathogenic live vectors have been used to deliver DNA into cells as efficient delivery tools in gene therapy. Recently, a non-pathogenic protozoan, *Leishmania tarentolae (L. tar)*, has attracted attention as an in situ protein-delivery vehicle. Cervical cancer is the second largest cause of cancer deaths among women worldwide and human papillomaviruses (HPV) is reportedly a frequent cause of this type of cancer. **Methods:** In the current study, we compared the potential of live *L. tar*-based and DNA-based vaccines expressing HPV16 E7 linked to C-terminal fragment of gp96 in a tumor mouse model. **Results:** We found that subcutaneous DNA injection with E7-CT (gp96), followed by electroporation, generate a significant E7-specific IFN-γ immune response and *in vivo* protective effects compared to transgenic *L. tar*-E7-CT (gp96) in challenge experiments with TC-1. **Conclusion:** It could be concluded that the DNA vaccine showed higher efficacy compared to the non-pathogenic live parasite-based vaccine in the tumor mice model. *Vac Res, 2014, 1 (1): 21 - 24*

Keywords: DNA vaccine, Live vaccine, *Leishmania tarentolae*, Glycoprotein 96, Electroporation.

INTRODUCTION

Cervical cancer is the second largest cause of cancer deaths among women worldwide. Human papilloma-viruses (HPV), particularly HPV16, is reportedly an important etiologic agent of cervical and oral cancer and more than 80% of all deaths due to this type of cancer occur in the developing countries [1, 2]. However, even under optimal treatment conditions (mainly surgery as well as chemotherapy and radiotherapy), 40% of cervical cancer patients die of the disease [3]. Immunotherapy, potentiating host immunity against HPV, is a promising strategy for the prevention and treatment of cervical cancer [4]. The pathogenic principle for the transforming activity of the high-risk HPV types has been mainly attributed to the E7 oncoprotein of the virus which has been targeted as an ideal antigen for a therapeutic vaccine [5]. DNA-based and live vector-based vaccines are among different developmental approaches which are capable of generating antigen-specific immune responses in vaccinated animals and humans [6, 7].

However, DNA vaccines despite ease of manufacturing, cost effectiveness and safety have poor immunogenicity records. Two main strategies to enhance DNA-based vaccines potency are the use of immuno-adjuvants such as heat shock

***Corresponding Author:** Azam Bolhassani
Department of Hepatitis and AIDS, Pasteur Institute of Iran, No. 69, Pasteur Ave, Tehran, Iran, 1316943551.
Email: Azam.bolhassani@yahoo.com
Tel: +98 21 66953311 Ext. 2240, **Fax:** +98 21 66465132

proteins (e.g., Gp96 or its fragments (N-/C-Terminal)) and optimization of cellular uptake of plasmid DNA by electroporation. Furthermore, the attenuated or non-pathogenic live vectors have also been used to deliver DNA into cells as efficient delivery tools in gene therapy [8]. Recently, *Leishmania tarentolae (L. tar)*, a unicellular eukaryotic protozoan, has been established as a candidate for heterologous genes expression and as an in situ protein-delivery vehicle [9, 10]. In this study, the protective effects of vaccination with recombinant *L. tarentolae* expressing HPV16 E7 fused to the C-terminal domain of gp96 (HPV16 E7-CT (gp96)) and DNA vector expressing E7-CT (gp96) delivered by electroporation were investigated in a tumor mouse model.

MATERIALS AND METHODS

Plasmid DNA. DNA constructs containing E7-GFP and E7-CT (gp96)-GFP (pLEXSY-E7, pLEXSY-E7-CT (gp96)) [11-12] were purified by Midi-kit (Qiagen) according to the manufacturer's instruction. DNA concentration and purity was determined spectrophoto-metrically by measuring the absorbance at 260/280 nm.

Parasite growth. The transgenic *L. tar*-E7 and *L. tar*-E7-CT (gp96) strains [12] were grown at 26°C in complete M199 medium. Promastigote forms were harvested by centrifugation at 3000 rpm for 10 min and were washed in PBS. Expression of E7 and E7-CT (gp96) proteins in promastigote stage of the recombinant L. tar was evaluated by Epi-fluorescent microscopy and Western blot analysis using anti-HPV16 E7 monoclonal antibody (1:10000 v/v, USBiological) under standard procedures as described previously [12].

Cells. TC-1 (ATCC number: CRL-2785) cancerous cell line [13] was cultured in complete RPMI 1640. On the day of tumor challenge, TC-1 cells were harvested by trypsinization, counted and re-suspended in PBS.

Mice immunization. Inbred C57BL/6 female mice, 6–8 week old were obtained from the breeding stocks maintained at the Pasteur Institute of Iran. All animal procedures were performed in accordance with the recommendations for the proper use and care of laboratory animals. Five groups of 6 mice were selected (Table 1). For DNA immunization, C57BL/6 mice (n = 6) were subcutaneously injected using electroporation twice at 3-week intervals with 50 µg of pEGFP-E7 [G1] and pEGFP-E7-CT (gp96) [G2]. Electroporation was performed with field strength of 60 V/cm (constant), 8 pulses for 200 ms each, using an ECM 830 electroporator (BTX). For live strategies, groups were immunized subcutaneously (s.c.) in the footpad twice at 3-week intervals with 2×10^7 stationary-phase recombinant *L. tar*. promastigotes expressing E7 [G3] and E7-CT (gp96) [G4] proteins. The control mice were treated with PBS [G5].

Table 1. DNA and Live vaccine modalities in C57BL/6 mice model

Group	First immunization	Second immunization	Challenge
1	pEGFP-E7	pEGFP-E7	TC-1
2	pEGFP-E7-CT (gp96)	pEGFP-E7-CT (gp96)	TC-1
3	*L. tar*-E7	*L. tar*-E7	TC-1
4	*L. tar*-E7-CT (gp96)	*L. tar*-E7-CT (gp96)	TC-1
5	PBS	PBS	TC-1

***In vivo* tumor protection.** Three weeks after the second immunization, mice were challenged subcutaneously with 1×105 TC-1 cells/mice in the right flank and tumor progression was monitored for 50 days after the challenge. The tumor growth was monitored by palpation twice a week and survival rates documented. At each time point, tumor size was determined by measuring the smallest diameter (a) and the largest diameter (b) by caliper.
The tumor volume was calculated using the formula: V = (a2b)/2 [14].

Cytokine assay. Six weeks after TC-1 challenge, two mice from each group were euthanized and the spleens were removed and 2×10^6 cells/ml of red blood cell-depleted pooled splenocytes were re-suspended in complete RPMI medium 1640, supplemented with 5% FCS. The cells were incubated in U-bottomed, 96-well plates (Costar, USA) in the presence of 10 µg/ml of rE7 or 10 µg/ml of rE7 + rCT (gp96) proteins and RPMI + 5% FCS and 5 µg/ml of concanavalin A (ConA) were used as negative and positive controls respectively. The cells were cultured for 3 days at 37°C and 5% CO2 and their supernatants were collected and frozen at −70°C till used. The presence of interferon-γ was measured using a DuoSet ELISA system (R&D Systems, USA) according to the manufacturer's instructions. All data were represented as mean ± SD of duplicate tests for each set of samples. The detection limit was 2 pg/ml for IFN-γ.

Statistical analysis. Statistical analysis was performed using Prism 5.0 software (GraphPad, San Diego, CA). One-way ANOVA and Student's t-test were performed to analyze cellular immune responses as well as the tumor volume measurements and compare individual data points, respectively. In the tumor protection experiment, the percentage of the tumor-free mice in different groups was analyzed by log-rank analyses. For all analyses, $p < 0.05$ was considered statistically significant. Data are presented as mean ± standard deviation (SD).

RESULTS

E7-CT (gp96) fusion DNA vaccine delivered by electroporation significantly increased E7-specific IFN-γ responses compared to live *L. tar*-E7-CT (gp96) vaccine. The levels of antigen-specific IFN-γ, induced by vaccination with DNA and live construct at 6 weeks post TC-1 challenge, as a critical indicator of anti-tumor immunity was evaluated. As shown in Fig. 1, mice immunized with the E7-CT (gp96)

DNA vaccine formulation exhibited significantly higher E7-specific IFN-γ response in comparison with *L. tar*-E7-CT (gp96) live vaccine [$p < 0.05$].

Mice were immunized with E7 DNA, E7-CT (gp96) DNA, *L. tar*-E7, *L. tar*-E7-CT (gp96) and PBS twice at 3-week intervals. TC-1 cancerous cells were injected 3 weeks after second immunization. At six weeks after TC-1 challenge, splenocytes from two mice were prepared and stimulated with rE7 and rE7 + rCT (gp96) proteins. The levels of IFN-γ-producing T cells against each recombinant protein were determined using ELISA. Data are presented as the mean ± SD. * means statistically significant results.

Vaccination with pEGFP-E7-CT (gp96) increases protection of mice as compared to *L. tar*-E7-CT (gp96) against TC-1 tumors. To evaluate the anti-tumor effects of DNA and live vaccine candidates, in vivo tumor protection experiments were performed by challenging vaccinated mice with TC-1 tumor cells. The data showed that ~43% of mice receiving E7-CT (gp96) DNA vaccination [G2], remained tumor-free 50 days after the TC-1 challenge, whereas only ~14% of mice receiving E7 DNA vaccination [G1] remained tumor-free (data not shown). In addition, mice receiving PBS [G5] developed tumors within 7–30 days after the challenge.

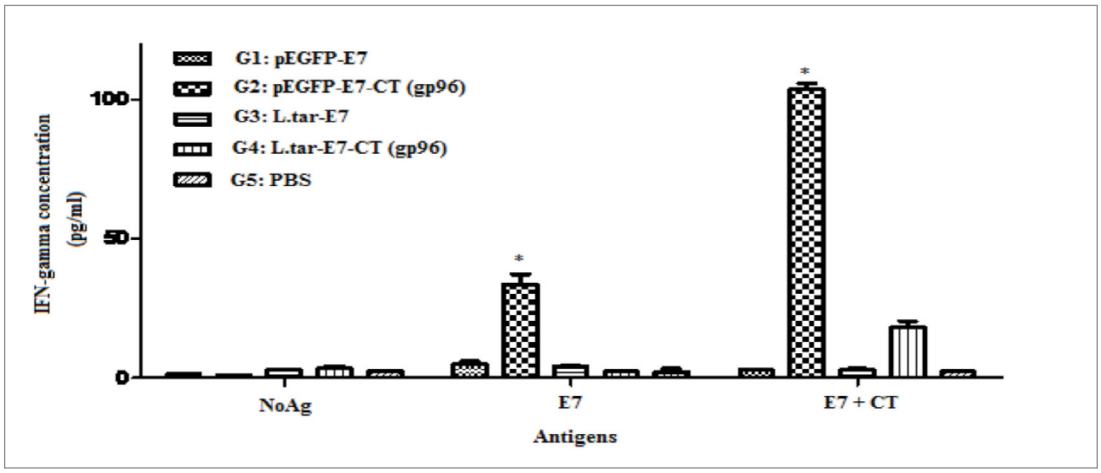

Fig. 1. E7-CT (gp96) fusion DNA vaccine delivered by electroporation significantly increased E7-specific IFN-γ responses compared to live *L. tar*-E7-CT (gp96) vaccine (*$p<0.05$).

E7-CT (gp96) DNA vaccination [G2] resulted in significant survival rates in comparison with the control [G5] and groups immunized with *L. tar*-E7 and *L. tar*-E7-CT (gp96) [G3 and G4; $p < 0.05$]. The tumor growth was delayed approximately 25 days in mice vaccinated with the E7-CT (gp96) DNA after TC-1 challenge. C57BL/6 mice immunized with *L. tar*-E7-CT (gp96) [G4] were significantly better protected compared to the control groups even after 50 days ($p < 0.05$) (Fig. 2). In addition, no significant protection was observed in the group vaccinated with *L. tar*-E7-CT (gp96) in comparison with groups immunized with E7-CT (gp96) DNA vaccine after TC-1 challenge. These results showed that E7- CT (gp96) fusion DNA could significantly enhance the anti-tumor immunity compared to live *L. tar*-E7-CT (gp96) vaccine.

Three weeks after the last immunization, the mice were subcutaneously challenged with 1×105 TC-1 tumor cells. The tumor growth was then measured twice a week and the tumor volumes were calculated. Rapid and exponential tumor growth was observed in mice in PBS group, with tumors appearing only 7 days after TC-1 challenge. Mice in the electroporated group [E7-CT (gp96) DNA] showed a significantly higher protection rate compared with other groups at 50 days after TC-1 challenge. The tumor volumes are presented as the mean ± SD. *$p < 0.05$.

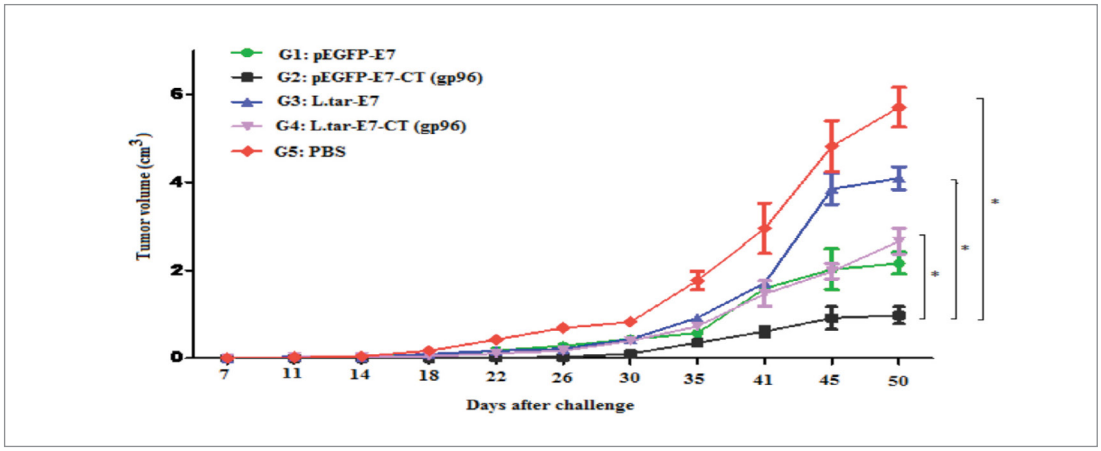

Fig. 2. Prophylactic anti-tumor effects of DNA and live candidate vaccines for HPV.

DISCUSSION

The objective of this study was to compare two different means of antigen delivery, namely live transgenic *L. tar* and naked DNA-based vaccines for protection against TC-1 tumor in C57BL/6 mice. DNA vaccines are attractive tools for the development of HPV vaccines. However, due to their low immunogenicity, there is a need to enhance DNA-based vaccine potency by the use of immuno-adjuvants such as heat shock proteins [14-16] and electroporation delivery systems [16, 17]. We have previously shown that subcutaneous DNA injection with E7-CT (gp96), followed by electroporation can generate a significant E7-specific IFN-γ immune response as well as a high protective effect in vaccinated mice compared to E7 or E7-NT (gp96) DNA vaccines [11]. Attenuated or non-pathogenic live vectors such as *L. tar* have been developed and used specifically to deliver DNA into cells as efficient delivery tools in gene therapy [18]. In this study, *L. tar*-E7-CT (gp96) live vaccine produced a significant protective effect in immunized groups compared to the non-vaccinated as well as the group vaccinated with *L. tar*-E7 [12]. We also measured and compared the production of IFN-gamma, the tumor growth and the survival rates in groups of mice which received transgenic *L. tar* or DNA-based candidate vaccines and challenged them with TC-1 tumor cells, to determine the best strategy for providing protection against HPV. Our results demonstrated that the E7-CT (gp96) DNA vaccine induced higher E7-specific IFN-γ response compared to the live vaccines at 6 weeks after TC1 challenge. In addition, DNA vaccine expressing E7-CT (gp96) was more effective prophylactically than the *L. tar*-E7-CT (gp96). The DNA formulation conferred ~43% protection compared to 0% tumor-free mice for *L. tar*-E7-CT (gp96) in the challenge experiments. This result indicated that *L. tar*-E7-CT (gp96) cannot produce a stable protection. The previous studies have demonstrated that unlike other pathogenic Leishmania strains, *L. tar* lacks the potential to replicate within the targeted APCs and is eliminated after several days from the infected murine host [18]. Therefore, non-pathogenic *L. tar* genome needs to be modified to enable the parasite to replicate and survive in eukaryotic hosts for long term protection *in vivo*.

Plasmid DNA vaccination using skin electroporation to the sites of plasmid DNA injection has allowed for dramatic increases in the immune responses compared with the plasmid DNA injection alone while limiting invasiveness of the delivery [19]. Electroporation has been successfully used to administer HPV DNA vaccine to mice as well as rhesus macaques which has prompted its use in an ongoing phase I clinical trial such as VGX-3100, a vaccine that includes plasmids targeting E6 and E7 proteins of both HPV subtypes 16 and 18, for treatment of patients with CIN 2 or 3 [16]. In summary, this study confirmed our previous results regarding the potential value of E7-CT (gp96) DNA vaccination [pEGFP-E7-CT (gp96)] using electroporation as well as its high potency compared to the live parasite vaccination. Further studies are required to generate more potent DNA vaccine candidates using immuno-adjuvants along with physical delivery systems.

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CONFLICT OF INTEREST

No potential conflicts of interest were disclosed.

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