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VLP production in *Leishmania tarentolae*: A novel expression system for purification and assembly of HPV16 L1

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ABSTRACT

Viral like particles (VLPs) have been used as immunogen for improvement of preventive vaccines against several viral infections in preclinical and clinical trials. These constructs can stimulate both cellular and humoral immunity. Two prophylactic HPV L1 VLP vaccines known as Gardasil and Cervarix were commercialized worldwide. However, there are main problems for expression and purification of VLPs in eukaryotic expression systems such as *baculovirus* and yeast leading to high cost of these vaccines. A novel *Leishmania* protozoan system has been applied to produce different recombinant proteins due to unique properties including generation of similar proteins with mammalian, easy handling, and large-scale culture. In the current study, we developed a novel strategy to produce HPV L1 VLP using stably transfected *Leishmania* cells. The positive transfectants were analyzed by SDS–PAGE and Western blot analysis. The assembly of purified L1 protein was detected by TEM microscopy. Finally, C57BL/6 mice were immunized by crude VLPs and antibody responses were assessed. The results of electronic microscopy revealed average 55–60 nm for L1 VLP. Furthermore, high IgG1 and IgG2a antibody responses were generated by L1 VLPs in mice similar to L1 VLPs produced in *baculovirus*-infected insect cells. Regarding the results, the amount of recombinant protein generated by *Leishmania* was 2–3 mg/500 ml media, suggesting further optimization of this system for using in large animals and human.

1. Introduction

Viral like particles (VLPs) are produced by structural proteins of viruses which have an inherent property for self-assembly, and imitate the morphology of the pathogen [1,2]. Generally, human papillomavirus (HPV) virions contain 360 copies of L1 and up to 72 copies of L2, which assemble into an icosahedral structure with one L2 molecule being at the central opening of each capsomere [3]. VLPs demonstrate a main development in subunit vaccines with enhanced immunogenicity [1,2]. VLPs possess the size and morphology of viruses (~22–200 nm), depending on the specific viral proteins being included [4]. Up to now, two HPV vaccines (quadrivalent Gardasil and bivalent Cervarix) based on the VLP structures were approved by FDA for human use. In these vaccination strategies, the recombinant HPV L1 major capsid protein was self-assembled into VLPs in yeast and/or insect cell expression systems. The size of VLPs was ~60 nm in diameter, similar to native virions [2,5]. Regarding some studies, there are different recombinant protein expression systems such as *E. coli*, yeast, insect, plant, and mammalian cells for generation of VLP-based candidate vaccines targeting various viral, bacterial, parasitic and fungal pathogens, as well as non-infectious diseases [5,6]. These expression systems have shown some disadvantages including lack of post-translational modifications, improper protein folding, and formation of inclusion bodies in prokaryotic systems; contamination, high cost, low yield, hyper-glycosylation in eukaryotic systems [4,6]. Recently, the ability of the eukaryotic parasite "*Leishmania tarentolae*" was confirmed to express different recombinant eukaryotic and biomedical proteins [7]. *Leishmania tarentolae* (*L. tarentolae*) shows some benefits compared to the mammalian cell lines such as: (a) the parasite is non-pathogenic for human; (b) it is fairly easy to cultivate; (c) it produces suitable recombinant protein yields (0.1–30 mg/L or more than), and (d) it can easily be improved to an industrial scale [8–11]. Furthermore, development of constitutive and inducible-integrative expression vectors for *Leishmania* led to the generation of secretory or intracellular recombinant proteins. By homologous recombination, the expression cassette is

Abbreviations: HPV, Human papillomavirus; VLP, Viral like particles; *odc*, Ornithin decarboxylase; DAB, Diaminobenzoic Acid; TEM, Transmission electron microscopy; OPD, O-Phenylenediamine; LM, Laminin; EMCV, Encephalomyocarditis virus.

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either inserted into the chromosomal 18S ribosomal RNA locus (*ssu*: constitutive system) or into the ornithin decarboxylase locus (*odc*: inducible-integrative system) of *L. tarentolae*. *Ssu* is a repetitive locus of the *L. tarentolae* genome with high rates of transcription by the host RNA polymerase I, whereas the *odc* locus is transcribed by RNA polymerase II [12]. In current study, we attempted to generate the recombinant L1 protein of HPV16 in the *L. tarentolae* expression system and also its assembly as viral like particle. In addition, its immunogenicity was studied in C57BL/6 mice model compared to VLPs produced in insect cells. The production of VLP in parasite (*L. tarentolae*) was done for first time in the world.

2. Material and methods

2.1. Gene construct

HPV16 L1 gene (Accession number: AJ313179.1) from pUF3-L1 vector (provided kindly by Prof. Martin Muller) was cloned into a cloning vector pGEM-7Zf (+) and sequenced. Then, the correct insert was subcloned into *Xho*I and *Kpn*I sites of an inducible LEXSY expression vector pLEXSY-I-blecherry3 (JenaBioscience). The resulting construct was designated as pLEXSY-I-L1 (Fig. 1). The plasmid was produced at high concentration using Midi DNA extraction kit (Qiagen).

2.2. Generation of stable Leishmania clones expressing HPV16 L1

Digestion of 15 μg pLEXSY-I-L1 was performed by *Swa*I restriction enzyme. This treatment generated a 2000 bp fragment representing the *E. coli* part and a large fragment containing the linearized pLEXSY-I-L1 integrated into the chromosomal *odc* locus of the LEXSY host (T7-TR). The EGFP control plasmid (pLEXSY-I-egfp-blecherry3: Cat. No. EGE-246) was used as a positive control. Gel extraction of the linearized plasmid was performed with an agarose Gel Extraction Kit (Qiagen). Then, the promastigotes of *L. tarentolae* (*L. tar*: T7-TR) were grown in Brain–heart infusion medium (BHI, Sigma) and also Medium 199 (M199, Sigma) at pH = 7.2 and 26 °C for comparison of culture medium efficiency. For transfection, the linearized pLEXSY-I-L1 (8–10 μg) was electroporated into 4×10^7 log phase parasites in 2 mm cuvettes at 450 V and 500 μF using Bio-Rad Gene PulserEcell. After two pulses, the recombinant stable transfectants were selected on two different solid media (BHI-agar and M199-agar) containing 50 μg/ml of bleomycin (Jena Bioscience, Germany). For maintaining T7 polymerase and TET repressor genes in the host genome, two antibiotics (LEXSY NTC and LEXSY Hygro) were added in final concentration of 50 μg/ml for each marker. For genomic analysis, transcription and expression assays, the recombinant clones were cultured in BHI and also M199 media containing 50 μg/ml of bleomycin at 26 °C.

2.3. Confirmation of genomic integration

The genomic DNA of recombinant *L.tar*-L1 from 1 ml of culture was prepared by the DNeasy® Blood and Tissue Kit (GF-1). Integration of pLEXSY-I-L1 into the genome was performed by diagnostic PCR using L1 primer pairs (L1F/L1R: below) and also 5′*odc* forward/

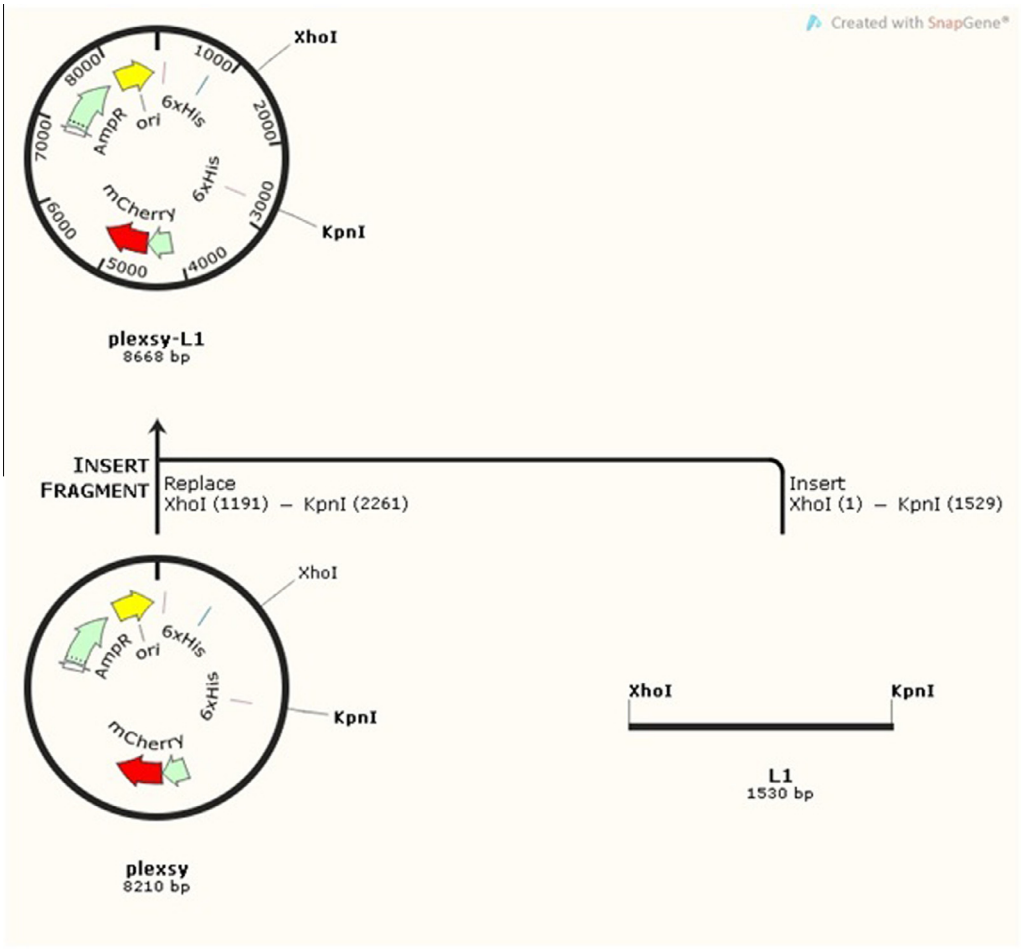

Fig. 1. Schematic model of pLEXSY-I-L1 construct by SnapGene.

utr1 (*aprt*) reverse primers as mentioned in Jena Bioscience manual.

Forward L1: 5′ATA **TCT AGA CTC GAG** ATG AGC CTG TGG CTG CCC 3′ [***Xba*I-*Xho*I**].

Reverse L1: 5′TAT **GGT ACC AAG CTT** CAG CTT CCT CTT CTT CCT 3′ [***Kpn*I-*Hind*III**].

2.4. Expression, purification and detection of the assembled L1 protein

Total RNA samples were extracted from 10^8 recombinant promastigote as well as wild type *Leishmania* cells using RNeasy mini-kit (Qiagen) according to the manufacturer's instructions. The cDNA synthesis was performed using Omniscript Reverse Transcriptase kit (Qiagen) from 1 mg of RNA. Amplification of the L1 fragment (1515 bp) was carried out by L1 primers under standard conditions. Furthermore, the expression of L1 protein in *Leishmania* was confirmed by SDS–PAGE and western blotting of cell extracts. Induction of protein expression was performed by 10 μg/ml Tetracycline in final concentration. For obtaining optimal expression, different cultivation/induction conditions, and times of harvest (24, 48, 72 h) were checked. For western blot analysis, cell lysate was separated on 12% SDS–PAGE and electrophoretically transferred onto nitrocellulose membrane. The membrane was blocked with 1% BSA at room temperature. The specific L1 monoclonal antibody (MD2H11, kindly provided by Prof. Martin Muller, German Cancer Research Center) was used in 1:5000 dilutions. The expected band of L1 protein was detected by DAB (Diaminobenzoic Acid, Sigma) as the substrate. Finally, the recombinant L1 protein was purified by affinity chromatography on Ni–NTA resin column using 6xHis-tag according to the manufacturer's instructions (Qiagen). Purification of the recombinant L1 protein was done under native conditions. Protein concentration was measured using BCA assay kit (Pierce, Rockford, USA). The purified VLP from insect cells (provided kindly by Prof. Martin Muller) was used as a control. For detection of L1 assembly and VLP formation, the structure and size of particles were determined by transmission electron microscopy (TEM).

2.5. Mice immunization

Three groups of 6–8-week-old female C57BL/6 mice ($n = 5$) were obtained from breeding stock maintained at the Pasteur Institute of Iran. All mice were maintained under specific pathogen-free conditions and all procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. Mice were immunized three times with two-week intervals as: group 1 (Test: 10 μg VLP L1/VLP L1 produced in *Leishmania*), group 2 (Control: 10 μg VLP L1/VLP L1 produced in insect cells), and group 3 (Control: PBS/PBS).

2.6. Assessment of antibody response

Mice sera from each group were collected 3 and 6 weeks after the third injections. The levels of IgG1 and IgG2a were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, a 96-well flat-bottom ELISA plate (Greiner) was coated overnight at 4 °C with L1 VLP as an antigen (5 μg/ml) diluted in PBS (pH = 7.2). Then, the plate was rinsed with washing buffer (0.5% (v/v) Tween-20 in PBS), incubated with blocking buffer (1% BSA in PBS) for 2 h at 37 °C. The pooled sera were diluted 1:50 in dilution buffer (0.5% (v/v) Tween-20 in blocking buffer), added to the plate and incubated for 2 h at 37 °C. After rinsing with washing buffer, the plate was incubated with biotin-conjugated goat anti-mouse IgG1 or IgG2a (diluted 1:1000 in 1% BSA/PBS-Tween, Southern biotechnology Association Inc, USA) for 2 h at 37 °C. Then, the plates were washed and incubated with streptavidin-horseradish peroxidase diluted in PBS (1:100,000; Sigma) at 37 °C for 1 h. Detection was done with 100 μl of O-Phenylenediamine (OPD, Sigma) as the substrate in citrate phosphate buffer (pH = 4.5), followed by incubation for 30 min at 37 °C. The enzyme reaction was stopped by 1 M H_2SO_4 and the absorbance was measured at 492 nm.

2.7. Statistical analysis

Statistical analysis was performed using Prism 5.0 software (GraphPad, San Diego, California, USA). One-way ANOVA was performed to analyze humoral immune responses. For all comparisons, $p < 0.05$ was considered statistically significant. Data are presented as mean ± standard deviation (SD).

3. Results

3.1. Production and assembly of HPV16 L1 protein in Leishmania system

The recombinant pLEXSY-I-L1 plasmid was prepared in large scale with high purity, linearized and electroporated into parasites. The transformants were selected by plating on solid medium containing bleomycin. Two culture media, M199 and BHI was used to evaluate the influence of culture medium on the production of HPV16 L1 protein in recombinant *Leishmania*. Integration of L1 gene in the genomic DNA of the recombinant cells was confirmed by PCR analysis. The expected 1.1 kb band was only observed from transformed cells indicating the correct integration of expression constructs into the *odc* locus. The expected PCR product of L1 was appeared as ~1515 bp fragment for L1 positive clones. To verify mRNA synthesis, RT-PCR was performed on total mRNA extracted from wild and transformed cells. The desired band of L1 (~1515 bp) was obtained in transgenic *Leishmania*. Protein expression was also detectable in cell extracts of transgenic parasites compared to wild type by western blotting. The dominant band of 60 kDa was detected in transgenic parasites expressing L1. No such corresponding band was revealed in the wild-type *Leishmania*. The best time of protein expression was obtained 48 h after induction by tetracycline. In addition, the results showed that recombinant protein expression was higher in M199 culture medium than that in BHI media. Immobilized metal-affinity chromatography (IMAC) was used to purify L1 protein. This method is based on the interaction between a transition metal ion (e.g., Ni^{2+}) immobilized on a matrix and specific amino acid side chains (e.g., Histidine). Herein, L1 protein containing sequences of consecutive histidine residues were efficiently retained on IMAC. Following washing of the matrix material, L1 protein containing polyhistidine sequences could be easily eluted by adding free imidazole. Indeed, the protein was purified by affinity chromatography under native condition (300 mM Imidazole) and the purified protein migrated as a 60 kDa protein in SDS–PAGE (Fig. 2A). The amount of recombinant protein generated by *Leishmania* was 2–3 mg/500 ml media. Microscopic analysis showed the formed particles similar to viral capsid named as VLP with 55–60 nm size (Fig. 2B).

3.2. Immunization with HPV16 L1 VLP induces the mixture of IgG1 and IgG2a with high intensity toward IgG2a in C57BL/6 mice

We evaluated the levels of IgG1 and IgG2a antibodies against VLP antigen using ELISA, 3 and 6 weeks after third immunization. As shown in Fig. 3, IgG2a and IgG1 levels were significantly higher in groups I and II injected with VLPs than those in control group ($p < 0.05$). Interestingly, the levels of antibody did not have any dif-

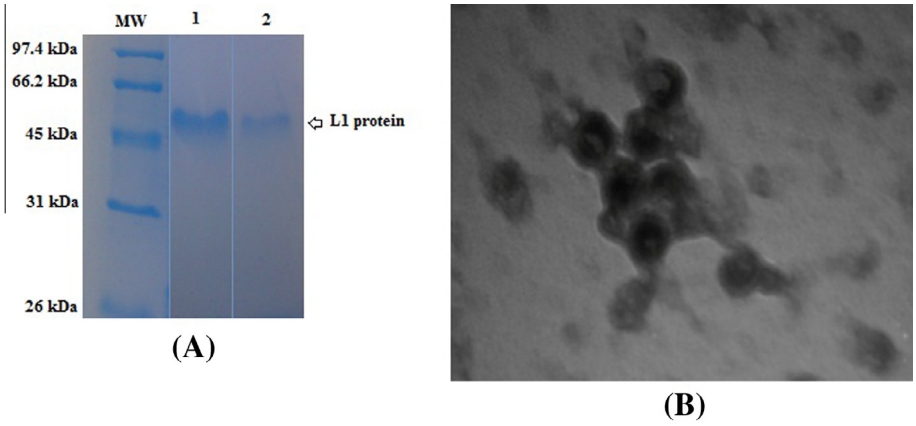

Fig. 2. (A) L1 protein purified by affinity chromatography migrated as a 60 kDa protein in SDS–PAGE. Lane 1: purified protein from insect cells, Lane 2: purified protein from *Leishmania*; (B) TEM analysis for detection of VLP formation.

ferences 3 and 6 weeks after immunization indicating the stability in humoral response. In addition, the ratio of IgG2a/IgG1 was twice in VLP-injected group indicating the direction of response toward IgG2a.

4. Discussion

Self-assembly of the recombinant viral proteins into empty capsids is an excellent strategy for generation of VLP-based vaccines and stimulation of a protective immune response similar to virions. VLPs present antigenic epitopes in the correct conformation resulting in B cell activation and antibody production. Regarding the complexity of VLP, it can be generated in either a prokaryotic or eukaryotic expression system using target gene-encoding recombinant vectors [6]. Recently, a *Leishmania*-based expression system was developed to produce different recombinant proteins (secretory or cytoplasmic) such as human laminin (LM)-332, FVII [13,14], human p53 [15], and LPG3 [16]. In current study, for first time, we improved a novel strategy to produce HPV VLP using stably transfected *Leishmania* cells. We could transfect *Leishmania* cells, with plasmid encoding HPV L1 protein, and a selection marker (bleomycin). After determination of stably transfected clones, the assembly of the crude VLPs and their immunogenicity were carefully evaluated in mice model. The size of VLP produced in *Leishmania* and also the levels of antibody responses were comparable to those generated by insect cells infected with recombinant *baculoviruses*. In addition, there was no severe reaction in injection site after VLP-based immunizations. Many studies indicated the advantages of VLP-based vaccines in eliciting immune responses. In these experiments, some agents such as type of protein for VLP assembly, VLP doses for immunization, type of vectors and expression systems were evaluated. Herein, we mention some VLPs produced in insect cells as an efficient expression system:

Porcine encephalomyocarditis virus (EMCV) virus-like particles were generated from Sf9 cells infected with recombinant *baculovirus* and were confirmed to be about 30–40 nm by TEM. Mice immunization with crude protein containing the VLPs led to 90% protection from EMCV infection [17]. In addition, immunization of BALB/c mice with EV71 VLP elicited potent and long-lasting humoral immune responses as shown by the high total IgG titer [18]. In a study, the evaluation of *influenza* vaccine candidate antigens manufactured using Sf9/*baculovirus*-based VLP technology showed a dose-dependent effect in VLP-vaccinated groups [19]. On the other hand, mice immunization with the RSV VLPs, without adjuvant, induced high anti-RSV F and G protein antibody responses. IgG2a/IgG1 ratios were very high, suggesting mostly Th1 responses. Also, immunization with a single dose of VLPs led to the complete protection of mice from RSV replication in lungs [20]. In other study, two types of *flaviviruses* virus-like particles were identified including F-VLPs (40–50 nm) and S-VLPs (20–30 nm) as a promising vaccine candidate. The data showed that IgG2a was major isotype versus IgG1 by immunization with F-VLPs. Also, mice vaccinated with a low dose of F-VLPs indicated higher protective efficacy (~83%) than mice immunized with S-VLP (~17%) against WNV infection. Indeed, F-VLPs resemble the virions and can produce an effective immune response as WNV vaccine candidates [21]. However, comparing to these systems,

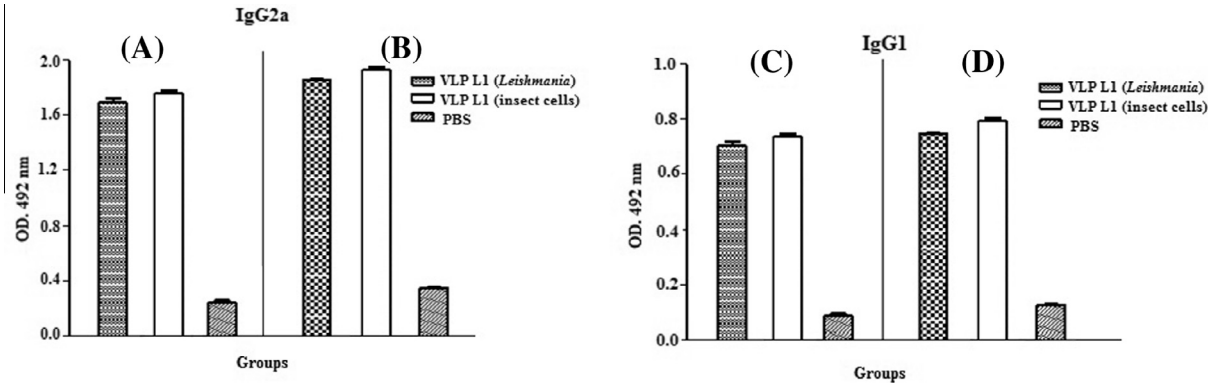

Fig. 3. Analysis of IgG2a (A & B) and IgG1 (C & D) antibodies against L1 VLP in each group, 3 (A & C) and 6 weeks (B & D) after third immunization. Data represent means ±SD and each assay was performed in duplicate. The p values <0.05 have indicated statistically significant differences between G1/G2 and G3 immunized mice.

Leishmania cells are easy to maintain, requiring no CO_2 incubator or shaker, can be grown in standard tissue culture media, can be readily transfected with plasmid DNA by electroporation and the recombinant clones can easily selected with antibiotic resistance. Indeed, this system can be used as an alternative to other eukaryotic systems because of simple genetic manipulation, suitable protein folding, and low-cost production. Briefly, *L. tarentolae* has been used to screen for anti-leishmanial drugs, to study RNA editing and gene amplification, to express eukaryotic recombinant proteins as well as to immunize against leishmaniasis in preclinical trials [12]. However, its use for VLP production was done for the first time. Regarding our results, VLPs produced in this system could increase humoral immune responses, with intensity toward IgG2a similar to other studies. In addition, the immune responses generated by *Leishmania*-based VLPs were similar to insect-based VLPs suggesting the same structures for displaying antigenic epitopes. However, further optimization will be needed to provide high amounts of VLPs and also induce virus-neutralizing antibodies in near future.

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