

Cloning and Expression of *Leishmania infantum* LPG3 Gene by the Lizard *Leishmania* Expression System

Leila Pirdel¹, Ahmad Zavarani Hosseini^{1*}, Bahram Kazemi², Manoochehr Rasouli³,
Mojgan Bandehpour², and Sara Soudi¹

1. Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

2. Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

3. Department of Immunology, Clinical Microbiology Research Centre, Shiraz University of Medical Sciences, Shiraz, Iran

Abstract

Background: Various prokaryotic and eukaryotic expression systems have been developed for the production of recombinant proteins. In the present study, we used a new protein expression system based on the Iranian Lizard *Leishmania*, a trypanosomatid protozoan as a host, for the expression of LPG3 gene from *Leishmania infantum* (*L.infantum*).

Methods: The LPG3 gene was cloned in the expression cassette for integration into the small subunit of the ribosomal RNA locus of Lizard *Leishmania* genome by electroporation. Expression of the recombinant LPG3 protein was confirmed by western blotting and immunofluorescence staining.

Results: Western blotting confirmed the expression and production of rLPG3 protein. Immunofluorescence analysis also revealed the staining throughout the cytoplasm of transfected parasites, indicating that the protein has been expressed.

Conclusion: These results demonstrate that *Leishmania* cells can be suggested an expression system for the production of recombinant LPG3 (rLPG3) to further research in vaccine designing against leishmaniasis.

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Keywords: *Leishmania infantum*, *Leishmania*, Recombinant proteins, Vaccines

* **Corresponding author:**
Ahmad Zavarani Hosseini,
Ph.D., Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
Tel: +98 21 82883090
Fax: +98 21 88220144
E-mail:
zavarana@modares.ac.ir
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Introduction

The family Trypanosomatidae (Euglenozoa, Kinetoplastida), which includes genera *Leishmania*, is one of the oldest groups of eukaryotes with a number of species that causes a range of debilitating or fatal diseases⁽¹⁾. About 200 million people are at risk of VL in 70 countries with estimated annual incidence of 500,000, and 50,000 deaths a year⁽²⁾. Lack of an effective vaccine and the emergence of drug-resistant strains have made most intervention attempts extremely challenging for the identification of new targets for development of vaccine⁽³⁾. Therefore, the need for

novel systems for the expression of recombinant proteins from protozoan parasites has become a pressing matter.

Leishmania produces a range of glycoconjugates containing phosphoglycan (PG) and two of the most studied abundant surface constituents are the GPI-anchored molecules lipophosphoglycan (LPG) and GP63 zinc metalloprotease⁽⁴⁾ that play important roles in parasite survival and pathogenicity⁽⁵⁾.

LPG3 is one of the class II LPG genes that encodes the *Leishmania* homolog of the mammalian Endoplasmic Reticulum (ER) chaper-

one GRP94, which belongs to HSP90 family⁽⁶⁾. It is involved in variety of processes including antigen presentation, folding and assembling of proteins, and secretory pathway. It also contains antigenic and immunogenic properties similar to other conserved antigens of the parasite^(7,8).

Although a variety of prokaryotic and eukaryotic expression systems have been developed for the synthesis of recombinant proteins such as bacteria, yeast, fungi, insect cells, mammalian cells, transgenic animals, and transgenic plants, none is universally applicable⁽⁹⁾. *Leishmania* has many advantages to use in biotechnological applications. Recently, *Leishmania tarentolae*, a nonpathogenic parasite of the gecko *Tarentola annularis*, has been established as a new eukaryotic expression system for the production of recombinant proteins. In addition, it has already been used successfully for the expression of erythropoietin⁽¹⁰⁾, tissue plasminogen activator⁽¹¹⁾, IFN- γ ⁽¹²⁾, human laminin-332⁽¹³⁾ and proprotein convertase 4 (a member of Ca²⁺ dependent mammalian subtilases)⁽¹⁴⁾. Most recently, the Iranian Lizard *Leishmania* has been used as an expression system for producing rFVII⁽¹⁵⁾.

Here, cloning and expression of LPG3 gene from *L.infantum* in the Iranian Lizard *Leishmania* are carried out for producing rLPG3 as a preliminary step for further investigation into vaccine development.

Materials and Methods

Amplification of LPG3 gene

Genomic DNA of *L.infantum*, cultivated in RPMI 1640 medium (Gibco-BRL, UK) and supplemented with 10% heat-inactivated FBS (Gibco-BRL, UK), 10 mM HEPES, 15 μ g/ml hemin, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Gibco-BRL, UK) at 26 °C, was extracted by phenol: chloroform: isoamyl alcohol extraction (1:1:1) and ethanol precipitation.

Then the LPG3 gene was amplified with forward primer, F-LPG3 (5' AGATCTATGG

CGAACTCGAGCTTGC3') and reverse primer, R-LPG3 (5'GCTAGCCCAGATCGTCCTCGCCGACTG3'), containing *Bgl*III and *Nhe*I restriction sites in each 5' end (underlined). PCR reaction was performed under the following conditions: 95 °C (5 min), 35 cycles of 95°C (1 min), 58°C (1 min), 72°C (2 min) and 72°C (10 min) for final extension. The 2316 bp desired band was cleaned up by QIAquick Gel Extraction Kit (Qiagen, USA) following the manufacturer's protocols.

Cloning of LPG3 gene

The purified PCR product was cloned in pTZ57R cloning vector using InsT/Aclone™ PCR Product Cloning Kit (Fermentas, Lithuania) following the manufacturer's procedures and transformed into competent *Escherichia coli* (*E.coli*) DH5 α . The transformed cells were screened for the presence of recombinant plasmid with the LPG3 insert by gene-specific PCR and analysis with *Bgl*III and *Nhe*I restriction enzymes. Isolated positive clones were sequenced using M13 forward and reverse primers by MWG Operon's Sequencing Service (Germany). The insert was removed by *Bgl*III and *Nhe*I digestion and subcloned into the *Bgl*III-*Nhe*I insertion site of *Leishmania* expression vector pLEXSY-hyg2 to create the recombinant pLEXSY-LPG3 plasmid. The presence of the LPG3 gene in pLEXSY-hyg2 was confirmed by *Bgl*III and *Nhe*I restriction enzymes and PCR amplification.

Cultivation and transfection of Lizard *Leishmania*

Lizard *Leishmania* promastigotes were cultivated in RPMI 1640 supplemented with 5 μ g/ml hemin, 100 IU/ml penicillin and 100 μ g/ml streptomycin (sigma, USA) at 26°C⁽¹⁶⁾. For integration of the expression cassette into the 18S ribosomal RNA (*ssu*) locus, the pLEXSY-hyg2 plasmid containing LPG3 gene was digested by *Swa*I restriction enzyme (Fermentas, Lithuania) and 10 μ g of the heavier fragment was transformed into cultivated Lizard *Leishmania* promastigotes by electroporation *in vitro*. Transgenic cells

were selected as single colonies on the supplemented RPMI 1640-agar medium containing 100 $\mu\text{g/ml}$ hygromycin B (Jena Bioscience, Germany) as a selective antibiotic. To confirm the integration of the LPG3 containing cassette into the *Leishmania* genome, PCR was performed on the genomic DNA of wild type and transgenic cells with LPG3 forward and *ssu* reverse primers (Jena bioscience, Germany):

LPG3 Forward: 5'-AGATCTATGGCGAAC TCGAGCTTGC3 -3'

ssu Reverse: 5'-CTGCAGGTTACCTACAG CTAC -3'

Western blot analysis

The wild type and transgenic cells were harvested and suspended in 50 μl of SDS-PAGE sample buffer containing 1 *Mm* PMSF. Then they were sonicated twice with 70 *Hz* for 20 *s* and boiled for 5 *min*. Twenty μl of the obtained sample was loaded on 10% polyacrylamide gel. Western blotting was performed on similarly prepared acrylamide gel and electrophoretically transferred onto the nitrocellulose membrane. After UV cross-linking for protein fixation, the membrane was blocked with 5% skim milk at room temperature. Mouse monoclonal HRP-conjugated anti-His tag antibody (Abcam, UK) was used in 1:1000 dilutions for 2 *hr* at room temperature. The protein band was detected by Diamino Benzoic Acid (DAB) and H_2O_2 .

Indirect immunofluorescence assay (IFA)

Lizard *Leishmania* promastigotes were fixed with 4% paraformaldehyde for 15 *min* at room temperature, washed three times in PBS, permeabilized by 0.1% Triton X-100 for 5 *min* at room temperature and washed three times in PBS. Subsequently, the cells were blocked for 30 *min* in PBS containing 10% goat serum. After three washes in PBS, the cells were incubated overnight with mouse monoclonal anti-His tag antibody diluted in PBS/10% goat serum (1:40), and FITC conjugated anti mouse IgG antibody diluted in PBS/10%goat serum (1:100) for 1 *hr* at room temperature. After three washes in PBS and

counterstaining with DAPI (4', 6'-diamidino-2-phenylindole), the cells were mounted in glycerol/PBS solution (1:1). Finally, they were examined for fluorescence under Nikon immunofluorescence microscope.

Results

Subcloning of LPG3 gene

A PCR reaction with LPG3-specific primers on the genomic DNA of *L.infantum* resulted in a single band with the expected size of 2316 *bp*. The desired band was ligated into T/A cloning vector, pTZ57R, excised by *Bgl*III

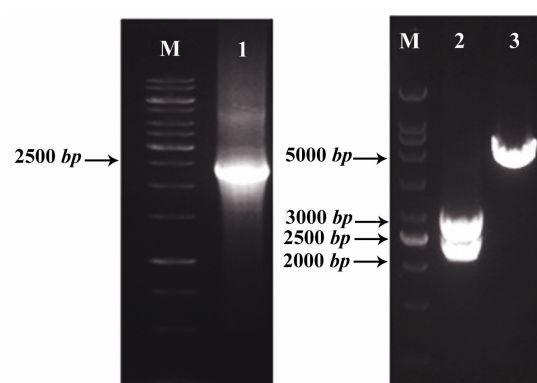


Figure 1. Detection of the recombinant plasmid pTZ57R vector-LPG3 by PCR and restriction enzyme digestion: amplified LPG3 gene (Lane 1), restriction analysis of pTZ57R-LPG3 vector (Lane 2), undigested vector (Lane 3), and 1 *kb* DNA size marker (Lane M). The products were electrophoresed on 1% agarose gel

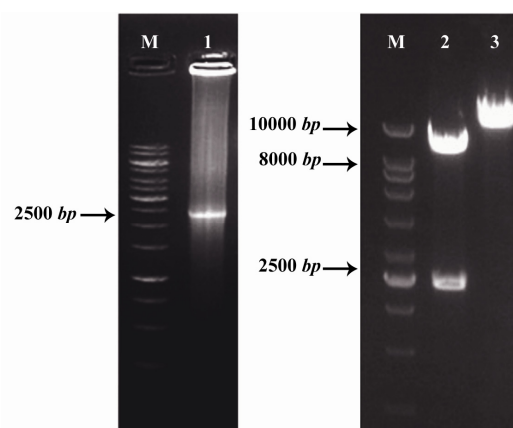


Figure 2. Detection of the recombinant pLEXSY-LPG3 vector by PCR and restriction enzyme digestion: amplified LPG3 gene (Lane 1), restriction analysis of pLEXSY-LPG3 vector (Lane 2), undigested vector (Lane 3), and 1 *kb* DNA size marker (Lane M). The products were electrophoresed on 1% agarose gel

and *NheI* enzymes (Figure 1) and subcloned into the expression vector, pLEXSY-hyg2 containing antibiotic resistance gene, hygromycin B (Figure 2). The sequence of the LPG3 was confirmed by restriction analysis and DNA sequencing (GenBank: HQ400675.1). BLAST assessment of the obtained sequence showed 98% identity with the data available in the GenBank (Figure 3).

Transfection of lizard leishmania cells

The hyg2-LPG3 fragment containing one

copy of the LPG3 obtained from the digestion of pLEXSY-LPG3 with the *SwaI* restriction enzyme was used for integration into the small subunit of Lizard *Leishmania* rRNA gene for homologous recombination by electroporation. After transfection, hyg-resistant cells were selected on semisolid RPMI 1640-agar medium containing 100 $\mu\text{g/ml}$ hygromycin and cultured in supplemented RPMI 1640 containing 100 $\mu\text{g/ml}$ hygromycin B for the second selection. Integration of the ex-

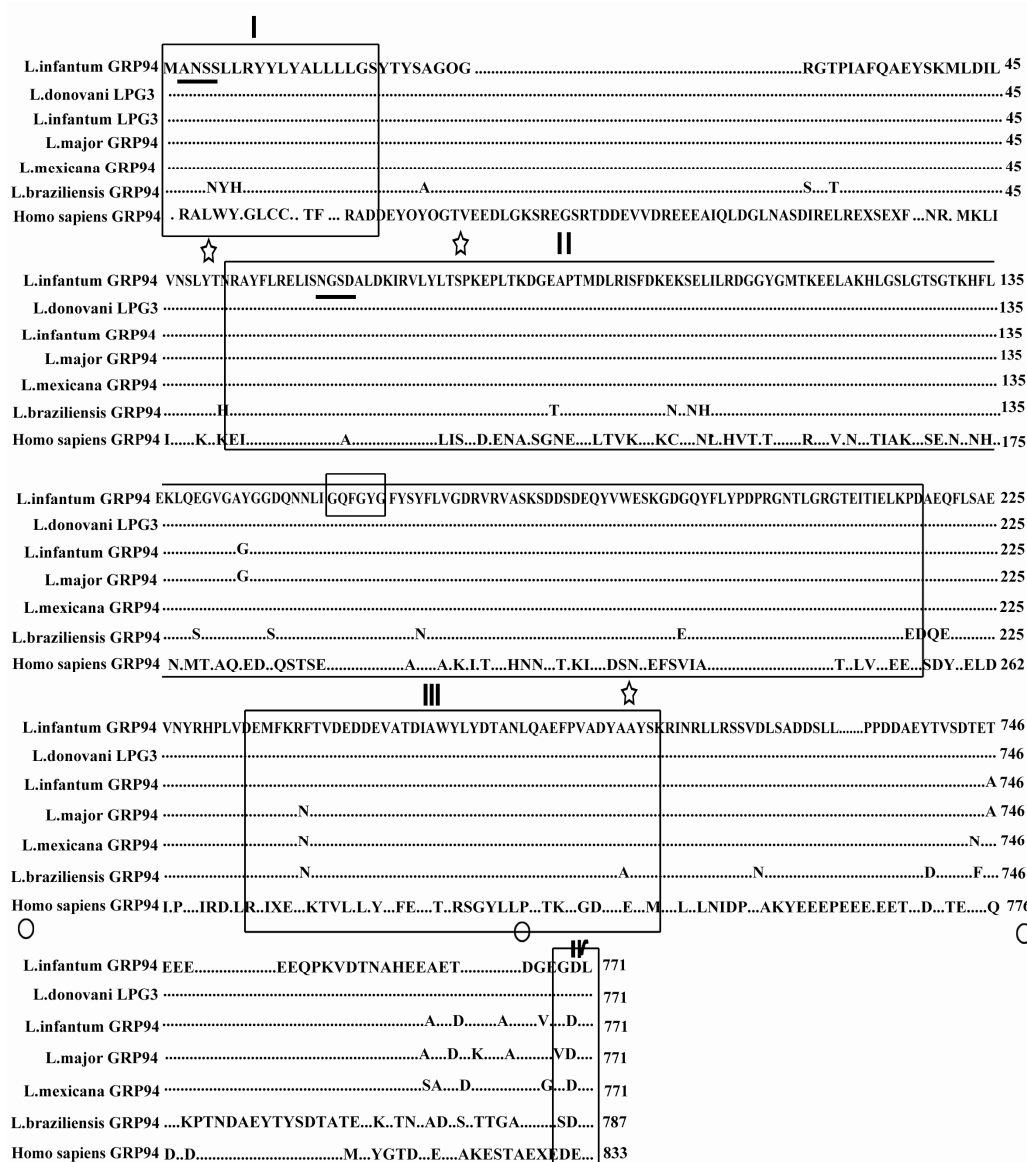


Figure 3. Sequence alignment of the deduced amino acid sequence of *L. infantum* LPG3 with other related proteins. The sequences were aligned with CLC protein workbench 5.5.1 software. The identical residues are indicated by dots. The boxes indicate the signal sequence at the N-terminus (box I), ATPase_c domain (box II), dimerization domain (box III), and the C-terminal ER retention signal (box IV). The GXXGXXG motif (grey letters in box II) is also conserved in all Hsp90 family proteins. Potential N-linked glycosylation sites are indicated by gray underlines and protein kinase C phosphorylation sites are marked by asterisks. Hyphens represent the introduced gaps for the optimum alignment.

pression cassette into the *ssu* locus was confirmed by PCR on the genomic DNA of the recombinant cells using forward LPG3 and reverse *ssu* primers, which generated a 3.5 kb band, demonstrating the occurrence of homologues recombination in the 18 S rRNA locus with the right orientation (Figure 4).

Western blotting

Expression of rLPG3 in Lizard *Leishmania* was investigated by western blot analysis. The expressed rLPG3 was detected in the lysate of transfected cells with mouse monoclonal HRP-conjugated anti-His tag antibody as a single band with an apparent MW of 97 kDa, which is slightly high due to the addition of a hexa-His tag sequence whereas the lysate of wild type cells did not react with monoclonal antibody (Figure 5).

Indirect immunofluorescence

It was necessary to determine the percentage of the transfected and selected cells that expressed the rLPG3 protein. As shown in figure 6, immunofluorescence analysis on the fixed and permeabilised Lizard *Leishmania* promastigotes showed that rLPG3 is present in all of the cells at approximately equal levels. This also demonstrates that LPG3 is largely present throughout the cytoplasm of

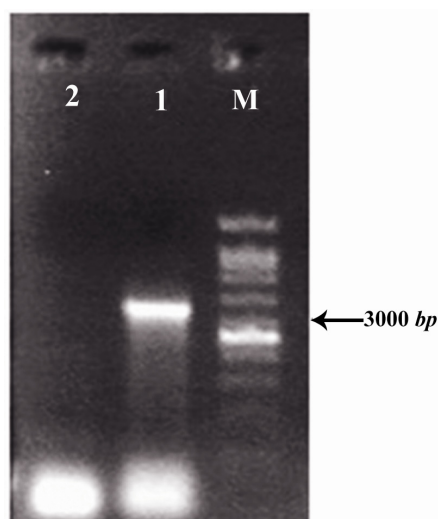


Figure 4. Confirmation of genomic integration into the *ssu* locus of Lizard *Leishmania* by diagnostic PCR with the forward LPG3 and *ssu* reverse primers: transfected cells with pLEXSY-LPG3 vector (Lane 1), wild type cells (Lane 2), and 1 kb DNA size marker (Lane M). The products were electrophoresed on 1% agarose gel

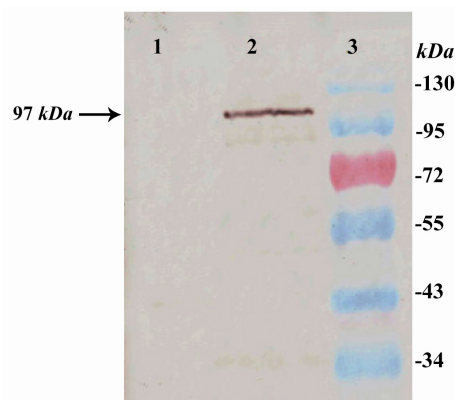


Figure 5. Western blot analysis of the transfected *Leishmania* cells lysate: lysate of wild type cells (Lane 1), lysate of cells transfected with pLEXSY-LPG3 expression vector blotted with anti-His tag monoclonal antibody (Lane 2), and molecular weight markers (Lane 3)

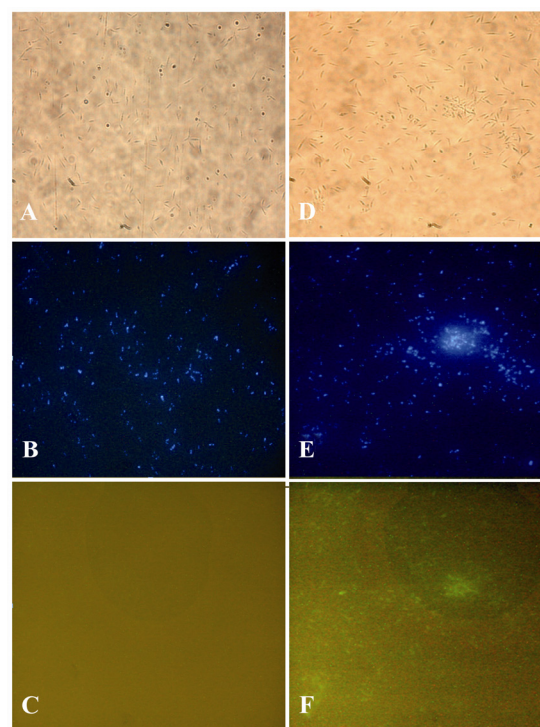


Figure 6. Indirect immunofluorescence analysis: (A, B, C), wild type cells; (D, E, F), transfected cells; (A, D), phase-contrast images; (B, E), DAPI staining, indicating the localization of DNA in the nucleus and kinetoplast in blue, and (C, F) FITC staining (green) using the anti-His tag monoclonal antibody diluted (1:40) for wild type and transfected cells ($\times 40$).

the cells, indicating the cells expressing the rLPG3 protein.

Discussion

In the current study, we have used a non-pathogenic trypanosomatidae, Lizard *Leish-*

mania, to represent a beneficial eukaryotic expression system for the production of recombinant proteins. One reason for using trypanosomatidae species is that high level expression of protein-coding genes driven by RNA polymerase I is obtained by integration into the 18 S rRNA locus ⁽¹⁷⁾. The main advantages of *Leishmania* expression system are as follows: (i) inexpensive growth condition, (ii) fast growth rate, (iii) safety for human, (iv) production of recombinant proteins and (v) posttranslational modification of target proteins with a mammalian-type N-glycosylation pattern and correctly protein folding ⁽¹⁸⁾ result to make Lizard *Leishmania* an attractive host for high level production of heterologous proteins such as human p53 ⁽⁹⁾, erythropoietin ⁽¹⁰⁾, IFN- γ ⁽¹²⁾, and human tissue plasminogen activator ⁽¹⁹⁾. Thus, the expression cassette containing the LPG3 gene was integrated into the *ssu* rRNA locus, which could enhance the expression level. Among the posttranslational modifications, glycosylation can be critical, not only for immunogenicity that it can enhance or conceal immunogenic epitopes but also to modulate biological activity ⁽²⁰⁾. Since Trypanosomatidae species naturally produce large amounts of glycoproteins, then this feature could be advantageous for the production of glycosylated proteins ⁽²¹⁾. For these reasons, Lizard *Leishmania* has attracted extensive attention to produce this valuable molecule (LPG3) in a different expression system.

The results showed that rLPG3 can be expressed in Lizard *Leishmania* to a level that allows detection by western blotting in the transfected cells, but this protein was absent in the wild type cells. The critical role of LPG3, homolog of the mammalian ER chaperon GRP94, is restricted to the synthesis of glycoconjugates implicated in parasite virulence, including LPG (lipophosphoglycan) ⁽⁶⁾. In addition, due to high immunogenicity of LPG3, it can be used as a valuable molecule for diagnostic purposes and a promising candidate for vaccine development against leishmaniasis ^(7,8).

Alignment of the amino acid sequence of LPG3 with the native signal sequence revealed several conserved regions, including the C-terminal ER retention signal, to localize in the ER. Moreover, immunofluorescence staining determined that the expressed protein was largely present throughout the cytoplasm of parasites, indicating the cells ability to express the recombinant protein. The Iranian Lizard *Leishmania* expression system has also been used to produce human coagulation factor VII with biological properties ⁽¹⁵⁾. This confirms that Lizard *Leishmania* represents potential advantages including full glycosylation, disulfide bond formation and proper folding.

Conclusion

In conclusion, this study has demonstrated that *Leishmania* cells can be used as a suitable host for the production of rLPG3 protein. We could successfully express the rLPG3 in this host to perform the future experiments on the usage of rLPG3 as a vaccine candidate by different vaccination strategies (Protein/ Protein and DNA/Protein) against leishmaniasis.

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