World J Microbiol Biotechnol
DOI 10.1007/s11274-010-0648-4

ORIGINAL PAPER

Cloning and expression of human IFN-γ in *Leishmania tarentolae*

Noushin Davoudi · Azam Hemmati · Zahra Khodayari · Ahmad Adeli · Mahdi Hemayatkar

Received: 2 August 2010 / Accepted: 29 December 2010

Abstract Increasing therapeutic applications for recombinant human interferon-gamma (rhIFN-γ), an antiviral pro-inflammatory cytokine, has broadened interest in optimizing methods for its production. We herein describe a unicellular eukaryotic system, *Leishmania tarentolae*, a Trypanosomatidae protozoan parasite of gecko *Tarentola annularis*, which has recently been introduced as a candidate for heterologous gene expression. In this study, the *hIFN-γ* cDNA was amplified from phyto-hemagglutinin-stimulated peripheral blood mononuclear cells of a healthy blood donor using RT–PCR. In order to express, the rhIFN-γ protein, the resulting cDNA was cloned in two expression cassettes (each containing one copy of *hIFN-γ* cDNA) and integrated into the small subunit of ribosomal RNA gene of *L. tarentolae* genome by electroporation. Transformed clones were selected in the presence of appropriate antibiotics. Western blotting of rhIFN-γ and ELISA confirmed the expression and production of 9.5 mg of rhIFN-γ protein/l respectively.

Keywords Recombinant human interferon-γ · rhIFN-γ · Expression · *Leishmania tarentolae*

Introduction

There are variety of prokaryotic and eukaryotic expression systems which have been developed for the synthesis of recombinant proteins (Zhu et al. 1986; Kim et al. 1997) including those from bacteria, yeasts, fungi, mammalian cells, insect cells, transgenic animals, and transgenic plants. Bacterial expression systems are generally unable to assemble glycan branches and disulfide bonds of glycoproteins and the expressed proteins fail to fold properly. Attempts to produce recombinant proteins in insect cells and *Saccharomyces cerevisiae* have been unsatisfactory due to poor secretion into the culture medium, hyperglycosylation and improper folding (Ogrydziak 1993). Thus, development of an alternative eukaryotic expression system capable of improving these problems is needed.

The family Trypanosomatidae (Euglenozoa, Kinetoplastida) includes several of the most serious vector-borne parasites of humans. The major human parasites include a number of species in the genera *Leishmania* and *Trypanosoma* (Hughes and Piontkivska 2003). Since 1986, it has been shown that *Leishmania* protozoan, a trypanosomatidae flagellate, could be used to express foreign genes (Zhang et al. 1995; Hughes and Piontkivska 2003). Among Trypanosomatidae family, *Leishmania tarentolae* is a non-pathogenic parasite of the gecko *Tarentolae annularis* and has been developed as a new potential eukaryotic expression system, as proven in the expression of erythropoietin and tissue plasminogen activator (Clayton et al. 1995; Breitling et al. 2002; Hemayatkar et al. 2010).

Human Interferon-γ is a secretory glycosylated protein with the total molecular weight of 25 kD and two potencial glycosylation sites (Sareneva et al. 1995). This glycoprotein is the product of activated T-lymphocytes and natural killer cells, and was originally described as an antiviral agent. As a result of its numerous functions, recombinant human IFN-γ (rhIFN-γ) is finding increasing therapeutic applications. In the treatment of chronic granulomatous disease (Seeger et al. 1998) and in severe malignant osteopetrosis (Van Slooten et al. 2000; Kocher and Kasser 2003;

N. Davoudi (✉) · A. Hemmati · Z. Khodayari · A. Adeli · M. Hemayatkar
Biotechnology Research Center, Pasteur Institute of Iran, No. 69, Pasteur Ave., Tehran, Iran
e-mail: davoudi@pasteur.ac.ir; noushin_d@yahoo.com

Published online: 12 January 2011

Marciano et al. 2004), rhIFN-γ has clinically accepted for beneficial effectiveness (US patent 6/333032). In the years 1982–1996 Interferon-γ has been expressed in *E. coli*. But as a bacterium, *E. coli* is unable to finalize the post-translational modifications (as mentioned above), thus, the protein produced was unglycosylated (Arora and Khanna 1996; Khalilzadeh et al. 2003). In 1983, Interferon-γ was cloned in *Saccharomyces cerevisae* under control of phosphoglycerate kinase (PGK), but the expression yield was not satisfactory (Derynck et al. 1983). Similarly, expression yield of *hIFN-γ* in chinese hamster ovary (CHO) cells was also unpromising (Zamani et al. 2006).

In the present study, cloning and expression of human *IFN-γ* cDNA was used to evaluate the usefulness of protozoa in the production of a small human recombinant pharmaceutical protein.

Materials and methods

Amplification of *IFN-γ* cDNA

The *IFN-γ* gene was isolated from phytohemaglutinin-stimulated peripheral blood mononuclear cells of an Iranian healthy individual blood donor using the RT–PCR technique. The cells were cultured as mentioned and incubated at 26°C for 72 h. Total RNA was extracted from 3×10^8 cells using TRIzol reagent (Gibco, UK) as follows. After washing the cells, the sediment was dissolved in 1.5 ml TRIzol, centrifuged for 10 min at 12,000*g* and 4°C, and kept at room temperature for 5 min. Then 0.2 ml chloroform was added for each 1 ml TRIzol and after shaking, it was kept at room temperature for 15 min. It was centrifuged for 15 min at 12,000*g* and 4°C, and the aqueous phase was collected. Then 0.75 ml isopropanol was added to the mixture and after keeping at room temperature for 10 min, it was centrifuged for 10 min at 12,000 *g* and 4°C. The RNA sediment was washed using ethanol 75% and finally dissolved in 30 µl RNase free (DEPC-treated) water. The cDNA was synthesized using Single-Stranded cDNA Synthesis kit (Fermentas, Lithuania) as recommended by the manufacturer: 1 µl oligo dT (50 pmol/µl) was added to 1–5 µg total RNA and the reaction volume was increased to 11 µl using RNase-free water. The reaction mixture was kept at 70°C for 5 min and then cooled rapidly on ice. Then 2 µl of 10 mM dNTPs, 2 µl RNase inhibitor and 4 µl 5x reaction buffer were added and the volume was increased to 19 µl using RNase-free water. The reaction mixture was kept at 37°C for 5 min and then 40 units of Moloney murine leukemia virus reverse transcriptase enzyme added. The reaction mixture was kept at 37°C for 60 min and at 70°C for 10 min, and finally frozen at −70°C.

Construction of recombinant expression vectors

The purified amplicon was cloned in pTZ57R using InsT/Aclone™ PCR Product Cloning Kit (Fermentas, Lithuania) as instructed by the manufacturer. The recombinant plasmid was analysed by sequencing. The cloned *IFN-γ* gene was amplified with the primers F-IFN 5′GGCGGATCCATGCAGGACCCATATGTA3′ and R-IFN 5′GGCGGATCCTTACTGGGATGCTCTTCGACC3′ which contained *BamH*I restriction sites in each 5′ end (underlined). The PCR product obtained was cloned in pET-32a cloning vector as intermediate vector. The *hIFN-γ* included vector (pET-32a-IFNγ) was digested with *BamH*I restriction enzyme and purified IFN-γ fragment was cloned into pFX1.4sat and pFX1.4hyg plasmids (Jena Bioscence, Jena, Germany) which had been digested by *Bgl*II previously. The recombinant pFX1.4sat-IFNγ and pFX1.4hyg-IFNγ plasmids were purified by a commercial plasmid extraction kit (Macherey–Nagel, Germany). The presence of the *IFN-γ* gene was confirmed by several restriction-enzyme analysis and sequencing using M13 forward and reverse primers.

Cultivation and transfection of *L. tarentolae*

Leishmania tarentolae was cultivated in Brain Heart Infusion (BHI) broth medium (DIFCO, USA) supplemented by 5 µg/ml hemin (Sigma, USA). Transfections were performed by electroporation of in vitro cultivated promastigotes as described by Beverley and Clayton (1993). The Leishmania expression vectors containing IFN-γ cDNA were digested by *Swa*I restriction enzyme (Fermentas, Lithuania) and the heavier fragment containing IFN-γ was purified. Of this purified fragment 10 µg was transformed into *L. tarentolae* cells by electroporation. Selection of single colonies would be on solidified BHI media containing nourseothricin (ClonNAT, Jena, Germany) or hygromycin B (Sigma, USA) and both.

Screening of transformed *L. tarentolae* colonies

To confirm the integration of the IFN-γ-containing cassettes into the *Leishmania* genome, PCR was performed on the genomic DNA of nourseothericin- and hygromicin-resistant colonies using IFNγ, sat- and hyg-specific primers. For further confirmation of homologous recombination integration in the 18 s rRNA site, an amplification of the fragments with hyg or sat forward and ssu specific reverse primers was performed.

SDS–PAGE and Western blotting

Total protein was obtained from the cell lysate of the transformed and the wild colonies (Hemayatkar et al. 2010).

20 µg of each acquired sample was loaded on 12% SDS–PAGE. The polyacrylamide gel was stained with Coomassie blue G-250 and de-stained with MQ water. Western blotting was performed on similarly prepared acrylamide gel according to the standard procedure (Coligan et al. 2003). Rabbit polyclonal antibody to human IFN-γ (Abcam UK) was used as the primary and the goat polyclonal anti-rabbit IgG HPR (Dako, UK) as the second antibody.

Immunoassay quantification of rhIFN-γ

In order to detect the rhIFN-γ, solid phase was coated with the rabbit polyclonal anti-human IFN-γ antibody (Abcam, UK). The standard enzyme-linked immunosorbent assay (ELISA), washing and blocking processes were performed. Serial dilution of standard IFN-γ and cell lysate of double transformed clone was added into each anti-IFN-γ-coated well. Antigen–antibody detection was carried out by conjugation with horseradish peroxidase (HRP; Pharmacia, Sweden) which was added to each reaction well after the washing step. The substrate 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB) (Sigma–Aldrich, USA) was added to each well followed by 20–30 min incubation and the reaction was stopped with 1 M H_2SO_4 solution. The standard rhIFN-γ; Imukin (Boehringer Ingelheim, Germany) was used as a positive control.

Results

Cloning of the IFN-γ cDNA

Human IFN-γ cDNA was synthesized by RT–PCR (as mentioned in Materials and Methods) and PCR was performed on the DNA extracted from cells using primers containing restriction sites for *BamH*I in both 5′ ends. A single 450 bp band was seen on agarose gel after electrophoresis (Fig. 1a). This band was extracted from the gel and ligated in pET-32a vector. The correct gene cloning was confirmed by restriction analysis (Fig. 1b) and DNA sequencing. BLAST assessment of the sequence obtained showed 100% similarity to human IFN-γ mRNA (GenBank: AF506749.1).

Construction of IFN-γ expression cassettes

In order to assess the relationship between integrated gene copies in the 18 s rRNA locus and protein expression rate, this gene was sub-cloned in pFX1.4hyg and pFX1.4sat expression vectors. IFN-γ gene was cut out of the pET-32a-IFNγ by *BamH*I restriction enzyme and eluted from the agarose gel. In addition, the pFX1.4hyg and pFX1.4sat

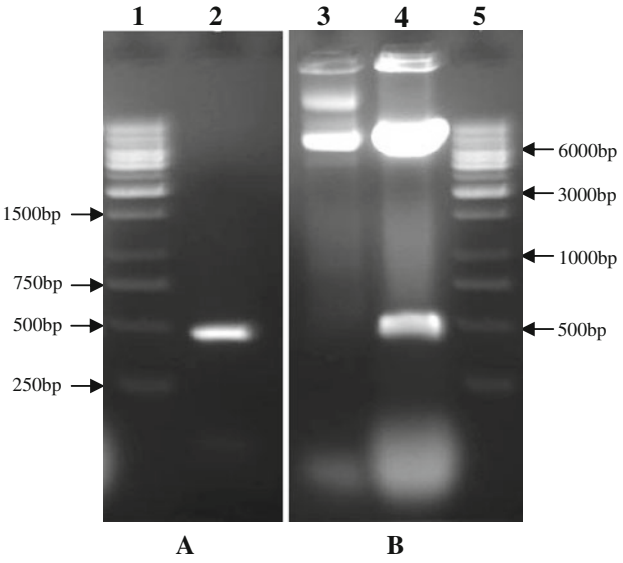

Fig. 1 **a** Agarose gel electrophoresis of PCR amplification of *hIFN-γ* gene. *Lane 1* 1 kb size marker, *lane 2* 460 bp *IFN-γ* gene. **b** Restriction analysis of pET-32a-IFNγ vector. *Lane 3* undigested vector, *lane 4* digested vector using *BamH*I restriction enzyme, *lane 5* 1 kb size marker

vectors were also digested using the *Bgl*II enzyme and electrophoresed on agarose gel for confirmation of the proper digestion. This enzymatic digestion cut out the 0.7 kb fragment from the 8.2 kb plasmid (pFX1.4sat) and 8.7 kb plasmid (pFX1.4hyg). Figure 2 shows the schematic picture of the ligation of IFN-γ gene in 7.5 kb fragment of specific *Leishmania* pFX1.4sat vector. As the difference between two final expression vectors is only the antibiotic resistance gene, the pFX1.4hyg vector has not been shown.

Transfection of *L. tarentolea* cells

Two DNA fragments, sat-IFNγ and hyg-IFNγ, containing either a *sat* or a *hyg* gene and one copy of the *IFN-γ* gene which was between two non-translated regions (NTRs) obtained from digestion of pFX1.4sat-IFNγ and pFX1.4hyg-IFNγ by *Swa*I restriction enzyme used to transform the *Leishmania* cells by electroporation. After the first round of transfection with sat-IFNγ, the cells were grown in semisolid BHI media containing 60 µg/ml nourseothricin to select the resistant clones. Individual clones were selected and transferred sequentially into 24-well plates and then into 25-ml tissue culture flasks, containing 1 and 10 ml selective medium, respectively. Genomic DNA isolated from nourseothericin-resistant clones was analysed by PCR. After confirmation by PCR, a second round of transfection with the hyg-IFNγ fragment was done and transformed cells were cultured on semisolid media containing two antibiotics nourseothricin-hygromycin,

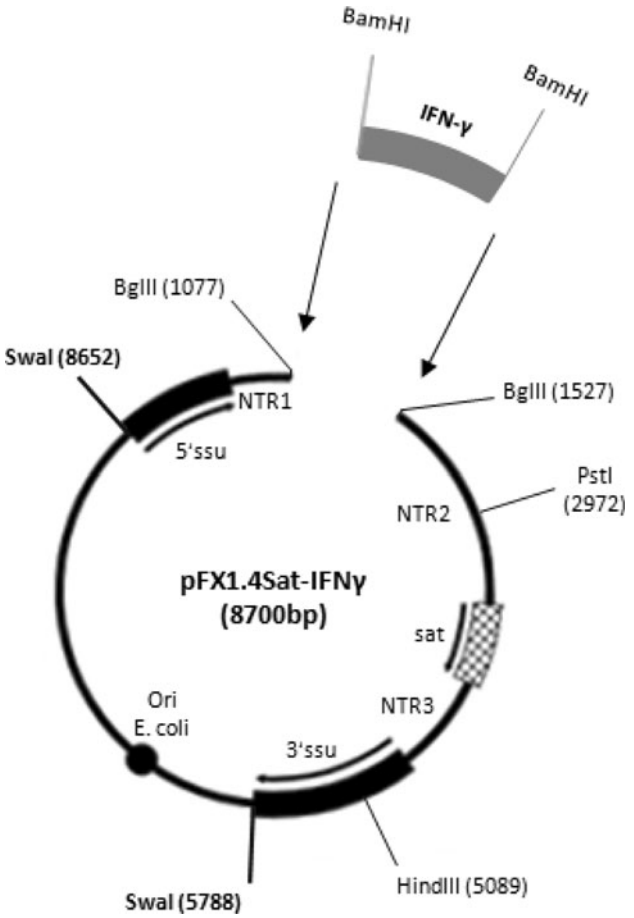

Fig. 2 Schematic diagram of pFX1.4-IFNγ vector used for expression of rhIFN-γ in *L. tarentolae*. *Abbreviations*: 5′ssu, 5′-portion of the small subunit of the *L. tarentolae* rRNA gene; NTR1 (0.4 k-IR cam BA), NTR2 (1.4 k-IR cam CB) and NTR3 (1.7 k-IR) are optimized gene flanking non-translated regions providing the splicing signals for post-transcriptional mRNA processing in *L. tarentolae*; *IFN-γ*, cDNA of the *IFN-γ* gene; sat, norseothricin resistance gene; 3′ ssu, 3′-portion of the small subunit of *L. tarentolae* rRNA gene

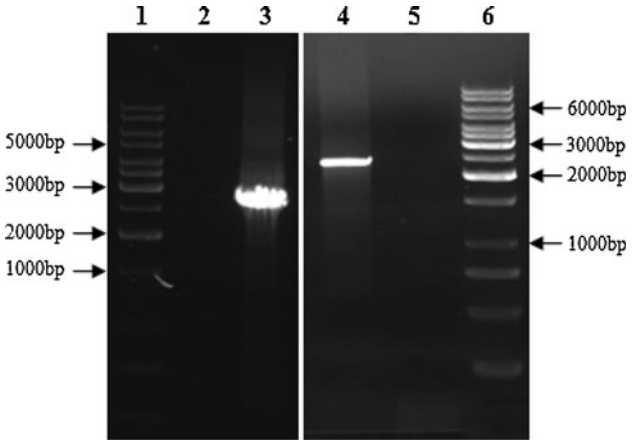

Fig. 3 Agarose gel electrophoresis of PCR amplification in a doubly transformed *L. tarentolae* clone to confirm the integration of the expression cassettes into the parasite genome. *Lane 1* 1 kb size marker, *lane 2* PCR on non-transformed cells with hyg forward and ssu reverse primers (control negative), *lane 3* 2.8 kb band related to the integration of hyg-IFNγ cassette into the ssu locus, *lane 4* 2.3 kb band related to the integration of sat-IFNγ cassette into the ssu locus resulted from sat forward/ssu reverse primers, *lane 5* PCR on non-transformed cells with sat forward and ssu reverse primers (control negative), *lane 6* 1 kb size marker

60 and 50 μg/ml, respectively. Double resistant clones were cultured in BHI broth medium containing both two antibiotics.

Integration confirmation

Integration of *hIFN-γ* (460 bp), *sat* (500 bp) and *hyg* (1,000 bp) in the genomic DNA of the recombinant cells was confirmed by PCR analysis (data not shown). Furthermore, to verify the right orientation of the integrated sat-IFNγ cassette, a PCR reaction using the *sat* forward and *ssu* reverse primers was performed on both wild type cells and the cells transformed with pFX1.4sat-IFNγ cassette. The expected 2.3 kb band was only observed from transformed cells, indicating the right orientation of the sat-IFNγ cassette. The same procedure was performed on genomic DNA of the cells with sat-IFNγ, after second transformation with hyg-IFNγ cassette using *hyg* forward and *ssu* reverse primers. The desired bands, 2.8 kb, were observed on the agarose gel (Fig. 3).

SDS–PAGE and Western blotting

Expression of rhIFN-γ in *L. tarentolae* was also investigated by western blot analysis. IFN-γ was detected in the cell lysate of transformed cells. The commercial IFN-γ reacted with rabbit polyclonal anti-IFN-γ antibody produced a 17 kD band representing the intact structure of the protein (Fig. 4, lane 1). Existence of the similar band confirmed the presence of rhIFN-γ in the cell lysate obtained from the double transformed clone (Fig. 4, lane 2) whereas in wild type cells IFN-γ was not detected with polyclonal antibody (Fig. 4, lane 3).

Estimation of the rhIFN-γ expression level

The rhIFN-γ was detected in the cell lysates by ELISA. The amount of the heterologous protein in each preparation was determined by sandwich ELISA using standard IFN-γ for comparison. From 100 ml of the culture of double transformed colony in the shake flask (OD: 2 at 600 nm), the production yield was 0.945 μg of rhIFN-γ (app. 0.8% of total protein).

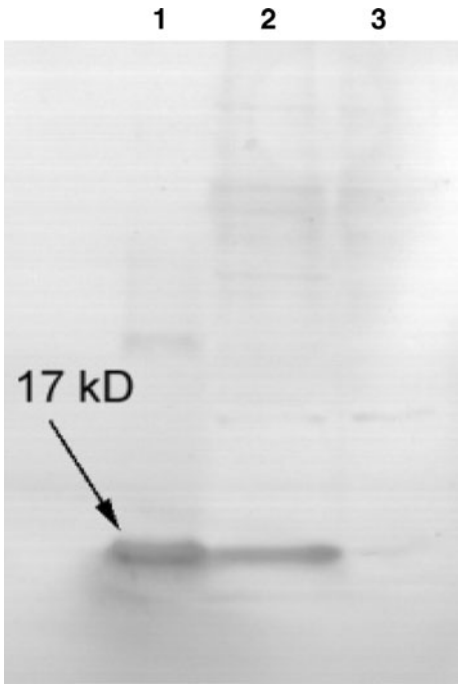

Fig. 4 Western blot analysis of double transformed Leishmania cell lysate. *Lane 1* 1 μg commercial IFN-γ, *lane 2* 20 μg of total protein from cell lysate of double transformed cells, *lane 3* 20 μg of total protein from cell lysate of wild cells

Discussion

Leishmania tarentolae is a parasite of the gecko *Tarentolae annularis* and a feasible eukaryotic expression system for high level production of active recombinant biopharmaceuticals (Soleimani et al. 2007; Breitling et al. 2002) because of its several unique features, including higher specific growth rate compared to mammalian cells, cultivation in low cost media, safety for humans, possibility to introduce several copies of a foreign gene into the parasite genome and production of recombinant proteins with an animal-like N-glycosylation pattern. These advantages, in addition to feasibility for constitutive or regulative protein production, make *L. tarentolae* an attractive host for high level production of heterologous proteins (Kushnir et al. 2005; Fritsche et al. 2007; Breitling et al. 2002; Hemayatkar et al. 2010). Moreover, *Leishmania* do not have some of limitations including safety considerations, requirements for serum-supplemented culture media and low growth rate. A couple of recombinant pharmaceutical and non-pharmaceutical glycoproteins such as human erythropoietin, tissue plasminogen activator and laminin-332 have already been produced in this expression system and in all cases, the expressed proteins were biologically active (Hemayatkar et al. 2010; Phan et al. 2009; Breitling et al. 2002).

Leishmania possess unusual mechanisms for gene expression. The recent sequencing of the *Leishmania* spp. genomes indicates that protein-coding genes are organized as polycistronic units (Ivens et al. 2005; Clayton and Shapira 2007; Haile and Papadopoulou 2007; Orlando et al. 2007). Transcription in this organism initiates at strand the switch regions on each chromosome, without defined RNA polymerase II promoters or other typical transcription factors (Martinez-Calvillo et al. 2003).

Collectively, the studies demonstrate that the basic features of the Trypanosomatidae secretory-endocytic pathways are very similar to those found in higher eukaryotic expression systems such as mammalian cell lines (Ralton et al. 2002; Clayton and Shapira 2007; Basile and Peticca 2009). Hence, in this study an attempt was made to express the recombinant human IFN-γ in *L. tarentolae*. Since the intergenic NTRs have an important role in the post-transcriptional modifications in Trypanosomidae (Teixeira and daRocha 2003), IFN-γ cDNA was cloned between two NTRs in commercial expression vectors. In order to increase the expression rate, two expression cassettes each one containing one copy of IFN-γ cDNA were constructed and transformed into the *Leishmania* cells, sequentially. In addition, constructs were integrated in chromosomal 18 s ribosomal RNA locus (ssu) that is a repetitive locus of the *L. tarentolae* genome with high rates of transcription by the host RNA polymerase I (Soleimani et al. 2007) which could enhance the expression level.

Results showed that rIFN-γ can be expressed in *L. tarentolae* cells to a level that allows detection by Western blotting. Moreover, increasing of the gene copy number by transforming the transgenic clones with the second expression cassette affects the expression level which investigated based on the intensity of the acquired bands (data not shown). Similar observations have been reported previously by several authors (Hemayatkar et al. 2010; Soleimani et al. 2007; Breitling et al. 2002).

IFN-γ is a pharmaceutical protein with a broad range of biological activities including activation of B and T-cell differentiation and increasing the expression of the major histocompatibility complex (MHC I, II) molecules, therefore, it has application in the treatment of a number of immunological, viral, and neoplastic diseases.

For these reasons it has attracted extensive attention, and scientists have tried to produce this valuable protein in different expression systems. Proper glycosylation of IFN-γ increases the survival time of biologically active IFN-γ in the circulation as the native protein shows resistance to crude protease preparations obtained from human granulocytes (Sareneva et al. 1995). Although it has been shown that *E. coli* might be considered as a suitable host with a relative high level of expression (Khalilzadeh et al. 2003; Zhang et al. 1992), lack of posttranslational modifications

and the need for refolding stages are still big challenges. In contrast, *L. tarentolae* is eukaryotic gene expression machinery, which includes full glycosylation and disulfide bond formation, and thus represents a potential advantage over other expression systems (Fernandez-Robledo and Vasta 2010), Therefore, expressed rhIFN-γ in this host could be properly folded, highly active and protease resistant. Our productivity was 1.6 ± 0.05 mg g^{-1} of dried cell (9.5 mg/l) that is seen acceptable for heterologous protein expression in this host in comparison with the other studies (Kushnir et al. 2005; Breitling et al. 2002). It was more than what was reported by Zamani et al. in CHO cells (100 ng/ml) (Zamani et al. 2006).

The above-mentioned facts confirm that the gene-expression system using *Leishmania* parasites combines many of the advantages of both prokaryotic and eukaryotic expression systems. We could successfully express the rhIFN-γ in *L. tarentolae* and plan to produce higher amount for assay of glycosylation pattern by performing the future experiment and optimizations.

Acknowledgments The authors thank Dr. Anis Jafari, of Molecular Biology Dept. of Pasteur Institute of Iran for her comments and suggestions. This work was supported by grant No.397 of Pasteur Institute of Iran.

References

Arora D, Khanna N (1996) Method for increasing the yield of properly folded recombinant human gamma interferon from inclusion bodies. J Biotechnol 52(2):127–133

Basile G, Peticca M (2009) Recombinant protein expression in *Leishmania tarentolae*. Mol Biotechnol 43:273–278

Beverley SM, Clayton CE (1993) Transfection of Leishmania and Trypanosoma brucei by electroporation. Methods Mol Biol 21:333–348

Breitling R, Klingner S, Callewaert N, Pietrucha R et al (2002) Non-pathogenic trypanosomatid protozoa as a platform for protein research and production. Protein Expr Purif 25(2):209–218

Clayton C, Shapira M (2007) Post-transcriptional regulation of gene expression in trypanosomes and leishmanias. Mol Biochem Parasitol 156(2):93–101

Clayton C, Hausler T, Blattner J (1995) Protein trafficking in kinetoplastid protozoa. Microbiol Rev 59(3):325–344

Coligan JE, Dunn BM, Speicher DW, Wingfield PT (2003) Short protocol in protein science: a compendium of methods from current protocols in protein science. Wiley, New York, pp 43–47

Derynck R, Singh A, Goeddle DV (1983) Expression of the human interferon-gamma cDNA in yeast. Nucleic Acids Res 11(6): 1819–1837

Fernandez-Robledo JA, Vasta GR (2010) Production of recombinant proteins from protozoan parasites. Trends Parasitol 26(5):244–254

Fritsche C, Sitz M, Weiland N, Breitling R, Pohl HD (2007) Characterization of the growth behavior of *Leishmania tarentolae*: a new expression system for recombinant proteins. J Basic Microbiol 47(5):384–393

Haile S, Papadopoulou B (2007) Developmental regulation of gene expression in trypanosomatid parasitic protozoa. Curr Opin Microbiol 10(6):569–577

Hemayatkar M, Mahboudi F, Majidzadeh-A K, Davami F, Vaziri B, Barkhordari F, Adeli A, Mahdian R, Davoudi N (2010) Increased expression of recombinant human tissue plasminogen activator in *Leishmania tarentolae*. Biotechnol J 5:1198–1206

Hughes AL, Piontkivska H (2003) Phylogeny of Trypanosomatidae and Bodonidae (Kinetoplastida) based on 18S rRNA: evidence for paraphyly of Trypanosoma and six other genera. Mol Biol Evol 20(4):644–652

Ivens AC, Peacock CS, Worthey EA et al (2005) The genome of the kinetoplastid parasite, *Leishmania major*. Science 309(5733): 436–442

Khalilzadeh R, Shojaosadati SA, Bahrami A, Maghsoudi N (2003) Over-expression of recombinant human interferon-gamma in high cell density fermentation of *E. coli*. Biotechnol Lett 25:1989–1992

Kim CH, Oh Y, Lee TH (1997) Codon optimization for high-level expression of human erythropoietin (EPO) in mammalian cells. Gene 199(1–2):293–301

Kocher MS, Kasser JR (2003) Osteopetrosis. Am J Orthop (Belle Mead NJ) 32(5):222–228

Kushnir S, Gase K, Breitling R, Alexandrov K (2005) Development of an inducible protein expression system based on the protozoan host *Leishmania tarentolae*. Protein Expr Purif 42(1):37–46

Marciano BE, Wesley R, De Carlo ES, Anderson VL (2004) Long-term interferon-gamma therapy for patients with chronic granulomatous disease. Clin Infect Dis 39(5):692–699

Martinez-Calvillo S, Yan S, Nguyen D, Fox M, Stuart K, Myler PJ (2003) Transcription of *Leishmania major* Friedlin chromosome 1 initiates in both directions within a single region. Mol Cell 11(5):1291–1299

Ogrydziak DM (1993) Yeast extracellular proteases. Crit Rev Biotechnol 13:1–55

Orlando TC, Mayer MG, Campbell DA, Sturm NR, Floeter-Winter LM (2007) RNA polymerase I promoter and splice acceptor site recognition affect gene expression in non-pathogenic Leishmania species. Mem Inst Oswaldo Cruz 102(7):891–894

Phan HP, Sugino M, Niimi T (2009) The production of recombinant human laminin-332 in a *Leishmania tarentolae* expression system. Protein Expr Purif 68:79–84

Ralton JE, Mullin KA, McConville MJ (2002) Intracellular trafficking of glycosylphosphatidylinositol (GPI)-anchored proteins and free GPIs in *Leishmania mexicana*. Biochem J 363:365–375

Sareneva T, Pirhonen J, Cantell K, Julkunen I (1995) N-glycosylation of human interferon-y: glycans at Asn-25 are critical for protease resistance. Biochem J 308:9–14

Seeger RC, Rosenblatt JD, Duerst RE, Reynolds CP et al (1998) A Phase I study of human gamma interferon gene-transduced tumor cells in patients with neuroblastoma. Hum Gene Ther 9(3):379–390

Soleimani M, Mahboudi F, Davoudi N, Amanzadeh A et al (2007) Expression of human tissue plasminogen activator in the trypanosomatid protozoan *Leishmania tarentolae*. Biotechnol Appl Biochem 48:55–61

Teixeira SMR, daRocha WD (2003) Control of gene expression and genetic manipulation in the Trypanosomatidae. Genet Mol Res 2:148–158

Van Slooten ML, Storm G, Zoephel A, Kupcu Z et al (2000) Liposomes containing interferon-gamma as adjuvant in tumor cell vaccines. Pharm Res 17(1):42–48

Zamani A, Pour-Jafari H, Elahi SM, Moghadam-Nazem N, Massie B (2006) Inducible expression of human gamma interferon. Iran Biomed J 10(4):197–202

Zhang Z, Tong KT, Belew M, Pettersson T, Janson JC (1992) Production, purification and characterization of recombinant human interferon gamma. J Chromatogr 604(1):143–155

Zhang WW, Charest H, Matlashewski G (1995) The expression of biologically active human p53 in Leishmania cells: a novel eukaryotic system to produce recombinant proteins. Nucleic Acids Res 23(20):4073–4080

Zhu J, Contreras R, Fiers W (1986) Construction of stable laboratory and industrial yeast strains expressing a foreign gene by integrative transformation using a dominant selection system. Gene 50:225–237