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Original Article

In vivo/In vitro immune responses to *L. major* isolates from patients with no clinical response to Glucantime

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Abstract

Background: Leishmaniasis is a major health problem in some endemic areas of tropical and subtropical areas of the world. Interleukin-12 (IL-12) and interferon gamma (IFN-γ) are essential cytokines associated with initiation of Th1 response. The main objective of this study was to evaluate of the type of immune response to *L. major* isolates from patients with no clinical response to antimonite (Glucantime).

Materials and Methods: This experimental study was carried out during 2013–2014. In the current study *Leishmania major* were isolated from 10 CL patients with a history of at least one course of treatment with Meglumine antimonate (Sb5). The isolates were used to evaluate *in vitro* and *in vivo* response to Sb5. J774 murine macrophage cell line was used for *in vitro* tests and Balb/c mice was used for *in vivo* studies. IL-12 gene expression was evaluated using Real-time PCR and IFN-γ serum level was quantified using ELISA technique. SPSS (version: 20), analysis of Covariance (ANCOVA) was used for statistical analysis.

Results: PCR results confirmed that all 10 isolates were *L. major*. The mean of IL-12 gene expression *in vitro*, *in vivo* and IFN-γ serum levels (pg/ml) after 2 and 3 weeks treatment *in vivo*, increased significantly following the treatment with Glucantime in the two groups of Balb/c mice infected either with patients' isolates or standard L. major. No significant difference was seen between the patients' isolates and standard species.

Conclusions: Although the L. major were isolated from patients with active lesion and no clinical response to Glucantime after at least one courses of Glucantime treatment but *in vivo* and *in vitro* immune response of L. major isolates showed no difference between the patients' isolates and standard L. major.

Key Words: Clinical resistant, gene expression, glucantime, il–12, ifn–γ, immune responses, leishmania major

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INTRODUCTION

Leishmaniasis is a zoonotic disease reported from various parts of the world. Leishmaniasis induced by various species of *Leishmania*, and transmitted by bite of different species of sand flies.[1] All pathogenic species of *Leishmania* infect mononuclear cells.[2]

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Host cells, e.g., neutrophils/macrophages, transfer *Leishmania* parasites from the site of infection to the lymph nodes where parasite antigens are introduced into primary T-cells.[3] Immunity to *L. major* infection depends upon activation of T cell responses. In fact, while both CD4 + and CD8 + cells are involved in this pathway, the role of CD4 + cells is more important.[4-6]

Interleukin-12 (IL-12) and interferon gamma (IFN-γ) are essential cytokines for initiation of Th1 response.[7] IL-12, mainly produced by antigen-presenting cells (APC) such as macrophages and dendritic cells (DC), seems to be crucial for initiation of Th1 response and resistance to intracellular parasites.[8] The role of IL-12 in resistance to infection has been revealed by using IL-12 knockout mice or using anti IL-12 antibodies.[9] Interferons are critical to intrinsic resistance not only due to their antiviral properties, but also due to the ability to regulate the innate and adaptive immune functions. IFN-γ activates the antimicrobial function of phagocytes.[10] Recombinant IFN-γ has been reported to activate infected macrophages and *in vitro* clear *L. major* in both resistant and susceptible mice.[11]

The present study carried out to evaluate immune response through IL-12 and IFN-γ titration following *L. major* infection and treatment with Sb5 as standard treatment for isolated species from patients who were under at least one course of Sb5 treatment.

MATERIALS AND METHODS

Leishmania parasites were collected from active lesions of 10 patients who had been treated with at least one course of Glucantime. The isolated *Leishmania* parasites were transferred into Novy-MacNeal-Nicolle (NNN) medium and then sub cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum (FCS). *Leishmania* identification was done using the Nested PCR method. DNA was extracted from the promastigotes isolated from the patients and standard *L. major* and *L. Tropica* parasites (MRHO/IR/75/ER and MHOM/IR/04/Mash10, respectively) by using High Pure PCR Template Preparation Kit (Roche, Germany). The first and second steps of primers were as follows respectively.[12]

CSB2XF: 5’CGA GTA GCA GAA ACT CCC GTT CA GC3’

CSB1XR: 5’ ATT TTT CGC GAT TTT CGC AGA ACG3’

Forward: 13Z: 5’ACTGGGGGTTGGTGTAAAATAG3’

Reverse: LiR: 5’TCGCAGAACGCCCCT

Briefly, the mixture contents of Nested PCR reaction were: $MgCl_2$ (25 mM) 1μ, dNTP (10 mM) 0.5 μ, PCR buffer 10 × 2.5 μ, Taq DNA polymerase (5 u/μl) 0.2 μ, D.D.W 15.80 μ, DNA sample 3 μ and the DNA amplification conditions were performed as follow: Denaturation 95°C/5 min, Denaturation 95°C/30 sec in 1 cycle, Annealing 56°C/1 min, Extention 72°C/1 min in 30 cycle, Final extention 72/10 min in 1 cycle. The PCR product obtained in the first step was used as a template in the second round. The materials used were as same as the first round with the exception that PCR product was used instead of primary DNA. After final stage of PCR, the products were electrophoresed on agarose gel 1% containing ethidium bromide [Figure 1].

The J774 Cell Line

The J774 murine macrophage cell line was purchased from the Pasteur Institute of Iran (Tehran, Iran). J774 cell line was cultured and viability of the cells was checked using trypan blue, 2×10^6 J774 cells were seeded on each 3.5 cm^2 well of six-well plates. The cells were cultured in 2 ml RPMI 1640 medium supplemented with 20% heat-inactivated fetal calf serum (FCS), 100 unit/ml of penicillin and 100 μg/ml of streptomycin.

Evaluating IL-12 gene expression

Six-well plates containing macrophages were prepared. Two plates were devoted to the pre-treatment phase, ten and two wells of the other two series (which were supposed to be treated with glucantime) were infected with metacyclic promastigotes of patients, isolates and standard strains respectively (in a ratio of seven parasites per macrophage). The plates incubated at 34°C with 5% CO_2 for 4–6 h, were washed with RPMI 1640 medium and added fresh culture medium or media containing glucantime with 7.2 μM pure

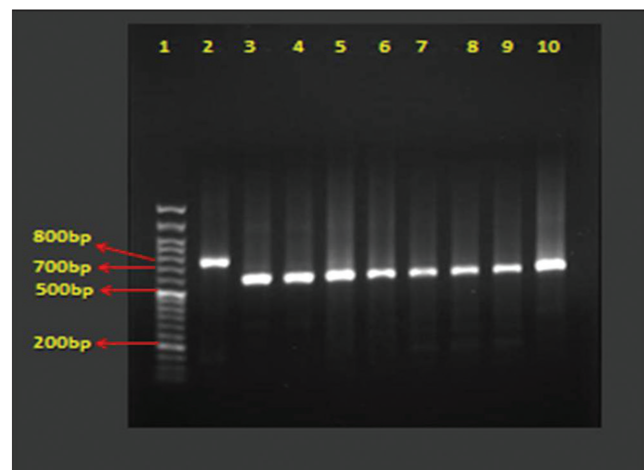

Figure 1: Nested PCR results. lane1: ladder marker, lane 2: *L. tropica* (positive control, MHOM/IR/04/Mash10), lanes 3, *L.major* positive control (reference strain MRHO/IR/75/ER). Lane 4-10, Leishmania isolates from patients. A band of 560bp is represented for *L. major*

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glucantime powder as ED50 (half maximal effective concentration) of the drug.[13] After 24 h of incubation at 37°C, macrophages were scraped and transferred into 1.5 ml micro tubes for RNA extraction.

RNA extraction and cDNA synthesis

RNA was extracted using RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Briefly, DNA was removed and RNA purification was done using RNase-Free DNase Set (QIAGEN, Germany). The integrity of the RNA was confirmed by denaturing agarose gel electrophoresis (data not shown), and the concentration of total RNA was quantified by determination of optical density at 260 nm (OD260). In order to design the primers (IL-12p40 and β-actin as housekeeping gene) numerous articles were studied, the selected sequences were matched, blasted in NCBI site and ordered.[14,15]

IL-12p40 primer: Forward: GGAAGCACGGCAGC AGAATA

Reverse: AACTTGAGGGAGAAGTAGGAATGG

β- Actin primer: Forward: GATGGTGGGAATGGGT CAGA

Reverse: GGGTCATCTTTTCACGGTTGG

First strand of cDNA synthesis was conducted using a "Revert Aid kit" (Fermentas, Canada) according to the manufacturer protocol. Briefly, 11 μl of Template RNA (total RNA) and 1 μl of Primer (Randome hexamer) was mixed gently and kept in 95°C for 5 min. Rapidly 4 μl of 5x Reaction buffer, 1 μl of Ribolock RNase inhibitor (20 U/μl), 2 μl of 10 mM dNTP mix and 1 μl of Revert Aid M-Mul V Reverse Trans Crip were added on the ice to reach to the total volume (20 μl). The obtained mixture was incubated in 25°C/5 min, 42°C/60 min and 70°C/5 min, respectively. Quantitative real-time PCR was used for quantification of cDNA after reverse transcription. PCR reactions were performed using the Maxima SYBER Green/ROX q PCR Master Mix (2x) from Thermo Scientific in 0.1 ml Micro Amp 8 strip tubes format adapted to ABI step one Plus. The total reaction volume was 20 μl (contacting SYBR Master 1x; 10 μl, RNase-free water; 7.5 μl, forward and reverse Primers each; 0.5 μl and cDNA; 1.5 μl) and the following conditions were preformed: 95°C/5 min for 1 Cycle, 95°C/10 sec and 60°C/30 sec both for 40 Cycles. Subsequent to real-time PCR, gene expression was quantified and analyzed by measuring threshold (CT) and applying the following formula:

ΔΔ CT= [(CT gene of treated sample–CT gene of internal control)–(CT gene of untreated sample–CT gene of internal control)].The results were analyzed by the comparative threshold cycle methods ($2^{-\Delta\Delta CT}$).

BALB/c mice *L. major* infection

A total of 110 female 4–6 weeks old BALB/c mice purchased from Pasteur Institute of Iran. The mice were divided into 11 groups (10 mice per group), 10 groups of mice were inoculated with 10 different *L. major* isolated from the patients and one group was inoculated with the standard *L. major* at the base of the tails. In each group, eight mice were treated with Sb5, and two of the mice were left untreated. After the culture and injection of 2×10^6 metacyclic parasites isolated from the patients or *L. major* standard strain (MRHO/IR/75/ER), small, hard nodules appeared at the site of injection within 2–3 weeks which developed into ulcer about 2 weeks later. Direct smear, Giemsa staining, and microscopic evaluations were carried out to confirm the presence of Leishmania. The mice were treated with topical Glucantime (100 mg/kg) using carbomer gel which is polymer of acrylic acid cross-linked with polyalkenyl ether or divinyl glycol.[13] Carbomers gel have a potential wide range of applicability in the pharmaceutical and dermocosmetic fields due to their high viscosity at low concentration, wide viscosity interval and characteristic flow behavior, compatibility with many active ingredients, good thermal stability, excellen to rgano-leptic characteristics, good patient acceptance and a potential candidate for use in controlled release drug delivery system.[16] The carbomer gel was kindly provided by Dr. Shahtalebi (Participating laboratories doctor Shahtalebi, Isfahan, Iran). Prepared gel was applied on lesions once a day, for 2 weeks in four mice and 3 weeks in four other mice in each group. On the due date, the lesions of labeled mice in the treatment and control groups were sampled. Subsequently, RNA extraction, cDNA synthesis, and real-time PCR were performed. Mouse INF-γ ELISA Kit (R and D systems, Abingdon, UK) was used to evaluate INF-γ. Retro-orbital blood samples were collected 2–3 weeks after the treatment was completed. The sera were kept at -20°C until used.

RESULTS

Nested PCR results

The result of Nested PCR is presented in Figure 1, as it is seen all the isolates were *L. major*.

In vitro studies

The mean IL-12 gene expression in untreated infected macrophages with isolates from the patients was 7.82 ± 2.80, which increased to 9.72 ± 2.2 following the treatment with glucantime. The corresponding values for the standard species were 7.7 ± 0.21 before treatment and 10.09 ± 1.6 after treatment with the medicine [Table 1]. The expression of IL-12 gene in

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Table 1: The mean IL-12 gene expression in untreated and treated groups with glucantime for the standard and patients isolated species *In vitro* and *In vivo*

	In vitro		*In vivo*			
	Glu⁻	Glu⁺	Glu⁻		Glu⁺	
			2w	3w	2w	3w
Patients sp (*n*=10)	7.82±2.80	9.72±2.2	8.34±1.42	7.79±0.98	10.72±2.5	10.64±2.39
Standard sp (*n*=3)	7.7±0.21	10.09±1.6	8.89±0.02	7.09±0.02	11.82±0.04	12.34±0.02

All results were expressed as means±standard deviation. According to the Covariance analysis treatment with Glucantime (GLU^+) comparing with untreated groups (GLU^-) significantly increased the mean expression of IL-12 in both *In vitro* and *In vivo* phase ($P < 0.05$). There was no significant difference between 2 and 3-week treatment (2w and 3w, respectively) and also between the standard and patients isolated species ($P > 0.05$)

patients, isolates and standard strains groups which were treated with glucantime was two-fold higher than untreated in this phase.

In vivo studies

This phase included 2- and 3-week treatment courses as follows:

Results of the two-week treatment course

The mean IL-12 gene expressions in groups of mice inoculated with patients' isolatesin glucantime treated group and untreated group of mice were 8.34 ± 1.42 and 10.72 ± 2.58, respectively. In the group of mice which were inoculated with the standard *L. major*, treatment with glucantime induced higher IL-12 gene expression than untreated group (11.82 ± 0.04 vs. 8.89 ± 0.02 in untreated mice *vs.* 8.89 ± 0.02 in untreated group. The mean overall IL-12 gene expression was 11.73 ± 4.10.

Results of the three-week treatment course

Similar to the 2-week treatment course, 3 weeks treatment with glucantime increased IL-12 gene expression in comparison with the untreated group [Table 1]. The mean overall IL-12 gene expression over the 3-week course of treatment was 11.84 ± 4.90. Analysis of covariance (ANCOVA) was applied to compare the *in vitro* and *in vivo* phases. When making comparisons within the *in vitro* phase, shifting from the *in vitro* group to the 2- and 3-week treatment courses significantly increased the mean IL-12 gene expression by 1.4 ± 0.7 ($P = 0.03$) and 1.54 ± 0.7 ($P < 0.05$) fold, respectively. However, pairwise comparisons using the Bonferroni correction revealed that the difference between the 2- and 3-week treatment courses was not significant ($P > 0.05$). On the other hand, shifting from the absence of treatment to the presence of treatment with Glucantime increased IL-12 gene expression by 2.8 ± 0.7 ($P < 0.05$). Meanwhile, ANCOVA results did not suggest any significant difference in IL-12 gene expression between the standard and isolated *L. major* species ($P = 0.2$).

The expression of IL-12 gene in infected macrophages with patients, isolates and standard strains groups which were treated with glucantime was four fold higher than untreated in both 2 and 3-week treatment.

Results of IFN-γ serum level measurements

Titration of IFN-γ serum levels after2 and 3 weeks Sb5 treatment was performed using ELISA method. The results showed a significantly higher ($P < 0.05$) level of IFN-γ in the group of mice treated for 2 weeks compared with untreated group (846.5 ± 6.7 pg/ml *vs*. 44.1 ± 1.1 pg/ml). Similar results were seen in groups of mice inoculated with standard *L. major*. The total mean IFN-γ level after 2-week treatment was 713.4 ± 5.0 pg/ml [Table 2]. Likewise, 3 weeks of treatment with glucantime elevated IFN-γ levels in the mice injected with isolated species (30.24 ± 8.30 pg/ml in untreated and 441.2 ± 3.7 pg/ml in treated samples). The total mean IFN-γ level after 3-weektreatment was 396.6 ± 2.8 pg/ml. According to the results of ANCOVA, the mean serum level of IFN-γ after 3 weeks of treatment was 316.8 ± 26.6 pg/ml lower than that after the 2-week treatment course ($P < 0.001$). In addition, treatment with glucantime increased the mean IFN-γ level by 609.7 ± 32.6 pg/ml in compare to untreated mice ($P < 0.001$). Finally, no significant difference existed between the standard and isolated species in this regard ($P = 0.4$).

DISCUSSION

Leishmaniasis is a critical health issue in tropical and subtropical areas of the world.[17] Due to the significance impact of vector-borne diseases such as leishmaniasis on overall health especially in poor parts of the world, World Health Organization (WHO) set the message of the World Health Day of 2014 as "small bite, big threat".[18]

According to the WHO, the efficacy of glucantime (the most common anti-leishmaniasis medicine) varies in different parts of the globe and among the methods of administration.[19] Antimony compounds have been used to treat leishmaniasis for over 60 years. However, there have been recent reports concerning drug resistance from various areas[20] In India, for instance, relapse or treatment failure is observed in 65% of leishmaniasis cases treated with these compounds.[21] Nevertheless, pentavalent antimonial (SbV) compounds are still the first line treatment and the medicine of choice against leishmaniasis.[22] These compounds have been

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shown to kill intracellular *Leishmania* species through activating the host's immune system. Sodium antimony gluconate (SAG) has multifaceted mechanism of action, *i.e.*, it can activate both innate and adaptive immunity by stimulating effective anti-leishmaniasis responses and thus cure the infection and eliminate the chance of relapse.[23] Croft and Yardley revealed the crucial effects of antimony compounds on the reticuloendothelial system and its shift toward recovery.[24]

In the present study it was shown that Glucantime treatment increases IL-12 gene expression. Such increments were also observed following 2- and 3-weeks treatment. IL-12 is an essential cytokine in initiation of Th1 response and enhancement of recovery in leishmaniasis.[25] Murray *et al.* showed that while antimony therapy was not very effective in IL-12 knockout mice, but it was literally ineffective in mice without T cells.[26] In a study to compare the immune boosting effects of anti-leishmaniasis drugs in 2013, Ghosh *et al.*, assessed SAG and amphotericin B alone and in combination with either miltefosine or paromomycin. They reported that except for paromomycin, all other medications increased tumor necrosis factor alpha (TNF-α) and IL-12 in culture supernatants of peritoneal macrophages from BALB/c mice compared to the values before treatment. Administration of single-dose anti-leishmaniasis drugs, such as antimony gluconate (60 mg/kg body weight), increased both TNF-α and IL-12 in blood serum of BALB/c mice.[27]

Based on our findings, IL-12 gene expression over both 2- and 3-week treatment *in vivo* were higher than *in vitro*. INF-γ plays a major role in the induction of Th_1 immune response and activation of macrophages. INF-γ produces and secrets by various sources such as NK cells, activated T cells, $TCD8^+$, and even macrophages.[6,28] Besides, INF-γ activates macrophages and enhances anti-leishmaniasis properties; IFN-γ is capable of releasing IL-12. It is believed that IFN-γ promotes Th_1 differentiation through collaboration with IL-12 and inhibition of IL-4 production and activities.[29] In comparisons of glucantime untreated and treated mice, it was shown that IFN-γ level was increased at 2 and 3 weeks after glucantime treatment [Figure 2].

In an experiment Wadhone *et al*., *in vitro* exposed peritoneal macrophages of BALB/c mice to miltefosine, and after 24 h of exposure, the INF-γ level in culture supernatants was increased.[30] Murray *et al.* evaluated the effects of SbV compounds and amphotericin B in INF-γ knockout BALB/c mice. It was concluded that although INF-γ acted as an androgen (or cofactor) in regulating responses to SbV compounds, it was not essential for response to treatment with amphotericin B.[31] Therefore, the raised levels of INF-γ

Table 2: The mean serum level of IFN-γ (pg/ml) in untreated and treated groups with glucan time after 2 and 3 week treatment for the standard and patients isolated species *In vivo*

	In vivo			
	2w		3w	
	Glu⁻	Glu⁺	Glu⁻	Glu⁺
Patients sp (*n*=10)	44.1±1.1	846.5±6.7	30.24±8.3	441.2±3.7
Standard sp (*n*=3)	60.1±3.7	920.3±1.8	49.5±0.01	413.3±0.12

All results were expressed as means±standard deviation. According to the Covariance analysis treatment with glucantime (GLU^+) comparing with untreated groups (GLU^-) significantly increased the meanserum level of IFN-γ (pg/ml) ($P<0.001$). There was significant reduction between 2 and 3-week treatment (2w and 3w respectively) ($P<0.001$) but not between the standard and patients isolated species ($P>0.05$)

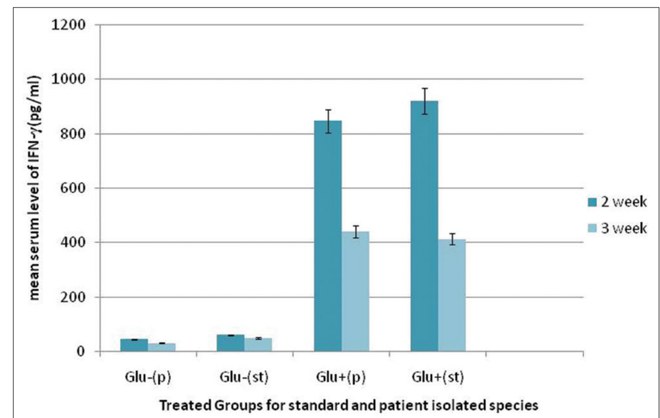

Figure 2: The mean serum level of IFN-γ (pg/ml) during 2 and 3 weeks in vivo treatment

after the administration of Glucantime in the present study seem logical. In fact, even after the reductions during the third week, the levels were still 14 times higher than those among untreated mice [Figure 1]. Asadpour *et al*. reported that owing to increased IL-4 levels, INF-γ gene expression in inguinal lymph nodes of BALB/c mice decreased during the first few hours after the infection with *L. major*. While mRNA levels were low during the first week, the levels had increased over the second and third weeks. However, the levels started to drop again in the fifth week.[32]

We did not detect a significant difference between isolated and standard species in terms of IL-12 gene expression or INF-γ levels. As it was mainly concerned about the probable role of *Leishmania* species in clinical resistance to Glucantime, and possibly presence of *L. major* subspecies in various parts of the world, we have proposed a number of explanations. Firstly, intrinsic resistance of the parasite and gene expressions can make the medicine ineffective through several mechanisms. Secondly, the person's immune system may fail to produce an appropriate Th1 response to eradicate the parasite within the macrophages. Finally, incorrect administration of the medicine may prevent its actual impact. Assessment of the interactions between clinical treatment-resistant species and the

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immune system and medicine in a particular host in the current study, it can be concluded that glucantime was able to promote the immune system even in the presence of species with clinical resistance. Hence, as failure of treatment might have been caused by other factors (*e.g.*, intrinsic resistance of the parasite or not delivering the medicine to the target cells and tissues); further comprehensive studies are required in this field.

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