Effects of heterogeneity of *Leishmania tropica* isolates in infected human macrophages

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Abstract: Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania*. The significance of *Leishmania tropica* (late ulcerative) in human cutaneous leishmaniasis has been recognized only recently, whereas can visceralize and cause systemic illness. Virulence variability was investigated by analyzing the experimental pathogenicity of nine *Leishmania tropica* strains. BALB/c mice were injected in the hind footpad with metacyclic promastigotes and lesion progression was recorded weekly intervals. Parasite burden, in tissue or draining lymph nodes, was determined by impression smears and limiting-dilution assay. Furthermore, human macrophages were infected with metacyclic promastigotes for 48 and 72 h, afterwards the percentage of infected macrophages and the average numbers of amastigotes per one infected macrophage were determined. A great variety of infection profiles between strains were observed; five strains showed a progressive infection about 5 weeks prior to being controlled (parasite loads in footpads swelling and lymph nodes were 10^4-10^5 and 10^6-10^8 parasites/mg, respectively). One strain produced only moderate and transient swelling, three strains never produced lesions, after which time, nonhealing and nonulcerative lesions persisted for over 6 months. The relationship was observed between profile and growth characterization in vitro. The high virulence strain was characterized by a significantly higher ability to infect human macrophages and to survive with higher replication levels within the infected macrophages. These results suggest that *L. tropica* isolates from the field may differ in virulence. The characterization of parasite virulence appears to be related to intrinsic parasitic factors that can affect the pathology of leishmaniasis.
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Introduction

Leishmania tropica is the causative agent of Old World anthroponotic cutaneous leishmaniasis (CL), a disfiguring parasitic disease that recently was found to be viscerotropic (1) and in Middle Eastern, Kenyan and Indian patients with classic kala-azar (VL) (2). This form is characterized by lesions that take a long incubation period, tend to form dry ulcers and an extended period of time to heal. *L. tropica* are more refractory to treatment than leishmaniasis caused by *Leishmania major*. Following healing, papules can occur around the periphery of the original lesion, termed recidivans type (3). This clinical polymorphism, which may be observed even within a small endemic region, may reflect either variability in the host immune response and/or in the parasite virulence. Early studies have shown that *L. tropica* promastigotes, grown in vitro, are pathogenic to BALB/c and C57BL/6 mice only when collected at the stationary phase as metacyclic parasites (4).

The surface molecules, particularly glycoprotein of 63 kDa (GP63) and lipophosphoglycan (LPG), were found to be crucial for parasite survival in mammalian and sand fly hosts. The dominant cell surface glycoconjugate of *Leishmania* is LPG, which has been involved in a large range of functions. In the mammalian host, this virulence factor's main functions include: attachment and entry into macrophages, modulation of nitric oxide production (5), induction of neutrophil extracellular traps (NETs) (6) and inhibition of protein kinase C (7).

The aim of this study was to assess the variability of virulence in 9 strains of *L. tropica* isolated in Iranian patients with cutaneous leishmaniasis through the evaluation of in vitro growth characteristics and analyzing infectivity in BALB/c mice.

Materials and methods

Parasites culture, growth curve and purification of metacyclic promastigotes

Leishmania tropica were isolated from cutaneous lesions of patients from Iran and were identified by using internal transcribed spacer 1 (ITS1) DNA sequencing-based (8). We used nine strains of *L. tropica* with less than five passages that showing respond to pentavalent antimony (SbV) treatment with EC50 values of less than 10 µg/ml (8). Parasites were grown at 26°C in medium 199 (Sigma, St. Louis, MO, USA) supplemented with 20% (v/v) heat-inactivated

FBS, 2 mM L-glutamine, 40 mM HEPES (complete M199) (9). In order to set up a growth curve and reach a high cell density, antibiotics were absent in the medium. Growth kinetics of strains were determined by inoculation of 10^6 parasites/ml in medium followed by daily quantification of promastigotes/ml over 8 days by Neubauer chamber counting. The experiment was repeated three times. The viability of promastigotes was estimated by trypan blue.

Metacyclic promastigotes at infective stage were isolated from stationary cultures by using a procedure based on a method of density gradient (10). In brief, a 40% stock solution of Ficoll Type 400 (Sigma, St. Louis, MO) was prepared. In a conical tube, 2 ml of 40% Ficoll was successively overlaid by 2 ml 10% Ficoll prepared in M199 medium and 2 ml DMEM containing stationary-phase parasites at a density of 2×10^8 cells/ml. The tube was centrifuged and parasites were recovered from the upper interface. The purity of metacyclic promastigotes verified by staining the parasites on a slide using Giemsa stain and examined the cells under light microscopy. Metacyclic promastigotes identified by morphological criteria like short and slender cell body with a tapered anterior end and a long flagellum twice the body length (9).

Experimental infection in BALB/c mice

6-8-weeks-old female BALB/c mice were purchased from the animal breeding facility of Pasteur Institute of Iran. Infective-stage metacyclic promastigotes were inoculated subcutaneously into the left hind footpad of mice at a dose of 10^6 parasites in 5–10 µl of saline (4, 11). A group of 5 mice were injected by prepared parasites. Observations of the inoculation sites were made at weekly intervals, and footpad swelling was measured in millimeters by a Vernier caliper (Thomas Scientific). The footpad swelling size was defined as the increase in the footpad thickness after reducing the size of the contralateral uninfected footpad. Parasite loads in footpad lesions were determined by microscopic examination of Giemsa-stained impression smears. Parasite titrations, in tissue or draining lymph nodes, were determined by limiting-dilution assay (LDA) (12). In brief, weighed footpad lesions were homogenized by using grinder in a tube containing 100 ml of M199. The tissue homogenate or lymph node single-cell suspensions were serially diluted in a 96-well flat-bottom microtiter plate containing biphasic medium. The number of viable parasites in each sample was determined from the highest dilution at which promastigotes could be detected after 7 days of incubation at 25°C.

Isolation and culture of human macrophages

Peripheral blood was obtained by venipuncture from adult healthy donors using heparin as an anticoagulant. Peripheral blood mononuclear cells (PBMC) were separated on a Ficoll-Hypaque density gradient. The cells were plated on an eight chamber Lab-Tek tissue culture slides (Miles Laboratories, Elkhart, IL) and incubated at 37°C, 5% CO2 for 60 min. Non-adherent cells were removed by gentle washing with warm culture medium, adherent cells were cultured in DMEM medium supplemented with 10% heat-inactivated human AB serum, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100µg/ml streptomycin (complete DMEM) for 4-5 days. Generated macrophages were used in co-culture experiments.

Macrophages were co-incubated with metacyclic promastigotes of three strains (high, intermediate and low virulence) at a parasite to a macrophage ratio of 5:1 in complete DMEM medium. After 4 h, monolayers were extensively washed and cultures were incubated at 37°C, 5% CO2 for a further 48 and 72 h. Slide chambers fixed with ethanol, and stained with Giemsa. In all settings intracellular amastigotes were counted by randomly counting at least 200 macrophages per slide. Results were expressed as the percentage of infected macrophages and the average number of amastigotes per one infected macrophage.

Statistical analysis

One-way analysis of variance was performed in order to evaluate the variety of different *L. tropica* strains according to the size of lesions induced in the BALB/c mice. Fisher's exact χ^2 test permitted comparison of the proportion of human macrophages infected by each strain. All statistical techniques were performed by GraphPad Prism statistical program software. Results are expressed as mean ± standard deviation of three independent experiments. P value < 0.05 were considered significant.

Results

Pathogenicity of *L. tropica* isolates in BALB/c mice

Considering that promastigotes, even when collected at the stationary phase, are heterogeneous populations and in order to eliminate the possibility that alteration in the pathogenicity showed by promastigotes could be generated by variation in the efficiency of in vitro metacyclogenesis, experimental infections were performed using the metacyclic promastigotes. The pathogenicity of nine *L. tropica* isolates was tested in BALB/c mice. Mice were inoculated subcutaneously in the hind footpad with 10^6 metacyclic promastigotes. Figure 1 shows the kinetics of footpad swelling development induced in groups of mice (five mice per group). It is very important that, in all strains, the cutaneous lesions were nonprogressive, nonulcerative and nonhealing.

Variety in the development of the experimental disease induced by the different strains was observed. Footpad swelling was detectable in all mice infected with the 527 and 375 strain of *L. tropica* by week 10, whereas, for 460 and 482 strains; the lesions were not

detectable until by week 16 also for 936 strain by week 21 post challenge. The cutaneous lesions developed progressively about 5 weeks prior to being controlled, after which time, nonhealing and nonulcerative lesions persisted for as long as 6 months. 384 strain produced only moderate and transient footpad swelling. 848, 665 and 175 strains of *L. tropica* never produced footpad lesions, although parasites were recovered from aspirated tissue after 6 months (Figure 2). The differences in footpad thickness, observed 10 and 21 weeks after infection, were statistically significant ($P < 0.05$).

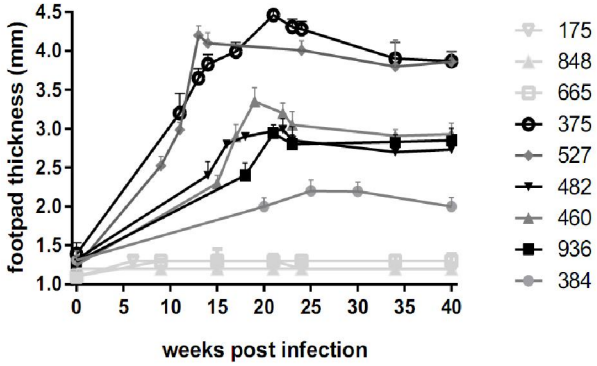

Fig.1. Course of lesion development in BALB/c mice inoculated with metacyclic promastigotes derived from nine *L. tropica* isolates. Mice were inoculated subcutaneously with 10^6 metacyclic promastigotes in the left hind footpad. Lesion size was monitored using a Vernier caliper and calculated by subtracting the size of the contralateral uninfected footpad. Values represent the mean lesion size of five mice per group (mm) ± the standard deviation.

Parasite loads in footpad lesions were determined by impression smears and by limiting-dilution assay (Figure 2). Mice with footpad swelling displayed high parasite loads in footpads and draining lymph nodes (10^4-10^5 and 10^6-10^8 parasites/mg, respectively) 6 months after inoculation, confirming the persistence of active infections in mice.

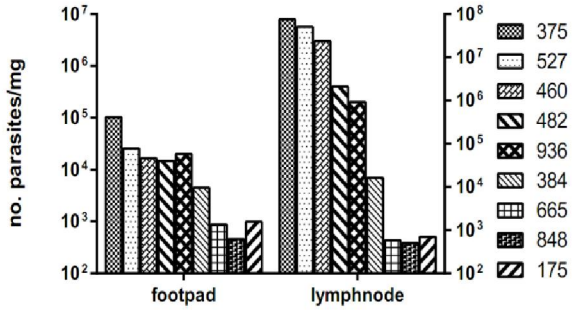

Fig.2. Tissue parasite burdens in BALB/c footpads and draining lymph nodes 6 months after sc inoculation of metacyclic promastigotes. Values represent the geometric mean number of parasites in each tissue, three mice per group.

Promastigote growth rates in vitro

We investigated the parasite development stage. All wild strains of *L. tropica* showed a typical growth curve, with well-defined logarithmic and stationary phases (Figure 3). *Leishmania* cells divided about every 6-7 hours. There was significant active division until 2 days of culture. In the late log phase and stationary phase *Leishmania* promastigotes differentiate into metacyclics. Interestingly, the strains highly pathogenic in mice, 527 and 375 strains, showed good adaptation to culture growth medium and reached higher concentrations on the 4th day of culture (about 107×10^6 and 91×10^6 promastigotes/ml, respectively). In contrast, the less virulent isolates, 665, 848, and 175 strains, grew very slowly and reached a maximum cell density about 21×10^6 promastigotes/ml on day 5. The 936 strain, which had an intermediate pathogenicity, also showed a growth rate, which was intermediate between those expressed by the high or the low virulence strains. The growth characteristics of these strains were reproducible over two different experiments.

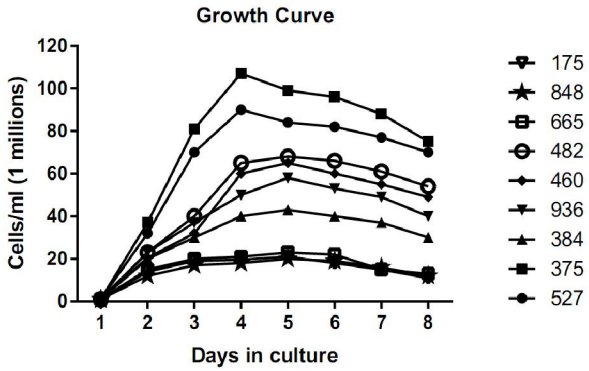

Fig.3. Growth kinetics of nine *L. tropica* isolates: 527 and 375 (high virulence), 936 (intermediate virulence), and 665, 848, and 175 (low virulence). Cultures were initiated in liquid medium at 10^6 cells/ml. The results represent the mean of parasite quantification of three culture samples per strain per day. The experiment was repeated three times.

Human macrophage infection with *L. tropica* and metacyclic promastigotes

Three *L. tropica* isolates; 527 (*L.t* HV, high virulence), 936 (*L.t* IV, intermediate virulence) and 665 (*L.t* LV, low virulence), selected on the basis of their different pathogenicity in BALB/c mice were used for further experiments. Four hours after addition of the metacyclic promastigotes, human macrophages were washed and the rate of macrophage infection was measured. Results are expressed as the percentage of infected macrophages and the mean number of amastigotes detectable within one infected

macrophage. At 4h post infection, *L.t* HV strain presented higher infectivity (76%±18) and *L.t* IV strains showed lower percentages of infected macrophages (44%±18), and *L.t* LV presented the lowest levels of infection (19.8%±4) (Figure 4A). After 48 and 72 hours post-infection, there was no change in the percentage of infected macrophages. Furthermore, Parasite burden was about twice as high in *L.t* HV than in *L.t* IV-infected macrophages and about threefold higher than in *L.t* LV-infected macrophages, and these differences were statistically significant ($P < 0{\cdot}05$) (Figure 4B).

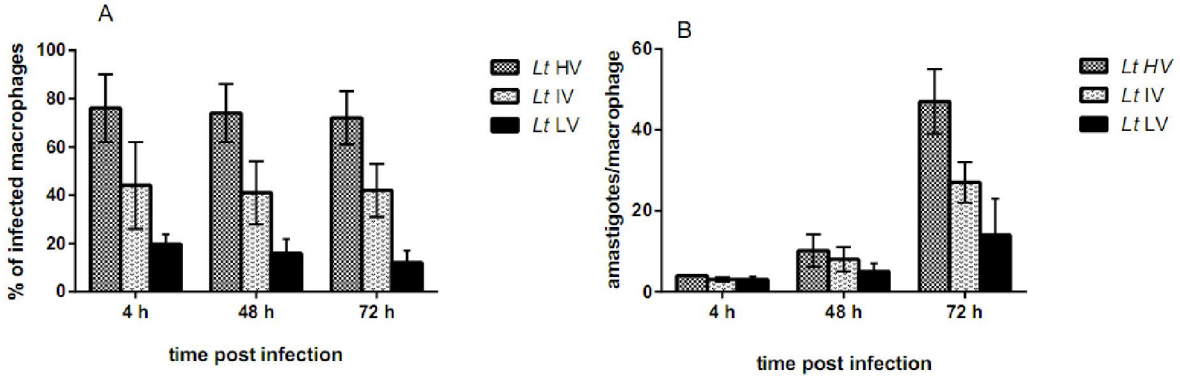

Fig.4. Infection of human macrophages by three *L. tropica* strains. Macrophages were infected with metacyclic promastigotes derived from one of three isolates at a ratio of five amastigotes/macrophage. At the indicated time, the fraction of infected macrophages (A) and the mean number of intracellular parasites per macrophage (B) were determined. Results are reported as the mean ± the standard deviation of duplicate samples.

Discussion

The present study has tried, using the BALB/c mouse model of *L. tropica* infection, to verify the variability in the natural virulence of nine *L. tropica* strains isolated in the field from human lesions. Afterward, the variability of virulence in *L. tropica* strains were evaluated through the assessment of their in vitro growth dynamics and infectivity in human macrophages. Although these isolates are likely heterogeneous, we decided to use nonclonal parasite populations in order to mimic the actual condition in the host cell. Using metacyclic promastigotes in infectivity rules out the possibility that differences in pathogenicity might merely reflect variable levels of in vitro metacyclogenesis made by the different strains.

Our results showed that the nine strains expressed significant variability in the pathogenicity induced in BALB/c mice. Some isolates were able to induce nonulcerative lesions, as commonly reported in BALB/c mice (4, 11) and three strain without any changes in footpad thickness. In contrast with the quickly progressing, nonhealing and destructive lesions produced by *L. major* (13), the *L. tropica* lesions were delayed in onset, nonulcerative and finally controlled. However, they were persisting for as long as about 40 weeks after challenge. The behavior of *L. tropica* in BALB/c mice determines this species from other cutaneous species including *L. major*, *L. mexicana*, *L. amazonensis* and *L. braziliensis* that have been shown to produce progressive, uncontrolled cutaneous lesions in BALB/c mice (14, 15). In similar studies, showed that different strains of *Leishmania major* (16), *Leishmania infantum* (17) and *Leishmania amazonensis* (18) isolated from patients promote different courses of infection in BALB/c mice.

In our study, three strains of *L. tropica* never produced footpad lesions, however, 6 months after subcutaneously inoculation of metacyclic promastigotes, parasites were recovered from aspirated footpads tissue and draining lymph nodes. Because cutaneous leishmaniasis caused by *L. tropica* is an anthroponosis in Iran, without any known nonhuman reservoirs, it is reasonable, that BALB/c mice are in general nonpermissive hosts for *L. tropica* and low virulence strains cannot able to produce lesions.

Correlation between virulence of *Leishmania* strains and ability to survive in murine macrophages has already been reported (16, 17), but few study is investigated about the macrophage response (19). Three strains 527, 936 and 665 (expressing high, intermediate and low virulence levels, respectively) were selected and studied for their capacity to infect human macrophages. We found that the high virulence strain was characterized by a significantly higher ability to infect human macrophages and to survive with higher replication levels within the infected macrophages. Parasites from high virulence strain were about two or three times more copious within macrophages than strains with lower virulence. These results suggest that although all strains of *Leishmania tropica* are ingested similarly by macrophages, high virulence strain replicates more rapidly inside the phagolysosome. Bodskyn *et al.* found that unlike the wild-type parasite, an avirulent *L. major* mutant was

phagocytized but unable to replicate in human macrophages (20). Similarly, the growth curve in liquid medium of the three strains was extremely different. The higher virulence strain grew more rapidly in culture medium. Moreover, the plateau phase reached significantly higher levels with the virulent strain compared to the two others.

Several parasite molecules have been shown to play an important role in *Leishmani*a virulence. Lipophosphoglycan (LPG) is a major surface component of the parasite glycocalyx, which was reported alteration in its structure correlate with alteration of the parasite virulence (21). Thus, avirulent clones are defective in LPG synthesis and go back to virulence when incubated with LPG (22, 23). Furthermore, return to virulence after passing *Leishmania* strains into BALB/c mice is correlated to higher expression of LPG (24). The higher virulence of metacyclic promastigotes compared to the procyclic parasites also correlates with changes in LPG structure (25). LPG roles are a receptor for attachment to macrophage cell membrane, a cofactor of survival within the phagolysosome, a key factor mitigating host responses by deactivation of macrophage signaling pathways and a modulator of several macrophage cytokines (26). Considering these important functional roles of LPG in host-parasite interactions, it was considered a major determinant of parasite virulence (27). Our results suggested that changes in the kinetics of in vitro parasite growth are classically associated with changes in parasite virulence and can be correlate to expression variation of LPG. This information could be useful in assessing possible virulence alterations induced by prolonged treatments that usually apply to *L. tropica*.

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