Original Article

Route of Infection Affects Pathogenicity of *Leishmania major* in BALB/c Mice

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Introduction: The term Leishmaniasis applies to a group of parasitic diseases caused by the genus *Leishmania*. Route of infection is one of the variables that have been reported to influence the immune responses as well as the disease outcome in experimental models of leishmaniasis. This research aims to study the effect of route of infection on the pathogenicity of *Leishmania major* in BALB/c mice. **Methods:** Low (10^3 parasites/mouse) and high (10^6 parasites/mouse) doses of *L. major* was injected into footpad or ear dermis of BALB/c mice. Lesion diameter was determined throughout the study. Parasite load of draining lymph nodes and spleen were assessed at three intervals. **Results:** Footpad in comparison to ear route showed higher pathogenicity of *L. major* in BALB/c mice as assessed by lesion diameter, parasite load in the draining lymph node, and dissemination of the parasite to the spleen. **Conclusion:** Our findings suggest that substantial differences between footpad and ear route need particular attention when we use experimental models for the study of *Leishmania* infections. *J Med Microbiol Infec Dis, 2017, 5 (1-2): 26-30.*

Keywords: *Leishmania major*, Pathogenicity, Administration Routes, Viscera, BALB/c mice.

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INTRODUCTION

Leishmaniasis includes a group of diseases caused by the parasites of the genus *Leishmania*. These parasites are transmitted to humans by the infective bites of sand flies. There are three primary forms of leishmaniasis: visceral, cutaneous, and mucocutaneous. Visceral leishmaniasis is the most severe form of the disease. There is a need for the development of new methods for prevention and treatment of these diseases. Experimental models can help achieve these goals. Several variables have been reported to influence the immune responses as well as the disease outcome in experimental models of leishmaniasis. These variables include the developmental stage, dose, species, strain, and substrain of the parasites used for injection, and the routes of inoculation as well [1-3]. Route of infection affects disease outcome [4] and immune response to *Leishmania* parasites [5]. Some reports have indicated the impact of infection route on the pathogenicity of *L. major* in C57BL/6 mice [5, 6].

The infectious dose is another crucial variable in BALB/c models of *L.* major infection; high doses result in exacerbation while low doses proceed to the protection of vertebrate hosts [7]. Also, high doses commonly lead to early dissemination of parasites to the spleen [8]. The traditional infectious dose for *Leishmania* infections in experimental animal models ranges from 10^5 to 10^7 parasites [9], while the infectious dose in natural transmission is much less, *i.e.*, <600 parasites in 75% of sand fly bites (within the range of 10-100000 parasites) [10, 11]. So, the results obtained from high dose infection may not have relevance to natural infection.

An essential difference between the cutaneous and visceral forms of leishmaniasis is the dissemination of parasites to internal organs and the ensuing damages in the latter form. The visceralization of *Leishmania* parasite is an index of its virulence, which is defined as "the degree of pathogenicity as indicated by case fatality rates and/or the ability of the organism to invade the tissues of the host" <http://www.ncbi.nlm.nih.gov/mesh/68014774>.

There is no information regarding comparison of the footpad and ear infection route for *L. major* in BALB/c mice. In this research, we studied the effect of infection route on the pathogenicity of *L. major* in BALB/c mice.

MATERIAL AND METHODS

Mice. Female BALB/c mice, 5-7 weeks old, were purchased from Pasteur Institute of Iran and maintained under conventional conditions in the animal care facility. Mice were housed in cages in a ventilated room with unlimited access to food and water under 12 h light and 12 h darkness in every 24 hours.

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Mice were euthanized by cervical dislocation before removal of spleen and lymph nodes. All experiments on the mice were approved by ethical committee of the Pasteur Institute of Iran (license number 95/0201/20704).

Parasite. *Leishmania major* strain MRHO/IR/75/ER was received from M. Mohebali (School of Public Health, Tehran University of Medical Sciences, Tehran, Iran). The identity of the parasite was previously confirmed by ITS1 PCR followed by RFLP [12]. The parasites were cultured in Novy Mac Neal Nicolle (NNN) media supplemented by RPMI medium, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The virulence of the parasites was preserved by injecting into BALB/c mice and retrieving it from draining lymph node of mice. Parasites were passaged *in vitro* by addition of new culture media every 4-5 days, and in order to maintain the parasites virulence, the parasites not grown in more than three consecutive *in vitro* cultures were used for injection to BALB/c mice.

Infection of BALB/c mice. Promastigotes were harvested at the stationary phase. They were ~35% morphologically metacyclic, acquired from metacyclic separation by Ficoll enrichment as described by others [13]. Volumes of 10 µl of the stationary phase promastigotes of *L. major* were injected subcutaneously by use of 0.5 ml insulin syringe (BD Company, USA Cat No. 329404) into the footpad or intradermally into the ear dermis. The low and high dose of promastigotes (10^3 and 10^6/mouse) were used for infection of mice. The lesions diameter was measured by assessing the thickness of ear or footpad with a Vernier caliper (Mitutoyo, Kawasaki, Kanagawa, Japan) at weekly intervals after infection. The diameter of lesions was calculated by subtracting the thickness of infected footpad or ear from contralateral uninfected footpads or ears. Lesion measurement was discontinued when the lesions became necrotic.

Parasite load assay. Parasite load was assayed in the spleen and draining lymph node of four mice of each experimental group. The number of parasites was estimated by quantitative limiting dilution assay according to Sacks & Melby [9]. Briefly, the lymph node and spleen of each mouse was mechanically homogenized in 1 and 3 ml of liquid culture media, respectively, and serially diluted (2 or 4-fold dilutions depending on the expected parasite load) in a 96-well microtiter plate containing NNN medium supplemented by liquid culture media. Each homogenized tissue was assayed in triplicate. The liquid culture media consisted of RPMI medium, 20% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The number of viable parasites in each organ was estimated from the highest dilution at which promastigotes were grown at 26°C after 10 days of incubation.

Study design. Four randomly divided experimental groups of mice (each consisted of 15 mice) were used in this study. Low (10^3 parasites/mouse) or high (10^6 parasites/mouse) doses of *L. major* were injected into either footpad or ear dermis of mice. The groups included low-dose/ear route, high-dose/ear route, low-dose/footpad route, and high-dose/footpad route. The criteria assessed in experimental groups included the thickness of lesions in footpad and ear, and parasite loads of draining lymph node and spleen at three intervals of one week, one month, and four months post infection. Four mice from each group were euthanized for assessment of parasite load at each interval. The study was carried out twice, and results of one representative experiment were presented.

Statistical analysis. Student's t-test was used to compare lesion diameter and parasite load results between two experimental groups. Multiple comparisons between more than two groups were performed by analysis of variance (ANOVA). A p-value of ≤ 0.05 was considered to be significant.

RESULTS

Effect of route of infection on lesion development. Our data showed that footpad route in comparison to ear route resulted in higher lesion diameters with a high infection dose of parasite dose, *i.e.*, 10^6 parasites/mouse (Fig. 1). The differences were statistically significant from week 4 after infection (p-value ≤ 0.05) up to the end of measurement of the lesions (16 weeks post-infection). The results of low dose infections did not show a significant difference between lesions size of the two infection routes. Also, both in footpad and ear dermis, the high infectious dose resulted in higher lesion diameters in comparison to the low infectious dose.

Effect of route of infection on parasite load of lymph node. The footpad infections, in comparison to ear infection, resulted in higher parasite load one month after infection by both high and low infectious doses ($p \leq 0.05$). The same differences were observed four months after infection, though not statistically significant (Fig. 2). Our data also showed, as it was predictable, that a high dose of the parasite, regardless of infection route, resulted in higher parasite load in draining lymph nodes ($p<0.05$) (Fig. 2).

Effect of route of infection on parasite load in spleen. We observed the parasite dissemination to spleen only in the footpad infection route (Fig. 3). The parasite dissemination occurred in one out of four mice at one month as well as four months after infection (Fig. 3). The visceralization tendency of the parasite is a reliable finding as it occurred in the repeated assays in 3 out of four mice 4 months after challenge. This finding shows that the parasite was of higher pathogenicity when inoculated via footpad compared with ear dermis.

DISCUSSION

High and low doses (10^3 and 10^6 parasites/mouse) were used in this study. The low dose in this study was used to simulate the infective bite by sand flies in nature [10, 11]. The high dose of 10^6 promastigotes/mouse was used to make our data comparable to the studies that used high doses [6-8, 14].

Our data showed that infection of *L. major* through footpad, in comparison to ear, resulted in higher pathogenicity.

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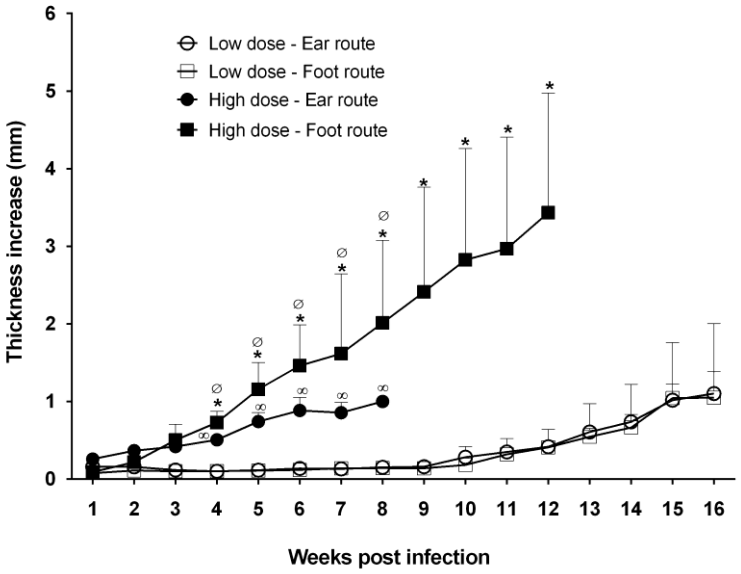

Fig. 1. Effect of infection route and dose of *L. major* on lesion development in BALB/c mice

Each point shows mean ± standard deviation in a group of 5 to 15 mice. ø, statistical significant difference between high dose foot and high dose ear. *****, statistical significant differences between high dose foot and low doses foot. ∞, statistical significant differences between high dose ear and low doses ear

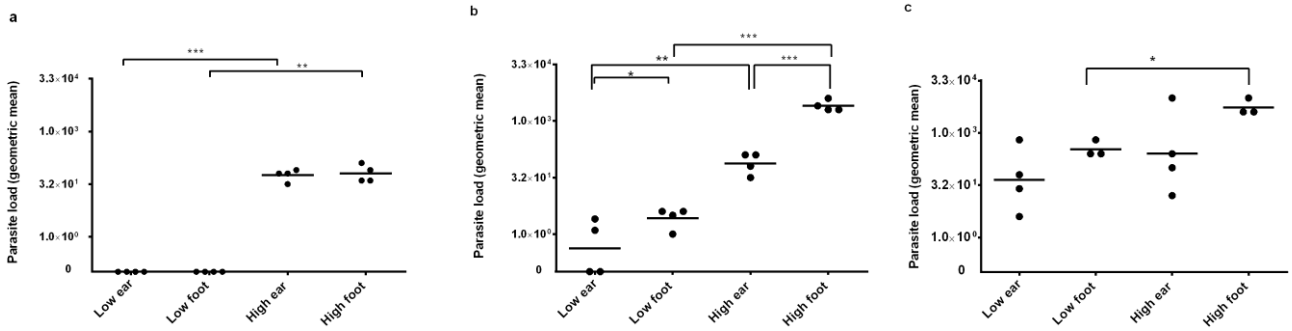

Fig. 2. Effect of infection route and dose of *L. major* on parasite load in lymph node of BALB/c mice

a, one week after infection. b, one month after infection. c, four months after infection. Each dot shows parasite load of lymph node in one mouse. Each bar shows the geometric mean in group of 3 or 4 mice. Asterisk shows statistical significant differences (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$)

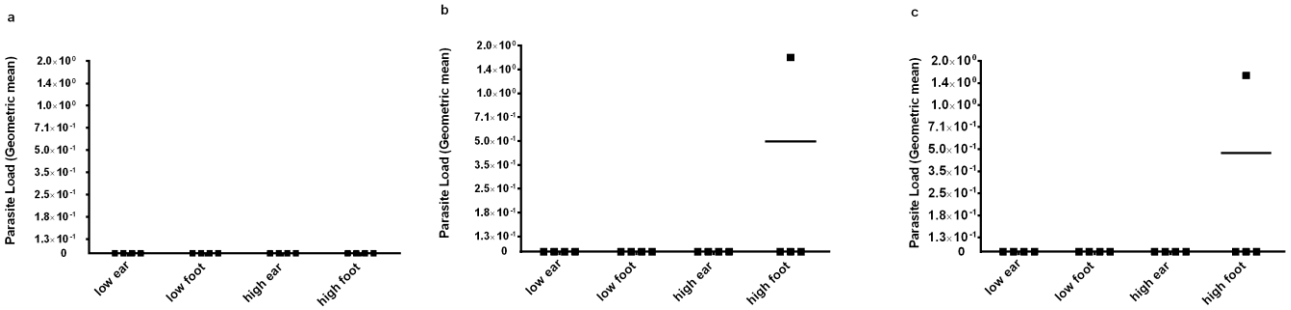

Fig. 3. Effect of infection route and dose of *L. major* on parasite load in spleen of BALB/c mice

a, one week after infection. b, one month after infection. c, four months after infection. Each dot shows parasite load in spleen of one mouse. The bars show geometric mean of groups

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The higher pathogenicity is shown by higher lesion diameter, higher parasite load in the draining lymph node, and dissemination of the parasite to the spleen. In our study, inoculation of *L. major* into BALB/c mice via footpad in comparison to ear resulted in a higher parasite load in draining lymph nodes, which is in agreement with the results of a similar study that used C57BL/6 mice [5]. Combining results of C57BL/6 mice with our results of BALB/c mice suggests that higher parasite loads of lymph node in footpad route in comparison to ear routes may be a characteristic of infection route and not a characteristic of mouse strain. It is noteworthy that these two mouse strains are entirely different regarding the pathogenicity of *L. major*. Hence, our findings suggest that route of infection may be responsible for the higher pathogenicity of *L. major* and not the genetic background of mice, even in the strains showing an utterly different susceptibility pattern to *L. major*.

Our data showed that infection through footpad route resulted in the visceral growth of *L. major* at a high dose while ear infection route with the same dose did not. The parasite dissemination to the spleen in footpad route was observed only in 1 out of 4 (25%) in the first experiment and 3 out of 4 (75%) in the repeated experiment 4 months after infection. The reasons for lack of visceralization in all mice are not clear for us, and further detailed experiments are required to confirm this preliminary data. Our findings regarding the absence of *L. major* in the spleen one week after low-dose infections are in agreement with another report [8]. However, our results of absence of parasites in the spleen at one week after high-dose infection are not in agreement with other reports [8, 15], which showed parasite dissemination to spleen from the weeks 1 and 2 after infection of the animal with *L. major*. These differences may be due to three possibilities: 1) higher infectious doses used by the two reports (2×10^6 stationary promastigotes and 10^7 amastigotes), 2) difference between the strains used, and 3) advantage of quantitation of alive parasite in our "limiting dilution assay" over "polymerase chain reaction" used in the two reports [8, 15].

The variation in the pathogenesis of a parasite once inoculated through different routes, *i.e*., footpad vs. ear can be explained by the cellular characteristics of different layers of the skin in footpad and ear; the inoculation via ear is intradermal while the footpad route is subcutaneous. The skin comprises three primary layers from outside to inside, epidermis, dermis, and hypodermis. In contrast to the epidermis and dermis, the hypodermis is naturally devoid of resident immune cells [16]. So, while the intradermal route targets dermal dendritic cells and macrophages, the subcutaneous injection do not directly involve the skin-resident antigen-presenting cells (Langerhans and dendritic cells), but instead, result in leukocyte attracted by inflammatory molecules secreted at the injection site [16].

Another difference between footpad and ear route is their anatomical position. Although the effect of different parts of the trunk (as anatomical position) has been reported for *L. major* infection of BALB/c mice [17], no difference has been shown between the ear dermis and the base of tail in the infection [18]. The potential effect of anatomical position in the present study requires further study.

The difference between footpad and ear route of infection should be brought into consideration when experimental animal models used for the study of *Leishmania* infections. Natural infection route of *Leishmania* by the sand fly is intradermal [19] and the data acquired through subcutaneous footpad route needs to be confirmed in more relevant intradermal ear route.

In summary, our findings suggest that *L. major* infection of BALB/c mice through footpad in comparison to ear results in higher pathogenicity. Leishmaniasis includes complexities that seem to be disarrangements of simplicities. Further studies will hopefully arrange these complexities in a simple order.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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