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Original Article

Pathogenicity Variations of Susceptibility and Resistance to *Leishmania major* MRHO/IR/75/ER Strain in BALB/c and C57BL/6 mice

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Abstract

Background: To compare the pathogenicity differences in two susceptible Balb/c and resistant C57bl/6 mice infected with *Leishmania major* MRHO/IR/75/ER as a prevalent strain of zoonotic cutaneous leishmaniasis in Iran.

Methods: Mice were assigned into four groups as control and infected BALB/c and C57BL/6 mice. Experimental leishmaniasis was initiated by (s. c) injection of the 2×10^6 *L. major* promastigotes into the basal tail of infected groups. The development of lesions was determined weekly by measuring the two diameters. After 10 weeks, all mice were killed humanly, target tissues including lymph node, spleen and liver from each mouse were removed, weighted, and their impression smears were prepared.

Results: Proliferation of amastigotes inside macrophages, pathogenicity signs in two susceptible, resistant hosts was varied, and these variations were depended on mice strain.

Conclusion: Host immunity may modify clinical signs and could affect the proliferation of amastigotes inside macrophages, the size of lesions, the survival rates, the degree of hepatomegaly and splenomegaly and the percentage of amastigotes in lesion, liver, spleen, lymph node and brain smears.

Keywords*:* Balb/c, C57bl/6, Pathogenicity, Iran, *Leishmania major,* MRHO/IR/75/ER

Introduction

Leishmania is a protozoan parasite that causes a spectrum of cutaneous, mucocutaneous or visceral clinical manifestations in host, depending on the parasite species, the host's immune response and genetics (1). Leishmaniasis is one of the most important infectious diseases worldwide. Currently, 12 million people in 88 countries are infected, with almost 2 million new infections per year. Unfortunately, 60,000 annual deaths result from leishmaniasis. Recently, the number of affected individuals has increased even in Europe due to co-infections with HIV. A vaccine does not exist at present and some treatment options are expensive and can cause resistance and major side effects (2). The disease is caused by an obligate intracellular parasite inoculated into the skin by the bite of a sand fly (3).

Healing in cutaneous leishmaniasis (CL) is thus dependent on the generation of immune cells (e.g. macrophages, neutrophils), cytokines (e.g. TNF-α, IFN-γ) and mediators, e.g. nitric oxide (NO) (3-5). Inducible NO production is required for control of *L. major* infection in mice (6). In addition, macrophages (MΦ) activate the expression of genes responsible for the high-

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output synthesis of intermediates, which contribute to the regulation of the inflammatory response. Most of these molecules, when synthesized at high concentration, exert pro-apoptotic effects and prevent the development of parasite (7). Interestingly, in *L. major* infections, MΦ were also responsible for the apoptosis (8), suggesting that in CL, MΦ critically contribute to parasite clearance and protective immunity (4).
Host genetic factors play an important role in resistance or susceptibility to infection with *Leishmania* (9). The disease phenotype observed in human CL can be mimicked in the laboratory by infection of different inbred strains of mice with *L. major* (5). The resistant C57bl/6 mouse, in particular, is believed to be a relevant model of *L. major* infections in humans, which are characterized by the development of localized dermal lesions that spontaneously heal. Balb/c mice produce Th2-type cytokines, which is associated with disease progression and susceptibility (5, 10). In contrast, recovery from infection in resistant C57bl/6 mice depends upon the induction of a Th1- type response resulting in activation of MΦ and killing of the intracellular organisms (11, 12). T-helper cells are characterized by the production of chemokines related to Th1 and Th2 responses (13, 14).
In spite of a large number of publication explaining the variations between susceptible and resistant strains during infection with *Leishmania* parasites (5, 9-12), there are no studies to investigate entire pathogenicity variations including the rate of lesion size, visceralization of parasite in target organs, proliferation of amastigotes inside macrophages, hepato/ splenomegaly degree, body weight and survival rate in a single study. This is a novel idea to test endemic Iranian strain of CL in two genetically different inbred mice by evaluation of pathogenicity parameters. Therefore, this study has been carried out to clarify pathogenicity patterns in both susceptible Balb/c and resistant C57bl/6 mice infected by *L. major* MRHO/IR/75/ER; as a prevalent strain of CL in Iran.

Materials and Methods

Animals

Female inbred Balb/c and C57bl/6 mice supplied by the Karaj Laboratory Animal unit, Pasteur Institute of Iran were used in this study. The initial body weight of control Balb/c were (27.6±1.6), test Balb/c (28.1±1.3), control C57bl/6 (19.3±0.3) and test C57bl/6 (18.3±1.6) (mean±standard error of mean, SEM). Mice were housed at room temperature (20-23 °C) on a 12 h light and 12 h dark cycle, with unlimited access to food and tap water. Experiments with animals were done according to the ethical standard formulated in the Declaration of Helsinki, and measure taken to protect animals from pain or discomfort. It was approved by Ethical Committee of the Pasteur Institute of Iran, in which the work was done.

In vitro cultivation of L. major IR/75

The *L. major* MRHO/IR/75/ER (IR/75) used in this study was the standard strain; the infectivity of the parasites was maintained by regular passage in susceptible Balb/c mice. The parasites were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 292 mg/ml L-glutamine and 4.5 mg/ml glucose (all supplied by Sigma). Under these culture conditions as determined by Kavoosi *et al.*, (2006), the stationary phase of parasite growth was obtained in 10 days (15).

Infection of Balb/c and C57bl/6 mice with L. major IR/75

Promastigotes of *L. major* IR/75 strain were harvested from culture media, counted and used to infect test groups of Balb/c and C57bl/6 mice. The base of the tail was injected intradermally with inoculums of 2×10^6 promastigotes in 0.2 ml.

Experiments and groups

The animal experiments were performed in four groups (n=5 mice / group) considering time, budget and long- period monitoring of animal according to the ethical issues for sample size and replication. The animal used in this expe-

riment including control 1 (naïve Balb/c), test 1 (Balb/c infected with *L. major*), control 2 (naïve C57bl/6) and test 2 (C57bl/6) infected with *L. major*.

Assessment of pathology

Measurement of lesion size

Lesion size was measured in millimeters (mm) by a digital caliper (Chuan Brand, China) in two diameters (D and d) at right angles to each other, and the size (mm) was determined according to the formula: S=(D+d)/2 at every week after inoculation (16).

Microscopical examination & smear preparation

The clinical diagnosis was confirmed by laboratory demonstration of the parasite in the lesions by making stained smears at the end of experimental period. Lesions were cleaned with ethanol and punctured at the margins with a sterile lancet and exudation material was smeared. Impression smears were prepared from liver, spleen, lymph node and brain by placing a small piece of tissue between two glass slides and pushing them in different directions. The smears were dried in air, fixed by methanol and stained with Giemsa for detection of amastigotes by light microscopy (16).

Measurement of amastigotes proliferation

The proliferation of parasites was evaluated by counting of amastigotes inside macrophages on Giemsa-stained lesion smears at the end of the experimental period. Five random MΦ were selected; counted and mean percentages were calculated as indicators for degree of proliferation in amastigotes inside each MΦ (16).

Assessment of hepato / splenomegaly degree

Entire livers and spleens were removed *post-mortem* at the end of the experimental period from mice after induction of terminal general anesthesia by inhalation of diethyl ether (Sigma). Organ wet weights were measured and compared with controls as indices for degree of hepatomegaly and splenomegaly (16).

Measurement of body weight and survival rate

Body weight was measured initially and at different times of experiment, using a top pan balance (OHAUS Scale Corp., USA). Survival rate was presented as the percentage of surviving experimental mice at every week after inoculation; test groups were compared with related controls and with each other.

Statistical analysis

The significance of differences was determined by Student's *t*- test and Analysis of variances (ANOVA) using Graph Pad Prism Software (Graph Pad, San Diego, CA, USA).

Results

Progress of cutaneous lesions was studied by measurement of lesion size in both susceptible Balb/c and resistant C57bl/6 test and control groups. Comparing the lesion size in these groups showed that the progress of lesion size in resistant mice is much lower than in susceptible ones. Lesions in C57bl/6 mice remained stable 0.3±0.2 mm until week 10 post infection, whereas in Balb/c mice, lesion sizes sharply increased (P<0.001) after week 5 to reach 14.2±1.5 mm (a mean diameter ~15 fold higher than in C57bl/6 mice). Macroscopically, the cutaneous lesions were ulcerated in Balb/c mice (Fig. 1).

Comparative proliferation of amastigotes inside macrophages was made by observation of parasites in Giemsa-stained smears in *Leishmania* groups. Percentages of positive smears were counted from lesion, liver, spleen, lymph node and brain of both Balb/c and C57bl/6 mice infected with *L. major*. In Balb/c groups, amastigotes were found in all lesion, liver, spleen and lymph node smears, whereas in C57bl/6 groups, no parasite was observed. Leishman bodies were more abundant in smears of lesion, liver, and lymph node than spleen (Fig. 2).

Proliferation of amastigotes inside MΦ was compared in lesions of Balb/c and C57bl/6 mice infected with *L. major*. It was made by observation of Giemsa-stained smears of lesions in *Leishmania* groups at the end of the experimental period .The proliferation of para-

site was evaluated by calculation of mean amastigotes inside five random MΦ. In Balb/c groups, a large number of amastigotes was found in MΦ of lesion, however in C57bl/6 groups, all MΦ were observed with no amastigotes inside (P<0.001) (Fig. 3).

Pathogenicity signs including hepatomegaly, splenomegaly, survival rate, and body weight were evaluated in four groups including two groups of controls and two groups of *L. major* infected Balb/c and C57bl/6 mice. An increase of liver and spleen weight was apparent in infected Balb/c mice; whereas no increase of spleen and liver weight were observed in C57bl/6 strain. Although, *Leishmania* represented its pathological effects by increasing hepatomegaly and splenomegaly because of disease in infected Balb/c mice (P<0.001); however, no pathological signs of hepatomegaly and splenomegaly were observed in C57bl/6 groups. All *L. major* infected C57bl/6 mice survived, whereas 40% of infected Balb/c mice died up to week 12 post infections. In addition, no significant alterations were observed in body weight of experimental groups (Fig. 4).

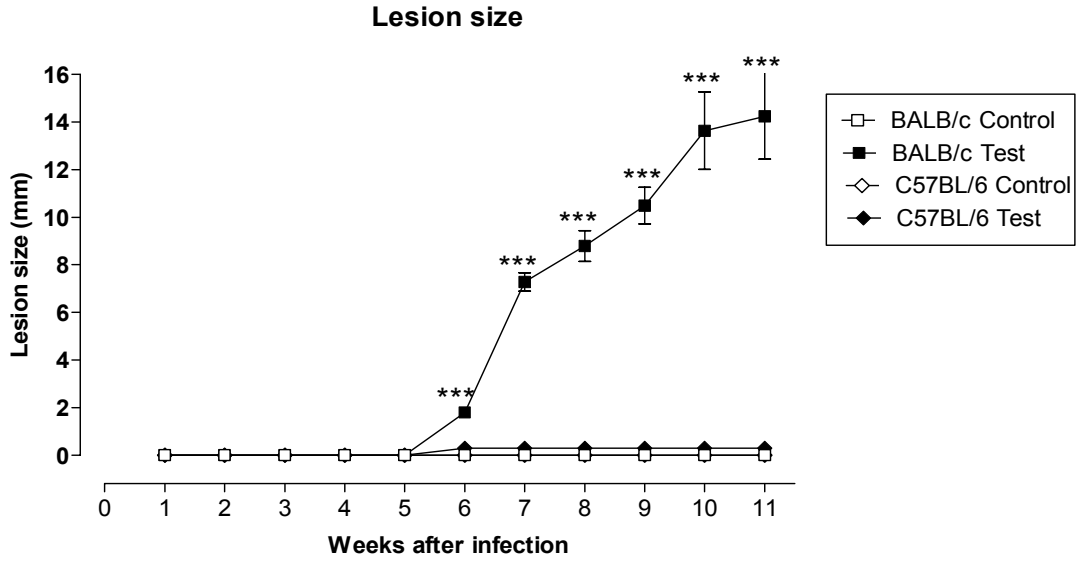

Fig. 1: Progress of lesion size of cutaneous leishmaniasis in groups of Balb/c and C57bl/6 mice infected with *L. major* .Lesion size was measured (in mm) by a digital caliper in two diameter (D and d) at right angles to each other, and the size was determined according to the formula: $S = (D+d)/2$. Significance of differences (P<0.001) was determined by Student's t-test using Graph Pad Prism (n=5 mice/group)

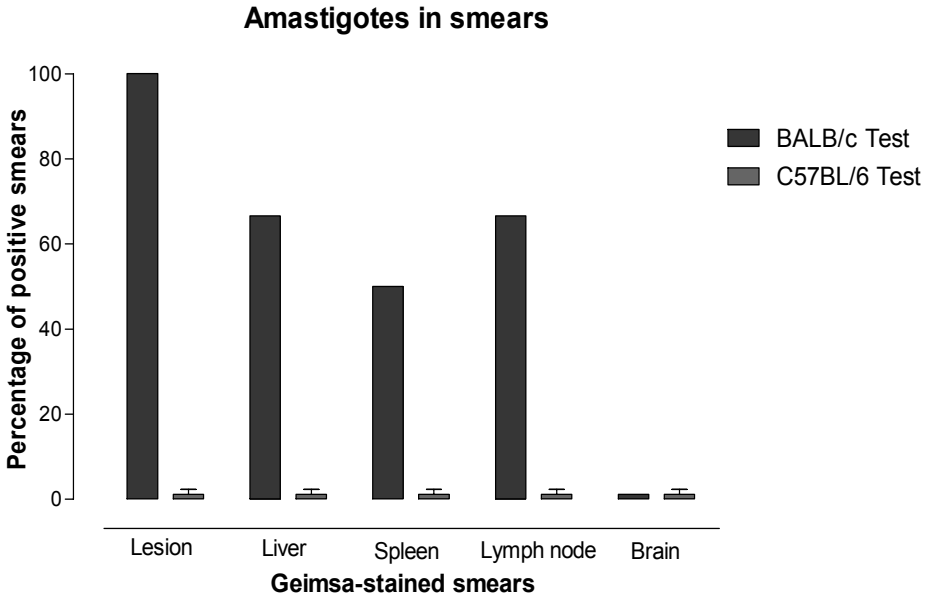

Fig. 2: Percentages of positive Geimsa-stained smears from lesions and tissues of Balb/c and C57bl/6 mice infected with *L.major*. Positive Geimsa-stained smears were counted from lesion, liver, spleen, lymph nodes and brain of test groups of mice infected with *L. major* at the end of the experimental period. Lesions were cleaned with methanol and punctured at the margins with a sterile lancet and exudation material was smeared. Impression smears were prepared from liver, spleen, lymph nodes and brain by placing a small piece of tissue between two glass slides and pushing them in different directions. Smears were dried in air, fixed by methanol and stained with Giemsa for examination by light microscopy (*n*=5 mice/group)

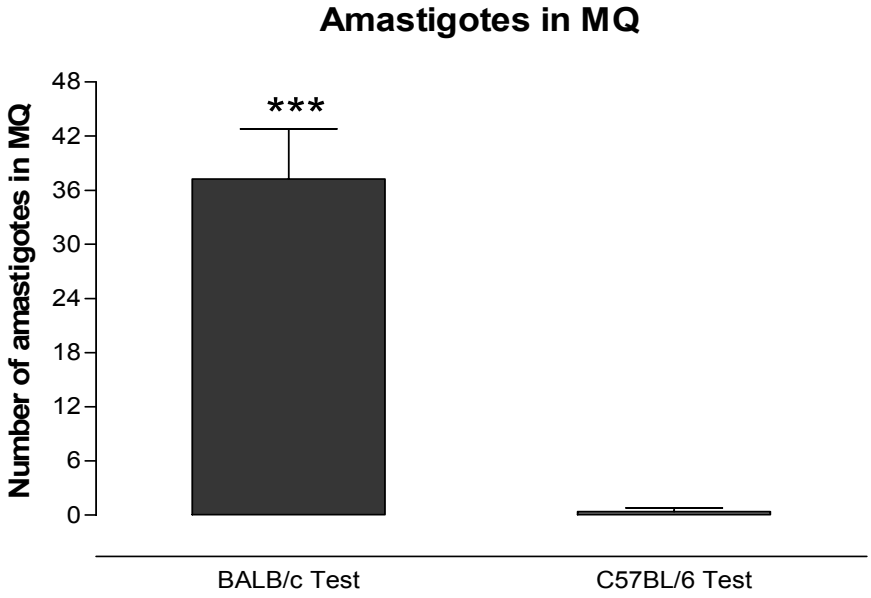

Fig. 3: Comparative proliferation of amastigotes inside macrophages from cutaneouse lesions of Balb/c and C57bl/6 mice infected with *L.major*. Proliferation of amastigotes inside macrophages was made by observation of Giemsa-stained smears of cutaneouse lesions in *Leishmania* groups at the end of the experimental period .The proliferation of parasite was evaluated by counting and calculation of mean amastigotes inside five random macrophages on Giemsa stained lesion smears. Analysis of differences ($P<0.001$) was determined by student's *t*-test using Graph Pad Prism (*n*=5 mice/group).

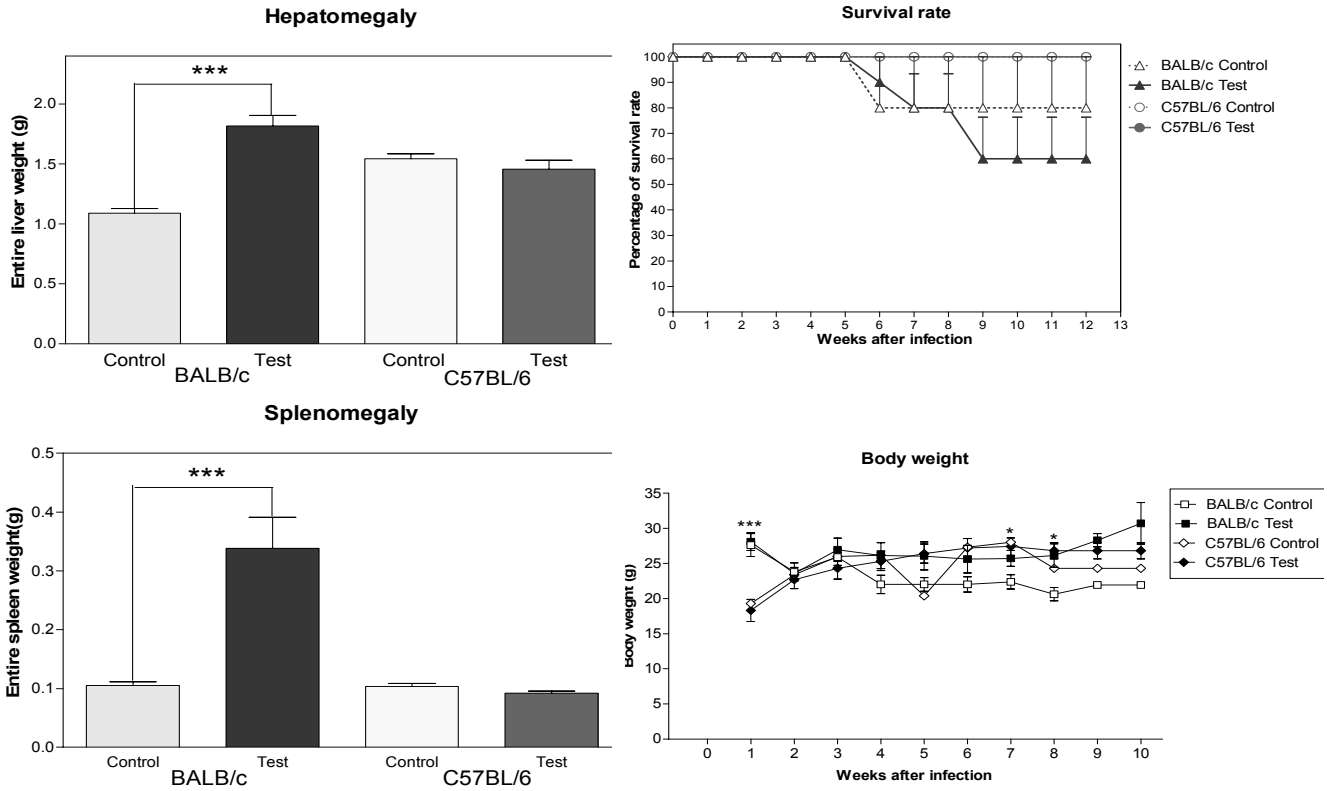

Fig. 4: Pathogenicity evaluation on four groups of Balb/c and C57bl/6 mice. Pathogenicity signs including hepatomegaly, splenomegaly, survival rate (at the end of the experimental period) and body weight (at every week after inoculation) all were evaluated in 4 groups of Balb/c infected with *L.major,* control Balb/c, C57bl/6 infected with *L.major* and control C57bl/6. For measurement of hepato/splenomegaly, entire livers and spleens were removed *post-mortem* from mice after terminal general aneastasia. Organ wet weights were measured as indices of possible hepatomegaly and splenomegaly. Survival rate was presented as the percentage of survival experimental mice at different weeks after infection. Body weight was measured using a top pan balance at different time of experiment. Significance of differences (P<0.001) was determined by One-Way ANOVA test using Graph Pad Prism (n=5 mice / group)

Discussion

The present study has revealed the course of infection and pathogenicity signs in susceptible Balb/c and resistant C57bl/6 mice infected with *L. major* IR/75, a prevalent strain of CL in Iran. In this study, liver, spleen, lymph node and brain as targets organs were studied to detect amastigotes, in order to evaluate visceralization of this cutaneous form of *Leishmania* parasite in rodent host. *Leishmania* parasites were detected in the spleen, liver and lymph node of infected Balb/c groups, whereas no parasite detected in target organs of C57bl/6 mice. Variation of parasite load in liver, spleen and lymph node has clarified a strain- specific difference of *Leishmania* localization.

In relevant publications, differences of parasite load between liver and spleen were related to the production of chemokines (17), mediators and MΦ (18). Activated MΦ released NO, which is believed to have leishmanicidal activity against *L. major* (16). The continuous presence of *L. major* makes possible spreading of the amastigotes to the lymph nodes and finally

to other visceral organs (19). Positive correlation between NO levels and evaluation of disease affect pathological signs of *L. major* infected Balb/c mice (16).

The course of *L. major* infection is markedly different in Balb/c and C57bl/6 mice (3, 20). This likely relates to differences in both the murine genetic profiles influencing the outcome of this infection (21, 22) and the immune responses directed by the individual pathogen species. There are some loci were described which controlled the size of *L. major*-induced lesions, and the degree of hepato / splenomegaly of the visceral consequences of advanced disease (9, 23, 24).

The high density of parasites that observed in spleen, liver and lymph node of Balb/c strain are a consequence of visceralization of parasite by blood stream dissemination into other target organs. The small and tiny cutaneous lesions in C57bl/6 mice were appeared at the site of inoculation, which were stable in size during infection. This is in agreement with previous reports indicated no progressive lesions in resistant C57bl/6 mice infected with CL strains (5, 9-11). Lesion development, especially necrosis, was related primarily to an acute infiltration of immune cells into the parasite-loaded dermis (25). Inherent properties of the MΦ in the lesions of resistant and susceptible mice determine the outcome of the infection via the mutual interaction of MΦ and T-cells (19).

In conclusion, this study on Iranian experimental CL infection revealed a variation responses in Balb/c and C57bl/6 mice infected with *L. major*. This variation may be associated with different genetic profiles of these two strains of inbred mice. Some of these results are in agreement with the previously reports in *L. major* infection (3, 20). Analysis of data revealed that differences in host immunity can modify clinical signs and could affect the proliferation of amastigotes inside MΦ, lesion size, survival rates, degree of hepato / splenomegaly and percentage of amastigotes in smears of lesion, liver, spleen, lymph node and brain.

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