Immunotherapy with Imiquimod Increases the Efficacy of Glucantime Therapy of *Leishmania major* Infection

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ABSTRACT

Background: Leishmaniasis is a complex disease which presents as visceral, cutaneous and mucocutaneous forms. The current treatment options for this infection are very limited and the immunological state of the host appears to play an important role in the efficacy of the treatment. Immunostimulation through immune response activating agents such as Imiquimod is another rational approach for this purpose. **Objective:** We assessed the efficacy of immunotherapy with Imiquimod alone or combined with Glucantime for treatment of *Leishmania major* infection in BALB/c mice. **Methods:** Treatment efficacy was monitored by determination of thickness and parasite load of infected footpad of mice. **Results:** The footpad thickness revealed that treatment with Imiquimod plus Glucantime has the highest efficacy. The results were confirmed by parasite load of infected footpad. Our data shows that treatment of *Leishmania major* infection in BALB/c mice by the combined Imiquimod and Glucantime is more efficient than each drug alone. **Conclusion:** The combination of Imiquimod with chemotherapy may offer a way for more efficient treatment of leishmaniasis.

Keywords: BALB/c Mice, Glucantime, Imiquimod, *Leishmania major*

INTRODUCTION

Leishmaniasis is an infectious disease caused by different species of Leishmania and is placed among the six most important tropical diseases by world Health Organization, with about 12 million people at risk of infection (1,2). It is a complex disease manifesting as visceral, cutaneous and mucocutaneous forms, where cutaneous leishmaniasis is the most common form of the disease and results in severe skin infection caused by several species, including *Leishmania major* (2,3).

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Despite great efforts, there is no acceptable vaccine against leishmaniasis up to now, and current treatment options are very limited. The first line treatment for all clinical forms of leishmaniasis is pentavalent antimonial stibogluconate and meglumine antimoniate, which has been used since almost 50 years ago (4,5). These drugs have shown several disadvantages such as toxicity, limited to parenteral administration and also developing of resistance to Antimony (6). In addition, antimonials are not approved by the Food and Drug Administration (FDA) for treatment of leishmaniasis (7). Another recommended drug is Amphotericin that should also be used parenterally and has other limitations including high cost, severe toxicity, variable efficiency, and restricted suppliers (8). Therefore, it is necessary to search for new drugs for the treatment of leishmaniasis in order to improve chemotherapy, and reduce the side effects of traditional treatments (9). In leishmaniasis, the immunological state of the host appears to play an important role in clinical pattern of the disease and on efficacy of the treatment; in this manner the parasite can establish infection through host immunosuppression. The fine balance of host immunosuppression suggests that immunostimulation is another rational approach to the treatment of the disease (10,11). It was therefore suggested to administer immunochemotherapy (immunotherapy plus chemotherapy) to patients suffering from severe forms of the disease, who did not respond to chemotherapeutic treatments (12).

Several immunomodulators have been studied alone or combined with chemotherapy up to now, either in cutaneous or visceral leishmaniasis, resulting in accelerated efficacy. In murine leishmaniasis, recombinant Th1 stimulating cytokine IL-12 given alone or combined with either Sodium Stibogluconate or Paromomycin cured the mice infected with *Leishmania major* (12-14). Recently, a combination of Z-100, a polysaccharide obtained from *Mycobacterium tuberculosis* combined with pentavalent Antimonial, was found effective against *L. amazonensis* in vitro (14). Thus, immunotherapy combined with chemotherapy may rapidly induce the effective immune response, resulting in improved treatment efficacy. The imidazoquinolines, of which Imiquimod is the best known, are low molecular weight substances and novel immune response-activating agents that can regulate immune responses through interactions with toll-like receptors (TLRs) on cells (15). Imiquimod acts via its immunomodulatory activity on various cell types such as epidermal Langerhans cells considered to be involved in immune responses by releasing a number of cytokines including IFN-γ, TNF-α, IL-1β, IL-1α, IL-6, IL-10, IL-1 receptor antagonist, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte CSF (G-CSF) (15-17). It was also found to induce and increase phagocytosis by production of nitric oxide (7,18). In the present study, we assessed the efficacy of immunotherapy with Imiquimod alone or combined with Glucantime for treatment of Leishmania major infection in BALB/c mice.

MATERIALS AND METHODS

Leishmania major strain MHROM/IR/75/ER was cultured in Schneider medium supplemented with 10% inactivated fetal calf serum, 292 µg/ml L-glutamine, 4.5 mg/ml glucose, 100 µg/ml streptomycin and 100 µg/ml penicillin at 23-25˚C. Virulence of the parasite was maintained by its repeated passage in BALB/c mice. The parasite was harvested

at stationary phase of growth, washed three times by PBS, counted and re-suspended in PBS. BALB/c mice, 4-6 weeks of age, were provided by animal production facilities of Pasteur Institute of Iran.

Study Protocol. Mice were divided into four groups (15 mice per group). One group was treated by Imiquimod (group IMQ), second group by Glucantime (group GLU), third group by Imiquimod plus Glucantime (group IMQ+GLU), and group 4 which did not receive any treatment was used as control (group Cont). In this study, maintenance and care of experimental animals complied with National Institute of Health guidelines for the humane use of laboratory animals.

Challenge Test. All groups were challenged by 2×10^6 stationary phase *Leishmania major* promastigote injected subcutaneously into the left hind footpad. Footpad thickness was assayed by a metric caliper at weekly intervals for 8 weeks after challenge.

Treatment Protocol. Treatments started 15 days after challenge. Imiquimod was utilized as a cream (Aldara, 3M Pharmaceuticals, St. Paul MN). The cream was applied on skin surface of the challenged site (coverage extended to 2 mm outside of the lesion border). The cream was used once every three days for 2 weeks. Glucantime was injected intraperitoneally at a dose of 100 mg/kg/day for 12 days (7).

Parasite Load Assay. Parasite load was assayed in popliteal lymph nodes by limiting dilution as described elsewhere (19). In brief, popliteal lymph nodes were removed under sterile conditions, homogenized and resuspended in culture media composed of RPMI1640 (Biosera) plus 15% heat-inactivated fetal calf serum (Biosera), penicillin (100 U/ml), and streptomycin (50 mg/ml) and the cell suspensions were serially (10 fold) diluted in a 96-well plates and kept at 24°C for 1 week. Eight repeats were used for each dilution. After 1 week of incubation, plates were examined with an inverted microscope. The presence or absence of mobile promastigotes was recorded in each well. The final titer was the last dilution for which the well contained at least one parasite. Parasite load for each sample was calculated, by SAS PROC IML program based on Taswell method (19) and assayed in three intervals: before treatment (two weeks after challenge), 15 days, and 45 days after the beginning of the treatment.

Statistical Analysis. Footpad thicknesses and parasite loads were compared between different groups by t-test or ANOVA. The p values lower than 0.05 were considered significant.

RESULTS

Treatment Efficacy Assay by Inhibition of Footpad Increase. Footpad thickness increase in this experimental model shows more pathogenicity of the parasite, i.e. lower efficacy of treatment. Comparison of footpad thickness increase among different groups had revealed that treatment with Imiquimod plus Glucantime (group IMQ+GLU) had the highest efficacy (lowest increase of the footpad thickness). The treatment efficacy decreased in mice treated with Glucantime alone (group GLU). The mice receiving Imiquimod alone (IMQ) had the lowest treatment efficacy as assessed by an increase in the footpad thickness (Figure 1). The footpad thickness difference between different groups was evident 4 weeks after the beginning of the treatments onwards. However, these differences were statistically significant only 6 weeks after the beginning of the treatment onwards.

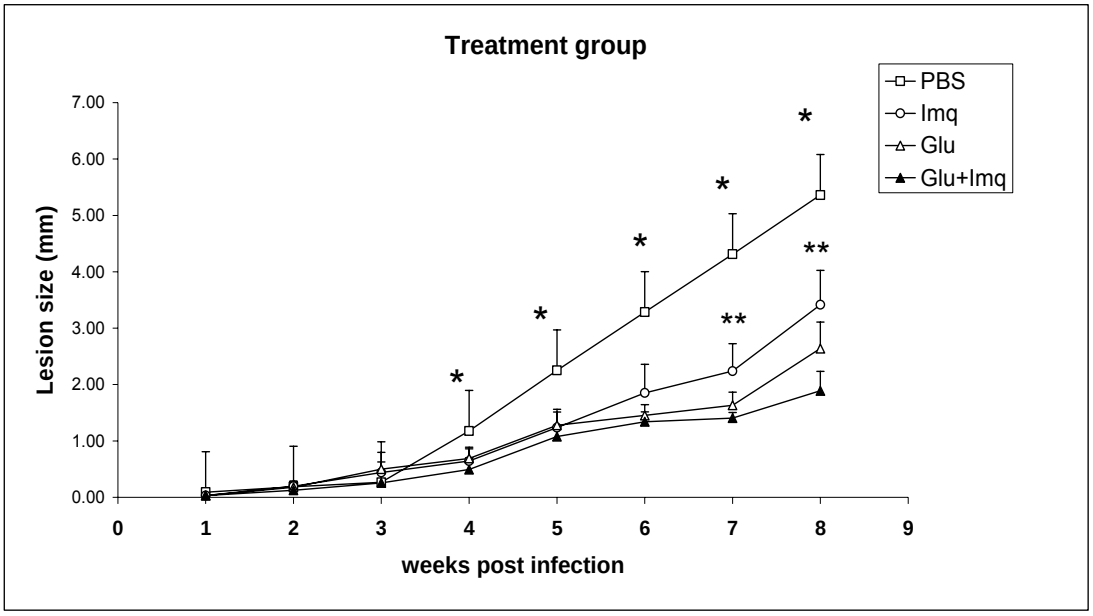

Figure 1. The effect of Imiquimod and Glucantime treatment on footpad thickness increase of *Leishmania major* infected BALB/c mice. Mice were infected with 2×10^6 stationary phase *Leishmania major* promastigotes in the footpad. Treatment started 15 days after infection. Values show footpad thickness ± SD of eight mice per group. "*" shows significant difference ($p<0.05$) between control group and all other treated groups. "**"shows significant difference ($p<0.05$) between Imiquimod treated group and mice treated with Glucantime alone or Glucantime plus Imiquimod.

Treatment Efficacy Assay by Parasite Loads. The number of the parasite in the lymph node was indicative of more pathogenicity of the parasite. Comparison of parasite loads between different groups at 45 days after the beginning of the treatment confirms the results of footpad thickness. Figure 2 demonstrates that treatment efficacy decreases in the order of Glucantime plus Imiquimod (group GLU+IMQ), Glucantime alone (group GLU), and Imiquimod alone (group IMQ). The difference of parasite loads between different experimental groups at 45 days after the beginning of the treatment was statistically significant between GLU+IMM and all the other groups, and also between GLU and all the other groups. The IMQ group showed lower parasite load in comparison to the control group. However, this difference was not statistically significant up to the last parasite load assay in the experiment (45 days after the beginning of the treatment).

DISCUSSION

In leishmaniasis, the immune response of the host plays an important role in both the clinical patterns of the disease and on the efficacy of the treatment. Therefore, immunotherapy or immunochemotherapy has been suggested for patients suffering from severe forms of the disease, not responding to usual chemotherapeutic treatments (10-13).

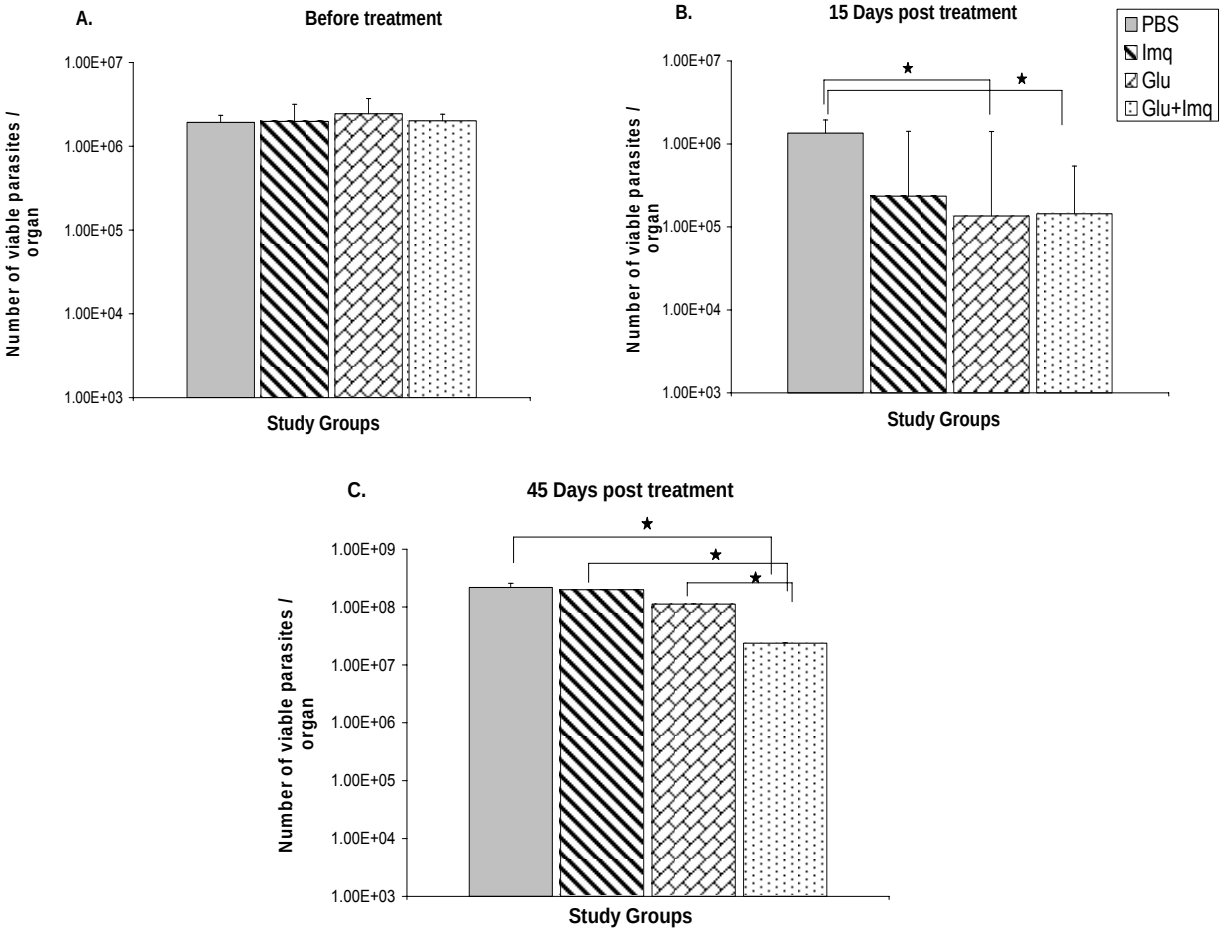

Figure 2. Effect of Imiquimod and Glucantime treatment on lymph node parasite load of *Leishmania major* infected BALB/c mice. Mice were infected with 2 × 10^6 stationary phase Leishmania major promastigotes in the footpad. Treatment by Imiquimod (IMQ), Glucantime (GLU), or the combination of the two drugs (GLU+IMQ) started 15 days after infection. Values show geometric mean titer ± SD of parasite loads of 3 mice per group before treatment (A), 15 days after (B) or 45 days after (C) the beginning of the treatment. "*" shows significant difference (p≤0.05) between the groups.

The combination of chemotherapy with immunomodulators may improve treatment by increasing the efficacy of anti-Leishmania drugs through triggering Th1 immune response and/or decreasing the toxicity of drugs via relatively low dose administration (14). It is assumed that imidazoquinoline compounds are immunomodulators, inducing Th1 responses and activating dendritic cells and macrophages when applied on the skin. Previous studies have shown that Imiquimod and its more potent analog Resiquimod directly activate macrophages and mediate intracellular killing of Leishmania amastigotes in the absence of T cells by activating the NF-kB and API signaling pathways through binding to Toll-Like receptor 7 (7,15). This results in the production of NO from macrophages and the secretion of proinflammatory cytokines, inducing IL-12 which plays an essential role in the control of Leishmania infection (7,18,21-23).

Buates et al. have shown that Imiquimod can induce leishmanicidal activity in vitro in

bone marrow macrophages infected by *L. donovani*. They confirmed that treatment of *Leishmania major* infected BALB/c mice with 5% Imiquimod topical cream can reduce parasite burden and the severity of lesions (7).

Our data shows that combined treatment of *Leishmania major* infected BALB/c mice with Imiquimod plus Glucantime is more efficient than each drug used alone as assessed by the inhibition of footpad thickness increase and by lower parasite burden. However, none of the treatment protocols resulted in complete cure in the mice as shown by footpad thickness increase in all treated groups, up to the end of the experiment. This study reveals that Imiquimod alone did not result in statistically significant cure of *Leishmania major* infected BALB/c mice. Our data differ from those of Bautes and Matlashewski, who had shown that Imiquimod alone was able to control *Leishmania major* infection in BALB/c mice, resulting in significant cure of the lesion (7). This discrepancy may be related to the onset of treatment which was started at the time of challenge by Bautes while in our study, treatment started 15 days after challenge.

Our result indicated that co-administration of Imiquimod with Glucantime was more effective than either one alone for the treatment of *Leishmania major* in BALB/c mice. These results suggest that Imiquimod can increase the leishmanicidal activity of Glucantime. Meanwhile, there is a possibility of a synergistic effect in the leishmanicidal pattern, because it has been recently reported that endogenous IFN-γ is involved in leishmanicidal response to some leishmaniasis drugs (21,22). Similar results are reported in literature when Imiquimod is combined with paromomycin (24). Our results are in agreement with another report that showed a 5% Imiquimod topical cream alone barely affected the parasite load or size of lesions in BALB/c mice infected with *Leishmania major*, but combination therapy with paromomycin and topical Imiquimod cream was effective (24).

In conclusion, the combination of chemotherapy with immunomodulators offers a way for the treatment of leishmaniasis decreasing the toxicity of drugs with relatively low dose of chemotherapy, reducing the time of treatment and decreasing the incidence of drug resistance. However, this combination should be further evaluated and optimized in experimental models and in humans.

ACKNOWLEDGMENT

This study is funded by grant No. 329 from the Pasteur Institute of Iran.

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