Treatment of \textit{Leishmania donovani}-infected hamsters with miltefosine: analysis of cytokine mRNA expression by real-time PCR, lymphoproliferation, nitrite production and antibody responses

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Objectives: Miltefosine, an orally effective antileishmanial drug, works directly on the parasite by impairing membrane synthesis and subsequent apoptosis of the parasite and has also been reported to have macrophage-activating functions that aid parasite killing. We investigated the type of immunological responses generated in miltefosine-treated \textit{Leishmania} donovani-infected hamsters, which simulate the clinical situation of human kala-azar.

Methods: Twenty-five-day-old infected hamsters, treated with miltefosine at 40 mg/kg for 5 consecutive days, were euthanized on days 30 and 45 post treatment (p.t.) and checked for parasite clearance and for real-time analysis of mRNAs of the Th1/Th2 cytokines interferon-\textgreek{g} (IFN-\textgreek{g}), interleukin-12 (IL-12), tumour necrosis factor-\alpha (TNF-\alpha), inducible nitric oxide synthase (iNOS), IL-4, IL-10 and transforming growth factor-\beta (TGF-\beta), nitric oxide (NO) production, the lymphocyte transformation test (LTT) and antibody responses. Responses were compared with the normal and \textit{Leishmania} infected groups at the same time points.

Results: By day 45 p.t. there was a significant increase in the mRNA expression of iNOS, IFN-\textgreek{g}, IL-12 and TNF-\alpha, whereas there were significant decreases in IL-4, IL-10 and TGF-\beta in cured hamsters as compared with their infected counterparts. \textit{In vitro} stimulation of lymphocytes with concanavalin A and soluble \textit{Leishmania} donovani antigen showed a maximum LTT response and there was a gradual increase in the NO level (\textapprox 7-fold compared with infected counterparts). Anti-\textit{Leishmania} IgG and IgG1 levels, found to be elevated in the infected group, decreased significantly after treatment but there was a significant increase in IgG2 isotype.

Conclusions: Treatment of \textit{Leishmania}-infected hamsters with miltefosine reverses the Th2-type response into a strong Th1-type immune response.

Keywords: Th1 response, Th2 response, immune responses

Introduction

Visceral leishmaniasis (VL) is one of the most important infectious diseases caused by the parasite \textit{Leishmania donovani} and is fatal if left untreated. The currently available drugs for the treatment of VL have several limitations. The disease outcome of VL is associated with immunological dysfunctions of T cells, natural killer cells and macrophages and as a result the treatment of VL is compromised. Therefore, an antileishmanial drug that can effectively and quickly reverse the immunosuppression of the infected host, besides killing the parasite, is desirable. A major milestone in the chemotherapy of VL was the discovery of miltefosine, initially developed as an anticancer agent, which directly affects the parasite by impairing its membrane synthesis and subsequent apoptosis. A report by Murray and Delph-Etienne suggested that miltefosine did not require any immune contribution to exert its antileishmanial functions in mice. In contrast, Constantinescu et al. attributed the importance of miltefosine’s immunomodulatory activity for the interleukin-12 (IL-12)-dependent production of interferon-\textgreek{g} (IFN-\textgreek{g}), which activates macrophages for inducible nitric oxide synthetase-2 (iNOS2)-dependent elimination of \textit{Leishmania}. Wadhone et al. observed that the antileishmanial function of miltefosine was significantly compromised in IFN-\textgreek{g}-deficient macrophages, suggesting the importance of endogenous IFN-\textgreek{g} in the miltefosine-induced antileishmanial function of macrophages. The authors further concluded that \textit{L.} donovani-infected macrophages induced a Th2 response but miltefosine treatment reversed this to a Th1 type response.
These findings prompted us to explore whether miltefosine is able to reverse the immunosuppression occurring in L. donovani-infected hamsters (Mesocricetus auratus), a model that, unlike the mouse model, largely correlates with the human manifestation of the disease and provides a perfect experimental model. We aimed to investigate the changes in various immunological parameters, particularly the mRNA expression of Th1/Th2 cytokines [tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)), IFN-\(\gamma\), IL-12, IL-10, IL-4, transforming growth factor-\(\beta\) (TGF-\(\beta\))] and iNOS, lymphoproliferative responses (lymphocyte transformation test, LTT), nitrite (NO) production and antibody responses, particularly those of the IgG isotypes, in this experimental host during infection and after treatment with miltefosine. Since this model has been hampered by the lack of commercial analytical reagents for cytokines we used a quantitative real-time PCR (qRT–PCR) method to determine intracellular Th1/Th2 cytokines.

Materials and methods

L. donovani strain (MHOM/IN/80/DD8) has been maintained as promastigotes in vitro and as amastigotes through serial passages in hamsters. All animal care and experimental use conformed to CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals) guidelines for laboratory animal facilities and were approved by the institutional animal ethics committee.

Twenty-five-day-old infected hamsters were treated with miltefosine at 40 mg/kg for 5 days consecutively and were checked on days 30 and 45 post treatment (p.t.) for the clearance of L. donovani amastigotes by splenic biopsy as described elsewhere, and the parasite load was assessed in comparison with the untreated control. The effect of miltefosine on the immunological responses was studied in these cured hamsters and compared with the untreated infected as well as untreated normal controls. Each group contained 8–10 animals. Two to three animals from each group were euthanized on days 60 and 75 post infection (p.i.), i.e. days 30 and 45 p.t. Blood, spleen and lymph node samples were taken for various immunological assays to characterize cell-mediated immunity (CMI) following treatment. To determine the cytokine profile, total RNA was extracted from spleen cells by the Trizol method and cDNA was synthesized according to the manufacturer’s protocol (Fermentas).

Primers for the quantification of the Th1/Th2 cytokine mRNA (TNF-\(\alpha\), IFN-\(\gamma\), IL-12, IL-10, IL-4, TGF-\(\beta\)) and iNOS mRNA were designed with Beacon Designer Software (Bio-Rad) on the basis of mRNA sequences available on PubMed (Table 1). qRT–PCR was conducted using SYBR Green PCR Master Mix (Bio-Rad) on an iQ5 multicolor real-time PCR system (Bio-Rad) according to the protocol of Samant et al., and data were analysed by the comparative \(C_t\) method. cDNAs from infected hamsters were used as comparator samples. All quantifications were normalized to the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT). A no-template control cDNA was included to detect contamination or non-specific reactions.

The cellular responses of lymph node cells of experimental animals to the mitogens concanavalin A (ConA) and lipopolysaccharide were assessed by the LTT and NO production, respectively, and soluble L. donovani antigen (SLD) as described elsewhere, with some modifications in which we used XTT (Roche Diagnostics) instead of \(^{3}H\)thymidine. Eighteen hours prior to termination of the experiment, XTT (50 \(\mu\)L) was added to 100 \(\mu\)L of the supernatant of each well and absorbance was measured at 480 nm with 650 nm as the reference wavelength. To estimate NO production, nitrite accumulation in culture supernatants of hamster peritoneal macrophages was determined by the Griess reaction as described elsewhere.

Antileishmanial antibody levels of IgG and its isotypes IgG1 and IgG2 were assessed using sera from all the experimental groups collected on day 30 and day 45 p.t. as described by Samant et al., with slight modifications. Briefly, 96-well ELISA plates (Nunc) were coated with SLD (0.2 \(\mu\)g/100 \(\mu\)L/well) overnight at 4°C and blocked with 1.5% bovine serum albumin at room temperature (RT) for 1 h. Sera were used at a dilution of 1/100 and kept for 2 h at RT. After washing, biotin-conjugated mouse anti-Armenian and Syrian hamster IgG, IgG1 and biotinylated anti-Syrian hamster IgM (BD Pharmingen) were added for 1 h at RT and were further incubated with peroxidase-conjugated streptavidin at 1/1000 (BD Pharmingen) for 1 h. Finally, the substrate 3,3,5,5-tetramethylbenzidine (Sigma–Aldrich) was added and the plate was read at 492 nm.

Two sets of experiments were performed in which two or three animals were used at each timepoint, i.e. days 30 and 45 p.t., in each set of experiments and the results were analysed by one-way ANOVA followed by Tukey’s post test using Graphpad Prism (version 3.03). The level of significance was set at \(P<0.05\).

Table 1. Sequences of forward and reverse primers used for quantitative real-time-PCR

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<th>Number</th>
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<tr>
<td>1</td>
<td>HPRT forward 5′ GATAATCCACCTCCTCCATAACTG 3′</td>
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<tr>
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<tr>
<td>12</td>
<td>iNOS reverse 5′ AGGATCAGAGGAGCACATC 3′</td>
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Results and discussion

Almost complete cure with miltefosine was observed by day 45 p.t. in L. donovani-infected hamsters compared with untreated infected hamsters when checked by microscopic examination of Giemsa-stained splenic dab smears. This was further confirmed by culturing spleen tissue of treated hamsters in complete RPMI medium; no promastigotes were seen at day 7 post culture. In untreated L. donovani-infected animals increases in mRNA transcripts of IL-4, IL-10 and TGF-\(\beta\) were observed and transcripts of iNOS, TNF-\(\alpha\), IFN-\(\gamma\) and IL-12 were decreased. With miltefosine treatment there was a noteworthy increase in the expression levels of Th1 cytokines by day 45 p.t. (Figure 1a). Significant among these were iNOS, IFN-\(\gamma\), IL-12 (\(P<0.001\)) followed by TNF-\(\alpha\) (\(P<0.01\)). In contrast, among the Th2-type cytokines there was a significant decrease in the expression of mRNAs for TGF-\(\beta\) (\(P<0.01\)), IL-4 and IL-10 (\(P<0.05\)) at this timepoint. Our findings endorsed the observations of Wadhone et al., who reported that miltefosine treatment of Leishmania-infected cells in vitro enhanced IFN-\(\gamma\), iNOS and IL-12 production.
Figure 1. (a) Real-time analysis of Th1/Th2 mRNA cytokines on days 30 and 45 post treatment (p.t.). *P < 0.05 indicates a significant difference in response between day 30 infected versus day 45 p.t. cured groups. (b) Lymphoproliferative response of mononuclear cells (lymph nodes) from normal, L. donovani infected and cured hamsters in response to ConA and SLD at 10 μg/mL. Proliferation is represented as mean optical density (OD) of stimulated culture/mean OD of unstimulated control. Each bar represents pooled data (mean ± SD) of five hamsters and the data represent the means of triplicate wells ± SD. (c) Nitrite production (μM) on days 30 and 45 p.t. by peritoneal macrophages of hamsters (n=5) primed with supernatants of normal, infected and cured hamster lymphocytes, respectively, in response to lipopolysaccharide (LPS) and SLD at 10 μg/mL after 24 h stimulation. The absorbance of the reaction product was measured at 540 nm using Griess reagent; (d) Leishmania-specific IgG and its isotype response on days 30 and 45 p.t. Each bar represents pooled data (mean ± SD value) of three replicates. Asterisks indicate significances of differences between the cured and infected groups (*P < 0.05; **P < 0.01; and ***P < 0.001). The data represent the results of two independent experiments; two or three animals were used in each experiment.
The other measures of CMI, which almost always accompanies the control of parasite growth and healing, are Leishmania-specific LTT and NO production. However, it is not LTT itself that is behind the primary effector mechanism of immunity, but rather the stimulation of Leishmania-specific T cells to produce macrophage-activating factors, including IFN-γ, which in turn activate macrophages to kill the intracellular parasites. The generation of NO also supports the up-regulation of iNOS by Th1 cell-associated cytokines. We also observed that in vitro stimulation of the lymphocytes with ConA, as well as SLD, showed maximum lymphoproliferative responses on day 45 p.t. in the cells of animals cured with miltefosine (Figure 1b). There was a gradual increase (~5-fold on day 30 p.t. and ~7-fold on day 45 p.t) in NO production compared with their infected counterparts (Figure 1c). These findings confirm that an NO-mediated macrophage effector mechanism is critical in the control of parasite replication in the animal model.

Apart from diminished cellular responses, VL is associated with the production of a high level of antibody, which is observed prior to the detection of a parasite-specific T-cell response. Following miltefosine treatment, the anti-Leishmania IgG, found to be elevated in the infected group, decreased significantly (P < 0.001) at both timepoints (Figure 1d). Similarly, the level of IgG1 isotype was also found to be significantly decreased (P < 0.01) on day 45 p.t. In contrast, the level of IgG2 isotype increased progressively with time in the treated group (P < 0.05 on day 30 p.t. and P < 0.001 on day 45 p.t). It is well established that titres of IgG and IgG1 antibodies increase with L. donovani load. The low level of these antibodies is thus consistent with the decreasing parasite loads seen in the treated group. The significant increase in IgG2 levels in cured animals is also indicative of enhanced CMI. Through this study we have ascertained that miltefosine treatment in Leishmania-infected hamsters boosted as well as restored both cellular and humoral immunity with production of high levels of Th1 cytokines—particularly IFN-γ, TNF-α and IL-12—along with the elevation of Leishmania-specific IgG2 isotype. Thus, in view of severe immunosuppression in VL, a better and effective strategy for the optimum efficacy of future antileishmanial drugs would be not only direct killing of parasites by the drug, but also simultaneous generation of immunity against the disease.

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Transparency declarations
None to declare.

References