Immunomodulatory effects of antileishmanial drugs

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Objectives: The commonly used antileishmanial drugs are sodium antimony gluconate (SAG), amphotericin B, miltefosine and paromomycin. There are a number of reports that antileishmanial drugs show immunomodulatory properties. Here, we attempt to understand how the innate arm of the immune system is modulated in response to these antileishmanial drugs.

Methods: BALB/c peritoneal macrophages were treated with miltefosine, SAG, amphotericin B or paromomycin. The membrane fluidity of macrophages following drug treatment was studied in terms of fluorescence anisotropy. The T cell-stimulating ability, production of cytokines and nitrogen and oxygen metabolite production in drug-treated macrophages were also studied. The study was also carried out using peritoneal macrophages from drug-treated BALB/c mice.

Results: The antileishmanial drugs altered macrophage membrane fluidity, except amphotericin B. The drug-treated macrophages showed enhanced T cell-stimulating ability and generation of reactive oxygen species, nitrite, interleukin-12 and tumour necrosis factor-α.

Conclusions: Antileishmanial drugs can stimulate the innate arm of the immune system, which may have a significant bearing on the cellular arm of the immune system.

Keywords: miltefosine, amphotericin B, SAG, paromomycin, immunomodulation, anisotropy, antigen presentation

Introduction

The usual drugs recommended for the treatment of leishmaniasis are pentavalent antimonials, amphotericin B, lipid formulations of amphotericin B, miltefosine and paromomycin.1 Pentavalent antimonials were the first drugs introduced against leishmaniasis.1 The polyeone antibiotic amphotericin B is currently recommended as a second-line treatment for leishmaniasis.1 Perhaps the most significant recent advance has been the effective oral treatment of leishmaniasis by miltefosine, an alkylphosphocholine.2 The T cell compartment is believed to be needed for the action of both miltefosine and pentavalent antimonials [sodium antimony gluconate (SAG)].3,4 Interferon (IFN)-γ is a major T cell-derived lymphokine for host defence. Thus, an adjunctive therapy of a combination of SAG and IFN-γ proved to be more effective.5 Paromomycin, an aminoglycoside antibiotic, has been used in clinical trials for leishmaniasis.6 Paromomycin inhibits protein synthesis and induces modification of membrane lipid metabolism.1 Previous reports show immunomodulation by miltefosine in vitro and in vivo in Leishmania-infected animals.5,6 In this study, we report novel data concerning the in vitro and in vivo effects of commonly used antileishmanial drugs on innate immunity and their bearing on the cellular arm of the immune system.

Methods

Ethics

The use of mice was approved by the Institutional Animal Ethics Committee of the Indian Institute of Chemical Biology, India. All animal experiments were performed according to the National Regulatory Guidelines issued by the Committee for the Purpose of Supervision of Experiments on Animals, Ministry of Environment and Forest, Government of India.

Isolation of peritoneal exudate cells (PECs) and drug treatment

For convenience, PECs were defined as macrophages. Macrophages were isolated from 4% starch-boosted BALB/c mice and plated on tissue culture Petri dishes in complete RPMI medium, for 48 h at 37 °C in the presence of a 5% CO2 atmosphere. Macrophages were treated with miltefosine, amphotericin B, SAG or paromomycin at the indicated doses for 24 or 72 h.

Drug treatment

Groups of BALB/c mice (five animals/group) were treated intravenously with a single dose of SAG (16 mg/kg body weight),7 amphotericin B (2.5 mg/kg body weight),8 or paromomycin (16 mg/kg body weight)9 or orally with five consecutive doses of miltefosine (30 mg/kg body weight/day).10
Two days after the completion of treatment, the animals were sacrificed to isolate PECs.

**Cell viability assay using MTT**

After 72 h of drug treatment, the cell viability was determined using MTT (Sigma).\(^{11}\)

**Measurement of fluorescence anisotropy (FA)**

The membrane fluorescence and lipid fluidity of cells were measured using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH; Invitrogen). The FA value was calculated using the equation: \( \text{FA} = [(I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)] \), where \( I_\parallel \) and \( I_\perp \) are the fluorescence intensities oriented, respectively, parallel and perpendicular to the direction of polarization of the excited light.\(^{12}\)

**Antigen presentation assay**

The antigen presentation assay was performed as described previously.\(^{13}\) The immunodominant 12–26 amino acid residues (LEDARRKAIYEKKK) of the bacteriophage λ repressor protein [defined as λR(12–26)] were used as an antigen.

**Measurement of interleukin (IL)-12 and tumour necrosis factor (TNF)-α from drug-treated macrophages**

The antileishmanial drug-treated macrophages were cultured in a 96-well plate at a density of 2.5 \( \times \) 10\(^7\) cells/mL. The supernatant was collected after 24 h of treatment and IL-12 and TNF-α were quantified by ELISA (BD Biosciences).

**Measurement of reactive oxygen species (ROS) and nitric oxide (NO)**

In one set of experiments, the drug-treated macrophages were subsequently treated with a suboptimal dose of IFN-γ (10 U/mL) for another 24 h before ROS and nitrite estimation. Another set received the drug alone and were kept with medium for another 24 h instead of IFN-γ. The suboptimal dose was selected as described by others.\(^{14}\) ROS were measured using 2',7'-dichlorofluorescin diacetate (H\(^2\)DCFDA, Sigma).\(^{12}\) NO generation was monitored by using the Griess reaction (Sigma) as described previously.\(^{12}\)

**Statistical analysis**

All statistical analyses were performed using Prism 5 (GraphPad Software).

**Results and discussion**

In this report, we describe the host cell-dependent mechanism of antileishmanial drugs for the killing of intracellular parasites. We used SAG, miltefosine, amphotericin B and paromomycin. The cytotoxic effect of the drugs on macrophages was studied. It was observed that at up to 20 \( \mu \)M miltefosine, 60 \( \mu \)g/mL SAG, 1 \( \mu \)M amphotericin B or 200 \( \mu \)M paromomycin the macrophages remained essentially viable after 72 h, beyond which the general condition of the cells deteriorated (Figure 1).

The macrophage membrane fluidity was studied in terms of FA (Figure 2a). It was observed that there was a significant increase in the FA value following treatment with 20 \( \mu \)M miltefosine. Similar studies were done with amphotericin B at concentrations of

**Figure 1.** Macrophage cell viability following antileishmanial drug treatment. BALB/c peritoneal macrophages were treated with 10–30 \( \mu \)M miltefosine, 0.5–2 \( \mu \)M amphotericin B, 30–120 \( \mu \)g/mL SAG or 100–400 \( \mu \)M paromomycin. Cell viability was measured using MTT after 72 h of drug treatment. **\( p < 0.001 \), representing a significant difference between macrophages treated with the highest drug concentration and untreated macrophages.
0.01–1 µM. Interestingly, there was hardly any change in the FA value with increasing amphotericin B dose. However, there was a statistically significant increase in the FA value at higher doses of SAG or paromomycin.

Previously, we have shown that alteration of the macrophage membrane fluidity has a bearing on T cell-stimulation ability. Thus, the effect of drug treatment on the macrophage-mediated antigen-presenting function was analysed (Figure 2b). It was observed that miltefosine at 20 µM (the highest dose tested) indeed enhanced the T cell-stimulating ability, as evident from significantly higher IL-2 production from a T cell hybridoma as compared with untreated macrophages. On the other hand,

![Figure 2.](https://academic.oup.com/jac/article-abstract/68/12/2834/696477)

(a) Membrane fluorescence and lipid fluidity of macrophages treated with antileishmanial drugs were studied in terms of anisotropy using DPH as a probe. The open bars and the filled bars represent 24 and 72 h of drug treatment, respectively. **P < 0.001, ***P < 0.0001. (b) Antigen presentation of macrophages treated with antileishmanial drugs. The ability of macrophages to drive a T cell hybridoma 9H3.5 was studied in terms of IL-2 production. The open triangles and the filled triangles represent 24 and 72 h of drug treatment, respectively. **P < 0.001, ***P < 0.0001. (c) Nitrite generation by macrophages representing significant difference between drug-treated and untreated macrophages. (d) Generation of ROS was measured using the H2O2-sensitive probe H2DCFDA. The extent of H2O2 generation was defined as the extent of ROS generation. For each antileishmanial drug treatment, either alone or in combination with IFN-γ, fluorimetric measurements were performed and the results are expressed as the mean fluorescence intensity per 10⁶ cells. LPS is taken as a positive control. **P < 0.001, ***P < 0.0001 reflecting a significant difference in ROS production between drug-treated and untreated macrophages. (e) Production of IL-12 and TNF-α from macrophages treated with antileishmanial drugs. **P < 0.001 and #P < 0.05, representing a significant difference in IL-12 and TNF-α production, respectively, between drug-treated and untreated macrophages. (f) Groups of BALB/c mice (five animals/group) were treated intravenously with a single dose of SAG (16 mg/kg body weight), amphotericin B (2.5 mg/kg body weight) or paromomycin (16 mg/kg body weight) or orally with five consecutive doses of miltefosine (30 mg/kg body weight/day). Two days after the completion of treatment, the animals were sacrificed to isolate peritoneal macrophages (PECs). (i) Membrane fluorescence and lipid fluidity of PECs from drug-treated animals. (ii) Antigen presentation of PECs from animals treated with antileishmanial drugs. The ability of macrophages to drive a T cell hybridoma 9H3.5 was studied in terms of anisotropy using DPH as a probe. The open bars and the filled bars represent 24 and 72 h of drug treatment, respectively. **P < 0.001, ***P < 0.0001 reflecting a significant difference in ROS production between drug-treated and untreated macrophages. (iii) Production of IL-12 from PECs from animals treated with antileishmanial drugs. The data represent a 1:10 dilution of serum. (iv) TNF-α production from PECs from animals treated with antileishmanial drugs. The data represent a 1:10 dilution of serum. **P < 0.001, ***P < 0.0001.
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macrophages treated with amphotericin B at any given dose failed to show any appreciable increase in IL-2 production from a T cell hybridoma compared with normal macrophages. Similarly, macrophages treated either with SAG or paromomycin at a higher dose showed enhanced T cell-stimulating ability. As miltefosine is a phospholipid-like molecule, it would be expected to be inserted into lipid membranes.13 A previous study showed that lipids with a single fatty acid chain, like miltefosine, do not induce the formation of sterol-rich domains in the membrane, but stabilize these domains.2 It has been demonstrated that the drug inhibits the esterification of free cholesterol and also stimulates an increase in cholesterol synthesis.7 An increase in cholesterol stabilizes cholesterol-rich domains within the membrane. Amphotericin B treatment of macrophages does not affect membrane fluidity, as demonstrated by anisotropy (Figure 2a). Thus, no significant change was found in the antigen-presenting ability of macrophages in terms of T cell activation. Positively charged paromomycin interacts with negatively charged phospholipids in the membrane, causing rigidifying effects within the membrane.16 The increase in anisotropy and elevated antigen-presenting function of the macrophages treated with either miltefosine or SAG or paromomycin essentially remains the same even after 72 h of treatment (Figure 2a and b). The drugs not only change the membrane properties of the macrophages in vitro, but also in vivo. Peritoneal macrophages from miltefosine-, SAG- or paromomycin-treated BALB/c mice showed an increase in anisotropy and T cell-stimulating ability (Figure 2f and ii). However, peritoneal macrophages from amphotericin B-treated BALB/c mice did not show any changes in anisotropy and T cell-stimulating ability (Figure 2f and ii).

The generation of nitrite and superoxides are the main intracellular killing mechanisms for leishmanial parasites within macrophages.17 We tested the ability of antileishmanial drugs either alone or in combination with a suboptimal dose of IFN-γ to induce NO production by macrophages. Here, we used a suboptimal dose of IFN-γ15 as a surrogate of T cell involvement in the activation of macrophages in combination with drugs. There was essentially not much NO production in response to antileishmanial drugs. However, in combination with a suboptimal dose of IFN-γ, there was a huge surge of NO production in response to all the drugs. Thus, for NO generation from macrophages, IFN-γ signalling is required along with antileishmanial drug action. A previous report showed that exogenously administered IFN-γ along with SAG eliminated the Leishmania parasite effectively.6 Our observations similarly show the synergistic effect of IFN-γ and antileishmanial drugs in the immune stimulation of macrophages (Figure 2c). There was enhanced ROS generation in macrophages in response to treatment with all the drugs alone, irrespective of the addition of a suboptimal dose of IFN-γ (Figure 2d).

Macrophages treated with antileishmanial drugs showed increased IL-12 and TNF-α production (Figure 2e). Miltefosine-, amphotericin B- or SAG-treated macrophages significantly produced IL-12 and TNF-α. However, the macrophages treated with paromomycin failed to produce significant levels of IL-12 or TNF-α compared with untreated macrophages. The serum collected from drug-treated BALB/c mice also showed a significant increase in IL-12 and TNF-α (Figure 2f and iv). Endogenous IL-12 and TNF-α play a complex role in inducing macrophage activation and enhancing host antimicrobial defence by ROS and nitrite generation.8 A previous report showed a 50% reduction in the proinflammatory response in TLR4 or TLR2 knockout primary murine macrophages compared with wild-type macrophages following amphotericin B treatment.18 Other reports showed the in vitro activity of miltefosine and paromomycin derived TNF-α production is increased by almost 75% when TLR4 or TLR2 is engineered to express on TLR4-deficient HEK 293 cells.19 Pretreatment with TLR4 siRNA or in combination with TLR9 siRNA causes >30% and >60% inhibition of miltefosine-mediated parasite killing, respectively, in Leishmania-infected THP-1 cells.20 We observed an almost 20% decrease in TNF-α production in a TLR4-deficient 23-ScCr cell line following amphotericin B or miltefosine treatment as compared with wild-type PECs (data not shown). The miltefosine- and amphotericin B-driven cytokine production is not solely dependent on TLR4.18,19 Thus, our observation has a close bearing on the action of TLRs other than TLR4.

In conclusion, this study showed that antileishmanial drugs, in general, do have immunomodulatory ability.

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Transparency declarations

None to declare.

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