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Short Communication

Evaluation of antileishmanial drugs activities in an *ex vivo* model of leishmaniasis

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ABSTRACT

Leishmaniasis is a poverty-related disease, the chemotherapy of which is based on few drugs. The *in vitro* macrophage-amastigote model using mouse peritoneal cells, human-monocyte transformed macrophages and immortalized cell lines have been used to test new and safe antileishmanial drugs. Considering the differences for drug sensitivities between these *Leishmania* infected cells, the efficacy of amphotericin B, pentavalent antimonial, miltefosine and resveratrol was evaluated in a recently developed *ex vivo* culture of macrophages isolated from mouse lesion induced by *L. amazonensis* ($CD11b^+F4/80^+CD68^+CD14^+$) compared with infected peritoneal macrophages ($CD11b^+F4/80^+CD68^+CD14^+$). The results show that IC50 values of amphotericin B, miltefosine and pentavalent antimonial for parasites in lesional and peritoneal macrophages were similar, although high doses of these compounds did not result in total clearance of parasites in lesional cells (amphotericin B), peritoneal cells (miltefosine) and both cell cultures (pentavalent antimonial). Amastigotes infecting lesional macrophages were more resistant to resveratrol as compared to parasites in peritoneal macrophages. The cytoxicity of miltefosine and resveratrol was higher in infected peritoneal macrophages than in lesional cells. These data suggest that the antileishmanial effect and citotoxicity of some anti leishmanial compounds are dependent of macrophage source and mouse peritoneal macrophages loaded with amastigotes do not represent the lesion cell.

Leishmaniasis is a poverty-related disease caused by several species of *Leishmania*. Presenting in cutaneous, mucocutaneous, or visaceral forms, it shows 0.7–1 million new cases per year [1]. Current therapy includes pentavalent antimonial, amphotericin B, miltefosine and paromomycin [2]. Drug resistance, side effects and cost issues has been previously reported [1–3]. In this scenario, studies are undertaken to identify new and safe antileishmanial drugs. Natural and synthetic compounds have been tested against *Leishmania,* mainly on *in vitro* model, macrophage-amastigote with primary macrophages (mouse peritoneal and bone marrow macrophages, human-monocyte transformed macrophages) and immortalized cell lines that mimic intracellular amastigotes condition even though they may not adequately represent lesional cells [4–9]. Recently, we developed a new approach to establish an *ex vivo* model of cells isolated from lesions of Balb/c mice infected with *L. amazonensis* identified as $CD11b^+CD14^+F4/80^+CD68^+$ myeloid cells that allowed us to analyze their phenotipical and functional capacities [10]. Considering the fact that differences for drug sensitivity among cell models infected *in vitro* with *Leishmania* have been reported [5,6], for the first time the efficacy of three antileishmanial reference drugs (amphotericin B, pentavalent antimonial and miltefosine) and a drug candidate resveratrol (natural polyphenol found in plants and active against *Leishmania* and bacteria) [11,12] have been tested to isolated macrophages from mouse skin lesions and compared with mouse peritoneal macrophages.

Amphotericin B (Sigma Aldrich, USA), miltefosine (Cayman Chemical Company, Ann Arbor, MI, USA) and pentavalent antimonial (*N*-methyl glucamine antimoniate) (Sanofi, France) were prepared in phosphate buffer saline or RPMI medium just before use, and resveratrol (Sigma-Aldrich) was dissolved in RPMI medium using dimethyl sulfoxide (Sigma-Aldrich) (0,01%).*Leishmania amazonensis* (MHOM/BR/73/M2269) promastigotes were cultured at 26 °C in RPMI medium, containing 10% inactivated fetal bovine serum (FBS) and 0,1% gentamicin, and amastigotes were isolated from skin lesions of female Balb/c mice as described previously [10]. All animal experiments were performed following the approved protocol of the Institute of Biology/Ethical Committee for Animal Research, UniversidadeEstadualde

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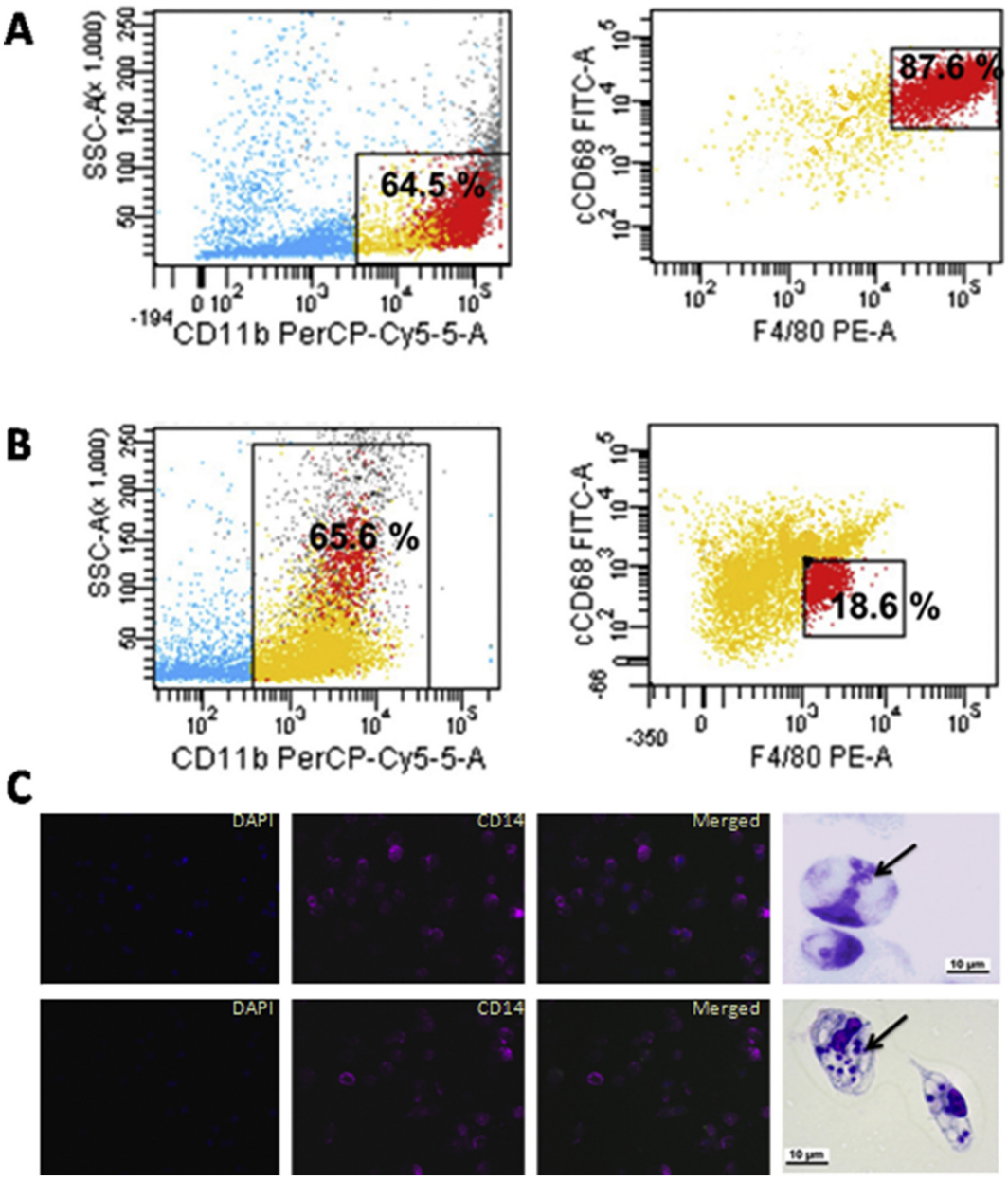

Fig. 1. Expression of macrophage markers in lesional cells and peritoneal macrophages infected with *L. amazonensis*. Lesional and peritoneal cells were sorted by CD11b magnetic beads and then peritoneal macrophages were infected with amastigotes. Flow cytometer analyses of infected peritoneal macrophages (A) and lesional macrophages (B) with CD11b, F4/80 and CD68 gating. Epifluorescence microscopy (DAPI and CD14) and Giemsa staining of infected peritoneal macrophages (images on the top) and lesional macrophages (lower images). Arrows indicate amastigotes within vacuoles.

Campinas, Brazil (protocol n. 2715-1). Peritoneal macrophages were obtained from normal Balb/c mice as described by Terreros et al. [10], cells were sorted with magnetic separation column using anti-CD11b antibody conjugated to magnetic beads (MiltenyiBiotec, Germany) and $Cd11b^+$ cells were cultivated in RPMI medium containing 10% FBS and 0.1% gentamicin at 37 °C, 5% CO2/95% humidified air. Lesional macrophages were obtained from Balb/c mice subcutaneously infected with 1×10^7 *L. amazonensis* promastigotes [10]. After 7–8 weeks of infection, the animals were euthanized, small pieces of lesion were collected and processed as previously described [10]. The cells obtained from lesions were also sorted with a magnetic separation column using anti-CD11b antibody conjugated to magnetic beads. $CD11b^+$ macrophages were cultivated in RPMI medium containing 10% FBS and 0.3% gentamicin at 37 °C, 5% CO2/95% humidified air. For assessment of drugs effects on macrophages, lesional cells were cultured on 24-well plates (5×10^5 cells/well) or 6-well plates (10×10^5/well) containing 13 mm diameter coverslips. Peritoneal macrophages were cultured under the same conditions and infected with amastigotes at a parasite ratio of 3:1. After 12 h, peritoneal cells were washed to remove extracellular parasites and replaced with fresh medium. The antibodies used to detect phenotypic markers in flow cytometer assay were anti-mouse CD11b, PerCP/Cy5.5 rat IgG2b κ, anti-mouse F4/80 PE rat IgG2a κ, anti-mouse CD68 Alexa Fluor 488 rat IgG2a and isotype controls (BioLegend). Cells were analyzed using FACS Canto II (BD Biosciences) (10,000 events for tube). Data analysis was performed with the software BD FACS Diva™ [10].

For immunofluorescence experiments, purified anti-mouse CD14 rat IgG2a κ, Alexa Fluor 488 goat anti-rat IgG H ± L (Life Technologies), isotype controls and DAPI (Sigma) were used. Dry-air slides were analyzed under an epifluorescence microscope (Leica DM LB). These assays were described by Terreros and coworkers [10]. Drugs were added to plates containing lesional or peritoneal macrophages and after

Table 1
Drug toxicity against *L. amazonensis* amastigotes and different macrophage populations[a].

Drug	Lesional macrophages		Peritoneal macrophages	
	IC_{50}	LD_{50}	IC_{50}	LD_{50}
	μg/ml		μg/ml	
Amphotericin b	0.18 (0.19–017)	3.42 (3.40–3.44)	0.19 (0.21–0.17)	2.67 (2.65–2.69)
Miltefosine	1.04 (1.09–0.98)	13.13[b] (13.00–13.24)	1.81 (1.90–1.72)	5.31 (5.73–4.89)
Resveratrol	0.31[c] (0.32-0.29)	28.47[d] (29.89-27.09)	0.09 (0.08–0.09)	10.36 (10.88–9.84)

[a] Drug concentration that inhibit 50% of infected macrophages (IC_{50}) and 50% of the macrophages viability (LD_{50}) at 48 h incubation time. 95% confidence intervals in parenthesis.

[b] A significant difference ($p = .04$) was observed with respect to infected peritoneal macrophages.

[c] A significant difference ($p = .03$) was observed with respect to infected peritoneal macrophages.

[d] A significant difference ($p = .02$) was observed with respect to infected peritoneal macrophages.

48 h the parasite load (% of infected cells and the number of intracellular amastigotes) were evaluated on coverslip stained with Giemsa by counting at least 200 macrophages per coverslip [10]. Macrophage adherence as a direct measurement of the cell viability and integrity was assessed by counting the adherent cells in 20 random fields per coverslip [9,10]. The drug concentration that caused a 50% reduction of viable macrophages (LD50 lethal dose) and the drug concentration that caused a 50% reduction of infected macrophages (IC50 inhibitory concentration) were calculated by using Microsoft excel ed50plus v1.0. A two-way ANOVA analysis was used to determine significant differences among samples. All analyses were performed using GraphPad Prism Software (version *6.04*; *GraphPad Software* Inc.). Experiments were repeated at least three times, each performed in duplicate or triplicate.

The majority of mouse lesional cells cultivated *ex vivo* exhibited large size, macrophage morphology, retained viability and contained intracellular amastigotes (5 to 14 parasites per cell and 90% of infected cells) [10]. They expressed macrophage markers CD11b, F4/80, CD68 and CD14 (65.6% $CD11b^{+++}$ cells; 18.6% $F4/80^{+}$ $CD68^{+}$ cells, 81,4% $F4/80^{+/-}$ $CD68^{+}$ cells and ≥60% $CD14^{+}$ cells (Fig. 1 and [10]). Peritoneal mouse macrophages were successfully *in vitro* infected with *L. amazonensis* (1 to 6 amastigotes per cell and 85% of infected cells) and expressed CD11b, F4/80, CD68 and CD14 markers (Fig. 1 and [10]). The anti-*Leishmania* efficacy of drugs was based on IC50 values (the drug concentration that caused a 50% reduction of infected cells)

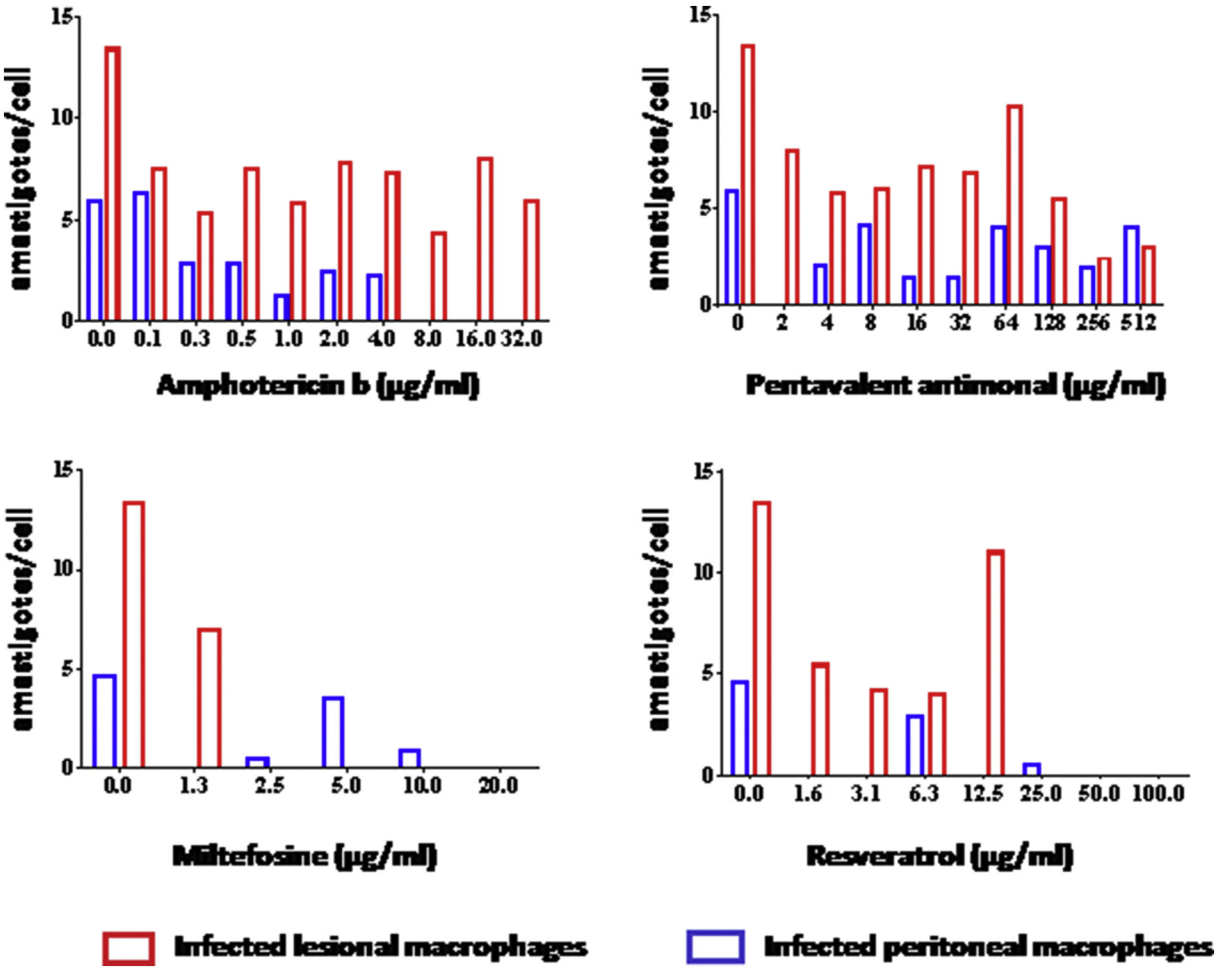

Fig. 2. Effect of antileishmanial drugs on different macrophage populations. The *in vitro* peritoneal macrophages infected with *L. amazonensis* (blue) and lesional macrophages (red) were treated with amphotericin B (A), pentavalent antimonial (B), miltefosine (C) or resveratrol (D) for 48 h and the number of intracellular amastigotes evaluated in on coverslip stained with Giemsa. These are the results of a typical experiment, representative of a group of three. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and reduction of intracellular amastigotes. The IC50 values of amphotericin B for amastigotes in lesional and peritoneal macrophages were similar (Table 1) although amastigotes still were found in lesional cells treated with doses higher than 4 μg/ml (8–32 μg/ml) (Fig. 2A). The cultures of infected peritoneal macrophages were free of amastigotes when treated with 8 μg/ml amphotericin B (Fig. 2C). The IC50 values of miltefosine for amastigotes in lesional macrophages were approximately twice lower than the IC50 values for parasites in peritoneal macrophages (1.81 μg/ml *versus* 1.04 μg/ml) (Table 1), even though this difference is not statistically significant ($p = .55$). It should be noted that both macrophage cultures reduced the number of infected cells when treated with 2.5 μg/ml miltefosine, but this dose completely eliminated intracellular amastigotes only in lesional macrophages (Fig. 2C). On the other hand, the IC50 values of resveratrol for amastigotes in lesional macrophages were three times higher than IC50 values for amastigotes in peritoneal macrophages (0.31 μg/ml *versus* 0.09 μg/ml), thereby demonstrating a statistically significant difference ($p = .03$) (Table 1).Pentavalent antimonial had antileishmanial activity on both macrophage populations; due to the variability on the dose-response data the IC50 values ranging from 64 to 128 μg/ml for lesional macrophages and 16–32 μg/ml for infected peritoneal macrophages; even high doses (512 μg/ml) did not result in total clearance of parasites in both macrophage cultures (Fig. 2B).In the main, the viability of untreated lesional and peritoneal macrophages was maintained 90–95% during 48 h period. However, the macrophage populations were differently sensitive to drugs. The LD50 values (the drug concentration that caused a 50% reduction of viable macrophages) of miltefosine and resveratrol for lesional macrophages were higher than LD50 values for peritoneal macrophages (Table 1). Because lesional and peritoneal macrophages could be exposed to high pentavalent antimonial concentrations (2048 μg/ml did not cause 50% cell death), LD50 could not be computed.

One can speculate that different infection levels observed in both macrophage cultures could affect drug efficacy. However, despite highest infection levels (3-fold) observed in lesional macrophages compared with peritoneal macrophages, amastigotes inside both cell populations were equally susceptible to amphotericin B, miltefosine and penvalent antimonial (Table 1 and Fig. 2). Parasites within lesional cells were more resistant to resveratrol. There are few reports comparing the activities of drugs against *Leishmania* within different macrophage populations cultured *in vitro*; none of these studies examined macrophages isolated from lesions. For example, Seifert and coworkers demonstrated that amphotericin B was more active in *L. donovani* infecting mouse peritoneal- and bone marrow-derived macrophages compared with parasites infecting human primary macrophages and THP1 cell line, and miltefosine was more active in parasites within human macrophages compared with parasites in mouse peritoneal- and bone marrow-derived macrophages [6]. Koniordou and coworkers reported the cell dependent efficacy of antimonials against *L. donovani* infecting human CD14+ primary macrophages compared to parasites in THP1 cells [7]. Although different *Leishmania* species were used in these assays, a macrophage source dependent *in vitro* efficacy of some antileishmanial drugs was observed in our study as well as Seifert and Koniordou reports [6,7]. Furthermore, we found differential citotoxicity of drugs in two macrophage populations; lesional cells are more resistant to miltefosine and resveratrol than the peritoneal macrophages. This could permit the determination of citotoxicity to high concentration of drugs in lesional cells. Although this *ex vivo* lesional model requires more complex culture conditions than those of cell lines and primary macrophages, and thus may not be used in drug screening tests, it may be useful for validation of leishmanial drug candidates and to clarify questions involving *Leishmania*-macrophage interactions.

In conclusion, using this *ex vivo* lesional model, it was possible demonstrate that the antileishmanial effect and citotoxicity of some anti leishmanial compounds are dependent of macrophage source, and mouse peritoneal macrophages loaded with amastigotes do not represent the lesion cells.

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