RESEARCH NOTES

J. Parasitol., 94(6), 2008, pp. 1415–1417

Efficacy of Pentavalent Antimony, Amphotericin B, and Miltefosine in *Leishmania amazonensis*-Infected Macrophages Under Normoxic and Hypoxic Conditions

D. C. Ayres, L. A. Pinto, and S. Giorgio*, Departamento de Parasitologia, Instituto de Biologia, Universidade Estadual de Campinas, Cx. Postal 6109, Campinas, SP, Brazil, Cep 13083-970; *To whom correspondence should be addressed. *e-mail: sgiorgio@unicamp.br*

ABSTRACT: Recently, our group demonstrated that mouse lesions infected with *Leishmania amazonensis* are hypoxic. Evidence indicates the negative impact of hypoxia on the efficacy of a variety of chemotherapeutic agents against tumors, fungi, bacteria, and malaria parasites. In the present study, comparison of the effect of antileishmanial drugs on *L. amazonensis*-infected macrophages under normoxic and hypoxic conditions was performed. We compared the effect of 5% oxygen tension with a tension of 21% oxygen on peritoneal murine macrophage cultures infected with the parasite and treated with glucantime, amphotericin B, or miltefosine. Analysis of the infection index (percentage of infected macrophages × number of amastigotes per macrophage), dose-dependent efficacy of drugs, and IC_{50} values demonstrated that hypoxia conferred a small, but significant, resistance to all 3 antileishmanial drugs. The present finding suggests that in vitro assays under hypoxia should not be neglected in drug studies.

Leishmaniasis is a neglected disease caused by several species of *Leishmania*; it is currently endemic in 88 countries (Desjeux, 2004). The severity of the disease produced by several *Leishmania* species varies enormously, ranging from cutaneous or mucosal to visceral or diffuse cutaneous infection. The former is generally caused by *Leishmania amazonensis*, a species transmitted mainly in the Amazon region of South America, which is associated with localized cutaneous lesions (Grimaldi and Tesh, 1993). Current therapies frequently fail to eradicate the parasite from infected tissues, while also presenting serious side effects (Berman, 2003). The first line of therapy for all forms of the disease is pentavalent antimonial (Sb^v), sodium stibogluconate, and meglumine antimoniate glucantime (Berman, 2003). The polyene antibiotic amphotericin B and diamidine pentamidine are second-line drugs, and miltefosine, a lysophospholipid analog, is the most recent drug approved for leishmaniasis treatment (Berman, 2003; Mishra et al., 2007). *Leishmania* spp. are protozoan parasites that replicate in mammalian cells, principally in macrophages (Desjeux, 2004).

Several characteristics of the disease include microcirculatory impairment, parasite proliferation, secondary bacterial infection, and expression of hypoxia-inducible transcription factor-1α in the murine cutaneous lesions infected with *L. amazonensis*. These features are strong indications of a reduced intralesional oxygen tension (hypoxia) (El-On et al., 1992; Grimaldi and Tesh, 1993; Arrais-Silva et al., 2005). In fact, hypoxia is a primary attribute of tissues experiencing infection, inflammation, or tumor cell proliferation (Ikeda, 2005). The effectiveness of drugs such as amphotericin B against *Candida albicans*, aminoglycoside antibiotics against *Escherichia coli*, antibiotics and antimalarial products against *Plasmodium falciparum*, and alkylating agents against tumor cells under hypoxic conditions has been investigated experimentally (Divo et al., 1985; Sokol-Anderson et al., 1986; Krungkrai and Yuthavong, 1987; Bryant et al., 1992; Brown, 2002; Koch et al., 2003; Grigoryan et al., 2005; Cao et al., 2007). Under hypoxic conditions, these drugs were less effective. To the best of our knowledge, no information is available for the effect of hypoxia on the efficacy of antileishmanial drugs. In the present study, the efficiency of glucantime, amphotericin B, and miltefosine on *L. amazonensis*-infected macrophages under normoxic and hypoxic conditions were evaluated.

Leishmania amazonensis (MHOM/BR/73/M2269) amastigotes were isolated from active skin lesions of BALB/c mice as described previously (Colhone et al., 2004). Primary mouse macrophages were obtained from normal BALB/c mice by peritoneal lavage as described previously, cultured in 24-well cell culture plates containing 13-mm-diameter glass coverslips (5×10^5 cells per well), and maintained in RPMI medium supplemented with antibiotics and 10% heat-inactivated fetal calf serum (Colhone et al., 2004). Macrophages were exposed to *L. amazonensis* at a parasite–macrophage ratio of 3:1 for 2 hr. After the exposure period, the cultures were washed to remove extracellular parasites and then incubated in the presence of different concentrations of the drugs at 37 C for 48 hr. For the evaluation of the infection index (IF) (percentage of infected macrophages × number of amastigotes per macrophage), cells on coverslips were stained with Giemsa and examined microscopically at ×1,000 magnification (Colhone et al., 2004). The drug concentration that caused a 50% reduction in IF (IC_{50}) was estimated by regression analyses using SigmaPlot 2001 (Systat Software Inc., San Jose, California). All experiments were repeated at least 3 times in triplicate wells, and the results are expressed as the mean ± SD. Statistical analyses were performed using the 2-tailed Student's *t*-test and Origin 6.0 (OriginLab Corp., Northampton, Massachusetts). A $P < 0.05$ value was considered to be significant.

Hypoxic cell culture conditions were established, as described previously (Colhone et al., 2004). The plates containing *L. amazonensis*-infected macrophage cultures were placed in a gas-tight modular chamber (Billups-Rothenberg, Del Mar, California). In all experiments, the exposure of cells to 5% O_2, 5% CO_2 and balanced N_2 is referred to as hypoxia, and the exposure of cells to 21% O_2, 5% CO_2 and balanced N_2 is referred to as normoxia (Colhone et al., 2004). The oxygen tension in the culture medium was 37 mm Hg under hypoxia conditions and 150 mm Hg under normoxia condition (O_2 analyzer YSI/53, YSI Inc., Yellow Springs, Ohio). The medium pH was 7.4 and did not change significantly during the course of the experiments. Glucantime (pentavalent antimony: *N*-methyl glucamine antimonate) (Aventis Pharma, São Paulo, Brazil) was diluted in RPMI medium; amphotericin B (Sigma-Aldrich, St. Louis, Missouri) and miltefosine (hexadecylphosphocholine) (Cayman Chemical, Ann Arbor, Michigan) were prepared in phosphate-buffered saline just before use.

To assess the effects of low oxygen tension on the efficacy of antileishmanial reference drugs, the infection index values (percentage of infected macrophages × number of amastigotes per macrophage), and the IC_{50} values obtained in *L. amazonensis*-infected macrophage cultures treated with drugs and maintained in normoxic or hypoxic conditions were evaluated and compared.

As expected, under normoxic conditions, glucantime, amphotericin B, and miltefosine were found to be toxic to intracellular parasites and exhibited dose-dependent efficacy in a 48-hr intracellular amastigote assay (Fig. 1). Under normoxic conditions, the mean IC_{50} value of glucantime, amphotericin B, and miltefosine was 30.4, 0.40, and 16.0 μg/ml, respectively (Table I). These data are consistent with previously reported results (Callahan et al., 1997; Santa-Rita et al., 2004). Exposure of *L. amazonensis*-infected macrophage cultures to hypoxia increased resistance to various concentrations of drugs, significantly (Fig. 1). Hypoxia caused a modest, but statistically significant, effect on the IC_{50} values of all the drugs tested (Table I). The IC_{50} value of glucantime against *L. amazonensis*-infected macrophages maintained in hypoxia increased 1.5-fold compared with normoxic conditions. The increase in IC_{50} values under hypoxia compared with normoxia was 1.25 for amphotericin B and 2.12 for miltefosine (Table I).

Previous evidence regarding the negative impact of hypoxia on the efficacy of a variety of chemotherapeutic agents (with unrelated modes of action) have been provided in numerous studies (Sokol-Anderson et al., 1986; Bryant et al., 1992; Teicher, 1994; Brown, 2002; Koch et al., 2003; Hyun et al., 2004; Grigoryan et al., 2005; Song et al., 2006; Cao et al., 2007). For example, the IC_{50} values of cisplatin, oxaplatin, gemcitabine, etoposide (Etopophos), bleomycin, mitomycin C, irinotecan, and paclitaxel show a greater than 5-fold increase under hypoxia against

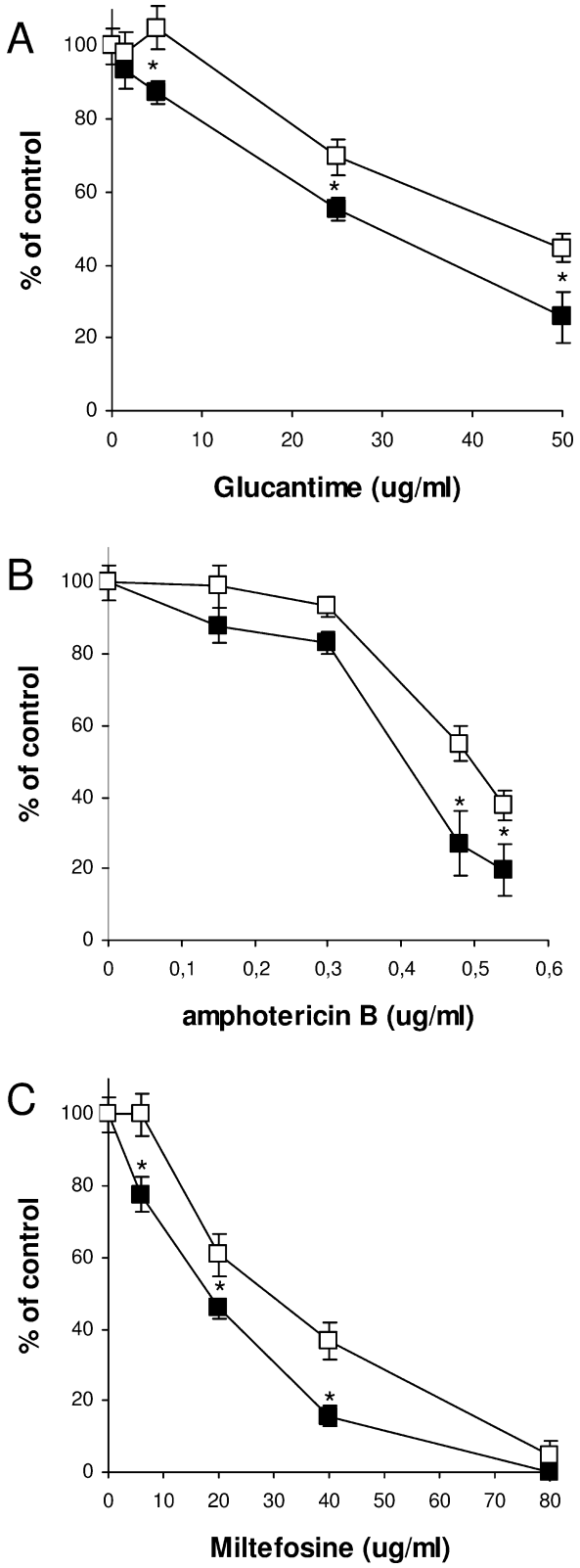

FIGURE 1. Effect of hypoxia on *Leishmania amazonensis*-infected macrophages treated with antileishmanial drugs. Murine peritoneal macrophages were infected with *L. amazonensis* amastigotes for 2 hr. Cell cultures were washed and immediately treated with glucantime (**A**), amphotericin B (**B**), or miltefosine (**C**), under normoxic (□) or hypoxic (■) conditions for 48 hr. The percentage of control values were calculated by dividing infection index in the presence of drug with infection index in the absence of drug and multiplying by 100. The results represent the mean ± SD of 3 experiments. The significance of the difference between cell cultures in hypoxia and normoxia is indicated in the figure. $*P < 0.05$.

TABLE I. Mean values of IC_{50} in normoxia and hypoxia of antileishmanial drugs*.

Drug	IC_{50} (mean ± SD) in μg/ml		
	Normoxia	Hypoxia	Relative increase in IC_{50}
Glucantime†	30.4 ± 4.8	43.5 ± 1.2	1.50
Amphotericin B	0.40 ± 0.01	0.50 ± 0.01	1.25
Miltefosine	16.0 ± 2.72	34.0 ± 3.15	2.12

* The IC_{50} values for a 48-hr intracellular *L. amazonensis* amastigote assay.
† Values for the antimonial agent glucantime are in micrograms of Sb per milliliter.

human embryonal carcinomas (Koch et al., 2003). Hypoxia confers a 2-fold higher resistance to daunorubicin in colon cancer cells lines (Cao et al., 2007), and one report found that amphotericin B-induced *C. albicans* protoplast lysis showed a 5-fold reduction at a concentration of 2 μg/ml of amphotericin B in a hypoxic atmosphere (Sokol-Anderson et al., 1986).

Although there is no previous investigation of the effect of hypoxia on the efficacy of drugs against trypanosomatid parasites, an oxygen-dependent effect of a variety of antibiotics and artemisinin, an antimalarial herb product, has been reported on *P. falciparum* in culture (Divo et al., 1985; Krungkrai and Yuthavong, 1987). The authors did not correlate low potency of drugs in hypoxic conditions with clinical and pathological circumstances; instead, they concluded that the compounds increasing the oxidant stress on the parasite and infected red cells.

In the present study, hypoxia displayed a small, but significant, effect on the IC_{50} values of glucantime, amphotericin B, and miltefosine for *L. amazonensis* amastigotes within macrophages. Although the exact modes of action of these antileishmanial drugs are yet to be completely defined, their mechanisms have been considered to be nonoverlapping. Pentavalent antimonials are generally regarded as prodrugs that require conversion to the trivalent form (Sb^{III}), which inhibits glycolysis, macromolecular biosynthesis, and trypanothione reductase activity (Wyllie et al., 2004). It is accepted that the damaging action of amphotericin B to *Leishmania* spp. originates from its binding to ergosterol, the major sterol incorporated into the cell membrane by the parasite, presenting the immediate consequence of membrane disorganization and depolarization (Beggs, 1994). The mode of action of miltefosine has been associated with perturbation of the alkylphospholipid metabolism, biosynthesis of alkyl-anchored glycolipids, and glycoproteins and apoptosis-like death in the parasite (Lux et al., 2000).

Because hypoxia has been shown to induce relative resistance to a broad spectrum of cytotoxic agents with unrelated modes of action, it has been suggested that this resistance may be caused by altered stability of the drug molecule in a hypoxic microenvironment (Teicher, 1994; Koch et al., 2003; Grigoryan et al., 2005). Furthermore, the hypoxia effect may be caused by a defective uptake mechanism in the cell. This may be the case for antileishmanial compounds; thus, macrophages under hypoxia may concentrate less drug within the parasitophorous vacuole harboring the parasites. Hypoxia can also lead to relative drug resistance through indirect effects via altered cellular metabolism, which decreases drug cytotoxicity (Teicher, 1994).

In summary, the data reported here show that hypoxia confers a relative resistance to antileishmanial drugs and suggests that in vitro assays under normoxia might overestimate the novel and potential antileishmanial drugs. Although the clinical relevance of the present results and

experiments using different *Leishmania* species need to be considered further, the present study indicates that hypoxia, a condition associated with tumors, inflammation, and infections, should not be neglected in drug studies.

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo, Conselho Nacional de Desenvolvimento Cientifico e Tecnológico and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil.

LITERATURE CITED

ARRAIS-SILVA, W. W., V. A. PAFFARO, JR., A. T. YAMADA, AND S. GIORGIO. 2005. Expression of hypoxia-inducible factor-1alpha in the cutaneous lesions of BALB/c mice infected with *Leishmania amazonensis*. Experimental Molecular Pathology **78:** 49–54.

BEGGS, W. H. 1994. Physicochemical cell damage in relation to lethal amphotericin B action. Antimicrobial Agents and Chemotherapy **38:** 363–364.

BERMAN, J. D. 2003. Current treatment approaches to leishmaniasis. Current Opinion in Infectious Diseases **16:** 397–401.

BROWN, J. M. 2002. Tumor microenvironment and the response to anticancer therapy. Cancer Biology and Therapy **1:** 453–458.

BRYANT, R. E., K. FOX, G. OH, AND V. H. MORTHLAND. 1992. Betalactam enhancement of aminoglycoside activity under conditions of reduced pH and oxygen tension that may exist in infected tissues. Journal of Infectious Diseases **165:** 676–682.

CALLAHAN, H. L., A. C. PORTAL, R. DEVEREAUX, AND M. GROGL. 1997. An axenic amastigote system for drug screening. Antimicrobial Agents and Chemotherapy **41:** 818–822.

CAO, X., L. FANG, S. GIBBS, Y. HUANG, Z. DAI, P. WEN, X. ZHENG, W. SADEE, AND D. SUN. 2007. Glucose uptake inhibitor sensitizes cancer cells to daunorubicin and overcomes drug resistance in hypoxia. Cancer Chemotherapy and Pharmacology **59:** 495–505.

COLHONE, M. C., W. W. ARRAIS-SILVA, C. PICOLI, AND S. GIORGIO. 2004. Effect of hypoxia on macrophage infection by *Leishmania amazonensis*. Journal of Parasitology **90:** 510–515.

DEGROSSOLI, A., M. C. BOSETTO, C. B. LIMA, AND S. GIORGIO. 2007. Expression of hypoxia-inducible factor 1alpha in mononuclear phagocytes infected with *Leishmania amazonensis*. Immunology Letters **114:** 119–125.

DESJEUX, P. 2004. Leishmaniasis: Current situation and new perspectives. Comparative Immunology, Microbiology & Infectious Disease **27:** 305–318.

DIVO, A. A., T. G. GEARY, AND J. B. JENSEN. 1985. Oxygen- and time-dependent effects of antibiotics and selected mitochondrial inhibitors on *Plasmodium falciparum* in culture. Antimicrobial Agents and Chemotherapy **27:** 21–27.

EL-ON, J., R. SNEIER, AND E. ELIAS. 1992. Leishmania major: Bacterial contamination of cutaneous lesions in experimental animals. Israel Journal of Medical Sciences **28:** 847–851.

GRIGORYAN, R., N. KESHELAVA, C. ANDERSON, AND C. P. REYNOLDS. 2005. In vitro testing of chemosensitivity in physiological hypoxia. Methods in Molecular Medicine **110:** 87–100.

GRIMALDI, G., JR., AND R. B. TESH. 1993. Leishmaniasis of the new world: Current concepts and implications for future research. Clinical Microbiology Reviews **6:** 230–250.

HYUN, J. Y., Y. S. CHUN, T. Y. KIM, H. L. KIM, M. S. KIM, AND J. W. PARK. 2004. Hypoxia-inducible factor 1alpha-mediated resistance to phenolic anticancer. Chemotherapy **50:** 119–126.

IKEDA, E. 2005. Cellular response to tissue hypoxia and its involvement in disease progression. Pathology International **55:** 603–610.

KOCH, S., F. MAYER, M. HONECKER, M. CCHITTENHELM, AND C. BOKEMEYER. 2003. Efficacy of cytotoxic agents used in the treatment of testicular germ cell tumours under normoxic and hypoxic conditions in vitro. British Journal of Cancer **89:** 2133–2139.

KRUNGKRAI, S. R., AND Y. YUTHAVONG. 1987. The antimalarial action on *Plasmodium falciparum* of qinghaosu and artesunate in combination with agents which modulate oxidant stress. Transactions of the Royal Society of Tropical Medicine and Hygiene **81:** 710–714.

LUX, H., N. HEISE, T. KLENNER, D. HART, AND F. R. OPPERDOES. 2000. Ether–lipid (alkyl-phospholipid) metabolism and the mechanism of action of ether–lipid analogues in *Leishmania*. Molecular and Biochemical Parasitology **111:** 1–14.

MISHRA, J., A. SAXENA, AND S. SINGH. 2007. Chemotherapy of leishmaniasis: Past, present and future. Current Medicinal Chemistry **14:** 1153–1169.

SANTA-RITA, R. M., A. HENRIQUES-PONS, H. S. BARBOSA, AND S. L. DE CASTRO. 2004. Effect of the lysophospholipid analogues edelfosine, ilmofosine and miltefosine against *Leishmania amazonensis*. Journal of Antimicrobial Chemotherapy **54:** 704–710.

SOKOL-ANDERSON, M. L., J. BRAJTBURG, AND G. MEDOFF. 1986. Amphotericin B-induced oxidative damage and killing of *Candida albicans*. Journal of Infectious Diseases **154:** 76–83.

SONG, X., X. LIU, W. CHI, Y. LIU, L. WEI, X. WANG, AND J. YU. 2006. Hypoxia-induced resistance to cisplatin and doxorubicin in non-small cell lung cancer is inhibited by silencing of HIF-1alpha gene. Cancer Chemotherapy and Pharmacology **58:** 776–784.

TEICHER, B. A. 1994. Hypoxia and drug resistance. Cancer Metastasis Reviews **13:** 139–168.

WYLLIE, S., M. L. CUNNINGHAM, AND A. H. FAIRLAMB. 2004. Dual action of antimonial drugs on thiol redox metabolism in the human pathogen *Leishmania donovani*. Journal of Biological Chemistry **279:** 39925–3932.