In vitro Antileishmanial Drug Susceptibility of Clinical Isolates from Patients with Indian Visceral Leishmaniasis—Status of Newly Introduced Drugs

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Abstract. Regional variations in susceptibility of *Leishmania donovani* clinical isolates have been reported to antimonials but not other antileishmanial drugs. Therefore, we evaluated the susceptibility of four antileishmanial drugs in clinical use in 28 clinical isolates from endemic and non-endemic regions in the J774A.1 macrophage cell line, and we found increased tolerance of miltefosine and paromomycin in isolates from a patient from a high endemic region. Effective dose for 90% killing (ED₉₀) values were significantly higher for miltefosine ($P = 0.005$) and paromomycin ($P = 0.02$) in isolates from the high endemic region, although there were no significant differences between ED₅₀ values for paromomycin, miltefosine, and amphotericin B in the non- versus endemic region isolates. This report is the first of higher ED₉₀ values for miltefosine and paromomycin indicating susceptibility difference between regions for these newly introduced drugs by the parasite, and their use should be carefully monitored through directly observed therapy or multidrug treatment to preserve their efficacy for longer periods.

INTRODUCTION

Visceral leishmaniasis (VL), also known as kala-azar, continues to be an important public health problem in certain regions of the world.¹ More than 90% of the estimated 500,000 new cases reported per year occur in endemic areas of India, Bangladesh, Nepal, Sudan, and Brazil. After development of extensive resistance to sodium stibogluconate (SSG),^{2,3} amphotericin B is being used extensively, and recently, miltefosine and paromomycin have been approved for treatment of VL in India. There are sporadic reports of variability in sensitivity to these newly introduced antileishmanial drugs,^{4,5} and the experience with SSG points to the fact that we urgently need to develop an assay that can correlate the clinical response to the experimental results. A number of reports describe *in vitro* susceptibility assays to assess the activity of chemotherapeutic agents against the amastigotes of leishmania parasites,^{6,7} but whether these assays correlate with the clinical outcome needs to be further investigated. The present study was undertaken to evaluate the *in vitro* macrophage drug sensitivity assay to determine intrinsic drug sensitivity of field isolates from different regions for VL in India.

MATERIALS AND METHODS

Ethics statement. The study was approved by the ethical committee of Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. Written informed consent was taken from all the subjects.

Patients. Clinical isolates from eastern Uttar Pradesh (UP) region with low endemicity (few cases per 10,000 populations; $N = 12$) were obtained from patients referred to Sir Sunderlal Hospital, Varanasi, UP, whereas all isolates from the high endemicity zone (≥ 30 cases per 10,000 populations⁸; $N = 16$) were obtained from patients treated at Kala-azar Medical Research Center, Muzaffarpur, Bihar. Diagnosis of VL was parasitologically confirmed by demonstration of amastigotes in splenic smears.⁹

Drugs. Miltefosine and SSG were obtained in crystalline form from Zentaris Weismuellerstr, Frankfurt, Germany and Albert David Ltd, Kolkatta, India, respectively. Amphotericin B in powder form was received as a gift from Bharat Serum and Vaccines Limited, Mumbai, India. Paromomycin was gifted by Gland Pharma, Hyderabad, India.

Parasite culture. Splenic aspirates obtained before treatment were used as the source of material for primary culture of promastigotes. Splenic aspirates were initially maintained at 26°C in biphasic Novy McNeal Nicolle (NNN) media for conversion of amastigotes to promastigotes in the presence of 500 μ L complete Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS; Gibco, Invitrogen Life Technologies, Grand Island, NY) as described previously.¹⁰ Primary culture was done in complete RPMI after 5–6 days of incubation. All parasites were used within two to three passages starting from primary isolation. *Leishmania donovani* species identification was done by restriction fragment length polymorphism (RFLP) of the HSP70 gene as previously described.^{11,12}

Stationary-phase promastigotes of *L. donovani* strains from UP and Bihar were used for the experiments; 5- to 6-day-old cultures were washed two times by centrifugation at $2,500 \times g$ for 15 minutes at 4°C in phosphate buffer saline and resuspended to a final concentration of 1×10^6 /mL in cRPMI (Gibco, Invitrogen Life Technologies). Stationary-stage promastigotes were counted in a hemocytometer, and their concentration was adjusted for macrophage infection as previously described.¹³

Raising intracellular amastigote infection in macrophage cell line. The J774A.1 macrophage cell line was commercially obtained from the National Center for Cell Science, Pune, India. The cell line was cultured in cRPMI supplemented with 15% heat-inactivated FBS (Gibco, Invitrogen), 60 U/mL penicillin, 100 μ g/mL streptomycin, 2 mM gentamicin, and 25 mM Hepes (pH 7.2; HIMEDIA, New Delhi, India); 5×10^4 macrophage cells were plated in 250 μ L cRPMI media in eight-well chamber Labtek tissue culture slides (Nunc, Thermo Fischer Scientific, Rochester, NY) in serum-free RPMI media and allowed to adhere for 4 hours at 37°C in 5% CO₂. After 4 hours, non-adherent cells were removed by washing two times in serum-free RPMI media. The adherent macrophages were infected with 5×10^5 stationary-phase promastigotes at an approximate leishmania to macrophage ratio of 10:1 in 0.3 mL

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cRPMI and incubated for 24 hours at 37°C in 5% CO₂. After 24 hours, macrophage infection was confirmed by Giemsa staining and counting of intracellular amastigotes. At least 100 cells per well (four corners and the center) were counted to determine the percentage of macrophages infected and the number of parasites per infected cell.^{13,14} All experiments were performed in duplicate.

Susceptibility of intracellular amastigotes to SSG, amphotericin B, miltefosine, and paromomycin. The J774A.1 macrophages in eight-well Labtek culture slides in serum-free RPMI media were infected with stationary-stage parasites from UP or Bihar in a ratio of 10:1 (leishmania to macrophage). Cells were incubated for 24 hours at 37°C in 5% CO₂. Non-internalized promastigotes were removed, and infected cells were treated with different concentrations of miltefosine (1, 2, 3, 5, 8, 10, or 25 µg/mL),¹⁵ amphotericin B (0.00125, 0.0025, 0.005, 0.010, 0.015, 0.020, or 0.025 µg/mL),¹³ paromomycin (25, 50, 100, 150, 175, 200, or 250 µg/mL),¹⁶ and SSG (4, 10, 20, 30, 40, or 50 µg/mL)³ along with two control wells for each drug. The cultures were incubated at 37°C in 5% CO₂ for 72 hours, and the slides were fixed in absolute methanol for 5 minutes and stained with Giemsa dye. At least 100 cells per well were counted to determine the percentage of macrophages infected and the number of parasites per infected cell. The percentage inhibition relative to untreated macrophages was calculated on the basis of the comparison of total amastigotes per 100 macrophages. Mean ED₅₀ and ED₉₀ values were determined for each strain as described.¹⁷

Statistical analysis. Unpaired *t* tests and Welch *t* tests were used to analyze the data. Statistical significance was accepted at *P* < 0.05.

RESULTS

Intracellular amastigote infection in macrophage cell line.

The level of infection obtained at 24 hours post-infection was 60–80% in plated macrophages. The infection level was approximately one to two amastigotes per macrophage.

***In vitro* drug sensitivity against field isolates of *L. donovani* using the intracellular amastigote assay.** For each strain, different concentrations of SSG, amphotericin B, miltefosine, and paromomycin were tested *in vitro* using the intracellular amastigote J774A.1 macrophage susceptibility assay.

Drugs were added to the experimental wells for 72 hours of incubation. The results from the macrophage sensitivity assay for SSG are shown in Table 1. The mean ED₅₀ value for SSG in amastigotes in the high endemic region was 24.1 ± 5.4 µg/mL, whereas in non-endemic region, it was 17.2 ± 2.9 µg/mL. The mean ED₉₀ values were 56.6 ± 9.6 and 42.0 ± 5.1 µg/mL in high and non-endemic regions, respectively. ED₅₀ and ED₉₀ values for other drugs have been given in Table 1.

DISCUSSION

Leishmaniasis is still one of the most neglected diseases affecting mainly the poorest to poor people of developing countries. In the endemic region of India, SSG, amphotericin B, and miltefosine drugs have been used, whereas in the non-endemic region, only SSG and amphotericin B are being used for the treatment of VL patients. Resistance to antimonials has been evident in the endemic region since the mid-1990s, and because of the use of miltefosine at a higher level

TABLE 1

ED₅₀ and ED₉₀ values for *in vitro* susceptibility of clinical isolates to antileishmanial drugs

Geographical status and drug concentration	Endemic region	Non-endemic region	<i>P</i> value
Miltefosine			
ED ₅₀ (µg/mL) amastigotes			
Range	5.3–10.9	3.5–10.1	0.08
Mean ± SD	7.1 ± 1.6	5.7 ± 2.2	
ED ₉₀ (µg/mL)			
Amastigotes	14.1–23.8	9.8–19.1	0.005
Mean ± SD	17.5 ± 2.4	13.7 ± 3.6	
Paromomycin			
ED ₅₀ (µg/mL) amastigotes			
Range	90.8–200.9	99.6–174.86	0.15
Mean ± SD	139.8 ± 33.6	122.1 ± 23.5	
ED ₉₀ (µg/mL)			
Amastigotes	163.8–380.3	189.8–265.7	0.02
Mean ± SD	279.6 ± 54.1	232.9 ± 24.8	
Amphotericin B			
ED ₅₀ (µg/mL) amastigotes			
Range	0.007–0.014	0.008–0.016	0.92
Mean ± SD	0.011 ± 0.002	0.011 ± 0.003	
ED ₉₀ (µg/mL)			
Amastigotes	0.023–0.031	0.019–0.035	0.05
Mean ± SD	0.027 ± 0.003	0.024 ± 0.005	
SSG			
ED ₅₀ (µg/mL) amastigotes			
Range	14.2–34.4	12.6–21.1	0.0007
Mean ± SD	24.1 ± 5.4	17.2 ± 2.9	
ED ₉₀ (µg/mL)			
Amastigotes	32.9–74.1	33.2–50.8	0.0001
Mean ± SD	56.6 ± 9.6	42.0 ± 5.1	

since 2004, there is a risk that drug pressure could lead to an increase in resistance to this drug in endemic areas. We used an *in vitro* susceptibility assay based on intracellular amastigotes on host macrophages to assess the susceptibility of *L. donovani* clinical isolates from different geographical regions of India to antileishmanial drugs, with main emphasis on miltefosine, paromomycin, and amphotericin B. There was no significant difference between the drug sensitivities of parasites isolated from different endemic regions against amphotericin B. Interestingly, higher ED₉₀ values observed for isolates of endemic regions for miltefosine and paromomycin compared with non-endemic regions might be a prelude to increasing tolerance of these drugs, which might result in increasing unresponsiveness in the future when there is greater use of these drugs. However, the limitation of this study is the number of isolates tested from two different regions, but these numbers are sufficient to describe the pattern of drug susceptibility for clinical isolates in both regions.³ Ideally, greater numbers would strengthen statistics and confirm the observed pattern. Susceptibility was clearly shown for amphotericin B, which is known to have antileishmania action independent of cell-mediated microbicidal activity.^{18,19} It is, therefore, reasonable to speculate that J774A.1 infection with promastigotes could, indeed, be used for resistance determination against miltefosine, amphotericin B, and paromomycin in field isolates.^{20,21} This finding could become particularly relevant for large-scale monitoring of miltefosine resistance in the Indian subcontinent, because it was recently introduced as a first-line medication for VL in the current Kala-Azar Elimination Program.²²

Here, we report, for the first time, higher ED₉₀ values for miltefosine in the endemic regions, where there is likely to be high drug pressure with the current extensive use of

miltefosine. Higher ED₉₀ values for paromomycin do not argue well for its use in future. Our finding about susceptibility of the newly introduced antileishmanial drugs necessitates the need to rethink the strategy to treat VL in the Indian subcontinent and elsewhere. This different strategy could be either a single-dose liposomal amphotericin B therapy^{23,24} or multidrug therapy instead monotherapy to prevent the loss of any of the other currently used antileishmanial drugs.²⁵ With no new antileishmanial drugs in the pipeline and no vaccine in sight, it is imperative to prevent the emergence of resistant strains, which may hinder the treatment of this disease. Our findings do indicate a correlation between *in vitro* drug susceptibility and endemicity of different regions, and thus, they indicate that the J774A.1 macrophage sensitivity assay could be useful for monitoring drug susceptibility in absence of markers for molecular resistance; additionally, this assay could assist clinicians in treatment decision making of VL in different endemic regions.

Received January 9, 2012. Accepted for publication March 29, 2012.

Acknowledgments: The authors thank the Kala-azar Medical Research Center, Sitaram Memorial Trust, Muzaffarpur, Bihar, India for constant support in conducting this study. V.K.P., S.M., and S.G. are thankful to Indian Council of Medical Research and Council of Scientific and Industrial Research, New Delhi, India for financial support.

Financial support: This study was supported by National Institute of Allergy and Infectious Disease, National Institutes of Health Tropical Medicine Research Center Grant 1P50AI074321.

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