CHARACTERIZATION OF NATURAL ANTIMONY RESISTANCE IN LEISHMANIA DONOVANI ISOLATES

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Abstract. Clinical resistance to pentavalent antimonial compounds has long been recognized as a major problem in the treatment of visceral leishmaniasis in India. However, mechanisms of natural resistance are unclear. In this study, we observed that Leishmania donovani clinical isolates not responsive to sodium stibogluconate showed resistance to antimony treatment in both in vitro and in vivo laboratory conditions. The resistant isolates have increased levels of intracellular thiols. This increase in thiol levels was not mediated by the amplification of γ-glutamylcysteine synthetase, but was accompanied by amplification of trypanothione reductase and an intracellular ATP-binding cassette transporter gene MRPA. The resistance of parasites to antimony could be reversed by the glutathione biosynthesis-specific inhibitor, buthionine sulfoximine, which resulted in increased drug susceptibility. These results suggest the possible role of thiols and MRPA in antimony resistance in field isolates.

INTRODUCTION

The protozoan parasite Leishmania is responsible for several pathologies ranging from self-healing cutaneous lesions to visceral infections that can be fatal if untreated. Along with Brazil, Sudan, and Bangladesh, India contributes to 90% of the global burden of visceral leishmaniasis. In absence of effective vaccines, chemotherapy is the main weapon to control infections. The first-line drug against all forms of Leishmania infection consists of pentavalent antimony (SbV) that contains drugs such as sodium stibogluconate (SSG) and glu- cantime, despite its requirement for long courses and cardiac toxicity. Unfortunately, the clinical value of antimony therapy is now challenged because an increasing rate of treatment failure is observed in several field sites and it has reached to epidemic proportion in the state of Bihar, India. The geographic and temporal grouping of SbV treatment failures suggests the emergence of antimony-resistant strains. In MuzaffARPur (Bihar), clinical resistance in L. donovani field isolates has been demonstrated. Amphotericin B (normal or liposomized) is used as an alternative, but has significant disadvantage of severe toxicity and high cost for a disease prevalent in third-world countries. The demonstration of the efficacy of miltefosine is a breakthrough, but single point mutations can lead to resistance, which suggests that resistance to this drug may occur rapidly. Monitoring and prevention of spreading of drug resistance is now a priority for the control of leishmaniasis (www.who.int/infections-disease-report/2000). Within this context an insight into the mechanism of natural drug resistance could contribute to the development of efficient strategies for monitoring SbV resistance at sites where it is endemic.

The mechanism of antimony resistance in field is unknown and most information comes from the work on laboratory mutants, mostly L. tarentolae, in which resistance was introduced in vitro by use of trivalent antimony (SbIII) or related metals such as AsIII. Use of these resistant mutants demonstrated that metal resistance is associated with reduced accumulation of drug, gene(s) amplification, and overproduction of trypanothione, the parasite-specific spermidine glutathione conjugate. It is generally agreed that the drug in clinical use, i.e., SbV, is a prodrug that needs to be converted into SbIII. Reduction of the metal was shown to occur non-enzymatically by thiols, as well as by a parasite-specific enzyme, thiol-dependent reductase (TDRI) and a new anti-monite reductase (ACR2). The exact site of conversion is still unclear. It may take place in either the parasite, in host macrophages, or in both. The decreased levels of SbIII in resistant strains seem to be caused either by decreased uptake of SbIII caused by lower expression of the parasite aquaglyceroporin gene (AQPI), which codes the protein responsible for uptake of trivalent metalloid, or by inhibition of intracellular reductase activity. Once the SbIII is within cell, it would be conjugated to trypanothione, which would then either be sequestered inside a vacuole by ATP-binding cassette (ABC) transporter MRPA (previously known as p-glycoprotein A; [PGPA]), or excluded by a thiol-X pump, which may correspond to other ABC transporters in the same family as PGPA (www.Genedb.org). Recently, transfection of the MRPA gene was shown to confer antimony resistance in axenic amastigotes of L. infantum selected for SbIII resistance. Because these data are being generated on in vitro stepwise-induced resistant Leishmania strains, it remains to be seen whether similar resistance mechanism operates in field isolates. One study conducted with clinical isolates showed significant decreased expression of AQPI and γ-glutamylcysteine synthetase 1 (γ-GCS1), a key enzyme of glutathione synthesis. Conversely, in another study, the resistant strains expressed higher levels of γ-GCS1 compared with sensitive strains. Furthermore, no reports are available on amplification of reported gene(s) and thiol levels in clinical isolates. Consequently, we focused on these aspects to explore the mechanism of resistance prevailing in field isolates of L. donovani that exhibit stable resistance phenotype.

MATERIALS AND METHODS

Reagents. Cysteine, reduced glutathione, gentamicin, RPMI 1640 medium, medium 199, HEPES, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), amphotericinB, SbIII (potassium antimony tartarate), and fetal calf serum were obtained from Sigma (St. Louis, MO). Trypanothione was obtained from

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Clinical isolates. Patients were selected from Kala-azar Medical Center of the Institute of Medical Sciences, Banaras Hindu University (Varanasi, India) and from its affiliated hospital at Muzzafarpur, Bihar. The ethics committee of the Kala-azar Medical Research Center (Muzzafarpur, India) reviewed and approved the study protocol. The criteria of diagnosis of visceral leishmaniasis was the presence of Leishman Donovan bodies in spleenic aspirates, which were graded according to standard criteria. After diagnosis, the patients were administered intravenously one course of SSG (20 mg/kg of body weight/day for 30 days). Response to treatment was evaluated by repeating spleenic aspiration at day 30 of treatment. The designation of patients was based on the absence of fever, clinical improvement with reduction in spleen size, and the absence of parasites in aspirates. Patients who had parasites were considered to be unresponsive to therapy. These patients were subsequently treated with amphotericin B.

Reference strain. Leishmania donovani Dd8 promastigotes (World Health Organization designation MHOM/IN/80/Dd8), which was originally obtained from (late) P. C. C. Garnham (Imperial College, London, United Kingdom), was used as the sensitive reference strain. It was maintained at the Central Drug Research Institute in golden hamsters.

Culture conditions. The spleenic aspirates of patients were inoculated into NNN medium, grown at 25°C, and subcultured every sixth day. The positive cultures were then adapted to medium 199 (Sigma) supplemented with 10% fetal calf serum (Gibco/Invitrogen, Carlsbad, CA) and 1% penicillin (50 U/mL) and streptomycin (50 μg/mL) solution (Sigma) for mass cultivation.

Antimony susceptibility of clinical isolates. In vitro assay. The trivalent antimony compounds are presumed to be the active form of the drug because they are highly active against both stages of the parasite. The 50% inhibitory concentration (IC₅₀) of SbIII was determined as an index of antimony resistance phenotype of the isolates. The effect of drugs on promastigote multiplication was evaluated by the cell-counting method. Exponentially growing parasites (10⁶ cells/mL stock) were seeded in 96-well microplates (0.2 mL/well) in the presence of drug(s) and allowed to grow for 48 hours at 26 ± 1°C. The relative number of viable Leishmania cells per well was determined microscopically and the IC₅₀ value was calculated by probate analysis. Amphotericin B was used as the reference drug.

In vivo assay. For establishment of resistant strains in golden hamsters (Mesocricetus auratus), animals were first given prednisolone (5 mg/liter in drinking water) for 10 days, inoculated intracardially with 1 x 10⁷ lade log phase promastigotes in 0.1 mL of phosphate-buffered saline (PBS), and given two booster doses at an interval of 10 days. Once the hamsters were infected with the promastigote forms, further passages in hamsters were carried out with spleenic amastigotes. Briefly, spleens were removed from highly infected animals under aseptic conditions, homogenized in Locke's solution, and centrifuged at 900 rpm for five minutes at 4°C to remove cell debris. Parasites were centrifuged at 2500 rpm for 10 minutes. The pellet was washed twice with cold Locke's solution, suspended in Locke's solution to give a concentration of 1 x 10⁷/0.1 mL, and used for intracardial infection. Animals with infections for 18–20 days (most with an infection level of 15–20% as determined by spleen biopsy) were used for treatment with sodium antimyglucanate (SAG) at intraperitoneal doses of 20 mg/kg and 40 mg/kg for five 5 days. Five infected control animals received placebo. Seven days post-treatment, spleen biopsies were performed. Parasite burden in both treated and untreated infected animals was assessed and percentage parasite inhibition in treated animals was calculated.

Analysis of thiols. Total intracellular thiols in promastigotes were estimated in de-proteinized cell extracts. Briefly, cells at mid log phase (3 x 10⁷/mL) were harvested, washed with PBS (pH 7.4), suspended in an equal volume of 10% trichloroacetic acid, and frozen and thawed once before centrifugation at 10,000 g for 10 minutes at 4°C. The thiol content of the supernatant solution was determined with 0.6 mM DTNB in 0.1 M sodium phosphate buffer (pH 8.0). The yellow color that developed from the production of p-nitro-m-carboxy-benzenethiol by the reaction of reduced thiols and DTNB was measured at a wavelength of 412 nm. The reduced glutathione was taken as the standard and total cell thiols were represented as total glutathione.

Effect of L-buthionine-(SR)-sulfoximine (BSO) on thiols and parasite survival. L-buthionine-(SR)-sulfoximine was added to the promastigote suspension at a concentration of 5 mM for 48 hours in the presence and absence of SbIII. After drug treatment, total intracellular thiols and the inhibitory effect of the compound on parasite growth were measured.

Trypanothione reductase assay. Trypanothione reductase activity in crude cell extracts of both sensitive and resistant strains was assayed spectrophotometrically at 340 nm, as previously described.

Protein estimation. The protein concentration was determined by the Bradford method using bovine serum albumin as the standard.

Nucleic acid isolation and Southern blot analysis. Total genomic DNA was isolated from parasites according to the protocol of Maniatis. DNA (5 μg) was digested with Xho I or Pst I and separated by electrophoresis on 0.8% agarose gels. Southern hybridization was performed with ψ-labeled γ-GCS1, MRPA (previously known as PGPA, an ABC transporter gene) (obtained from Dr. M. Ouellette, Centro Internacional de Ensenamiento e Investigaciones Medicas, Cali, Colombia), and trypanothione reductase. Probes were radiolabeled using a random prime labeling kit (BRIT, Bombay, India) to a specificity of 2 x 10⁶ cpm/μg.

Statistical analysis. Significance of the data was calculated using the Student t-test.

RESULTS AND DISCUSSION

Resistance to antimony in Leishmania is widespread in several geographic regions and has reached epidemic proportion in parts of India. Acquired resistance has been studied in the laboratory for several decades and has provided much formation but few studies have been conducted with field isolates. Therefore, we investigated the mechanism of resistance to pentavalent antimonials in field isolates from India.

Characterization of the antimony resistance phenotype in field isolates. Five isolates were collected from patients with...
visceral leishmaniasis who had undergone one course of treatment with antimony. These isolates were classified as resistant or sensitive on the basis of their clinical response to treatment (Table 1). Because determining the drug sensitivity of isolates is always open to criticism that removal of parasite from host and adaptation to culture media may lead to the selection of a population that is best suited for growth in medium which may or may not be resistant, we checked the resistance phenotype of promastigotes in vitro to SbIII (potassium antimony tartrate) and SbV (sodium stibogluconate) in animal model. SbIII significantly inhibited growth of promastigote in a dose-dependent manner (Figure 1). The IC_{50} values of responsive isolates were much less than the values for unresponsive isolates (Table 1). At a concentration of 25 μg/mL, growth inhibition of sensitive isolates varied from 35% to 50%, but was only 4–20% in resistant isolates. Resistant isolates exhibited nearly 2–3-fold higher IC_{50} values for SbIII compared with the sensitive reference strain (Dd8) (Table 1). Trivalent antimony compounds are believed to be the active form of SbV and are toxic to promastigotes of various Leishmania species.\(^\text{19}\) The relatively nontoxic SbV is considered to be a prodrug that is converted to highly toxic SbIII either in macrophages,\(^\text{45}\) the parasite,\(^\text{23}\) or both. Only 20% inhibition of promastigote growth was observed at a high concentration of SbV (data not shown). This is consistent with results of a study with L. donovani promastigotes.\(^\text{46}\) These results clearly demonstrate a correlation between the unresponsiveness of the patient to one full course of treatment with SSG (clinical resistance) and the refractory response of isolated parasites to SbIII under laboratory conditions (promastigote growth inhibition assay).

To validate the in vitro antimony resistance data, one sensitive and two resistant isolates were evaluated for their SSG susceptibility in an animal model. Treatment with SSG at a dose of 40 mg/kg (given five times intraperitoneally) resulted in an approximately 95% decrease in parasitic burden in animal spleen(s) infected with sensitive strains or the reference strain. However, this dose failed to cure animals infected with resistant strains (Table 1). Animals had a significant parasite load (approximately 35–40%) even after drug treatment. Lira and others observed a correlation between clinical response and SSG (SbV) sensitivity of intracellular amastigotes in macrophages.\(^\text{9}\) Our data show a correlation between clinical response, insensitivity of promastigotes to SbIII, and a refractory response of parasites to SbV in an animal model. Our results show that the IC_{50} for SbIII may be used as a marker for antimony resistance. The data are further supported by the study on L. infantum isolates that required a two-fold or greater 50% effective dose compared with that sensitive strain corresponded to clinical failure.\(^\text{43}\) Furthermore, this decrease in susceptibility to SbIII is drug specific with no cross resistance to a second-line drug (amphotericin B) because all strains were highly sensitive to this drug (Table 1). This finding was consistent with that of a study that showed that in vivo susceptibility of L. donovani field isolates to SSG was drug specific.\(^\text{47}\)

### Table 1

<table>
<thead>
<tr>
<th>Strains</th>
<th>Clinical response of field isolates</th>
<th>IC_{50} (μg/mL)</th>
<th>% inhibition in parasite burden</th>
<th>% inhibition in parasite burden</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SbIII</td>
<td>Amphotericin B</td>
<td></td>
</tr>
<tr>
<td>Dd8</td>
<td>NA</td>
<td>26.20</td>
<td>0.024</td>
<td>1.00</td>
</tr>
<tr>
<td>S1</td>
<td>Cured</td>
<td>24.78</td>
<td>0.017</td>
<td>0.95</td>
</tr>
<tr>
<td>S2</td>
<td>Cured</td>
<td>25.91</td>
<td>0.012</td>
<td>0.99</td>
</tr>
<tr>
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<td>83.19</td>
<td>0.010</td>
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<tr>
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<td>R3</td>
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<td>71.40</td>
<td>0.012</td>
<td>2.73</td>
</tr>
</tbody>
</table>

\(\text{IC}_{50}\) = 50% inhibitory concentration; SbV = potassium antimony tartrate; RI = IC_{50} of field isolate/IC_{50} of reference strain Dd8; NA = not available because the reference strain is maintained in animals only; ND = not done.

\(\uparrow\) Mean data from three separate experiments.

**Determination of total intracellular thiol levels in Leishmania.** A 1.35–1.7-fold increase in total intracellular reduced thiol levels were observed in isolates unresponsive to SSG, and levels in sensitive strains were almost comparable to that of the sensitive reference strain (Figure 2). However, this increase was much less than the 10–20-fold increase observed in laboratory-selected mutants.\(^\text{16,17,48}\) This suggests that increase in thiol levels may be related to the fold resistance to metal ions. Laboratory-selected mutants normally show a 50–200-fold increase in resistance to metal ions and field isolates.

**Figure 1.** In vitro drug susceptibility of promastigotes of Leishmania donovani field isolates to trivalent antimony (SbIII). S = sensitive; R = resistant.
show at most a 2–7-fold increase in resistance to antimony (Table 1).

**Effect of BSO on total intracellular thiol concentrations and SbIII resistance.** Figure 3A shows the effect of BSO on total intracellular thiol levels in two *Leishmania donovani* field isolates (one sensitive, S1 and one resistant, R1). L-buthionine-(SR)-sulfoximine, a specific inhibitor of glutathione biosynthesis, at a concentration of 5 mM significantly depleted total intracellular thiol levels in both resistant and sensitive parasites in a time-dependent manner. Within 48 hours, nearly 50% of the thiols were depleted in a sensitive strain and 70% of the thiols were depleted in a resistant strain. This depletion was accompanied by a substantial increase in the sensitivity of resistant isolates to SbIII (Figure 3B). L-buthionine-(SR)-sulfoximine at a concentration of 5 mM did not show any significant growth inhibitory effect on either resistant or sensitive strains. Similarly SbIII at concentrations of 25 μg/mL and 10 μg/mL did not inhibit growth of resistant and sensitive isolates more than 20% (Figure 1). However, in the presence of BSO, the inhibitory effect of SbIII was significantly enhanced in resistant isolates. The synergistic inhibitory effect of BSO plus SbIII on growth was more than the individual and additive effects in resistant strains. Similarly SbIII at concentrations of 25 μg/mL and 10 μg/mL did not inhibit growth of resistant and sensitive isolates more than 20% (Figure 1). In antimony-resistant laboratory mutants, increases in thiol levels is partially linked to amplification of *GCS*, a key enzyme in glutathione synthesis. To determine whether such amplification is also present in clinical resistant isolates, Southern blot analysis was performed. Hybridization of *Xho* I-digested genomic DNA from both sensitive and resistant cell lines with a *GCS* probe identified a 3.6-kb band in all isolates (Figure 4A). The amount of DNA of each isolate loaded on the gel was estimated by re-hybridization of same blot with a control *tubulin* probe (Figure 4B). Densitometric analysis after normalization with the control confirmed that there was no amplification of *GCS* in resistant strains compared with sensitive strains (Figure 4C). This finding is consistent with those of previous studies,

**Gene amplification.** In antimony-resistant laboratory mutants, increases in thiol levels is partially linked to amplification of *GCS*, a key enzyme in glutathione synthesis. To determine whether such amplification is also present in clinical resistant isolates, Southern blot analysis was performed. Hybridization of *Xho* I-digested genomic DNA from both sensitive and resistant cell lines with a *GCS* probe identified a 3.6-kb band in all isolates (Figure 4A). The amount of DNA of each isolate loaded on the gel was estimated by re-hybridization of same blot with a control *α*-tubulin probe (Figure 4B). Densitometric analysis after normalization with the control confirmed that there was no amplification of *GCS* in resistant strains compared with sensitive strains (Figure 4C). This finding is consistent with those of previous studies,

**Thiols have also been shown to be required for MRPA-mediated resistance in *L. infantum* axenic amastigotes, which can also be reversed by treatment with BSO.

**In vivo resistance to sodium stibogluconate in animal models can be reversed by treatment with BSO.** Thus, thiol depletors may increase the leishmanicidal effect of drugs and decrease drug resistance in the parasite.

**Kapoor and others** have also shown inhibition of glutathione synthesis by treatment with BSO to be a chemotherapeutic strategy for treatment of leishmaniasis.
Increased expression of γ-GCS was recently demonstrated in SAG-resistant *L. donovani* strains from India. However, non-responsive field isolates from Nepal showed decreased expression of γ-GCS and ornithine dicarboxylase, which suggested that other mechanism(s) may be involved in the increase in trypanothione levels. Southern blot analysis of *Pst* I-digested genomic DNA with a trypanothione reductase probe showed a stronger 4.5-kb band in resistant isolates (Figure 5A). Densitometric analysis after normalization with the α-tubulin gene showed 2.0–2.5-fold amplification of the trypanothione reductase gene in resistant strains compared with sensitive strains (Figure 5C). This amplification was accompanied by an increase in the specific activity of trypanothione reductase (Figure 5D). Increased levels of trypanothione reductase will help to maintain increased levels of reduced trypanothione, which is required for conjugation with antimony for efflux and overcoming the inhibitory effect of SbIII. These data indicate the possible involvement of trypanothione reductase in drug resistance. However, this finding must be confirmed with additional isolates. Recently, another important enzyme of thiol metabolism, cytosolic trypaparedoxin peroxidase, was shown by DNA amplification to be over-expressed in the cytoplasm of an arsenite-resistant variant of *L. amazonensis*. These observations further sug-

Figure 4. γ-glutamylcysteine synthetase (γ−GCS) gene amplification in *Leishmania donovani* field isolates. Genomic DNA of various strains were isolated, digested with *Xho* I, and hybridized with A, γGCS and B, α-tubulin. C, Densitometric analysis of the γ−GCS gene after normalization with the α-tubulin gene. Values are the mean ± SD of at least three experiments. R = resistant; S = sensitive; ns = not significant with respect to Dd8.

Figure 5. Trypanothione reductase (TR) gene amplification in *Leishmania donovani* field isolates. Genomic DNA of various strains were isolated, digested with *Pst* I, and hybridized with A, TR and B, α-tubulin. C, Densitometric analysis of the TR gene after normalization with the α-tubulin gene. D, Specific activity of TR in crude lysate of *L. donovani* field isolates. Values are the mean ± SD of at least three experiments. ***P < 0.001; *P < 0.05; **P < 0.01. R = resistant; S = sensitive; ns = not significant with respect to Dd8.
role in antimony resistance. Recently, a decrease in expression of AQP1 has been demonstrated in SAG-resistant clinical isolates.\textsuperscript{32} Taken together, these observations demonstrate that clinical SSG non-responsiveness in field isolates of \textit{Leishmania} is a stable phenotype that is associated with thiol levels. They also suggest that thiol depletors may increase the leishmanicidal effect of drugs, which suggests a strategy to reverse resistance in field isolates.

Received September 12, 2006. Accepted for publication November 22, 2006.

Acknowledgments: We thank Dr. M. Ouellette for providing the MRPA and γ-GCS1 probe and A. Kauser for computational help.

Financial support: This work was supported by the Department of Biotechnology (grant no. BT/PR2792/Med/14/383/2001), India. Mukul K. Mittal and Ashutosh Ravinder were supported by the Council of Scientific and Industrial Research, India, and Smita Rai was supported by the Department of Biotechnology, India. The American Society of Tropical Medicine and Hygiene (ASTMH) assisted with publication expenses.

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