Gene Expression Analysis of the Mechanism of Natural Sb(V) Resistance in *Leishmania donovani* Isolates from Nepal

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Control of visceral leishmaniasis (VL) is being challenged by the emergence of natural resistance against the first line of treatment, pentavalent antimonials [Sb(V)]. An insight into the mechanism of natural Sb(V) resistance is required for the development of efficient strategies to monitor the emergence and spreading of Sb(V) resistance in countries where VL is endemic. In this work, we have focused on the mechanism of natural Sb(V) resistance emerging in Nepal, a site where anthroponic VL is endemic. Based on the current knowledge of Sb(V) metabolism and of the in vitro trivalent antimonial [Sb(III)] models of resistance to *Leishmania* spp., we selected nine genes for a comparative transcriptomic study on natural Sb(V)-resistant and -sensitive *Leishmania donovani* isolates. Differential gene expression patterns were observed for the genes coding for 2-thiol biosynthetic enzymes, gamma-glutamylcysteine synthetase (GCS) and ornithine decarboxylase (ODC), and for the Sb(III) transport protein aquaglyceroporin 1 (AQP1). The results indicate that the mechanism for natural Sb(V) resistance partially differs from the mechanism reported for in vitro Sb(III) resistance. More specifically, we hypothesize that natural Sb(V) resistance results from (i) a changed thiol metabolism, possibly resulting in inhibition of Sb(V) activation in amastigotes, and (ii) decreased uptake of the active drug Sb(III) by amastigotes.

Leishmaniasis is a disease complex caused by the protozoan parasites of the genus *Leishmania*. *Leishmania* spp. multiply as obligate intracellular amastigotes in the phagolysosome of vertebrate macrophages and are transmitted by bloodfeeding sand flies in which the parasites proliferate as extracellular promastigotes. The clinical manifestation of leishmaniasis can range from disfiguring cutaneous and mucocutaneous disease to lethal visceral disease, depending on the infecting species and the host’s immune response. The annual incidence worldwide is estimated to be 500,000 for visceral leishmaniasis (VL), of which 90% occurs in the Indian subcontinent, Sudan, and Brazil (7). Control of the disease relies primarily on chemotherapy, and the first-line treatment against all forms of leishmaniasis is pentavalent antimonials [Sb(V)] such as sodium stibogluconate (Pentostam) and meglumine antimonite (Glucantime). Unfortunately, the clinical value of anthomypotherapy is now challenged as an increasing rate of treatment failure is observed in several field sites (24, 30). The most alarming reports come from Bihar in northern India, where more than 60% of the patients do not respond to Sb(V) treatment (29). The geographical and temporal grouping of Sb(V) treatment failures suggests the emergence of anthomycin-resistant strains. Indeed, in Muzaffarpur (Bihar) Sb(V)-resistant *Leishmania* isolates were identified, and a correlation was found between clinical outcome of Sb(V) treatment and in vitro Sb(V) sensitivity of corresponding *L. donovani* isolates (19, 29). Monitoring and prevention of spreading of drug resistance is now a priority for the control of leishmaniasis. Within this context an insight into the mechanism of natural Sb(V) resistance could contribute to the development of efficient strategies for monitoring Sb(V) resistance at sites where it is endemic.

Although antimonials have been in clinical use for 60 years now, there are still some crucial aspects of Sb(V) metabolism in *Leishmania* that remain uncharacterized. It is generally agreed that Sb(V) is a prodrug that needs to be activated to trivalent antimony [Sb(III)] (12). The activation reaction involves a reduction by thiols, but the site of this activation (macrophage or amastigote) and the exact mechanism (enzymatic or nonenzymatic) is still uncertain (10, 25–28). The mechanism of action of the active form, Sb(III), is also poorly understood. A recent study suggested that Sb(III) would perturb the thiol redox potential of the cell (34), but other studies suggested inhibition of energy metabolism and macromolecular biosynthesis (1, 2). The mechanism of antimonial resistance, however, has been studied extensively. It was repeatedly demonstrated that in vitro stepwise induced Sb(III) [or the related metal As(III)] resistance can be associated with an upregulated Sb(III) detoxification pathway, involving overexpression of (i) thiol biosynthetic enzymes (14, 15, 17) and (ii) the Sb(III)/thiol conjugate sequestering pump MRPA (9, 14, 16). A recent study showed that these induced Sb(III)-resistant
strains also have decreased Sb(III) accumulation compared to wild-type strains, which seems to be caused by a lower level of expression of the gene AQP1, coding for the protein responsible for uptake of Sb(III) (13, 20). Other studies on in vitro-induced Sb(V) resistance have described a deficient intracellular reducing activity of Sb(V) to Sb(III) (28). However, it is possible for uptake of Sb(III) (13, 20). Other studies on in vitro-exposure of the gene AQP1, coding for the protein responsible for uptake of Sb(III) (13, 20).

### MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Function or relevance</th>
<th>Sequence</th>
<th>Forward primer and reverse primer</th>
<th>Final concn in quantitative PCR (nM)</th>
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<tr>
<td>GCS</td>
<td>γ-Glutamylcysteine synthetase</td>
<td>Thiol biosynthesis; overexpression linked to Sb(III) resistance (14)</td>
<td>AY371485</td>
<td>5’TTCGCCCTCTGTGCGCTC3’</td>
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<tr>
<td>GS</td>
<td>Glutathione synthetase</td>
<td>Thiol biosynthesis; overexpression linked to Sb(III) resistance (15)</td>
<td>LinJ14.0670</td>
<td>5’TGGAGGTCTCTCCGAGTGA3’</td>
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<td>ODC</td>
<td>Ornithine decarboxylase</td>
<td>Thiol biosynthesis; overexpression linked to Sb(III) resistance (17)</td>
<td>M81192</td>
<td>5’AACCCAGGATGCGATTCGAC3’</td>
<td>500</td>
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<tr>
<td>TR</td>
<td>Trypanothione reductase</td>
<td>Reduction disulfide form trypanothione, possibly inhibited by Sb(III) (34)</td>
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<td>TDR1</td>
<td>Thiold-dependent reductase 1</td>
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<td>LinJ33.0250</td>
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<td>ACR2</td>
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<td>AQP1</td>
<td>Aquaglyceroporin 1</td>
<td>Uptake of Sb(III)(13,20)</td>
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<td>5’AGAAGCGCTCCTACCCAC3’</td>
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<td>MRPA</td>
<td>Multidrug resistance protein A</td>
<td>Sequestration of Sb(III) thiol conjugate, overexpression linked to Sb(III) resistance (9, 14, 16)</td>
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<td>Pentamidine resistance protein 1</td>
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**Isolates and in vitro Sb(V) susceptibility testing. (i) Leishmania donovani isolates.** All strains were isolated from bone marrow aspirates from confirmed visceral leishmaniasis patients before treatment, and they were recruited at the B. P. Koirala Institute of Health Sciences, Dharan, Nepal. Patients corresponding to isolates BP206/0, BP9091, and BP087/0 responded to the subsequent Sb(V) treatment, while isolate BP9010/0 originates from an unresponsive patient. Promastigotes were grown in Tobie’s blood agar medium (32) at 26°C and harvested when log phase was reached. Leishmania species identification was done by cytochrome proteinase b PCR-restriction fragment length polymorphism analysis. Briefly, DNA was extracted with the QiAmp DNA mini kit (QIAGEN), PCR amplified, and digested as reported elsewhere (31). Restriction patterns were resolved using the DNA Labchip kit on a Bioanalyzer 2100 (Agilent Technologies) and compared to patterns of reference strains.

**In vitro Sb(V) sensitivity testing.** Promastigotes were maintained in M199 medium supplemented with 20% heat-inactivated fetal calf serum at 25°C. All strains were tested for their in vitro sensitivity to Sb(V) within seven passages at 37°C in a 5% CO2-air mix. Twenty-four hours after infection, one slide was methanol fixed and Giemsa stained to determine the initial level of infection. If the infection level was higher than 80%, the infected cultures were exposed to sodium stibogluconate [Sb(V) was from (i) GSK, (ii) Albrect David Ltd., and (iii) Viteco over a dose range of 60, 30, 10, and 3 μg/ml. After 5 days, the percentage of infected macrophages in each well was determined. The strain L. donovani MHOM/ET/67/HU3, a World Health Organization reference strain sensitive to sodium stibogluconate and meglumine antimoniate, was included in each assay as a reference. The ratio of the EC50 of treated cultures, the percent inhibition was calculated by sigmoidal regression analysis (Microsoft [MS] office). and 30% effective doses (E30) and E50 were determined. The strain L. donovani MHOM/ET/67/HU3, a World Health Organization reference strain sensitive to sodium stibogluconate and meglumine antimoniate, was included in each assay as a reference. The ratio of the EC50 of treated cultures, the percent inhibition was calculated by sigmoidal regression analysis (Microsoft [MS] office).
promastigotes were from the same passage as the concurrent in vitro sensitivity tests. The adherent, infected macrophages were then washed with ice-cold phosphate-buffered saline (PBS) to remove extracellular promastigotes. Three milliliters of 0.0125% sodium dodecyl sulfate-PBS was added to each flask of infected macrophages and gently agitated. As soon as the macrophages appeared to be lifting and started to disintegrate, 5 ml of ice-cold PBS was added to each flask and the contents were thoroughly mixed using a pastette. A cell scraper was also used to remove any further adherent cells. The contents were aspirated through a 25-gauge by 5/8-inch needle and transferred into a 50-ml centrifuge tube. The liquid was made up to 30 ml with ice-cold PBS and centrifuged at 3,100 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended and washed in ice-cold PBS a further two times. After the final wash, the pellet was resuspended in approximately 1.5 ml PBS, and the amastigotes were counted using a Thoma hemocytometer. The suspension was then transferred to a microcentrifuge tube for the final centrifugation step at 14,000 rpm for 10 min.

RNA isolation and analysis. All parasites cultured for RNA extraction were immediately upon harvest resuspended and disrupted in RNAqueous Lysis/Binding solution (Ambion) containing guanidinium isothiocyanate. The resulting cell lysates were immediately frozen at −80°C until RNA extraction. Total RNA was extracted using the RNAqueous kit (Ambion), and all samples were DNase treated using the DNA-free kit (Ambion) to remove possible contaminating genomic DNA. Quality and quantity of the resulting RNA were determined using the RNA 6000 Nano Labchip kit on the Bioanalyzer 2100 (Agilent Technologies).

cDNA synthesis and real-time quantitative PCR. Total RNA (≥150 ng/reaction) was reverse transcribed at 55°C with Transcripter Reverse Transcriptase (Roche) using conditions recommended by the manufacturer with a 15mer oligo(dT). The resulting cDNA was diluted 10 times, and 2 μl was added to 25 μl quantitative PCRs for expression profiling of nine genes coding for proteins involved in Sb(V) metabolism, referred to as target genes, and of seven additional genes included for normalization purposes, referred to as internal controls (Table 1). A quantitative PCR contained 1× iQ Sybr Green Supermix (Bio-Rad) and forward and reverse primers as specified in Table 1. Reactions were run on an iCycler (Bio-Rad) using the following thermal profile: initial denaturation at 95°C for 5 min followed by 30 cycles with denaturation at 95°C for 30 s, annealing at 60°C for 15 s, and extension at 72°C for 15 s. The PCR was immediately followed by a melt curve analysis using temperature increments of 0.5°C every 30 s to ascertain if the expected product was amplified and to ensure no non-specific products or primer dimers (which could bias the quantification) were formed. The following controls were included in each run for each gene: (i) two serial dilution points of the sample BPK206/0 promastigotes to ascertain consistent PCR efficiency, (ii) negative controls of cDNA synthesis (i.e., without reverse transcriptase), and (iii) no-template controls. All reactions were done in duplicate, with their arithmetic average threshold cycle (CT) used for data analysis.

Analysis of quantitative data and applied statistics. The raw (nonnormalized) expression levels were determined with the delta CT method, more specifically the CT value of a gene for a sample was related to the CT value of the same gene in the sample with the highest expression (or lowest CT value), taking the amplification efficiency of the PCR for that gene into account. The geNorm VBA applet for MS Excel was used to determine the 5 most stable expressed genes from the set of 16 tested genes (Table 1) in a given sample panel and were subsequently used to determine the normalization factor for each sample as described by Vandesompele et al. (33). The relative (normalized) expression levels were obtained by dividing the raw expression levels by the given normalization factor.

 Ninety-five percent confidence intervals (CI) were based on the quantitative data collected from four repeated experiments that included cDNA synthesis and quantitative PCR and were calculated on log-transformed normalized expression levels using the T.INV function in MS Excel to calculate the critical t value (CI = mean log-transformed normalized expression level ± SEM × t, where SEM is standard errors of the means and t is the critical t value of T.INV(0.05 df)) [22].

RESULTS

Expression profiling of target genes and internal control genes. We wanted to verify if any of the following hypothetical mechanisms of Sb(V) resistance was present in our natural Sb(V)-resistant L. donovani strains: (i) decreased uptake of Sb(III), (ii) decreased activation of Sb(V), (iii) decrease in target of Sb(III), or (iv) increased Sb(III) detoxification. All known genes coding for proteins putatively involved in any of these hypothetical mechanisms were thus chosen for gene expression profiling (Table 1). In addition, we chose seven internal control genes which were expected to have a stable expression level in all samples and could as such be used in the normalization process as described in Materials and Methods. Quantitative PCR assays were designed and individually optimized for the nine target genes and seven internal control genes (Table 1) with a specific view to obtain robust and reliable quantification assays. All PCRs had an efficiency above 90% and did not amplify primer dimers or any other nonspecific products.

Gene expression analysis in intracellular amastigotes. The results of the gene expression analysis of all the target genes in intracellular amastigotes are shown in Fig. 1. GCS has a three- to fourfold lower level of expression in the resistant strains than the sensitive strains. For ODC there was also a significantly lower level of expression (two- to threefold) in resistant than in sensitive strains, but the difference is much less pronounced than that for GCS. AQP1 was found to be six- to sevenfold less expressed in resistant strains than in sensitive strains. All the other genes had comparable levels of expression in all tested intracellular amastigotes.

Comparative gene expression analysis in intracellular amastigotes and promastigotes. We also determined the gene expression levels in promastigotes for the three genes that were

![FIG. 1. Relative gene expression levels of target genes GCS, GS, ODC, and TR (A) and TDR1, ACR2, AQP1, MRPA, and PRP1 (B) in intracellular amastigotes for the Sb(V)-sensitive strains BPK206/0 and BPK091/0 (white bars) and the Sb(V)-resistant strains BPK087/0 and BPK190/0 (gray bars) with 95% CIs. (All expression levels were rescaled versus expression levels of BPK190/0.).](http://aac.asm.org/)
shown to be differentially expressed in intracellular amastigotes (GCS, ODC, and AQP1) and the internal control genes. The promastigote data were added to the amastigote data and allowed the determination of relative gene expression levels in both stages in the four different strains. The results for the internal control genes showed the expected patterns for promastigotes and amastigotes, e.g., the data on α-tubulin showed a lower level of expression in amastigotes than in promastigotes in all four strains (data not shown). The resulting relative gene expression levels for GCS, ODC, and AQP1 are shown in Fig. 2. For GCS, a similar tendency of lower levels of expression in resistant strains is present in promastigotes as it is in amastigotes, although the difference is not significant, as the 95% CIs overlap. For ODC a different picture arises; the difference in gene expression levels we observed in sensitive and resistant amastigotes is absent in promastigotes. However, there is a significantly different expression level between promastigotes and amastigotes in Sb(V)-resistant strains which is absent in the two Sb(V)-sensitive strains. The last gene, AQP1, shows a profile similar to that of GCS. The significantly lower level of expression in resistant compared to sensitive intracellular amastigotes is also present in promastigotes, again less pronounced but still significant. Furthermore, it is clear that in sensitive parasites, AQP1 is upregulated when transforming from promastigotes to amastigotes; this regulation is absent in resistant strains as both life stages have similar low expression levels.

**DISCUSSION**

Differential gene expression profiles in natural Sb(V)-resistant and -sensitive *Leishmania donovani* strains were demonstrated for the following three genes: (i) GCS, coding for the enzyme that catalyzes the rate-limiting step in the synthesis of glutathione (thiol moiety of trypanothione); (ii) ODC, coding for the enzyme that catalyzes the rate-limiting step of the synthesis of the spermidine moiety of trypanothione; and (iii) AQP1, coding for the protein responsible for the uptake of Sb(III). In intracellular amastigotes, all three genes had a lower level of expression in Sb(V)-resistant strains than in sensitive strains. In promastigotes, however, the differences between resistant and sensitive strains were much less pronounced for GCS and AQP1 and were absent for ODC, which emphasizes the need to study Sb(V) resistance in intracellular amastigotes. The combined data from promastigotes and intracellular amastigotes shed light on how gene expression has changed in Sb(V)-resistant strains compared to that of Sb(V)-sensitive strains. For AQP1, and maybe also for GCS, we observed a higher level of expression in amastigotes than promastigotes in sensitive strains, while in resistant strains both life stages have the same expression level. Furthermore, the expression levels in resistant strains are significantly lower than those in the sensitive strains. Altogether, the data imply that there is a constitutive change in the resistant strains disrupting the regulation of gene expression. In contrast, the differential expression of ODC seems to be intracellular amastigote specific and could, as such, be the result either of a regulatory effect due to the changed GCS expression or to an amastigote-specific constitutive change.

The mechanism of antimonial resistance in *Leishmania* spp. has already been studied extensively during the past 10 years using in vitro resistant strains as models. The in vitro resistant strains were obtained by stepwise exposure to either Sb(III) or As(III). It was repeatedly shown that the mechanism of in vitro Sb(III) and As(III) resistance is associated with an upregulated thiol biosynthesis marked by increased GCS, GS, and ODC expression levels and concomitant increased intracellular thiol concentrations compared to those of the wild-type strains (14–18, 23). In this study, we focused on natural Sb(V)-resistant strains, which evolved by exposure to Sb(V) in the form of host treatment. The combined data from GCS and ODC expression...
levels suggest that in natural Sb(V)-resistant strains, the thiol biosynthesis is downregulated compared to that of Sb(V)-sensitive strains, although this still needs to be confirmed on the level of intracellular thiol concentrations. This difference between Sb(III) resistance (in vitro models) and Sb(V) resistance (natural resistance) reflects the dual role of thiols in antimony metabolism. On the one hand, thiols promote resistance to Sb(III) due to their role in the Sb(III) detoxification pathway (8, 23, 34), while on the other hand thiols sensitize amastigotes to Sb(V) as they promote the activation of the produg Sb(V) (6, 11, 12, 35, 36). This aspect of antimony/thiol metabolism highlights the importance of distinguishing between resistance provoked by exposure to Sb(III) and Sb(V). When Leishmania spp. are exposed in vitro to Sb(III) pressure, the most obvious putative mechanisms of resistance are decreasing Sb(III) uptake or increasing Sb(III) detoxification, and both have indeed been demonstrated in several species (9, 14, 16, 18, 20). However, upon exposure to Sb(V) (treatment) pressure, there is an extra putative resistance mechanism that is inhibiting the activation of Sb(V). Inhibition of Sb(V) activation is a more attractive and economic option than upregulation of Sb(III) detoxification pathways for an amastigote in natural circumstances, as (i) it requires no upregulation of gene expression, for which in vivo amastigotes might not have the metabolic flexibility, (ii) it avoids exposure to the active form Sb(III), and (iii) it requires no extra energy cost to sequester or extrude Sb(III) by ABC transporters. The results of this study support this hypothesis, as natural Sb(V)-resistant parasites seem to have the capacity to inhibit both enzymatical (decreased GCS/glutathione and thiol used by both TDR1 and ACR2) and nonenzymatical (decreased GCS and ODC/trypanothione) activation of Sb(V). This mechanism of decreased activation of Sb(V) in response to Sb(V) pressure was also described for Pentostam-induced resistant axenic L. donovani amastigotes (28). Furthermore, the lower expression levels of AQP1 in resistant compared to sensitive strains suggest that natural Sb(V) resistance is also associated with a decreased uptake of Sb(III). A similar phenomenon was recently described for Sb(III)-resistant strains, and this obviously protects the parasite against the direct applied Sb(III) pressure (20). However, in natural Sb(V)-resistant strains, a decreased uptake of Sb(III) would protect the intracellular amastigote against Sb(III) emanating from Sb(V) activation in the macrophage.

In summary, we hypothesize that the mechanism of natural Sb(V) resistance is multifactorial, involving a changed thiol metabolism leading to inhibition of the activation of Sb(V) inside the amastigote and a decreased uptake of Sb(III) resulting from Sb(V) activation in the macrophage (Fig. 3). As regulation of gene expression in Leishmania has been found so far to occur at the posttranscriptional level (4), it is as yet uncertain whether the differential RNA levels observed for the three genes are translated into differential protein levels. Therefore, further studies are required on protein and metabolic levels to confirm the proposed model for the Sb(V) resistance mechanism.

Sb(V)-resistant strains are emerging throughout the Indian subcontinent, and a new generation of molecular-based drug susceptibility tests are urgently needed to set up feasible and efficient monitoring strategies. As discussed above, our results imply that natural Sb(V) resistance is associated with a constitutive change and as such offer the first leads to start the search for genetic markers of natural Sb(V) resistance that can be used for the development of such a molecular test.

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