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Novel Intracellular Sb^{V} Reducing Activity Correlates with Antimony Susceptibility in *Leishmania donovani**

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The standard treatment of human visceral leishmaniasis involves the use of pentavalent antimony (Sb^{V}). Its mechanism of action is unknown because of the limited information available about intracellular antimony metabolism and about the genes that regulate these processes. Herein, flow injection-inductively coupled plasma mass spectrometry (ICP-MS), flow injection hydride generation ICP-MS, and ion chromatography ICP-MS were used to measure antimony accumulation and intracellular metabolism in the human protozoan parasite *Leishmania donovani*. Amastigotes (the intracellular form) and promastigotes (the extracellular form) accumulate Sb^{V} and Sb^{III} via separate transport systems. Stage-specific intracellular Sb^{V} reducing activity was apparent in amastigotes, which reduced the negligibly toxic Sb^{V} to highly toxic Sb^{III}. This amastigote-specific reducing activity was deficient in the Pentostam-resistant mutant *L. donovani* Ld1S.20. These data indicate that parasite susceptibility to Sb^{V} correlates with its level of Sb^{V} reducing activity. Also, in promastigotes of both wild-type *L. donovani* and the Pentostam-resistant mutant *L. donovani* Ld1S.20, Sb^{V} inhibited the toxicity of Sb^{III} but not of As^{III}. Both Sb^{V} and Sb^{III} were toxic to wild-type amastigotes. However, as observed in promastigotes, in mutant amastigotes Sb^{V} inhibits Sb^{III} but not As^{III} activity. Anion exchange chromatography showed that intracellular antimony metabolism occurred in both promastigotes and amastigotes. These data demonstrate that the interaction between the two antimony oxidation states occurs intracellularly, within the parasite. The results also indicate that Sb^{V} anti-leishmanial activity is dependent on its reduction to Sb^{III}. The mechanism of this novel intracellular Sb^{V} reduction has yet to be identified, and it may or may not be enzymatic. This is the first description of intracellular Sb^{V} reducing activity in *Leishmania* as well as in any prokaryotic or eukaryotic cell.

Leishmania donovani is the major causative agent of visceral leishmaniasis. In nature, the parasite exists either as an extracellular promastigote found in the alimentary tract of sandflies or as an obligatory intracellular amastigote found in phagolysosomes of mammalian macrophages(1–3). During the last several years, a number of laboratories have utilized axenic culture of *L. donovani* amastigotes for the direct evaluation of cell biological and biochemical processes in the amastigote, devoid of the host macrophage (4–7,8,9,10). This technique has also been used to investigate the mechanism of drug action and resistance as well as for screening of potential new drugs(11–13). The treatment of choice of human visceral leishmaniasis is the administration of pentavalent antimony (Sb^{V})-containing drugs such as sodium stibogluconate (Pentostam, Wellcome, Beckenham, UK) or meglumine antimoniate (Glucantime, Rhone-Poulenc, Paris, France). Despite extensive use of these compounds over the last decades, the mechanism of action remains unclear.

It has been hypothesized that Sb^{V} is not toxic to *Leishmania*, but rather that it is enzymatically reduced, probably by the host macrophage, to Sb^{III}, the form of antimony that is highly *Leishmania*-toxic (14–18). In contrast, it has been shown that Sb^{V} is directly toxic to axenic amastigotes (12, 13, 19), thus negating the necessity of the macrophage for Sb^{V} reduction. Furthermore, these data imply that either the parasite reduces Sb^{V} to Sb^{III} intracellularly, and thus Sb^{III} is directly toxic to amastigotes, or that both oxidation states of antimony are active against *Leishmania* amastigotes. The modes of action of the two oxidation states of antimony (Sb^{III} and Sb^{V}) on *Leishmania* are, as yet, not fully understood.

Several groups have shown obligatory cross-resistance between Sb^{V}, Sb^{III}, and arsenite (As^{III}) in *Leishmania tarentolae*, *Leishmania major*, *Leishmania mexicana, L. donovani* and *Leishmania infantum* (11, 20–22). In contrast, it has been suggested that, at least in *L. donovani,* such cross-resistance does not necessarily occur (12). Furthermore, it has been demonstrated that, when bound to organic compounds, structural similarities exist between Sb^{III} and Sb^{V} (23). For example, the trivalent antimony compound potassium antimonyl tartrate has a structure resembling that of the pentavalent antimony compound sodium stibogluconate. Thus it is conceivable that Sb^{V} and Sb^{III} act on a common parasite target molecule. It is also possible that, when given together, these compounds might act additively or synergistically, or might inhibit one another. It is also possible that at least part of the anti-leishmanial activity of As^{III} may not be mediated by a mechanism similar to that of antimony despite the fact that cross-resistance in other *Leishmania* species has been observed (11,20–22).

To understand the relationships between these compounds and their activity against *L. donovani,* antimony accumulation and its subsequent intracellular metabolism were investigated using novel antimony speciation methods.

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EXPERIMENTAL PROCEDURES

Materials

Sodium stibogluconate (powder) was a gift from The Wellcome Trust (Beckenham, UK). Potassium antimonyl tartrate was obtained from Sigma Chemical Co. (St. Louis, MO.). [^{3}H]Thymidine was obtained from PerkinElmer Life Sciences. Medium 199 and fetal calf serum were obtained from Biological Industries, Inc. (Bet Haemeek, Israel). Materials for antimony determinations were as follows: sodium tetrahydridoborate (puriss. p.a. for determination of hydride formers) and potassium fluoride (MicroSelect; >99.5% purity) were obtained from Fluka, Inc. (Seelze, Germany), potassium iodide (puriss, p.a.; >99% purity) was obtained from Sigma-Aldrich, Inc. (Deisenhofen, Germany). All other chemicals were of analytical grade.

Parasites

A cloned line of *L.donovani* 1SR (5,24) and the Pentostam-resistant mutant *L. donovani* Ld1S.20 (13) were used.

Culture

Promastigotes were grown in medium 199 at 26 °C and supplemented with 10% fetal calf serum. *In vitro* culture of amastigotes was performed as described by Saar *et al.* (5). Transformation of amastigotes to promastigotes was performed by centrifugation of amastigotes (1,200 × *g*, at room temperature for 10 min), suspension in promastigote medium, and incubation at 26 °C. Under these conditions, amastigotes differentiate to promastigotes within 48 h (5).

Dose Response Analyses

Parasite viability was measured using a radiolabeled thymidine incorporation assay as follows: mid-log phase cells (~5 × 10^5 cells/ml) were added in duplicate to 24-well flat-bottomed microtiter plates (2-ml final volume). Drugs were serially added to the cells (either promastigotes or amastigotes), and cells were then incubated for 48 h at either 26 °C (promastigotes) or 37 °C in 5% CO_2 (amastigotes). Subsequently, 10 μl of [^{3}H]thymidine (0.1 mCi/ml) were added to each well and incubated for another 3 h (promastigotes) or 24 h (amastigotes). 1-ml aliquots from each well were centrifuged (1,200 × *g* at room temperature for 10 min) and added to 5 ml of ice-cold trichloroacetic acid, vortexed, and incubated on ice for about 20 min. Samples were then filtered through a glass microfiber filter (GF/C, Whatman International Ltd., Springfield Mill, UK) and washed once with 10 ml of cold trichloroacetic acid followed by 10 ml of 95% ethanol. Radioactivity was measured using β-scintillation counting. Results were expressed as percentage viability.

Determination of Intracellular Antimony

Cell Preparation—L. donovani promastigotes and amastigotes were incubated for 3, 6, or 12 h with potassium antimonyl tartrate (34 μg of Sb^{III}/ml, 0.28 mM Sb^{III}), sodium stibogluconate (1 mg of Sb^V/ml, 8.2 mM Sb^V), or both. Subsequently, cells were washed twice with ice-cold phosphate-buffered saline and then extracted with concentrated nitric acid.

Determination of Total Antimony—The content of antimony in each sample was measured using flow injection-inductively coupled plasma-mass spectrometry (FI-ICP-MS)[1] at *m/z* 121 using water as solvent stream (flow rate was 1.5 ml/min, *n* = 5) under clean room conditions (class 1000). Each sample contained 100-μl aliquots of cell extract, which were diluted 1:100 with water (25, 26). For each measurement, aliquots of 10 μl were injected with five repetitions.

Determination of Sb^{III} and Sb^V—Intracellular Sb^{III} and Sb^V were measured using flow injection-hydride generation-inductively coupled plasma-mass spectrometry (FI-HG-ICP-MS), which has recently been developed (27). Briefly, Sb^{III} was reduced to antimony hydride (SbH_3) using 0.2% sodium tetrahydridoborate in de-ionized water and was directly quantified using mass spectrometry at *m/z* 121. To avoid Sb^V interference with the antimony hydride measurements, the Sb^V reduction was suppressed by the addition of 100 mg/liter of fluoride (as KF) to the solvent stream. Subsequently, Sb^V was reduced to antimony hydride in 2 steps; pre-reduction using 1.2% KI followed by reduction with 0.2% sodium tetrahydridoborate, both in de-ionized water as solvent stream. The Sb^V content in each sample was calculated as the difference between the absorbance determined after the second reduction (Sb^{III} + Sb^V) and first reduction (Sb^{III}). Nonreducible organic antimony is not detected by this method. For each measurement, aliquots of 100 μl were injected with five repetitions.

Anion Exchange Chromatography of Intracellular Antimony—Chromatographic separation of intracellular antimony was done as described by Ulrich *et al.* (28). Briefly, cell extracts were diluted 1:100 with distilled water, and 100-μl aliquots from each preparation were injected into a laboratory-packed PRP-X100 HPLC column. Sb^V and Sb^{III} were eluted from the column using 15 mM nitric acid and were directly injected to ICP-MS (*m/z* 121) for antimony absorbance. Each sample was filtered and degassed prior to chromatography.

[1] The abbreviations used are: FI-ICP-MS, flow injection-inductively coupled plasma mass-spectrometry; FI-HG-ICP-MS, flow injection-hydride generation-inductively coupled plasma-mass spectrometry; IC-ICP-MS, ion-chromatography-inductively coupled plasma-mass-spectrometry; WT, wild type; HPLC, high performance liquid chromatography; LD, lethal dose.

TABLE I
Accumulation of antimony by L. donovani promastigotes

Promastigotes were incubated with potassium antimonyl tartrate (Sb^{III}), sodium stibogluconate (Sb^V) or both for 12 h at 26 °C. Cell extractions and intracellular antimony concentration determinations using FI-ICP-MS (total $[Sb]_i$ column) and FI-HG-ICP-MS ($[Sb^{III}]_i$ and $[Sb^V]_i$ columns) were performed as described under "Experimental Procedures."

Parasites	Extracellular antimony	Intracellular antimony *ng Sb/1 × 10^6 cells*		
		Total $[Sb]_i$	$[Sb^{III}]_i$	$[Sb^V]_i$
Ld1SR (WT)	Sb^{III}[a]	50 ± 2	26 ± 2	3 ± 1
Ld1SR	Sb^V[b]	98 ± 4	1.5 ± 0.3	74 ± 4
Ld1SR	Sb^{III} + Sb^V	183 ± 12	29 ± 2	127 ± 5
Ld1SR	None	0.6 ± 0.1		
Ld1S.20 (mutant)	Sb^{III}	43 ± 2	24 ± 2	1.8 ± 0.2
Ld1S.20	Sb^V	87 ± 4	1.3 ± 0.2	67 ± 3
Ld1S.20	Sb^{III} + Sb^V	143 ± 6	25 ± 2	106 ± 5
Ld1S.20	None	1.0 ± 0.2		

[a] 34 μg of Sb^{III}/ml, corresponding to 0.28 mM Sb^{III}.
[b] 1 mg of Sb^V/ml, corresponding to 8.2 mM Sb^V.

RESULTS

Antimony accumulation in promastigotes and amastigotes of both the wild-type and the mutant *L. donovani* Ld1S.20 was determined using two different methods. First, total intracellular antimony content was measured with FI-ICP-MS (25, 26), and the results are summarized under "Total $[Sb]_i$" in Tables 1 and 2. The second method used to determine the content of antimony hydride (SbH_3) was FI-HG-ICP-MS (27, 28). In this method, Sb^V and Sb^{III} in cell extracts were separately reduced to SbH_3, which then flowed through the plasma to the mass spectrometer, where its concentration was subsequently determined (Tables 1 and 2, $[Sb^V]_i$ and $[Sb^{III}]_i$). This method enables the determination of the intracellular content of each of the antimony oxidation states.

As shown in Table 1 (Total $[Sb]_i$), in WT promastigotes, Sb^{III} or Sb^V each accumulated intracellularly after a 12-h incubation. Furthermore, when WT promastigotes were simultaneously incubated with both Sb^V and Sb^{III}, the total [Sb] measured intracellularly approximated the sum of the intracellular concentrations of the two antimony oxidation states when incubations were with Sb^V or Sb^{III} alone. The same was true with promastigotes of the mutant *L. donovani* Ld1S.20.

With a similar set of experiments using WT and mutant amastigotes, the data in Table 2 (Total $[Sb]_i$) show that both Sb^{III} and Sb^V were accumulated intracellularly. Sb^V accumulation was concentration-dependent, suggesting that its transport may be mediated by a permease. When compared with promastigotes, the transport of Sb^V into WT amastigotes is 6.7- and 4.9-fold greater, when incubation was with Sb^V alone or with both oxidation states concomitantly, respectively. Sb^{III} accumulation was similar in promastigotes and amastigotes of both WT and mutant *L. donovani* Ld1S.20. The change in Sb^V accumulation in mutant amastigotes resembles that of WT amastigotes although to a lesser extent, with Sb^V accumulation increasing by only 4.9-fold.

TABLE II
Accumulation of antimony by L. donovani amastigotes

Amastigotes were incubated with potassium antimonyl tartrate (Sb^{III}), sodium stibogluconate (Sb^V) or both for 12 h at 37 °C in 5% CO_2. Cell extractions and intracellular antimony concentration determinations using FI-ICP-MS (total $[Sb]_i$ column) and FI-HG-ICP-MS ($[Sb^{III}]_i$ and $[Sb^V]_i$ columns) were performed as described under "Experimental Procedures."

Parasites	Extracellular antimony	Intracellular antimony *ng Sb/1 × 10⁶ cells*		
		Total $[Sb]_i$	$[Sb^{III}]_i$	$[Sb^V]_i$
Ld1SR (WT)	Sb^{III} (34 μg/ml)	48 ± 2	42 ± 3	2.0 ± 0.5
Ld1SR	Sb^V (0.2 mg/ml)	187 ± 8	55 ± 4.0	103 ± 6
Ld1SR	Sb^V (1 mg/ml)	653 ± 40	118 ± 8	376 ± 15
Ld1SR	Sb^{IIIa} + Sb^{Vb}	890 ± 57	179 ± 12	638 ± 10
Ld1SR	None	1.5 ± 0.4		
Ld1S.20 (mutant)	Sb^{III} (34 μg/ml)	56 ± 3	45 ± 3	1.5 ± 0.5
Ld1S.20	Sb^V (200 μg/ml)	59 ± 3	2.5 ± 0.8	49 ± 3
Ld1S.20	Sb^V (1 mg/ml)	407 ± 18	2.9 ± 0.8	318 ± 16
Ld1S.20	Sb^{IIIa} + Sb^{Vb}	548 ± 39	15 ± 1	445 ± 22
Ld1S.20	None	1.7 ± 0.2		

[a] 34 μg of Sb^{III}/ml, corresponding to 0.28 mM Sb^{III}.
[b] 1 mg of Sb^V/ml, corresponding to 8.2 mM Sb^V.

To determine the intracellular fate of antimony in both promastigotes and amastigotes, cell extracts of parasites treated with Sb^V, Sb^{III}, or both, were analyzed for each of the antimony oxidation states using FI-HG-ICP-MS. The Sb^V concentrations used were those that in competition experiments (Figs. 2 and 3) resulted in 10 and 100% inhibition of Sb^{III} toxicity by Sb^V.

In WT promastigotes incubated with either Sb^{III} or Sb^V alone, only the specific oxidation state added to the extracellular medium was found intracellularly (Table 1, $[Sb^{III}]_i$ and $[Sb^V]_i$). Most of the accumulated Sb^V (76%) was identified as intracellular Sb^V, but only 52% of the accumulated Sb^{III} was recovered by FI-HG-ICP-MS as Sb^{III}. When the extracellular medium contained Sb^{III} and Sb^V, both these forms were found intracellularly as well with similar Sb^{III} concentrations. Sb^V accumulated at a 71% higher concentration when parasites were incubated with Sb^V and Sb^{III} (*versus* with Sb^V alone). Similar results were obtained when these experiments were performed using mutant *L. donovani* Ld1S.20 promastigotes.

In contrast to the above findings, when amastigotes of WT parasites were subjected to the same conditions (Table 2), both Sb^V and Sb^{III} were detected intracellularly when parasites were incubated with only Sb^V. Thus, reduction of Sb^V to Sb^{III} was observed in WT amastigotes incubated with Sb^V only. When incubated for 12 h with 0.2 mg of Sb^V/ml, WT amastigotes reduced 29% of the accumulated antimony to Sb^{III}; 18% was reduced when incubation was with 1 mg of Sb^V/ml. This may indicate that the latter concentration is at saturation for enzymatic Sb^V reduction. In contrast, only minor levels of reduction of Sb^V to Sb^{III} in mutant amastigotes occurred. Furthermore, the high level of Sb^{III} accumulation by the mutant amastigotes (Table 2) rules out a possible role for active Sb^{III} efflux in Ld1S.20 resistance to pentavalent antimony.

The results in Tables 1 and 2 indicate that Sb^V is stable. No spontaneous reduction of Sb^V to Sb^{III} was observed during the 12-h assays. Only 1% reduction was observed after a few weeks (not shown). This is in agreement with previous observations that Sb^V is stable in aqueous solutions even though the redox potential favors Sb^{III} (29). Hence, it is likely that rapid reduction of Sb^V requires intracellular catalytic activity of either an enzymatic or nonenzymatic nature.

To further delineate the nature of the intracellular reduction of Sb^V, $[Sb^{III}]_i$ was determined after 3-, 6-, and 12-h incubations of both WT and mutant promastigotes and amastigotes with Sb^V alone. As shown in Fig. 1, WT amastigotes showed a time-dependent reduction of Sb^V whereas mutant amastigotes exhibited less than 0.025 of the reducing activity. Promastigotes, both WT and mutants, did not reduce Sb^V to any extent.

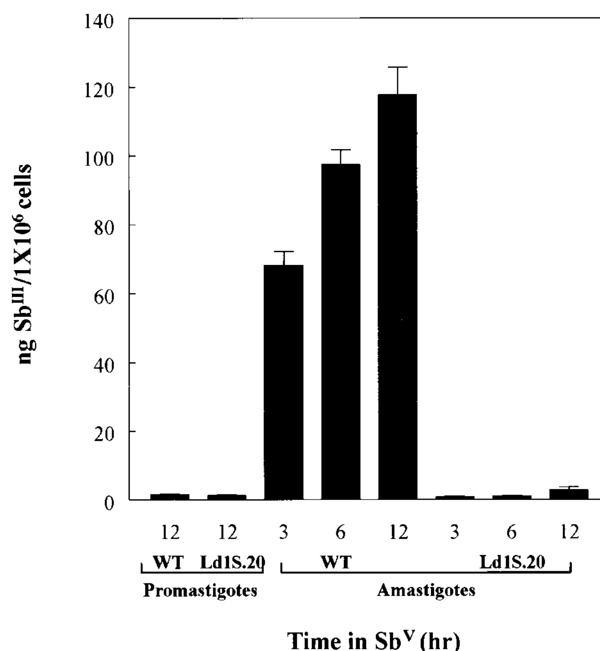

FIG. 1. **Sb^V reduction by *L. donovani*.** Promastigotes of both WT and mutant *L. donovani* Ld1S.20 were incubated for 12 h and amastigotes for either 3, 6, or 12 h with 1 mg of Sb^V/ml sodium stibogluconate. Cells were then extracted and analyzed for Sb^{III} content as in Tables 1 and 2. The results are expressed as mean ± S.D. (n = 5).

The results in Fig. 1 and Tables 1 and 2 indicate that Sb^V reducing activity correlates with the previously documented stage-specific susceptibility of WT amastigotes to pentavalent antimony(12). However, in amastigotes, the intracellular concentration of Sb^V was 5-fold higher than in promastigotes, suggesting that it may also be toxic to amastigotes. To assess the specific role of Sb^V in the anti *L. donovani* activity of antimony, the intracellular relationship between Sb^V and Sb^{III} was studied with competition assays.

Dose response analyses were performed by incubating wild-type *L. donovani* 1SR with variable concentrations of sodium stibogluconate (Sb^V) and a fixed potassium antimonyl tartrate (Sb^{III}) concentration equivalent to the LD_{90} for WT *L. donovani* promastigotes (100 μg/ml = 34 μg of Sb^{III}/ml). As shown in Fig. 2*A*, $[Sb^V]$ of ≥ 200 μg/ml neutralized the toxicity of Sb^{III}, resulting in no mortality at 1 mg/ml Sb^V. Also, the minimal Sb^V concentration required for reversion of Sb^{III} toxicity declined with decreasing Sb^{III} concentration (not shown). A similar relationship between Sb^V and Sb^{III} was also shown with promastigotes from the Pentostam-resistant mutant *L. donovani* Ld1S.20 (Fig. 2*B*).

In contrast, Sb^V failed to reverse arsenite (As^{III}) toxicity to *L. donovani* even at Sb^V concentrations as high as 5 mg/ml (Fig. 2, *A* and *B*). These data emphasize the specificity of the Sb^V-Sb^{III} interaction in *L. donovani* promastigotes, both WT and mutant.

For wild-type amastigotes, the dose-response curve for Sb^V in the presence of a fixed low dose of Sb^{III} (3.4 μg of Sb^{III}/ml, equivalent to the LD_{20}) showed additive toxicity of Sb^V and Sb^{III} (Fig. 3*A*). In the presence of 34 μg of Sb^{III}/ml (equivalent to the LD_{90}) no inhibition of Sb^{III} toxicity by Sb^V was observed. This is in agreement with the previously reported stage-specific susceptibility of *L. donovani* to Sb^V (12).

When the same dose-response analysis was performed using amastigotes of the *L. donovani* mutant Ld1S.20, Sb^V inhibited Sb^{III} toxicity in a fashion similar to that observed in promastigotes. As shown in Fig. 3*B*, $[Sb^V]$ ≥ 200 μg/ml reduced mor-

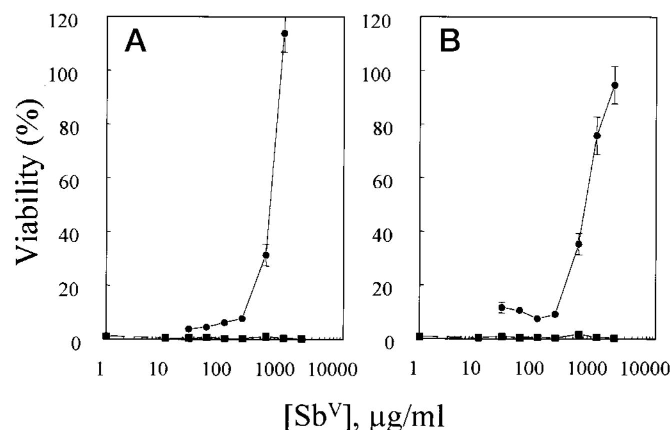

FIG. 2. **Effect of sodium stibogluconate (Sb^V) on the toxicity of potassium antimonyl tartrate (Sb^{III}) or arsenite (As^{III}) on *L. donovani* promastigotes.** *A,* wild-type (Ld1SR), *B,* Pentostam-resistant mutant (*L. donovani* Ld1S.20). Promastigotes were pretreated with 100 μg/ml potassium antimonyl tartrate ( ), or 8 μg/ml arsenite (■). Viability was determined using [^{3}H]thymidine incorporation. The results are expressed as mean ±S.D. (n = 3).

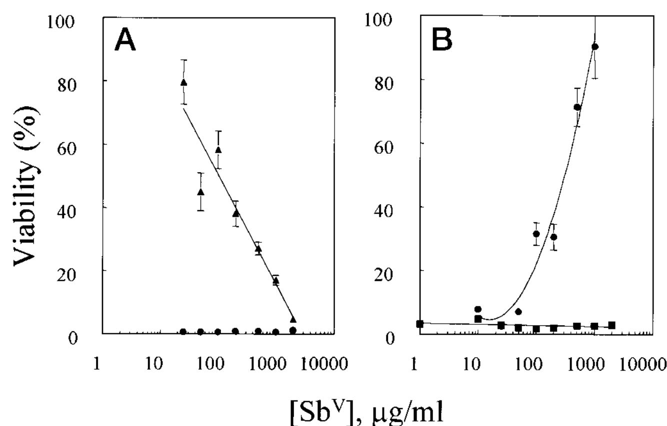

FIG. 3. **Effect of sodium stibogluconate (Sb^V) on the toxicity of potassium antimonyl tartrate (Sb^{III}) or arsenite (As^{III}) on *L. donovani* amastigotes.** *A*, additive toxicity of Sb^V (sodium stibogluconate) and Sb^{III} (potassium antimonyl tartrate) on wild-type *L. donovani* amastigotes. Cells were pretreated with either 10 μg/ml potassium antimonyl tartrate (▲, 3.4 μg/ml Sb^{III}) or 100 μg/ml potassium antimonyl tartrate (, 34 μg/ml Sb^{III}). Viability was determined as described under "Experimental Procedures." *B,* effect of sodium stibogluconate on the toxicity of potassium antimonyl tartrate and arsenite on amastigotes of the *L. donovani* mutant Ld1S.20. Amastigotes were pretreated with either 100 μg/ml potassium antimonyl tartrate (, 35 g/ml Sb^{III}) or 1 μg/ml arsenite (■). Viability was determined using [^{3}H]thymidine incorporation. The results are expressed as mean ± S.D. (n = 3).

tality caused by high dose Sb^{III} (34 μg/ml Sb^{III}), and at about 1 mg of Sb^V/ml no mortality was observed. When these mutant amastigotes were treated with As^{III} instead of Sb^{III}, Sb^V failed to reverse As^{III} toxicity, further emphasizing the specificity of the Sb^V-Sb^{III} competition.

Differences in antimony intracellular accumulation and reducing activity were observed between promastigotes and amastigotes and between WT and mutant parasites. Furthermore, often total $[Sb]_i$ was greater than $[Sb^V + Sb^{III}]_i$ (Tables 1 and 2). Therefore, to ascertain whether antimony metabolism other than oxidation or reduction occurs intracellularly, cell extracts from the above experiments were subjected to anion exchange chromatography, and fractions eluted from the column were directly subjected to ICP-MS.

When a parasite-free solution containing both sodium stibogluconate and potassium antimonyl tartrate was subjected to HPLC anion exchange chromatography, two distinct peaks (Sb^V and Sb^{III}), were identified. Sb^V and Sb^{III} retention times were 100 and 300 s, respectively (not shown). Fig. 4 shows chromatograms of WT and mutant amastigotes and promastigotes incubated with Sb^V. As shown, when amastigotes were incubated with Sb^V, the Sb^{III} peak was absent in the mutant but not in WT. This correlates with the lack of Sb^V reducing activity observed in the mutant amastigotes (Fig. 1). In both WT and mutant amastigotes, peaks other than free Sb^V and Sb^{III} were detected. Differences in peaks A through F were apparent between WT and mutant. These peaks might represent covalent interactions of either Sb^V or Sb^{III} with intracellular molecules.

No major differences were observed between WT and mutant promastigotes when incubations were performed with Sb^V (Fig. 4, *C* and *D*). Despite the qualitative nature of the chromatographic results, the size of both the promastigote Sb^{III} peaks (WT and mutant) was smaller than in WT amastigotes, further emphasizing the low level of Sb^V reducing activity in promastigotes (Fig. 1).

DISCUSSION

Bacteria and yeast that live in environments contaminated with arsenate (As^V) reduce the element intracellularly to arsenite (As^{III}) as part of the mechanism that was evolved to evade the toxic effects of this heavy metal (30). Given that *Leishmania* spp. are not necessarily exposed to heavy metals in their natural habitats (sandflies and vertebrates), they may not have developed this type of protective mechanism. In fact, by reducing nontoxic Sb^V *Leishmania*, they actually expose themselves to the lethal effects of Sb^{III}. This work aimed to assess the role of Sb^V reduction in antimony toxicity to and resistance in *L. donovani*.

The data presented herein show the following. 1) Intracellular stage-specific Sb^V reduction occurs in WT *L. donovani* 1SR and susceptibility to Sb^V correlates with the level of Sb^V reducing activity. 2) The Sb^V-resistant mutant amastigote of *L. donovani* Ld1S.20 is deficient in Sb^V intracellular reducing activity. 3) Antimony metabolism with stage-specific differences occurs in both promastigotes and amastigotes of WT *L. donovani* 1SR and 4) stage-specific intracellular competition between Sb^{III} and Sb^V, but not As^{III}, occurs in WT *L. donovani* 1SR. This competition is not related to antimony transport.

The results of the experiments described indicate that Sb^V reverses the toxicity of Sb^{III} to *L. donovani* promastigotes, in both the WT and the mutant *L. donovani* Ld1S.20. In addition, Sb^V and Sb^{III} are toxic in an additive fashion to WT amastigotes. These data support previous findings that described the stage-specific toxicity of Sb^V, but not of Sb^{III}, to *L. donovani* (12). In contrast, in mutant *L. donovani* Ld1S.20 amastigotes, a response similar to promastigotes was observed (inhibition).

In both wild-type and mutant promastigotes and amastigotes, no cross-inhibition by Sb^V occurs when As^{III} is substituted for Sb^{III}. This suggests that, despite similar activity in other biological systems (30, 31), in *L. donovani*, antimony and arsenic may not act via similar mechanisms.

FI-HG-ICP-MS and FI-ICP-MS data show that both antimony oxidation states, either individually or together, enter promastigotes and axenic amastigotes. The specific quantitative results for combined Sb^{III} and Sb^V incubation rule out the possibility that competition occurs at the plasma membrane transport level. This is true for both promastigotes and amastigotes of both WT and mutant *L. donovani* Ld1S.20 parasites.

Because Sb^V-Sb^{III} antagonism does not take place at the transport level, the assumption that it transpires intracellularly has been borne out. Stage-specific intracellular *L. donovani* Sb^V reducing activity exists in WT amastigotes, which

FIG. 4. **Anion exchange chromatography of antimony in *L. donovani.*** WT and mutants were treated for 6 h with 1 mg of Sb^V/ml sodium stibogluconate, and then extracted as described under "Experimental Procedures," subjected to a PRP-X100 HPLC column, and subsequently eluted using 15 mM nitric acid. The eluted samples were injected directly to ICP-MS. *A* and *B,* WT and mutant amastigotes, respectively; *C* and *D,* WT and mutant promastigotes.

reduces negligibly toxic Sb^V to highly toxic Sb^{III}. In WT promastigotes, the putative *L. donovani* stage-specific antimony reducing activity may be expressed at either a low level or may be of very low activity, thus explaining the negligible susceptibility of these promastigotes to Sb^V.

In the mutant *L. donovani* Ld1S.20 amastigote Sb^V reducing activity is present either in small amounts or may be nonexistent; thus Sb^V accumulates intracellularly but is not reduced to Sb^{III}. Consequently mutant amastigotes are less susceptible than WT to Sb^V. The data presented in Fig. 1 indicate that in WT amastigotes, stage-specific Sb^V reducing activity occurs, whereas in the mutant amastigote, Sb^V reduction is less than 0.025 that of WT, thus Sb^V accumulates intracellularly but is not reduced to Sb^{III}. Furthermore, the observation that both WT and mutant promastigotes and amastigotes accumulate Sb^{III} to the same level (Tables 1 and 2) rules out a possible role for antimonite efflux as the reason for low level of Sb^{III} reduction as well as for Ld1S.20 Sb^V resistance. The Sb^V reducing activity of *L. donovani* might resemble that of the arsenate reductase previously described in bacteria and yeast, which catalyzes reduction of As^V to As^{III} (30, 32, 33). It is possible that an *L. donovani* enzyme that catalyzes Sb^V reduction plays a key role in parasite susceptibility to antimony. This possibility is currently being investigated in our laboratory.

In WT amastigotes, despite Sb^V reduction, $[Sb^V]_i$ remains relatively high and theoretically should have inhibited the intracellular Sb^{III} present (Table 2), as seen in both WT and mutant promastigotes as well as in mutant amastigotes. However, the data presented in Fig. 3*A* show that this does not occur. This might suggest that Sb^V is directly toxic to WT amastigotes. If this is the case, it must be assumed that Sb^V toxicity is coupled with Sb^V reducing activity.

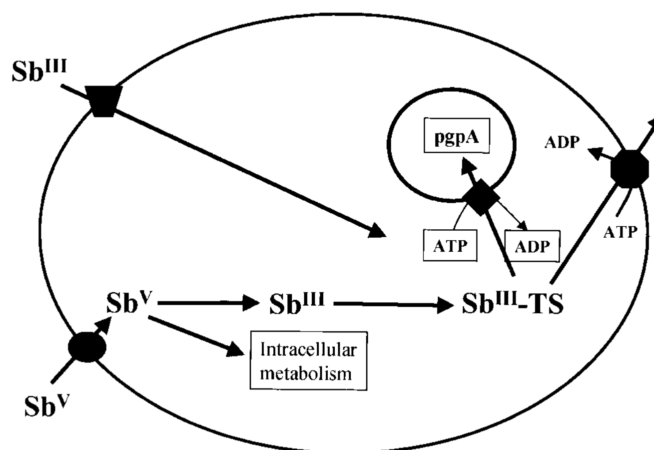

FIG. 5. **A proposed mechanism for antimony metabolism and its neutralization by *L. donovani.*** Both promastigotes and amastigotes accumulate Sb^V. Enzymatic reduction of Sb^V to Sb^{III} occurs in both life stages, but reducing activity is much higher in amastigotes (current data). Subsequent binding of Sb^{III} to trypanothione and the active efflux of this complex have been previously proposed (20, 34, 35).

Fig. 5 illustrates a model describing antimoniate reduction

and the subsequent neutralization of Sb^{III} by trypanothione. We propose that Sb^{III} accumulates in cells by transport across the plasma membrane and/or by the intracellular reduction of Sb^V to Sb^{III} via either an enzymatic or nonenzymatic mechanism. Sb^{III} then complexes with trypanothione, and this complex is then extruded from the parasite via specific transporters, as has been previously proposed (20, 34, 35). A mutation in a purported stage-specific Sb^V reductase would result in resistance of amastigotes to Sb^V but not to Sb^{III}. However, any mutation that occurs in enzymes involved in reactions downstream of the site of the proposed Sb^V stage-specific reducing activity should result in antimony as well as in arsenite-antimony cross-resistance. This could explain why others have reported Sb^V-Sb^{III}-As^{III} cross-resistance in various *Leishmania* species (11, 20, 22, 36). Furthermore, the additional role of macrophage-associated antimony reduction in natural infection, as previously suggested (11, 16, 17), cannot be ruled out.

In promastigotes, the observation that Sb^V inhibits Sb^{III} toxicity is surprising. No similar phenomenon has been reported before. The intracellular Sb^V antagonism of Sb^{III} toxicity in WT and mutant promastigotes as well as in mutant amastigotes could occur because of a number of possibilities. In solution, gluconic acid dissociates from antimony and is replaced by hydroxyl groups forming hexahydroxyl antimony, whereas tartrate remains conjugated to Sb^{III}. Thus, Sb^V antagonism of Sb^{III} is more likely to be a result of noncompetitive inhibition rather than of competitive inhibition (because of structural similarity). Alternatively, an inactive oxide hybrid is formed between potassium antimonyl tartrate and one-half of the sodium stibogluconate molecule. Because Sb^V is rapidly reduced to Sb^{III} in amastigotes, such hybrids will probably not form. These possibilities are currently being investigated.

The recently developed antimony anion exchange chromatography method (28) showed that Sb^V is not only reduced but also metabolized by *L. donovani*. Peaks separated from cell extracts after Sb^V treatment (Fig. 4) suggest the possibility of covalent interactions of either Sb^V or Sb^{III} with intracellular molecules. The bound antimony may not be reduced by BH_4 (27), thus explaining the differences between total $[Sb]_i$ and the reducible intracellular Sb^V and Sb^{III} measured in Tables 1 and 2.

The amount of antimony accumulated in *L. donovani* promastigotes and amastigotes are 20 and 300 times higher than that reported for *L. panamensis* (17). Furthermore, although this New World *Leishmania* species also exhibits stage-specific susceptibility to Sb^V, both developmental stages accumulate it to the same extent. Hence, it is likely that in New and Old World species, Sb^V acts via different modes of action.

The novel approaches of FI-HG-ICP-MS, FI-ICP-MS, and anion exchange chromatography facilitated the description of intracellular antimony metabolism in *L. donovani* promastigotes and amastigotes, both WT and mutant. To the best of our knowledge, this is the first description of intracellular Sb^V reduction in parasitic protozoa as well as in any intact cell.

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