Studies on stibanate resistant *Leishmania donovani* isolates of Indian origin

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Studies with 26 clones of *L. donovani* promastigotes derived from three different Indian isolates indicated that wild type parasites are mixture of stibanate sensitive and resistant cells. Both forms of the parasite were resistant to the drug. Infection with resistant parasites appears to be the primary reason of high rate of pentavalent antimony unresponsiveness among Indian kala-azar patients. It was observed that the resistant parasites originated as a result of irregular and often incomplete treatment of kala-azar patients with pentavalent antimonials.

Parasitic protozoa belonging to the genus *Leishmania* are the causative agents of a wide spectrum of diseases collectively known as leishmaniases. Visceral leishmaniasis (VL) or kala-azar is generally caused by members of *Leishmania donovani* complex, namely, *L. donovani*, *L. infantum* and *L. chagasi*. The disease is potentially fatal to humans and if remains untreated, mortality rate is over 95%. Organic pentavalent antimonials [Sb(V)] in the form of sodium stibogluconate (pentostam, solustibosan, stibanate) or meglumine antimonate (glucantime) are the drugs of first choice for treatment of VL and all other forms of leishmaniases\(^1\). When treatment with Sb(V) fails, pentamidine or amphotericin B are used as drugs of second choice\(^2\). Many and frequent side effects are the causes of their limited use. Allopurinals have also been used successfully for treatment of antimony unresponsive VL patients in Kenya and Bihar\(^3\). Drug resistance is a major barrier in the treatment and control of parasitic diseases. Unresponsiveness to antimony chemotherapy in VL and mucocutaneous leishmaniasis in particular has long been recognised as a serious clinical problem\(^4\). Increasing number of reports\(^5-11\) about initial failure to Sb(V) therapy or relapse after apparently successful prior treatment is the basis of this conclusion.

Objective of the present study was to generate Sb(V) resistant parasites from wild type natural isolates and to examine whether development of resistance in parasites is one of the main reason for this alarming rise in Sb(V) unresponsiveness among Indian kala-azar patients.

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Materials and Methods

**Cell culture**—*L. donovani* promastigotes were routinely maintained in M-199 (Life Technologies, USA) supplemented with 10% heat-inactivated fetal bovine serum (Innovar Biological Incorporated, USA) containing 25 mM Hepes (Life Technologies, USA, pH 7.5), streptomycin (100 µg/mL) and penicillin (100 units/mL; Sigma Chemical Company, USA). Parasites were subcultured at 5-7 days interval at an initial cell density of 2.5x10^5/mL. *L. donovani* isolates used in this study were, MHOM/IN/83/AG83 (AG83), MHOM/IN/88/GE1 (GE1), MHOM/IN/88/GE2 (GE2), MHOM/IN/94/RS (RS), MHOM/IN/95/MF (MF) and MHOM/IN/95/CK (CK). AG83, GE1 and GE2 were isolated from kala-azar patients responsive to Sb(V) therapy, whereas, RS was isolated from a post kala-azar dermal leishmaniasis (PKDL) patient who had kala-azar in 1991, treated with Stibanate and was completely cured. From 1992 onwards the patient showed typical symptoms of PKDL with progressively increasing depigmented patches along with appearance of nodules in face, neck and abdomen. Parasites were isolated from foreskin scrapings of right ear lobe before any treatment. MF and CK were isolated from bone marrow aspirates of Sb(V) unresponsive kala-azar patients.

**Development of stibanate unresponsive parasites**—Stibanate (sodium antimony gluconate) resistant promastigotes of AG83, GE1 and GE2 isolates were developed in four steps by *in vitro* passage of parasites (5x10^7/mL) in presence of 0.5, 1, 2 and 3 mg/mL of stibanate (Gluconate Health Limited, India) in the medium. In each step, parasites were cultured for at least 5-7 passages to attain steady and...
optimal cell growth. To generate resistant parasites in vivo, Syrian golden hamsters were intracardially injected with suspension (0.1 mL) of wild type AG83 promastigotes (1 × 10⁷) and after 7 days the animals were subcutaneously injected with suboptimal dose (100 mg/kg body wt/day) of stibanate for two consecutive days as described by Trotter et al. On day 7 after stibanate treatment, the animals were sacrificed and isolated spleen cells were cultured at 22-24°C in liquid medium in presence of different concentrations of stibanate for isolation of stibanate resistant parasites.

Cloning — Promastigotes of different isolates were cloned by limited dilution. In short, exponentially growing cells were serially diluted to less than 5 cells/mL in M-199 supplemented with 20% of fetal bovine serum (FBS) and 50% of conditioned medium. of cell suspension (100 μL) was added in 96 wells bored in culture plates (Costar, USA) and incubated at 22-24°C for 2-3 weeks. Any plate having more than 6 positive wells were discarded. Filter sterilised culture supernatant from exponentially growing AG83 promastigotes in M-199 containing FBS (20%) was used as conditioned medium.

Drug sensitivity assay — To evaluate stibanate sensitivity in vitro, promastigotes (5 × 10⁵ cells/mL) were incubated at 22-24°C in presence of various concentrations of the drug for 10-12 days and growth of the parasites was monitored by cell counting. To test drug sensitivity in vivo, two groups of Syrian golden hamsters (12 animals in each group) were infected intracardially with stibane sensitive and resistant clones derived from wild type GE1. After seven days, six animals in each group were injected subcutaneously with complete dose (400 mg/kg/day) of stibanate for five consecutive days as described by Trotter et al. and animals were monitored for 6 months.

Results

Two stibanate resistant cell lines were generated from wild type sensitive AG83 promastigotes that could survive and multiply in presence of 3 mg/mL of stibanate. Stibanate resistant cell line, AG83R1 was developed by repeated in vitro passages of promastigotes in presence of increasing concentration of stibanate; and another resistant cell line AG83R2 was isolated from in vitro culture of spleen cells of infected hamsters treated with suboptimal doses of stibanate. Results presented in Table 1 indicated that in absence of stibanate, wild type promastigotes of AG83, GE1 and GE2 multiply nearly 30-45 times in 8 days while more than 90% of the same promastigotes even failed to survive in presence of 3 mg/mL of the drug. Therefore, it appeared that stibanate had lethal effect on in vitro growth of three different wild type natural isolates of L. donovani promastigotes. Under identical conditions, the drug had no lethal effect on resistant promastigotes, namely, AG83R1 or AG83R2 (Table 1). Similarly, stibanate had no lethal effect on three other wild type promastigotes, namely, CK and MF isolated from two Sb(V) unresponsive kala-azar patients and RS isolated from a PKDL patient. It was observed that all resistant parasites, namely, AG83R1, AG83R2, CK, MF and RS multiplied nearly 15 to 35 times in presence of 3 mg/mL of stibanate and growth of all resistant parasites except RS was partially inhibited in presence of the drug (Table 1). Growth patterns of AG83, AG83R1 and RS representing one each of a sensitive, a resistant and a PKDL isolate in presence and absence of 3 mg/mL of stibanate over a period of 12 days have been shown in Figure 1. Growth patterns of other sensitive (GE1 and GE2) and resistant isolates (AG83R2, CK and MF) were similar to that of AG83 and AG83R1 respectively (data not shown).

Over 90% of wild type AG83 promastigotes died in presence of stibanate (3 mg/mL), but some parasites remained viable even after two weeks. Unlike normal promastigotes, morphologically these viable parasites

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Stibanate (mg/mL)</th>
<th>Cell count ( \times 10^8 )</th>
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<tbody>
<tr>
<td>AG83</td>
<td>0</td>
<td>14.6 ± 1.2</td>
</tr>
<tr>
<td>-do-</td>
<td>3</td>
<td>0.05</td>
</tr>
<tr>
<td>GE1</td>
<td>0</td>
<td>19.0 ± 1.2</td>
</tr>
<tr>
<td>-do-</td>
<td>3</td>
<td>0.05</td>
</tr>
<tr>
<td>GE2</td>
<td>0</td>
<td>22.0 ± 1.7</td>
</tr>
<tr>
<td>-do-</td>
<td>3</td>
<td>0.05</td>
</tr>
<tr>
<td>AG83 R1</td>
<td>0</td>
<td>11.6 ± 1.7</td>
</tr>
<tr>
<td>-do-</td>
<td>3</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td>AG83R2</td>
<td>0</td>
<td>25.0 ± 2.4</td>
</tr>
<tr>
<td>-do-</td>
<td>3</td>
<td>18.0 ± 1.6</td>
</tr>
<tr>
<td>CK</td>
<td>0</td>
<td>16.0 ± 2.2</td>
</tr>
<tr>
<td>-do-</td>
<td>3</td>
<td>11.0 ± 0.8</td>
</tr>
<tr>
<td>MF</td>
<td>0</td>
<td>21.6 ± 1.7</td>
</tr>
<tr>
<td>-do-</td>
<td>3</td>
<td>16.0 ± 2.9</td>
</tr>
<tr>
<td>RS</td>
<td>0</td>
<td>17.0 ± 2.1</td>
</tr>
<tr>
<td>-do-</td>
<td>3</td>
<td>17.0 ± 1.6</td>
</tr>
</tbody>
</table>

*Initial cell counts were \( \times 10^6/mL \) in case of AG83R2 and \( 5 \times 10^7/mL \) for all other isolates.
were oval shape with a very short flagellum and could hardly move. Ability of few promastigotes to survive in presence of stibanate raised the possibility that wild type natural isolates of \textit{Leishmania} were the mixed population of cells having varying degree of sensitivity towards the drug. To examine whether wild type isolates of \textit{Leishmania} were the mixture of stibanate sensitive and resistant cells, AG83, GE1 and GE2 promastigotes were cloned by limited dilution. Out of total 26 clones, 13 clones were obtained from AG83, 3 from GE1 and the rest were from GE2 respectively. Stibanate sensitivity assay indicated that nearly 33\% of the clones of both AG83 and GE1, and 80\% of the clones of GE2 were resistant to 3 mg/mL of stibanate in culture (Table 2). Therefore, it appeared that wild type natural isolates of \textit{Leishmania} parasites of Indian origin isolated from kala-azar patients responsive to Sb(V) therapy were the mixture of sensitive and resistant cells. In contrast, all clones derived from CK, MF, RS and AG83R1 were stibanate resistant indicating that unlike AG83, GE1 or GE2, wild type promastigotes CK, MF, RS and AG83R1 consisted of a homogeneous population of stibanate resistant cells.

To test whether the resistant promastigotes also remained resistant after being transformed to amastigotes \textit{in vivo}, hamsters were infected with two clones derived from GE1—stibanate sensitive (GE1C6S) and stibanate resistant (GE1F8R). \textit{In vitro} growth of these clones has been shown in Fig. 2. \textit{In vitro} growth of other sensitive and resistant clones were similar to that of GE1C6S and GE1F8R respectively (data not shown). Results presented in Table 3 indicated that 5 out of 6 hamsters infected with the sensitive clone, GE1C6S, survived following treatment with stibanate, whereas, under identical conditions only 1 out of 6 hamsters survived after

\begin{table}[h]
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\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Isolate} & \textbf{Total Number of clones} & \textbf{Sensitive (\%)} & \textbf{Resistant (\%)} \\
\hline
AG83 & 13 & 9 (69) & 4 (31) \\
GE1 & 3 & 2 (67) & 1 (33) \\
GE2 & 10 & 2 (20) & 8 (80) \\
AG83R1 & 7 & 0 (nil) & 7 (100) \\
CK & 3 & 0 (nil) & 3 (100) \\
MF & 4 & 0 (nil) & 4 (100) \\
RS & 4 & 0 (nil) & 4 (100) \\
\hline
\end{tabular}
\caption{\textit{In vitro} stibanate sensitivity of various clones of \textit{L. donovani} promastigotes}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Isolate} & \textbf{Treatment} & \textbf{Survival (\%)} \\
\hline
GE1C6S & None & 0/6 (nil) \\
& Stibanate & 4/6 (67) \\
GE1F8R & None & 1/6 (17) \\
& Stibanate & 1/6 (17) \\
\hline
\end{tabular}
\caption{\textit{In vivo} stibanate response of two clones of \textit{L. donovani} promastigotes}
\end{table}
infection with the resistant clone, GE1F8R. Therefore, these results indicated that in vitro stibenate sensitivity or resistance of parasites did not change following transformation within experimental hosts. As expected, without stibenate treatment, over 80% of hamsters died within 2-3 months irrespective of whether they were infected with the sensitive or resistant clone.

Discussion
Cases of VL as well as other forms of leishmaniasis refractory to Sb(V) therapy are known for a long time in many endemic areas including India\textsuperscript{15}. However, the exact correlation between Sb(V) resistance and treatment failure still remains obscure. In earlier studies\textsuperscript{14,15} clonal population of parasites have been used to generate Sb(V) resistant promastigotes. We have used wild type natural isolates of \textit{L. donovani} for development of Sb(V) resistant mutants as infection of naive hosts occurs with wild type parasites. Development of Sb(V) resistance was a slow process and was achieved only when the parasites in culture were exposed to stepwise increase in the drug concentrations for nearly 30-40 passages. All resistant promastigotes irrespective of their origin grew nearly 15-35 times in 7 days in presence of 3 mg/mL of the drug. Attempts to generate promastigotes that can grow in more than 3 mg/mL of stibenate was not successful. Using a Sudanese isolate of \textit{L. donovani} promastigotes, Ullman \textit{et al.}\textsuperscript{16} have generated two cell lines PENT 0400 and PENT 03200 that are resistant to 0.4 and 3.2 mg/mL of pentostam respectively. In contrast, resistant promastigotes generated from patients with American cutaneous leishmaniasis can grow in presence of 3-4 fold higher concentrations of Sb(V)\textsuperscript{14,15}. The degree of resistance exhibited by \textit{L. donovani} promastigotes of Indian origin was similar to that of Sudanese isolate of \textit{L. donovani}\textsuperscript{16}. We have observed that compared to wild type parasites, the resistant cells grew slowly and attained lower cell density in presence of the drug. However, when they were grown in absence of the drug for 2-3 passages, they remained resistant to Sb(V) but growth rate and optimal cell density became similar to that of wild type cells. Therefore, slower growth rate of the resistant parasites in presence of stibenate was probably due to non-specific inhibitory effect of the drug. \textit{In vitro} growth of PENT 0400 and PENT 03200 has also been found to be partially inhibited in presence of pentostam\textsuperscript{16}. Our result was also in agreement with Ullman’s findings where they have reported that pentostam resistance of PENT0400 and PENT03200 is a stable genetic trait. Analysis of stibenate sensitivity of various clones derived from 3 different isolates indicated that 13 out of 26 were resistant to the drug \textit{in vitro}. Our results demonstrated that natural wild type \textit{L. donovani} promastigotes of Indian origin were a mixture of stibenate sensitive and resistant parasites. Grogl \textit{et al.}\textsuperscript{17} have also observed that wild type promastigotes of \textit{Leishmania} spp, isolated from patients with American cutaneous leishmaniasis represent a heterogeneous population as demonstrated by a biphasic concentration response to Sb(V) which is typical of mixed population. It appeared that development of Sb(V) resistance in the present case was due to selection of resistant cells from the mixed population under continuous drug pressure. This conclusion was also supported by the fact that all clones derived from wild type AG83R1, the resistant cell line developed \textit{in vitro} were resistant to 3 mg/mL stibenate. Our results also showed that the proportion of Sb(V) resistant parasites present in wild type GE1 was nearly same in both AG83 and GE1 but was significantly higher in GE2. Apparent reason(s) for this difference was not clear as all three were isolated from kala-azar patients responsive to Sb(V) treatment. It might be possible that GE2 was isolated from a patient who responded to treatment with higher doses of Sb(V) or for a longer duration or combination of both. Whether these arguments were valid or not could not be ascertained as detail clinical history of the patient was not available.

Recently Ibrahim \textit{et al.}\textsuperscript{18} have demonstrated that a mutant of \textit{L. major} promastigote is resistant to 1 mg/mL of pentosan whereas amastigotes are sensitive to as low as 20 and 40 mg/mL of the drug. Using two clones of GE1, we have clearly demonstrated that \textit{L. donovani} promastigotes and amastigotes were equally affected by the particular concentration of stibenate. Previously drug sensitivity of amastigotes was tested \textit{in vitro} using isolated macrophage system\textsuperscript{19,20}. This is probably the first report using experimental animals that Sb(V) resistant promastigotes are also resistant to the drug \textit{in vivo}. Our results also suggested that steady increase in number of kala-azar patients unresponsive to treatment with Sb(V) was mainly due to infection with truly Sb(V) resistant parasites. This conclusion was strongly supported by the fact that all clones derived from CK and MF isolated from two Sb(V) unresponsive kala-azar patients were resistant to 3
mg/mL of stibenate. Since resistant parasites could be easily isolated from infected hamsters treated with incomplete doses of stibenate, irregular and incomplete treatment appeared to be the principal reason of Sb(V) unresponsiveness. Steady rise in number of Sb(V) unresponsive kala-azar cases was therefore, primarily due to infection with Sb(V) resistant parasites generated and selected out as a result of wide and at times abusive use of antimonial drugs.

Until recently nothing was known about the mechanism of Sb(V) resistance in Leishmania except that the resistant parasites accumulate less radioactive pentostam than the wild type cells. It has been proposed that Sb (V) / As (V) containing compounds, including antileishmanial drug pentostam, are reduced intracellularly to Sb (III) / As (III), conjugate to trypanothione, and extruded by As-thiol pump. In the present study, stibenate resistant promastigotes were found to be sensitive to both Sb (III) and As (III) [data not shown]. Similarly, pentostam resistant cell lines PENT0400 and PENT 03200 have also been found to be sensitive to growth inhibition and cytotoxicity caused by SbCl₃ and SbCl₅ as well as to a variety of other cations as Cd, Zn and As. Therefore, mechanism(s) other than extrusion as thiol conjugate was operative in stibenate resistance in L. donovani. Amplification of P-glycoprotein related genes has also been observed in arsenite resistant cells where P-glycoproteins are involved in extrusion of hydrophobic drugs from mammalian cells. Further studies are needed to determine the exact mechanism of Sb(V) resistance in L. donovani, a human pathogen.

Acknowledgement

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References

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