Studies on Stibanate unresponsive isolates of *Leishmania donovani*

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Visceral leishmaniasis, also known as kala-azar (KA) is generally caused by *Leishmania donovani*. Organic pentavalent antimonials (SbV) is the first line of treatment for KA. However, the number of KA patients unresponsive to treatment with Sb(V) is steadily increasing in India and elsewhere. The primary objective of this work is to determine the factor(s) associated with the rise of unresponsiveness. Analysis of the clonal population of parasites clearly indicated that wild type parasites isolated from KA patients who were clinically cured after treatment with Sb(V), were a mixture of resistant and sensitive cells. The resistant promastigotes were also resistant as amastigotes *in vivo*. It was further observed that Stibanate sensitive parasites can be made resistant to the drug by repeated passages in experimental animals followed by incomplete treatment with suboptimal doses of the drug. These results suggest that the steady rise in Sb(V) unresponsiveness of KA patients in India is due to infection with resistant parasites, generated as a result of irregular and often incomplete treatment of the patients.

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1. Introduction

Leishmania are a group of unicellular organisms and causative agents of a wide spectrum of human diseases collectively known as leishmaniasis. They are transmitted from one host to another by the bite of blood sucking sandfly vectors (Kala-azar Commission Report 1932). Visceral leishmaniasis also known as kala-azar (KA) is caused by *Leishmania donovani* and is fatal if it remains untreated. Chemotherapy is the only practical means for combating the disease. However, currently available drugs for treatment are far from satisfactory. In India, the pentavalent antimony compound, "Urea Stibamine", was first introduced as a potent antileishmanial drug against KA by the UN (Brahmachari 1928). Use of this drug reduced the mortality rate from 90–95% in untreated cases to less than 5% in treated ones (Zuckerman and Lainson 1977). Later on, this drug was replaced by antimony gluconate (Pentostam) and antimony methyl glutamine (Glucantine); and these compounds remain the most effective antileishmanials available today. 'Stibanate' is a commercially available drug in India for the treatment of KA. It is a pentavalent antimony compound with the chemical structure $C_6H_9Na_2O_9Sb$.

Drug resistance is a major barrier in the treatment and control of the disease. How frequently *Leishmania* becomes resistant to antimony has not been determined carefully, although such isolates from visceral leishmaniasis (VL) patients have been reported (Bryceson 1982). In Bihar, between 1994 and 1997, unresponsiveness to pentavalent antimonials have increased from 34% to 64% (Thakur *et al* 1998). Many explanations have been offered for failure of antimony treatment including undertreatment due to an inadequate supply of the drug and

Keywords. Drug resistance; *Leishmania donovani*; sodium stibogluconate; visceral leishmaniasis

Abbreviations used: KA, Kala-azar; PKDL, post kala-azar dermal leishmaniasis; Sb(V), sodium stibogluconate; VL, visceral leishmaniasis

immunologic or pharmacokinetic defects in the host. The present study was undertaken to address the different aspects of drug unresponsiveness.

2. Materials and methods

2.1 *Parasites*

The following parasites were mainly used in this study: *L. donovani* isolates AG83 (MHOM/IN/83/AG83), GE1 (MHOM/IN/89/GE1), GE2 (MHOM/IN/89/GE2), RS (MHOM/IN/94/RS), CK (MHOM/IN/95/CK), MF (MHOM/IN/95/MF). AG83, GE1 and GE2 were isolated from KA patients who were subsequently cured after sodium stiobogluconate [Sb(V)] therapy, whereas CK and MF were isolated from bone marrow aspirates of KA patients unresponsive to treatment with Stibanate. RS was isolated from a post kala-azar dermal leishmaniasis (PKDL) patient who had KA in 1991, treated with Stibanate and was completely cured. However, from 1992 onwards, the patient showed symptoms of PKDL. The isolates were cryopreserved in liquid nitrogen. For the experiments described below, the isolates were thawed and were injected into hamsters to check whether they had retained their virulency. Infected hamsters were sacrificed and transformation from amastigotes to promastigotes was carried out. The *in vitro* culture of *L. donovani* was maintained at 22°–25°C in Medium-199, supplemented with 10% FBS and was subcultured at 5–6 days interval when cell density was approximately 5–7 × 10^7/ml.

2.2 *Development of Stibanate unresponsive parasites*

Stibanate resistant promastigotes of Ag83, GE1 and GE2 were developed by *in vitro* passage of the parasite (5 × 10^5) with a stepwise increase in the concentration of Stibanate (0·5, 1, 2, 3, 4 and 5 mg/ml) in the medium. In each step, parasites were cultured for at least 5–7 passages to attain steady and optimal cell growth.

Attempts were also made to generate resistant parasites from GE1C6S *in vivo*. To achieve this, hamsters were injected with GE1C6S and then treated with suboptimal doses of Stibanate (200 mg/kg body wt. for 3 consecutive days). After 6 weeks, infected hamsters were sacrificed and transformation of amastigotes to promastigotes was carried out in the presence of different concentration of Stibanate. It was observed that transformation took place only in the presence of 0·3 mg/ml Stibanate. Promastigotes transformed in the presence of 0·3 mg/ml of Stibanate were again injected into the hamsters. After the second *in vivo* passage, followed by incomplete treatment with Stibanate, transformation took place in the presence of 0·7 mg/ml Stibanate. After five such *in vivo* passages followed by incomplete treatment with the drug, transformation of amastigotes to promastigotes readily took place in the presence of 3·0 mg/ml Stibanate.

2.3 *Parasite cloning*

Wild type promastigotes of different isolates were cloned as described by Pal *et al* (2001). In short, exponentially growing cells were serially diluted to less than 5 cells/ml in medium M-199 supplemented with 20% fetal bovine serum, 50% conditioned medium, 50 mg/ml gentamycin, 100 U/ml penicillin and 100 µg/ml streptomycin. Aliquots of 100 µl of the cell suspension were added in each well of 96 well tissue culture plates (Costar, USA) and incubated at 22–24°C for 2–3 weeks. Filter sterilized culture supernatants from exponentially growing promastigotes in medium M-199 containing 20% FBS were used as conditioned media.

2.4 *Drug sensitivity assay*

To evaluate Stibanate sensitivity *in vitro*, promastigotes (5 × 10^5/ml) were incubated at 22–25°C in the presence of various concentrations of the drug for 10–12 days, and the growth of the parasites was monitored by cell counting. To test drug sensitivity *in vivo*, Syrian golden hamsters were injected intracardially with Stibanate sensitive and resistant clones. After 7 days, half of the animals in each group were injected subcutaneously with the complete dose (400 mg/kg/day for five consecutive days) of Stibanate as described (Trotter *et al* 1980). The survival of the animals was monitored for 6 months.

2.5 *EC_{50} values of sensitive and resistant clones*

To determine EC_{50} values of Stibanate for different clones of *L. donovani*, promastigotes were grown (5 × 10^5/ml) in the presence of different concentrations of the drug and were counted on day 7. Cell counts were plotted against drug concentrations and EC_{50} values were extrapolated from the graph.

3. Results

Initially, Stibanate resistant cells were developed from sensitive wild type AG83 promastigotes, which can survive and multiply in the presence of the drug. It was observed that freshly transformed promastigotes could be adapted to grow up to 3 mg/ml Stibanate. In contrast, promastigotes well adapted to *in vitro* culture could be adapted to grow up to 5 mg/ml Stibanate (figure 1). It can be seen that while the wild type sensitive promastigotes

multiplied 50–70 times in the absence of Stibanate, over 90% of the parasites even failed to survive in the presence of the drug, indicating that Stibanate has a lethal and growth inhibitory effect on wild AG83 promastigotes. Under identical conditions it was observed that in the presence of the drug, the resistant cells grew slowly and the time for attaining optimal cell density was delayed by 24–48 h. Moreover, optimum cell density in the presence of the drug was only 50–60% that which was noticed when it was not present (figure 1).

While over 90% of wild type sensitive promastigotes died in the presence of Stibanate (figure 1), some parasites remained viable even after two weeks: unlike normal promastigotes, morphologically, these viable parasites are oval shaped with a very short flagellum having very little movement. The ability of a fraction of the promastigotes to survive in the presence of Stibanate raised the possibility that wild type natural isolates of *Leishmania* are a mixed population of cells that have different degrees of sensitivity towards the drug. To determine whether the wild type isolates of *Leishmania* are a mixture of Stibanate sensitive and resistant cells, promastigotes of AG83, GE1, GE2, CK, MF and RS isolates were cloned by limited dilution. A total number of 37 clones were obtained: 13, 3, 10, 3, 4 and 4 from AG83, GE1, GE2, CK, MF and RS, respectively (Pal *et al* 2001).

Out of these, 4 clones were selected for further study. Among these four clones, two were derived from GE1, one sensitive (GE1C6S) and another resistant (GE1F8R) to Stibanate *in vitro*. The other two clones were derived from CK (CK1R) and RS (RS1R) respectively. Figure 2 shows *in vitro* growth of these clonal populations of parasites in the presence of Stibanate. It was apparent from the results that while GE1C6S failed to survive in the presence of over 1·0 mg/ml, Stibanate up to 3·0 mg/ml had no effect on *in vitro* growth of CK1R or RS1R, while the growth of GE1F8R was inhibited by nearly 30–40% (figure 2).

To test whether the resistant parasites also remain resistant as amastigotes *in vivo*, hamsters were infected with promastigotes of GE1C6S, GE1F8R, CK1R and RS1R. Infected hamsters were then treated with complete doses of Stibanate and the survival of the animals was monitored for six months. Results (table 1) indicated that 5 out of 6 hamsters infected with the sensitive clone survived following treatment with Stibanate, whereas under identical conditions, none or only one out of six hamsters survived when infected with resistant clones, namely, GE1F8R, CK1R and RS1R following treatment with Stibanate. Also, as expected, without Stibanate treatment, over 80% of the hamsters died within 2–3 months, irrespective of whether they were infected with the sensitive or the resistant parasites (table 1). Therefore, it is clear from these results that *in vitro* Stibanate sensitivity or resistance of the clones has not changed, following transformation within experimental hosts.

In vitro growth of different clones in the presence of various concentrations of Stibanate indicated that EC_{50} values of Stibanate for GE1F8R, CK1R and RS1R are about 6–10 times higher than that of GE1C6S (table 2).

The GE1C6R promastigotes (derived from GE1C6S cells by repeated *in vivo* passages in the hamsters followed by incomplete treatment with Stibanate) were checked for their homogeneity in terms of drug sensitivity. For this, the cells were cloned by limited dilution. Four clones were obtained, and the clones were then

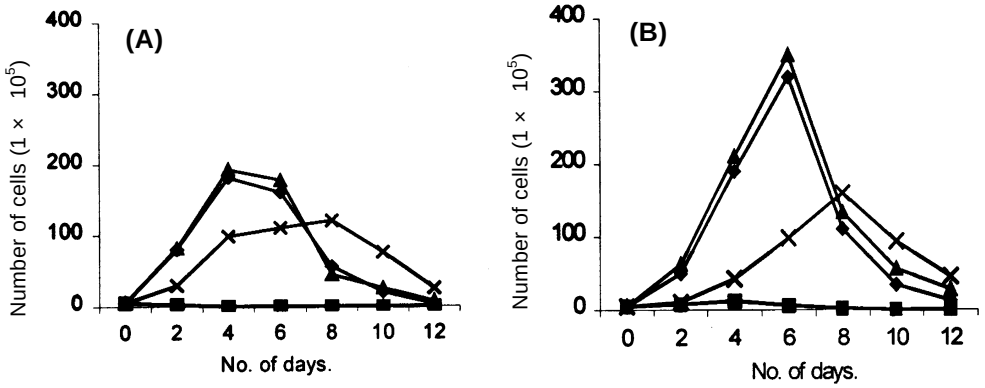

Figure 1. Effect of Stibanate on *in vitro* growth of Ag83 promastigotes. (**A**) Virulent (freshly transformed) AG83 promastigotes in the presence of 0 and 3·0 mg/ml Stibanate. (**B**) Adapted AG83 promastigotes in the presence of 0 and 5·0 mg/ml Stibanate. (♦, ■) Growth of wild type sensitive promastigotes in the absence and presence of Stibanate respectively. (▲, ×) Growth of *in vitro* generated resistant parasites in the absence and presence of Stibanate respectively.

checked for their growth in the presence of Stibanate. All the four clones were found to be resistant to 3 mg/ml Stibanate. Figure 3 shows the effect of 0, 1, 2 and 3 mg/ml Stibanate on one of the clones (GE1C6R1), derived from GE1C6R.

5. Discussion

Cases of VL and other forms of leishmaniasis refractory to Sb(V) therapy were known in endemic areas including India (Campbell and Rew 1986). Several factors, either alone or in combination, may be responsible for treatment failure. However, the exact correlation between Sb(V) resistance and treatment failure remains obscure. Previous workers (Ullman *et al* 1989), using a Sudanese isolate of *L. donovani*, have generated two cell lines PENT0400 and PENT03200 that were resistant to 0·4 and 3·2 mg/ml of Pentostam (sodium stibogluconate), respectively. Wild type natural isolates of *L. donovani* were used in this study as infections of naïve hosts occur with wild type parasites. Development of Sb(V) resistance was a slow process, and was achieved only when the parasites in the 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 the presence of 3 mg/ml of the drug, and their rate of growth was found to be relatively slower than that of wild type cells. The degree of resistance exhibited by *L. donovani* promastigotes of Indian origin used in this study is similar to that of Sudanese isolate of *L. donovani* (Ullman *et al* 1989).

Previously, drug sensitivity of the amastigotes was tested only *in vitro* using isolated macrophages (Berman and Lee 1984; Ibrahim *et al* 1994; Faraut-Gambarelli *et al* 1997; Lira *et al* 1999), which does not exactly reflect the *in vivo* conditions as the total immune system of the host. Moreover, Ibrahim *et al* (1994) also demonstrated that a mutant of *L. major* promastigotes is resistant to 1 mg/ml of pentostam whereas amastigotes are sensitive to as low as 20 and 40 μg/ml of the drug. Lira *et al* (1999) also observed that there is a strong correlation between clinical response and SAG sensitivity *in vitro* using intracellular amastigotes in macrophages, but that there is no correlation between clinical response and SAG sensitivity using extracellular promastigotes. In this

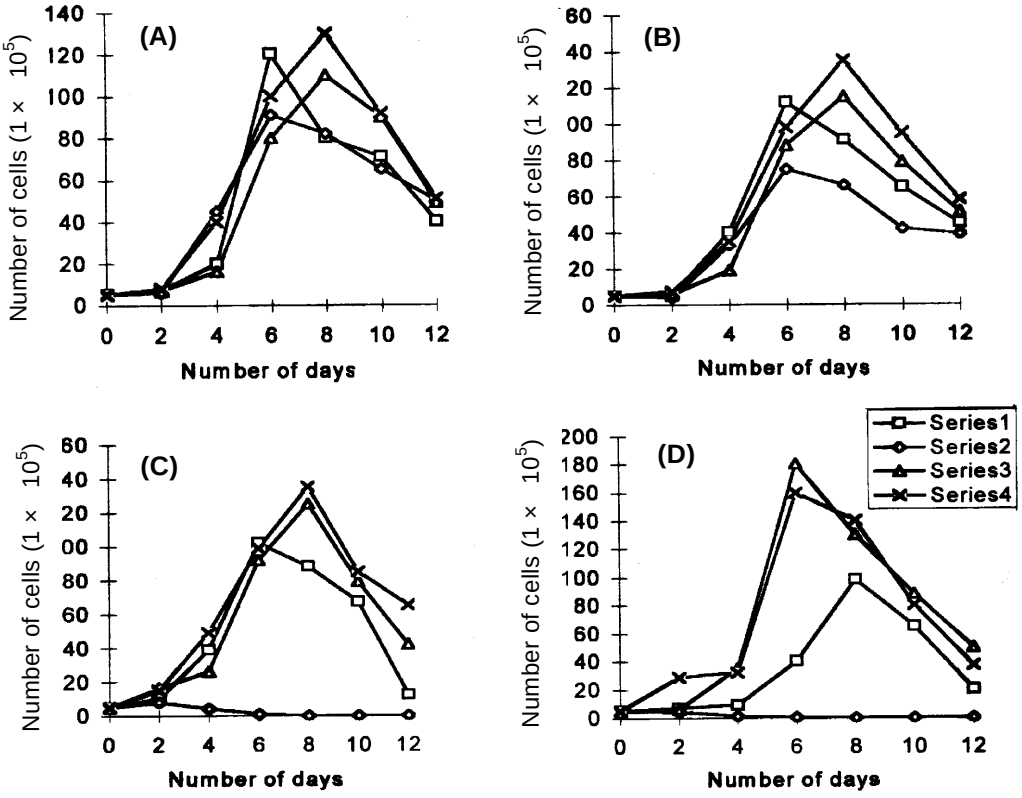

Figure 2. *In vitro* growth of various clones of *L. donovani* promastigotes in the presence of Stibanate. (**A–D**) Parasites in the presence of 0, 1, 2 and 3 mg/ml Stibanate and series 1–4 represent GE1F8R, GE1C6S, CK1R and RS1R respectively.

study, four clonal populations of promastigotes were used to study their Stibanate sensitivity as amastigotes *in vivo* using Syrian golden hamsters. It was observed that over 80% of hamsters infected with Stibanate sensitive promastigotes survived after treatment with the drug, whereas, over 80% of animals in each group infected with the Stibanate resistant parasites died irrespective of whether they were treated with the drug or not (table 1). Also, over 80% animals died without Stibanate treatment when they were infected with either the sensitive or the resistant clone. So contrary to previous studies, our results clearly indicate that the resistant parasites retained their phenotype *in vivo*.

The EC_{50} values of different clones (table 2) suggest that the resistant clone GE1F8R was about 10 times more resistant than the sensitive clone GE1C6S. Both these clones were derived from wild type GE1. As expected, EC_{50} values of CK1R and RS1R were also 3–4 times higher than that of GE1C6S. The EC_{50} values of sensitive cells, as reported earlier (Ullman *et al* 1989) were significantly less than the value of sensitive GE1C6S. This discrepancy in EC_{50} values may arise due to the fact that in India, over the last 50–60 years, KA patients were treated with Stibanate. So the basal level of tolerance of the drug has increased even for the sensitive clones. This statement is supported by the following facts. Over 95% of patients were cured after treatment with Sb(V) when the drug was first used in India more than five decades ago. In the early 80s the Manson-Bahr (1982) regimen of treatment was mainly followed; the drug was given in the dosage of 6 ml (1 mM) for 6–10 days. With this regimen of treatment, the rate of relapse and unresponsiveness was found to be over 30% in the mid 80s. However, increasing the duration to 20 days with the same dosage i.e. 6 ml (1 mM) daily, the relapse rate dropped down to 0·5% but a subset of patients did not respond to Sb(V) and were cured by Pentamidine, whereas, a daily dose of 20 mg/kg of sodium antimony gluconate for 20–30 days was effective in the 1980s (Thakur *et al* 1988). In a series of studies conducted on patients treated in Bihar between 1994 and 1997, 34–64% unresponsiveness was observed (Thakur *et al* 1998; Sundar 1997). These results clearly indicate that in India, unresponsiveness to Sb(V), is steadily on the rise. Also, the resistant parasite generated *in vivo* from the sensitive clone was homogeneously resistant (figure 3). Since resistant parasites can be generated from infected hamsters treated with incomplete doses of Stibanates, our findings make it plausible that irregular and incomplete treatment is the reason behind drug resistance. In rural areas where health care facilities are not adequate, irregular and incomplete treatment is common. In a recent report from our laboratory (Bhattacharyya

Table 1. *In vivo* Stibanate sensitivity of different clones of *L. donovani* promastigotes.

Isolate	Treatment[a]	Survival (%)
GE1C6S	None	0/6 (nil)
	Stibanate	5/6 (83)
GE1F8R	None	1/6 (17)
	Stibanate	1/6 (17)
CK1R	None	0/6 (nil)
	Stibanate	0/6 (nil)
RS1R	None	1/6 (17)
	Stibanate	1/6 (17)

[a]Subcutaneous injections of Stibanate (400 mg/kg body weight) for 5 consecutive days were administered 7 days after infection. The animals were monitored for over 6 months after infectionw, and in over 80% cases, deaths occurred between 2–3 months.

Table 2. EC_{50} values of Stibanate for different clones of *L. donovani*.

Isolate	EC_{50} (mg/ml stibanate)
L. donovani GE1C6S	0·4
L. donovani GE1F8R	4·0
L. donovani CK1R	3·5
L. donovani RS1R	3·0

Promastigotes (5×10^5 cells/ml) were incubated with various concentrations of Stibanate at 22°

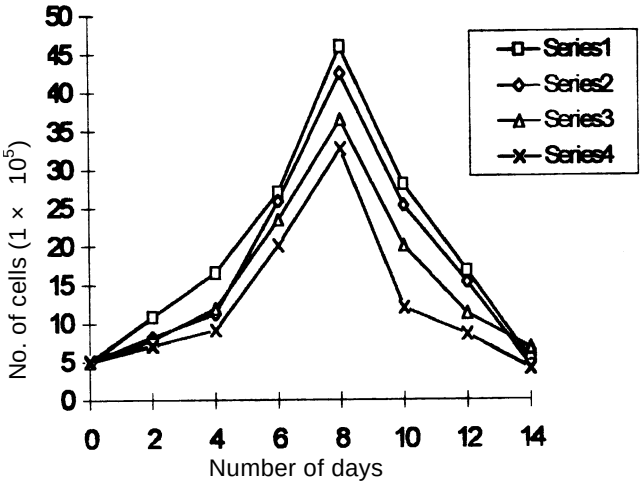

Figure 3. The effect of Stibanate on *in vitro* growth of resistant promastigotes derived from sensitive parasites. Series 1–4 represent the growth of GE1C6R clone 1 in the presence of 0, 1·0, 2·0 and 3·0 mg/ml of the drug. GE1C6R was derived by *in vivo* passages of GE1C6S as mentioned in the text.

et al 2001), we have reported that there is an 80 kDa multiple drug resistance associated protein (MRP) present in the pellicular membrane, which is 3–4 times more in the resistant parasites. Hence, drug resistance in *L. donovani* is due to the presence of transport activities, which are actively involved in the efflux of drugs from the cells.

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