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Effect of metal ions and other antileishmanial drugs on Stibanate-resistant *Leishmania donovani* promastigotes of Indian origin

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Pentavalent antimonial Sb(V)-resistant *Leishmania* promastigotes isolated from Indian kala-azar patients were found to be cross-resistant to other heavy metal ions and two antileishmanial drugs, pentamidine isethionate and amphotericin B, used for treatment of Sb(V)-unresponsive patients. Results strongly suggest the presence of transport activities in resistant cells which are actively responsible for efflux of these drugs.

IN general, parasites belonging to *Leishmania donovani* complex, namely *L. donovani*, *L. infantum* and *L. chagasi* are the causative agents of visceral leishmaniasis (VL) or kala-azar (KA), a fatal disease if it remains untreated. The digenic lifecycle of *Leishmania* consists of an extracellular promastigote form, present in the midgut of vector sandfly and an intracellular amastigote form, present within host macrophages. Promastigotes are transmitted from one host to another by the bite of sandfly vectors. KA poses a major health problem in most tropical countries, including the Indian subcontinent. Organic pentavalent antimonials Sb(V) (Stibanate, Pentostam, Glucantime) are the drug of first choice for treatment of KA. When these drugs fail, pentamidine isethionate or amphotericin B are used as drugs of second choice[1]. It was reported that in Bihar, unresponsiveness to Sb(V) has increased from 34 to 64% between 1994 and 1997 (ref. 2).

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Wild-type promastigotes of six different *Leishmania* isolates were cloned by limited dilution [3]. Among these, RS was isolated from a post kala-azar dermal leishmaniasis (PKDL) patient, CK and MF from Stibanate-unresponsive kala-azar patients and AG83, GE1 and GE2 from kala-azar patients who were successfully treated with Stibanate. It was observed that nearly 33% of the clones of AG83 and GE1 and 80% of the clones of GE2 were resistant to 3.0 mg/ml Stibanate in culture (Table 1), indicating that wild-type parasites isolated from Indian KA patients responsive to Sb(V) therapy are a mixture of drug-sensitive and resistant cells. These results are similar to the one reported by Grogl *et al.*[4], that wild type promastigotes of *Leishmania* species isolated from patients with American cutaneous leishmaniasis represented a mixed population, with different degrees of sensitivity to Sb(V). However, it is not apparent why per cent of resistant cells in GE2 is significantly higher than that in either AG83 or GE1 (Table 1). Unlike AG83, GE1 and GE2, all clones derived from CK and MF isolated from KA patients unresponsive to Sb(V) treatment and RS isolated from a PKDL patient were resistant to 3 mg/ml Stibanate (Table 1). It is generally believed that PKDL is caused by residual parasites surviving upon successful chemotherapy, which escape to the skin after the KA patient is clinically cured. This may be one of the reasons supporting the fact that all the clones derived from RS were resistant to Stibanate.

For further studies, three resistant clones (GE1F8R, CK1R, RS1R) derived from GE1, CK and RS and one sensitive clone (GE1C6S) derived from GE1 were selected. It was observed that in addition to Sb(V) resistance, the parasites were also cross-resistant to heavy metal ions such as Sb^{3+}, As^{3+} and Zn^{2+} (Figure 1), and three other mechanistically unrelated drugs, namely amphotericin B, pentamidine isethionate and colchicine (Figure 2). It was observed that the resistant cells can grow in presence of 1.5, 6.0 and 5.0 μM amphotericin B, pentamidine isethionate and colchicine. However, the same concentrations of these drugs are lethal to *in vitro* growth of the

Table 1. *In vitro* Stibanate sensitivity of clones derived from different isolates of *L. donovani* promastigotes

Isolate	Total	Number of clones	
		Sensitive (%)	Resistant (%)
AG83	13	9 (69)	4 (31)
GE1	3	2 (67)	1 (33)
GE2	10	2 (20)	8 (80)
CK	3	0 (Nil)	3 (100)
MF	4	0 (Nil)	4 (100)
RS	4	0 (Nil)	4 (100)

Promastigotes (5×10^5 cells/ml) were incubated at 22–25°C in medium M-199 supplemented with 20.0 mM HEPES, pH, 7.5 and 10.0% foetal calf serum in presence of 3.0 mg/ml Stibanate and viable cells were counted on day 7. Final cell counts were in the order of 10^7 and $< 10^4$ for resistant and sensitive parasites, respectively.

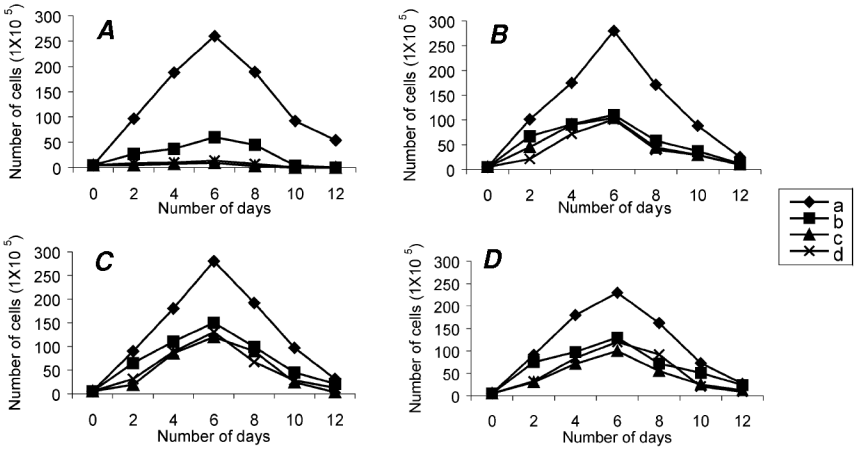

Figure 1. Effect of Zn^{2+}, As^{3+} and Sb^{3+} on *in vitro* growth of Stibanate-resistant *L. donovani* promastigotes. ***A–D*** represent *in vitro* growth of GE1C6S, GE1F8R, CK1R and RS1R, respectively in absence of any metal ions (a) and in presence of 4.0 mM $ZnCl_2$ (b), 200.0 nM Na_2HAsO_4 (c) and 100.0 nM $SbCl_3$ (d). Other conditions are same as in Table 1.

sensitive cells. Moreover, unlike in sensitive parasites, where intracellular drug concentrations rise throughout the experiment, in resistant cells, optimum levels were attained within 10–20 min and then started to decline steadily after 30 min and was reduced to a half of their optimal levels after 60 min (Figure 3). To examine whether a drug efflux system is operative in resistant cells, parasite proteins were analysed by immunoblotting experiments using monoclonal antisera raised against MRP[5], a plasma membrane drug transporter protein. Results (Figure 4) indicated that expression of a 80 kDa protein recognized by the monoclonal antisera directed against MRP is significantly higher in the resistant clones, i.e. in GE1F8R, CK1R and RS1R, than that of the sensitive clone GE1C6S. Quantitatively, levels of the 80 kDa protein are nearly 3–4 times higher in resistant clones, as determined by densitometric scanning of the anti-MRP reactive 80 kDa protein band. Moreover, it was observed that the 80 kDa protein like MRP is located entirely in the pellicular membrane of the parasite (Figure 5). In tumour cells, two plasma membrane drug transporter proteins, P-glycoprotein (PGP)[6] and MRP[5] were shown to confer multidrug resistance phenotype. Both proteins act as drug efflux pumps. Similarly, Essodaigui *et al.*[7] reported the existence of three transport activities in promastigotes of different *Leishmania* species which share several properties of MRP1. Grogl *et al.*[8] also reported increased expression of 96–106 and 23–25 kDa peptides recognized by PGP-specific monoclonal antibodies in Pentostam-resistant *L. enreiettii* and *L. panmensis* clonal parasites. The Pentostam-resistant cells were reported to be cross-resistant to Glucantime, another pentavalent antimonial and to other structurally and functionally unrelated compounds. Recently, Mukhopadhyay *et al.*[9] showed that promastigotes of *L. tarentolae*, resistant to As^{3+} and Sb^{5+}, including Pentostam, have increased levels of intracellular thiols, particularly trypanothione. It was proposed that in resistant promastigotes, Sb^{5+}/As^{5+} containing compounds

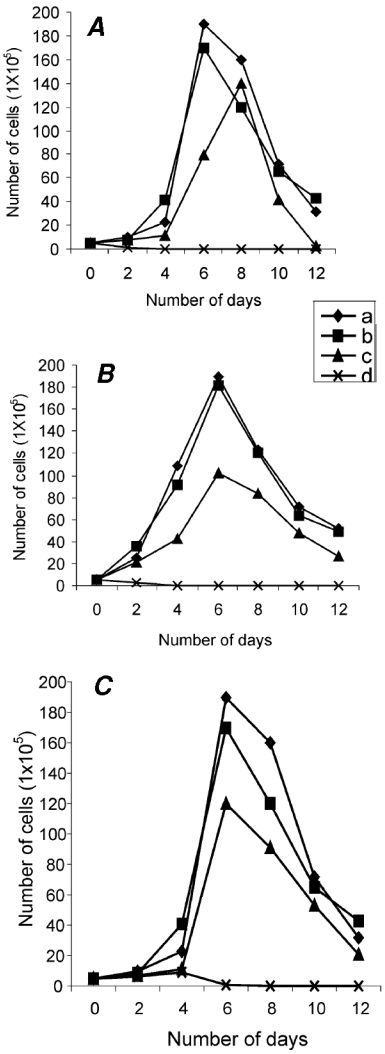

Figure 2. Effect of amphotericin B, pentamidine isethionate and colchicine on Stibanate-sensitive and resistant *L. donovani* promastigotes. Series a, d and b, c represent *in vitro* growth of GE1C6S and GE1F8R, respectively in absence and in presence of 1.5 μM amphotericin B (***A***), 6.0 μM pentamidine isethionate (***B***) and 5.0 μM colchicine (***C***). Other conditions are same as in Table 1.

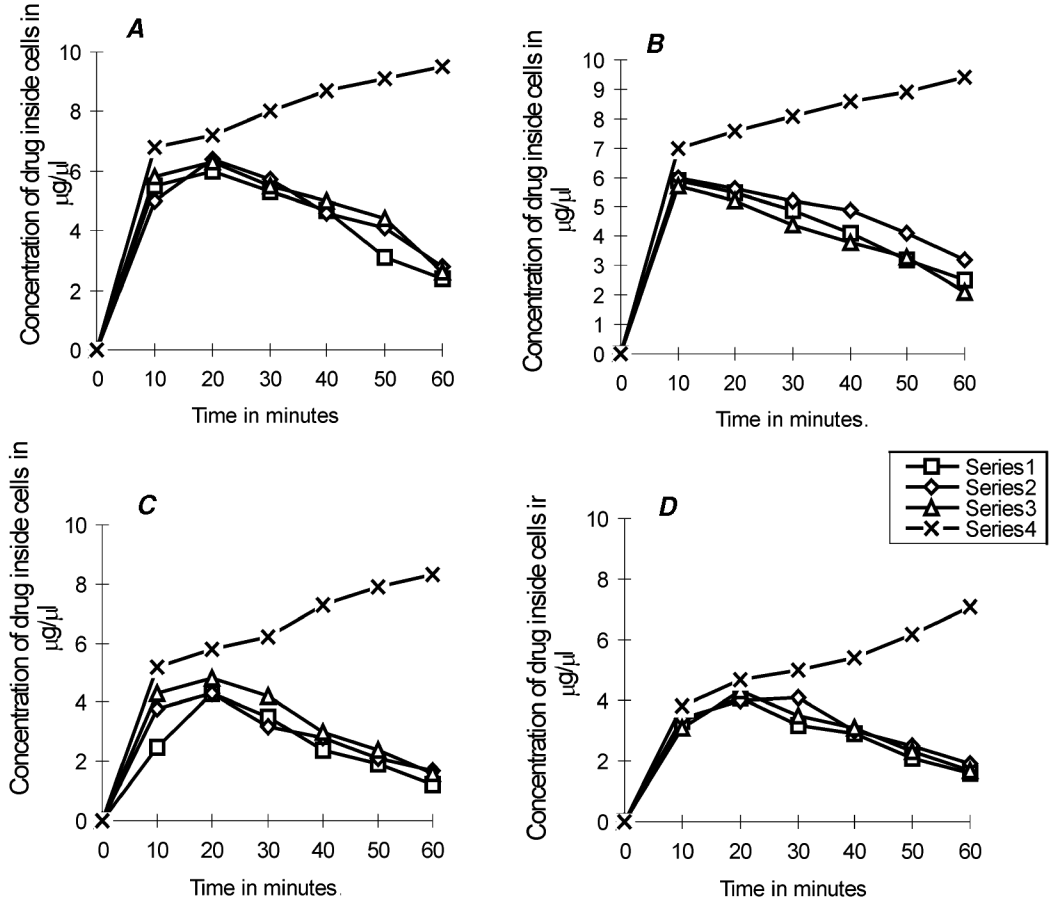

Figure 3. Intracellular concentration of various drugs in different *L. donovani* promastigotes. Cells (2×10^7/ml) suspended in 50.0 mM sodium phosphate buffer, pH 7.2, containing 100.0 mM NaCl were incubated at 22–25°C in presence of 10.0 µg of the various drugs. To determine intracellular concentrations of the drugs, cells were harvested at different time intervals, washed, sonicated and OD of 10,000 × g supernatants was measured at 212, 243, 406 and 265 nm, the respective λ_{max} of Stibanate, colchicine, amphotericin and pentamidine isethionate. Figures **A–D** represents pentamidine isethionate, sodium stibogluconate, amphotericin B and colchicine, respectively. Series 1–4 represents CK1R, RS1R, GE1F8R and GE1C6S, respectively.

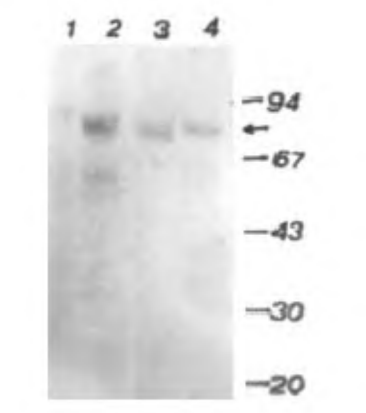

Figure 4. Western blot analysis of MRP-like protein of different *L. donovani* promastigotes. Parasite proteins (10.0 µg) were separated by SDS-PAGE[13] using 10.0% slab gel, transferred to nitrocellulose and immunoblotted with monoclonal antibodies against MRP (1 : 1000). Protein bands were detected by forming avidin–biotin complex followed by treatment with 4-chloro-1-naphthol[14]. Lanes 1–4 represent GE1C6S, GE1F8R, CK1R and RS1R, respectively.

are reduced intracellularly to Sb^{3+}/As^{3+}, conjugated to trypanothione and then extruded out by the As–thiol pump. In this study, Sb(V)-resistant parasites were also found to be resistant to Sb^{3+}, As^{3+} and Zn^{2+}. Therefore, like *L. tarentolae*, the mechanism of Sb(V) resistance in *L. donovani* appears to be due to intracellular reduction of Sb^{5+} to Sb^{3+} and effective efflux of Sb^{3+}–trypanothione conjugate by the As–thiol pump. In contrast, resistance to pentamidine isethionate and amphotericin B appears to be mediated by MRP[5].

In Bihar, both pentamidine isethionate and amphotericin B have been frequently used for treatment of antimony-unresponsive KA patients and like antimonials, indiscriminate and irregular treatment with these drugs cannot be ruled out, especially in rural settings. This may explain why antimony-resistant parasites isolated from KA and PKDL patients are also cross-resistant to these drugs. It has been reported that in the early 70s, most of the KA patients were cured with 15 injections of

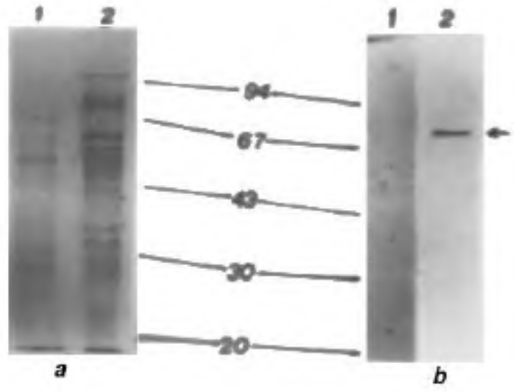

Figure 5. Subcellular localization of MRP-like protein. Lanes 1 and 2 represent proteins in cytosolic and pellicular membrane fractions. (*a*) Coomassie-stained gel, and (*b*) Western-blotted nitrocellulose paper, as in Figure 4.

pentamidine isethionate given in a dosage of 4 mg/kg body weight[10,11]. In the late 80s, a majority of them required 30 injections[12], indicating some degree of unresponsiveness to pentamidine isethionate. Although, unresponsiveness to amphotericin B has not been reported so far, patients may soon become unresponsive to this drug, if its use is not carefully monitored. Therefore, if these drugs are used indiscriminately, as the antimonials have been in the past, we may face a situation when no antileishmanial drugs will be available for treatment of kala-azar in India.

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Evidence for rare male mating advantage and sexual isolation in two karyotypically different strains of *Drosophila ananassae*

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Experiments were conducted to study rare male mating advantage in two karyotypically different strains of *Drosophila ananassae*: (a) ST/ST and (b) AL/AL (2L). Mating success of flies of two strains was scored by direct observation in Elens–Wattiaux mating chamber at five different ratios employing multiple-choice technique and two different experimental designs. The results indicate that there is significant one-sided rare male mating advantage in favour of AL males. The same data were analysed for a comparison between number of homogamic and heterogamic matings. With both experimental designs, the homogamic matings are significantly more frequent than heterogamic ones and isolation estimate remains low (0.55–0.62), which shows preferential mating between females and males with the same karyotype. These findings provide an evidence for one-sided rare male mating advantage in favour of AL males and sexual isolation between karyotypically different strains of *D. ananassae*.

IT is widely demonstrated that sexual selection in *Drosophila* species is often frequency-dependent, where the rare competing genotype is favoured for mating, while the common type is at a disadvantage in a population. Rare type mating advantage is a possible mechanism for maintaining genetic polymorphism in nature without genetic load at equilibrium, unlike heterozygous advantage model. Rare type mating advantage may be two-sided where both types of flies are favoured when rare or may be one-sided, when only one type is rare.

Since its first demonstration by Petit[1] in *Drosophila melanogaster* and later by Ehrman *et al.*[2] and Ehrman[3] in *D. pseudoobscura*, rare male mating advantage has so far been reported in 12 species of *Drosophila* as well as in

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