Is there true Sb(V) resistance in Indian kala azar field isolates?

Visceral leishmaniasis (kala azar) is a serious health problem in the eastern states of Bihar and West Bengal[1]. *Leishmania donovani* is the major causative agent of visceral leishmaniasis in India. The treatment of choice since long for kala azar has been the administration of a pentavalent antimony Sb(V) containing drug, sodium antimony gluconate (SAG). However, the focus is now shifting to the use of amphotericin B and the oral drug miltefosine. Resistance to antimony Sb(V) in *Leishmania* is widespread in several geographic regions, reaching epidemic proportions in parts of India[2]. In Muzaffarpur, the epicentre of the outbreak, more than 60% of previously untreated patients are unresponsive to antimonials[2,3]. A number of explanations for antimony treatment failures have been offered, like under-treatment due to premature termination of chemotherapy and immunologic or pharmacokinetic defects in the host. But is the parasite truly resistant to SAG, is a matter of concern and debate amongst Indian researchers. Although isolated studies have been carried out to confirm parasite resistance in Indian field isolates, no documented results can be found apart from a study carried out abroad[4], wherein Indian field isolates were collected from disease endemic areas between 1995 and 1998 from drug unresponsive and responsive patients. Unfortunately, these cultures are not available anywhere in India for Indian researchers to pursue further work towards unravelling mechanisms of resistance.

In order to consider policies to prevent or control drug resistance, parasite resistance should be monitored, rather than patient relapse rates. In the absence of genetic markers as yet, this can well be done by testing of drug sensitivity of amastigotes in macrophage cultures. In this study, we collected some field isolates relatively recently from patients admitted to the Kala Azar Medical Research Centre of the Institute of Medical Sciences, BHU, Varanasi and also from its affiliated hospital situated at Muzaffarpur, Bihar, India. We successfully managed to establish *in vitro* culture of three isolates. Isolates 39 and 41 were from patients unresponsive to SAG chemotherapy and isolate 2001 was from patients who responded to chemotherapy. The criterion for diagnosis was the presence of LD bodies in splenic aspirations performed and graded according to standard criteria[5]. After diagnosis, the patients were administered a course of SAG (Albert David, Kolkata), 20 mg/kg body weight intravenously once daily for 30 days. Response to treatment was evaluated by repeating splenic aspiration at day-30 of treatment. The designation of responsive patients was based on the absence of fever, clinical improvement with reduction in spleen size and the absence of parasites in the splenic aspirate. Patients who showed presence of parasites were labelled as unresponsive cases. These patients were subsequently treated successfully with amphotericin B. Parasites were cultivated initially at 26°C in triple N-agar tubes and subsequently promastigotes were cultivated in HEPES-buffered (pH 7.4) medium 199 (Sigma) with 10–20% heat-inactivated foetal bovine serum (Atlanta Biologicals) at 25°C in 25 cm^2 tissue-culture flasks. Establishing culture of parasites from micro quantity of biopsy material of patients is prone to many problems: material being scarce may not lead to successful culture; sometimes parasites do not grow, and inadvertently, as biopsy material is collected under not-so-stringent sterile conditions, there is frequent contamination.

In order to determine whether resistance is the intrinsic property of parasites and not the host, an axenic amastigote culture of these three field isolates collected from kala azar patients unresponsive and responsive to SAG chemotherapy, was used to infect J774 macrophages in order to evaluate SAG resistance *in vitro*. We preferred to use amastigotes rather than promastigotes, as reported previously[4], to obtain high and consistent infection rates over a 7–9-day period. The promastigote stage in the lifecycle is not susceptible to two major drugs used clinically, these being pentavalent antimonial drugs, sodium stibogluconate and meglumine antimoniate[6,7]. It has been shown that pentavalent antimony activity against *L. donovani* axenic amastigotes is stage-specific[8]. Promastigotes were subjected to axenic amastigote-forming conditions by growing them for 4–5 days at pH 5.3, 35°C in Grace's medium plus 20% HIFBS[9]. They were used to infect J774 macrophages at a host–parasite ratio of 1 : 10. Specifically, 4×10^6 macrophages were mixed with 4×10^7 axenic amastigotes in 4 ml of medium in a 25 cm^2 flask, to begin the infection at 35°C. After 24 h, the level of infection was quantitated and 0.5 ml of the cells (10^6) was plated in duplicate in 24-well, tissue culture-treated polystyrene plates. Different concentrations of the drug (SAG) obtained from the same commercial source as used for patient treatment, were added to final concentrations of 0, 12.5, 25 and 50 µg/ml. The cultures were incubated for an additional 24 h. The level of infection was quantitated by microscopic counting of macrophages and parasites. The total number of intracellular parasites per well was estimated for each drug concentration used in duplicate, as a product of the total number of macrophages, the percentage of infected cells and the average number of intracellular parasites/macrophages. The percentage of infected cells and the average number of intracellular parasites per cell were tallied microscopically, by continuous examination of consecutive areas in a given drug concentration, until the infected cells examined reached 50. In a separate experiment, the amastigotes were inoculated into M199 with 20% HIFBS with different drug concentrations as used in the earlier experiment and allowed to differentiate into promastigotes. Microscopic counting of parasites was done when the culture of promastigotes with no drug reached turbidity. Counting promastigotes again estimated the sensitivity of these isolates to SAG. A correlation between clinical response and SAG sensitivity *in vitro* was observed for both the experiments (Figure 1). Isolate 39 was more resistant than isolate 41, and isolate 2001 in comparison was drug-responsive. These experiments were repeated two more times independently and the same correlation was observed each time. These results are also in conformation with an earlier work[4], wherein isolates collected during 1995 and 1997 were used. Therefore, the circulation of parasites truly resistant to SAG along this time is established.

It has also previously been documented[10] that amastigote infection in macrophages responds to clinically used antileishmanial compounds. Pentavalent antimonial drugs are highly effective in

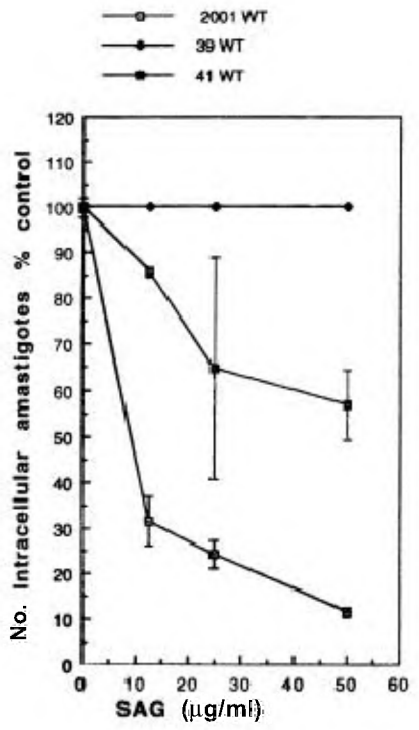

Figure 1. Sodium antimony gluconate sensitivity of Indian *Leishmania donovani* isolates assayed as amastigotes in J774 macrophages.

the *in vitro L. donovani*/macrophage system[11]. Mouse peritoneal macrophages were used in these studies. Similarly, human macrophages from transformed peripheral blood monocytes have been infected with amastigotes *in vitro* and were used for study of drug action in *L. tropica*[12]. We used J774 macrophage cell line with phagocytic properties[13], which gives high infection rates as peritoneal and blood macrophages and continuous replication of the amastigotes, intracellularly. Early experiments showed that the ratio of inoculum of amastigotes to macrophages affected the activity level of the drug. With a low amastigote inoculum, the infection rate declined after the third day. In view of these results, drug studies were carried out using infection ratios of ten amastigotes to one macrophage. Studies have also shown that SAG was partly effective only at very high concentrations of 5000–10,000 mg/l (ref. 14). 1, 3, 9, 27, 81 mg Sb^{v}/l was used by Neal and Croft[11]. Our aim was to check whether there was a correlation with clinical response when isolates were assayed for drug sensitivity as intracellular amastigotes. We selected the range of 12.5 to 50 μg SAG/ml. We believe that our model of using intracellular infections arising from promastigotes adapting to conditions for axenization of *L. amazonensis*, is useful in revealing differences in the drug sensitivity of *L. donovani* isolates from Indian patients with kala azar who were responsive or unresponsive to a full course of SAG treatment. When the axenic amastigotes were allowed to differentiate into promastigotes within the drug-containing medium, we again observed that the growth of promastigotes is according to drug sensitivity/resistance as when they were amastigotes within macrophages. We therefore observed a correlation between clinical response and SAG sensitivity *in vitro*, thus establishing parasite resistance to the drug. Most importantly, these isolates are now available for pursuing further work on drug resistance mechanisms.

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'Complementary' and 'mainstream' medicine: friend or foe?

Pal[1] has addressed an issue that is of greater significance than his brief on complementary and alternative medicine.

The West belatedly realized that many people used non-allopathic therapies. Logically, ayurveda may have to be considered as mainstream in India because it had been there for centuries before allopathy came along[2]. Western systems patronizingly define complementary and alternative medicine as 'therapies generally not taught nor practised in regular hospitals, lacking evidence of effectiveness and generally not reimbursable by third-party payer'[3].

Ayurveda emphasizes that lifestyle measures are integral to comprehensive management[4]. Homeopathy also seeks to allow the body's own systems to correct imbalances. There should be no controversy in applying these principles to preserve health and to treat diseases.

The biopsychosocial model, which integrates biological, psychological and social components of treatment process, has been described in mainstream journals and textbooks[5,6]. Medical care-seeking behaviour is being studied, to improve patient compliance with treatment. Physicians should be conversant