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Integrating genomics and proteomics permits identification of immunodominant antigens associated with drug resistance in human visceral leishmaniasis in India

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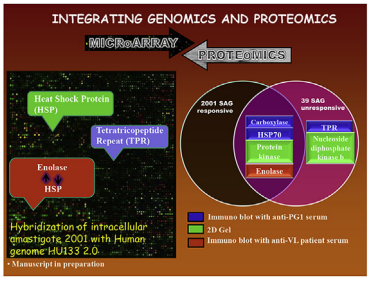
GRAPHICAL ABSTRACT



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ABSTRACT

Resistance of human pathogens like *Leishmania* to drugs is a growing concern where the multidrug-resistant phenotype renders chemotherapy ineffective. The acquired resistance of *Leishmania* to antimony has promoted intense research on the mechanisms involved but the question has not been resolved yet. In this study we have explored host-pathogen- drug interactions leading to identification of pharmacological determinants of host macrophages that resist the sodium antimony gluconate (SAG) mediated intracellular parasite killing. mRNA profiling of mammalian host stage amastigotes of sodium antimony gluconate (SAG) 'sensitive' and 'resistant' parasite lines was carried out using Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array. Patient sera was used to identify immunogenic proteins by two-dimensional gel analysis (2DE) and mass spectrometric analysis (LC-MS/MS). Immunofluorescence microscopy confirmed the identities on 'sensitive' and 'resistant' parasite lines. A total of nine immunogenic proteins whose intensities changed significantly and consistently in multiple experiments were detected, suggesting that a cohort of proteins are altered in expression levels in the 'resistant' parasites. Global expression profiling using microarrays revealed this regulation was not reflected by changes in the levels of the cognate mRNAs. Following identification of proteins by mass spectrometry, one such regulated protein, enolase, was chosen for more detailed analysis. Immunofluorescence microscopy employing antisera against this enzyme confirmed that its level was differentially regulated in the 'resistant' isolate. We show that high serum level of immunoreactive protein is associated with 'resistant' phenotype. Differentially expressed proteins with immunomodulatory activities were found to be associated with the 'resistant phenotype'.

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1. Introduction

Leishmania is a intracellular protozoan parasite causing the disease leishmaniasis, and is transmitted between hosts by the bite of the female sandfly. Cutaneous leishmaniasis (CL) is the most common manifestation of the disease, with between 700,000 and 1.2 million new cases every year. Visceral leishmaniasis, or kala-azar, is deadly if not treated, and accounts for 200,000 to 400,000 new cases and 20,000 to 40,000 deaths each year (WHO Fact sheet, 2014). More than 60% of world's visceral leishmaniasis (VL) is reported from India, Bangladesh and Nepal affecting the poorest population groups (WHO Fact sheet, 2014). Approximately 90% of patients with VL in India live in the northeastern states of Bihar from where the isolates used in the present study were collected. *Leishmania* have a dimorphic life cycle, surviving as flagellated promastigotes in the midgut of the phlebotomine sandfly vector and as rounded, non-motile amastigotes with in the macrophage of mammalian host. The bite of female sandfly is the entry point of *Leishmania* promastigotes into the bloodstream of mammalian host, where flagellated promastigotes via receptor mediated mechanism attach to mononuclear phagocytes and are taken into the parasitophorous vacuole. Due to the effect of decrease in pH and increase in temperature, flagellated promastigotes undergo significant biochemical and metabolic changes to form a non-flagellated, round amastigote (Handman and Bullen, 2002). In the absence of vaccines and the impact of vector control being limited, chemotherapy is the key target in VL control (Chappuis et al., 2007).

Pentavalent antimony therapeutic arsenals (sodium antimony gluconate; SAG) against leishmaniasis which have had a great history in the successful treatment of Kala Azar in India for the last sixty years, now result in treatment failure rates due to development of drug resistance reaching up to 65% (Sundar and Chatterjee, 2006). Elucidation of the mechanism(s) of antimony resistance in *Leishmania* is therefore important for the development of molecular tools to detect and monitor the levels of drug susceptibility, to optimize drug use, and to identify novel drug targets to block or circumvent existing resistance mechanisms. The knowledge gained will help to bring back the safe and cheap SAG drug treatment in a new 'avatar'. In a study conducted on VL in Sudan found that combination of SAG and paramomycin administered for 17 days was associated with higher cure and survival rates compared to SAG monotherapy administered for 30 days (Melaku et al., 2007).

Earlier work from our laboratory (Singh, 2006) and others (Decuypere et al., 2012) have established that multiple SAG resistance mechanisms are circulating in the Indian subcontinent. Although treatment failure is commonly attributed to parasite drug resistance, the effectiveness of chemotherapeutic drugs also relies strongly on parasite driven immune effectors of the host. *Leishmania* being an intracellular parasite, the antileishmanial drugs must cross host cell barriers to target the intracellular parasite. Sb^{V} is a pro drug which is reduced to the active trivalent form (Sb^{III}) inside both the host macrophage and the intracellular amastigote (Roberts et al., 1995). In the bloodstream there is a mildly reducing environment and the antileishmanial action is probably related to the interaction between the Sb^{III} and the Cys containing peptides, proteins and enzymes.

The aim of our present study was to determine the antigenic proteins involved in 'resisting' the leishmanicidal action of the antimonial drug by the intracellular parasite for which we employed a proteomic serological approach using patient sera collected from Kala Azar endemic area in India. Using sera and parasites isolated from visceral leishmaniasis (VL) patients in India, from a geographical area where *L. donovani* infections are frequently unresponsive to Sb, our results showed that on infection of host macrophages with drug 'sensitive' and 'resistant' clinical isolates, identified nine immunogenic proteins involved in host-pathogen-drug interactions leading to 'resistant' phenotype. Drug efflux emerged as an important host cell regulator of SAG mediated parasite resistance.

2. Materials and methods

2.1. Parasite culture

The collection and maintenance in culture of SAG 'resistant (39) and 'sensitive' (2001) *L. donovani* clinical isolates used in this study, has been detailed in our earlier publication (Singh, 2006). Infection of murine macrophage cell line, J774A.1 and isolation of intracellular amastigotes has been detailed in our earlier publication (Kaur et al., 2010).

Ethical Statement: Clinical isolates from parasitologically confirmed patients of Kala Azar from endemic zone of Bihar and Uttar Pradesh were collected as splenic aspiration performed by our authorized clinical collaborator and co-author Dr Shyam Sundar, with prior written consent of the patients. This study had been reviewed and approved by the institutional ethics committee of the Kala Azar Medical Research Centre, Banaras Hindu University, Varanasi.

2.2. Patient sera and western blotting

Details of patient sera used in this study is shown in Table 1. Sera is classified as VL (pre-treatment, sera obtained during active disease previous to treatment) and PT (post-treatment, sera from treated and cured patients). Uninfected volunteers i.e. endemic controls (EC) from the same endemic area were also included. Immunoreactivity profile of pool of patient sera to *Leishmania* protein extract from promastigotes (P) and amastigotes (A) of 'sensitive' and 'resistant' isolates as well as with mouse macrophage (J774A.1) cells, was determined by SDS PAGE and western blotting as previously described (Kumar et al., 2007). *Leishmania* parasites/macrophages were harvested, washed in PBS and lysed directly in the 5 × sample buffer (0.313 M Tris–HCl pH 6.8, 10% SDS, 0.05% bromophenol blue, 50% glycerol and 0.5 M DTT). Western blotting was performed using biotin labeled secondary antibody followed by the streptavidin conjugated to AP. In all blots, sera were used at 1:100, and secondary anti-human IgG was used at 1:2000.

2.3. Sample preparation for 2-DE

Isolated intracellular amastigotes of 'sensitive' and 'resistant' *L. donovani* clinical isolates were suspended in 2-DE lysis buffer (7 M urea, 2 M thiourea, 40 mM Tris, 4% CHAPS, 0.1 mg/ml phenylmethylsulfonyl fluoride). Lysis was allowed to proceed for 2 h at room temperature. The lysed samples were centrifuged to remove the insoluble material and supernatant was taken in fresh tube. Whole soluble proteome of 'sensitive' and 'resistant' *L. donovani* clinical isolates were acetone precipitated by adding four volumes of ice cold (−20 °C) to the supernatant and incubated at −20 °C for more than 1 h. The precipitated proteins were centrifuged at 13,000 g for 10 min at 4 °C and washed once with 80% cold acetone (−20 °C). The precipitated protein pellet of 'sensitive' and 'resistant' *L. donovani* clinical isolates was resolublised in rehydration buffer (8 M urea, 4% CHAPS, 0.002% bromophenol blue) 80 mM DTT and 0.5% (v/v) IPG buffer pH 3–10 for amastigotes and 0.5% IPG buffer pH 4–7 for promastigotes. The protein concentration of each of 'sensitive' and 'resistant' *L. donovani* clinical isolates were measured using 2D Quant kit (GE Healthcare Cat. NO. 806483).