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Epidemiological, clinical & pharmacological study of antimony-resistant visceral leishmaniasis in Bihar, India

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***Background & objectives*: Sodium antimony gluconate (SAG) is reported to be losing its efficacy in Bihar as a first line drug for treatment of visceral leishmaniasis (VL). Concerned with the increasing incidence of antimony-resistant VL patients in Bihar, we undertook an epidemiological, clinical and pharmacological study to formulate a scientific basis for choosing a suitable first line drug for VL.**

***Methods*: Consecutive, fresh and parasitologically confirmed patients of VL from different geographical areas of Bihar were considered for inclusion in the study. Parasites isolated from patients were tested *in vitro* to assess their response to sodium antimony gluconate (SAG) to 20 µg/ml, response to 20 mg/kg of SAG for 5 days in experimentally induced VL in BALB/c mice from those isolates, and response to SAG in patients treated with SAG for 28 days. Similarly response in culture (1µg/ml) to amphotericin B (AMB) of all 282 isolates, (1mg/kg body wt for 20 days) in patients and infected BALB/c mice (1mg/kg body wt for 5 days) was determined. Antimony levels of plasma were determined at 2, 8 and 24 h after intramuscular injection of SAG. Patients unwilling for SAG treatment or relapsed after SAG treatment and withdrawn from SAG group because of toxicity were treated with AMB. Plasma antimony levels were estimated by atomic absorption spectrometer.**

***Results*: Though antimony sensitive and resistant patient were distributed in all 14 districts of Bihar studied, there was a significant variation from district to district. Of the 123 patients included in the SAG treatment group, 19 were withdrawn due to development of toxicity and 2 died; 178 patients were treated with AMB. No patient in AMB group developed any toxicity or died. Only 47 (46%) of 102 patients, 106 (37.6%) of 282 infected macrophages, 90 (52.9%) of 170 experimental infections were cured with SAG. Mc Nemar's test on paired comparisons showed statistical significance difference ($P<0.01$) between infected macrophage and experimental infection. AMB cured all patients, infected mice and cleared parasites from all isolates.**

***Interpretation & conclusion*: Antimony resistant strains of *L. donovani* were wide spread over different geographical areas in Bihar. SAG cured lesser percentage of VL cases clinically compared to AMB and should be replaced by AMB as a first line drug.**

Key words Antimony resistant - BALB/c mice - *in vitro* - visceral leishmaniasis (VL)

Sodium antimony gluconate (SAG), a pentavalent antimonial introduced as a first line drug for the treatment of kala-azar visceral leishmaniasis, (VL) in Bihar in late 1940s cured 90 per cent of patients when given at a dose of 6 ml daily through intramuscular (im) route (approximately 10 mg/kg) for 6 to 10 days and repeated after a gap of 10 days[1] and with minimal toxic effects. Indian and Chinese

patients with kala-azar were suggested to be more sensitive to a single course of SAG 6 ml im daily for 6-10 days[2]. In order to minimize the adverse effects, the dose was started from 0.5 ml im daily, increasing by 0.5 ml every day till the dose of 6 ml was reached, then given daily or on alternate day in Bihar[3]. In 1977 epidemic of kala-azar when 100,000 cases were reported, 70 per cent of patients did not respond to this regimen of the drug[4].

During 1980s we achieved cure rate of about 90 per cent by increasing the duration of treatment to 20 days[5], more than 20 days if necessary[6], and changing to 20 mg/kg body weight for 30 to 40 days[7]. The efficacy rate of SAG decreased from 80 per cent in early 1990s[8] to 35 per cent in early 2000[9] even with WHO regimen of 20 mg/kg for 4 wk.

This study was undertaken to present a proper perspective of the current values of SAG in the treatment of VL in Bihar with the following objectives: (*i*) to find out *in vitro* sensitivity of parasites isolated from patients residing in different geographical areas in Bihar to SAG; (*ii*) the response of parasites to SAG in experimentally infected BALB/c mice; and (*iii*) response to SAG and estimation of antimony levels in treated patients. AMB was used for comparison.

Material & Methods

Patient: Fresh, consecutive and parasitological confirmed cases of VL coming from different areas of Bihar for treatment in Balaji Utthan Sansthan, Patna between October 2000 to September 2002, and who gave informed consent were included in the study.

Of a total of 310 patients, 282 were included in the study. Patients positive for HIV and other co-infections (n=28) were excluded.

Thorough clinical history and body weight were taken and spleen and liver size was measured. Total and differential count of WBC, Hb concentration, platelet count, prothrombin time, serum creatinine, blood sugar, serum Na and K, serum aspartate and alanine aminotransferase (AST, ALT) were estimated using standard methods. ECG, HIV positivity test and chest X-ray were done in each patient. Bone marrow aspiration was done and aspirates were used for smear examination, for infected macrophage culture, and for production of visceral leishmaniasis in BALB/c mice. Serum antimony levels at 2, 8 and 24 h after im injection were estimated by atomic absorption spectrometer[10]. Clinical cure was defined as abatement of fever and regressions in the size of spleen at the end of full course of treatment. Parasitological cure was defined as absence of parasites in bone marrow/ splenic aspirates at the end of treatment and final cure meant no clinical or parasitological relapse at the end of 6 months of follow up.

The patients were withdrawn from the study if they developed cardiac toxicity – prolongation of QT interval corrected for heart rate (QT interval) > 0.50 msec and marked ST segment changes after SAG treatment, serum creatinine more than 3 mg/dl, ALT more than 5 times the upper limit. Patients withdrawn due to toxic effects of SAG, patients relapsing after clinical cure, or not responding to a full course of treatment, and those unwilling to be treated by SAG were treated with amphotericin B (AMB).

SAG manufactured in India (Albert David, Ltd., Kolkata) found comparable to the best brand pentostam[11] (Burrows Wellcome, England) was used at a dose of 20 mg/kg body weight daily for 4 wk im. AMB (Fungizone manufactured by Sarabhai Chemicals, India) was used at a dose of 1 mg/kg body weight daily from the first day for 20 days given in 2 h as slow intravenous infusion[12]. The drug (as dry powder) was dissolved in 10 ml of sterile water and diluted in 500 ml of 5 per cent dextrose and infused through a scalp vein canula. A small amount of hydrocortisone was also given with infusion to ward off phlebitis and infusion related shivering or rigor.

Hydrocortisone and paracetamol were kept ready to treat infusion related reactions. Certain precautions were taken before starting AMB to prevent complications[13,14].

The patients were followed up for 6 months. If a patient did not turn up for follow up, a card or telegrams or messenger was sent.

Infected macrophage culture: Infected macrophages were derived from the bone marrow aspirate of patients and by *in vitro* cultivation and maintenance in RPMI 1640 medium (Hi-Media, Mumbai) with 20 per cent heat inactivated foetal calf serum (FCS) (Biological Industries, West Israel) containing (per ml) 25 µg gentamycin (Nicholas Piramal, India), 50 units Penicillin (Alembic Chemicals, India) and 50 µg streptomycin (Sarabhai Chemicals, India). The culture technique of Berman *et al*[15] was followed except that infected macrophages were isolated from bone marrow material. Macrophages were counted by haemocytometer and finally total number of amastigotes per culture was determined[16]. In each experiment 20 µg/ml of SAG was added to one well of tissue culture plate (NUNC, Denmark) and next one with no drug. Antileishmanial activity *in vitro* was calculated as percentage inhibition of parasites by SAG and AMB[17].

Experimental infection: Inbred male BALB/c mice (4 wk old with 16-18 g body weight, purchased from Central Drug Research Institute, Lucknow, India) were used in this study. All mice were given balanced diet (Pranave Agro Industries, New Delhi) and water *ad libitum*. For establishment of visceral leishmaniasis in these mice (5/batch/ isolate), promastigotes of stationary phase of growth and 6 and 7 sub-passage were inoculated intravenously by tail vain. The inoculum size being adjusted so that each mouse was inoculated with 2×10^8 promastigotes/g weight. After 60 days of inoculation amastigotes burden of spleen of each batch was determined from Giemsa stained impression smears[18] as well as in monophasic and biphasic culture media. The number of amastigotes per spleen was calculated[19]. After achieving parasitic load of 1×10^6 parasites spleen the drug (SAG) at a dose of 20 mg/kg or AMB at a dose of 1 mg/kg were administered through intraperitoneal route once daily for 5 days[20]. After completion of treatment the mice were allowed for 4 wk rest and then amastigotes burden of spleen was determined[16] in smear and culture also. Serum antimony assay was done with atomic absorption spectrometer[10].

Statistical analysis: The statistical significance of difference between means was assessed by the Z test and that of differences between proportions by Chi-square test (unpaired, paired). Antimony level data relating to various time periods were normalized using a base 10 log transformation. To determine the effects of time and plasma levels of two groups of patients (sensitive and resistant) and their interaction, repeated measures analysis of variance (ANOVA) was performed.

Results

Baseline characteristics of 282 patients with VL are shown in Table I. Intermittent fever, shivering or rigor, diminution in appetite, loss of weight, enlargement of spleen and liver, leucopenia and anaemia were the main features of the disease.

District-wise distribution of patients from 14 districts of Bihar showed that maximum number of patients were from Patna, followed by Bhojpur. There was a significant variation from district to district (X^2=51.16, P<0.001). ANOVA for eight districts with more than 10 patients also showed a significant difference among the number of patients coming from these districts (X^2=18.87, P<0.01) (Table II). Isolates from 106 (37.6%) patients were sensitive and 176 (62.4%) were resistant to SAG. Chi square test was applied to assess the variation in the proportion of

Table I. Baseline characteristics of patients, with visceral leishmaniasis (n=282)

Male	65
Age, yr	28 (6-68)
Duration of illness, months	2.4 (0.5-6.5)
Spleen size, cm	6.5 (1.0-13.2)
Liver size, cm	2.3 (0.0-10.4)
Parasite density score*	2.4 (1.0 -5.0)
Haemoglobin, g/dl, geometric mean (range)	7.5 (4.4 -12.5)
WBC count x 10^3, cells/mm^3, geometric mean (range)	3.8 (2.1-8.6)
Platelet count x 10^3, cells/mm^3, geometric mean (range)	119 (90-250)

Values are mean (range), unless otherwise indicated

*The density of the parasites was graded by microscopical examination at x 100, on a log scale from 0 (no. parasites/ 100 fields) to 6 (>100 parasites/ field)

Table II. District-wise distribution of patients and sensitivity of their isolates to SAG *in vitro* (n = 282)

District (N)	Sensitive (%)	Resistant (%)
Muzaffarpur (14)	4(28.5)	10(71.5)
Chapra (24)	10(41.7)	14(58.3)
Vailshali (34)	14(41.1)	20(58.9)
Sitamarhi (4)	4(100)	0
Samastipur (34)	4(11.8)	30(88.2)
Motihari (4)	4(100)	0
Madhubani (2)	0	2(100)
Begusarai (10)	6(60)	4(40)
Siwan (8)	6(75)	2(25)
West Champaran (12)	2(16.7)	10(83.3)
Patna (96)	40 (41.7)	56(58.3)
Bhojpur (30)	6(20)	24(80)
Gopalganj (4)	0	4(100)
Jehanabad (6)	6(100)	0
Total (282)	106(37.6)	176(62.4)

x^2=51.16, df=13, *P*<0.001 (ANOVA for all 14 districts)

x^2=18.87, df=7, *P*<0.01 (ANOVA for 8 districts with >10 patients)

Table III. Drug response of isolates in infected macrophage culture, experimental infection of visceral leishmaniasis produced by these isolates, and in patients

Category	Sensitive (%)	Resistant (%)
Infected macrophages	41 (40)	61 (60)*
Experimental infection	56(55)	46 (45)
Clinical (in VL patients)	47(46)	55(54)

No. of samples tested in each group = 102

**P*<0.01 compared to experimental infection (Mc Nemar's test)

sensitivity with respect to the endemic area of kala-azar. The difference between two groups was not significant (X^2 =10.97, *P*>0.05).

Table III shows the drug (SAG) response of isolates in infected macrophages, experimental infection of VL produced by these isolates and in patients. In order to compare the proportion of resistance in infected macrophages, experimental infection and VL patients, 102 isolates which were common to all the three categories, were taken into consideration. Mc Nemar's test on paired samples was applied between infected macrophage and experimental infection, infected macrophage and VL patients, and VL patients and experimental infection. A statistically significant difference in proportion of resistance to SAG was observed between infected macrophages and experimental infection (*P*<0.01). AMB cleared parasites from all isolates, cured infection in all animals and cured all patients.

Geomatric mean values (transformed back to original scale) of plasma antimony levels in the groups of antimony sensitive and resistant patients, at different time points are shown in Table IV. *P* values for the interaction between time and groups and main effects of time and group obtained by using repeated measures ANOVA are also shown. The effect of time was significant for all pairs of time intervals (*P*<0.001). However, the differences in mean antimony levels between the sensitive and resistant groups was not significant. The group by time

Table IV. Antimony level in plasma of sensitive and resistant patients collected at 2, 8 and 24 h after intramuscular injection in SAG

Plasma of patients	Antimony level in µg/ml*(95%CI)			*P* values**		
	2 h	8 h	24 h	Group	Time	Groups and Time
Sensitive (n=47)	11.5 (11.16-11.94)	6.78 (6.47-7.08)	2.74 (2.45-3.04)	0.33	0.001	0.001
Resistant (n=55)	11.02 (10.33-11.75)	6.32 (5.83-6.85)	3.47 (3.09-3.89)			

*Values are geometric means and 95 per cent confidence intervals (CI), are transformed back to original scale.

**Repeated measures ANOVA.

SAG, sodium antimony gluconate

interaction was significant ($P<0.001$) which indicated that the difference between sensitive and resistant groups varied significantly between the three time points of blood collection (Table IV). The mean antimony levels showed a decreasing trend over time in both the groups of patients.

Of the 282 patients, 123 were enrolled in SAG treatment group. All patients who were reluctant to be included in SAG group, 19 withdrawn patients due to development of toxicity and 8 patients who had relapsed were treated with AMB and all were cured.

In SAG group 21(17%) patients developed serious cardiac toxicity and were withdrawn from the study. 19 patients after a gap of 10 days when cardiac problem stabilized were treated with AMB. Two patients could not be saved even after withdrawal and died of cardiac arrhythmia and cardiac arrest.

Ten (8.1%) patients developed arthralgia, 5 (4%) developed metallic taste in mouth, 13 (10.6%) developed rise in liver enzymes, 5 (4%) fall in haemoglobin and 5 (4%) rise in serum creatinine in SAG group. In some patients with cardiac toxicity, rise in liver enzymes and serum creatinine also occurred. In AMB group shivering or rigor during infusion was observed in 86 (48.9%), rise in serum creatinine in 8 (4.5%), rise in liver enzymes in 2 (1.1%) and fall in haemoglobin only in 2 (1.1%) patients. No patient was withdrawn from AMB group due to toxicity and no patient died.

Discussion

Our study indicated that patients of VL coming from various districts of Bihar to Patna for treatment carried SAG resistant strains of *Leishmania donovani*. Earlier studies[21,22] showed that these resistant cases of VL were caused by resistant strain of *L.donovani*, and not by *L. tropica*[23]. This study also indicated that plasma levels of antimony were not different in the SAG resistant and sensitive groups of patients suggesting that resistant patients were not hyperexcreter of the drug.

Resistance to antimony was more apparent in infected macrophages than in mouse model. This difference might be due to two factors (*i*) antileishmanial activity of SAG in man and animal is supported by body immune system which is not available to macrophages *in vitro*; and (*ii*) that parasites become less resistant to SAG as a result of cultures in 6-7 subpassages[24].

The reason for this high resistance of parasite to SAG in Bihar might be due to haphazard and low doses of the drug used in Bihar. It has been shown in experimental studies that discontinuous exposure of *L. donovani* to SAG can lead to emergence of drug resistant clones of *L. donovani*[25,26].

It has been shown experimentally that drug resistance in parasite can be reversed by inhibiting glutathione biosynthesis[27]. Such drugs need to be developed. Comparing AMB was found to be a much superior drug than SAG as it cured all patients, cleared all isolates of parasites and cured VL in all mice. Toxicity of amphotericin B has been minimized by taking some precautions and could be used in rural set up[28].

In the laboratory it was also shown that amphotericin B niclosamide and clofazimine caused strong inhibition of oxygen uptake by *L. donovani* promastigotes[29]. Such compounds need to be evaluated for drug development for VL. Experimentally it was also shown that combination of compounds achieved higher rate of killing of promastigotes[27] but till now such useful combinations had not been found with the available drugs in the market. The mechanism of drug resistance is complex. The mechanism operative in laboratory producing resistant strains of *L. donovani* was not operative in field isolates obtained in India[30].

We conclude that SAG resistant strains of *L. donovani* were widespread in Bihar causing drug resistant disease and till other suitable effective drug is available, AMB can replace SAG as a first line drug.

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