Original Articles

Leishmania donovani: a model of unresponsiveness to sodium stibogluconate

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Abstract

Objective: To develop a model of sodium stibogluconate (SSG)-unresponsive L. donovani.
Design: Experimental study.
Setting: Institute of Endemic Diseases, University of Khartoum.
Subjects: Isolates of L. donovani from visceral leishmaniasis endemic areas in eastern Sudan.
Methods: Thirty leishmania donovani complex isolates were made unresponsive to sodium stibogluconate (SSG) by exposure to increasing concentrations of the drug in a cell-free system.
Results: The initial drug concentrations that inhibited the growth of 50% and 90% (IC50 and IC90) of the isolates were calculated as 0.4±0.04 mg/ml and 0.8±0.1 mg/ml respectively. Following induction of resistance, IC50 and IC90 rose to 50 and 35 folds of the initial values respectively ($p<0.05$). The time and the number of passages required for the isolates to recover to early log phase following exposure to SSG increased initially with increasing SSG concentrations and later declined despite continued increasing concentrations. SSG unresponsiveness was abolished when the SSG concentration was increased to three folds.
Conclusion:In vitro resistance to SSG was successfully induced in L. donovani isolates that can serve as a model for studying underlying molecular basis of SSG-unresponsiveness. SSG-unresponsiveness probably develops in the wild over time as a result of repeated exposure to low SSG concentrations.

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Introduction

Visceral leishmaniasis (VL) is a chronic febrile illness caused by a protozoan parasite of the genus Leishmania. It is mainly a disease of tropical and subtropical regions of the world with 0.5 million new cases reported annually[1]. Pentavalent antimonial agents are the treatment of choice for all forms of leishmaniasis[2,3]. Treatment failure in patients with VL is becoming a common problem in many endemic areas[4, 5]. Mechanisms of resistance to pentavalent compounds have not been fully elucidated, but changes in the pharmacokinetics of the drug, the use of suboptimal doses, concurrent diseases and host immune status, have been cited as reasons for apparent resistance/failure of treatment[6, 7, 5]. In vitro development of drug-resistant parasites considerably facilitates studies on the nature, molecular basis of drug resistance and the mode of action of given drugs. The advantages of using an in vitro system for the induction of drug resistance include the development of parasite cell lines with drug levels comparable to those in vivo. These drug-resistant parasite cell lines can tolerate levels of the drug that are lethal in animal models.

This study aimed to induce/augment unresponsiveness to sodium stibogluconate(SSG) in L. donovani isolates from visceral leishmaniasis endemic areas of Sudan. The study also aimed to show that resistance to SSG can be abolished by increasing the drug concentration/ dose.

Materials and methods

Leishmania parasite isolates:

Thirty Leishmania isolates were collected from lymph nodes/bone marrow aspirates as part of routine parasitological investigation for VL suspected patients. Samples collected were from eastern, southern and western Sudan. Part of the aspirate was smeared onto slides and stained with Giemsa stain, the rest was dispensed into lysis buffer for molecular characterization of the isolates.

Molecular characterization of the isolates:

The Polymerase chain reaction (PCR) was performed using a set of gene specific primers for mini-circle kinetoplast kDNA[AJS3 5`GGGGTTGGTGTAAAATAGGG-3` and DBY 5`CCAGTTTCCCGCCCCGAG-3`; Inqaba Biomedical Industries, Hatfield, South Africa].The reaction volume was 50µl per sample in 0.2 ml thin walled micro-centrifuge tubes, the mixture contained 5µl of 10X reaction buffer (Promega, Madison WI, USA) with a final concentration of 1X, 2µl of 20 mMdNTPs mixture (0.2 mM of dTTP, 0.2 mM

dATP, 0.2 mM dCTP & 0.2 mM dGTP), 3µl of 25 mM MgCl2 (Promega, Madison WI USA), 2.0µl of primers mixture and 0.25µl of thermo-stable Deoxyribonucleic acid (DNA) polymerase (Promega, Madison WI, USA) (5U/µl).To each PCR tube, 3µl of Deoxyribonucleic acid (DNA) templates were added, and the PCR mixture was completed to 50µl with double distilled water. The following programme was fed to the PCR machine and was run for 35 cycles: initial denaturation at 94° C for 3 minutes, annealing at 64°C for 1 minute, extension at 72°C for 1 minute and denaturation at 94°C for 30 seconds. A final extension cycle at 72°C for 10 minutes was included. Reference strains of Leishmania donovani (RS) were kindly donated by Dr AH Sharief, Tropical Diseases Research Institute, Khartoum, Sudan, and Dr. S. Croft, London School of Hygiene & Tropical Medicine, UK) were included in the analysis.

Isolation and cultivation of parasites:

Leishmania parasite isolates were injected into culture bottles containing biphasic media (Novy, McNeal, Nicole NNN) consisting of solid-phase agar mixed with defibrinated rabbit blood and overlaid with RPMI-1640 (Rosen Park Memorial Institute) supplemented with 10% Faetal Calf Serum (FCS) and 1% of penicillin/streptomycin solution (10,000 units penicillin & 10 mg streptomycin per ml). All cultures were incubated at 24°C. Log phase promastigotes were transferred into a 50 ml tissue culture flasks containing RPMI-1640 supplemented with 10% FCS and 1% penicillin/streptomycin solution.

Induction of resistance:

Early log phase promastigotes were counted and made to a concentration of $2.5x10^6$ parasites/ml in FCS-free medium. One ml of the promastigotes suspension was exposed to increasing concentrations of SSG in 6-wells plates (SSG®, Albert David, Kolkata, India) for 12 hours at 25°C. The SSG concentrations were as follows: 0.05 mg/ml, 0.15 mg/ml, 0.45 mg/ml, 1.35 mg/ml, 4.05 mg/ml, 12.15 mg/ml and 36.45 mg/ml.Following 12-hours incubation, cultures were centrifuged at 2800 rpm for 10 minutes at 4°C and the parasite pellets were re-suspended in fresh medium supplemented with 20% FCS. On day 15, live parasites were expanded to early log phase (10-15 x 10^6 promastigotes/ml) to be exposed again to the higher next concentration of SSG. Control cultures were maintained in drug-free complete medium simultaneously with each batch.

Determination of resistance levels:

Induction of resistance was shown by failure of the drug to reduce parasite count after incubation. Inhibitor concentrations IC50 and IC90 were determined for study isolates before and after induction of resistance. In brief: 106 promastigotes/ml were exposed to SSG over a range of concentrations in serial dilutions in 96 well plates. The total number of promastigotes/ml was counted using a haemocytometer and the IC50 and IC90 values for each resistant isolate were calculated.

Results

Induction and selection of SSG-unresponsive L. donovani isolates:

The mean drug concentrations that inhibited the parasites growth by 50% and 90% (IC50 and the IC90) were initially determined (wild type) and were found to be 0.4 ± 0.04 mg/ml and 0.8± 0.1 mg/ml, respectively. Following successful induction of resistance/unresponsiveness, the IC50 and the IC90 were found to be 50 and 35 folds higher than those of the wild type, respectively ($p<0.002$) (table 1).

Table 1: Sensitivity of study wild type (WT) and resistant (P-12.15R) isolates to SSG expressed as IC_{50} & IC_{90}

Sodium Stibogluconate concentration	*Wild Type isolates (30 isolates)*	*Resistant isolates(30isolates)*
IC_{50} (mg/ml)	*0.4 ± 0.04*	*19.5 ± 0.04*
IC_{90} (mg/ml)	*0.8 ± 0.1*	*34.4 ± 0.6*

IC50 & IC90 were expressed as means ± SD

IC50=the drug concentration that inhibits 50% of parasite growth.

IC90=the drug concentration that inhibits 90% of parasite growth.

The mean parasite counts (2.5x106 parasites/ml) for the isolates did not change when incubated for 12 hours with the first SSG concentration (0.05 mg/ml). The mean counts for the recovered parasite from the previous experiment dropped to 2,000,000 parasites/ml when incubated with 0.15 mg/ml of SSG for 12 hours ($p>0.05$). The same isolates after recovery from the drug pressure and incubation with SSG at 0.45 mg/ml dropped to a mean parasite count of 1,800,000 parasites/ml ($p>0.05$). Following complete recovery, the isolates were subjected to SSG concentration of 1.35 mg/ml, where the mean parasite count dropped significantly to 800,000 parasites/ml ($p<0.001$). Following recovery and incubation with the next SSG concentration of 4.05 mg/ml, the mean parasite count dropped slightly to 2,200,000 parasites/ml) ($p>0.05$). At 12.15 mg/ml of SSG, the recovered isolates from the previous experiment did not change at all and the mean parasite count remained at 2,500,000 parasites/ml ($p>0.05$), in all SSG concentration the different sensitivity between 30 isolates was observed (Fig. 1). The later experiment was repeated three times. The mean parasite counts for the isolate that were unaffected by SSG at a concentration 12.15 mg/ml fell to 00 parasites/ml when the SSG concentration was increased to 36.45 mg/ml in three consecutive experiments using the same isolates frozen backup (Fig. 1). Control cultures (drug-free complete medium)

did not show any significant change in parasite counts with time.

The time and the number of passages required for isolates to recover and expand adequately to early log phase (10-15x106 parasites/ml) gradually increased and then dropped. Four passages over 3 weeks were needed to move from SSG concentrations of 0.15 mg/ml to 0.45 mg/ml. Three passages over 3 weeks, were required to move from 0.45mg/ml to 1.35 mg/ml. Six passages over 5 weeks were needed to move from 1.35 mg/ml to 4.05 mg/ml. Eight passages over 7 weeks were needed to move from 4.05 mg/ml to 12.15 mg/ml. Five passages over four weeks were needed for adequate expansion after exposure to 12.15 mg/ml. Three trials to increase the level of resistance to 36.45 mg/ml were unsuccessful.

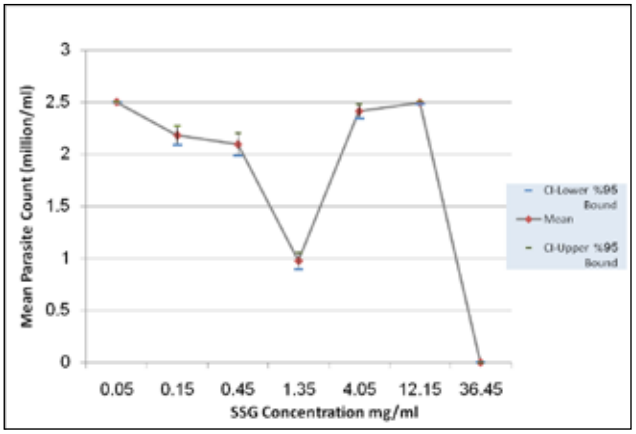

Fig. 1.Dose-response curve of study isolates exposed to increasing concentrations of SSG.Parasite counts are mean counts for the 30 study isolates expressed as parasites/ml

Discussion

Drug unresponsive visceral leishmaniasis is a major problem in the Indian subcontinent and is predicted to rise markedly in the HIV/AIDS era especially in Africa, India & Southern Europe. Induction of pentavalent antimonials (SbV) resistance using promastigotes was successfully reported by many investigators[8, 9]. The use of drug resistant promastigotes is believed to obviate the problem of starting with a heterogeneous population from which an initial selection of a parasite population with innate resistance/unresponsiveness to the drug could have occurred. The use of promastigotes instead of amastigotes could be justified because promastigotes are inherently partially resistant to pentavalent antimonials and so the resistance mechanism is already in place. Since SSG (SbV) resistance mechanisms in promastigotes are not clear, drug-resistant promastigotes provide a useful tool in experimental chemotherapy and potential application in the primary screening of candidate anti-leishmanial drugs. Furthermore, studying the processes that involve genetic changes and mechanisms that originate in the nucleus is not usually affected by the close developmental stages of the organism. A theoretical problem may arise in interpretation of results obtained using a parasite stage (promastigotes) that is not directly related to the situation in nature[10]. However, synonymy could be drawn between in vitro situations of culture and subculture in the laboratory and natural situations. In nature, sand flies carry parasites between patients or between patients and the reservoir host.In addition, in vitro amastigotes cannot be claimed to be identical to those developing inside the human host. Genetic changes involved in the development of promastigotes resistant to SSG are stable after differentiation into amastigotes and passage through animals[11]. This finding furthermore justifies our use of promastigotes as an in vitro model for induction/augmentation of unresponsive to SSG.

The experimental use of drug-resistant promastigotes of Leishmania can serve a number of purposes: it provides a tool for the investigation of the mechanisms of SSG resistance; it may suggest chemotherapeutic strategies that can overcome drug resistance and makes it possible to include an SSG (SbV)-resistant line in drug testing programs. Culture/in vitro studies have an advantage over animal studies because parasites can be exposed to drug levels which are lethal to animals.

Successful induction/augmentation of unresponsivenessin the study isolates was supported by the fact that the IC_{50}& the IC_{90} for SSG of the resistant isolates were significantly greater (50 time & 35 times) than those of the wild isolates. Furthermore, the time and number of passages needed for the isolates to recover to early log phase following exposure to increasing SSG concentrations were shorter when it was expected to be longer. Failure of the parasite to grow when the SSG concentration was tripled probably indicated that the degree of drug unresponsiveness that developed could be overcome by an increase in the SSG concentration. The study findings provide support for claims that parasite unresponsive to SSG could develop when low doses of SSG are used. The findings also provide support to the Sudanese National Leishmaniasis Control Programme of not adhering to the upper dose limit of 850 mg/day suggested by World Health Organization. Emergence of the SbV-resistant phenotypes could happen early during the initial treatment phase, or later during the first treatment and/or sometime during a period of multiple courses of treatment in stepwise manner depending on the rate of elimination of the SbV (SSG) sensitive strains. Alternatively, sub-curative tissue levels of SSG (SbV) could enhance the survival of tolerant parasite strains whose mutation to drug resistant may be facilitated (genetic selection). Care should be taken to ensure that the initial clinical treatment is adequate in dose and duration to prevent emergence of resistant strains in the wild.

The in vitro isolation and characterization of parasite

strains unresponsiveness to SSG considerably facilitates studies on the nature and the molecular basis of drug resistance and the mode of action of given drugs. This could be elusive since studies on miltefosine-resistant promastigotes failed to show the presence of any DNA expression either as circular or liner amplicons. The previous study confirmed the stability of the resistant phenotypes for at least 3 months in the absence of drug pressure. Stable mutations in one or more proteins may have accounted for the resistant phenotype[12].

Conclusion

In vitro resistance to SSG was successfully induced in L. donovani isolates. SSG unresponsiveness probably develops in the wild over time as a result of exposure to low SSG concentrations. These isolates could act as a useful model to studying the molecular basis of SSG unresponsiveness.

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