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The P2 adenosine transporter as an indicator of drug resistant Trypanosoma brucei

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Abstract

We have analysed the *Trypanosoma brucei* gene TbAT1, which codes for the P2 adenosine transporter, to investigate a possible link between the presence of mutations in this gene and trypanocidal drug resistence. About 70% of *T. b. gambiense* isolates from a focus in North Western Uganda with high treatment failure rates following melarsoprol therapy had a mutations within TbAT1. Unexpectedly, all individual isolates contained the same set of nine mutations in their TbAT1 genes. Identical sets of mutations were also found in a multi-drug resistant *T.b.rhodesiense* isolate from South Eastern Uganda and in a *T.b.gambiense* isolate from a relapsing patient from Northern Angola. Quantitative analysis of isolates from North western Uganda revealed that melarsoprol relapse patients have significantly more infections with mutant TbAT1 ($c^2 = 6.70$; d.f = 1; p = 0.0097, two tailed). Genetic knock out of TbAT1 of bloodstream trypanosomes did no affect in vitro cell proliferation, as well as mouse infectivity. However, the cells were rendered up to four times less susceptible to melarsoprol, melarsene oxide, and diminazene aceturate. In vivo experiments revealed that mouse infections of TbAT1 knock out clones could not be treated with four injections of 2mg/kg melarsoprol, while the same dose cured all the animals infected with the wild type clone. We conclude that loss of P2 transport activity significantly contributes to the increased drug resistence in African trypanosomes.

Key words: *Trypanosoma brucei*, P2 Adenosine transporter, Drug resistance

Introduction

There are increasing reports of relapses following melarsoprol therapy in some *T. b. gambiese* endemic areas of Africa namely Sudan, Uganda, Congo and Angola. In Uganda, up to 30% of treated patients are reportedly non-responsive to Melarsoprol therapy (Legros et al. 1999). Recent data from in vitro sensitivity studies has revealed that the relapses in North Western Uganda are due to reduced drug susceptibility of the trypanosomes to melarsoprol (Matovu et al. 2001). Resistance to Melarsoprol and diamidines was associated with a deficiency in the P2 transporter of *T. brucei* (Carter and Fairlamb, 1993; Barret et al. 1995). More recent studies have revealed several mutations in the gene which codes for the P2 transporter, TbAT1, in a laboratory-derived arsenical-resistant stock of *T. brucei*. When expressed in yeast, this mutated P2 transporter could no longer import Melarsoprol, as well as other compounds (Mäser et al. 1999). In these trypanosomes, resistance resulted from a reduced net drug uptake.

In view of the challenges to HAT management by Melarsoprol treatment failures, the aim of this study was to investigate a possible link between mutations in the TbAT1 transporter gene and the occurrence of relapses

after Melarsoprol treatment, and to demonstrate if mutations in TbAT1 might contribute to the high rates of treatment failures after Melarsoprol chemotherapy. Further confirmation of the TbAT1 involvement in drug resistance was generated from knock-out of the gene in a *T. brucei* clone.

Sfa NI RFLP analysis

Earlier work on the laboratory-derived Melarsoprol-resistant stock STIB 777R had demonstrated that a SfaNI restriction site present in the wild-type TbAT1 gene had disappeared, while a new site was generated 323 bp further downstream by an independent mutation (Mäser et al. 1999). In order to validate and expand these findings, a 678 bp fragment from portions of the same DNA as used for SSCP was analyzed by SfaNI digestion. Three different RFLP banding patterns were obtained with these samples (Fig. 1). The banding characteristic of the wild type STIB 777S was seen in 27 (41.5%) of the patients, while the pattern characteristic of the mutated STIB 777R was found in 12 patients (18.5%). The third pattern, which was evident in 26 patients (40%), represented a composite of the wild type and the mutated patterns, containing all fragments from both prototype patterns (Fig. 2). A perfect correlation between the results of RFLP and SSCP analyses was observed for all of the 65 samples analyzed.

Fig. 1. SfaNI RFLP of TbAT1 reveals three different banding patterns. Lanes 1 and 2: DNA from cultured Melarsoprol-sensitive (777S) and -resistant stocks (777R), respectively; lanes 3 - 6: representative samples for the analysis of PCR fragments amplified from patient CSF (patients F015, R027, R015 and R026). Lanes 1 and 3 represent the RFLP of wild type TbAT1; lanes 2 and 4 represent the RFLP of the mutated TbAT1 present in stock 777R; lanes 5 and 6 represent a mixed RFLP containing all bands from both prototype patterns.

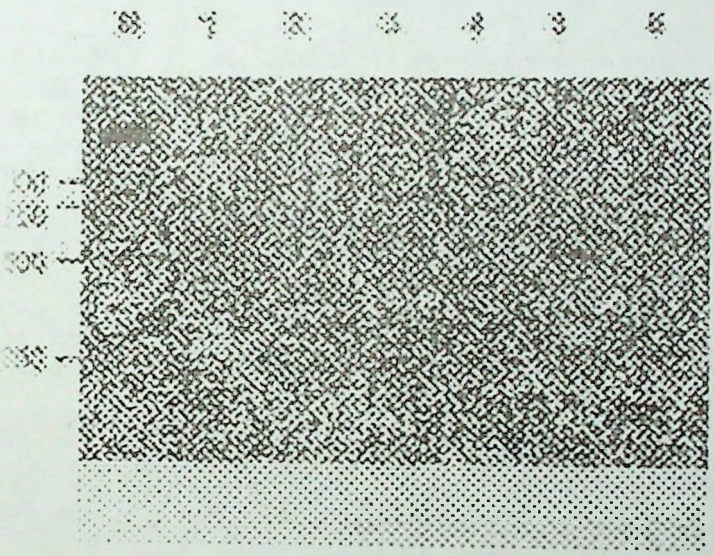

Table 1: Distribution of SfaNI RFLPs in newly infected and relapse cases. Wild-type: RFLP pattern represented by STIB777S; mutant: RFLP pattern represented by STIB777R; mixed: both patterns present simultaneously.

CASES	wild-type	mutant	mixed	
newly infected	14 (64)	5 (23)	3 (14)	22 (100)
relapsed	13 (30)	7 (16)	23 (54)	43 (100)
TOTAL	**27 (41)**	**12 (19)**	**26 (40)**	**65 (100)**

A quantitative analysis of the RFLP data is summarized in Table I. Relapse patients have significantly more often infections with trypanosomes with either the mutant or the mixed SfaNI RFLP than do the newly infected patients (likelihood ratio $c^2 = 6.70$; d.f. = 1; p = 0.0097, two-tailed).

DNA sequencing

Full-size TbAT1 was amplified and sequenced from all CSF samples where RFLP and analysis had indicated the presence of mutations, as well as representative relapse samples which displayed wild type patterns. DNA sequencing of the TbAT1 genes from all isolates indicated as mutant revealed the presence of nine mutations when compared to the sequence of STIB 777S (Fig. 2). Unexpectedly, all 12 sequences analyzed contained the same set of nine mutations. The same set was also detected in the TbAT1 gene of a multi-drug resistant isolate from South Eastern Uganda (STIB 871). Eight of the nine of these mutations were also found in the laboratory-derived Melarsoprol-resistant stock STIB 777R. Five of the nine mutations lead to an amino acid substitution (Leu71Val, Ala178Thr, Gly181Glu, Asp239Gly and Asn276Ser), while a sixth mutant represents a deletion of the entire codon for F316. The remaining three mutations are silent (C21T, T144C and C471T). The laboratory-derived stock STIB 777R contained five of the six expressed mutations, was lacking the deletion of the Phe316 codon, but contained an additional expressed mutant Leu380Pro. It also contained all three silent mutations, plus one additional one (C501T). DNA analysis of TbAT1 from the CSF of six relapse patients where RFLP/SSCP analyses had given a wild-type pattern fully confirmed the absence of mutations and demonstrated that the DNA sequence of the entire open reading frame is identical to that of the reference stock STIB 777S.

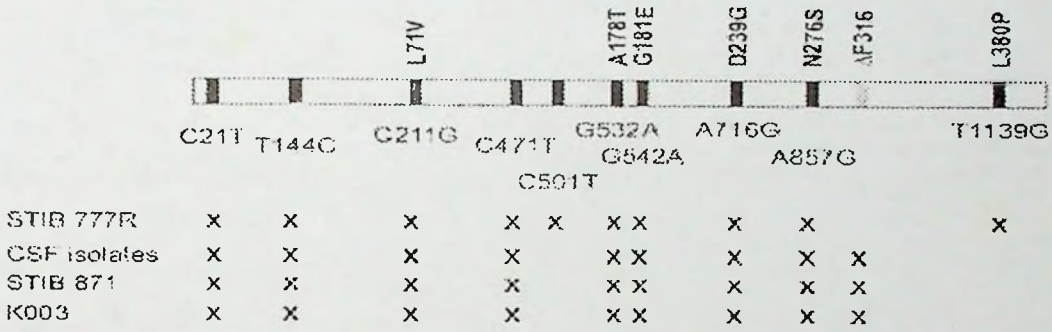

Fig. 2: Position and sequence of mutations is highly conserved between the laboratory-derived Melarsoprol-resistant stock STIB 777R and field isolates.Mutations L71V, A178T, G181E, D239G and N276S are conserved between the laboratory-derived Melarsoprol-resistant stock STIB 777R and the various field isolates. The deletion of an entire codon (DF316) is absent in 777R, but is present in all field isolates, while L380P was detected only in 777R. The transition G532A eliminates a SfaNI site present in the wild-type sequence, while the transition A857G generates a new SfaNI site.

Genetic knock-out of TbAT1

A genetic knockout of TbAT1 was performed to investigate whether it plays a significant role in drug import across the trypanosomal cell membrane. This was done by replacement of the gene with markers for neomycin and puromycin resistance. Fragments 700 and 300bp of the TbAT1 un-translated regions respectively were amplified by the PCR. They were ligated into a vector to flank genes for neomycin or puromycin resistance. Linearlised constructs with either marker were introduced into the trypanosomes by electropolation. The construct for the first round electropolation contained the neomycin resistance marker. The trypanosomes were then cultured in presence of neomycin, which selected out all the non-transformed individuals. Two clones arising from this selection were then transformed with the construct containing the puromycin resistance gene. The trypanosomes were then subjected to selection with both neomycin and puromycin added in the medium, with the expectation that double resistant cells would be those in which the TbAT1 was successfully replaced.

Correct integration of the antibiotic resistence markers was confirmed by Southern blotting.

Effects of TbAT1 deletion on the phenotype

The TbAT1 knock-out strains did not show any obvious differences in morphology, motility or proliferation in culture when compared to wild-type BS221. The population doubling time did not significantly vary and remained around eight hours, clearly indicating that TbAT1 is not essential for cell survival. This points to existence of alternative routes for purine salvage in trypanosomes.

To investigate whether loss of TbAT1 had an effect on drug susceptibility of the derivative clones, a long-term viability assay was performed (Kaminsky et al. 1989) in which the trypanosomes were exposed to serial drug dilutions over a period of ten days. The results given in Table 1 demonstrate that the knock-out clones exhibited minimum inhibitory concentrations (MICs) for melarsoprol, melarsene oxide and diminazene aceturate which were no more than four-fold higher than those of wild-type BS221. This observation was surprising: it implied that there are additional routes of drug entry into the cells besides the P2 transporter.

Table 2: Minimum inhibitory concentrations of melarsoprol, melarsene oxide and diminazene aceturate for wild-type BS221 and heterozygous and homozygous TbAT1 knock-out strains. The 10 day assay was performed twice with each drug concentration represented in triplicate

Clone	Melarsoprol (ng / ml)	Melarsene oxide (ng / ml)	Diminazene (ng / ml)
wt BS221	18	4.5	25
01 (TbAT1 +/-)	18	4.5	50
02 (TbAT1 +/-)	18	18.0	50
11 (TbAT1 -/-)	18	9.0	50
12 (TbAT1 -/-)	36	18.0	100
21 (TbAT1 -/-)	36	9.0	100
22 (TbAT1 -/-)	36	4.5	100

We then exposed the cells to a high excess of melarsene oxide to monitor drug induced cell lysis in the knock-out clones and wild-type BS221. Fig. 3 presents the pooled data from two independent, very similar sets of experiments. Wild type cells rapidly absorbed the drug and were essentially completely lysed within 30 minutes ($t/2 = 22.6$ min). A heterozygous strain from which one allele of TbAT1 had been deleted was considerably more resistant to lysis than the wild type ($t/2 = 33.5$ min), and deletion of the second allele of TbAT1 further increased the survival time in melarsene oxide ($t/2 = 42.5$ min). This indicates that both copies of TbAT1 are required for full drug sensitivity (haploid insufficiency), demonstrating that the deletion of TbAT1 reduces drug sensitivity of the cells to some extent, but does not confer full drug resistance.

Effects of TbAT1 deletion on melarsoprol resistance in mouse infections

To investigate if the rather slight increase in drug resistance observed for the knock-out clones in culture would translate into melarsoprol resistance in the infected animal, mice were infected with wild-type BS221 and with two independent TbAT1 knock-out clones. After the onset of parasitemia, animals were treated intraperitoneally with melarsoprol at 2mg/kg and 10 mg/kg for four consecutive days.

There was a drastic reduction of parasitaemia in all treated mice, while the untreated controls died within 5 days post-infection. Of the mice infected with wild type BS 221, there was no re-appearance of parasitaemia at either dose of melarsoprol applied. On the other hand, all mice infected with the two knock-out clones relapsed after treatment with 2mg/kg melarsoprol and died due to parasitaemia by day 20 post–treatment. None of the mice infected with the two knock-out clones and treated with 10mg/kg melarsoprol has relapsed.

Discussion

Our observations indicate presence of an identical, complex set of mutants within TbAT1 in trypanosomes of different species, and from geographical locations which are hundreds of kilometers apart. In addition, a very similar set of mutations was also present in STIB 777R which was selected in the mouse for Cymelarsan resistance (pospichal et al. 1994).

A correlation of the clinical status of the patients (newly infected vs relapse) with the TbAT1 genotype of their trypanosomes demonstrated a strong propensity of relapse patients to harbour parasites with mutant TbAT1 genes. However, about 30 % of relapse patients analyzed showed a wild-type pattern in RFLP analysis which was confirmed by sequencing. This observation strongly indicates that TbAT1 may contribute to, but is not the only gene responsible for conferring refractoriness to Melarsoprol. This view corresponds with ample experimental evidence that trypanosomes isolated from relapse patients after Melarsoprol treatment failure are not necessarily highly resistant to Melarsoprol in culture conditions (R. Brun, personal communications), and

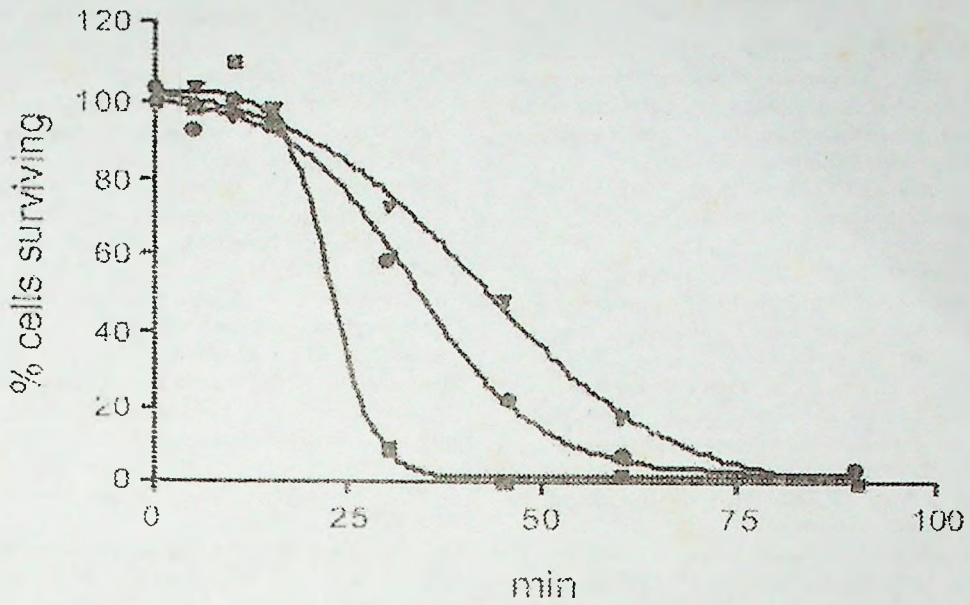

Fig. 3. Trypanosome lysis in the presence of 10mM melarsene oxide is more rapid for wild type than TbAT1 knock-out cells. Trypanosomes were incubated in growth medim in the presence of 10 mM melarsene oxide, and cell lysis was determined at the times indicated. Average values of two independent experiments are given. Black squares: wild type BS221, filled circles: first round knock-out (heterozygous for TbAT1), filled triangles: double knock-out (both alleles of TbAT1 deleted).

many patient-related variables such as concomitant infections, nutritional status and others may well contribute to the 3 - 9 % treatment failure rate which are considered normal (Wang 1995; Pepin and Milord 1994). However, genetic mechanisms in the parasite may contribute to the marked increase, over short time spans and in individual regions, of relapse rates to up to 30 % (Legros et al. 1999).

Drug sensitivity assays in culture did not portray the knock-out clones as absolutely resistant, but rather as having reduced drug susceptibility. It is therefore apparent that even in complete absence of P2 activity, the drug still finds its way into the cytosol and exerts effects on its still existent target. The observation that resistance to melarsene oxide in culture is already considerably increased in cells haploid for TbAT1 might be of particular relevance to the in vivo situation. Mutations in only one copy of the gene might already confer a considerable selective advantage to the mutated parasites within a patient under melarsoprol treatment.

On a pratical note, the demonstrated sensitivity of SfaNI RFLP analysis for the detection of mutated TbAT1 may be developed into a method of rapidly screening patients for the presence of trypanosomes with a mutated transporter. This will allow to identify infections that will not respond to treatment with drugs that rely on TbAT1 (melarsoprol and diminazene). Such may be treated with alternative compounds e.g. DFMO, isometamidium, or with combination chemotherapy.

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