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☐ **Brief Communication** ☐

Towards developing a diagnostic regimen for the treatment follow-up of *Trypanosoma brucei gambiense*

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Abstract: BALB/c mice infected with a high virulent strain of *Trypanosoma brucei gambiense* IL3707 were treated intraperitoneally (ip) with either Melarsoprol (Mel-B) or PSG(+) buffer as controls. The mice were subsequently monitored regularly for parasites by direct microscopic examination of their tail blood or buffy coat and by polymerase chain reaction (PCR). Mel-B was found to be an effective drug for treatment against *T.b. gambiense* because at the end of the first treatment schedule, all treated mice were negative for parasites even by PCR, while all the control animals were positive. Three of the five Mel-B treated mice, while parasitologically negative, were PCR positive between 53 and 80 days post infection (DPI), indicating that they still harbored an infection. All treated mice were subsequently negative for parasites even by PCR at 88 DPI. A combination of conventional microscopic examination and PCR offers a good prediction of cure following treatment of trypanosomosis.

Key words: *Trypanosoma brucei gambiense*, mice, Mel-B, treatment follow-up, diagnosis, PCR

Human African trypanosomosis (sleeping sickness) constitutes one of the major problems of public health in many African countries. The recommended treatment follow-up period for this infection is at least two years during which time no trypanosomes should be detected in blood or cerebrospinal fluid. Further, white cell counts in cerebrospinal fluid should remain less than 5/ml and the protein content less than 25 mg/dl on repeated examinations (WHO, 1998). However, it is often difficult to effect this follow-up, because many patients do not return for check-up after they feel better. Also, re-infection is possible during this lengthy follow-up period. The purpose of this study was to try to establish a procedure that would shorten the treatment follow-up period by combining conventional techniques that diagnose circulating trypanosome parasites in blood with PCR in infected mice treated with Melarsoprol.

Two groups of 5-week old male BALB/c mice

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each were individually challenged with $5x10^3$ trypomastigotes of *T.b. gambiense* IL3707 intraperitoneally (ip) in an inoculum of 0.5 ml of PSG(+) (phosphate, sodium, glucose) buffer. This parasite is a highly virulent strain as described earlier by Inoue et al. (1998). The mice were separated into two groups of 5 mice each and labeled as experimental and control groups respectively. At four day post-infection (DPI), mice from the experimental group were started on ip treatment with Melarsoprol (Mel-B) (obtained from WHO, Geneva) at a dose of 10 mg/kg/day (Poltera et al., 1981). The treatment was by 3 series of 3 injections (1 injection per day during 3 consecutive days) separated by 7 days of rest. In the control group, PSG was administered using a similar protocol.

Two methods were used to detect parasites from infected mice. In the first instance, a drop of blood was obtained by tail snip, spread as a thin smear on a microscope slide, and observed under a light microscope at X400 magnification. Negative samples were further examined by the more sensitive buffy coat technique as described by Murray et al. (1977). Briefly, infected blood was collected into a heparinized hematocrit tube and centrifuged at 15,000 *g* for 5 min. The buffy coat was collected onto a glass slide and examined through phase contrast microscopy (Inoue et al., 1998). The first series of diagnoses was done at 4 DPI (just before the start of the first schedule of treatment), 7 DPI (one day after the first schedule of treatment), 14 DPI (just before the second schedule of treatment), 17 DPI (a day after the second schedule of treatment), 24 DPI (just before the third and last schedule of treatment), and 27 DPI (a day after the last schedule of treatment). Thereafter, these diagnostic tests were conducted at regular intervals.

About 10 μl of blood from the tail of a mouse was pipetted into 100 μl of lysis buffer containing a final concentration of 10 mM Tris HCl (pH 8.0), 10mM EDTA (pH 8.0), 100 mM NaCl, 0.5% SDS and 300 μg/ml of proteinase K (Promega Biotech., USA). After incubation for 2 hr at 55°C (Eyela, Dry Thermobath MG-1100, Tokyo, Japan), 100 μl of phenol: chloroform (1:1) was added and then vortex mixed. The samples were then centrifuged at 4,000 *g* for 5 min at room temperature and the supernatant (containing DNA) was transferred to a new tube. This phenol:chloroform extraction procedure was repeated twice. To the supernatant was added 10 μl of 3 M sodium acetate (pH 5.2) and 250 μl of 100% ethanol, and frozen at -80°C for 30 min. These samples were subsequently thawed at room temperature. After centrifugation at 4,000 *g* for 15 min at 4°C, the supernatant was removed and 500 μl of 70% ethanol was added to the DNA pellet. The samples were again centrifuged at 4,000 *g* for 5 min at 4°C and the supernatant was removed. The micro-tubes with DNA samples were then air-dried (Eyela, Centrifugal Vaporizer, Tokyo, Japan). Each DNA sample was dissolved in 10 μl of Tris-EDTA buffer and kept frozen at –30°C until use.

DNA preparations were amplified in a final volume of 30 μl in a Perkin Elmer PCR machine (Gene Amp 2400, CA, USA). In preparation of the master mix, the following volumes were used; 20.4 μl DDW, 3 μl of 10x PCR buffer (Perkin Elmer, PE Applied Biosystems, CA, USA), 3 μl of 2 mM dNTPs (Perkin Elmer), 0.3 μl of VSG 117 primer (Science Tanaka Co. Ltd., Hokkaido, Japan) (Bromidge et al., 1993) (50 pmol/μl each of sense and antisense), 0.3 μl Taq DNA polymerase (Perkin Elmer), and 3 μl of DNA sample was used as template per reaction program. In the PCR reaction program, samples were incubated at 94°C for 10 min in an initial denaturation step, followed by 40 cycles at 94°C (30 sec), 51°C (1 min) and 72°C (2 min). The PCR reaction products were analysed by agarose gel electrophoresis and detected visually by UV transillumination (Atto Bioinstrument, Tokyo, Japan) of the ethidium bromide-stained gel.

A summary of the results from various diagnostic tests used to detect *T.b. gambiense* IL3707 in BALB/c mice that were either treated with Mel-B or PSG(+) is shown in Table 1. *Trypanosoma brucei gambiense* IL3707 is a highly virulent parasite in BALB/c, and because parasites were identifiable in blood as early as 4 DPI, both techniques by parasite

Table 1. Results of various diagnostic test used to detect *Trypanosoma brucei gambiense* (IL 3707) in BALB/c treated with either Mel-B or PSG(+)

		Days post-infection												
Test	Group	4	7	14	17	24	27	41	53	67	74	80	88	104
Tail blood[a)]	PSG(+)	4/5[b)]	1/1	—[c)]										
	Mel-B	4/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Buffy Coat[a)]	PSG(+)	4/5	1/1	—[c)]										
	Mel-B	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
PCR	PSG(+)	4/5	1/1	—[c)]										
	Mel-B	5/5	1/5	0/5	0/5	0/5	0/5	ND[d)]	2/5	3/5	1/5	3/5	0/5	ND

[a)]Parasitological observation of parasites by direct microscopy of blood smear and buffy coat, respectively

[b)]Results are shown as a proportion of the: $\frac{\text{Number of mice in which parasites were detected}}{\text{Number of mice tested}}$

[c)]All mice dead. [d)]Not done.

examination of tail blood and PCR were positive. All the mice that were treated by Mel-B were positive for parasites by both PCR and parasitological diagnosis at 4 DPI and just before the start of the first schedule of treatment. By 7 DPI, four mice from the control group died of trypanosome infection while the surviving animal was positive for infection by both parasitological diagnosis and PCR. By 14 DPI, all control mice died of trypanosomosis.

After the first dose of Mel-B (7 DPI), all treated mice were parasitologically negative till the end of the experiment at 104 DPI. Three of the five treated mice during the period between 53 and 80 DPI were PCR positive, indicating that they still harbored an infection. Throughout the experimental period, Mel-B treated mice maintained a normal packed cell volume (PCV) and retained a healthy general appearance (Mbati, 1997).

Detection of trypanosomes in the blood of an infected individual is the most ideal method for diagnosis of African trypanosomosis. However, it has very low sensitivity, though its variant the buffy coat technique is more sensitive (Kanmogne et al., 1996) with a sensitivity of about 59% (Desquesnes, 1997). PCR is exquisitely sensitive and can provide specific and direct information regarding the presence of parasites in infected individuals. Amplification of DNA equivalent to that in a single parasite has been achieved, thus permitting identification of parasites at levels far below the detection limit of the standard parasitological techniques (Masake et al., 1997).

Between 53 and 80 DPI some Mel-B treated mice were parasitologically negative but positive by PCR, and thereafter at 88 DPI were PCR negative. It is conceivable that some trypanosomes escape the early treatment by Mel-B, but are rendered non-pathogenic and are occasionally detected by PCR. These parasites are ultimately destroyed by the host immune response, and thereafter are not detected either parasitologically or by PCR. The results obtained from this study point to the difficulty of establishing a golden standard in the development of an appropriate diagnostic regimen that would assist in the treatment follow-up of trypanosomosis. It is imperative that future studies establish a suitable cut-off time frame between treatment and follow-up (during which time PCR and parasitological diagnosis is negative) to declare an individual cured. Such data would be invaluable in designing a diagnostic regimen for shortening the current 2 year period of treatment follow-up in trypanosomosis patients. Although PCR diagnosis is too expensive for routine diagnosis, this study points to the potential of developing a diagnostic regimen for treatment follow-up that combines conventional microscopy and PCR.

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