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The relapse of *Trypanosoma brucei brucei* infections after chemotherapy in rabbits

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Summary

Infections of *T. brucei* in the rabbit were found to relapse after chemotherapy. The results indicated that 25 mg/kg diminazene aceturate given 3 days after infection resulted in a complete cure but if given 7 days after infection relapses frequently occurred. However, treatment was apparently successful if delayed until 14 or 21 days. Six of the rabbits originally treated with diminazene aceturate on day 7 were treated with suramin 21 days later; in 3 rabbits the infections relapsed. In all rabbits in which drug treatment was not curative, the clinical condition nevertheless improved. An attempt to locate a cryptic focus of infection in rabbits was unsuccessful.

Key words: *Trypanosoma brucei brucei;* diminazene aceturate; suramin; relapse infections; cryptic foci of infection.

Introduction

Unlike the African trypanosomes of the subgenus Nannomonas and Duttonella which are predominantly haemoparasites, trypanosomes of the subgenus Trypanozoon also invade the body tissues. This contributes to the somewhat different clinical picture and pathology of the diseases caused by these parasites (Losos and Ikede, 1972). The brain is one such extravascular site of infection and trypanosomiasis caused by the Trypanozoon, or brucei, group of trypanosomes, known in man as 'sleeping sickness', is typified by involvement of the central nervous system (CNS) (Apted, 1970; Poltera et al., 1985). In animals too, infected with *Trypanosoma b. brucei,* involvement of the CNS has been reported (Losos and Ikede, 1972; Morrison et al., 1983; Whitelaw et al., 1985).

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Various authors have described experimental models of cerebral trypanosomiasis in the mouse using *T. b. brucei* (Poltera et al., 1980) or *T. b. gambiense* (Poltera et al., 1985), the mountain vole using *T. b. gambiense* (Rudin et al., 1983) and the vervet monkey using *T. b. brucei* and *T. b. rhodesiense* (Rudin et al., 1983; Poltera et al., 1985). Jennings et al. (1977, 1979) showed by organ subinoculation that infection relapse after chemotherapy in mice was caused by parasites originating in the brain. This was probably due to the inability of most trypanocides to cross the blood-brain barrier (Williamson, 1970) although Poltera et al. (1980) demonstrated the persistance of trypanosomes in the brain even after chemotherapy with melarsoprol, the drug of choice for cerebral trypanosomiasis in man (Poltera et al., 1985). In the experiments of Jennings et al. (1977) the timing of treatment was shown to be important. They found that treatment of mice 3 or 7 days after infection with several *T. b. brucei* stabilates gave complete cures but delaying treatment until day 14 or 21 invariably resulted in infection relapse. Poltera et al. (1985) also failed to cure monkeys infected with either *T. b. brucei* or *T. b. gambiense* and treated with diminazene aceturate (Berenil, Hoechst, Germany) at 5 mg/kg for 3 or 4 days late in infection, and again they showed the persistance of intracerebral trypanosomes.

The rabbit as a model for trypanosomiasis caused by the brucei group of trypanosomes has some advantages over the murine model in that *T. b. brucei* in rabbits generally causes an infection with low and sporadic parasitaemia. This more closely mirrors the situation occurring naturally in domestic ruminants infected with *T. b. brucei* and in man infected with *T. b. gambiense.* The infection in the rabbit also leads to the appearance of many of the typical signs of extravascular chronic trypanosomiasis including oedema of the genital organs, nasal and lacrimal discharges and loss of weight (Losos and Ikede, 1972).

The purpose of the present study was to investigate possible relapse of *T. b. brucei* infections after chemotherapy in the rabbit.

Materials and Methods

Rabbits. – The rabbits used in these experiments were Dutch rabbits, mostly male, obtained from Cheshire Rabbit Farms (Tarporley, Cheshire), and weighing about 2 kg at the beginning of each experiment.

Mice. – Female outbred CD-1 mice for subinoculation of rabbit blood and other tissues, were obtained from Charles River (Margate, Kent) at 6–8 weeks of age when they were about 25 g in weight. The mice were sublethally irradiated with 600 rads using a ^{60}Co source, on the day prior to subinoculation.

Trypanosomes. – The trypanosomes used were a stabilate designated *T. b. brucei* GVR 35/2, a working stabilate prepared from *T. b. brucei* GVR 35 previously described by Jennings et al. (1982). The rabbits were infected with approximately 1×10^4 parasites intraperitoneally (i.p.).

Trypanocides. – Two trypanocides were used – diminazene aceturate (Berenil; Hoechst, Germany) at a dose rate of 25 mg/kg body weight, given by intramuscular injection, and suramin (Germanin; Bayer, Germany) at a dose rate of 20 mg/kg given by intravenous injection.

Table 1. Treatment regimens for Experiment 1 and Experiment 2

	Rabbit No.	Days after infection											
		3	7	14	19	21	28	42	49	62	64	70	86
		Experiment 1											
Group A	1	–	–	K									
	2	–	–	K									
	3	–	–	–	K								
	4	–	–	–	–	–	K						
Group B	5	–	D	–	–	–	S	–	K				
	6	–	D	–	–	–	S	–	K				
	7	–	D	–	–	–	S	–	K				
	8	–	D	–	–	–	S	–	–	–	–	K	
	9	–	D	–	–	–	S	–	–	–	–	K	
	10	–	D	–	–	–	S	–	–	–	–	K	
Group C	11	–	–	–	–	D	–	S	–	–	K		
	12	–	–	–	–	D	–	S	–	–	K		
	13	–	–	–	–	D	–	S	–	–	K		
	14	–	–	–	–	D	–	S	–	–	–	–	K
	15	–	–	–	–	D	–	S	–	–	–	–	K
	16	–	–	–	–	D	–	S	–	–	–	–	K
		Experiment 2											
Group E	1	D	–	–	–	–	–	–	–	K			
	2	D	–	–	–	–	–	–	–	K			
	3	D	–	–	–	–	–	–	–	K			
Group F	4	–	D	–	–	–	–	–	–	K			
	5	–	D	–	–	–	–	–	–	K			
	6	–	D	–	–	–	–	–	–	K			
Group G	7	–	–	D	–	–	–	–	–	K			
	8	–	–	D	–	–	–	–	–	K			
	9	–	–	D	–	–	–	–	–	K			

D = diminazene aceturate (Berenil) 25 mg/kg
S = suramin (Germanin) 20 mg/kg
K = killed

Treatment regimens. – The rabbits were treated according to the protocol in Table 1. A fourth group of non-infected, non-treated controls were included in Experiment 1.

Clinical examination. – The rabbits were clinically examined 3 times weekly for changes in body weight, rectal temperature, lymph node and spleen size, testicular or vaginal appearance and concurrent illness.

Blood sampling. – Blood samples were taken in heparinised capillary tubes from the marginal ear vein 3 times weekly. The blood was examined for the presence of trypanosomes by the wet film 'rapid matching' method of Herbert and Lumsden (1976) and then centrifuged in a microcentrifuge for duplicate haematocrit readings. The buffy coat was also examined for the presence of trypanosomes (Murray et al., 1977). As subinoculation of blood into rodents is considered to be a sensitive test to detect sub-microscopic levels of circulating *T. b. brucei,* periodically 0.2 ml of blood was inoculated i.p. into groups of 3 irradiated mice.

Subinoculation of rabbit organs. – Rabbits were killed by cervical dislocation, and immediately afterwards a blood sample was taken, and the spleen, brain, kidneys, lymph nodes and testicles removed. A sample of each organ was macerated in isotonic saline by gently teasing apart through a wire gauze using a rubber tipped syringe plunger, and each organ suspension or blood sample (0.2 or 0.5 ml) was inoculated i.p. into groups of irradiated mice. The mice were then checked 3 times weekly for 30 days for the development of a patent parasitaemia. In the first experiment only samples of blood and brain tissue were subinoculated.

Results

The levels of parasitaemia recorded throughout the experiments were very low, many only detectable using the buffy coat method. In Experiment 1, the days on which rabbits in the control infection group (A) were patently parasitaemic can be seen in Table 2. In 3 of these rabbits parasitaemias were very low, whilst in the fourth, parasitaemia reached a level of more than 10^6 per ml.

By day 7, the day of diminazene treatment of Group B, 4 out of the 6 rabbits in this group had been parasitaemic. However, after diminazene therapy in this group all 6 rabbits exhibited a relapse parasitaemia occurring between 5 and 12 days after treatment. These parasitaemias were usually transient but in most cases were seen on more than one day after treatment, and they were never detectable by any method other than examination of the buffy coat layer.

In Group C, treated with diminazene of day 21 after infection, all the rabbits had been previously parasitaemic on more than one day. After treatment, only 3 of the 6 rabbits had a relapse parasitaemia before termination of the experiment and this was detectable just on a single occasion.

The rabbits in Groups B and C of Experiment 1 were given a second treatment, this time using suramin (Table 1). After the rabbits in Group B were treated with suramin the 2 that had a patent parasitaemia on the day of treatment remained parasitaemic for a further 7 and 9 days respectively (Table 2). Only one of the remaining 4 rabbits had a detectable parasitaemia 40 days after treatment. None of the Group C rabbits had a relapse parasitaemia after suramin treatment.

In Experiment 1, blood from rabbits was on occasions subinoculated into mice (Table 2). Parasitaemia was not often subsequently detected in these mice even where rabbits had a patent parasitaemia at or near the time of subinoculation. Subinoculation into mice of parts of the brain of rabbits in Experiment 1 (Table 2) also did not usually result in the development of parasitaemia in the mice although subinoculation of part of the brain from one control rabbit led to the development of parasitaemia in the mice one week before that detected following blood inoculation from the same rabbit.

In order to determine whether diminazene could be curative in rabbits infected with this strain of *T. b. brucei* if given early enough in the infection, a second experiment (Experiment 2) was performed in which 3 rabbits were treated as early as 3 days after infection (Group E). These 3 rabbits never

Table 2. The development of parasitaemias in rabbits infected with *T. b. brucei* 35/2 before and after treatment with diminazene aceturate on day 7 or 21 after infection, and suramin 21 days later (Experiment 1)

	Rabbit No.	Days after infection																												
		2	5	7	9	12	14	16	19	21	23	26	28	30	33	35	37	40	42	44	47	49	50–63	64	66	68	70	71–85	86
Group A	1	–	+	+	–	+	$+^{K●▲}$																						
	2	–	+	–	–	+	$+^{K●▲}$																						
	3	–	–	+	–	+	+	+	$+^{KO△}$																				
	4	–	–	$-^{○}$	–	+	–	–	$-^{○}$	–	+	–	$-^{KO△}$																
Group B	5	–	+	$+^{d}$	–	–	+	+	$+^{○}$	+	+	–	$+^{s}$	+	–	+	–	–	–	–	–	$-^{KO△}$							
	6	–	+	$+^{d}$	–	–	+	+	$-^{○}$	–	–	–	$-^{s}$	–	–	–	–	–	–	–	–	$-^{KO△}$							
	7	–	+	$-^{d}$	–	+	–	–	$-^{○}$	–	–	–	$+^{s}$	+	+	–	+	–	–	–	–	$-^{KO△}$							
	8	–	–	$-^{d●}$	–	–	–	–	$+^{○}$	–	–	–	$-^{s}$	–	–	–	–	–	–	–	–	–	–	–	+	$-^{KO△}$		
	9	–	+	$+^{d}$	–	+	–	+	$-^{○}$	–	–	–	$-^{s}$	–	–	–	–	–	–	–	–	–	–	–	–	$-^{KO△}$		
	10	–	–	$-^{dO}$	–	–	+	+	$+^{○}$	–	–	–	$-^{s}$	–	–	–	–	–	–	–	–	–	–	–	–	$-^{KO△}$		
Group C	11	–	–	+	–	+	+	–	–	$-^{d}$	–	–	–	–	–	–	–	–	$-^{sO}$	–	–	–	–	$-^{KO△}$					
	12	–	–	+	–	–	+	+	–	$+^{d}$	–	–	+	–	–	–	–	–	$-^{sO}$	–	–	–	–	$-^{KO△}$					
	13	–	–	$-^{●}$	+	+	+	+	+	$-^{d}$	–	–	+	–	–	–	–	–	$-^{sO}$	–	–	–	–	$-^{KO△}$					
	14	–	–	$-^{○}$	–	+	+	–	–	$-^{d}$	–	–	–	–	–	–	–	–	$-^{sO}$	–	–	–	–	–	–	–	–	–	$^{KO△}$
	15	–	+	+	–	+	+	+	–	$+^{d}$	–	–	–	+	–	–	–	–	$-^{sO}$	–	–	–	–	–	–	–	–	–	$^{KO△}$
	16	–	+	+	–	+	+	–	+	$-^{d}$	–	–	–	–	–	–	–	–	$-^{sO}$	–	–	–	–	–	–	–	–	–	$^{KO△}$

\+ parasitaemia observed
– no parasitaemia observed
^{d} treatment with 25 mg/kg diminazene aceturate
^{s} treatment with 20 mg/kg suramin
^{K} killed
$^{●○}$ subinoculation of blood with/without development of parasitaemia in mice
$^{▲△}$ subinoculation of brain with/without development of parasitaemia in mice

Table 3. The development of parasitaemias in rabbits infected with *T. b. brucei* 35/2 before and after treatment with diminazene aceturate on day 3, 7 or 14 after infection (Experiment 2)

	Rabbit No.	Days after infection															
		1	3	6	7	8	10	13	14	15	16–37	38	41	43	45	46–61	62
Group E	1	–	–[d]	–		–	–	–		–	–	–	–	–	–	–	K○△
	2	–	–[d]	–		–	–	–		–	–	–	–	–	–	–	K○△
	3	–	–[d]	–		–	–	–		–	–	–	–	–	–	–	K○△
Group F	4	–	–	+	[d]	–	–	–		–	–	+	+	–	–	–	K○△
	5	–	–	+	[d]	+	–	–		–	–	–	–	–	–	–	K○△
	6	–	–	–	[d]	–	–	–		–	–	–	–	–	+	–	K○△
Group G	7	–	–	+		+	–	+	[d]	+	–	–	–	–	–	–	K○△
	8	–	–	+		–	–	+	[d]	–	–	–	–	–	–	–	K○△
	9	–	–	–		+	–	+	[d]	–	–	–	–	–	–	–	K○△

\+ parasitaemia observed
– no parasitaemia observed
[d] treatment with 25 mg/kg diminazene aceturate
[K] killed
●○ subinoculation of blood with/without development of parasitaemia in mice
▲△ subinoculation of brain with/without development of parasitaemia in mice

developed a patent parasitaemia (Table 3). Two of the 3 rabbits (Group F) treated on day 7 were parasitaemic on day 6. After treatment 2 rabbits displayed a scanty parasitaemia in only one of which a patent parasitaemia was detected before treatment. All of the rabbits (Group G) treated on day 14 after infection had been parasitaemic on at least two days before treatment. From the day after treatment, parasites were never detected in the blood of any of these rabbits, either microscopically or by mouse subinoculation.

In both experiments, rabbits treated with diminazene on or before day 7 after infection did not show a deterioration in health or a decrease in weight, even if infection relapse subsequently occurred.

Clinical examination and measurement of the haematocrit in the rabbits of both experiments that were not treated by day 7 revealed many of the signs typical of *T. b. brucei* infections. For example, in nearly all these animals haematocrit levels decreased from a initial level of about 0.41 to a minimum level of 0.23, body temperature was frequently elevated and there was loss of weight. Many rabbits had swelling of the genital organs and nasal and lacrimal discharges were common signs of infection. In some rabbits a secondary pneumonia developed which required treatment with antibiotics. There was no obvious enlargement of the lymph nodes, but palpation of the spleen revealed some enlargement in most of the infected rabbits. Treatment of the clinically affected rabbits on day 14 (Group G) or day 21 (Group C) resulted in an improvement of their condition.

The non-infected control rabbits in Group D thrived throughout the period of the experiment.

In Experiment 2, rabbits were killed 62 days after the initial infection at a time when parasites had not been detected for at least 2 weeks. Subinoculation of blood and various organs into mice failed to demonstrate any evidence of cryptic foci of infection.

Discussion

Rabbits infected with *T. b. brucei* GVR 35/2 developed many of the clinical signs associated with *T. b. brucei* infection in domestic animals (Losos and Ikede, 1972). Parasitaemia was often recorded sporadically, even in those rabbits in which clinical signs were severe. In rabbits in which treatment did not appear to be curative, and a relapse parasitaemia was recorded, the clinical condition of the rabbits improved after drug therapy.

The experiments reported here investigated the relapse of *T. b. brucei* GVR 35/2 infections after chemotherapy in the rabbit. Trypanocidal drug treatment with 25 mg/kg diminazene aceturate, given early after infection (3 days), resulted in a complete cure, but if treatment was not given until 7 days, relapses were common. Unexpectedly, if treatment was delayed until 14 or 21 days after infection it apparently led to a cure as far as could be determined by the limited

observation period. These results are in contrast to the results of Jennings et al. (1977, 1979) in mice, using several *T. b. brucei* stabilates, in which treatment with the same drug at 40 mg/kg successfully cured infections up to 7 days after infection, but when delayed until day 14 or later usually led to a relapse. Furthermore, Jennings (unpublished results), using *T. b. brucei* GVR 35/2, the same stabilate as in this study, treated groups of 5 mice with 25 mg/kg diminazene on days 0, 3, 5, 7 and 14 after infection and again achieved a complete cure with treatment up to 7 days after infection. However, in all 5 mice, a relapse parasitaemia developed when treatment was delayed until 14 days after infection. Similarly vervet monkeys chronically infected with *T. b. rhodesiense* or *T. b. brucei,* and treated with 5 mg/kg diminazene for 3 or 4 days, also relapsed (Poltera et al., 1985).

The relationship between the cure of the infection and the timing of the drug administration appears to be rather more complex in the rabbit than that reported in mice by Jennings et al. (1977) (vide supra). In the limited number of rabbits used in these experiments, treatment on or after day 14 appeared to be more efficient than treatment on day 7, although the early treatment on day 3 elicited a cure. The reasons for this are unclear. Diminazene is reputed not to cross the blood-brain barrier (Williamson, 1970) but the integrity of this barrier may be reduced by day 14 post-infection due to encephalitis, allowing therapeutic levels of drug to enter the brain. Trypanosomes in the brain may have 'shifted' to an extravascular intracerebral site (Poltera et al., 1981) and relapses may therefore have occurred later than our observation period. Alternatively it is possible that the period in which viable trypanosomes can be found in the brain is transient and that by day 14, in some rabbits at least, the brain may no longer be a focus of infection.

In an attempt to cure the rabbits with relapsing parasitaemias after diminazene treatment, a second treatment was given in Experiment 1, 3 weeks later, using another trypanocidal drug, suramin, which is also unlikely to cross the blood-brain barrier (Williamson, 1970). Again, treatment was more effective in the group in which the initial diminazene therapy had been delayed until 21 days post-infection in that none of the Group C rabbits had a relapse parasitaemia after the second drug treatment.

On several occasions throughout the experiment attempts were made to determine whether failure to observe parasitaemia was due to the parasite numbers present being too low to be observed by microscope observation on whole blood or the buffy coat layer. It has been reported under field conditions (Godfrey and Killick-Kendrick, 1961) that low levels of *T. b. brucei* infection can be readily detected by subinoculation into mice. In the experiments recorded here, parasitaemias were not always detected after subinoculation even in situations where a patent parasitaemia was observed in the rabbits. Similarly Parkin (1935), cited by Losos and Ikede (1972), stated that subinoculation of blood from horses known to be infected with *T. b. brucei* from

microscope observation, did not always lead to development of a parasitaemia in recipient rats. One possibility, in the treated rabbits in our experiments, is that the trypanosomes observed in the blood after trypanocide treatment, had been affected by residual drug and were incapable of replication in mice (Gray et al., 1982).

In the second experiment a tentative attempt was made to locate a possible extravascular source of infection after chemotherapy by subinoculation of organ samples, although at the time of the subinoculation, 62 days after the experiment had begun, there had been no evidence of circulating parasites for at least two weeks. None of the recipient mice developed a patent parasitaemia. There are several possible explanations for this. The rabbits may have been entirely aparasitaemic at this stage; parasites may not have been able to multiply in mice because they were affected by residual drug (vide supra); or parasites could have been in an organ not subinoculated or not in the portion of each organ which was subinoculated. Histopathological studies might be useful in any future studies on the rabbit to identify a possible localisation of trypanosomes (Poltera et al., 1981).

In conclusion, these experiments have demonstrated the occurrence of infection relapse after drug treatment in the rabbit. However, the relationship between the efficacy of drug treatment and the stage of infection would appear to be more complex in the rabbit than that reported earlier in mice and requires further investigation.

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