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Total Protein and White Cell Changes in the Cerebrospinal Fluid of Vervet Monkeys Infected with *Trypanosoma rhodesiense* and the Post-treatment Reaction

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ABSTRACT

In an attempt to elucidate the events leading to the development of post-treatment reactive encephalopathy in human African trypanosomiasis (HAT), a group of vervet monkeys (*Cercopithecus aethiops*) were experimentally infected with *Trypanosoma rhodesiense*. When terminally sick on day 42, they were treated with either diminazene aceturate (Berenil®), suramin or melarsoprol. Trypanosomes appeared in the cerebrospinal fluid (CSF) by day 14 of infection and increased in numbers with progress of the disease. However, only marginal increases in CSF total proteins and white cells occurred during the same period. Treatment with Berenil resulted in persistence and increase in numbers of CSF trypanosomes, a dramatic increase in proteins and white cells, culminating in clinical encephalitis. Suramin cleared CSF trypanosomes within 4 weeks, with marginal increase in proteins and white cells up to 8 weeks after treatment, followed thereafter by a gradual and prolonged fall to pre-infection levels. Melarsoprol eliminated trypanosomes from the CSF in less than a week but the white cell and protein levels increased for another 4 weeks before finally falling. The post-treatment increase in white cell numbers and total proteins was therefore dependent on the trypanocidal drug, and was highest and most prolonged when Berenil was used and lowest with suramin. The present studies demonstrate that trypanocidal treatment of infected animals is followed by a post-treatment reaction in the central nervous system, the severity of which is related to the drug used and the presence of trypanosomes in the CSF. The vervet monkey therefore appears to be a good model for studying the reaction in HAT.

INTRODUCTION

Sleeping sickness, or human African trypanosomiasis (HAT), caused by either *Trypanosoma rhodesiense* or *T. gambiense*, runs a complex course which if untreated leads to late-stage disease and death. The late stage of the disease is characterised by invasion of the CNS with trypanosomes and an abnormal cerebrospinal fluid (CSF). At this stage, treatment can only be achieved using melarsoprol (Arsobal®) for both *T. rhodesiense* and *T. gambiense* infections or difluoromethylornithine (DFMO; Ornidyl®) for the *T. gambiense* disease. Treatment with melarsoprol results in an unpredictable, often fatal arsenical encephalopathy in at least 10% of treated patients (Adams et al., 1986).

Studies using a *T. brucei* mouse model indicate that the encephalopathy may be related to the development of a post-treatment meningoencephalitis, whose severity is related to the trypanocidal compound used (Jennings et al., 1989). A *T. rhodesiense* vervet monkey (*Cercopithecus aethiops*) model of HAT has been established at the Kenya Trypanosomiasis Research Institute (KETRI) that provides an excellent opportunity to carry out in-depth investigations into the mechanisms leading to the development of the reactive encephalopathy (Schmidt and Sayer, 1982).The model was therefore used in the present studies to determine the CSF changes in white cells and total proteins that occur following infection with *T. rhodesiense* and after treatment with various trypanocidal drugs.

MATERIALS AND METHODS

Animals

Fifteen vervet monkeys of both sexes and weighing between 2.7 and 4.5kg were acquired from the Institute of Primate Research in Kenya. They were initially housed in quarantine for a minimum of 90 days while being screened for evidence of disease, including zoonotics. During that time they became accustomed to handling and staying in individual squeeze-back stainless steel cages. They were fed twice daily with commercial monkey pellets, fresh fruits and vegetables, and water was provided ad libitum. Before the study, the animals were transferred to experimental wards and allowed to settle for another 2 weeks.

Trypanosomes

The stabilate of *T. rhodesiense* used was KETRI 2537, a derivative of EATRO 1989 which was isolated from a patient in Uganda by direct inoculation of blood and lymph node aspirate into a monkey (Fink and Schmidt, 1980) and later cryopreserved.

Trypanocidal compounds

The trypanocidal drugs used included melarsoprol (MelB; Arsobal® Specia), suramin (Bayer) and diminazene aceturate (Berenil®, Hoechst). Melarsoprol was presented as a 36 mg/ml solution. The total volume of administration was made up to 1ml using water of injection. Suramin and Berenil were weighed out and reconstituted in distilled water to give a total volume of 1ml for injection.

Cerebrospinal fluid sampling and analysis

Cerebrospinal fluid (CSF) was collected by lumbar puncture of the anaesthetised monkey. Some of the free-flowing CSF was collected into a capillary tube, immediately transferred onto a haemocytometer chamber and the number of white cells counted. If trypanosomes were not seen in the counting chamber, approximately 1ml of CSF was collected in a pipette whose tip had been sealed by heating. This was centrifuged and the sealed end examined using a microscope as described by Gould and Sayer (1983). In case this was also negative, 0.2ml CSF was inoculated intraperitoneally into each of two mice and development of parasitaemia assessed for 60 days post-inoculation.The total protein concentration in the CSF was determined from 100μl of sample using the Biorad method.

Experimental design

Eleven vervet monkeys were infected by intravenous injection with approximately 10^4 trypanosomes. Before and during the course of the disease, a daily clinical evaluation was done and the presence of trypanosomes in ear-prick blood determined. The parasitaemia was estimated using the method of Herbert and Lumsden (1976). At weekly intervals, the animals were anaesthetised with diazepam (1mg/kg bwt) and ketamine hydrochloride (10-15mg/kg bwt), weighed, and a detailed clinical examination carried out. Five millilitres of blood was collected by inguinal venipuncture for haematology, biochemistry and serology, and 2ml CSF by lumbar puncture.

When the monkeys were terminally sick on day 42, they were treated with either melarsoprol (3.6 mg/kg bwt iv) for 4 days, suramin (20 mg/kg bwt iv) for 5 days or Berenil (5 mg/kg bwt im) for 3 days (Table 1). Animals treated with Berenil were not cured but developed clinical meningoencephalitis and were re-treated with melarsoprol (3.6 mg/kg bwt iv) from day 130 to 133. All the animals were then monitored during a follow-up period of at least 36 weeks. Four monkeys that were not infected and did not receive any treatment served as controls.

Table 1

Treatment regimes in *Trypanosoma rhodesiense* infected vervet monkeys.

Number of monkeys	Trypanocidal drug used	Drug dosage (mg/kg) and days (D) of treatment
4	Melarsoprol	3.6X4 iv (D42-45)
4	Suramin	20X5 iv (D42-46)
3	Berenil Melarsoprol	5X3 im (D42-44) 3.6X4 iv (D130-133)
4	Uninfected	

RESULTS

Pre-treatment clinical findings

The animals developed clinical signs of disease 5 to 7 days after infection. These were characterised by dullness, reduced appetite, raised hair coat, periorbital oedema, enlargement of the spleen and superficial lymph nodes, progressive anaemia, rapid wasting and weight loss. Parasitaemia developed on day 5 and after the first wave, remained elevated during the pre-treatment period.

Auscultation of the heart revealed various abnormalities including tachycardia, mitral and tricuspid incompetence, second degree heart block and terminal bradycardia.

Pre-treatment CSF changes

The mean CSF white cell changes before and after *T. rhodesiense* infection are shown in Figure 1. In all the animals, the mean pre-infection values ranged between 2.5±1.29/μl (mean±1SD) and 5.11±6.11/μl. After week 2 of infection, the number of white cells increased gradually, reaching 12±8.99/μl in terminal disease. Before infection, the mean total protein concentration was 17.36±5.84 mg/dl to 22.73±3.02mg/dl (Fig.2). After week 3 of infection there was a marginal increase in proteins to 24.19±5.60 mg/dl at week 6.

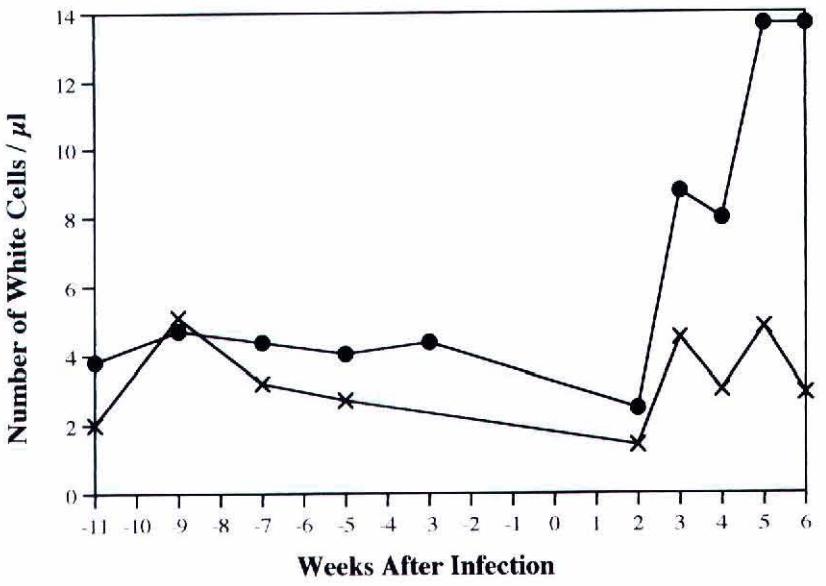

Figure 1. The number of white cells (No/μl) in the cerebrospinal fluid of normal (—✕—) and *T. rhodesiense* infected (—●—) vervet monkeys.

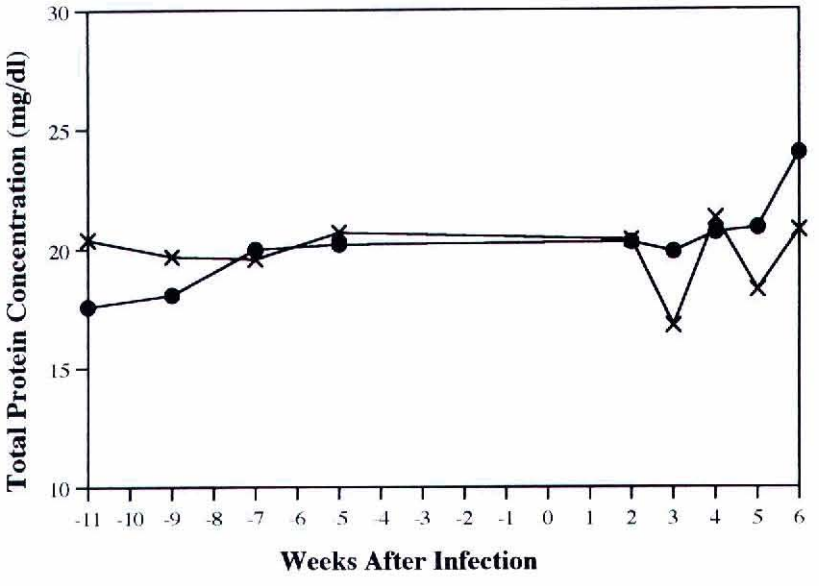

Figure 2. Total protein concentration (mg/dl) in the cerebrospinal fluid of normal (—✕—) and *T. rhodesiense* infected (—●—) vervet monkeys.

Post-treatment CSF changes

Melarsoprol treatment

Trypanosomes had been cleared from the CSF by day 7 after treatment with melarsoprol. No significant changes in CSF total proteins and white cells occurred during the 2 weeks following treatment (Fig.3). From week 3 after treatment however, increases in both total proteins and white cells occurred, and were highest in week 4 (total proteins: 32.7±12.54 mg/dl, white cells: 17.67±12.22/μl). Thereafter, a temporary fall in total proteins occurred, up to week 8 after treatment, followed by another rise in week 10 (30.88±7.04 mg/dl). Normal protein levels were established 16 weeks after treatment. The number of white cells remained high for up to 10 weeks after treatment, then fell rapidly to normal in the following 4 weeks. As such both total proteins and white cells had become normal 16 weeks after treatment.

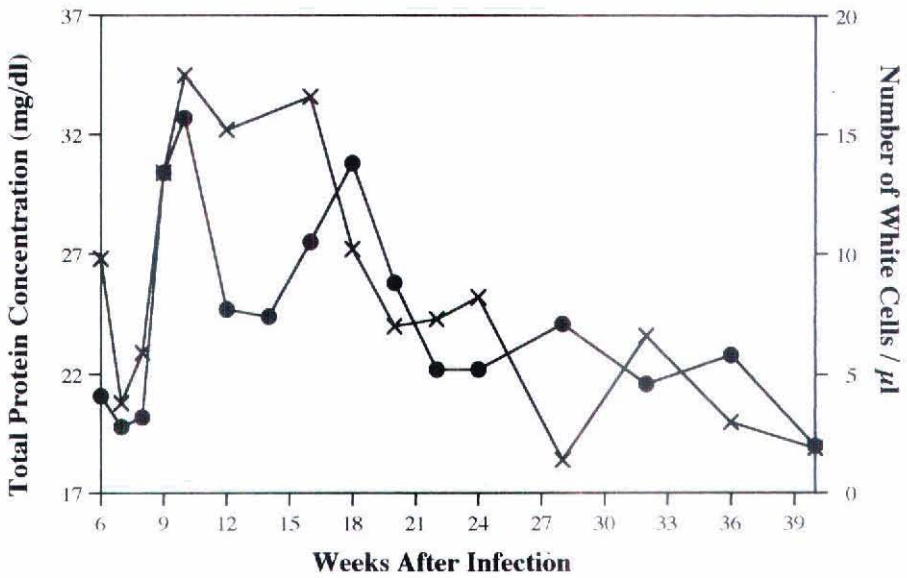

Figure 3. Total protein concentration (—●—) and number of white cells (—×—) in the cerebrospinal fluid of *T. rhodesiense* infected vervet monkeys following treatment with melarsoprol.

Suramin treatment

Trypanosomes persisted in the CSF for 3 weeks after treatment and then disappeared. Slight and gradual increases in total proteins occurred, reaching

peak values (24.92±4.67 mg/dl) 5 weeks after suramin treatment. This was followed by fluctuating levels during the subsequent period (Fig.4). Similarly, a slight increase in white cells occurred, reaching the highest number (14.25±10.14/μl) 9 weeks after treatment. By week 11 however, the number had gone back to normal.

Berenil treatment

Treatment with Berenil had no effect on trypanosomes in the CSF where their number continued to rise. As with melarsoprol and suramin treatments, no significant changes in total proteins and white cells occurred during the first 3 weeks after treatment with Berenil. From week 4, total proteins increased rapidly up to week 12 (56.9mg/dl), when the animals developed clinical encephalitis and had to be treated with melarsoprol (Fig. 5).

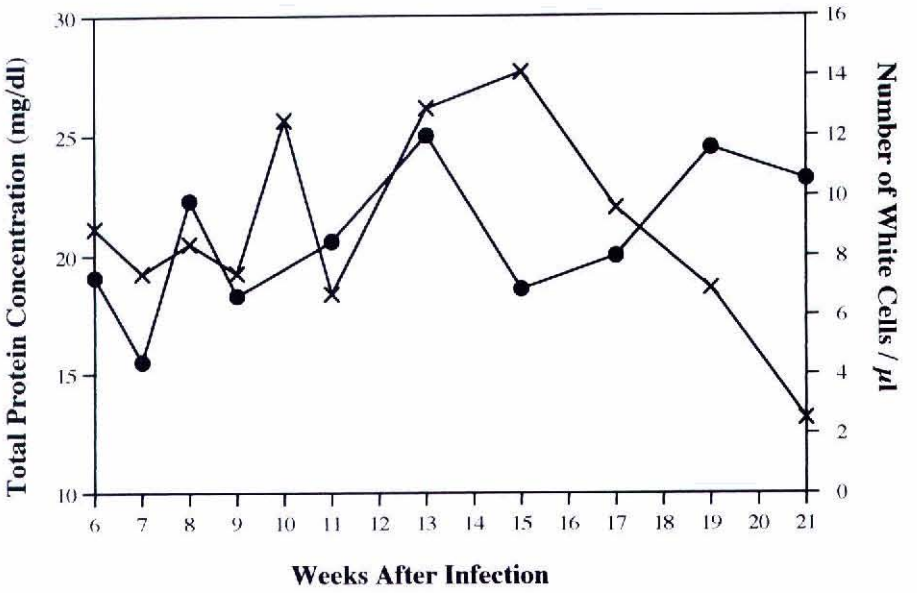

Figure 4. Total protein concentration (—●—) and number of white cells (—✕—) in the cerebrospinal fluid of *T. rhodesiense* infected vervet monkeys following treatment with suramin.

Subsequently there was an initial rapid fall in total proteins up to week 17 (27.8 mg/dl) after Berenil. Normal values were established 12 weeks later. Likewise, white cells increased rapidly from 4 weeks after Berenil treatment and were highest (678/μl) at the onset of encephalitis. Melarsoprol caused a

rapid fall in cell numbers during the following 8 weeks to stabilise at 20 to 30 cells/μl. However, this number was higher than the pre-infection normal value and was maintained during the next 10 weeks follow-up period.

When the mean post-Berenil treatment protein and white cell values were compared to the ones from melarsoprol and suramin treated animals, they were several orders of magnitude higher. Values for melarsoprol treated monkeys were twice as high as those for suramin treated ones.

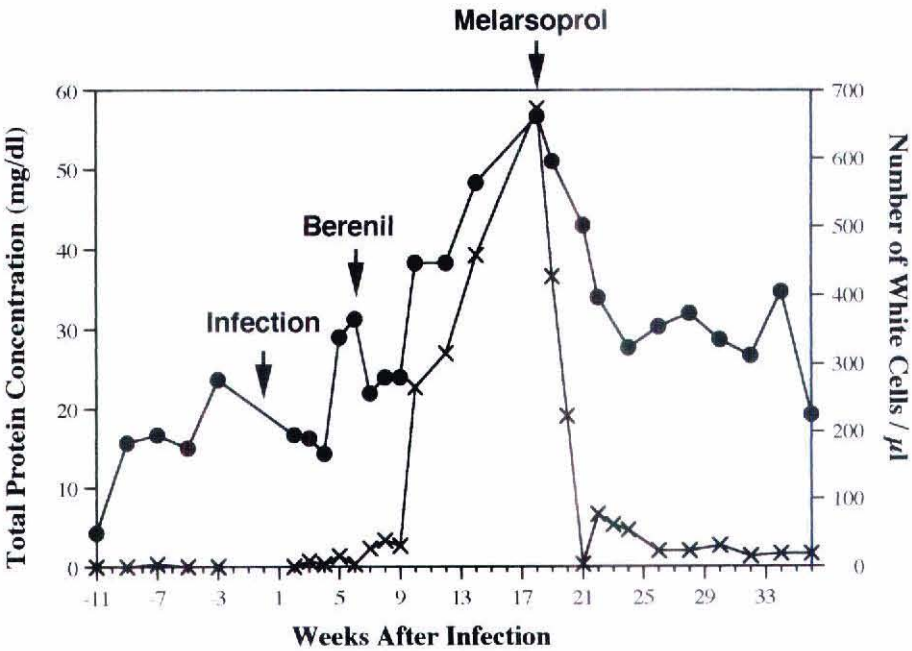

Figure 5. Changes in total protein concentration (—●—) and number of white cells (—×—) in the cerebrospinal fluid of *T. rhodesiense* infected vervet monkeys following treatment with Berenil, then melarsoprol.

DISCUSSION

Infection of monkeys with *T. rhodesiense* resulted in an acute disease syndrome and presence of trypanosomes in the CSF by day 14. In terminal stages in week 6, slight increases in total proteins and white cells occurred. In rats and mice infected with *T. brucei*, similar early invasion of the CNS was confirmed by demonstration of trypanosomes in spinal and trigeminal ganglia, the median eminence and the neural lobe of the pituitary gland by

day 13 of infection (Schultzberg et al., 1988). Similarly in humans infected with *T. rhodesiense*, apart from the presence of trypanosomes in the CSF only marginal increases in proteins and white cells occur during the first two months of infection (Dumas and Boa, 1988).
Treatment of terminally sick animals with either melarsoprol, suramin or Berenil caused alterations in the CSF composition starting from the third week after treatment. The degree of change was variable, being highest in Berenil and lowest in suramin treated monkeys. Raised CSF total proteins and white cell numbers in sleeping sickness patients is regarded as an indication of brain involvement and an existing meningoencephalitis (De Raadt, 1984; Dumas and Boa, 1988). While Berenil is not recommended for treatment of sleeping sickness, its use for precipitating early meningoencephalitis in various animal models of HAT is well established (Schmidt and Sayer, 1982; Poltera et al., 1985). The dramatic increase in total proteins and white cells in Berenil treated animals was therefore an indicator of an underlying meningoencephalitis. The events leading to the encephalitis have not been properly understood. Jennings et al. (1989) have postulated that since Berenil does not cross the blood-brain barrier, its use causes sudden removal of all circulating trypanosomes as well as those in the other organs of the body, while leaving those in the CNS. Immunologically active cells then migrate to the CNS, causing intensification of meningoencephalitis.

The increase in total proteins and white cells in vervet monkeys treated with a curative regime of melarsoprol indicates that post-treatment encephalitis did develop. This was delayed as it occurred after week 3 of treatment, and less dramatic than in Berenil treated animals. In sleeping sickness patients, reactive arsenical encephalopathy develops from 10 days after institution of treatment (Adams et al., 1986; Arroz, 1987), which is in agreement with the findings of the present studies.

Treatment with a curative regime of suramin caused only minimal and transient changes in CSF composition, indicating that encephalitis might not have developed. Likewise in human patients treated with either suramin or pentamidine, encephalopathy was never seen (Pepin and Milord, 1991). Recent work has shown that suramin has high immunosuppressive properties that it exerts through a number of mechanisms. One of the mechanisms is inhibition of the binding of interleukin-2 (IL-2) to its cell surface receptor (Mills et al., 1990). Interleukin-2 is the major factor involved in regulating lymphoid differentiation and proliferation, and thus regulates the magnitude and duration of the immune response. It is possible that when vervet monkeys and sleeping sickness patients are treated with suramin, there is temporary suppression of any subsequent immunological response and thus the post-treatment reaction is prevented.

From the foregoing, it appears that development of post-treatment encephalitis is unrelated to the ability and rate at which a particular drug

kills trypanosomes in the CNS. Berenil, which has no effect on brain trypanosomes induced the most severe reaction. At the same time encephalitis due to melarsoprol was more pronounced than that caused by suramin, yet melarsoprol kills trypanosomes faster than suramin. If the rate of killing of trypanosomes was important, the post-treatment reaction would have occurred within a few days after melarsoprol treatment and not 3 weeks later. As indicated by Jennings et al. (1989), the effect due to Berenil may be related to its inability to cross the blood-brain barrier. That caused by suramin and melarsoprol appears to be related to the characteristics of the particular compound. Haller et al. (1986) could not discern any relationship between the reaction and the drug dosage or administration schedule. The reaction cannot be explained by direct drug toxicity, or by a specific Jarisch-Herxheimer reaction. Both the arsenic moiety in melarsoprol and its toxic propylene glycol solvent have been incriminated as possible factors contributing to the reaction. Minimal changes occurred in the CSF following suramin treatment. This observation, coupled with the immunosuppressive properties of suramin, highly supports the idea of using suramin in combination with other compounds during treatment of sleeping sickness patients.

When meningoencephalitis was already established, treatment with melarsoprol caused no further deterioration in CSF composition. Instead there was rapid recovery to near-normal levels in 5 weeks. This was unexpected, in view of the observations made when treatment was carried out with melarsoprol at 6 weeks of infection. In the light of these findings, the observation by Pepin and Milord (1991) that patients who have suffered an attack can receive more drug without showing a similar reaction needs further investigation.

The present investigations indicate that the post-treatment reaction develops 2 to 3 weeks after trypanocidal treatment and may be related to the number of immune cells and total protein concentration in the CNS. In an attempt to prevent its occurrence, clinicians have used corticosteroids prior to, during or soon after trypanocidal treatment, with inconclusive responses (Pepin et al., 1989). It would appear that for the steroids to achieve any effect they should be administered after trypanocidal treatment, which is closer to the expected period for encephalopathy to develop. The work also supports the approach of treating sleeping sickness patients with suramin in combination with melarsoprol in order to prevent the post-treatment reaction. The establishment of the current vervet monkey model of sleeping sickness provides and excellent opportunity for carrying out such investigations.

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