OBSERVATIONS ON THE RELEVANCE OF FILTERABLE FORMS OF TRYPANOSOMES TO LATENT INFECTION OF *TRYPANOSOMA EVANSI* IN MICE.

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CONTENTS

ABSTRACT

An investigation of tissue forms of T. evansi and their relevance to latent infections. Organ transfers and filtration procedures were carried out before and after treatment. Filterable forms did not appear to be of importance in the maintenance of latent infections. Extravascular Trypanosomes were demonstrated in the choroid plexus by electron-microscopy. Such forms may have been too large to pass through the 0.8μ pore size filter.

Introduction

This thesis presents the results of an investigation into the behaviour of Trypanosoma evansi in laboratory mice. The study was designed to extend the findings of Abebe, Jones, and Boyd (1983) in relation to the distribution of trypanosomes during the establishment of infection and following drug treatment.

The control of trypanosomiasis in domestic livestock depends to a large extent on the effective use of chemotherapy. This is particularly so in the case of T. evansi infections in camels and horses because control of the principal vectors, flies of the Family Tabanidae and of the Genus Stomoxys (Family Muscidae) (Nieschulz, 1929, Sergent and Donatien, 1922) is impractical. Therefore, a clearer appreciation of the factors influencing the appearance of relapses following treatment is of importance in understanding the epidemiology of T. evansi and in the management of an infected herd.

Trypanosome infections of mammals are often characterised by marked changes in the numbers of parasites in the peripheral blood. The fluctuations may have a periodicity associated with fever and other clinical episodes (Evans, 1881, Fantham & Thompson 1911, Fiennes, Jones & Laws, 1946, Apted 1972). Sergent and Donatien (1921) described long periods of latent

infection of T.evansi in the camel during which parasites could not be detected in the circulation. Similarly after drug treatment, it is common for trypanosomes to be absent from the peripheral circulation for a time only to be followed by their re-appearance. Although this behaviour may be due to drug resistance, sometimes the relapsed population retains the same sensitivity as the earlier infection.

The nature of such relapses has been the subject of much study because of its implication for the interpretation of results obtained from studies of drug resistance (Ehrlich, 1907, Calver 1945, Fiennes 1950a and b, Maclennan and Na'Isa 1970, Buyst, 1975, Kazyumba, 1979, Jennings, Whitelaw & Urquart, 1977, Evans & Brightman, 1980). In the field, such relapses have often been attributed to drug resistance or re-infection. However, studies of laboratory animal infections have resulted in the formulation of three other hypotheses to explain this phenomenon:

(1) The trypanosomes occupy sites which are less accessible to drugs (Jennings et al, 1977).

(2) The trypanosomes occur as stages or forms which are less vulnerable to drug action than are the blood-stream forms (Jennings et al, 1977, Evans & Brightman, (1980).

(3) Drugs may persist in concentrations sufficient to have a trypanostatic effect (Ercoli & Iudice, 1980).

Previous studies on suramin-sensitive stocks of Trypanosoma evansi in mice have showed that relapses occurred following treatment with suramin at dose rates which apparently cleared the parasites from the bloodstream (Abebe, Jones & Boid 1983). Such relapsed populations of trypanosomes were shown to be equally susceptible to the curative action of suramin as the original inoculum, indicating that the relapses were not due to the development of drug resistance.

REVIEW OF LITERATURE

This review of literature is presented because the interpretation of any results from the present study must be made in the light of existing knowledge. Much of this relates to T. brucei because most of the work on latent infections has been done using this species and it is believed that T. evansi is closely allied to it (Hoare, 1922, Mahmoud and Gray 1980).

A. THE RELAPSE OF T. BRUCEI AND T. EVANSI INFECTIONS AFTER DRUG TREATMENT.

Relapses are a common feature of T. brucei infections in man, apparently occurring at the rate of 5% of cases in rhodesiense areas and up to 30% in gambiense areas (Poltera, Hochmann & Lambert, 1981, quoting Buyst, 1975 and Kazyumba, 1979).

They occur following apparent cure with a number of drugs, including tartar emetic, suramin, pentamidine, berenil and melarsoprol (Apted, 1970, Abolarin, Evans, Tovey and Ormerod, 1983, Kazyumba, 1979). In fact, Apted (1970) considered that total cure in Man could be claimed until careful monitoring had shown that trypanosomes have been absent from the peripheral circulation for four years following treatment. Hawking (1940 a & b) observed that relapses occurred in man during or shortly after treatment with suramin. He demonstrated that the relapsed trypanosomes retained their sensitivity to suramin and suggested that the relapses occurred because the drug

failed to reach curative levels in the plasma of a proportion of patients.

The majority of studies on the pathogenesis and treatment of trypanozoon infections have used the mouse as a model. Jennings et al, (1977) were the first to draw attention to the fact that successful chemotherapy was related to the stage of infection of T. brucei in mice, although this phenomenon was familiar to those involved in the treatment of human sleeping-sickness (Apted 1970). Jennings et al (1977) concluded that the observed relapses were not due to drug resistance because the relapsed trypanosomes remained as sensitive to the treatment as the parent inoculum. They considered that such relapses were due to trypanosomes occurring in sites which were less favourable to drug penetration or existing in a developmental stage insensitive to drug action.

In a later study, Jennings, Whitelaw, Holmes, Chizyuka and Urquart, (1979) demonstrated that the brain was the main site for the maintenance of the infection during the aparasitaemic interval often observed following treatment with diminazine aceturate. They concluded that the subsequent relapses originated from the brain. Poltera (1980) described similar relapses following treatment of T. brucei infection with ethidium bromide and melarsoprol in which the relapsed populations remained drug sensitive. He emphasised the importance of

demonstrating a prolonged aparasitaemic interval before claiming a cure.

Drug resistance in T. evansi has been studied by Gill (1971a and b). He induced resistance to quinapyramine, suramin, stilbamidine and tryparsamide by treating infected mice with gradually increasing sub-curative doses and repeated passage of the relapsed population. He also studied the development of resistance to quinapyramine and suramin in stocks of T. evansi from ponies which had been treated prophylactically with these drugs. He appears to have been observing true drug resistance rather than the type of relapse described by Jennings et al. Ercoli and Iudice (1980) attributed a trypanostatic effect to diamidines and suramin used to treat T. evansi (T. venezuelense) infections in rats and mice. They claimed that waning drug levels exerted a continued effect on the trypanosomes which prevented binary fission. They considered that eventually the drug concentration declined to a point where multiplication could occur with subsequent re-appearance of parasitaemia. They also found that attempts to impose a second infection only resulted in a rapid parasitaemia if a sufficient interval had elapsed since the drug treatment. There is some difficulty in interpreting their description because they refer to a parasite-free latent period and yet claim to have isolated trypanosomes from the blood by inoculating other animals. Ercoli and Iudice

(1980) postulated that this trypanostatic effect may also account for the observations of Jennings et al (1977). However, in man relapses may occur after treatment long after any possible residual drug action (Apted, 1970, Losos, 1972).

Evans and Brightman (1980) described the effect of salicylhydroxamic acid (SHAM) and glycerol on infections of T. brucei and T. evansi in mice. They showed that pleomorphic stocks of T. brucei relapsed after treatment whereas monomorphic infections of T. evansi and T. brucei were radically cured. This led them to suggest that either the stumpy forms of pleomorphic stocks of trypanosomes were resistant to the action of SHAM and glycerol or that the monomorphic stocks did not produce forms which occurred outside the circulation (i.e. in situations inaccessible to the drug). Abebe et al, (1983) demonstrated relapses with one West African and three Sudan stocks of T. evansi following treatment with suramin. In this particular study mice were infected with T. evansi and then treated 48 hours later. Mice which received 1 or 2mg/Kg. suramin relapsed between fourteen and twenty one days later while those treated with 4mg/Kg remained aparasitaemic for thirty days as judged by the micro-haematocrit method. In this case it would appear that a short interval between infection and treatment was sufficient for the establishment of a latent infection. In contrast, Jennings et al, (1977) found that with

T.brucei a minimum of fourteen days was needed to establish a latent infection.They concluded that the brain itself or cerebro-spinal fluid (C.S.F.) were the likely sites of persistent infection. Evans and Brightman (1980) found that a twenty eight day infection of T.evansi was completely cured with SHAM and glycerol. One of the difficulties of comparing these studies is the different sensitivity of the methods used for detecting parasitaemia, the varying duration of the experiments, and the different stocks of trypanosomes.

A delay in the involvement of the central nervous system (C.N.S.) occurs with T.brucei infections in Man. In the rhodesian form of sleeping-sickness, invasion of the C.N.S. does not occur until three or four weeks have elapsed following infection (Apted, 1970). The chances of obtaining a cure are much greater if treatment is begun before the C.N.S. is involved because many drugs do not reach sufficiently high concentrations in the brain (Evans 1981). For instance, suramin will cure nearly 100% of early cases but with increasing numbers of relapses the prognosis deteriorates (Apted, 1970). Relapses are also common in T. evansi infections of camels following treatment with suramin and samorin (Losos, 1980, Rottcher pers. comm), but there is no information as to the site of persistent latent infection in the camel. Resistance of T. evansi to suramin has also been reported in camels in Ethiopia (Scott, 1973) and

Russia (Petrovskii and Khamiev, 1974). However, it is not clear from these studies to what extent the relapsing phenomenon, as opposed to real drug resistance, might have been involved.

B. THE EXISTENCE OF TISSUE FORMS OF T. BRUCEI AND T. EVANSI.

T. brucei is considered to differ fundamentally from T. vivax and T. congolense in being essentially a parasite of the tissues of the mammalian host (Wolbach and Binger, 1912, Yorke, 1911). This important characteristic was established in early histopathological studies and the subject has been reviewed by Goodwin (1970), Ormerod (1970) and Losos and Ikede (1972). A direct comparison of the distribution of T. congolense and T. brucei in the mouse and rat has been made by Ssenyonga (1980). T.brucei was found in the connective tissue and body cavities from the eighth day of infection. The numbers in the tissues remained high in contrast to the fluctuating parasitaemia. T. congolense, however, was only found in the blood-vessels, particularly the capillaries. Similarly, Morrison, Murray, Sayer and Preston (1981) found widespread extravascular invasion and tissue damage with T. brucei in the dog with the heart, eyes and C.N.S. being particularly affected.

The main points of controversy concerning tissue (i.e. extravascular) forms of T. brucei are their morphology and their location in the host. Salvin-Moore

and Breinl, (1907) described the appearance of round forms of T. gambiense in rats in the lung, bone marrow and spleen. They, along with Fantham (1911), were convinced that these were an integral part of a development cycle in the vertebrate host. Buchanan (1911) described similar forms of T. brucei in the gerbil. Hoeppli and Regendanz (1930) also observed tissue forms of T. gambiense widely distributed in the rat. In particular, they mentioned large numbers of round forms, as well as normal trypanosomes in the choroid plexus. Peruzzi (1928) gave a detailed description of T. brucei infection in monkeys in which he mentioned the widespread occurrence of trypanosomes in situations outside blood-vessels, being particularly abundant in the heart, kidney, adrenal gland and choroid plexus. He considered that the choroid plexus was an important staging post in the invasion of the C.N.S. However, he considered that the leishmanial forms which he observed within muscle fibres and phagocytic cells, as well as in tissue spaces, were degenerating trypanosomes.

Le Port, (1935), relating pathology to clinical findings of sleeping-sickness in Man, again drew attention to the possible importance of the choroid plexus. He considered that swelling of the choroid plexus might interfere with the circulation of cerebro-spinal fluid (C.S.F) and lead to ventricular hypertension and, hence, headache which is an early sign

of T. brucei infection in Man. In a more recent experimental study of T. brucei in the horse, Neitz and McCully (1971) describe the development of clinical signs and associated histopathology resulting from the involvement of the C.N.S. They mention apathy, hind-quarter paralysis and, later, excitement. Trypanosomes were found in the C.S.F. and the brain showed perivascular cuffing and diffuse gliosis. The choroid plexus was infiltrated with lymphocytes, plasma cells and morula cells.

The possibility of the occurrence of developmental tissue forms of T. brucei in the vertebrate host was raised again by Soltys and Woo (1969a). They described round forms in impression smears of liver and spleen of mice infected with T. brucei and T. congolense. Later Soltys et al (1969b) claimed to have separated these forms from blood-stream forms by means of filtration. They passed suspensions of infected spleen and liver through a range of cellulose acetate filters of different pore sizes. They found, by subsequent inoculation of the filtrate into mice, that the tissue forms would pass through a 1.0μ pore size whereas blood forms were held back. In a later study (Soltys and Woo, 1970) claimed to have observed leishmanial forms (now termed amastigotes by Hoare, 1972) by staining filter discs of 0.45μ pore size. They supported their claim by citing the fact that

amastigotes were known to be part of the life cycle of other trypanosome species such as T. cruzi in Man, T. nabiasi in the rabbit (Grewal,1957) T. microti in mice and T. evotomys in voles (Molyneux, 1968).

Ormerod (1963) described a range of morphological forms of T. lewisi in the rat kidney and suggested the possibility of circulating trypanosomes being replenished from extravascular sites. Deriving ideas from this work, and from the earlier studies, Ormerod and Venkatesan (1971a & b) drew attention to the possible importance of the amastigote form in the choroid plexus as a source of relapsing infection. They observed amastigotes in impression smears of spleen and liver of mice with three day infections of T. brucei but the greatest number were seen in the choroid plexus. Poltera(1980) working with T. brucei in mice claimed to have demonstrated trypanosomes in the interstitium of the heart and choroid plexus by means of immuno-fluorescence and electronmicroscopy. He considered that invasion of these sites did not occur until the third week of infection but was able to obtain relapses when ethidium and melarsoprol were given as early as the sixth day. He also found trypanosomes persisting in the choroid plexus when they had been cleared from the blood. Poltera, Hochmann and Rudin (1980) went on to describe the occurrence of trypomastigotes in the space between the blood-vessels and the ependyma of the choroid plexus.

However, in contrast to Ormerod and Venkatesan (1971), they did not see amastigotes. In a further study, Poltera, Hochmann and Lambert (1982) demonstrated a changed distribution of trypanosomes in the C.N.S. following treatment. In untreated mice the infection appeared to be preferentially localised in the choroid plexus. Following melarsoprol treatment, however, there was a diffuse distribution of trypanosomes through the brain. They concluded that tissue forms were an important source of relapsing trypanosomes whether treatment was given early or late in the course of infection. Van Marck, Le Ray,Beckers, Jacob, Lery and Gigase (1981) studied the choroid plexus of rodents infected with T. brucei gambiense. They stressed the difficulty of observing extravascular trypanosomes by light microscopy and therefore considered that electronmicrocsopy gave useful additional information. They found that the space between the blood vessels and ependyma was much increased during trypanosome infections compared with that found in normal mice. They found many trypomastigotes in this space but, in contrast to Ormerod and Venkatesan (1971b), no amastigotes. They concluded that the possibility of intra-cellular trypanosomes was as yet hypothetical.

Abolarin et al (1982) produced electron-micrographs which they claimed demonstrated the presence of

intra-cellular trypomastigotes in the ependymal cells of the mouse choroid plexus. They considered that these forms might be the postulated cryptic phase of T. brucei. After clearance of trypanosomes from the blood-stream with SHAM and glycerol, they demonstrated by tissue transfer experiments that the organism persisted only in the brain. They also showed by electron-microscopy that, following treatment, the perivascular spaces of the choroid plexus were cleared of trypanosomes. They postulated that the trypomastigotes from the perivascular space penetrated the ependymal cells, underwent a process of multiplication and caused disruption of the cells. They then returned to the blood-stream via the perivascular space. Poltera (1983) criticised this conclusion on the grounds that whole brain homogenates were used in the transfer inoculations instead of only the choroid plexus. He quoted further evidence (Rudin, Poltera and Jenni, 1983 and Van Marck, Malumba, Beckers, Gigase and Lery, 1983) that intracellular forms occurred also in plasma cells and macrophages in the choroid plexus.

Rudin et al (1983) provided evidence that the brain contained more extravascular forms of T. brucei than did the choroid plexus. This was particularly the case following treatment. Poltera (1983) therefore considered the case for ependymal cell involvement to be unproven.

The precise role of the choroid plexus in infections with T. brucei thus remains unresolved.

Little attention has been paid to the pathology of T. evansi so that even less is known about possible CNS involvement in infections with this species. Holmes, (1904) described amoeboid forms of T. evansi occurring particularly in the spleen, liver and bone marrow of infected ponies in India. He included a series of drawings of these round forms transforming into elongate, flagellate organisms. They appear very similar to those of Fantham, (1911) and Salvin-Moore and Breinl, (1907) of T. brucei.

Clinical observations of infected animals have provided some evidence of invasion of the CNS by T. evansi. Ataxia commonly occurs in T. evansi infections in horses. In the first description of the disease in India, Evans, (1881) mentioned locomotor disturbance in fore and hind limbs. There was also dullness but continued appetite. Hutyra and Makek(1916) described a staggering gait progressing to paralysis of the hind-quarters in Surra of horses. In T. evansi infections of horses in South America (Mal De Cadeiras) Hutyra and Marek (1916) recorded hind-limb weakness as the main sign. Infected horses tended to stand with their legs apart and in walking, dragged their hooves on the ground. Persistent appetite was also mentioned. Similarly inco-ordination and paralysis of the hind-quarters also occurs in dourine

(Smith, and Hunt and Jones 1972), an infection of horses caused by T. equiperdum. This organism is thought to be closely related to T. evansi (Hoare, 1972). Hutyra and Marek (1916) mentioned that inco-ordination also occurred in the dog with T. evansi infection.

Seegert, (1930),in a study of the disease in South America, showed a series of photographs demonstrating the marked ataxia in the horse. Innes and Saunders, (1962), while lamenting the dearth of knowledge about CNS pathology mention that T. evansi has been found in the grey and white matter, and C.S.F. of dogs. The trypanosomes were found between the cells in areas adjacent to, as well as at some distance from, blood vessels. They make a point of saying that they were not present within cells. There was a general meningo-encephalitis with necrotic foci and the choroid plexus was infiltrated with cells.

Innes and Saunders (1962) stated that C.S.F. from infected horses caused infections when inoculated into rats. Furthermore, the C.S.F. of horses with Mal de Cadeiras contained increased amounts of protein and inflammatory cells (c.f.T. brucei in Man and horses).

In a recent review of T. evansi infections, Losos, (1980) again mentioned the hind-limb paresis in the South American disease which leads to ataxia, and then inability to stand. There is also relaxation of the anal sphincter. Losos describes hind-leg paresis in natural

and experimental infections of cattle and buffalo elsewhere.

Seiler, Omar and Jackson, (1981) described a series of T. evansi infections in horses in Malaysia. Significantly, the C.N.S. clinical signs and histopathology were remarkably similar to those seen in experimental T. brucei infections in the horse (Neitz and McCully, 1971).

Neither Leese, (1927) nor Curasson, (1947) made particular reference to the importance of central nervous involvement in T. evansi infections in the camel. Leese recorded that occasionally swaying of the hind-quarters was seen but this may have been a sign of general weakness. He said that terminally there might be paralysis or a sudden episode of excitement. Curasson recorded that at post-mortem, infected camels had increased quantities of C.S.F. It is very curious that the camel, which is probably the most important species to be affected by T. evansi , appears to show so little clinical evidence of CNS involvement. No reference to CNS histo-pathology in the camel has been found.

Finally, it is interesting to note three clinical manifestations (not constant) of sleeping-sickness in Man which are paralleled in the symptomatology of T. evansi infection in animals: persistent hunger, (Ormerod, 1972) episodes of confused behaviour, and abortion (Apted, 1970).

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS.

The mice were random bred, albino females weighing between 28 and 34g. They were known as the CFI strain and were obtained from the Small Animal Centre, University of Edinburgh.

STOCKS OF T. EVANSI

Kassala	1	All isolated in Sudan in 1977 from camels by inoculation of blood into mice. After further passages in mice, they were stored as stabilates at -196 deg.C.
"	2	
"	3	
"	4	
Khartoum	3	
Kosti	2	
Kisima		Both isolated from camels in Kenya in 1982 by the same method as above.
Ol Maisor		

INFECTIONS IN MICE

All inoculations were given by the intraperitoneal route, using a 25g 5/8" or 23g 1" needle.

Detection of infection.

(1) Wet film of tail blood (Lumsden et al, 1976). At least twenty fields were examined at X 400 magnification.

(2) Microhaematocrit (H.C.T.) method (Bennett, 1962, Woo, 1969). Care was taken to ensure that the whole circumference was examined because the depth of focus of the optical system was not sufficient to allow inspection of the whole buffy coat face.

(3) Sub-inoculation of mice with:-

(a) Blood.

This was obtained by cardiac puncture under deep ether anesthesia. Filtered and unfiltered aliquots (volume not standardised) were inoculated.

(b) Tissue homogenates.

Mice were killed by exsanguination under deep ether anaesthesia and the organs were dissected out immediately after death and placed on ice. Each organ was then mixed with a small volume of P.B.S. (see below) and macerated thoroughly in a borosilicate glass tissue homogeniser (Jencons Scientific Ltd.) of 30 ml. capacity. The whole of the brain, spleen and lungs were thus treated but only about half the liver since too thick a suspension clogged the filter.

Part of each homogenate was put back on ice until inoculated while approximately 1 ml. was filtered. Filtration was performed using 25 mm.diameter discs of mixed esters of cellulose acetate and nitrate of various pore sizes, made by Millipore. They were held in Swinnex polypropylene filter holders. On most occasions a fibre glass pre-filter (Millipore AP25 Depth Filter) was used. In the later experiments, 1 ml. of P.B.S. was used to wash through the filter. The filtrate was kept on ice until needed.

On certain occasions the choroid plexus was removed from the brain with fine forceps under a dissecting

microscope. It was found essential to use a petri-dish filled with P.B.S. so that the flimsy choroid plexus floated out. Maceration was performed in a 1 ml.tissue homegeniser.

Filtered and unfiltered aliquots (approximately 1 ml.) of each organ were inoculated into separate mice.

Recipient mice were monitored for the appearance of parasitaemia by wet film, HCT, and occasionally by a second transfer of brain homogenate. They were followed for periods of between twenty four and seventy five days.

(4) Impression Smears. These were only made on a few occasions. After drying on filter paper, the cut surface of the organ was dabbed several times on a glass slide. After methanol fixation, staining was by the standard Giemsa method, at pH 7.2.

(5) Histology. This was only used in studies of the choroid plexus. The tissue was fixed in 10% formol saline and mounted in araldite before sectioning.

Standard histological methods were followed and staining was with Giemsa.

(6) Electron-microscopy. The choroid plexus was placed in glutaraldehyde immediately after dissection. After at least twenty four hours, it was transferred to osmium tetroxide before sectioning in araldite and mounting on grids. Sections were stained with lead citrate according to the method of Reynolds (1963).

SOLUTIONS AND DRUGS.

Phosphate buffered saline (P.B.S.) was made up in distilled water with the following constituents: sodium chloride (8.0g/litre), potassium chloride (0.2g/litre), disodium hydrogen phosphate (1.15g/litre), potassium dihydrogen phosphate (0.2g/litre). The pH was adjusted to 7.2.

Suramin was obtained as Antrypol from Imperial Chemical Industries Ltd., Cheshire. It was made up in distilled water at 0.5g/ml. It was administered by the intraperitoneal route.

Salicylhydroxamic acid (SHAM) was obtained from Aldrich Chemical Co.,Inc., U.S.A. It was made up and used in conjunction with glycerol according to the method described by Evans and Brightman (1980).

EXPERIMENTS

Part I

BEHAVIOUR OF BLOODSTREAM FORMS AFTER FILTRATION.

OBJECTIVE: To determine which pore size prevented the passage of these forms.

A. Blood was collected from two mice which had been infected for four days with Kassala 2. After centrifugation, the buffy coat was removed and re-suspended in P.B.S. Aliquots of this were then passed through filter pore sizes of 1.2, 1.0 and 0.8µ. The filtrates were examined for live trypanosomes by dark field microscopy.

B. Blood was collected from a three day infection of Kassala 2 and passed through a diethyl amino ethyl cellulose column. The eluate was collected and aliquots filtered through pore sizes 1.2, 1.0 and 0.8µ. Matching counts of trypanosomes (Lumsden et al, 1979) were performed on the unfiltered and filtered samples which were then each inoculated into a separate mouse.

PART II.

THE BEHAVIOUR OF TRYPANOSOMES IN ORGANS AFTER FILTRATION.

OBJECTIVE: To determine whether infective forms in organs pass through a pore size which blocks the passage of blood forms.

Brain, lung, liver and spleen were taken from a mouse with a three day infection of Kassala 2 and the organs macerated as described above. Each organ

homogenate was filtered through 1.2, 1.0, and 0.8μ pore size filters. All the filtrates were then inoculated separately into mice. Unfiltered samples were also transferred as controls.

PART III.

THE ESTABLISHMENT OF FILTERABLE FORMS IN ORGANS.

OBJECTIVE: To determine the stage of infection at which filterable forms become established in organs.

Mice were killed on different days following infection. Their blood and organs were treated as described above and inoculated into mice.

PART IV.

THE ROLE OF THE CHOROID PLEXUS.

OBJECTIVE: To determine whether the choroid plexus acted as a source of filterable, infective forms.

On six occasions, involving infections with four different sticks, the choroid plexus was removed, macerated and half the suspension filtered through 0.8μ pore size. Both unfiltered and filtered portions were inoculated into mice. All the donor mice were showing trypanosomes in the blood at the time of transfer.

PART V.

CONTROL EXPERIMENTS.

OBJECTIVE:

A. To investigate the effect of inoculum size on the development of parasitaemia and the effect of different treatments on blood-stream forms.

Blood with a known concentration of trypanosomes (counted by the haemocytometer method of Lumsden et al, (1976), was diluted with P.B.S. to give concentrations ranging from 10^6 to 10^1 per ml. Groups of mice were inoculated with 1 ml.of each dilution. Different stocks of trypanosomes were also used. Recipient mice were followed by wet film and HCT and, in some cases, by sub-inoculation of pooled brain.

B. To investigate the effect of mixing blood-stream forms with organ homogenate.

2×10^6 bloodstream forms (Kassala 3) taken from a parasitaemic mouse, were added to 1 ml. aliquots of organ homogenates from a normal mouse. These were filtered and each aliquot was inoculated into a separate mouse.

C. To investigate the effect of maceration on blood-stream forms.

Diluted blood containing 10^5 trypanosomes /ml(Kosti 2) was treated in a homogeniser in the same way as organs. Filtered and unfiltered 1 ml.aliquots were put separately into two mice.

D. To investigate the effect of brain tissue on blood-stream forms during maceration and filtration.

A known concentration of trypanosomes in blood was added to a brain suspension from a normal mouse such that the mixture contained 10^5 trypanosomes per ml. After

maceration filtered and unfiltered 1ml. aliquots were put separately into two mice.

PART VI.

THE EFFECT OF TREATMENT ON FILTERABLE FORMS IN ORGANS.

OBJECTIVE: To determine whether infective filterable forms persisted in organs following treatment.

A. Mice were infected with 10^6 trypanosomes and treated with either suramin or SHAM with glycerol at different times following infection. Three stocks of T. evansi were used and the drugs were each used at two different dose rates. The mice were killed at varying intervals following treatment and their tissues examined by sub-inoculation as described above.

B. Mice were infected with 10^6 trypanosomes and treated with either suramin or SHAM and glycerol. On different occasions, whole (i.e unmacerated) choroid plexus and filtered macerated choroid plexus was transferred to mice.

PART VII.

THE OCCURRENCE OF AMASTIGOTES IN ORGANS.

Impression smears were made from the brain, lung, liver and spleen of a three day infection with Kassala 2 and of an eight day infection with Kosti 2.

PART VIII.

THE DEMONSTRATION OF EXTRA-VASCULAR TRYPANOSOMES.

The choroid plexus from a mouse infected for fifty three days with Kassala 2 was removed and examined by histology and electron-microscopy.

RESULTS

In the tables which follow, the symbol + means that one recipient mouse developed infection from one donor organ homogenate as indicated. The symbol 0 means that no infection was detected in the stated period of monitoring.

PART I. FILTRATION OF BLOOD-STREAM FORMS.

A. The result of passing blood-stream forms through filters of different pore sizes and examining the filtrate by dark ground microscopy is shown in Table 1.

Table 1

Pore size (μ)	Observation
1.2	Many motile trypanosomes
1.0	Many motile trypanosomes
0.8	No motile trypanosomes

B. The results of passing a suspension of blood-stream forms through different pore sizes and inoculating mice are shown in Table 2 on the following page.

Table 2

Pore Size (μ)	Result
Unfiltered suspension	+
1.2	+
1.0	+
0.8	0

Recipient mice were monitored for seven days.

Experiments IA and IB suggested that blood-stream forms did not pass through a 0.8μ pore size.

PART II

FILTRATION OF ORGAN HOMOGENATES.

The results of passing homogenates through filters of different sizes are shown in Table 3.

Table 3

Pore size (μ)	Brain	Lung	Liver	Spleen
1.2	+	+	+	+
1.0	+	+	+	+
0.8	+	+	+	+

The result suggested that organs contained forms which passed through the 0.8μ pore size.

Table 4 summarises all transfers of filtered tissues carried out during the course of this study.

Table 4

Tissue	No. of transfers	No. infective
Blood	21	1
Brain	25	7
Lung	17	3
Liver	17	8
Spleen	17	1
Myocardium	1	0
Choroid plexus	7	0

The results suggested that the brain and liver were the most important sources of forms which passed through the 0.8μ pore size.

PART III FILTERABLE FORMS AT DIFFERENT STAGES OF INFECTION.

The results of tissue transfers at different stages of infection are shown in Table 5 on the following page.

Table 5

Stage of Infection (days)	Stock of T.evansi	Unfilt. Blood	Filt. Blood	Filt. Brain	Filt Lung	Filt. Liver	Filt. Spleen
1-2	Kassala2	4/4	0/5	0/5	0/5	0/5	0/5
3-10	Kassala2						
	Khartoum3	7/8	1/8	5/10	3/9	8/9	1/9
>10	Kassala2	3/3	0/3	2/8	0/3	0/3	0/3

This result suggested that forms which passed the 0.8μ pore size did not become established until the third day. Thereafter, the brain and liver appeared to be the most important sites.

PART IV. INVESTIGATION OF THE CHOROID PLEXUS.

The results of inoculating choroid plexus homogenate from untreated mice are shown in Table 6. All donor mice were showing parasitaemia at the time of transfer.

Table 6 follows on the next page.

Table 6.

Stock of T. evansi	Stage of inf'n.(days)	Filt. Choroid plexus	Unfilt. Choroid plexus
Kassala 2	3	0	+
Kisima	5	0	+
OL Maisor	6	0	+ #
Kassala 2	43	0	+ #
Kassala 2	43	0	+
Kassala 4	5	0	+ #
Kassala 2	5	0	+ #

Recipient mice died before parasitaemia confirmed.

+ Recipient mice monitored for an average period of forty nine days.

These results did not implicate the choroid plexus as an important source of forms passing the 0.8μ pore size.

PART V CONTROL EXPERIMENTS

A. Effect of the inoculum size of different trypanosome stocks on infectivity. The results are shown in Table 7.

Table 7

Stock of T.evansi	Inoculum Size (No.of organisms)	No.of mice inoculated	No infected
Kassala 2	10^6	39	38
Kassala 2	10^4	5	1
Kassala 2	10^2	5	0*
Kassala 2	10^1	5	0*
Kosti 2	10^6	25	25
Kosti 2	10^3	3	3
Kosti 2	10^2	3	3
Kassala 3	10^5	3	3
Kassala 3	10^4	3	2
Kassala 3	10^3	3	3
Kassala 4	10^6	3	3
Kisima	10^6	5	5
OL Maisor	10^6	5	5

* Pooled brains of the recipients were inoculated into mice with negative result.

The infectivity of Kassala 2 appeared to be lower than that of Kosti 2.

B. Effect of mixing blood-stream forms with normal organ homogenates prior to filtration.

The results are shown in Table 8 on the following page.

Table 8.

Inoculum (Organ homogenate and Blood-stream forms)	Result
Brain	+
Lung	0
Liver	0
Spleen	0

Recipient mice were monitored for 31 days. The presence of brain homogenate appeared to facilitate the passage of blood-stream forms through the 0.8μ pore size filter.

C. Effect of maceration on blood-stream forms. The results are shown in Table 9.

Table 9.

Inoculum	Result
Macerated, filtered blood	0
Macerated,unfiltered blood	+

Maceration appeared to have no effect on the infectivity of blood-stream forms.

D. Effect of brain homogenate on blood-stream forms during maceration and filtration.

The result is shown in Table 10. on the following page.

Table 10.

Inoculum Blood-stream forms and normal brain	Result
Macerated and filtered	0
Macerated and not filtered	+

Recipient mice monitored for 20 days.

The presence of brain tissue did not appear to influence the effect of maceration on blood-stream forms.

PART VI. THE EFFECT OF TREATMENT.

A. Investigation of effect of treatment on forms in organs which pass the 0.8μ pore size filter.

The results of treatment with suramin at 2mg/Kg are shown in Table 11, as shown on next page.

Recipient mice were monitored for an average period of 38 days.

It was difficult to demonstrate the presence of forms passing the 0.8μ pore size filter in any organ during the early stages of infection.

Table 11

Stage of infection (days)	Stock of T.evansi	Time after treatment (days)	Blood Unfilt.	Blood Filt.	Brain Unfilt.	Brain Filt.	Lung	Liver	Spleen	Kidney
2	Kassala 2	1	0	0	-	0	0	0	0	-
2	"	3	0	0	-	0	0	0	0	-
2	"	5	0	0	-	0	0	0	0	-
2	"	8	0	0	-	0	0	0	0	-
2	"	38	-	-	+	0	-	-	-	-
6	OL Maisor	16	+	-	+	0	-	-	-	0
6	"	28	0	-	0	0	-	-	-	-
12	Kassala 2	17	0	-	-	-	-	-	-	-
12	"	25	0	-	0	0	-	-	-	-
12	"	38	0	-	0	0	-	-	-	-
18	"	43	+	-	+	0	-	-	-	-
20	Kosti 2	7	3/3	-	3/3	3/3	-	-	-	-

0 indicates negative result. - indicates transfer not performed.

The results of the other treatments are shown in Table 12.

Table 12

Stage of Infect'n (days)	Stock of T.evansi	Treatment	Days after treatment	Unfilt: blood Unfilt	Brain Unfilt	Brain filt
20	Kosti 2	suramin 6mg/Kg	7	1/3	0/3	0/3
20	Kosti 2	SHAM 300mg/Kg & glycerol 4g/Kg	7	2/3	1/1	0/3
28	Kosti 2	SHAM 400mg/Kg & glycerol 4g/Kg	3	1/2	1/1	0/2

Recipient mice were monitored for 41 days.

B. The results of transferring the choroid plexus following treatment are shown in Table 13.

This Table is set out on following page.

Table 13

Stage of infect'n (days)	Stock of T.evansi	Treat-ment	Days after treatment	Blood uflt	Choroid Plexus uflt	flt	whole
12	Kassala2	suramin 2mg/Kg	17	0	0	0	
20	Kosti 2	suramin 6mg/Kg	32	2/2			0/2
20	Kosti 2	SHAM 300mg/Kg & glycerol 4g/Kg	32	1/2			0/2

The result is given as:-

Number of recipients infected/number inoculated.

Recipient mice were monitored for a period of 20 days.

Neither filterable forms passing through the 0.8μ pore size filter nor any other form could be demonstrated in the choroid plexus after treatment.

PART VII

DEMONSTRATION OF ROUND FORMS (AMASTIGOTES).

Many round forms were seen in the brain with rather fewer in the spleen and liver, of a three day infection with Kassala 2 and in the brain and spleen of an 8 day infection with Kosti 2. Diagrams of examples of these forms

are shown in Fig.1. Plate 1 shows an example of an amastigote in the brain of the 8 day infection with Kosti 2. The diameter of these organisms appeared to range from three to 5μ.

PART VIII

HISTOLOGICAL AND ELECTRON-MICROSCOPIC EXAMINATION OF CHOROID PLEXUS.

Giemsa stained sections demonstrated trypomastigotes both in the capillaries and in the perivascular space. Such forms of trypanosome that were seen in sections appeared to be elongate i.e. trypomastigote rather than amastigote.

Examination by the electron-microscope of the same choroid plexus revealed changes very similar to those observed in T.brucei infections (Ormerod, pers.comm.). Many trypomastigotes were seen in the perivascular space but none were observed within the ependymal cells. Plate 2 demonstrates large numbers of trypomastigotes situated between the ependymal cells and an adjacent capillary. Plate 3 shows the normal structure of the choroid plexus.

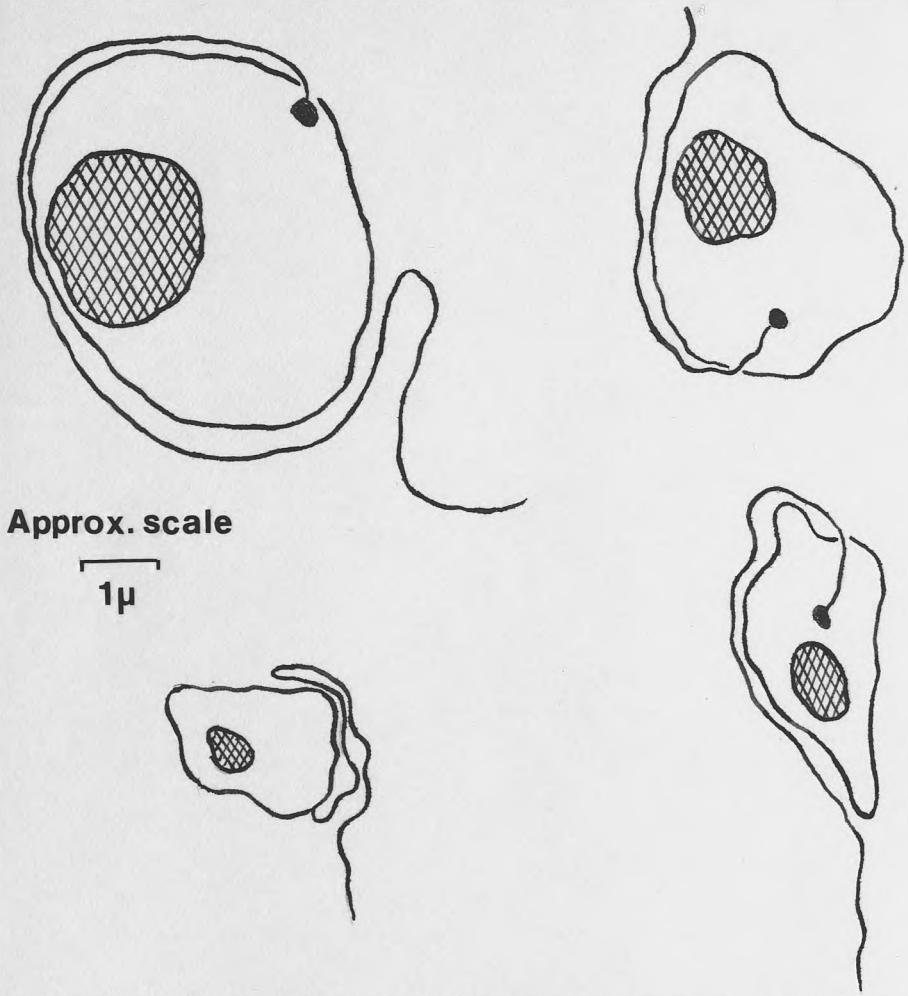


Fig 1 Round forms in brain and liver of 3 day infection with T. evansi (Kassala 2)

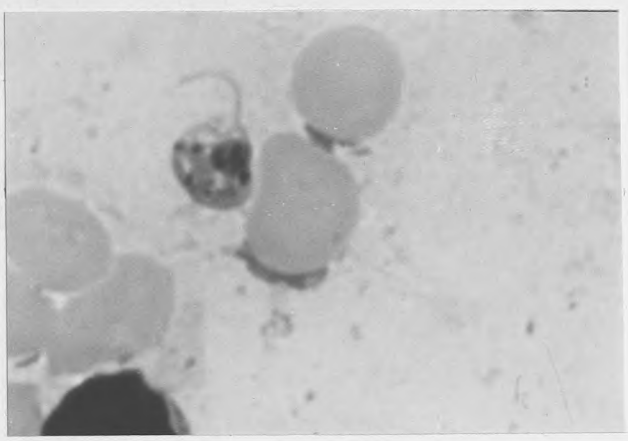


Plate 1 Round form in brain of 8 day infection with T. evansi (Kosti 2)

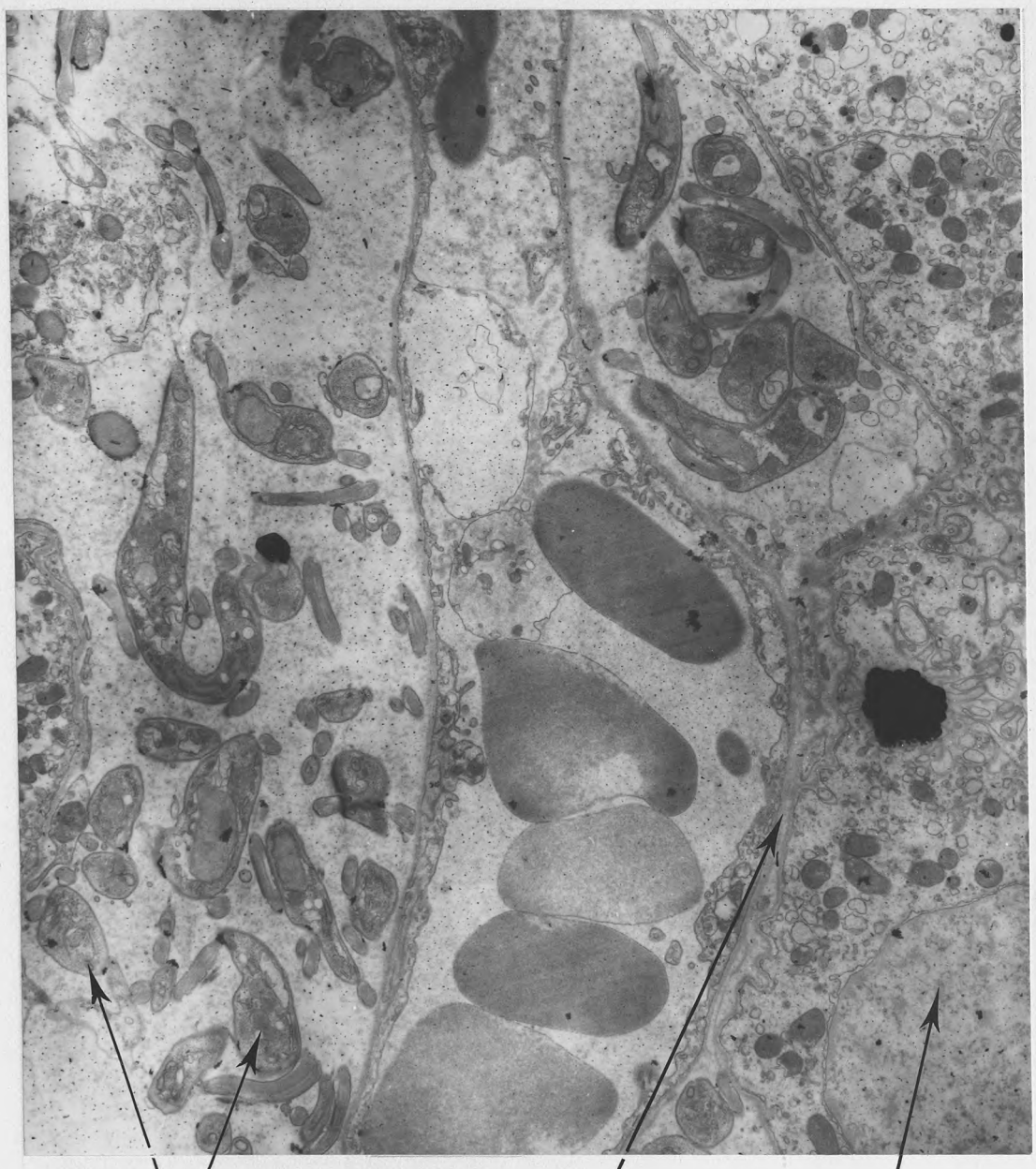


Plate 2 Choroid plexus of 53 day infection with T. evansi (Kassala 2) x 7500

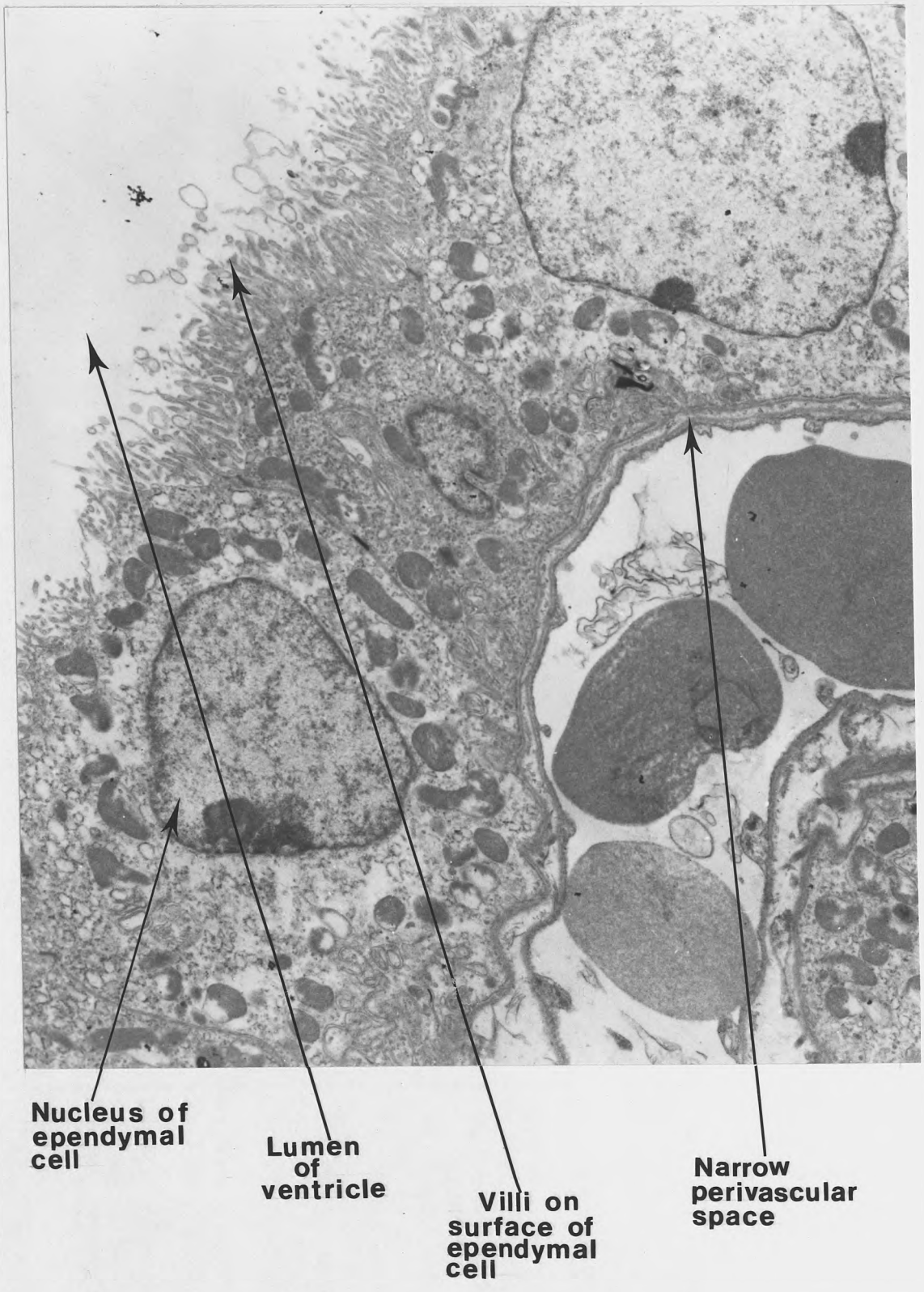


Plate 3 Relationships in choroid plexus

Table 14 Summary of Relapses Found In Different Studies.

Stock of T.evansi	Drug & Dose	Day of infection	No. rel./No. treated (days)	Time to relapse (days)	Test used	Source
Kassala 2	suramin 2mg/Kg	2	0/6*		Wet film & HCT	#
Kassala 2	suramin 2mg/Kg	12	0/3		Wet film & HCT	#
Kassala 2	suramin 2mg/kg	2	5/5	15-21	Wet film daily HCT weekly	**
Kassala 2	suramin 2mg/kg	18	2/2	43	HCT	#
Kassala 2	suramin 4mg/Kg	2	0/5		Wet film daily HCT weekly	**
Kosti 2	suramin 2mg/Kg	20	5/5	3-18	HCT	#
Kosti 2	suramin 6mg/Kg	20	3/5	7-31	blood inoc'n.	#
Kosti 2	SHAM 300mg/Kg & glycerol 4g/Kg	20	3/5	7-31	HCT & blood inoculation	#
Kosti 2	SHAM 400mg/Kg & glycerol 4g/Kg	28	1/2	3	HCT	#
+ LSHTM 34/51/6	SHAM 300mg/Kg & glycerol	3	0/25		Wet film twice weekly	##
LSHTM 33/78/1	SHAM 300mg/Kg & glycerol 4g/Kg	28	0/25		Wet film twice weekly	##
OL Maisor	suramin 2mg/Kg	6	1/2	16	HCT	#

** Abebe et al (1983).
# Present study.
Evans & Brightman (1980).
* Brain remained infected.
+ LSHTM: London School of Hygiene & Tropical Medicine.

DISCUSSION

This study was based on the premise that a size difference would allow a filtration technique to separate blood-stream forms of T. evansi from those occurring extravascularly in organs. Since any infected tissue might contain blood-stream forms within its capillary bed, it was essential to remove these in order to reveal the presence of other forms. It was therefore important to establish which pore size of filter prevented the passage of blood-stream trypanosomes while allowing smaller kinds to pass through.

Results from Part 1A and B suggested that blood-stream forms of T. evansi did not pass through the 0.8μ pore size filter. This differed slightly from the result obtained by Soltys and Woo (1970), who found that blood-stream forms of T. brucei did not pass through the 1.2μ per size.

Earlier, Reich and Beckwith (1922) and Reich (1924) quoted by Ormerod (1979), had demonstrated that forms of T. brucei in organs would pass through filters which held back both blood-stream forms of trypanosome and a Bacillus sp. used as a marker. Ormerod (1979) was unconvinced by the filtration technique on the grounds that it gave inconsistent results, perhaps because some of the pores in the filter may have coalesced.

It was demonstrated in the present study (Table V) that blood-stream forms of T. evansi occasionally passed through the 0.8μ pore size.

In the present discussion, the term filterable forms refers to trypanosomes which pass through a 0.8μ pore size Millipore filter and are then capable of infecting mice. The results from Parts II and III suggested that filterable forms occurred in the brain, lung, liver and spleen by the third day of infection, which was in agreement with the findings of Soltys et al (1969) in relation to T. brucei. Impression smears (Part VII) showed that round forms of T. evansi were present in liver, brain and spleen by the third day of infection. However, the diameter of these amastigotes suggested that they would be unlikely to pass the 0.8μ filter. Tables 4 and 5, showing the results of tissue transfers, indicate that the liver and brain contained more filterable forms than the lung and spleen. The peak of infectivity of organs appeared to be between the third and tenth day. Thereafter the number of filterable forms appeared to decline, though with a tendency to persist in the brain. The choroid plexus did not appear to be infected with filterable forms aduring the early stages of infection. However, transfers were carried out only up to day 43 (Table 6) so the importance of this tissue during long established infections may have been missed. Some evidence for this was obtained from the examination of

sections of the choroid plexus from a 53 day infection with Kassala 2 (Part VIII). This clearly demonstrated that many trypomastigotes (Plate 2) occurred in the perivascular area of the choroid plexus at this stage of the infection. Rudin et al (1983) showed that in T. brucei infections, extravascular parasites could be detected in the choroid plexus of the mouse by day 28. Abolarin et al (1982) demonstrated that extravascular T. brucei occurred in the rat choroid plexus by day 24. However, in the present study, the choroid plexus from two mice infected for 43 days failed to yield filterable forms (Table 6). This suggested that these were not important in this site in T. evansi infections. Although impression smears of the brain showed round forms of T. evansi by day 3, (Fig. 1) the extravascular parasites seen in the choroid plexus at day 53 appeared to be trypomastigotes (Plate 2). If these were the organisms relevant to latent infections they might not pass through pore size 0.8μ filters.

Unlike the findings of Abebe at al (1983), the present study did not show a clear-cut pattern of relapse following clearance of trypanosomes from the blood with drug treatment. Table 14 (p.39) compares the relapses which occurred in three different studies. In the present investigation, using suramin at 2mg/Kg in 48 hour infections with Kassala 2 (Table 11), no filterable forms

were detected in brain, lung, liver or spleen during the first eight days following treatment. In contrast to the findings of Abebe et al (1983), no parasitaemia was detected up to thirty eight days following treatment and yet, at that stage, unfiltered brain homogenate was infective. Unfortunately, blood and other organs were not transferred at the same time as the brain. It was therefore not possible to deduce that (a) The persistent infection was extravascular (though the donor mice had been aparasitaemic by the HCT method) or (b) The brain was the only site where infection had persisted. However, as the filtered brain was not infective, it appeared that the filterable forms of T. evansi were not important in the maintenance of latent infection.

Because of the initial finding that the lung, liver and spleen contained no filterable forms after treatment, only the brain and choroid plexus were studied during the remaining experiments. Treatment with suramin, at 2mg/Kg, at later stages of infection, produced relapses as indicated in Table 14. Unfortunately, in many cases relapses occurred too soon after treatment to allow transfers to be carried out during the aparasitaemic interval. However, in only three out of eight mice treated later than Day 2, were filterable forms demonstrated by inoculation of brain. Furthermore, the brains of the three mice recorded as positive, had been pooled so the overall result may have been only one out

of eight. Blood and unfiltered brain were also infective on this occasion. This was not convincing evidence of the importance of filterable forms. Relapse following treatment with suramin at 6mg/Kg was only demonstrated by sub-inoculation of blood. Simultaneous transfer of brain, both filtered and unfiltered, failed to reveal latent infection (Table 12).

Similarly, treatment with SHAM at 300mg/Kg and 400mg/Kg with glycerol at 4g/Kg was followed by relapsed parasitaemia but, although unfiltered brain was infective, filtered brain was not (Table 12). This again suggested that filterable organisms were not playing an important role in latent infections. However, the results of Part VI A suggested that latent infection might have persisted in the brain as larger forms (perhaps the trypomastigotes mentioned earlier) which did not pass through the 0.8μ pore size filter.

The failure to transfer infection with both filtered and unfiltered choroid plexus (See Table 13 in Part VI B) after treatment with suramin and SHAM with glycerol did not suggest a special role for this tissue in maintaining latent T. evansi infection. However, a different result might have been obtained if a series of transfers had been carried out at a much later stage of infection.

Of more importance than filterability is the actual site in the brain where latent infection persists and

whether different drugs can reach it in sufficient concentration to kill the organisms.

The following locations are possible sites for latent infection:-

(1) In the perivascular space of the choroid plexus i.e between the blood vessels and the ependymal cells (Plate 3).

(2) Within the ependymal cells of the choroid plexus.

(3) Within the ependymal cells lining the ventricles.

(4) Between the cells in the grey and white matter of the brain (i.e. in the extra cellular fluid (E.C.F.) of the brain).

(5) Within other cells in the brain and choroid plexus.

(6) In capillary shunts.

The occupation of site 1 by T. brucei has been demonstrated (Van Marck et al 1981, Abolarin et al 1982). The present study showed that T. evansi also occurred here. However, following treatment with SHAM, melarsoprol and diminazine aceturate T. brucei appeared to be eliminated from this site (Abolarin et al 1982, Rudin et al 1983). Sufficient electron microscopic examination of choroid plexus material has not been performed during the present study to confirm that the same occurs following treatment of T. evansi infections.

Sites 2 and 3 postulated by Abolarin et al(1982) were thought to be inaccessible to suramin and SHAM and hence, a to be likely sites from which recrudescence might occur. The possible occurrence of T. evansi in 2 and 3 remains uncertain. Only one choroid plexus was examined for intracellular forms in the present study and none were seen. If they were important sites, it might be expected that the organisms occurring in them would have passed through a 0.8μ pore size filter. However, such forms might not be infective. Nonetheless, failure to transfer injection with whole, unfiltered choroid plexus suggested that T. evansi does not occur in 2 (Table 13).

T. evansi trypanosomes have been observed in site 4 (Innes and Saunders, 1962) but the necessary examination of brain sections were not carried out during the present study. However, the negative results from the choroid plexus, taken in conjunction with the positive results from unfiltered brain during aparasitaemic periods, suggested that this site may be important in the persistence of T. evansi infections. Rudin et al (1983) showed that T. brucei persisted in site 4 in mice and monkeys following treatment with melarsoprol and diminazine aceturate.

Apart from a few organisms seen in plasma cells and macrophages (claimed by Rudin et al, 1983), there is no evidence for trypanosomes occurring in site 5. As with sites 2 and 3, organisms here might have been expected to

pass through a 0.8μ pore size, but there was little evidence for this.

The present study has neither proved nor disproved that T. evansi occurs in site 6. A hint that it might be important was given by one observation. A mouse with an eight day infection of T. evansi was aparasitaemic by the HCT and yet impression smears of the brain showed vast numbers of blood-stream forms.

The distribution of drugs in the C.N.S. was beyond the scope of this study but a brief consideration is pertinent to a discussion on the importance of the above mentioned sites. The blood-brain barrier is a rather different concept from the blood - C.S.F. barrier (Davson 1967) and this may explain how extravascular trypanosomes can persist in the brain itself when they have been eliminated from the choroid plexus perivascular space by drug treatment (Rudin et al, 1983). The blood-brain barrier comprises capillary endothelium and a covering of processes from adjoining astrocytes. The blood - C.S.F. barrier consists of capillary endothelium, connective tissue and choroid plexus epithelium (or ependyma). However, Dobbing (1961) considered the so-called barriers to be physiological rather than structural and hence they had a different effect on different substances.

Whatever the mechanism affecting it, the relative distribution of drugs between the following fluid compartments within the brain is clearly of importance to the possible persistence of trypanosomes:-

Plasma - E.C.F.

E.C.F. - intra-cellular fluid (I.C.F.) in ependymal cells } choroid plexus

I.C.F.

Plasma - E.C.F.

E.C.F. - I.C.F.

E.C.F. - C.S.F. } brain

Amongst the many factors affecting this distribution are (Barlow, 1964, Williamson, 1970 and Dobbing, 1961):-

(1) Ionic character of the drug.

(2) Ability to combine with protein.

(3) Lipid solubility.

(4) Capillaries in brain being surrounded by astrocyte processes.

(5) Rate of drug breakdown.

(6) Varying character of the blood-brain and blood - C.S.F. barriers in different parts of the brain.

(7) The very small volume of the brain E.C.F.

Examples of drug distribution (inferred from Williamson, 1970, Evans, 1981 and Rudin et al 1983) are given below:-

	Brain E.C.F.	C.P. E.C.F.	C.S.F.
Diminazine Aceturate (Berenil):	-	+	-
SHAM with glycerol	-	+	-
Melarsoprol	±	+	+
Suramin	-	+	-
Homidium	-	?	-

C.P. - choroid plexus

\+ Reaches sufficient concentration to kill T. brucei

\- Reaches insufficient concentration to kill T.brucei

± Renowned for activity when C.N.S. infection established and yet Rudin et al, 1983, noted persistence of extravascular forms after treatment.

There may be species differences in distribution e.g. homidium will enter the C.S.F. of cats, melarsenoxide does not enter the C.S.F. of rabbits but does so in monkeys. A full consideration of this topic is felt to be beyond the scope of this discussion.

The present study found T. evansi infections to relapse after treatment with suramin and SHAM with glycerol. This indicated that infection was present in an extravascular situation. The failure to transmit infection with choroid plexus, whereas successful transfer was achieved with whole brain homogenate, suggested that the infection persisted in the E.C.F. of the brain parenchyma.

Evans and Brightman (1980) showed that in their experiments, monomorphic infections (whether stocks of *T. brucei* or *T. evansi*) were cured with SHAM and glycerol whereas pleomorphic infections invariably relapsed. They suggested that either extravascular forms might be lacking in *T. evansi* infections or that such as did exist, did not possess the same metabolic capability as the stumpy forms of *T. brucei*. The present study did not seem to uphold these suggestions.

In summary, it seems that the precise area of the brain where trypanosomes persist may vary according to host species, drug used, species and strain of trypanosome, and local character of the various brain barriers.

CRITICISM OF METHODOLOGY.

A. The use of sub-inoculation as a means of demonstrating infection.

(1) The control experiments (Part V) showed that that number of organisms and the stock of *T. evansi* affected the infectivity of an inoculum. An added variable in the case of tissue transfers might have been the infectivity of the extravascular organisms themselves. The use of recipient mice treated with cyclophosphamide would have provided a more sensitive indicator system.

An alternative method would have been extensive use of the electron-microscope, but as the distribution of tissue forms is often focal, some infections would doubtless have been missed by this method also. Immunological methods (fluorescent antibody staining or serological monitoring) would have been too sophisticated for the circumstances.

(2) The method used for the detection of parasitaemia both in recipient mice, and following treatment, is critical in assessing the results of this study and comparing them with others. Initially, wet films were used but when it was realised that very low parasitaemias developed, increasing reliance was put on the HCT. Other studies (Ercoli & Iudice, 1980, Tchalim, 1980, Poltera et al 1981) have used only the wet film.

Fig.2 shows diagrammatically the minimum number of organisms detected by different methods. Apart from Bennett's study of avian trypanosomes, the methods relate to T. brucei and T. evansi.

B. Inherent defects in the filtration procedure. e.g. adverse effect on trypanosomes and pore size variation.

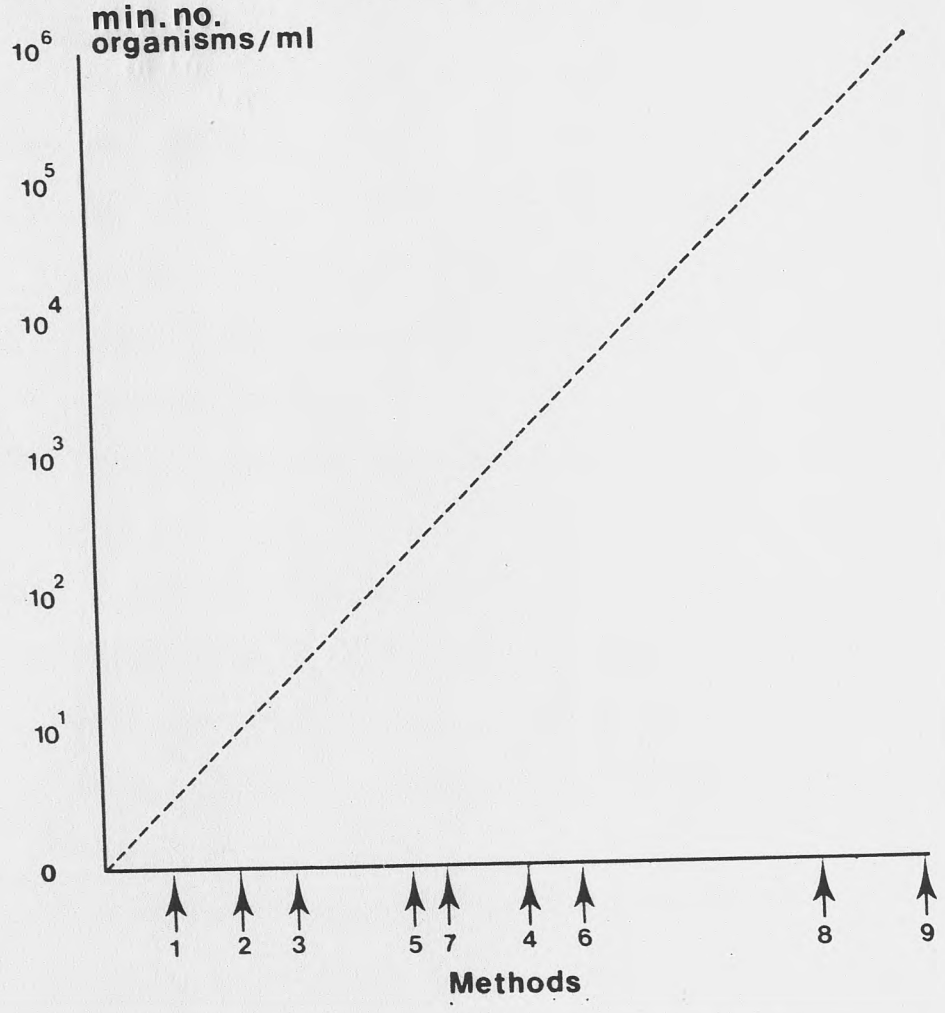
C. Defects in filtration technique. e.g. too much pressure applied; temperature variation and washing procedure not constant.

D. Lack of standard monitoring procedure. e.g. regular intervals between HCT examinations; total period of monitoring.

E. Failure to consistently transfer unfiltered tissue and blood as controls.

F. Failure to carry out transfers of organ homogenates at later stages of infection both before and after treatment.

Fig 2 Methods of detecting parasitaemia



1 Miniature anion exchange (Lumsden et al 1979)
2 Micro-haematocrit with 3 tubes (Woo & Rogers 1974)
3 Micro-haematocrit with 1 tube (Bennett 1962)
4 Wet film-1/200 fields x 400 (Lanham 1977)
5 Mouse inoculation (Paris et al 1982)
6 Buffy coat by dark ground (Paris et al 1982)
7 Micro-haematocrit with 1 tube (Paris et al 1982)
8 Wet film-1/20 fields x 400 (Herbert & Lumsden 1976)
9 Wet film-1 – 9/60 sec. search (Lumsden et al 1973)

CONCLUSIONS

(1) Filterable forms were found in brain, liver, lung spleen. Their significance was not clear and their morphology was not determined.

They might have been:-

(a) Blood-stream forms forced through the filter by faulty technique.

(b) Small trypomastigotes of amastigotes in tissue spaces.

(c) Small trypomastigotes or amastigotes within cells.

(d) Small blood-stream forms in capillary shunts.

(2) Filterable forms were not regularly found following treatment. (Some negative results may have been due to reduced infectivity or low inoculum size).

(3) Persistent latent infection occurred in T. evansiinfections but did not appear to be due to filterable forms.

(4) The brain was not shown to be the only important site for persisting infections.

(5) Trypomastigotes were found outside the blood-vessels in the choroid plexus.

(6) The choroid plexus was not implicated in the maintenance of latent infections.

(7) Amastigote (round) forms of T. evansi occured in brain, liver and spleen.

(8) The failure of SHAM with glycerol, and suramin, to effect a cure might have been linked with the occurence of extravascular trypomastigotes.

(9) The difficulty of demonstrating that the blood was free of trypanosomes, coupled with the frequent failure of filtered brain to infect mice meant that tissue trypanosomes were not proven to be responsible for persisting latent infection.

(10) Intra-cellular forms were not demonstrated.

(11) This study suggested that some of the persisting infections in the camels at Rumuruti might be due to latent infections and that this should be looked for in the liver, lung and spleen as well as brain and and choroid plexus.

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