Trypanosomiasis and the brain

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SUMMARY

Neurological involvement following trypanosome infection has been recognised for over a century. However, there are still many unanswered questions concerning the mechanisms used by the parasite to gain entry to the CNS and the pathogenesis of the resulting neuroinflammatory reaction. There is a paucity of material from human cases of the disease therefore the majority of current research relies on the use of animal models of trypanosome infection. This review reports contemporary knowledge, from both animal models and human samples, regarding parasite invasion of the CNS and the neuropathological changes that accompany trypanosome infection and disease progression. The effects of trypanosomes on the blood-brain barrier are discussed and possible key molecules in parasite penetration of the barrier highlighted. Changes in the balance of CNS cytokines and chemokines are also described. The article closes by summarising the effects of trypanosome infection on the circadian sleep-wake cycle, and sleep structure, in relation to neuroinflammation and parasite location within the CNS. Although a great deal of progress has been made in recent years, the advent and application of sophisticated analysis techniques, to decipher the complexities of HAT pathogenesis, herald an exciting and rewarding period for advances in trypanosome research.

Key words: Human African trypanosomiasis, CNS, neuroinflammation, mouse model, cytokine, chemokine, sleeping sickness.

INTRODUCTION

Human African trypanosomiasis (HAT), or 'sleeping sickness', results from infection with the protozoan parasites *Trypanosoma brucei rhodesiense* or *T.b.gambiense*. Both forms of HAT are fatal if untreated. There are two clinical stages associated with the disease. The first is the early or haemolymphatic stage where the parasites proliferate and spread in the blood and lymphatic system. This is followed by the late or CNS-stage of the disease which is marked by trypanosome invasion of the CNS. This pattern is found in both *T.b.rhodesiense* and *T.b.gambiense* infections; however *T.b.rhodesiense* is a more acute disease with rapid progression to the late stage whereas *T.b.gambiense* follows a more protracted course that can last several years before death occurs (Apted, 1970). Once the parasites have established within the CNS many of the neurological features characteristic of the infection develop. Clinical manifestations suggestive of neurological involvement include mental and psychiatric disturbances and a wide range of motor symptoms, and signs of sensory involvement can become apparent. Visual impairment can also occur. In the latter stages of the disease the characteristic disruption of the normal sleep-wake cycle, from which the disease derives its popular name, develops with the presence of nocturnal insomnia and daytime somnolence, progressing to coma and ultimate death (Atouguia and Kennedy, 2000). Treatment of early-stage disease is relatively effective; melarsoprol is the only drug available that can be used to treat both *T.b.rhodesiense* and *T.b.gambiense* infections once the CNS has become involved (Barrett *et al*. 2007). Unfortunately melarsoprol treatment is associated with severe adverse reactions and can result in the generation of a post-treatment reactive encephalopathy (PTRE) in up to 10% of treated patients with a 50% mortality rate (Pepin and Milord, 1994). The mechanisms culminating in this severe adverse reaction remain unclear (Hunter and Kennedy, 1992; Kennedy, 2006).

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NEUROPATHOGENESIS

Pathological changes

The vast majority of the data on neuropathological changes associated with trypanosome infection and drug treatment, is derived from animal models of the disease with only a few post-mortem reports examining CNS material from human cases available. Many animal species have been used in investigations but most of the information comes from mouse, rat and primate models. In *T.b.gambiense* infections of vervet monkeys, the presence of perivascular cuffing,

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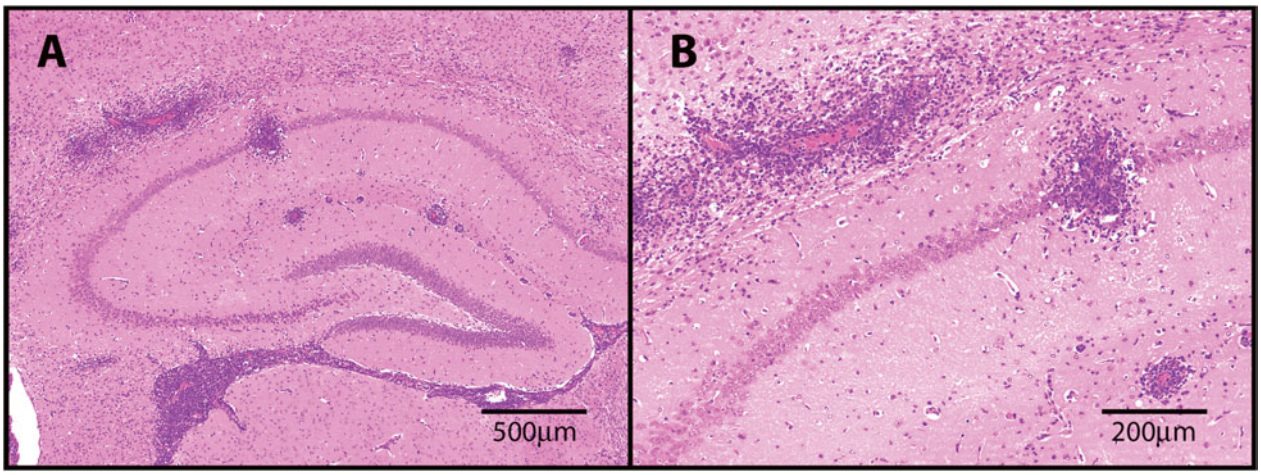

Fig. 1. H&E stained sections through the hippocampal brain region prepared from a mouse infected with *T.b.brucei* and treated sub-curatively with diminazene aceturate to exacerbate the neuroinflammatory response. Note the presence of severe perivascular cuffing and encephalitis (A). The extravasion of the inflammatory cells from the perivascular region into the parenchyma is shown in greater detail (B).

meningitis and encephalitis accompanied by an intense astocytosis has been reported. The inflammatory infiltrate was comprised of mononuclear cells, lymphocytes, plasma cells and immunoglobin containing Mott or morular cells (Ouwe-Missi-Oukem-Boyer *et al*. 2006). In experimental *T.b.rhodesiense* infections of vervet monkeys, a continuous chronic inflammatory process characterized by three distinct phases has been described. In phase 1, the animals developed a chronic meningitis with plasma cells, lymphocytes and monocytes in the subarachnoid and pial connective tissue. In phase 2, the neuroinflammation progressed from the meninges to the cerebral vessels, particularly those entering the brain, followed by the development of encephalitis in phase 3. In this model, trypanosomes appeared to spread together with the inflammatory cells, being first located in the choroid plexus, then spreading to the perivascular space and final to the brain parenchyma (Schmidt, 1983). When mice were infected with *T.b.rhodesiense* a very similar pattern of neuroinflammation, characterized by the development of meningitis, perivascular cuffing and finally encephalitis, was described (Fink and Schmidt, 1979). Again, the cell types involved were lymphocytes, plasma cells and macrophages with trypanosomes disseminating in concert with the inflammatory cell infiltrate. This inflammatory pattern was also echoed in a rat model of *T.b.gambiense* infection with the infiltration of plasma cells, Mott cells, lymphocytes and macrophages. In this study, a breakdown of T cell subsets showed that $CD4^+$ cells outnumbered $CD8^+$ cells in the infiltrate (Anthoons *et al*. 1989). A very similar neuroinflammatory reaction developed following *T.b.brucei* infection of mice marked by the infiltration of lymphocytes, plasma cells, macrophages and Mott cells leading to a diffuse meningoencephalitis (Poltera *et al*. 1980). In the Glasgow model of HAT, mice infected with *T.b.brucei* GVR35 develop a mild meningitis as the infection enters the CNS-stage. This neuroinflammatory response can be exacerbated by sub-curative trypanocidal drug treatment to generate either a moderate neuroinflammatory reaction with the presence of inflammatory cells in the meninges and perivascular space or a severe meningoencephalitis involving infiltration of the neuropil by lymphocytes, plasma cells and macrophages (Fig. 1) (Hunter *et al*. 1992*b*; Jennings *et al*. 1997; Kennedy, 1999). A marked astocytosis develops in parallel with the inflammatory cell infiltration and accompanying the onset of CNS disease (Fig. 2) (Hunter *et al*. 1992*b*). An increased neuroinflammatory reaction as a result of sub-curative drug treatment is not an idiosyncrasy of our model since this phenomenon has been reported by several researchers (Schmidt and Sayer, 1982; Poltera *et al*. 1985) and could relate to the development of the PTRE found in human cases of HAT (Hunter *et al*. 1992*a*).

In general, these models present a largely similar picture of the development of CNS disease with a stepwise infiltration of the brain by lymphocytes, plasma cells and macrophages (Kennedy, 2006). Mott cells were frequently detected however neutrophils rarely appeared. Activation of microglia (Chianella *et al*. 1999) and astrocytes (Hunter *et al*. 1992*b*; Kennedy *et al*. 1997; Ouwe-Missi-Oukem-Boyer *et al*. 2006) was frequently described. A general sparing of neuronal elements with little evidence of demyelination until the terminal stages of the infection are also a common feature (Fink and Schmidt, 1979; Hunter *et al*. 1991). Examination of human post-mortem material showed a similar pattern of neuroinflammation to that described in the animal models. Macrophages, lymphocytes and plasma cells were detected in the meninges, perivascular cuffs and

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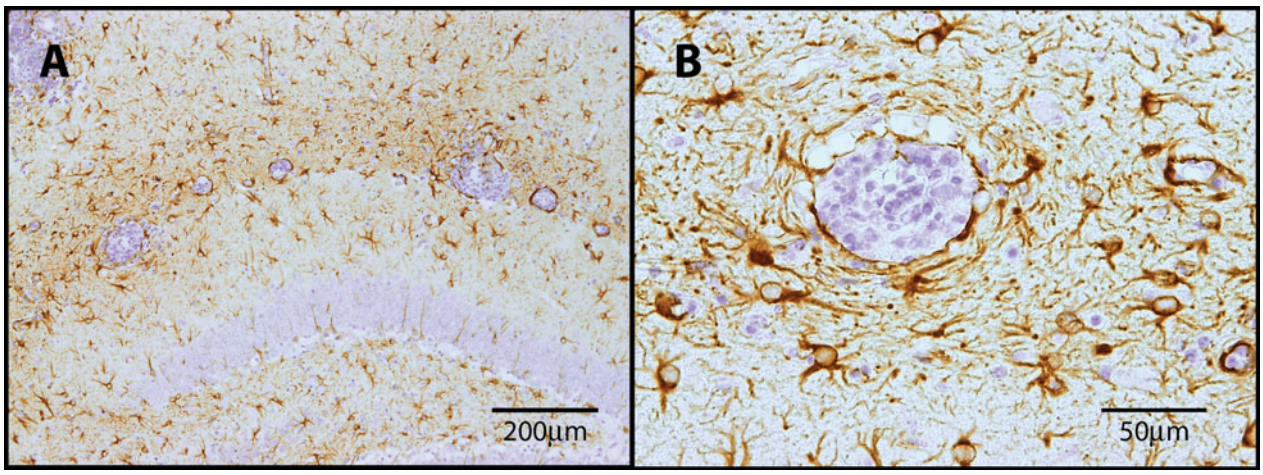

Fig. 2. Glial fibrillary acid protein (GFAP) staining of the hippocampal brain region prepared from a mouse infected with *T.b.brucei* and treated sub-curatively with diminazene aceturate to exacerbate the neuroinflammatory response. A wide spread astrocyte activation, present throughout the hippocampal region, is apparent (A). Note the large reactive astrocytes surrounding the inflamed blood-vessel and the prominent glia limitans (B).

infiltrating the neuropil. Mott cells were also present (Adams *et al*. 1986; Adams and Graham, 1998). Evidence of an inflammatory reaction was found only occasionally in the ventricles and choroid plexus (Adams *et al*. 1986). Neuroinflammatory changes were noted in areas adjacent to the ventricles including the thalamus, hypothalamus and supraoptic nuclei as well as in the cerebral white matter (Adams *et al*. 1986; Atouguia and Kennedy, 2000). Diffuse microglial hyperplasia and astrocyte activation were also reported (Adams *et al*. 1986; Pentreath, 1989). In spite of the severity of the inflammatory reaction only nominal demyelination occurred with a general sparing of the nervous tissue (Bentivoglio *et al*. 1994; Atouguia and Kennedy, 2000). The PTRE is characterized by a marked increase in the severity of the reaction and in some instances can take the form of an acute haemorrhagic leucoencephalopathy (Adams *et al*. 1986). Overall, the pathological changes reported following examination of human post-mortem samples are in accord with the data extrapolated from the animal models of CNS HAT and highlight the value of such model systems in pathological studies of this disease.

The role of CNS cytokines in the disease

Information on the expression of cytokines within the CNS during the course of the disease has been obtained through the use of animal models. Although these studies suggest an association between cytokine expression and the development of the neuroinflammation, care must be taken when interpreting the data to determine cause and effect in this highly complex system. Using comparative RT-PCR, up-regulated expression of TNF-α, MIP-1, IL-1α, IL-4, IL-6 and IFN-γ was found in the brains of *T.b.brucei* GVR35 infected mice (Hunter *et al*. 1991). The induction of IL-6, MIP-1α, TNF-α and IFN-γ and up-regulation of IL-1α mRNA expression corresponded with the onset of astrocyte activation in this model whereas GM-CSF transcripts were only detected during the PTRE (Hunter *et al*. 1992*b*). Protein levels of cytokines within the brain have been examined in a more recent study again utilising the *T.b.brucei* GVR35 mouse model of the disease. In these experiments, CNS levels of IFN-γ and TNF-α increased with the severity of the neuroinflammatory response while there was strong evidence of an exponential decline in the severity of the CNS reaction with increased levels of IL-10 and IL-6. A significant increase in IL-1β concentration in the brain was detected early after infection and remained high through the early and late-CNS stages of the disease although there was no correlation between IL-1β levels and the development of the neuroinflammatory response (Sternberg *et al*. 2005). Intraventricular injection of IL-1 receptor antagonist in a rat model of trypanosome infection resulted in a restoration of body weight in the animals but had no effect on the neuropathology whereas injection of soluble TNF-α receptors ameliorated the CNS reaction (Quan *et al*. 2003). These findings provide further evidence to support a correlation between TNF-α but not IL-1β concentrations in the severity of the neuroinflammatory reaction. *In situ* hybridisation (ISH) has also been employed to demonstrate the presence of a variety of inflammatory mediators in the brain of *T.b.brucei*-infected rats during the course of the infection (Quan *et al*. 1999). IL-1β, TNF-α, IL-1β converting enzyme and inhibitory factor κBα (IκBα) mRNA was first detected in the median eminence, choroid plexus and arcuate nucleus eventually spreading throughout the parenchyma as the disease progressed while mRNA for IL-6 and IFN-γ was found most commonly in the

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choroid plexus. TUNEL staining showed the presence of apoptotic cells in the regions of cytokine overexpression suggesting a potentially cytotoxic action (Quan *et al*. 1999). An increase in the number of cells expressing IFN-γ, TNF-α, TGF-β, and, to a lesser degree, IL-4 and IL-10, has also been detected in the rat brain by 6 hours following infection with a monomorphic strain of *T.b.brucei* (Sharafeldin *et al*. 1999). This was paralleled by a significant rise in the level of IFN-γ and TGF-β found in the CSF. In a further study Sharafeldin *et al*. used immunocytochemistry to demonstrate increased staining of the chemokines; MIP-2, RANTES, MIP-1α and, to a lesser extent, MCP-1 in the brain early after infection (Sharafeldin *et al*. 2000). Initially astrocytes and microglia were the main source of these chemokines with T cells and macrophages taking over production later in the infection. This early production of chemokines by the resident glial cells suggests that the initial steps in the development of the CNS inflammatory disease could be controlled from within the CNS and that production of these factors may be responsible for initiating inflammatory cell infiltration. In both of these studies, the production of the inflammatory mediators was detected only hours following infection. It is possible that these changes are related to the extremely acute disease pattern associated with infections using this strain of trypanosome and therefore may not constitute a true reflection of the pathogenesis found in human disease.

In HAT patients, CSF concentrations of cytokines have been used to estimate the levels of these inflammatory mediators within the brain. Significant increases in the concentration of a number of cytokines and chemokines in the CSF have been detected in both *T.b.rhodesiense* and *T.b.gambiense* infections. In the Democratic Republic of Congo, significantly elevated levels of IL-6, IL-8 and IL-10 were found in the CSF of late-stage *T.b.gambiense* patients with the concentrations reducing after melarsoprol chemotherapy (Lejon *et al*. 2002). Significant increases in CSF IL-10 and IL-6 levels have also been shown in *T.b.rhodesiense* infection in Uganda. Again, the concentration of these cytokines was reduced after drug treatment (MacLean *et al*. 2001, 2006). Further investigation of serum/CSF concentration quotients, indicated substantial intrathecal synthesis of IL-10 in 29% of patients, although the source of IL-6 could not be determined due to insufficient sample volume (MacLean *et al*. 2006). In contrast to the cytokine profiles attained from rodent models of HAT, neither of these groups found significant changes in CSF TNF-α or IFN-γ concentrations (MacLean *et al*. 2001, 2006; Lejon *et al*. 2002). However, evidence linking high plasma IFN-γ concentrations with disease progression to the CNS has been shown in a study examining the virulence of trypanosome infections in two distinct districts, Tororo and Soroti, in Uganda (MacLean *et al*. 2007). It is possible that the apparent disparity between the CNS cytokine profiles found in the human disease and the mouse model reflect either differences between the cytokines present in the brain and the CSF or variations in the sensitivity of the assay systems used. Changes in chemokine concentrations have been found in studies conducted in Gabon and Angola (Courtioux *et al*. 2006). Here, CSF from late-stage patients infected with *T.b.gambiense*, was shown to contain elevated levels of MIP-1α, MCP-1, IL-8 and IL-1β. Furthermore, high concentration of these inflammatory mediators correlated with the presence of neurological signs including sleep, gait and sensory disturbance, abnormal reflexes and psychiatric disturbances.

Potential treatment adjuncts in HAT chemotherapy

There is an overwhelming need for immediate improvements in the chemotherapy of HAT. Therefore the use of drugs that are already licensed for other diseases may provide the most timely and cost-effective method to increase the safety and efficacy of existing trypanocidal therapies. Recent studies have shown that several compounds may be suitable for this purpose. Accumulating evidence suggests that the neuropeptide Substance P (SP) may play a role in the pathogenesis of a range of neurological disorders including late-stage trypanosomiasis (Kennedy *et al*. 1997; Kennedy *et al*. 2003). Using a mouse model of late-stage HAT Kennedy *et al*. showed that administration of a highly selective non-peptide SP NK-1 receptor antagonist (RP,67-580, Rhone-Poulenc Rorer) precipitated a significant amelioration of the CNS inflammatory reaction and reduced the degree of astrocyte activation in treated animals compared to non-treated or enantiomer-treated controls. This indicates that SP may play a role in the generation of the neuroinflammatory reaction. A humanised SP receptor antagonist, aprepitant (EMEND® Merck & Co. Inc.) has been tested in the HAT mouse model in conjunction with melarsoprol treatment (Rodgers *et al*. 2007). No additional or unexpected CNS reactions were apparent indicating that the use of aprepitant as an adjunct to melarsoprol administration may be possible.

The second generation tetracycline antibiotic, minocycline, has also shown potential in this area. Minocycline treatment impedes the passage of leucocytes into the CNS when administered in experimental autoimmune encephalitis indicating that it may have therapeutic potential in multiple sclerosis. When minocycline was given to mice infected with *T.b.brucei*, beginning on the day of parasite inoculation, both leucocyte and trypanosome transmigration into the brain parenchyma were reduced together with astrocyte and microglial activation (Masocha *et al*. 2006). This was accompanied by a

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decrease in the expression of adhesion molecules; ICAM-1 and E-selectin, matrix metalloproteases; MMP-3, MMP-8 and MMP-12 and cytokines; TNF-α, IFN-γ, IL-1α, IL-1β and IL-6 as measured by comparative real-time reverse transcription PCR (Masocha *et al*. 2006). Furthermore, when minocycline was administered in combination with the trypanocidal drug suramin, given at a sub-curative dose, an extended aparasitaemic period was achieved (Amin *et al*. 2008). Although more work is required in this area it may be possible to use minocycline as an adjunct to early-stage drugs to prevent relapse in 'intermediate' stage disease. The presence of this 'intermediate' or 'early late-stage' of infection was suggested following successful treatment of patients presenting with trypanosomes in their CSF and white blood cell counts of no higher than 20, with the early stage drug pentamidine (Doua *et al*. 1996; Lejon *et al*. 2003*a*, *b*). These criteria are above the threshold figure of 5 white blood cells suggest by the WHO guidelines to define CNS-stage disease (WHO, 1998).

The kynurenine pathway, the major pathway in the metabolism of tryptophan, has been little explored with regard to trypanosome infections. There are numerous catabolites formed along this pathway, some have neuroprotective properties while others are neurotoxic. A recent study employing an experimental compound, Ro-61-8048, that inhibits kynurenine-3-monooxygenase and manipulates the pathway towards the production of neuroprotective kynurenic acid found a reduction in the severity of the neuroinflammatory response in mice infected with *T.b.brucei* following inhibitor treatment (Rodgers *et al*. 2009). These findings suggested that the kynurenine pathway is a valid target for future investigations in the development of novel chemotherapeutic adjuncts.

Magnetic Resonance Imaging (*MRI*)

Although sophisticated neuroimaging techniques are generally unavailable in HAT-endemic areas, on a few occasions the increase in international travel has provided an opportunity to use MRI to investigate the CNS-stage of the disease. Using this technology, symmetrical focal lesions in the deep white matter of the internal capsules, cerebellum and splenium of the corpus callosum (Sabbah *et al*. 1997) as well as abnormalities in the basal ganglia, external capsule and extreme capsule (Gill *et al*. 2003) were detected. The possible use of MRI to distinguish between relapsed CNS disease and the development of the PTRE has also been reported following scanning of a comatose HAT patient receiving melarsoprol treatment (Braakman *et al*. 2006). In this case, MRI showed the presence of multiple white matter lesions and lesions in the central grey matter and cortex. Fluid-attenuated inversion recovery T2-weighted MRI (T_2*) did not reveal haemosiderin within these areas indicating that the lesions were not attributable to the presence of microhaemorrhages (Braakman *et al*. 2006). In another case, a patient became confused and apraxic followed by the development of generalised tonic-clonic seizures during a course of melarsoprol (Checkley *et al*. 2007). MRI investigation showed widespread bilateral abnormalities involving the supratentorial and infratentorial white matter in T_2-derived images. In some of these areas, including the midbrain and brainstem, multiple microhaemorrhages were detected following T_2* examination suggestive of the PTRE (Checkley *et al*. 2007). Although the data presented within these studies indicate that MRI can provide valuable information in the diagnosis and management of the PTRE, it is highly unlikely that this technology, while commonplace in the developed world, will become available to the majority of those patients affected by HAT.

TRYPANOSOMES IN THE BRAIN

Although CNS involvement in HAT is well recognised in the progress of the disease there have been few reports of parasites detected within the brain (Mott, 1907). This could be the result of inadequate fixation of the brain allowing degradation of the parasites before examination of the tissue or trypanocidal drug treatment prior to the death of the patient clearing the parasite from the brain (Calwell, 1937). Many questions therefore still exist regarding the precise route and mechanisms used by the trypanosomes to facilitate CNS invasion.

Blood-brain barrier systems

The concept of the brain as an 'immuno-privileged site', with little or no capacity to mount an immune response, has altered drastically in recent times. Effective immune surveillance within the CNS, producing a parenchymal immune-mediated reaction, has been reliably demonstrated following peripheral immunisation with autoantigens or pathogens sequestered within the brain and the passage of leucocytes into the CNS is now an accepted process (Man *et al*. 2007). However, in order to preserve normal brain functions it is essential to maintain the specialized nature of the environment present within the CNS. Therefore barrier mechanisms exist to control the trafficking of cells and most molecules between the peripheral and central compartments. These barriers prevent or severely impede this exchange and exist in three forms; the blood-brain barrier (BBB), the blood-CSF barrier and the arachnoid barrier. These have been described in detail elsewhere and only a brief description will be given in this article (Abbott *et al*. 2006; Man *et al*. 2007; Saunders *et al*. 2008). The BBB is situated between

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the lumen of the cerebral blood vessels and the brain parenchyma. The physical barrier is formed by the endothelial cells comprising the vessels walls. These endothelial cells are held in close apposition to each other via tight junctions formed by four transmembrane proteins, occludin, claudins, junctional adhesion proteins (JAMs) and endothelial selective adhesion molecules (ESAMs) together with a number of cytoplasmic accessory molecules (Abbott *et al*. 2006). Adherens junctions are also present between the endothelial cells and stabilize the cell-cell interactions in the junctional zone (Abbott *et al*. 2006). The endothelial cells are surrounded by basement membrane and astrocyte foot processes, which form the glia limitans. Pericytes lie within the basement membrane between the endothelial cells and the astrocytes. These cells have long cytoplasmic processes that encircle the endothelial cells and make contact with them through specialized junctions (Zlokovic, 2008). In addition, neurons and microglia found adjacent to the astrocytes are thought to be important in barrier maintenance. These components collectively form the neurovascular units that constitute the classic BBB (Hawkins and Davis, 2005; Abbott *et al*. 2006). This barrier prevents paracellular crossing of most molecules with the exception of oxygen and carbon dioxide. Small lipophilic molecules, such as barbiturates, or ethanol can also diffuse across the barrier; movement of most other molecules requires specific transport systems present on the luminal and abluminal surfaces of the cells. There are a variety of pathways available to allow the exchange of molecules including specific transport proteins, receptor-mediated endocytosis and adsorptive mediated endocytosis (Abbott *et al*. 2006).

The blood-CSF barrier is found between the choroid plexus blood vessels and the CSF. The choroid plexus is a highly vascularised tissue located in the cerebral ventricles. In contrast to the brain parenchyma, the blood vessels here are fenestrated and the tight junctions linking the endothelial cells are discontinuous; therefore these cells form a non-restrictive barrier. Tight junctions do exist between the epithelial cells of the choroid plexus and these cells effectively constitute the blood-CSF barrier (Johansson *et al*. 2008).

The arachnoid barrier is probably the least familiar of the three barrier types. This barrier is situated between the dura and the pia and appears to be structurally the most complex of the three. The blood vessels entering the dura are fenestrated and as such form a poor barrier. However, the endothelial cells of the blood vessels entering the arachnoid and the pia are connected by tight junctions in a similar fashion to those found in the parenchyma although they lack the presence of astrocytes and pericytes. In addition, there are tight junctions between the epithelial cells forming the basal layer of the arachnoid membrane and this layer acts as a barrier between the dura and the CSF filled sub-arachnoid space. It is worthy of note that neurons are typically no more than 8–20 μm from a brain capillary but this distance can increase to centimetres when considering neuronal proximity to CSF compartments. This fact highlights the importance of the classical BBB, or neurovascular unit, in comparison with the blood-CSF and arachnoid barriers in the maintenance of the CNS microenvironment (Abbott *et al*. 2006; Zlokovic, 2008).

Leucocyte transmigration into the brain can occur through these barriers and involves a complex series of interactions between the leucocytes and the epithelial cells. The process is initiated when the leucocytes form loose connections with the endothelial cells via selectin-integrin interactions. This loose binding or 'tethering' allows the leucocyte to 'roll' along the endothelial cell barrier with the flowing blood testing for the presence of surface-bound luminal chemokines. When these chemokines are encountered by the leucocyte chemokine receptors signalling pathways within the leucocyte are activated precipitating conformational changes in the leucocyte integrins leading to high-affinity binding to the endothelial cell typically via adhesion molecules. The leucocytes then move to the inter-endothelial junction where they extend protrusions through the junction sampling for abluminal chemokines. Transmigration of the leucocyte occurs in response to the presence of these chemokines following a chemotactic gradient. Once the cells have crossed the endothelial cell layer they are sequestered in the perivascular space between the endothelial cell basement membrane and the parenchymal basement membrane. Completion of the transmigration into the brain parenchyma requires the action of matrix metalloproteinases (MMP's) that degrade the cellular matrix and facilitate leucocyte passage through the basement membrane (Man *et al*. 2007).

Trypanosome distribution in the brain

Parasites have been detected in the brain early after infection with *T.b.brucei* in rodent models (Schultzberg *et al*. 1988; Masocha *et al*. 2004). In a rat model of *T.b.brucei* infection, trypanosomes were first detected in the spinal and trigeminal ganglia, the median eminence and hypothalamic area as well as the neural lobe of the pituitary. The stroma of the choroid plexus was also highly parasitized. As the infection progressed, the parasites could be detected in the pineal gland and the area postrema. Trypanosomes were only rarely detected in the parenchyma until the terminal stage of the infection when many parasites were found in the brain parenchyma and in the spinal cord (Schultzberg *et al*. 1988). It has been suggested that trypanosome infection causes progressive damage to the BBB as the disease advances (Pentreath *et al*. 1994; Philip *et al*. 1994).

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To investigate this, fluorescent dye was injected into the jugular vein of *T.b.brucei* infected rats. The dye was first detected in the thalamus and hypothalamus. At later time points the dye was found permeating the white and grey matter of the cortex indicating a progressive loss in BBB integrity as the disease developed (Philip *et al*. 1994). Trypanosomes were also detected in the brain parenchyma. A later study, again utilising *T.b.brucei* infection of rats, showed large numbers of parasites in the choroid plexus, median eminence and pineal gland (Mulenga *et al*. 2001). Trypanosomes were also found in the meninges but only rarely in the sub-pial tissue. The numbers of parasite increased as the disease progressed and their distribution spread to the brain parenchyma. No disruption to the tight junctions of the BBB was found following staining with occludin and ZO-1 indicating that the trypanosomes had entered the brain without triggering a permanent breakdown in the integrity of the BBB tight junctions (Mulenga *et al*. 2001). This suggests that the trypanosomes enter the CNS in a regulated fashion and do not simply diffuse into the CNS as a consequence of barrier breakdown. Furthermore, studies by Masocha and colleagues found early invasion of the circumventricular organs in rats and mice following infection with *T.b.brucei*. From here the trypanosomes spread to the parenchyma via intracerebral vessels rather than through CSF (Masocha *et al*. 2004). Areas such as the circumventricular organs contain neurons that are specialized in neurosecretion or chemosensitivity; therefore the endothelium in these areas is leaky to allow tissue blood exchange of molecules. These areas are separated from the rest of the brain by a glial barrier and a barrier at the ependyma isolates them from the CSF (Abbott *et al*. 2006). This could explain the apparent delay in parenchymal invasion until the later stages of the infection and equate to the recently suggested 'early-late-stage' of the disease in human infection (Doua *et al*. 1996; Lejon *et al*. 2003*a*, *b*; Kennedy, 2004).

There is also evidence to suggest that the pro-inflammatory cytokine IFN-γ may play a significant role in trypanosome invasion of the CNS. In experiments utilising IFN-γ or IFN-γ receptor-deficient mice, a reduced number of trypanosomes was detected within the CNS compared to their wild-type counter-parts (Masocha *et al*. 2004). In these knockout mice, the parasites traversed the epithelial cell layer but failed to penetrate the parenchymal basement membrane. Whether administration of IFN-γ to the IFN-γ knockout strain would allow the trypanosomes to cross into the brain remains to be determined. Studies also indicate that the laminin composition of the basement membrane plays an important role in trypanosome transmigration into the CNS (Masocha *et al*. 2004, 2007). Masocha *et al*. have shown that basement membrane containing laminin α4 is permissive to trypanosome transmigration into the brain while that containing laminin α5 is inhibitory to parasite entry of the parenchyma (Masocha *et al*. 2004) mirroring the restrictions seen in T cell transmigration into the CNS (Sixt *et al*. 2001). These studies also demonstrated that trypanosomes fail to reach the CNS in infections of recombinant activating gene (RAG)-1-deficient mice, which lack both T and B cells, strengthening the hypothesis that lymphocyte transmigration plays a crucial role in the ability of the trypanosomes to penetrate the brain parenchyma. Studies investigating the effects of minocycline in trypanosome infections, as described above, provide further evidence linking trypanosome and T cell transmigration into the CNS (Masocha *et al*. 2006, 2007; Amin *et al*. 2008).

In vitro *BBB studies*

It is now possible to mimic the BBB using *in vitro* BBB systems and these can be useful tools in identifying the cellular and molecular pathways important to trypanosome penetration of the brain. In these systems, brain microvascular endothelial cells (BMECs) prepared from various species, including humans, can be grown on collagen-coated membranes that remain suspended in culture medium. This effectively isolates the fluid in the upper chamber, equivalent to the lumen of the blood vessel, from that in the bottom of the well, mimicking the abluminal surface of the blood vessel. The transwell system allows agents to be added to either side of the barrier and their ability to penetrate to the opposite side assessed. In addition, the effect of the agent on the real time transendothelial electrical resistance (TEER) can be monitored to measure the effect of the agent on the tightness of the barrier (Grab and Kennedy, 2008). Studies utilising this system to investigate the interaction of trypanosomes with the endothelial cells have suggested that *T.b.rhodesiense* [originally classified as *T.b.gambiense* (Nikolskaia *et al*. 2008)] appears to cross the human BMECs more readily than *T.b.brucei*. Data from multiple experiments found that 18·7% of the total *T.b.rhodesiense* inoculate traversed the barrier compared to 2·8% of the *T.b.brucei* inoculate (Grab *et al*. 2004). However, further investigation broadening the range of strains tested is required as these attributes could be peculiar to the individual strains included in this study. Only a transient reduction in TEER was seen between 3 and 6 hours following introduction of either *T.b.rhodesiense* or *T.b.brucei* to the transwell culture system suggesting that the parasites do not penetrate the cell layer through damaging the barrier integrity. Fluorescence microscopy demonstrated that the trypanosomes attach to the BMECs at the inter-endothelial cell borders (Grab *et al*. 2004). Further studies, employing confocal microscopy, have shown the presence of trypanosomes within the endothelial cell. Neither the

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viability nor the significance of these intracellular forms is currently known (Nikolskaia *et al*. 2006*b*). The importance of parasite-derived substances in the ability of trypanosomes to traverse BMECs has been demonstrated in a recent study investigating trypanosome cysteine protease enzymes (Nikolskaia *et al*. 2006*a*). These enzymes belong to the papain family and are important for the growth and survival of a number of protozoan pathogens (Sajid and McKerrow, 2002). Two forms of cysteine protease, cathepsin B-like enzyme and cathepsin L-like enzyme, also known as brucipain, are present in *T. brucei*. Transient alterations in intracellular calcium concentrations ($[Ca^{2+}]_i$) were detected in human BMECs exposed to either trypanosomes or trypanosome-conditioned medium. If a specific cathespin L-like cysteine protease inhibitor K11777 was added to the culture medium prior to exposure to the parasites the changes in $[Ca^{2+}]_i$ were prevented. This was not the case when cathepsin B-like cysteine protease inhibitors were introduced to the medium indicating a causal role for brucipain in the $[Ca^{2+}]_i$ fluctuations. The importance of the $[Ca^{2+}]_i$ changes in parasite traversal of the barrier was demonstrated in a series of experiments using BMECs pretreated with either a $[Ca^{2+}]_i$ chelator (BAPTA-AM), a phospholipase C inhibitor (U73122) or a protein kinase C inhibitor (Calphostin C) to prevent alteration in the $[Ca^{2+}]_i$. Use of these compounds precluded trypanosome crossing of the BMECs in a dose-dependent fashion. Additionally, enhanced crossing of the barrier was found if brucipain-enriched culture medium was added to the system again indicating the central role of brucipain in trypanosome penetration of the *in vitro* BBB system (Nikolskaia *et al*. 2006*a*). The potential functions of cathepsin-L and cathepsin-B were further investigated using trypanosomes electroporated with plasmids containing either brucipain or cathepsin-B transgenes designed to produce RNAi following induction with tetracycline. These parasites were used in murine infections. Induction of RNAi to inhibit cathepsin-L (brucipain) extended the survival period of 50% of the animals compared with non-induced controls and had no effect on the parasitaemia. However, induction of RNAi targeting of cathepsin-B cleared the parasites from the bloodstream and prevented a lethal infection with survival of the animals until the endpoint of the experiment. These findings indicate the cathepsin-B may be a promising chemotherapeutic target to attain curative treatments while cathepsin-L may be play a role in disease progression to the CNS (Abdulla *et al*. 2008).

SLEEP DISTURBANCES

The most commonly recognised clinical symptom associated with late-stage HAT is an alteration in sleep patterns, with the presence of daytime somnolence and night-time insomnia, which occurs following infection and becomes more pronounced as the infection advances (Buguet *et al*. 2005). Indeed these symptoms led to the disease becoming commonly known as 'sleeping sickness'.

HAT and circadian rhythms

In HAT patients, alterations in the normal circadian variation of growth hormone secretion, prolactin and cortisol levels and plasma renin activity have been described (Radomski *et al*. 1994; Lundkvist *et al*. 2004). The master circadian pacemaker, responsible for the control of these endogenous recurring rhythms in mammals, is localised to the suprachiasmatic nuclei (SCN) situated in the anterior, ventral hypothalamus of the brain (Lundkvist *et al*. 2004; Coogan and Wyse, 2008). The rhythms controlled by the SCN are entrained through light signals conducted to the SCN via the neurons of retinohypothalamic tract. In normal rats, light stimulation produces a rapid expression of c-*fos* in the SCN but this induction is strikingly reduced in *T.b.brucei* infected rats (Peng *et al*. 1994). Although the retinohypothalamic tract remains intact in these animals, a decrease in the expression of glutamate receptor subunits in the area of the SCN innervated by the retinal fibres has been demonstrated (Lundkvist *et al*. 1998*a*). In addition, a decrease in the frequency but not the amplitude of excitatory post-synaptic activity, an event normally under circadian control, has been detected in the SCN in brain slices prepared from *T.b.brucei*-infected rats (Lundkvist *et al*. 2002). Furthermore, Lundkvist *et al*. demonstrated that this reduction in firing frequency could be mimicked by treatment of brain slices prepared from normal rats with a cocktail containing the pro-inflammatory cytokines IFN-γ and TNF-α together with LPS (Lundkvist *et al*. 2002). The influence of the immune system in the control of the circadian clock has been comprehensively reviewed elsewhere (Coogan and Wyse, 2008) and diurnal variation in the expression of cytokines including IL-1β, TNF-α, and IFN-γ as well as TNF-α receptors and IFN-γ receptors within the brain and SCN have been described together with the rhythmic expression of the cell signalling molecules JAK1, JAK2 and STAT1 (Lundkvist *et al*. 1998*b*; Coogan and Wyse, 2008). Many of these pro-inflammatory mediators are highly expressed in HAT and animal models of the disease, as described above. Moreover, IL-1β and TNF-α in particular are known to influence sleep (Krueger *et al*. 2001; Obal and Krueger, 2003; Opp, 2005).

HAT and sleep structure

In addition to changes in circadian sleep-wake profiles, alterations in the sleep structure have been

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described in late-stage HAT patients (Montmayeur *et al*. 1994; Buguet *et al*. 2001, 2005) and animal models of the disease (Toth *et al*. 1994; Grassi-Zucconi *et al*. 1996). Under normal conditions, sleep can be divided into two sequential phases; REM (rapid eye movement) sleep and non-REM or slow-wave sleep (SWS). REM sleep is connected with vivid dreaming while non-REM is associated with reduced neuronal activity. On falling asleep, individuals generally enter a non-REM stage followed by a period of REM sleep. This cycle repeats throughout the night (McCarley, 2007). In polysomnographs of late-stage HAT patients, striking changes to this pattern are seen with the frequent occurrence of sleep on-set REM (SOREM) where patients go from wakefulness straight into REM sleep without passing through a preceding non-REM stage (Buguet *et al*. 2001, 2005). Polysomnography has also confirmed that the total time spent asleep, and the duration of each of the sleep stages, remain largely unaltered in HAT patients irrespective of disease severity (Buguet *et al*. 2001). Trypanosome infections therefore result in a dysregulation and fragmentation of sleep patterns rather than hypersomnia. Changes in EEG events are also evident in late-stage HAT such as the slowing of the EEG or the presence of periodic slow waves during periods of wakefulness. Altered K complexes, degraded spindals, hypersynchronic slow waves and hypnopomic delta bursts occurring during slow wave sleep have been described (Buguet *et al*. 2005). These abnormalities ameliorate following trypanocidal chemotherapy. The potential use of polysomnography, both as a diagnostic tool to distinguish between early and late-stage disease and to monitor the efficacy of chemotherapy, has been suggested although further studies in this area are required (Buguet *et al*. 2005). In a rat model of trypanosomiasis, the onset of sleep changes characterized by numerous awakenings from slow-wave sleep, a reduction in the average length of slow-wave sleep periods and reduced REM sleep latency were predictive of the terminal stage of the infection (Bentivoglio *et al*. 1994; Grassi-Zucconi *et al*. 1995). Significant decreases in slow-wave sleep and delta wave amplitude during slow-wave sleep were also apparent in a rabbit model of HAT (Toth *et al*. 1994).

The initial invasion of the brain by accumulations of trypanosomes is thought to occur in regions including the circumventricular organs and choroid plexus as described above. These areas lie in close proximity to the SCN and sites involved in sleep regulation (Bentivoglio and Kristensson, 2007) raising the possibility that host parasite interactions could result in the production of mediators such as cytokines or neurotransmitters that can act on these brain regions to alter both the circadian control of sleep-wake cycles and sleep structure itself.

CONCLUDING REMARKS

The interactions between trypanosomes, the host immune system and the CNS are numerous and complex. In recent years, some progress has been made in unravelling the mysteries of the disease but many questions still remain. Further studies investigating the apparent link between inflammatory cell and trypanosome transmigration across the BBB into the CNS are required to elucidate molecules either suitable for drug targeting or to provide markers for disease progression. The application of advanced molecular analysis techniques to define these host-parasite interactions, with the ultimate goal of manipulating the response to a more favourable outcome, provides the basis for significant advances in HAT research in the coming years.

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