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Delineating neuroinflammation, parasite CNS invasion, and blood-brain barrier dysfunction in an experimental murine model of human African trypanosomiasis

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ABSTRACT

Although *Trypanosoma brucei* spp. was first detected by Aldo Castellani in CSF samples taken from sleeping sickness patients over a century ago there is still a great deal of debate surrounding the timing, route and effects of transmigration of the parasite from the blood to the CNS. In this investigation, we have applied contrast-enhance magnetic resonance imaging (MRI) to study the effects of trypanosome infection on the blood-brain barrier (BBB) in the well-established GVR35 mouse model of sleeping sickness. In addition, we have measured the trypanosome load present in the brain using quantitative Taqman PCR and assessed the severity of the neuroinflammatory reaction at specific time points over the course of the infection.

Contrast enhanced–MRI detected a significant degree of BBB impairment in mice at 14 days following trypanosome infection, which increased in a step-wise fashion as the disease progressed. Parasite DNA was present in the brain tissue on day 7 after infection. This increased significantly in quantity by day 14 post-infection and continued to rise as the infection advanced. A progressive increase in neuroinflammation was detected following trypanosome infection, reaching a significant level of severity on day 14 post-infection and rising further at later time-points. In this model stage-2 disease presents at 21 days post-infection.

The combination of the three methodologies indicates that changes in the CNS become apparent prior to the onset of established stage-2 disease. This could in part account for the difficulties associated with defining specific criteria to distinguish stage-1 and stage-2 infections and highlights the need for improved staging diagnostics.

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1. Introduction

Human African trypanosomiasis (HAT), also known as sleeping sickness, is caused by infection with the parasitic protozoans *Trypanosoma brucei gambiense (T.b.gambiense)* or *Trypanosoma brucei rhodesiense (T.b.rhodesiense)* and is spread by the bite of the tsetse fly insect vector [1]. The disease is usually fatal if not diagnosed and treated with appropriate chemotherapy. *T.b.gambiense* is by far the more prevalent of the two infections and is responsible for approximately 97% of all reported cases with *T.b.rhodesiense* accounting for the remaining 3%. The parasites are restricted to sub-Saharan Africa where approximately 70 million people are at risk of infection. However, following the application of effective control and surveillance strategies the number of reported new cases fell to less than 10,000 in 2009 and has continued to decline with less than 3000 new cases recorded in 2015 [2]. These figures suggest that elimination of the disease, defined as less than one case per 10,000 population in at least 90% of endemic areas, by 2020 is an achievable target. Nevertheless, the reported case number likely depicts a significant under representation of the scale of the problem and WHO estimate that the actual case number is closer to 20,000 [1]. Re-emergence of the disease to epidemic levels has occurred historically [3] and this recurrence emphasises the necessity to sustain current control strategies.

Following infection the disease progresses through two stages. During stage-1 the parasites proliferate in the blood, lymphatic system and peripheral organs. However, the most serious form of HAT, stage-2, occurs when the trypanosomes circumvent the blood-brain barrier (BBB) to enter and establish within the CNS.

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Gambiense infections are associated with a chronic progressive course and can take months to years before stage-2 disease is reached, while *rhodesiense* infections are more acute with parasites entering the CNS within a matter of weeks [4]. The progression of the infection to the CNS-stage is associated with the development of a neuroinflammatory reaction described in only a limited number of human cases [5,6]. This neuroinflammatory response to trypanosome infection has been mirrored in both rodent and primate models of the human disease and is characterised by inflammatory cells, including lymphocytes, macrophages and plasma cells, infiltrating the meninges and choroid, followed by inflammation of the parenchymal vessels and lastly the development of encephalitis. Astrocyte and microglial cell activation accompany this response although little neuronal damage or demyelination occur until the terminal stages of the disease are reached [5,7–9].

The precise microenvironment required within the brain to conserve optimal function is maintained by the presence of specialised barriers situated between the neural tissue and the circulating blood [10]. These barriers protect the brain from the vast majority of toxins and pathogens as well as regulate the exchange of nutrients, metabolites, molecules and ions between the brain parenchyma and the blood by means of specific transporters and ion channels [11]. The most extensive barrier type is found between the blood and the brain parenchyma and is formed through a complex functional interplay between brain microvascular endothelial cells, which are bound together by sophisticated 'tight junctions', pericytes, astrocytes, neurons and microglial cells. Together these cells constitute the neurovascular units that comprise the 'classical' parenchymal BBB [11]. There are additional barriers in the choroid plexus separating the blood from the ventricular CSF, and between the blood and subarachnoid CSF. These barriers have endothelial cells joined by tight junctions but lack the other cellular components of the parenchymal BBB [12].

Numerous neurological conditions, of both infectious and non-infectious aetiology, can initiate various degrees of BBB impairment [13–15]. However, the impact of trypanosome infection on BBB function remains controversial [16–19]. Rhodamine dye, injected into the jugular vein of rats during the advanced stages of *T.b.brucei* infection, has been found permeating the brain cortical white and grey matter, indicating the presence of BBB dysfunction in these animals [17]. In a similar rat model, Mulenga et al. [16] detected increasing numbers of parasites in the brain parenchyma as the infection progressed, though no changes were seen in the staining patterns of the tight junction proteins occludin and zonula occludens 1, or the penetration of fibrinogen or IgG. In this instance, the findings suggest that trypanosome infection and transmigration into the CNS does not result in loss of BBB integrity. The application of an *in vitro* BBB model utilising human brain microvascular endothelial cells has provided further evidence suggesting that trypanosomes do not cause lasting damage to the BBB [19]. This study showed that *T.b.rhodesiense* induced only a transient reduction in transendothelial electrical resistance (TEER), which was most pronounced around 3 h following introduction of the parasites [19].

More recent studies, employing contrast-enhanced magnetic resonance imaging (CE-MRI) to investigate BBB function in a murine model of HAT, demonstrated significant and widespread BBB dysfunction during CNS-stage disease [20]. Furthermore, the barrier impairment was present in animals displaying only mild to moderate neuroinflammatory changes in the brain; typically comprised of inflammatory cells in the meninges and the development of perivascular cuffs around some of the blood vessels. In the investigation presented here we have extended these findings to ascertain when BBB impairment becomes apparent following *T.b.brucei* infection and measured the severity of the dysfunction. In addition, the degree of neuroinflammation and the trypanosome burden in the brain has been determined during the progression of the disease.

2. Materials and methods

2.1. Animals and infections

All animal experiments were approved by the University of Glasgow Ethical Review Committee and performed in accordance with the ARRIVE guidelines, UK Animals (Scientific Procedures) Act, 1986 and EU directive 2010/63/EU.

The well-established *Trypanosoma brucei* (*T.b.*) *brucei* GVR35 mouse model of human African trypanosomiasis was used throughout this study. Briefly, 45 female CD-1 mice were infected by intraperitoneal injection of 2×10^4 parasites in 100 µL phosphate buffered saline glucose (PBS-G). The animals were divided into three cohorts and assigned to study; the neuroinflammatory reaction (n = 20), the trypanosome load (n = 20) or BBB function (n = 5). Each cohort was further divided into sub-groups (n = 5) to investigate disease progression at 7, 14, 21 and 28 days post-infection. Only one group was allocated to MRI as serial scans were performed on individual animals at each time point. Uninfected animals were included with the neuroinflammation (n = 4) and MRI (n = 3) studies to act as normal controls.

2.2. Histopathology

The severity of the neuroinflammatory reaction was assessed in groups of mice sacrificed at each time-point. At sacrifice the animals were perfused transcardially with approximately 120 mL sterile saline. The brains were then excised, fixed in 4% neutral buffered formalin, and paraffin-wax processed. Coronal sections, taken through the hippocampal brain region, were then prepared and stained with haematoxylin and eosin. The stained sections were assessed in a blinded fashion and the severity of the neuroinflammatory reaction graded using a previously described grading scale [21]. Briefly, a score of 0 describes a normal brain, grade 1 describes sections where a mild meningitis is present while grade 2 shows a moderate meningitis with perivascular cuffing of some vessels. Grade 3 is characterised by more severe meningitis and perivascular cuffing with a few inflammatory cells infiltrating the neuropil, and grade 4 describes a severe meningoencephalitis with inflammatory cells throughout the brain parenchyma.

2.3. Quantitative PCR

Trypanosome load in the brain was determined using Taqman real-time PCR as described previously [22,23]. Mice were euthanased at 7, 14, 21 and 28 days -post-infection and perfused transcardially with 120 mL of sterile saline to remove peripheral blood from the CNS. The brains were then excised, immediately placed in dry ice and stored at −70 °C until required. DNA was prepared from a 25 mg sample of whole brain homogenate (DNeasy Tissue kit; Qiagen) and Taqman real-time PCR performed [22,23]. Briefly, Taqman PCR was carried out in a 25 µL reaction volume comprising 1 x Taqman Brilliant II master mix (Agilent), 0.05 pmol/µL forward primer (CCAACCGTGTGTTTCCTCCT), 0.05 pmol/µL reverse primer (GAAAAGGTGTCAAACTACTGCCG), 0.1 pmol/µL probe (FAM-CTTGTCTTCTCCTTTTTTGTCTCTTTCCCCC T-TAMRA) (Eurofins MWG Operon) and 100 ng template DNA. The primers and probe were specifically designed to detect the trypanosome *Pfr*2 gene. A standard curve, constructed using a serial dilution of pCR®2.1 vector containing 1×10^6–1×10^1 copies the cloned *Pfr*2 target sequence (Eurofins MWG Operon), was include in each PCR plate. The amplification was performed using a MxPro

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