Glial cells response during human african trypanosomiasis infection

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Abstract. Human African Trypanosomiasis is a neglected tropical disease whose infection rate is currently estimated at over 300,000 cases annually. The disease has two forms – acute and the chronic form. The chronic form involves the central nervous system with parasite entering the brain. The aim of the study was to find out how the glial cells respond during HAT infection as well as after treatment. This was done by experimentally infecting 14 vervet monkeys *(Chlorocebus aethiops)* with *Trypanosoma brucei rhodesiense* then treating them at 28 days post infection (DPI) with diminazene aceturate for early stage disease. Two animals were then serially euthanized on a fortnight interval from day 42 to 98 post infection. The remaining animals were treated at 140 DPI for late stage (CNS disease) with melarsoprol then monitored up to 462 DPI when the surviving animals were euthanized. The animal's brains were perfused with formalin, sectioned at the level of the third ventricle then wax embedded and then subjected to immunohistochemical staining to stain for the glial cells; astrocytes, oligodendrocytes (OGs) and microglia.

From the immunohistochemistry, astrocytosis was detectable at 42 DPI at the area next to choroid plexus which indicated the entry point of the parasites to the brain. The number and spread of astrocytes in the brain increased with progression of the disease to peak at 98 DPI. The astrocytes were mainly distributed in the white mater unlike microglia which were mainly in the grey mater. The density of microglia was notably high in the meningeal area and reduced deeper in the brain. High densities of microglia were also noted around blood vessels in advanced infection. OGs were reactive in both the white and the grey mater. All the cells responded to the infection with a proliferation as well as increase in size with progression of infection.

Keywords: Astrocytes; Microglia; Oligodendrocytes; Trypanosomiasis.

Introduction

The late stage Human African Tripanosomiasis (HAT) involves the central nervous system (CNS) and a key event is the activation of astrocytes (Kennedy, 2004). Activation of astrocytes has been linked to neuroinvasion of the trypanosomes in the brain tissue (Holash et al., 1993). In a normal brain tissue, these cells are invisible but in case of an injury they

become activated hence increase in size. This is what is referred to as astrocyte activation or astrocytosis (Pekny and Nilsson, 2005). Early astrocyte activation is likely to be a useful indicator of the initiation and progression of the CNS inflammatory response (Kennedy, 2004). The vervet monkey model of trypanosomiasis mimics the human disease both clinically and immunologically (Farah et al., 2005; Ngotho et al., 2006; 2009). In addition to astrocytes, microglia and oligodendrocytes (OGs) are the major glial cells in the brain (Streit, 2000; Giaume et al., 2007).

Microglia are a type of glial cells that are the resident macrophages of the brain and spinal cord, and one of their main functions is to monitor and sustain neuronal health (Streit, 2000). Oligodendrocytes, another type of glial cells are involved in production of myelin (Otero and Merill, 1997). These cells can respond either by activation or degeneration during brain injury (Gomes-Leal et al., 2004). OGs are the major cell type of white matter, which in humans constitutes about 50% of the total brain volume. The brain pathology, is, to a very great extent, a pathology of glia, which, when falling to function properly, determines the degree of neuronal death (Giaume et al., 2007). Malfunction of glia therefore is fatal for the nervous system; all in all glial cells can survive and operate in the presence of dead or dying neurones; neurones, however, cannot survive in the absence of glia (Giaume et al., 2007). Glial cells also have Ca2+-permeable receptors and channels, as well as store-operated channels and pumps, all of which determine Ca2+ homeostasis. In addition, glial cells detect functional activity in neighboring neurons and respond to it by means of Ca2+ signals that can modulate synaptic interactions. Ca2+ overload resulting from dysregulation of channels and pumps can be deleterious to glia (Alberdi et al., 2005). Microglia and astrocytes could contribute to excitotoxicity by releasing excitatory neurotoxins such as quinolinic acid and glutamate following brain and spinal cord trauma (Blight et al., 1995; 1997; Giulian et al., 1993; Obrenovitch, 2001). There is strong experimental evidence that OGs degeneration can be a very important pathological outcome, which can give rise to significant functional deficit following white mater (WM) damage in

both experimental and human diseases (Fowler et al., 2003; Irving et al., 1997; Matute et al., 2001; McDonald et al., 1998; Stirling et al., 2004; Zehr et al., 2004). It has been shown that astrocytes respond to infection by increase in size, both cytoplasm and the processes and then a reduction in the processes and increase in cytoplasm during healing (Maloba et al., 2011). The response of glial cells as a whole during trypanosomiasis has not been studied. This study therefore intends to investigate the response of the glial cells and quantify them during *Trypanosoma brucei rhodesiense* HAT infection in the vervet monkey.

Methodology

Ethics

This work was approved by the Institutional Animal Care and Use Committee (IACUC) of the Trypanosomiasis Research Centre of Kenya Agricultural Research Institute (TRC – KARI) where the work was carried out.

Study animals

Thirteen vervet monkeys *(Chlorocebus aethiops)* of mixed sexes (9 females and 4 males all adults) were used in the experiment. The animals were trapped from the wild in an area non endemic for HAT. They were subjected to a 90 day quarantine period during which they were screened for zoonotic diseases and treated for ecto and endoparasites before being recruited to the experiment. The animals were fed twice daily with commercial monkey pellets (Unga Feeds Ltd, Nakuru, Kenya) and fresh fruit and vegetables including carrots tomatoes and bananas. They were provided with water *ad libitum.* They were housed in individual stainless still cages measuring 90x60 cm^3 at temperatures of between 23-25°C.

Experimental Design

Twelve of the thirteen animals were each infected with 104 *T.b. rhodesiense* (Isolate KETRI 2537), delivered in 1ml of phosphate saline glucose (PSG) intravenously. One monkey was used as a negative control. The monkeys were treated at 28DPI with

diminazene aceturate (Veriben®, Ceva Sante Animale, France) intramuscular at a dose/day of 5mg/kg body weight for three days. Two vervets were then randomly selected and euthanized with pentobarbitone sodium, (Eutha-naze®, Bayer – Germany) at 50 mg/kg body weight serially on a fortnight interval from day 42 to 98 post infection and perfused brain samples obtained. The remaining animals were treated with the second treatment for late stage trypanosomiasis administered at 140 DPI. This was done with Melarsoprol (Arsobal®, Sanofi-Aventis, France) which was administered at a dose rate of 3.6 mg/kg/day infused intravenous via saphenous vein for four consecutive days. The remaining animals together with the control were monitored until 462 DPI when they were euthanized. However one animal died at 187 DPI. The experimental design was according to Ngotho et al. (2009).

Pathology

Brain samples collected from experimentally infected vervet monkeys with HAT in a previous study (Ngotho et al., 2009) were used in this experiment. During the perfusion, the animals under deep anesthesia were infused with physiological saline followed by 10% formalin in order to fix the brain tissue *in situ.* The vervet brain samples were collected at the level of the third ventricle containing the choroid plexus, suprachiasmatic nucleus, and brain stem. The brain samples were then fixed with formalin, wax embedded and stored till use. The samples were in pairs from each animal – one from the left and the other from the right side of the brain. These brain samples were then analyzed by immunohistochemistry using Glial fibrillary acidic protein (GFAP) (rabbit polyclonol anti-glial fibrillary acidic protein, Dako, Denmark) which is specific for astrocytes as a primary antibody. The sections were also stained for OGs using Glutathione S-Transferase pi Ab-1 (GSTpi) (rabbit polyclonal, Lab Vision, Thermscientific, UK) and for microglia using CD 68 (Mouse Monoclonal, Dako, Denmark).

Immunohistochemistry

Immunohistochemistry was performed on the wax-embedded frontal serial sections from which 15 μm thick slides were prepared. The slides to be used for immunohistochemistry were prepared by coating with chrome alum-gelatin (subbing) solution then incubated at 50°C for one hour and left overnight at room temperature. This aided in adhesion of the tissue to the slide. The brain sections were placed on superfrost slides (Fisher Scientific, Town, USA), deparaffinized through xylene (Riedl-de Haën® Sigma-Aldrich, Germany) for 5 minutes followed by re-hydration through serial dipping in ethanol (Pancreac, Pancreac Quimica SAU, Spain, E.U.) of following dilutions: 100% 5 minutes, then 95% 5 minutes, 80% 3 minutes. The slides were then washed with running tap water for 5 minutes. They were then washed with phosphate buffered saline (PBS) (Fisher Scientific, UK) at pH 7.5. The sections were then rinsed in tap water in a coupling jar. This was followed by blocking for endogenous peroxidase activity by incubating the sections in the dark for 15 minutes in 0.6% hydrogen peroxide in methanol. The sections were washed with phosphate buffered saline for 5 minutes and incubated for 20 minutes with horse normal serum PBS (Vectastain® Vector Laboratories, Inc., USA) diluted at 1:100 in. Excess serum was then blotted from the sections and thereafter incubated for 30 minutes in primary antiserum diluted in PBS. This was followed by staining with primary antiserums for 30 minutes. The primary antiserums used were antibody against Glial Fibrillary Acidic Protein (GFAP) diluted in PBS pH 7.5 at 1:2000 for staining astrocytes, GSTpi diluted at 1:100 in PBS pH 7.5 for OGs and CD68 diluted at 1:100 in PBS at pH 7.5 for microglia and all left to stand for 1 hour before use. The slides were washed in PBS followed by incubation of sections for 30 minutes with biotinylated secondary antibody solution (Vectastain® Vector Laboratories, Inc., USA) which was raised in horse.

They were then washed in PBS for 5 minutes. This was followed by incubation for 30 minutes in Vectastain® ABC reagent (Vectastain® Vector Laboratories, Inc., USA) a mixture of ragent A and B diluted in buffer at 1:25. The slides were washed again in buffer for 5 minutes. They were then incubated in peroxidase substrate solution (Vectastain®

Vector Laboratories, Inc., USA) until the desired stain intensity developed. The slides were then rinsed in tap water and counter stained with Mayer's haematoxylin for 45 seconds. The slides were washed under running tap water before they were dehydrated using 95% and 100% ethanol twice in each concentration with 14 dips in each coupling jar. The slides were rinsed with tap water and then incubated in xylene overnight at room temperature to clear alcohol. They were then mounted and examined under a light microscope (Leica DM 500 with attached camera – Leica ICC 50) starting with a magnification of X100 then X400. Cells were counted according to method described by Maria et al. (2006) but this time using a grid measuring 0.05 mm^2 at a magnification of X250.

Results

From the staining, astrocytosis was detectable at 42 DPI at the area next to choroid plexus which indicated the entry point of the parasites to the brain. The size of astrocytes increased with progression of the disease to peak at 98DPI (figure 1). One animal died at 187 DPI and this could have been due to melarsoprol effect which is said to post treatment reactive encephalopathy (PTRE). At this time the astrocytes appeared to be shrinking in size and had almost disappeared by 462 DPI which signified healing. At 42 DPI the astrocytes were approximately 20 μm full length with long processes of about 10 μm. The astrocytes had mostly 2 processes. At 56 DPI the astrocytes were slightly bigger with a size of about 30 μm in full length while at 70 DPI astrocytes were of size 35 μm long with at least four processes. At 84 the astrocytes sizes were about 40 μm with prominent processes. At 98 DPI, the astrocytes length was about 50 μm with long and numerous processes. For samples taken on day 187 post infection, the processes were very short (2.5-5 μm long) with large cell bodies of 15-20 μm. The staining intensity was also darker than the previous time points which indicated loss of viability. At 462 DPI, samples had very small astrocytes measuring 10 μm and short processes measuring 2.5-5 μm. Generally, the astrocytes were detected in the white mater and the pathology was more severe in this region than in the grey mater. The astrocytes were mainly distributed in the white mater unlike microglia which were mainly in the grey mater. The density of microglia was notably high in the meningeal area and reduced deeper in the brain during early infection starting from around 42 DPI just like the astrocytosis.
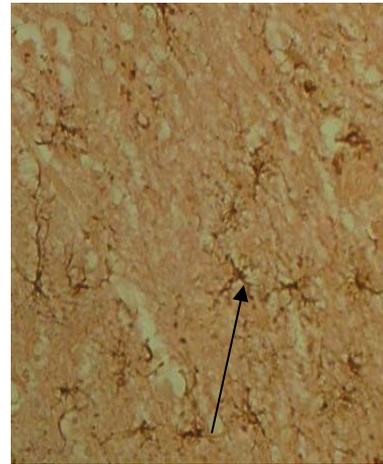
Figure 1. Astrocytosis severe pathology 98 dpi (x400). The arrow indicates an activated astrocyte
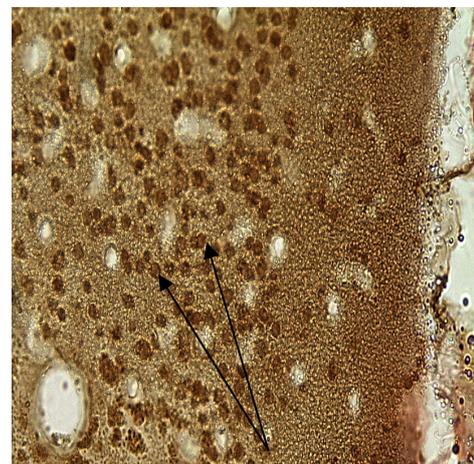

Figure 2. Reactive microglia at 98 dpi (x400). The arrows indicate reactive microglia

High densities of microglia were also noted around blood vessels in advanced infection. Microglia were reactive (increased in size and numbers) after infection and this progressed with days post infection. The reactivity was seen to start from the meningeal area where there was most reactivity and lessening deeper in the parenchyma. The cells were mainly seen in the gray mater unlike the astrocytes which are mainly found in the white mater. OGs were responsive both in the white matter and grey mater. They were responsive as the infection

progressed starting from 42 DPI which coincided with astrocyte activation i.e. parasite entry into the brain, but by day 98 post infection, most of them were degenerative (figure 3).

All the cells were responsive to the infection with a proliferation as well as increase in size with progression of infection.
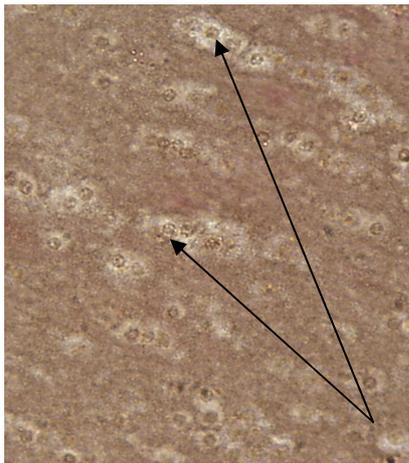
Figure 3. Pycknotic oligodendrocytes at 98 dpi (x400). The arrows indicate pycknotic oligodendrocytes

As Indicated in table 1, there was a gradual increase in number of astrocytes as measured around the region bordering the third ventricule to peak at 70 DPI and then "decline" at 84 DPI and 98 DPI. There was a high number of microglia at 42 DPI but just around the region bordering the third ventricle with most of the brain parenchyma having none. There was a noticeable increase in these cells number in the brain parenchyma from 70 DPI to peak at 98 DPI as well as an increase in their sizes with 42 DPI diameter size being 10 μm, while at 56 DPI being 20 μm and at 98 DPI having a diameter of 40 μm. There was a prominence of the cells in the meninges at the later stages of infection mainly at 98 DPI. OGs were also high in density in the same areas as microglia at day 42 and then scanty in the parenchyma at day 56 post infection then increased to peak at 98 DPI with most of them being degenerative at this time then decline by 462 DPI.

Discussion

Astrocytosis was detectable by day 42 post HAT infection at the area next to choroid plexus which indicates the entry point of the parasites to the brain.

Table 1. Cell counts at various time points – days post infection

Cells/field	DPI							
	control	42	56	70	84	98	187	462
Reactive astrocytes/field	0±0	13.3±2.7	26.3±5.2	37.8±6.5	23.6±6.2	14.9±3.1	21.0±2.4	16.1±4.6
Reactive Microglia/ field	0±0	12.8±5.0	32.5±4.3	54.3±7.9	71.1±11.3	140.4±12.9	52.3±12.5	15.6±5.1
Oligodendrocytes/field	50±3.2	55.5±5.2	64.3±6.2	70.6±10.1	88.3±4.2	102±8.3	48.5±3.2	58.2±3.4

NB: No. of cells – mean±SD.

The size of astrocytes have been shown to increase with progression of the disease to peak at 98 DPI which also coincides with severe pathology (Maloba et al., 2011). At this time, the astrocytes appeared to be shrinking in size and had almost disappeared by 462 DPI which signified healing. The astrocytes were mainly distributed in the white mater unlike microglia which were mainly in the grey mater. The density of microglia was notably high in the meningeal area and reduced deeper in the brain. High densities of microglia were also noted around blood vessels in advanced infection. OGs were responsive in both the

white and the grey mater. All the cells responded to the infection with a proliferation as well as increase in size with progression of infection to peak at 98 DPI then decline in density. Oligodendrocyte proliferation is associated to neuronal damage through cytokine release during brain pathology (Wesselingh and Thompson, 2001). The increase in density could have been associated with demyelination due to infection hence recruitment of more cells while at the same time were undergoing degeneration then cell death. This could explain why there was decline in density at 187 DPI. This was then

followed by an increase in the numbers OGs by 462 DPI which could indicate a remyelination as shown by Lucchinetti et al. (1999) during multiple sclerosis.

Late stage HAT is characterized by encephalitis (Schmidt, 1983). In the rat model, early infiltration of the brain by astrocytes occurs in areas in which the blood brain barrier (BBB) is not well developed: the sensory ganglia, area postrema, pineal gland, and median eminence (Giese et al., 1998). Later, the BBB is disrupted more diffusely (Philip et al., 1994). Astrocyte activation is one of the first signs of neurological involvement (Giese et al., 1998). Late-stage HAT and the development of PTRE are histologically characterized by perivascular cuffing, nonspecific lymphoplasmacytic meningoencephalitis, microglial hyperplasia, reactive astrocytes, and infrequent demyelination (Giese et al., 1998; Pentreath et al., 1994). Although early activation of astrocytes occurs diffusely, marked activation of microglial cells occurs in a discrete distribution in advanced disease (Chianella et al., 1999). Areas of activation include the cerebral cortex, septum, and hypothalamus and are not associated with neuronal damage histologically (Chianella et al., 1999). The onset and progression of microglial cell activation also correlates with the onset and progression of sleep disturbances, leading to speculation about the role of microglia in this prominent clinical manifestation of HAT (Chianella et al., 1999; Pentreath et al., 1994). The microglia undergo changes in morphology, numbers, cell surface receptor expression and production of growth factors and cytokines (Streit, 2000). OGs on the other hand undergo degeneration which present as pyknotic bodies (Gomes-Leal et al., 2004). Damage of OGs coupled with destruction of myelin sheath eventually lead to impaired nerve impulse transmission (Káradóttir and Attwell, 2007).

The late stage of the disease is characterized by influx of inflammatory cells resulting in meningitis, which culminates in severe meningoencephalitis when the trypanosomes invade the brain parenchyma (Kennedy, 2004). In many cases, inflammation results in thickening of leptomeninges which become adherent to the dura mater. The meningitis is accompanied by severe perivascular cuffing of the blood vessels with inflammatory cells infiltrating the parenchyma. The cellular infiltration is composed of lymphocytes, macrophages – microglia and plasma cells. In addition, Mott cells, which are plasma cells containing immunoglobulin are frequently encountered. A pronounced astrocytosis and diffuse microglial hyperplasia are significant features of the neuroinflammatory reaction. (Sternberg et al., 2005; Kennedy, 2004).

Being the defense cells, microglia's high density indicate the fight they launch to the parasite entry. Resting microglia possess the general function of CNS surveillance and present a down regulated state (Kreutzberg, 1996; Perry and Gordon, 1991; Raivich et al., 1999; Stoll and Jander, 1999). Microglia are considered very sensitive to minor pathological conditions, such as variation in the extracellular ions concentration (Kreutzberg, 1996). Resting microglia become activated in all pathological conditions such as stab wound, degeneration, stroke, ischemia, brain and spinal cord trauma, brain infection, and neurodegenerative diseases (Ishizawa et al., 2004; Kreutzberg, 1996; Perry and Gordon, 1991; Raivich et al., 1999; Stoll and Jander, 1999).

Acknowledgements

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