

# ***Trypanosoma brucei brucei* crosses the blood–brain barrier while tight junction proteins are preserved in a rat chronic disease model**

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## ***Trypanosoma brucei brucei* crosses the blood–brain barrier while tight junction proteins are preserved in a rat chronic disease model**

African trypanosomiasis, sleeping sickness in humans, is caused by the systemic infection of the host by the extracellular parasite, the African trypanosome. The pathogenetic mechanisms of the severe symptoms of central nervous system involvement are still not well understood. The present study examined the routes of haematogenous spread of *Trypanosoma brucei brucei* (*Tbb*) to the brain, in particular on the question whether parasites can cross the blood–brain barrier, as well as their effect on tight junction proteins. Rats were infected with *Tbb* and at various times post-infection, the location of the parasite in the central nervous system was examined in relation to the brain vascular endothelium, visualized with an anti-glucose transporter-1 antibody. The tight junction-specific proteins occludin and zonula occludens 1, and the possible activation of the endothelial cell adhesion molecules

ICAM-1 and VCAM-1 were also studied. At 12 and 22 days post-infection, the large majority of parasites were confined within blood vessels. At this stage, however, some parasites were also clearly observed in the brain parenchyma. This was accompanied by an upregulation of ICAM-1/VCAM-1. At later stages, 42, 45 and 55 days post-infection, parasites could still be detected within or in association with blood vessels. In addition, the parasite was now frequently found in the brain parenchyma and the extravasation of parasites was more prominent in the white matter than the cerebral cortex. A marked penetration of parasites was seen in the septal nuclei. In spite of this, occludin and zonula occludens 1 staining of the vessels was preserved. The results indicate that the *Tbb* parasite is able to cross the blood–brain barrier *in vivo*, without a generalized loss of tight junction proteins.

Keywords: blood–brain barrier, endothelial cells, inflammation, nervous system, occludin, *Trypanosoma brucei*

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## **Introduction**

African trypanosomiasis, sleeping sickness in humans and nagana in animals, is caused by the systemic infection of the host by the extracellular parasite, the African trypanosome. African trypanosomes have evolved a sophisticated process of antigenic variation, by which the parasite continuously changes its variant surface glycoprotein (VSG) coat, thus enabling evasion of the host antibody immune responses (reviewed by

Donelson *et al.* [4]). Although African trypanosomiasis presents itself in two different forms, the chronic (predominantly occurring in central and west Africa) and the acute (east, central and parts of southern Africa), these versions are at the opposite ends of a spectrum of the same disease. Similar pathological and neuropathological hallmarks of the disease are observed in both forms, in which they are extended over months to years in the chronic form, and weeks to months in the acute form (reviewed by Kristensson and Bentivoglio [11]). In humans, the chronic form is caused by the subspecies *Trypanosoma brucei gambiense*, while the acute form is

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caused by *T. brucei rhodesiense*. *Trypanosoma brucei brucei* (*Tbb*), which is non-infective for humans, causes a mild form of the disease in wild life and game, but a severe disease in domesticated animals. *Tbb* is infective for rodents, hence its use in experimental models of African trypanosomiasis.

African trypanosomiasis is characterized by a number of distinct neurological symptoms, which include disruption of sleep and extrapyramidal motor disturbances as well as neuropsychiatric changes (reviewed by Pentreath [16]). Although the histopathological reactions in the brain have been well described, the pathogenetic mechanisms behind the nervous system disease remain to be clarified. In particular, it is not known whether or not the parasites can penetrate the blood–brain barrier (BBB), which is a question of critical importance for therapeutical considerations (reviewed by Pentreath [17]). In experimental animals, *Tbb* parasites have been found at early stages of infection in the choroid plexus, as well as in other circumventricular organs and peripheral ganglia, which all lack a BBB or blood–nerve barrier [22]. The present study was undertaken to analyse, in a chronic experimental model of African trypanosomiasis in rats, whether the parasites cross the BBB or not, and their effect on tight junction proteins.

## Materials and methods

The experiments were conducted following protocols that received institutional approval and authorization by the local animal ethical committee (Stockholms Norra Djurförsöksetiska Nämnd, project N152/98), and all efforts were made to minimize animal suffering and the numbers of animals used. Animals were supplied by authorized breeders (B & K Universal AB, Sollentuna, Sweden). The rats were kept under a 12–12 h-light/dark cycle, with food and water *ad libitum*.

### Trypanosome infection

Male rats (200–250 g) were infected by intraperitoneal (i.p.) injection of a pleomorphic stabilate (1 × 3 day passage in a CB57 mouse) of *T. brucei* (AnTat 1:1, derived from stabilate EATRO 1125, obtained from Dr N. van Meirvenne, Laboratory of Serology, Institute of Tropical Medicine 'Prince Leopold', Antwerp, Belgium), diluted in 60 mM phosphate-buffered saline (PBS) containing 40 mM glucose. A volume of 0.5 ml, containing

25 000–30 000 trypanosomes, was injected into each animal. On the day of sacrifice, blood samples were taken from the tip of the tail to assess parasitaemia, and the animals were weighed.

### Preparation of tissue

In one set of experiment, rats were deeply anaesthetized with chloral hydrate (i.p.; 30 mg/100 g body weight) and rats were killed by decapitation on day 11 ( $n=4$ ), 22 ( $n=4$ ), 42–45 ( $n=4$ ), and 55 ( $n=2$ ) post-infection (p.i.). The brains were quickly dissected out, immediately frozen on dry ice, and kept at  $-70^{\circ}\text{C}$  until sectioning.

In another set of experiments, seven rats were deeply anaesthetized with chloral hydrate after a p.i. time of 11, 25, 42, 49, and 55 days and perfused through the ascending aorta with 50 ml 0.9% saline, followed by a cold fixative composed of 4% formalin and 14% picric acid in PBS (pH 7.4). The brains were dissected out, post-fixed for 2 h at  $4^{\circ}\text{C}$ , and cryoprotected overnight in 12% sucrose in PBS.

Two uninfected rats in each set of experiments were included as controls.

Fourteen micrometre frontal serial sections were cut on a cryostat and thaw-mounted on chrome–alum gelatine-coated slides, two sections per slide. Sections were collected from the level of the third ventricle containing the choroid plexus, the septal nuclei, the suprachiasmatic nuclei and the median eminence, and from the level of the pineal gland.

### Immunofluorescence histochemistry

The fresh frozen sections were fixed for 30 s in cold ( $4^{\circ}\text{C}$ ) 4% formalin and 14% picric acid in PBS, rinsed three times in PBS, fixed for 30 s in  $-20^{\circ}\text{C}$  acetone, and rinsed twice in PBS prior to immunohistochemical processing. Most of the analyses were made from fresh frozen brains. For the plasma proteins (fibrinogen and rat IgG) and glucose transporter-1 (GLUT-1)/parasite immunohistochemistry perfused material was also examined.

Most of the sections were taken from the level of the septal nuclei with the choroid plexus clearly visible in the lateral ventricle, and from the level of the pineal gland. All sections were pre-incubated with 1% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS (all primary and secondary antisera were diluted in this solution) for 1 h at room temperature. One section per

slide, from each of the aforementioned levels at each time point p.i. was then incubated with a mixture of rabbit polyclonal anti-VSG (1 : 5000; AnTat 1 : 1, prepared from a lyophilizate, 1 : 1 in double distilled water; a kind gift from Dr N. van Meirveinne), and goat polyclonal anti-GLUT-1 (1 : 40; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antisera for 24–48 h at 4°C. The adjacent section was incubated with a rabbit polyclonal anti-occludin antibody (1 : 200; Zymed Laboratories Inc., San Francisco, CA, USA). In two cases, 45 and 55 days p.i., every 20th section was stained for GLUT-1 and the *Tbb* parasite, and adjacent sections for cresyl violet. A second series of sections were incubated with mouse anti-intercellular cell adhesion molecule (ICAM-1; 1 : 200; Serotec, Raleigh, NC, USA), and the adjacent section with mouse anti-vascular cell adhesion molecule (VCAM-1; 1 : 200; PharMingen, San Diego, CA, USA) antisera. A third series of sections were incubated with either a mixture of anti-VSG and rat anti-zonula occludens (ZO-1; 1 : 100; Chemicon International Inc, Temecula, CA, USA), anti-VSG and mouse anti-occludin (1 : 200; Zymed Laboratories Inc.), anti-ZO-1 and rabbit anti-occludin, or anti-GLUT-1 and rabbit anti-occludin antisera. A fourth series was incubated with a rabbit anti-fibrinogen (1 : 5000; Dako A/S, Glostrup, Denmark) antiserum. Following incubation in the primary antisera, the sections were rinsed three times, 15 min each, and then incubated for 60 min at room temperature with the following secondary antibodies: for VSG, occludin (rabbit) and fibrinogen, donkey anti-rabbit IgG conjugated to rhodamine red (1 : 100; Jackson Immuno Research, West Grove, PA, USA), or goat anti-rabbit IgG conjugated to Alexa 488 (1 : 400; Molecular Probes, Eugene, OR, USA); for GLUT-1, donkey anti-goat IgG conjugated to Alexa 488 (1 : 400; Molecular Probe); for ZO-1, donkey anti-rat IgG conjugated to Cy3 (1 : 500; Jackson Immuno Research); for ICAM-1, VCAM-1 and occludin (mouse), donkey anti-mouse IgG conjugated to rhodamine red (1 : 100; Jackson Immuno Research). One series of sections was incubated directly with a donkey anti-rat IgG conjugated to Cy3 (1 : 500; Jackson Immuno Research). Following rinsing in PBS, the sections were cover-slipped in glycerol mixed with 2.5% diazabicyclanoctane (Sigma).

The material was examined in a Nikon fluorescence microscope. Black and white illustrations were prepared by scanning negatives in a Nikon Cool Scan III slide scanner and using the computer software Adobe

PhotoShop 5.0. Colour photomicrographs were taken with a Zeiss AxioCam digital camera, and illustrations made using PhotoShop 5.0. No digital alterations were made to any of the photomicrographs.

### Counting of *Tbb* parasites

Parasites in two ocular fields (viewed through a 10 × ocular, 20 × objective) on either side of the midline were counted from the cortex (grey matter), and the corpus callosum (white matter), in four animals from each p.i. time point, and an average of the number of parasites of the four fields calculated. The parasites were divided into two groups according to their relationship with the vessels; intravascular or extra-vascular.

## Results

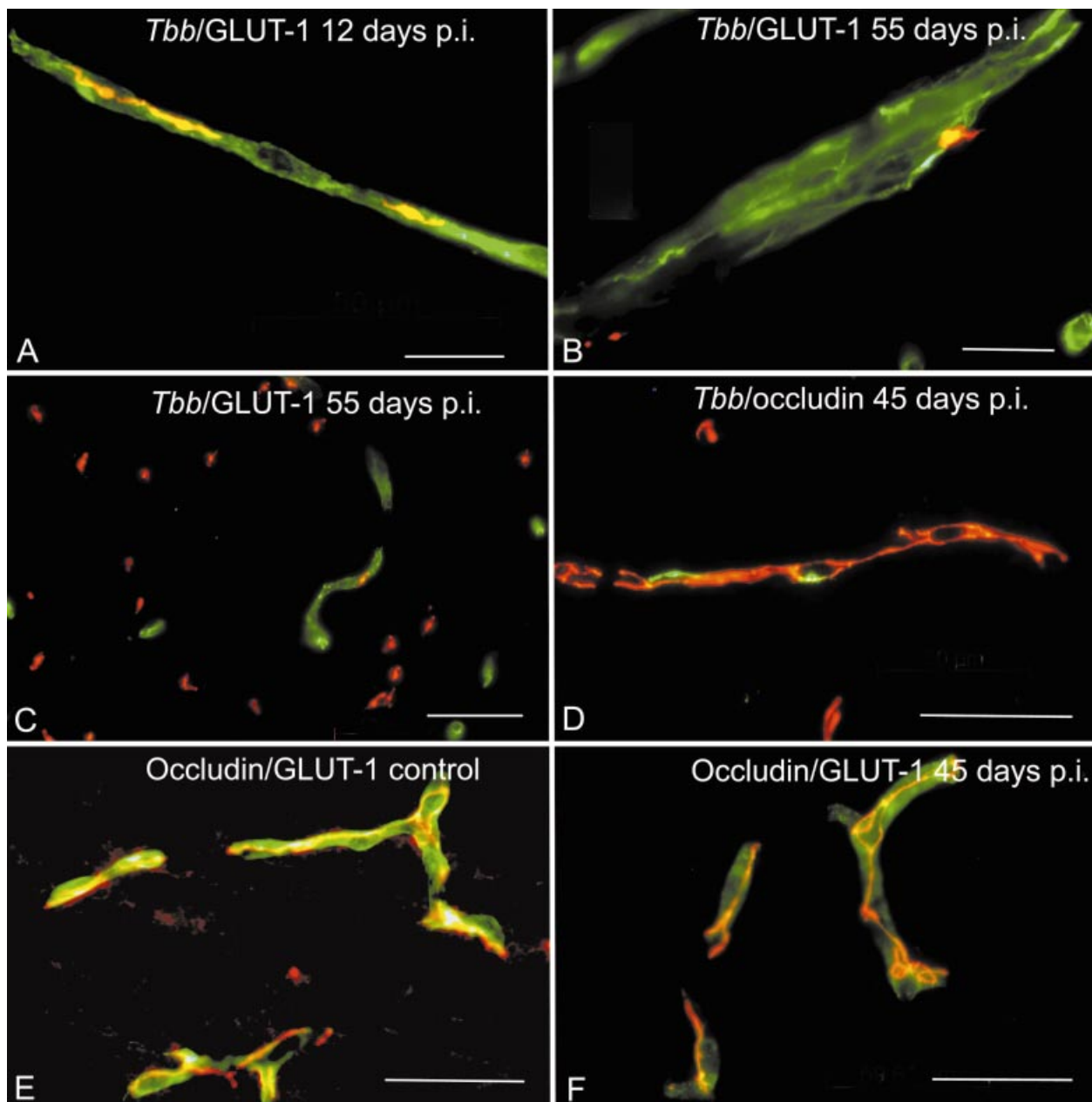
### Course of the disease

On examination, *Tbb* were found in the blood of all rats in which the parasite had been injected. The rats did not display any signs of disease during the first 30 days p.i. Later, the rats showed signs of reduced activity but otherwise no overt signs of disease were observed.

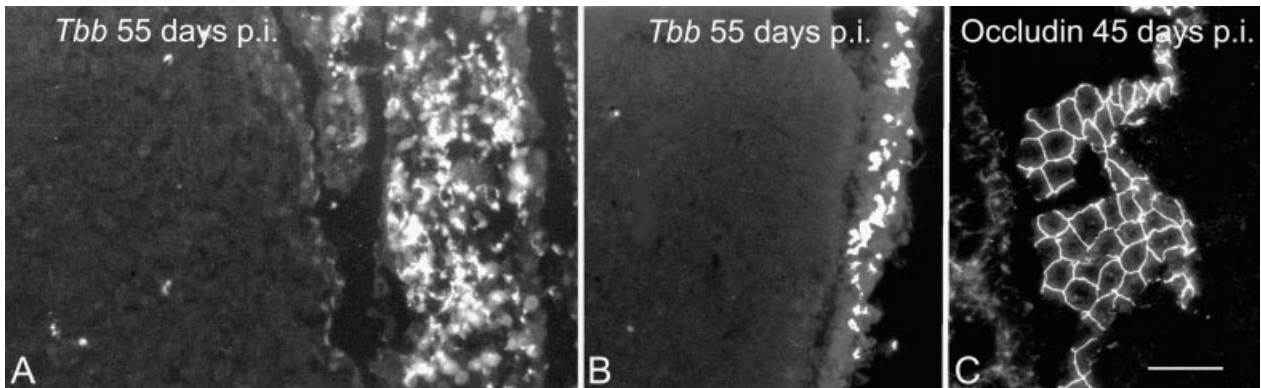
### Relationship between GLUT-1 and the *Tbb* parasite

GLUT-1 is the glucose transporter of the endothelial cells and, hence, of the blood–brain barrier [14, 24], and was in the present study used as a marker of the cerebral vascular endothelium. Both perfused and fresh frozen tissues were analysed with respect to the relationship between GLUT-1 and *Tbb*. The fresh frozen material that had been quickly fixed in formalin/acetone gave, however, a much brighter immunofluorescence than the perfused material. The GLUT-1 immunoreactivity could be observed in association with the brain vascular endothelium (Figure 1a–c,e–f) in all areas examined. No GLUT-1 immunostaining was, however, found in the pineal gland or in the choroid plexus (see Dobrogowska & Vorboldt [3]).

Immunostaining of *Tbb* VSG revealed a large number of parasites in the choroid plexus (Figure 2a), pineal gland and median eminence in all the infected animals, and they appeared to increase in number with longer p.i. times. There were, however, interanimal variations in



**Figure 1.** Photomicrographs from the level of the septal nuclei at different post-infection survival times. (a) A brain capillary in the cortex immunostained for glucose transporter-1 (green) and the *Tbb* parasite (red/yellow) at 12 days post-infection. At this stage most parasites are confined within the blood vessels. (b) Same immunostaining as in a at 55 days post-infection, from the cortex. A parasite can be seen traversing a capillary wall. (c) Septal nuclei 55 days post-infection immunostained for glucose transporter-1 (green) and the *Tbb* parasite (red). At this stage most parasites are localized to the brain parenchyma. (d) A brain capillary in the cortex immunostained for occludin (red) and *Tbb* parasite (green). No fragmentation of occludin staining could be observed, even when the parasites were in close association with the vessels. (e-f) Brain capillaries in the cortex immunostained for glucose transporter (green) and occludin (red/yellow) in a control brain (e), and at 45 days post-infection (f). No generalized loss of occludin staining could be observed in the infected material, as all vessels were double-labelled for both markers in control as well as in infected brains. Scale bars: a,b 20  $\mu$ m; c-f, 50  $\mu$ m.



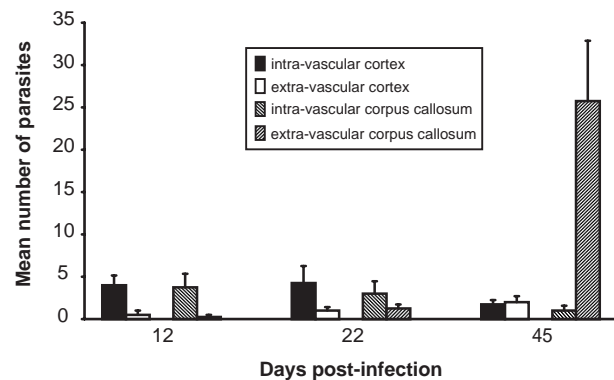
**Figure 2.** Photomicrographs from the level of the septal nuclei with an accumulation of the *Tbb* parasite in the choroid plexus of the lateral ventricle (a), and in the leptomeninges (b) 55 days post-infection. The septal nuclei are located to the left in (a). Note the paucity of parasites in the subependymal layer of the lateral ventricle (a), and beneath the pia (b). Occludin immunostaining of the choroid plexus is shown in (c) from a rat 45 days post-infection. Scale bar 50  $\mu$ m.

the number of parasites. There was no accumulation of the *Tbb* parasite in areas close to the lateral or third ventricles (Figure 2a). A large number of parasites were found in association with the leptomeninges, but there were no or only a few parasites in the subpial tissues of the brain (Figure 2b).

At 12 days p.i., the large majority of the *Tbb* parasites were found to be confined within the blood vessels (Figure 1a). Only an occasional parasite was seen in the brain parenchyma outside the vessels. Also at 22 days p.i., the majority of parasites were found within blood vessels. At later stages, 42, 45 and 55 days p.i., parasites could still be detected within or in association with blood vessels (Figure 1b). In addition, parasites were now frequently found within the parenchyma. Particularly in the white matter, a large number of parasites were observed clearly external to the vessels. Counting the *Tbb* parasites revealed that a larger number of parasites was found in the white matter parenchyma, compared to the cerebral cortex (Figure 3). At the latest p.i. time points studied (42, 45 and 55 days) a remarkable accumulation of parasites could be observed in the septal nuclei, with most of them localized to the brain parenchyma (Figure 1c). An accumulation of parasites was seen around large vessels in these nuclei. Most parasites in the parenchyma of the brain had a slender appearance.

### Relationship between occludin and the *Tbb* parasite

In order to determine whether *Tbb* infection had any effect on tight junction proteins, brain sections from



**Figure 3.** Histogram of mean numbers ( $n=4$  at each time point) of intra- and extra-vascular parasites in the cortex and corpus callosum at different time points post-infection. A significantly larger number of extra-vascular parasites is observed in the corpus callosum compared to the cortex at the latest time point ( $P<0.05$ ; Student's *t*-test). Bars show SEM.

control non-infected and infected rats were incubated with an antibody to the tight junction marker occludin, an integral membrane protein [5]. Occludin immunostaining could be detected only in the fresh frozen and not in the perfused material. In control rats, occludin immunoreactivity was observed in vessels throughout the whole brain section as thin lines forming the outline of the individual endothelial cells. Occludin staining could also be detected outlining epithelial cells of the choroid plexus of control and rats at all stages p.i. (Figure 2c), but was not observed in the pineal gland. In material double-labelled for occludin and the parasite,

the occludin staining had the same appearance in non-infected as in infected rats. Vessels, in which parasites were detected, were carefully analysed for signs of disruption. No clear fragmentation of the occludin could be detected in any of the brains sampled, not even in vessels closely associated with parasites (Figure 1d).

In sections double-labelled for GLUT-1 and occludin, all vessels immunopositive for GLUT-1 also showed occludin immunostaining in both control and infected rats (Figure 1e–f). Only a few small transversely cut capillaries and occasionally part of a longitudinally cut vessel immunostained for GLUT-1, seemed to lack staining for occludin. The fact that all GLUT-1 immunostained vessels were also immunostained for occludin, suggests that there was no general loss of occludin staining in the infected rats.

### Relationship between ZO-1 and the *Tbb* parasite

To further analyse the effect the *Tbb* parasite could have on tight junction proteins, sections from both control and infected rats were examined in material double-labelled for the parasite and ZO-1, a tight junction peripheral membrane protein [6]. As seen for occludin, ZO-1 immunoreactivity was observed in vessels as thin lines outlining endothelial cells of both control and infected rats. The ZO-1 staining was slightly weaker than that seen for occludin. There were no clear signs, however, of changes in the ZO-1 staining even when the parasites were in close apposition to the vessel wall. Double-labelling with ZO-1 and occludin showed that the same vessels were labelled with both markers.

### Expression of cell adhesion molecules

The expression of two cell adhesion molecules, ICAM-1 and VCAM-1 [12], was examined in control and infected brains. ICAM-1 was detected in sections from control rats showing faint staining in a few large vessels and the choroid plexus. VCAM-1 appears not to be constitutively expressed in rodent brain, and was not detected in control brains. Already at 12 days p.i., there was a clear upregulation of both ICAM-1 and VCAM-1 on the endothelium of both small and large vessels in all areas of the brain examined. At later stages, i.e. 22, 42, 45 and 55 days p.i., the up-regulation was more robust than at the earliest time point sampled (Figure 4a–d). There was

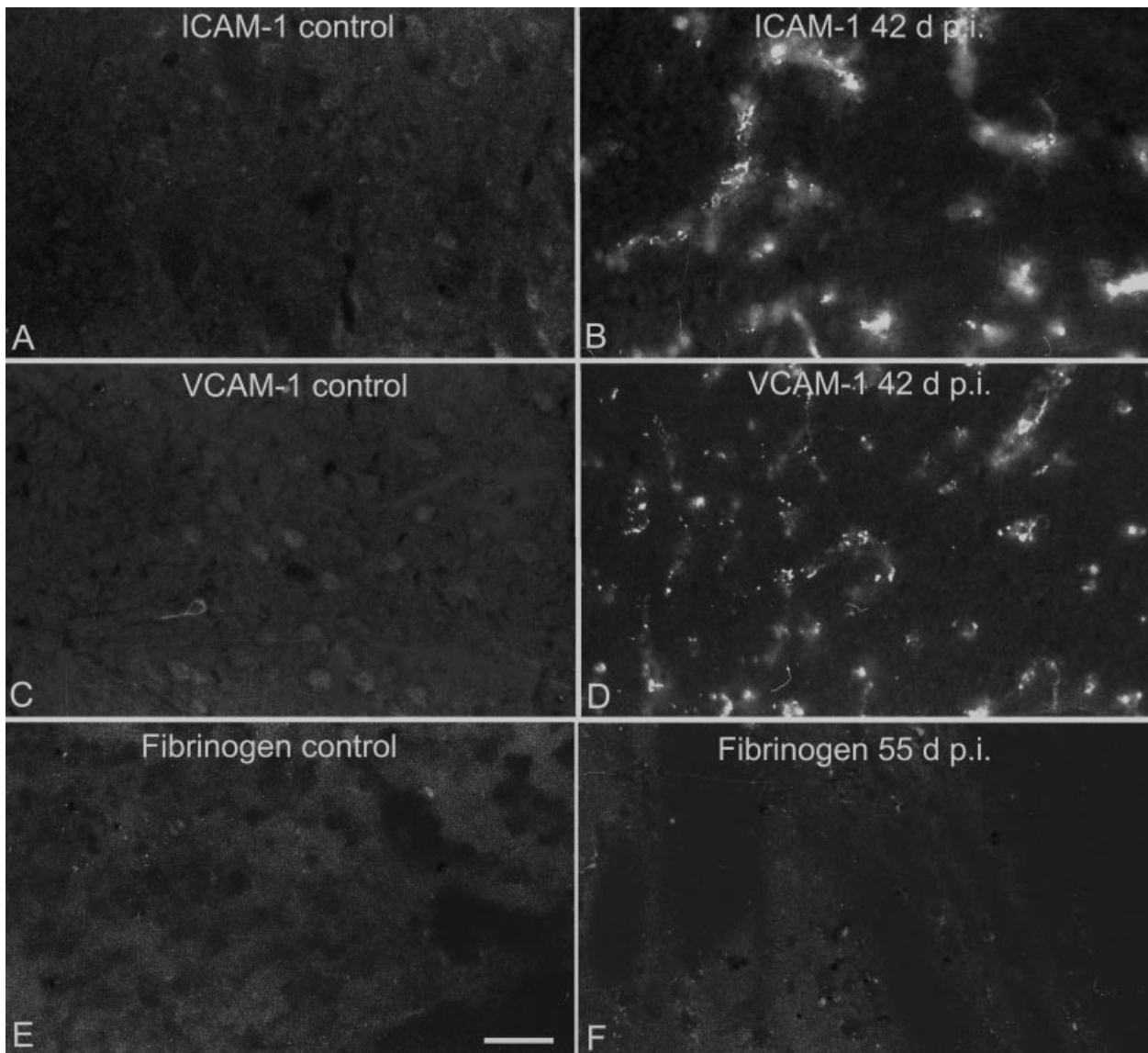
no apparent difference in the up-regulation of ICAM-1 or VCAM-1 in white matter vs. grey matter. Nor was the upregulation greater in areas with large accumulation of the *Tbb* parasite, e.g. the septal nuclei. The ICAM-1 and VCAM-1 immunostaining had the appearance of small and large granules (Figure 4b,d).

### Plasma protein distribution

To investigate changes in BBB permeability, the leakage of plasma proteins across the cerebral endothelium was investigated in both fresh frozen and perfused brains. Some fibrinogen immunostaining could be detected around vessels in control brains from fresh frozen material, but the distribution was limited and diffuse. In infected fresh frozen brains, there was a slightly greater immunoreactivity for fibrinogen within the brain parenchyma. In perfused brains, fibrinogen immunoreactivity was not detected in neither control nor infected brains (Figure 4e,f). IgG was virtually absent in control brains from both fresh frozen and perfused material, as well as in perfused infected brains. In infected fresh frozen brains, IgG immunostaining could be detected in cerebral vessel in both grey and white matter. The IgG immunostaining was associated with the vessels and not observed in the brain parenchyma.

### Discussion

In the present chronic model of African trypanosomiasis, a marked and early accumulation of parasites in the circumventricular organs was found, similar to the situation in a prior study [22], and that this invasion increased during the course of infection. Two routes by which blood-borne microbes can invade the brain parenchyma have been discussed. One is the direct passage through the cerebral capillaries, the other involves an early attack on the choroid plexus followed by seeding into the cerebrospinal fluid and passage through the subependymal or subpial tissues into the parenchyma (reviewed by Zhang and Tuomanen [26]). Based on the interpretation of the appearance of inflammatory cells in experimental vervet monkeys, the latter pathway has been suggested for *Tbb* infection, i.e. the trypanosome encephalitis develops as a result of parasites migrating into the brain from the subarachnoid space via perivascular spaces [20]. This would agree with the finding of an early parasite invasion of the



**Figure 4.** Photomicrographs of cerebral cortex (a–d) and the striatum (e–f) from brains of control (a,c,e) and infected rats 42 days (b,d) and 55 days (f) post-infection. Sections from the level of the septal nuclei have been stained immunohistochemically for ICAM-1 (a,b), VCAM-1 (c,d), and fibrinogen (e,f). Note the robust induction of both ICAM-1 and VCAM-1 on endothelial cells in the infected brain. Scale bar 50  $\mu$ m.

circumventricular organs described in previous and present studies. However, now that the relation between the *Tbb* and the cerebral capillary endothelium could be directly visualized, a few parasites were seen clearly outside the vessel lumina even at the earliest time points p.i. In brains sampled during the later time points, not only were parasites seen in the walls of vessels, but also on the outside of such vessels, and these extravascular parasites increased in number with time. Parasites did not localize beneath the pia in the cerebral cortex or the

ependymal cell layer around the ventricles, and there was no gradient of parasites from either the subarachnoid space, perivascular spaces of large vessels or the ventricles into the brain parenchyma. This indicates that the parasites in the brain parenchyma derive mainly from parasites that had penetrated directly through the cerebral vessels.

A number of microbes, especially bacteria, have been shown to pass across the BBB. Some can migrate by transendothelial endocytosis, e.g. group B streptococci

and *Streptococcus pneumoniae*, while others may pass through disrupted tight junctions, e.g. *Borrelia burgdorferi* and *Treponema pallidum* (reviewed by Zhang and Tuomanen [26]). Information on how blood-borne parasites can transverse the BBB is, however, sparse. In the present study, there were no signs of disruption of the tight junctions, i.e. there was no generalized loss of the tight junction proteins, occludin and ZO-1, as has been described during certain conditions with a breakdown of the BBB [1, 2]. The results therefore indicate that the *Tbb* parasite can migrate by transcytosis through the cerebral endothelial cells rather than through disrupted junctions between them. Whether this migration follows a receptor-mediated process or not remains to be examined. Bloodstream forms of *T. congolense* can attach to endothelial cells through lectin-like molecules at the anterior part of their flagella, but *Tbb* do not show similar interaction [7]. Thus, it will be of interest to analyse whether during the inflammatory process induced by the parasites, *Tbb* can bind to any of the adhesion molecules, ICAM-1 or VCAM-1, that were upregulated in the cerebral endothelial cells already at the very early time points examined.

Studies using fluorescent dyes as tracer have displayed an extensive leakage of dyes into the brain parenchyma and altered electrolyte concentrations characteristic of a vasogenic oedema at late or final stages of trypanosome infections in a rodent model [17]. In these brains only occasional parasites were observed in the parenchyma with no obvious association to areas with greater leakage of dyes. The observation, based on morphological and immunohistochemical criteria, of parasite penetration into the brain parenchyma, where tight junction proteins of the vessels appeared preserved, indicates that a breakdown of the BBB may not be a prerequisite for invasion of *Tbb*. This is consistent with the opinion that *Tbb* in the brain may escape being exposed to trypanocidal drugs that cannot penetrate the BBB. Thus, suboptimal treatment of *Tbb*-infected mice with the trypanocidal drug diminazene aceturate results in clearance of the parasite from the blood and visceral organs but not from the brain [8–10]. Since *Tbb* do not survive well in the cerebrospinal fluid [15] and *Tbb* introduced into the mouse brain may die [21], it has been suggested that trypanocidal drugs in theory are not required for treatment of trypanosomes in the brain [21]. The present study shows, however, that slender *Tbb* accumulated with time in the brain parenchyma;

especially in the white matter and the septal nuclei the parasites became more numerous outside than inside the vessels. This finding therefore supports the clinical evidence that drugs that pass the BBB are required for treatment of the cerebral forms of African trypanosomiasis [25].

The appearance of *Tbb* outside vessels was much more prominent in the white matter of the corpus callosum than in the grey matter of the cerebral cortex. This finding is of particular interest, since both in humans and experimental animals the inflammation in the brain during African trypanosomiasis is mainly localized to the white matter, i.e. a leukoencephalitis (e.g [18, 19]). Why the parasites penetrate vessels in the white matter more easily than in grey matter is not clear. The inherent properties or the reaction to inflammatory factors of the endothelial cells may differ between the regions or, alternatively, the large extracellular spaces within the white matter may facilitate movement of the parasites into the parenchyma once they have penetrated the vessels. In this respect, it is of interest to note that in human immunovirus (HIV)-1 infections, virus-infected macrophages show a predilection to migrate through the BBB in the cerebral white matter (reviewed by Nottet [13]) and that the white matter endothelial cells show a hyperplasia, which may facilitate transendothelial transport in HIV-associated leukoencephalopathies [23].

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