An optimized *in vitro* blood–brain barrier model reveals bidirectional transmigration of African trypanosome strains

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The transmigration of African trypanosomes across the human blood–brain barrier (BBB) is the critical step during the course of human African trypanosomiasis. The parasites *Trypanosoma brucei gambiense* and *T. b. rhodesiense* are transmitted to humans during the bite of tsetse flies. Trypanosomes multiply within the bloodstream and finally invade the central nervous system (CNS), which leads to the death of untreated patients. This project focused on the mechanisms of trypanosomal traversal across the BBB. In order to establish a suitable *in vitro* BBB model for parasite transmigration, different human cell lines were used, including ECV304, HBMEC and HUVEC, as well as C6 rat astrocytes. Validation of the BBB models with *Escherichia coli* HB101 and *E. coli* K1 revealed that a combination of ECV304 cells seeded on Matrigel as a semi-synthetic basement membrane and C6 astrocytes resulted in an optimal BBB model system. The BBB model showed selective permeability for the pathogenic *E. coli* K1 strain, and African trypanosomes were able to traverse the optimized ECV304–C6 BBB efficiently. Furthermore, cocultivation indicated that paracellular macrophage transmigration does not facilitate trypanosomal BBB traversal. An inverse assembly of the BBB model demonstrated that trypanosomes were also able to transmigrate the optimized ECV304–C6 BBB backwards, indicating the relevance of the CNS as a possible reservoir of a relapsing parasitaemia.

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INTRODUCTION

Human African trypanosomiasis is a vector-borne parasitic disease, which currently causes about 10 000 deaths each year. At the end of the last century the number of lethal cases was estimated as up to 300 000, according to WHO data. The disease threatens over 60 million people of 36 African nations, reaching lethality of 100 % without treatment (WHO, 2010). The parasites, called trypanosomes, are transmitted during a blood meal of tsetse flies of the *Glossina* genus and can infect humans as well as cattle. *Trypanosoma brucei brucei* is one of the causative agents of Nagana, a severe cattle disease, which hampers intensive cattle farming in endemic areas (Steverding, 2008). *T. b. gambiense* and *T. b. rhodesiense* are human pathogens and multiply within the blood circulation system. Therein, the

parasites evade the host immune system by different strategies, for instance by switching their surface-coat antigens (Dubois *et al.*, 2005). As the disease progresses, the parasites infect the central nervous system (CNS), leading to the severe outcome of the disease. Once inside the CNS, parasites are hardly reached by drugs or by the immune system. Depending on the trypanosome subspecies the parasites transmigrate through the human blood–brain barrier (BBB) within a few weeks (*T. b. rhodesiense*), some months or even years (*T. b. gambiense*). So far, the invasion into the CNS of African trypanosomes is poorly understood (Grab & Kennedy, 2008). It has been shown that secreted proteases (Nikolskaia *et al.*, 2006, 2008), in the case of *T. b. rhodesiense*, and the composition of the extracellular matrix as well as secreted IFN- γ (Masocha *et al.*, 2004), in the case of *T. b. brucei*, play a critical role in the process of BBB transmigration.

The human BBB consists of endothelial cells from brain capillaries and ensures the maintenance of the CNS microenvironment. It regulates selective transport of substances and protects the brain from most pathogens and toxins. The interaction of the endothelium with its

Abbreviations: BBB, blood–brain barrier; BBBi, inverse BBB; CNS, central nervous system; FESEM, field-emission scanning electron microscopy; HBMEC, human brain microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; PMA, phorbol 12-myristate 13-acetate.

Supplementary material is available with the online version of this paper.

associated cells, like astrocytes, pericytes and microglia, is important for the properties of the BBB (Abbott, 2005). Astrocytes are known to induce and regulate many BBB characteristics and functions, namely the formation of tight junctions as well as the expression and asymmetrical localization of special enzymes, e.g. within transport systems (Abbott *et al.*, 2006; Cecchelli *et al.*, 2007).

In vitro models of the BBB allow direct analysis and manipulation of the molecular mechanisms underlying the transmigration of African trypanosomes into the brain. In order to identify new targets for drug development, an increased understanding of the transmigration process is urgently needed (Grab & Kennedy, 2008). The complexity of BBB models ranges from three-dimensional model systems (Cucullo *et al.*, 2007; Parkinson *et al.*, 2003; Stanness *et al.*, 1997) to transwell-based models with one or more cell types (astrocytes, pericytes and endothelial cells) (Nakagawa *et al.*, 2007).

In the present study we generated and compared different *in vitro* BBB models, and here we describe an optimized BBB model consisting of the human cell line ECV304 and rat C6 astrocytes (Hurst & Fritz, 1996). Analysis of transmigration in this model revealed different BBB transmigration rates for different African trypanosome strains. Evaluation of macrophage transmigration also demonstrated that paracellular extravasation of macrophages does not facilitate trypanosomal CNS invasion. Furthermore, parasite transmigration across an inverse assembly of the optimized ECV304–C6 BBB indicated that the traversal process occurs bidirectionally, supporting the possibility of trypanosomal return to the bloodstream out of the CNS.

METHODS

Culture conditions of bacteria and trypanosomes. *E. coli* HB101 (Boyer & Roulland-Dussoix, 1969) and *E. coli* K1 (Korhonen *et al.*, 1985) were grown on Luria–Bertani (LB) agar plates. For transmigration assays an overnight culture was washed off the plate with phosphate-buffered saline (PBS, pH 7.4). Optical density was measured in a spectrophotometer (Libra S22; Biochrom) at 550 nm and the suspension was diluted in the cell media M199 or Quantum 286. *T. b. brucei* TC221 (Hirumi *et al.*, 1980) was grown in Baltz Medium complete solution (Hirumi & Hirumi, 1989) at 37 °C, 5% CO₂ and 95% humidity. The trypanosomes were cultured in 25 cm² cell culture flasks and divided every 2 days with rates of 1:100 and 1:500. Alternatively, trypanosomes were cultured in 24-well plates. They were divided 1:100 and 1:1000 every 2 days. *T. b. gambiense* STIB930 and *T. b. rhodesiense* STIB900 were cultivated in 24-well plates at 37 °C, 5% CO₂ and 95% humidity. They were subcultured depending on cell density with splitting rates of 1:5 or 1:10 every second or third day. HMEM medium was used for *T. b. gambiense* STIB930 and BMEM medium for *T. b. rhodesiense* STIB900. For medium compositions refer to Supplementary Table S1, available with the online version of this paper.

Cultivation of cell lines. All cell lines were cultured in a CO₂ incubator [Sanyo MCO-18AC(UV)] at 37 °C, 5% CO₂ and 95% humidity. The media compositions for the cell lines ECV304 (ECACC 92090409), C6 cells (ECACC 92091712), HBMEC (human brain

microvascular endothelial cells; Stins *et al.*, 1997), HUVEC (human umbilical vein endothelial cells; PromoCell) and J774 murine macrophages (J-774A.1 murine monocytes-macrophages, DSMZ ACC170) are listed in Supplementary Table S1. The cell lines were subcultivated by trypsinization with Trypsin-EDTA (PAA Laboratories) every 3–5 days up to the 10th passage. The cells were seeded in new culture flasks with splitting rates between 1:5 and 1:20. HUVEC were detached using 1:10 diluted Trypsin-EDTA in PBS (pH 7.4) and divided up to the fifth passage with splitting rates between 1:2 and 1:5. For co-cultivation assays 5 × 10⁵ J774 macrophages [not activated with PMA (phorbol 12-myristate 13-acetate)] were cultured together with 2 × 10⁵ *T. b. brucei* TC221 in 1 ml J774 medium in 24-well plates. Macrophages and trypanosomes were counted at time points 0, 24, 48 and 72 h with a Neubauer or a Fuchs–Rosenthal counting chamber respectively. For comparison, macrophages and trypanosomes were cultured alone in their standard media in 24-well plates for 72 h.

Generation of *in vitro* BBB models. ECV304, HBMEC, HUVEC and C6 cell lines were used to set up different BBB models. For optimization of the BBB models, cell seeding concentrations were varied. The number of C6 cells per insert varied from 1 × 10⁴ to 5 × 10⁴. The number of ECV304 cells was between 3 × 10⁴ and 1.1 × 10⁵ per insert. HBMEC and HUVEC were seeded at 6 × 10⁴ cells per insert. To generate the optimized BBB model, ECV304 and C6 cell lines were cultivated in 24-well inserts with membranes with 8 µm pores coated with a Matrigel layer (BD Biosciences #354480; precoated with Matrigel). ECV304 cells were grown inside the inserts and C6 cells on the outside, underneath the membrane. To seed C6 cells, the inserts were turned around and 5 × 10⁴ cells were incubated on the outer side of the PET (polyethylene terephthalate) membrane for 1 h, allowing the cells to attach to the membrane. After Matrigel rehydration according to the manufacturer's guidelines, 6 × 10⁴ ECV304 cells were seeded on top of the Matrigel layer. Alternatively, collagen-I-coated inserts (BD Biosciences #354490; precoated collagen-I inserts with 3 µm pores) were used to set up the ECV304–C6 BBB. The inserts were cultivated for 5 days in M199 medium with a medium exchange on the fourth day. BBB models containing ECV304 or HBMEC were used for transmigration assays on the fifth day of cultivation. BBB models containing HUVEC were assayed on the sixth day. To improve barrier tightness (see Results), M199 medium was replaced by the serum-free Quantum 286 medium (PAA Laboratories).

Transmigration assays. The different BBB models were infected with 1 × 10⁵ *E. coli* HB101. Prior to infection, bacteria were diluted in serum-free M199 or Quantum 286 and were incubated in the upper compartment of the transwell system for 5 h. Dilution series of specimens from the lower compartment were plated on LB agar plates, which were incubated at 37 °C. C.f.u. were determined after 12–18 h of cultivation. Transmigration assays with *E. coli* K1 were performed as described for *E. coli* HB101, with variation of inoculum and infection time. Inocula of 10⁵, 10⁶ and 10⁷ bacteria per insert were applied and an infection time of 5, 6, 8 and 10 h was performed. To evaluate if membrane pores of 3 µm diameter are sufficient for trypanosomal transmigration, cell- and Matrigel-free 24-well inserts with membrane pores of 3 µm diameter (BD Biosciences) were incubated for 4, 24 and 48 h with 10⁴ and 10⁵ *T. b. brucei* TC221 per insert. Transmigrated trypanosomes were counted with a Neubauer counting chamber.

Transmigration assays across the optimized ECV304–C6 BBB were performed with different trypanosome strains in infection medium (M199 medium containing 10% fetal calf serum, or Quantum 286 without serum). Grown trypanosome cultures were centrifuged for 10 min at 400 g and resuspended in infection medium. Trypanosomes with an inoculum of 1 × 10⁶ parasites per BBB insert were incubated for

6 h at 37 °C, 5 % CO₂ and 95 % humidity. Transmigrated trypanosomes were counted from the lower compartment using a Neubauer counting chamber.

J774 murine macrophages were activated with 200 nM PMA (Sigma Aldrich Chemie) 24 h prior to the transmigration assays. The macrophages were harvested with a cell scraper, centrifuged at 100 g for 5 min and resuspended in infection medium. Inocula of 1×10^6 J774 cells per insert were incubated for 5 h at 37 °C, 5 % CO₂ and 95 % humidity. Transmigrated J774 macrophages were counted from the lower compartment using a Fuchs–Rosenthal counting chamber.

Transmigration experiments were repeated in at least two independent test series with a minimum of three parallels each. Results were expressed as mean $\pm$ SD. Statistical significance was calculated with Student's *t*-test (Microsoft Excel 2007) and is represented in the figures by asterisks (*) indicating different *P*-values (*= $P \leq 0.05$; **= $P \leq 0.01$; ***= $P \leq 0.001$).

Field-emission scanning electron microscopy (FESEM). Cell culture inserts with infected or uninfected BBB cell layers were stored overnight in fixation buffer containing 5 % paraformaldehyde and 1 % glutaraldehyde in PBS. For electron microscopy the samples were transferred in cacodylate buffer (0.1 M cacodylate, 0.01 M CaCl₂, 0.01 M MgCl₂, 0.09 M sucrose, pH 6.9) containing 2 % glutaraldehyde and 5 % formaldehyde for 1 h on ice, washed with TE buffer (20 mM Tris, 1 mM EDTA, pH 6.9) before dehydrating in a graded series of acetone (10 %, 30 %, 50 %, 70 %, 90 %, 100 %) on ice (15 min for each step). Samples were then critical-point dried with liquid CO₂ (CPD 30; Bal-Tec) and covered with a gold film by sputter coating (SCD 500; Bal-Tec) before examination in a field-emission scanning electron microscope (Zeiss DSM 982 Gemini) using the Everhart–Thornley SE-detector and the inlens SE-detector in a 50:50 ratio at an acceleration voltage of 5 kV at calibrated magnifications. Images were stored on MO-disks and contrast and brightness adjusted with Adobe Photoshop 7.0 (Adobe Systems).

RESULTS

BBB model optimization leads to selective permeability

To establish a highly reproducible BBB model the endothelial cell lines HBMEC and HUVEC and the endothelial-like cell line ECV304 were seeded on Matrigel-covered inserts, either alone or in co-culture with C6 astrocytes. The number of ECV304 cells seeded was varied to obtain optimal confluence of the cells inside the insert, which is essential for a tight barrier. Moreover, the number of C6 astrocytes was altered to achieve optimal induction properties. The barrier tightness was evaluated by bacterial transmigration assays with *E. coli* HB101 (Fig. 1). This *E. coli* strain is deficient in active invasion, though transmigration can occur paracellularly through leaky cell layers (Berriman *et al.*, 2005; Huang *et al.*, 1995; Kim, 2000; Stins *et al.*, 1999). The optimal cell amounts were 6×10^4 ECV304 cells per insert and 5×10^4 C6 cells per insert. Optimal cell amounts found for ECV304 cells were then applied for HBMEC and HUVEC cell seeding. HUVEC failed to generate a tight barrier for *E. coli* HB101 either in monoculture or in co-culture with C6 astrocytes. HBMEC cell layers were completely impermeable for *E. coli* HB101

Fig. 1. Bacterial transmigration across different BBB models. An inoculum of 10^5 *E. coli* HB101 per insert was added and samples were taken after 5 h from the lower compartment of the transwell system. Dilution series were plated on agar plates for colony counting. The bars show the percentage of impermeable inserts. HBMEC and HUVEC seeded on Matrigel were not able to generate a tight barrier in monoculture or in co-culture with C6 astrocytes. ECV304 cells seeded on Matrigel in co-culture with C6 cells resulted in 80 % impermeable inserts, compared to ECV304 cells in monoculture on Matrigel, which produced only 25 % impermeable inserts. An exchange of Matrigel for collagen I leads to a completely permeable BBB model. Up to 100 % impermeable inserts were determined for the ECV304–C6 model system using Quantum 286 medium (indicated by *) and Matrigel.

in 25 % of the tested inserts. The percentage of impermeable inserts increased to 50 % after co-culture of HBMEC with C6 cells. Co-culture of ECV304 and C6 astrocytes decreased the permeability of the cell layers substantially. One-quarter of the transwell inserts with monocultures of ECV304 cells were impermeable, whereas the combination of ECV304 with C6 cells prevented the transmigration of *E. coli* HB101 in 80 % of the tested inserts. Changing the culture medium to serum-free Quantum 286 improved the barrier function against *E. coli* HB101 up to 100 %. In exchange for Matrigel as an underlying matrix, collagen-I coated inserts were used for the set up of an ECV304–C6 BBB, and this resulted in a completely permeable barrier system. Therefore, the use of Matrigel turned out to be essential.

To validate the selective permeability of the optimized ECV304–C6 BBB, transmigration of *E. coli* K1 was determined in comparison to *E. coli* HB101. *E. coli* K1 is a causative agent of meningitis in newborns and known to cross the human BBB. Comparative transmigration analyses were performed including the variation of infection time and bacterial inoculum. *E. coli* K1 and HB101 with an inoculum of 10^5 bacteria per insert transmigrated the optimized ECV304–C6 BBB in a time-dependent manner. After 5 h of infection no transmigrated *E. coli* K1 or *E. coli* HB101 bacteria were detected (Fig. 2).

Fig. 2. Time-dependent transmigration of pathogenic *E. coli* K1 and avirulent *E. coli* HB101 across the optimized ECV304–C6 BBB. *E. coli* in amounts of 10^5 bacteria per insert were added and c.f.u. from transmigrated bacteria were determined after 5, 6, 8 and 10 h. The transmigration rate of *E. coli* K1 increases with longer infection time whereas *E. coli* HB101 transmigration is not detectable after 5 h and remains low after 10 h.

Longer infection times of 6, 8 and 10 h resulted in a substantial increase of *E. coli* K1 transmigration, whereas only marginal transmigration was observed for *E. coli* HB101. In addition to different infection times the transmigration of *E. coli* K1 and HB101 was also analysed applying inocula of 10^5 , 10^6 and 10^7 bacteria per insert. At high inocula of 10^6 and 10^7 bacteria per insert and 5 h of incubation, *E. coli* K1 efficiently transmigrated across the optimized ECV304–C6 BBB (Supplementary Fig. S1). In contrast, this optimized BBB model remained impermeable for *E. coli* HB101 even with high bacterial numbers used for inoculation. Both *E. coli* HB101 and *E. coli* K1 transmigrated cell-free inserts with Matrigel, with similar transmigration rates (Supplementary Fig. S1). These results demonstrate that neither the membrane nor the Matrigel contributed to the selective barrier function of the BBB models.

In conclusion, the results obtained indicate that a selective and reliable ECV304–C6 BBB can be generated by co-culture of 5×10^4 C6 cells and 6×10^4 ECV304 cells seeded on Matrigel-covered membrane inserts with pores 8 μ m in size. The optimal culture conditions comprise Quantum 286 medium and an incubation of the inserts for 5 days at 37 °C, 5% CO₂ and 95% humidity with a change of medium on the fourth day.

Electron microscopy visualizes the structure of the BBB cell layers

Microscopic characterization of optimized ECV304–C6 BBB inserts was performed after 5 days of incubation. Examination of the structure of the cell layers indicated that

ECV304 cells grew as a confluent and tight cell layer (Fig. 3a). A side view of the cell layer was obtained by lifting fixed ECV304 cells from the insert membrane. The ECV304 cells grew in confluent layers with some overlapping regions of adjacent cells (Fig. 3b). The C6 cells formed a loose meshwork underneath the insert membrane (Fig. 3c). After 5 days of cultivation, the astrocyte cells maintained contact with the insert membrane as visualized by light microscopy of the 24-well plate bottoms. Membrane inserts with different pore sizes were applied in cell-free transmigration assays in order to analyse the contribution of the size of the membrane pores to the growth of the cells and transmigration efficiency of the trypanosomes. *T. b. brucei* TC221 trypanosomes were able to transigrate membrane pores of at least 3 μ m in diameter (Fig. 3d). In order to exclude the pore size as a limiting factor for transmigration and due to the ability of the ECV304 cells to span large pores, 8 μ m pore sized membrane inserts were chosen for the optimized ECV304–C6 BBB.

Bidirectional transmigration of trypanosomes through the BBB

T. b. gambiense and *T. b. rhodesiense* cause the two different subtypes of human African trypanosomiasis, whereas *T. b. brucei* is described as an animal pathogen. Transmigration data revealed that the two human-pathogenic strains and *T. b. brucei* TC221 are all able to traverse the optimized ECV304–C6 BBB (Fig. 4). With an inoculum of 10^6 trypanosomes per insert, the significantly highest transmigration rates were detected for *T. b. gambiense* STIB930, with $1.60 (\pm 1.32) \times 10^4$ trypanosomes ml⁻¹, followed by *T. b. rhodesiense* STIB900 with $2.99 (\pm 1.77) \times 10^3$ trypanosomes ml⁻¹ and *T. b. brucei* TC221 with a similar transmigration rate of $2.24 (\pm 3.35) \times 10^3$ trypanosomes ml⁻¹. Increasing the inoculum to 10^7 trypanosomes per insert led to a significant increase of the transmigration across the optimized ECV304–C6 BBB in the case of *T. b. brucei* TC221, with $2.55 (\pm 1.89) \times 10^4$ trypanosomes ml⁻¹, and *T. b. rhodesiense* STIB900, with $1.97 (\pm 1.76) \times 10^4$ trypanosomes ml⁻¹. In contrast, the transmigration rate of *T. b. gambiense* STIB930, $2.09 (\pm 1.03) \times 10^4$ trypanosomes ml⁻¹, showed an increase that is not significant. Growth analyses of the parasites in cell culture medium revealed that the trypanosomal strains did not multiply within 6 h of incubation.

The brain is considered as a site of immune evasion. Therefore, the parasites might be able to traverse the BBB inversely. In order to simulate the situation of inside-out transmigration, an inverse BBB arrangement (BBBi) was designed. The ECV304 cells were seeded on the outside of the transwell insert membrane and the C6 cells grew inside the insert. After 5 days of incubation transmigration assays with an inoculum of 10^6 trypanosomes per insert were performed. Determination of transmigration rates indicated that trypanosome strains cross the BBBi model in a similar manner as monitored for the original BBB model

Fig. 3. FESEM analysis of the optimized ECV304–C6 BBB. (a) The human ECV304 cells build a tight and confluent cell layer after 5 days of incubation. (b) ECV304 cells partly lifted from the insert membrane enable a side view. The cell layer consists of partly overlapping neighbouring cells. (c) C6 astrocytes growing upside down build a loose meshwork with gaps large enough for trypanosomal migration. (d) *T. b. brucei* TC221 transmigration through cell-free Matrigel-covered inserts with membrane pores 3 µm in diameter.

Fig. 4. Transmigration of *T. b. brucei* TC221, *T. b. gambiense* STIB930 and *T. b. rhodesiense* STIB900 across the optimized ECV304–C6 BBB. Inocula of 10⁶ trypanosomes were added to the inserts and samples were taken after 6 h from the lower compartment of the transwell system. Similar transmigration rates were determined for the animal-pathogenic *T. b. brucei* TC221 and the human-pathogenic *T. b. rhodesiense* STIB900. In comparison, a significantly higher transmigration efficiency was monitored for the human-pathogenic *T. b. gambiense* STIB930 ($P \leq 0.001$).

(Fig. 5). *T. b. gambiense* STIB930 transmigrated with $2.97 (\pm 3.61) \times 10^4$ trypanosomes ml⁻¹, significantly higher than *T. b. brucei* TC221 with $4.62 (\pm 4.06) \times 10^3$ trypanosomes ml⁻¹ and *T. b. rhodesiense* STIB900 with $2.91 (\pm 1.88) \times 10^3$ trypanosomes ml⁻¹.

Trypanosomes do not benefit from macrophage transmigration

Paracellular extravasation of macrophages into tissues is a well-described process (Marchesi, 1961; Wittchen, 2009). For African trypanosomes paracellular transmigration of endothelia has been reported as well (Grab *et al.*, 2004). To investigate whether paracellular transmigration of macrophages has synergistic effects on the transmigration of trypanosomes, as shown for lymphocytes in a rodent model (Masocha *et al.*, 2004), the transmigration of J774 murine macrophages alone and together with *T. b. brucei* TC221 was examined. Evaluation of the transmigration rates of J774 macrophages across the optimized ECV304–C6 BBB with an inoculum of 10⁵ cells per insert indicated that the J774 macrophages have the ability to cross this BBB model with a transmigration rate of $7.1 (\pm 3.6) \times 10^2$ cells ml⁻¹ (Fig. 6). Activation of macrophages with PMA did not alter the transmigration rate. In cell-free inserts the Matrigel

Fig. 5. Transmigration of trypanosomes across the inverse ECV304-C6 BBB (BBBi). Inocula of 10^6 trypanosomes were added to the inserts and samples were taken after 6 h from the lower compartment of the transwell system. The human-pathogenic *T. b. gambiense* STIB930 transmigrates across the BBBi significantly more effectively ($P \leq 0.001$) than the non-human-pathogenic strain *T. b. brucei* TC221 and the human-pathogenic *T. b. rhodesiense* STIB900, which shows similar transmigration rates to *T. b. brucei* TC221.

layer prevents transmigration of macrophages. In cotransmigration assays with J774 macrophages and *T. b. brucei* TC221 trypanosomes, both inoculated with 10^6 cells per insert, the transmigration rate of macrophages did not vary significantly. In contrast, the transmigration of the *T. b. brucei* TC221 in the presence of J774 macrophages decreased significantly (Fig. 7). In order to exclude

Fig. 6. Transmigration of J774 murine macrophages across the optimized ECV304-C6 BBB. J774 (ECV304-C6 -PMA) and PMA-activated J774 macrophages (ECV304-C6 +PMA) with an inoculum of 10^5 cells per insert show similar transmigration rates after 5 h. BBB-free inserts ('Cell-free') covered with Matrigel (+Matrigel) are not traversed by J774 macrophages, in contrast to BBB-free inserts without Matrigel (-Matrigel), which show an intermediate phenotype.

Fig. 7. Transmigration of *T. b. brucei* TC221 (Tbb) and J774 murine macrophages across the optimized ECV304-C6 BBB. Inocula of 10^6 macrophages and 10^6 trypanosomes were added to the inserts, and after 5 h samples were taken from the lower compartment of the transwell system. Transmigration of *T. b. brucei* TC221 trypanosomes (black columns) significantly decreases ($P \leq 0.05$) in the presence of J774 macrophages. The transmigration of J774 macrophages (grey columns) is not affected by *T. b. brucei* TC221 trypanosomes.

inhibitory effects from J774 macrophages on the viability of the trypanosomes, co-cultivation assays were performed. *T. b. brucei* TC221 reached similar cell densities after culture with and without J774 macrophages. During 48 h of co-cultivation the trypanosomes and the macrophages multiplied steadily (data not shown). Results of the growth assays revealed that the J774 macrophages had no negative effects on the viability of *T. b. brucei* TC221. Taken together these results show that the transmigration of J774 macrophages had no synergistic effects on the traversal of *T. b. brucei* TC221.

DISCUSSION

In animals and humans African trypanosomes traverse the BBB during infection, which leads finally to the fatal outcome of the disease. In the present study we established an improved *in vitro* BBB model, which is highly reproducible and suitable for the investigation of the trypanosomal attack on the brain. The optimized ECV304-C6 BBB consists of two closely interacting cell lines with a semi-synthetic extracellular matrix in between. C6 astrocytes are a cell line derived from a rat glioma and secrete soluble factors inducing BBB-specific gene expression in ECV304 cells (Hurst & Fritz, 1996; Hurst *et al.*, 1998; Kuchler-Bopp *et al.*, 1999). The BBB is a dynamic system and needs continuous induction processes, as evidenced by the fact that cells from brain microvessels can lose BBB features in monocultures (Reichel *et al.*, 2003). In our study, evaluation of *E. coli* HB101 transmigration was chosen as the criterion for a tight barrier. *E. coli* HB101 does not possess fimbriae and is also deficient in genes that

are necessary for invasive dissemination (Huang *et al.*, 1995; Kim, 2000; Stins *et al.*, 1999). Therefore, transmigration of *E. coli* HB101 across cell barriers occurs paracellularly through fissures and gaps within the cell layers. As shown by the transmigration assays with *E. coli* HB101 the combination of the interacting cell lines improved the barrier function of the optimized ECV304–C6 BBB. This BBB model was shown to be impermeable for bacterial amounts of 10^5 and 10^6 *E. coli* HB101 per insert. Electron microscopy analyses revealed a tight ECV304 cell layer with overlapping cell regions. Incubation of the optimized ECV304–C6 BBB with higher amounts of bacteria results in a loss of barrier function. This is possibly due to a decrease in pH to <6.4 , as indicated by the change of the medium colour after 5 h of infection. Another reason for unspecific transmigration could be LPS-mediated barrier opening, as shown for other endothelial monolayers (Nooteboom *et al.*, 2000). The invasive and meningitis-causing *E. coli* K1 strain was used as a positive control and showed a dose- and time-dependent BBB transmigration. The substantial differences in transmigration efficiencies between pathogenic and non-pathogenic *E. coli* strains demonstrated the selective permeability of the optimized ECV304–C6 BBB. This optimized model was also evaluated in permeation assays using different pharmaceutical compounds (Kühne *et al.*, 2011), showing good correlation of the penetration values with the reference data.

The optimized ECV304–C6 BBB was used for further transmigration analyses with the causative agents of human African sleeping sickness. *T. b. gambiense* and *T. b. rhodesiense* as well as the bovine-pathogenic *T. b. brucei* were able to transmigrate the optimized ECV304–C6 BBB. The latter showed the lowest transmigration rate. *In vivo*, *T. b. brucei* is not able to survive in the human bloodstream because of its sensitivity to trypanolytic factors in human serum (Hajduk *et al.*, 1989; Rifkin, 1978). If the trypanolytic factor is absent, non-human pathogenic trypanosomes can cause life-threatening infections, as shown in a case report from India (Vanhollebeke *et al.*, 2006). Therefore, the absence of the trypanolytic factor in the infection media is possibly the reason for the transmigration of *T. b. brucei* across the optimized ECV304–C6 BBB. *T. b. rhodesiense* causes East African sleeping sickness, which is an acute disease. In contrast, the West African sleeping sickness progresses more like a chronic disease and is caused by *T. b. gambiense*. Interestingly *T. b. rhodesiense* transmigrates with lower rates than *T. b. gambiense*, suggesting that the acute course of the disease is possibly not due to a stronger trypanosomal invasion into the brain. A tenfold increase of the trypanosomal inoculum resulted in an increased transmigration of *T. b. brucei* and *T. b. rhodesiense*, but not of *T. b. gambiense*, indicating that a certain density of parasites has to be reached in order to achieve maximum transmigration rates. The invasion process occurs by opening of the tight junctions without the destruction or degradation of tight junction proteins, and trypanosomes

transmigrate through the BBB paracellularly (Grab *et al.*, 2004). Opening of the tight junctions has been proven by real-time TEER (transendothelial electrical resistance) measurements, which revealed a transient loss of barrier integrity during transmigration (Grab *et al.*, 2004). A comparable transmigration mechanism is known from the paracellular exsation process of leukocytes, which are able to cross endothelial barriers without cellular damaging. This mode of extravasation has been shown for monocytes (Giri *et al.*, 2000) and neutrophils (Burns *et al.*, 2000), and leads to the assumption that the transmigration processes of leukocytes and trypanosomes across the BBB may be closely related (Masocha *et al.*, 2007). Masocha *et al.* (2004) described that the lack of B- and T-lymphocytes in RAG-1-deficient mice prevents *T. b. brucei* from crossing the BBB. Our data demonstrate that trypanosomes are not able to simply follow the macrophages during their transmigration process. Moreover, we found that approximately 60 % fewer *T. b. brucei* trypanosomes transmigrate in the presence of J774 macrophages, whereas the transmigration of J774 macrophages is not affected in presence of *T. b. brucei* trypanosomes. The low transmigration rate could be caused by a competitive blocking by macrophages of adhesion sites like laminin $\alpha 4$ chains in the extracellular matrix (Masocha *et al.*, 2004). Another possibility for getting across the BBB is the ‘Trojan horse’-like travelling inside macrophages, as shown for example for *Cryptococcus neoformans* (Charlier *et al.*, 2009). However, we found no evidence for intracellular African trypanosomes in our microscopic observations during transmigration or co-cultivation assays.

After multiplication within the bloodstream, the trypanosomal attack on the brain marks the second stage of the disease, which finally causes the death of the infected host. Correct identification of the disease stage of patients is essential for successful treatment in order to prevent relapsing parasitaemia. As shown in a rat infection model, the CNS can be a source of reinfection with parasites after clearance of bloodstream trypanosomes in consequence of treatment with drugs that cannot enter the CNS (Jennings *et al.*, 1979). Results of the present study showed that trypanosomes traverse an inverse ECV304–C6 BBB model system with similar transmigration rates as in the normal BBB model. These results support the data of the rat-infection model and indicate that the CNS invasion is not a one-way process and that trypanosomes are capable of returning to the blood circulation.

In conclusion, we have evaluated three different cell lines for the generation of an *in vitro* BBB model. The optimized ECV304–C6 BBB is a highly reproducible and selective permeable barrier model for pathogens and was established for the comparative analysis of different African trypanosomes. We were the first to show that *T. brucei* strains traverse the blood–brain barrier *in vitro* in both directions. The BBB model presented provides a reliable *in vitro* system that facilitates investigation of BBB-transmigration processes in order to understand the cellular and molecular mechanisms underlying CNS invasion. This is urgently

needed for improvements in therapy and for the development of new drugs.

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