Minocycline Impedes African Trypanosome Invasion of the Brain in a Murine Model

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Passage of Trypanosoma brucei across the blood-brain barrier (BBB) is a hallmark of late-stage human African trypanosomiasis. In the present study we found that daily administration of minocycline, a tetracycline antibiotic, impedes the penetration of leukocytes and trypanosomes into the brain parenchyma of T. brucei brucei-infected C57BL/6 mice. The trypanosome-induced astrocytic and microglial reactions were reduced in the minocycline-treated mice, as were the levels in the brain of transcripts encoding adhesion molecules, cytokines, and matrix metalloproteases (MMPs). Invasion of trypanosomes and leukocytes into the brain parenchyma most likely triggered the loss of weight and death of infected animals, since minocycline did not affect the growth of T. b. brucei either in vitro or in vivo or the levels of the transcripts encoding the cytokines and MMPs in the spleen. In conclusion, our data show that T. b. brucei invasion of the brain is related to that of leukocytes and that minocycline can ameliorate the disease in trypanosome-infected mice.

Human African trypanosomiasis (HAT) or sleeping sickness is caused by the inoculation of subspecies of Trypanosoma brucei by infected tsetse flies. T. b. gambiense, which occurs in West and Central Africa, causes a chronic form of the disease, whereas T. b. rhodesiense, which is found in East Africa, causes an acute variant of the disease. Clinically, the disease is divided into two stages: the early stage, in which the parasites invade the hemolymphatic system, and the late meningoencephalitic stage, with severe signs of nervous system involvement (3, 12). If not treated, the infection is lethal. Neuropathologically, the changes are characterized by an infiltration of inflammatory cells in the brain, which is most prominent in the white matter (leukencephalitis), accompanied by a marked activation of astrocytes and microglia (1, 11).

In a mouse model of the disease, parasites penetrate the BBB at a late stage and can enter the brain parenchyma with preserved tight junction proteins in the cerebral vessels (16). The penetration of trypanosomes across the BBB shows certain similarities to that of leukocytes since endothelial basement membranes, which form part of the BBB, containing laminin 8 are permissive for both T-cell and T. b. brucei transmigration, whereas those containing laminin 10 are restrictive (15, 23). Minocycline, a second-generation tetracycline antibiotic with multiple biological effects distinct from its antimicrobial actions, has been demonstrated to reduce the number of leukocytes invading the central nervous system (CNS) parenchyma in experimental allergic encephalitis (EAE) (2, 19), and this effect contributes to its therapeutic activity against the disease. The drug also reduces the expression or activity of molecules associated with leukocyte transmigration from the blood vessels into an inflammatory site, such as adhesion molecules (8), cytokines and chemokines and their receptors (10), and matrix metalloproteases (MMPs) (2, 19), which can degrade components of the extracellular matrix and basement membranes.

We investigated the effect of minocycline on the brain parenchymal invasion by T. b. brucei in a murine model of the infection. We report that treatment with minocycline reduced penetration of the parasite, as well as CD45+ leukocytes, into the brain parenchyma, prevented weight loss, and prolonged the survival of infected mice. This was paralleled by reduced levels of adhesion molecules, cytokines, and MMP transcripts, as well as reduced microglia and astrocyte activation, in the brain.

MATERIALS AND METHODS

Mice, parasites, infection, and minocycline treatment. All experiments were conducted according to protocols that received institutional approval and authorization by the local animal ethical committee (Stockholms Norra Djurförsökssetiska Nämnd, project N451/03). Efforts were made to minimize animal numbers and the suffering of the animals used. C57BL/6 mice were used and were supplied by the breeding unit at the Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden, and were kept with food and water ad libitum under specific-pathogen-free conditions.

Mice (8 to 12 weeks old) were infected by intraperitoneal (i.p.) injection with 2,000 to 3,000 parasites of a pleomorphic stabilate of T. b. brucei, AnTat 1.1E, derived from stablate EATRO 1125 (passaged in C57BL/6 mice; obtained from N. van Meirvenne, Laboratory of Serology, Institute of Tropical Medicine “Prince Leopold,” Antwerp, Belgium). Parasites were diluted in 60 mM phosphate-buffered saline (PBS) containing 40 mM glucose. Mice were treated i.p. daily with minocycline (Sigma, Steinheim, Germany) or its vehicle (PBS), commencing on the day of T. b. brucei inoculation. Control infected mice were injected with 200 µl of PBS daily, while the minocycline-treated infected mice received 50 mg of the drug/kg twice a day for the first 2 days and once daily for the next 5 days, followed by 25 mg/kg for the subsequent days until the animals were sacrificed. These doses were chosen based on those reported to reduce T-cell invasion of the CNS during EAE (19). Animals were weighed and checked...
at 37°C and 5% CO₂ as described previously (6, 13). The number of mobile 
separated by DEAE-cellulose chromatography and incubated in HMI-9 medium 
across the BBB, sections cut at a level of the lateral ventricles containing the choroid 
in vivo produced a peak concentration of 7 µg/ml. The concentration range of minocycline used 
for the in vitro experi-
ments was chosen taking into consideration that the doses of minocycline used 
72 h in the presence or in the absence of minocycline (covering a range of 1 to 
MMP-9 Sense TATTTTTGTGTGGCGTCTGAGAA NM-013599
MMP-11 Sense TGACCCCACTCACTTTCACTGA NM-008606
MMP-10 Sense TGCACCCTCAGGGACCAA NM-019471
MMP-8 Sense GATGGACCCAATGGAATCCTT NM-008611
MMP-3 Sense TCCTGATGTTGGTGGCTTCA NM-010809
MMP-2 Sense CCCATGAAGCCTTGTTTACCA NM-008610
TIMP-2 Sense ATAAAGATGTTCAAAGGACCT

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* GenBank accession numbers of sequences used for primer design.

daily for signs of disease. Blood samples were taken from the tip of the tail during 
the course of infection to assess parasitemia by using the Herbert and Lumsden 
chart (5).

In vitro studies. T. b. brucei, freshly isolated from infected C57BL/6 mice, were 
separated by DEAE-cellulose chromatography and incubated in HMI-9 medium 
at 37°C and 5% CO₂ as described previously (6, 13). The number of mobile trypanosomes was counted by using a Neubauer hemacytometer after 24, 48, and 
72 h in the presence or in the absence of minocycline (covering a range of 1 to 
20 µg/ml). The concentration range of minocycline used for the in vitro experi-
ments was chosen taking into consideration that the doses of minocycline used in 
vivo produced a peak concentration of 7 µg/ml in plasma in rats (2, 19).

Immunohistochemistry. Mice were deeply anesthetized with isoflurane and 
mice were divided into two groups according to their relationship with the vessels;
intravascular or extravascular. CD45+ leukocytes were counted in a manner similar to that described for the parasites.

Real-time reverse transcription-PCR. Gene transcripts of several adhesion 
molecules, proinflammatory cytokines, MMPs, and tissue inhibitors of metallo-
proteases (TIMPs) (Tables 1, 2 and 3) were quantified in the brains of minocy-
lcline-treated and PBS-treated uninfected and infected mice by real-time PCR.

The number of VSG-immunopositive T. b. brucei in five ocular fields (viewed through 10× ocular and 20× objective lenses) from the cortex and corpus callosum on either side of the midline was determined and in four fields from the septal 
nucleus in four animals from each postinfection (p.i.) time point. The parasites 
were divided into two groups according to their relationship with the vessels;
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TABLE 1. PCR primer sequences of cyclophilin, adhesion 
molecules, and cytokines

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T. b. brucei (Fig. 1A). Similar parasitemia were observed in PBS-treated and minocycline-treated infected mice (Fig. 1A). In line with this observation, minocycline did not show toxic effects on T. b. brucei in vitro since there were no significant differences in the number of mobile trypanosomes cultured axenically for 24, 48, and 72 h in the presence of 1 to 20 µg of minocycline/ml or medium alone (Fig. 1B).

There was a gradual continuous increase in body weight of uninfected mice during a 35-day observation period, which was not affected by minocycline treatment. Nontreated, infected mice showed decreased body weights from about 20 days p.i. In contrast, minocycline-treated, infected mice gained weight as the uninfected controls did (Fig. 1C). Nontreated, infected mice were sacrificed between days 30 to 35 p.i., before they became moribund. On the other hand, infected mice treated with minocycline for 30 days p.i. did not show signs of disease around that time period (30 to 35 days p.i.) and were sacrificed between days 40 and 45 p.i., before becoming moribund.

Minocycline reduces the number of trypanosomes invading the brain parenchyma. In order to assess the effect of minocycline on the parasite invasion of the brain parenchyma, double immunolabeling with antibodies to T. b. brucei and glucose transporter 1 (GLUT-1), a marker of cerebral blood vessel endothelial cells, was performed on brain sections. Daily treatment with minocycline reduced the density of T. b. brucei in the brain parenchyma in mice examined both at 20 and 30 days p.i. (Fig. 2A). In nontreated mice several parasites were seen extravascularly in the brain parenchyma (Fig. 2B), whereas in the minocycline-treated mice the parasites were mainly localized in the lumens of the blood vessels (Fig. 2C). There was no conspicuous cuffing of parasites around blood vessels in either nontreated or minocycline-treated mice as we previously described in IFN-γ, IFN-γ receptor-, and RAG-deficient mice (15).

Minocycline reduces the T-cell infiltration and glial reaction during T. b. brucei infection. To characterize the effect of minocycline on the T. b. brucei infection-induced brain inflammation, the density of CD45⁺ leukocytes in the brain parenchyma, as well as the activation of astrocytes and microglia, were evaluated. The number of CD45⁺ cells in the brain parenchyma of minocycline-treated mice was reduced compared to that of nontreated, infected mice both at days 20 and 30 p.i. (Fig. 3). In the nontreated, T. b. brucei-infected mice intense tomato lectin immunoreactivity appeared in mainly hypertrophic ramified microglia that was most pronounced in the white matter and hypothalamic periventricular areas (Fig. 4A and data not shown), and this immunoreactivity was markedly reduced in the minocycline-treated mice (Fig. 4B). In the nontreated, infected mice there was increased GFAP immunoreactivity of astrocytes, particularly prominent in the white matter, septum, and hypothalamic periventricular regions at both 20 and 30 days p.i (Fig. 4C and data not shown). This immunoreactivity was reduced in the minocycline-treated, infected mice (Fig. 4D).

Effect of minocycline on levels of transcripts encoding adhesion molecules, cytokines, and MMPs in the brain and spleen. The levels of mRNAs encoding intercellular adhesion molecule 1 (ICAM-1) and endothelial-leukocyte adhesion molecule 1 (E-selectin) were increased in the brains of infected animals and were reduced by minocycline treatment (Fig. 5A and B), whereas those of vascular cell adhesion molecule 1 (VCAM-1) and plate-
let endothelial cell adhesion molecule 1 (PECAM-1) were less, or not, affected by the infection (data not shown). Brains from trypanosome-infected mice showed significant increase in the levels of MMP-3, -8, and -12 mRNA titers compared to uninfected controls, whereas levels of MMP-1b, -2, -7, -9, -11, -13, -14, and -19 and TIMP-1 and -2 mRNA were unaltered, and MMP-10 was below the detection limit (Fig. 5C and D and data not shown).

Minocycline treatment reduced the infection-induced elevation of MMP-3, -8, and -12 mRNA levels in the brain at 30 but not at 20 days p.i. (Fig. 5C and D and data not shown). The levels of tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), interleukin-1β (IL-1β), IL-1β, and IL-6 mRNAs were also increased in the brains of infected mice and reduced by minocycline treatment (Fig. 6 and data not shown).

Spleens from infected and uninfected mice showed similar expression of mRNAs encoding ICAM-1 and E-selectin. Elevated TNF-α, IFN-γ, IL-1α, IL-1β, and IL-6 mRNA levels in the spleens from mice at 20 and 30 days p.i. were not altered by minocycline treatment (data not shown). Increased levels of MMP-8 and -9 transcripts were detected in spleens from infected mice at day 20 and 30 p.i., whereas the levels of MMP-2, -3, -11, and -12 were unaltered compared to uninfected controls. Minocycline treatment did not reduce the elevated MMPs transcript levels in the spleen (data not shown).

FIG. 2. Effects of minocycline treatment on trypanosome invasion into the brain parenchyma. (A) Reduction in density of parasites in the parenchyma of minocycline-treated compared to PBS-treated mice at 20 and 30 days p.i. Each bar represents the mean ± the SEM of the values obtained from five to six animals. *, P < 0.05 (Mann-Whitney U test). (B and C) Double immunofluorescence labeling of trypanosomes (red) and cerebral endothelial cells (green) at 30 days p.i. in PBS (B)- and minocycline (C)-treated mice. Note the presence of extravascular parasites in PBS-treated and intravascular parasites in minocycline-treated mice. Bar, 25 μm.

FIG. 3. Reduction in density of CD45+ cells in the parenchyma of minocycline-treated compared to PBS-treated mice at 20 and 30 days p.i. Each bar represents the mean ± the SEM of the values obtained from five to six animals. *, P < 0.05 (Mann-Whitney U test).
We have previously reported that the penetration of trypanosomes across the basement membranes of the BBB shows similarities to that of leukocytes (15). In the present study, minocycline reduced the invasion of the brain parenchyma not only by leukocytes (CD45<sup>+</sup> cells) but also by trypanosomes. This suggests that neuroinvasion of trypanosomes is related to that of leukocytes, whereby the latter may pave the way for the former. The transmigration of leukocytes from the blood into the brain parenchyma takes place in an integrated sequence of events, including rolling and activation of leukocytes, adhesion to the vascular endothelium, diapedesis across the endothelium, and ultimately penetration through the endothelial and astrocytic basement membranes (23, 27). Several molecules, including cell adhesion molecules, cytokines, chemokines, and MMPs, play a role during this multistep process (22, 27). Minocycline with its pleiotropic effects might impede the leukocyte transmigration into the CNS by regulating any of these molecules.

We observed that the infection-induced expression of the adhesion molecules ICAM-1 and E-selectin transcripts in the brains of <i>T. b. brucei</i>-infected mice was reduced by minocycline treatment, which also possibly curbed the brain invasion of the parasites and leukocytes by reducing the expression of these molecules. Minocycline has been reported to downregulate the expression of ICAM-1 and reduce the number of infiltrating cells during ischemic renal injury, as well as to impair leukocyte chemotaxis (8).

The reduction of cytokine expression in the brain by minocycline treatment could partly be due to the reduced influx of...
cytokine-producing leukocytes into the brain, since the levels of cytokine expression in spleen cells were not affected by the treatment. In line with this, no increase in IFN-γ transcripts was observed in the brains of T. b. brucei-infected RAG-1−/− mice, which lack B and T cells, suggesting that lymphocytes are the major source of this proinflammatory cytokine in the brains of infected wild-type mice (15). In addition, a direct inhibitory effect by minocycline on activation of astrocytes and microglia has been described (9, 24), and this could also have contributed to the reduced cytokine expression in the brain.

Minocycline reduced the increased levels of MMP-3, -8, and -12 transcripts in infected mice brains at 30 days p.i. This reduction could also reflect a suppressed influx of leukocytes into the brain. Minocycline abrogated MMP-2 expression in the CNS of rats with EAE and, in parallel, suppressed T-cell recruitment into the CNS (19). However, at 20 days p.i., where minocycline treatment reduced the numbers of leukocytes and trypanosomes in the brain parenchyma, there was no significant increase in MMP transcript expression in the brains, thus ruling out a role of MMP transcript regulation on the protective effect conferred by minocycline treatment. A direct inhibitory effect of minocycline on MMP enzymatic activity has, however, been described (2, 18, 21) and could play a role in the altered outcome of brain infection in minocycline treated mice.

The infected minocycline-treated mice did not lose weight as the nontreated mice did, in spite of the fact that the two groups of animals showed similar levels of parasitemia and transcripts for inflammatory molecules in the spleen. Probably, the reduction of infection-induced expression of TNF-α, IL-1α, IL-1β, IL-6, and IFN-γ in the brains of treated mice mediates the protective effect of minocycline. These proinflammatory cytokines have been demonstrated to be involved in weight loss associated with chronic infections, sepsis, and cancer (7, 25). Furthermore, intracerebroventricular infusion of IL-1 receptor antagonist or a combination of IL-1 receptor antagonist and soluble type-1 receptor of TNF restores or prevents the loss of weight in rats caused by infection with T. b. brucei (20). Thus, these findings suggest that the loss of weight and mortality caused by the T. b. brucei is due to brain involvement rather than to systemic effects of the infection.

The present study demonstrates that treatment with minocycline reduces T. b. brucei invasion of the brain parenchyma, ameliorates CNS inflammatory parameters associated with the infection, prevents weight loss, and prolongs survival of the animals.

Minocycline and other tetracycline antibiotics have been used in combination therapy against other parasitic diseases such as malaria (17, 26, 28) and toxoplasmic encephalitis (4). Our findings may therefore be of relevance for the development of treatment strategies with minocycline as a supplement to trypanocidal drugs used in the treatment of the disease.

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REFERENCES


