Nitric Oxide and Cytokine Synthesis in Human African Trypanosomiasis

Lorna MacLean, Martin Odiit, and Jeremy M. Sternberg

Plasma and cerebrospinal fluid (CSF) concentrations of nitrate and the cytokines interferon (IFN)–γ, tumor necrosis factor (TNF)–α, interleukin (IL)–10, and IL-4 were measured in 91 African trypanosomiasis patients before and after treatment. Nitrate levels overall were not significantly elevated over those for control persons, but a marginal increase in plasma nitrate was detected in patients reporting illness of <40 days’ duration. Plasma IFN–γ and total TNF–α concentrations increased during infection, but free TNF–α levels were low in all patients. The most dramatic cytokine response was for IL-10, which was significantly elevated in both plasma and CSF during infection but returned to control levels after treatment. The results indicate that human African trypanosomiasis leads to the development of a strong anti-inflammatory cytokine response.

African trypanosomes (Trypanosoma brucei species) provide an exquisite example of antigenic variation as a host immune evasion strategy, yet the disease is characterized by immunosuppression [1]. The mechanism underlying immunosuppression in human African trypanosomiasis poorly understood but has received considerable attention in a mouse model, in which it is associated with suppressor macrophages. In the early stages of murine infection with T. brucei, nitric oxide (NO) and prostaglandins produced by activated macrophages cause lymphocyte hyporesponsiveness in the spleen [2] and bone marrow [3]. The importance of suppressor macrophages in modulating the course of infection in mice was demonstrated by use of chemical inhibitors of NO synthesis or use of NO synthase (NOS) gene–deleted mice [4]. Improved control of parasitemia indicated that NO produced by activated macrophages cause disease-exacerbating factor; however, NO synthesis declines after ~10 days of infection [5]. In the subsequent chronic phase of infection, immunosuppression is associated with cytokines, such as interleukin (IL)–10, that down-regulate the inflammatory response [6, 7].

In contrast to infections in experimental mice, there is a paucity of data on the immunologic sequelae of human infection with African trypanosomes. Trypanosoma brucei rhodesiense, which occurs to the east of the Rift Valley, causes a milder form of African trypanosomiasis than T. b. gambiense, but the disease follows the same progression in both subspecies. The disease is characterized by an early hemolymphatic phase of parasite proliferation in the blood and lymphatic system, followed by penetration of the blood-brain barrier and a late meningoencephalitic phase of infection. These will be referred to henceforth as early- and late-stage infections. In T. b. rhodesiense infection, data have been presented indicating an early up-regulation of NO synthesis [8], and, in a small study of patients infected with T. b. gambiense, plasma levels of tumor necrosis factor (TNF)–α and IL-10 were found to be elevated [9]. The purpose of this study was to determine levels of NO, interferon (IFN)–γ, TNF–α, IL-4, and IL-10 in a well-defined cohort of patients with T. b. rhodesiense sleeping sickness and to test the hypothesis that infection is associated with NO up-regulation in early infection and IL-10 production in late infection.

Patients and Methods

Patients. Ninety-one sleeping sickness patients and 71 healthy control persons were recruited in Tororo, Iganga, and Soroti districts of southeast Uganda between November 1998 and July 2000. Blood samples were obtained from all subjects. In addition, cerebrospinal fluid (CSF) samples were taken from all sleeping-sickness patients or suspects. Blood samples were subjected to routine hematology tests and were tested for concurrent infections. Malaria-infected and microfilaricmic individuals were excluded from the study. In practice, trypanosomiasis is often diagnosed on the basis of an index of suspicion, but for the purpose of this study, only samples from individuals for whom a positive microscopic diagnosis of trypanosomes was obtained were included. Parasitemia was estimated from wet blood films. Infections were categorized as either early stage (trypanosomes in blood but not in CSF) or late stage (meningoencephalitic try-
were enrolled; 79 of the patients had early-stage (hemolymphatic) infection with microscopically diagnosed late-stage (meningoencephalitic) infections, and 12 had early-stage (hemolymphatic) infection. Patients with sleeping sickness were enrolled; 79 of the patients had early-stage (hemolymphatic) infection with microscopically diagnosed late-stage (meningoencephalitic) infections, and 12 had early-stage (hemolymphatic) infection. Patients with sleeping sickness were enrolled; 79 of the patients had early-stage (hemolymphatic) infection with microscopically diagnosed late-stage (meningoencephalitic) infections, and 12 had early-stage (hemolymphatic) infection. Patients with sleeping sickness were enrolled; 79 of the patients had early-stage (hemolymphatic) infection.

Blood samples were collected into tubes with heparin and were centrifuged at 100 g for 10 min. Plasma was aliquoted and was immediately frozen in liquid nitrogen. CSF samples were also frozen in liquid nitrogen.

Cytokine and nitrate assays. Nitrate concentration was determined by the use of the nitrate reductase-linked Griess assay, as described elsewhere [8]. Plasma nitrate can be influenced by renal dysfunction, so an alkaline pircate reagent (Sigma) was used to determine creatinine levels. Cytokine (IFN-γ, TNF-α, IL-4, and IL-10) concentrations were measured by using an analyte capture ELISA (Opti-EIA; BD-PharMingen). TNF-α–TNF receptor complexes interfered with the TNF-α ELISA (authors’ unpublished observations), so samples were additionally analyzed by using a competitive ELISA (Accucyte; AMS Biotechnology). All assays were done in triplicate. Seropositivity to human immunodeficiency virus (HIV) types 1 and 2 was tested by ELISA (Murex; Biotech).

Statistical analyses. Paired analysis of pre- and posttreatment parameters was done with the Wilcoxon signed-rank test. The Mann-Whitney U test was used for unpaired analyses by group.

Results

Clinical findings. Ninety-one T. b. rhodesiense–infected patients with sleeping sickness were enrolled; 79 of the patients had microscopically confirmed late-stage (meningoencephalitic) infection, and 12 had early-stage (hemolymphatic) infection with no parasites and CSF white blood cell counts of <5 cells/mm³. In addition, 71 healthy, uninfected volunteers from the study area provided control plasma samples. The clinical characteristics of the late- and early-stage infection groups on admission and after treatment are summarized in table 1. Both groups exhibited anemia typical of sleeping sickness, with a significant reduction in packed cell volume in comparison with that of control persons. No patients exhibited significant increases in body temperature. Parasitemia in infected patients ranged from 1 × 10⁴ to 2 × 10⁶ parasites/mL (median, 6 × 10⁵ parasites/mL). HIV seropositivity was similar in all groups. No significant differences were detected in plasma or CSF cytokines when the patients were grouped according to HIV status (data not shown).

Plasma nitrate concentrations. In a previous study with a smaller cohort of patients [8], we found plasma nitrate levels to be significantly elevated in patients with early-stage sleeping sickness. In contrast, in the current study, no significant differences were detected between plasma nitrate levels in infected versus control subjects or in infected subjects before and after treatment (figure 1A). However, we also asked study patients to indicate the duration of their illness before recruitment: The time ranged from 5 days to 3 months. When the early-stage patients were grouped according to duration of illness, those who reported illness of <40 days’ duration had higher plasma nitrate levels (median, 129.4 μM) than did posttreatment patients or controls. However, the small size of this group (n = 9) limits the statistical significance of this result (P = .08). On the other hand, the plasma nitrate levels of early-stage patients reporting an illness of >40 days’ duration did not significantly differ from levels in control persons (median, 81.3 μM). Median creatinine levels were within the normal ranges (male patients, 79.5–123.7 μM; female patients, 70.7–106.1 μM) in all samples, and no significant differences in nitrate/creatinine ratios were detected.

Plasma cytokine concentrations. In patients with early-stage sleeping sickness, plasma IFN-γ levels (figure 1B) were significantly elevated, compared with those in uninfected control persons (median, 29.1 pg/mL vs. 8.4 pg/mL; P < .0001), and they remained significantly elevated in posttreatment samples (median, 14.3 pg/mL; P < .01). IFN-γ levels were lower in late-stage infections (median, 17.6 pg/mL) than in early-stage infections, although they were still higher than levels for control persons (P < .0001).

TNF-α measurements in plasma can be confounded by complexes with soluble TNF receptors I and II [10]; thus, both free and total TNF-α levels were measured in plasma from human African trypanosomiasis patients. Free TNF-α levels were low in all patients, and no significant differences were evident between the pretreatment and posttreatment or control groups. Total TNF-α levels (figure 1C) were typically 100-fold greater

<table>
<thead>
<tr>
<th>Study group</th>
<th>Total no./% female</th>
<th>Age, mean years (range)</th>
<th>Packed cell volumea</th>
<th>Temperature, °C</th>
<th>% HIV infected</th>
</tr>
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<tbody>
<tr>
<td>Early-stage disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Before treatment</td>
<td>12/33</td>
<td>37.5 (19–60)</td>
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<td></td>
<td>36.4</td>
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<tr>
<td>After treatment</td>
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<td>Late-stage disease</td>
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<tr>
<td>Before treatment</td>
<td>79/41</td>
<td>33.8 (2–80)</td>
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<td></td>
<td>34.7</td>
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<td>After treatment</td>
<td></td>
<td></td>
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<tr>
<td>Uninfected control group</td>
<td>71/49</td>
<td>37.2 (13–74)</td>
<td>40.0 (9.0)</td>
<td>36.5 (0.5)</td>
<td>36.4</td>
</tr>
</tbody>
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NOTE: Early-stage disease is defined as the hemolymphatic phase of parasite proliferation in the blood and lymphatic system; late-stage disease is defined as penetrating the blood-brain barrier and as a late meningoencephalitic phase of infection. HIV, human immunodeficiency virus.

a Data are median (interquartile range).
than free TNF-α levels. In early-stage infections, the pretreatment levels were not significantly higher than posttreatment levels. In late-stage infection, the total TNF-α levels were higher before treatment than after treatment (median, 2.1 vs. 1.0 ng/mL; \( P < .05 \)).

IL-10 concentrations for early-stage patients with sleeping sickness were significantly higher than their posttreatment levels (median, 182.9 vs. 16.1 pg/mL; \( P < .01 \); figure 1) and levels of late-stage patients (median, 160.8 vs. 19.7 pg/mL; \( P < .0001 \)); they were also elevated in comparison with levels for the uninfected control group (median, 12.8 pg/mL; \( P < .001 \)). IL-4 levels showed no significant differences before and after treatment, regardless of the stage of the disease (data not shown).

**CSF nitrate and cytokine levels.** There was no significant change in CSF nitrate levels in pretreatment samples (5.8 \( \mu M \)), compared with levels in control (7.6 \( \mu M \)) or posttreatment samples (4.0 \( \mu M \)). However, pretreatment CSF IL-10 levels were significantly higher than those in posttreatment samples from both early-stage (median, 9.1 vs. 2.6 pg/mL; \( P < .05 \)) and late-stage (94.3 vs. 5.1 pg/mL; \( P < .0001 \); figure 1) patients, and IL-10 concentrations were 10-fold higher in the late-stage than in the early-stage CSF samples. TNF-α, IL-4, and IFN-γ con-
centrations in the CSF were low and did not differ significantly between the pre- and posttreatment samples (data not shown).

Discussion

Although the immunology of African trypanosomiasis has been studied extensively in experimental mice, little attention has been paid to natural infection in humans. In the mouse model, there is an early pro-inflammatory cytokine response and a Th1-driven macrophage activation, both of which lead to high levels of NO and prostaglandin synthesis by macrophages. Although it is not possible to measure NOS activity directly in human trypanosomiasis, measurement of plasma nitrate provides an indirect estimate of systemic NO synthesis [11].

In the present study, plasma nitrate was not elevated in late infection. Those patients in the early stage of disease who reported <40 days of illness exhibited marginally elevated plasma nitrate levels, whereas those with a longer illness did not. The small number of patients recruited in these 2 categories limits the statistical significance of these data. However, on the basis of our current and previous findings for early-stage patients [8], we propose that the duration of early-stage infection after a fly bite is critical in determining NO responses and that NO synthesis is strongly down-regulated at a point within the first 40 days of infection. Studies in mice have shown this to occur ~10 days after infection [5]. A decline in NOS activity as infection progresses is consistent with our finding of significantly elevated IFN-γ levels in the early stage of infection, with a subsequent decline in late-stage infection. The decline in the expression of this Th-1 cytokine was not accompanied by any increase in Th-2 cytokine levels in the plasma of sleeping sickness patients, as evidenced by the low level of IL-4 at all stages of infection.

IL-10 levels were high in plasma of sleeping sickness patients both in early- and in late-stage infection and declined to the levels of uninfected control persons after treatment. IL-10 down-regulates a range of inflammatory and activation markers on macrophages, including NOS and TNF-α, and it up-regulates the synthesis of soluble TNF receptors I and II [12]. This may account for the low levels of free TNF-α measured in this study.

Another recent study has demonstrated up-regulation of IL-10 synthesis in T. b. gambiense infection [9], and that finding, taken together with data presented here and those obtained with both murine [7] and bovine [6] T. congolense infections, suggests that IL-10 is a critical immunomodulator in both human and bovine trypanosomiasis.

There was no significant increase in nitrate levels in the CSF during infection. This is consistent with our previous data [8] and indicates that the inflammatory response associated with meningoecephalitic infection is controlled by IL-10, which was detected at high levels in patients with late-stage sleeping sickness. Indeed, the IL-10 level in the CSF was a powerful predictor of late-stage infection, and it will be of interest to study further its diagnostic potential. It is noteworthy that this indirect measurement of counter-inflammatory activity in the brain is inconsistent with results obtained in experimental rodents, for which a general activation of pro-inflammatory cytokines [13] and NO synthesis [14] has been described.

In conclusion, we propose that, in human African trypanosomiasis, an early inflammatory response is rapidly controlled through induction of IL-10 synthesis. Because studies in human subjects allow the analysis only of circulating cytokines and nitrate, it is not possible to determine the location of the synthesis of either inducible NOS or IL-10, although reverse-transcription–polymerase chain reaction analysis of mRNA isolated from peripheral blood mononuclear cells from infected subjects was negative for both of these (data not shown). It will, therefore, be necessary to study this delicate balance further in rodent and possibly primate models to determine the potential for immunological interventions during sleeping sickness.

References