Interleukin-6, IL-8 and IL-10 in serum and CSF of *T.b. gambiense* sleeping sickness patients before and after treatment.

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Abstract

Serum and cerebrospinal fluid (CSF) concentrations of interleukin (IL)-6, IL-8, IL-10, tumour necrosis factor-α and interferon-γ were determined in 46 T.b. gambiense sleeping sickness patients before and after treatment. According to their CSF cell number before treatment, patients were classified as early stage (0-5 cells/µl), intermediate stage (6-20 cells/µl) or late stage patients (> 20 cells/µl).

In serum, slightly higher IL-8 concentrations were found in early stage patients compared to intermediate or late stage patients. These high IL-8 levels dropped after treatment. Higher IL-10 concentrations were detected in serum of patients in intermediate or late stage compared to early stage patients. In both intermediate and late stage groups, serum IL-10 decreased after treatment. In CSF, elevated concentrations of IL-6, IL-8 and especially of IL-10 were observed in late stage T.b. gambiense patients. After treatment, these concentrations dropped to levels similar to those of the other patients. Tumour necrosis factor-α was detected only in a few serum and CSF samples, which were scattered over the different patient groups. Interferon-γ was detected in serum of 5 patients and remained undetectable in CSF.
Introduction

Infection with the protozoan parasite *Trypanosoma brucei (T.b.) gambiense* causes sleeping sickness or Human African Trypanosomiasis. Initially, trypanosomes proliferate in the lymph and blood compartments. Later, the parasites invade areas with a reduced blood-brain barrier such as the choroid plexus and circumventricular organs (SCHULTZBERG et al., 1988). From there, they spread further into the brain. These events are associated with a chronic meningo-encephalitis and are accompanied by development of perivascular cuffing, by infiltration and activation of plasma cells, Mott cells, T-cells, microglia and astrocytes (ANTHOONS et al., 1989; QUAN et al., 1999).

Cytokines are considered to play an important role during these inflammatory processes. In experimental trypanosomiasis models it has been shown that astrocyte activation is accompanied by increased mRNA expression for interleukin (IL)-1α, macrophage inflammatory protein 1, and tumour necrosis factor (TNF)-α, and a peak of IL-6 and interferon (IFN)-γ mRNA expression (HUNTER et al., 1992). Recently, QUAN et al. (1999) reported a chronic overexpression of pro-inflammatory cytokines in the brain of *T.b. brucei* infected rats. By *in situ* hybridisation, cells expressing IL-1β and TNF-α mRNA were observed already 22 days after infection in the meninges, the choroid plexus and circumventricular organs. IL-6 and IFN-γ mRNA expressing cells were rather restricted to the choroid plexus. No evidence for induction of IL-10, IL-12 or IL-4 was however found by these authors.

For human African trypanosomiasis on the other hand, only limited and fragmentary data on cytokines are available. In *T.b. gambiense* infected patients, high TNF-α levels, correlating with disease severity, were found in serum (OKOMO-ASSOUMOU et al.,
1995). In the plasma of 8 late stage patients, increased levels of TNF-α and IL-10 were observed, which both declined quickly after melarsoprol treatment (RHIND et al., 1997). Normal IL-1α levels were detected in the cerebrospinal fluid (CSF) of *T. b. gambiense* infected patients (PENTREATH et al., 1990). In *T. b. rhodesiense* infections, abnormally high plasma TNF-α and IL-6 levels were observed, decreasing after treatment, whereas the IL-1β levels were comparable to controls (REINCKE et al., 1994). Only recently, cytokine measurements on both plasma and CSF of *T. b. rhodesiense* patients before and after treatment, were reported by (MACLEAN et al., 1999). The authors observed elevated plasma IFN-γ, which remained significantly high after treatment, but no IFN-γ was detected in the CSF of the same patients.

Within the framework of a study conducted on alternative treatment protocols and stage determination, we had the opportunity to collect paired serum and CSF samples of *T. b. gambiense* sleeping sickness patients before and after treatment. In order to explore the alterations in the cytokine environment during human *T. b. gambiense* trypanosomiasis infection, we analysed the available samples for some selected cytokines. Our intention was to investigate 1° the cytokine profiles in paired serum and CSF samples of untreated patients, 2° whether these profiles are related to the disease stage, and 3° whether and how these profiles change after treatment. Here we present results on IL-6, IL-8, IL-10, TNF-α and IFN-γ.

**Materials and methods**

Of 46 parasitologically confirmed *T. b. gambiense* infected patients originating from Equateur Province in R.D. Congo, paired serum and CSF samples were taken before
treatment and the day after the last drug administration. Patients were examined and treated at the hospital of Bwamanda between February and May 1998, were between 17 and 48 years old (median 31) and male/female ratio was 1.09. Sleeping sickness patients with 0-5 cells/µl were treated with Suramine (6 IV injections of 20 mg/kg, 3 days of rest between each injection) and patients with 6-20 cells were treated with Melarsoprol (2 series of 3 consecutive days of Melarsoprol 3.6mg/kg/day, 7 days of rest between the series). Patients with more than 20 cells or trypanosomes in the CSF participated in a clinical trial on alternative treatment of second stage trypanosomiasis and were treated 1° with Melarsoprol (3 series of 3 consecutive days of Melarsoprol 3.6mg/kg/day, 7 days of rest between the series) or, 2° with a short course of Melarsoprol (day 1 0.6 mg/kg, day 2 1.2 mg/kg and day 3 to day 10 1.8 mg/kg) or, 3° with Nifurtimox (3x5mg/kg/day for 14 days) or 4° with a combination of Melarsoprol and Nifurtimox (day 1 0.6 mg/kg Melarsoprol, day 2 1.2 mg/kg Melarsoprol, day 3 to day 10 1.2 mg/kg Melarsoprol in combination with 2x7.5mg/kg Nifurtimox). The protocol was approved by the Bureau Central de la Typanosomiase (BCT) of the Ministry of Health of R.D. Congo. From each patient or accompanying relative, an informed consent was obtained. After preparation of the serum and centrifugation of the CSF, samples were frozen at −20°C for maximal 4 months, transported to Antwerp on dry ice and liquid nitrogen and stored at −70°C. Serum and CSF were kept frozen until cytokine analyses. No control serum or CSF from non-sleeping sickness patients originating from the same region was available for this study.

Samples were analysed for IL-6, IL-8, IL-10, TNF-α and IFN-γ with ELISA kits of Eurogenetics NV (Tessenderlo, Belgium), following the instructions of the manufacturer. All measurements were done in duplicate; pre- and post-treatment
samples of the same patient were run in the same plate. Detection limits, corresponding to the lowest concentration of the standards included in the assays, are given for serum and CSF in table 1 and 2. Values in serum or CSF below these were extrapolated. According to the manufacturer of the ELISAs, normal values for IL-6, IL-8, IL-10, TNF-α and IFN-γ in serum are below the detection limits. 

For interpretation of the results, patients were subdivided in 3 groups according to their CSF cell number before treatment: 13 Patients with 0-5 cells/µl (early stage, median 2 cells/µl), 9 patients with 6-20 cells/µl (intermediate stage, median 12 cells/µl) and 24 patients with more than 20 cells/µl (late stage: 21-980 cells/µl, median 72 cells/µl). Differences in cytokine concentrations between early, intermediate and late stage patients were analysed by ANOVA for normally distributed variables (Kolmogorov Smirnov, p>0.05), assuming equality of variance, or by the Kruskall Wallis test for non-normally distributed variables. Pre- and post-treatment concentrations within each group were compared by a T-test for paired samples in case of normally distributed values, otherwise by the Wilcoxon test.

**Results**

An overview of the IL-6, IL-8, IL-10, TNF-α and IFN-γ concentrations measured in the serum and CSF of the *T.b. gambiense* patients is given in respectively table 1 and 2.

In all patients before and after treatment, IL-6 serum concentrations were above the normal level (table 1). No significant differences were observed between early,
intermediate or late stage patient groups (fig 1A), or between the samples taken before and after treatment within each group.

In 13 CSF samples of trypanosomiasis patients before treatment (fig 1B), the IL-6 concentration was above the detection limit. The concentration of IL-6 in CSF of late stage patients before treatment was significantly higher (Kruskall Wallis test, p<0.001) than in the other patient groups, and decreased significantly after treatment (Wilcoxon test, p=0.011).

Only 3/46 serum samples taken before treatment contained normal IL-8 concentrations. Serum IL-8 concentrations were slightly higher in the early stage group (Kruskall Wallis test, p=0.08) (fig 2A), but dropped significantly after treatment (paired T test, p=0.033).

The IL-8 concentrations in the CSF samples of late stage patients (fig 2B) were significantly higher than in the other groups (Kruskall Wallis test, p=0.005). After treatment, levels significantly decreased (paired T test, p<0.001) to the levels observed in early and intermediate stage after treatment.

In serum, all IL-10 concentrations measured were above normal before as well as after treatment (table 1). IL-10 concentrations were significantly lower (fig 3A) in early stage than in intermediate or late stage (ANOVA, p=0.036). After treatment, the serum IL-10 concentrations in the intermediate and late stage group decreased significantly (paired T test, p=0.059 and p<0.001 respectively).

In the CSF, the pre-treatment IL-10 concentrations were above the detection limit in 26 samples. Of these 26 samples, 21 came from late stage patients, representing 88 % of
all late stage patients. The CSF IL-10 concentrations in late stage patients were significantly higher (Kruskall Wallis test, p<0.001) than in early and intermediate stage patients (fig 3B). After treatment, the IL-10 levels in late stage samples significantly decreased (Wilcoxon test, p<0.001).

Before treatment TNF-α was above the detection limit in 10 serum samples (table 1) and in 12 CSF samples. No differences in the TNF-α levels between disease groups were observed. After treatment, TNF was still detected in 3 serum and in 12 CSF samples.

IFN-γ (table 1) was above the detection limit in 5 pre-treatment sera, and in 8 post-treatment sera. Again, no clear differences between the groups were observed. IFN-γ remained undetectable in CSF samples.

**Discussion**

The abnormally high IL-6 serum concentrations in our study, confirm findings in *T.b rhodesiense* patients (REINCKE et al., 1994). We could however not find a decrease in IL-6 concentration after treatment or a difference in serum IL-6 concentration between the patient groups, which could reflect a normal high background level in a population exposed to many other infections. Unfortunately, no control sera were available to verify this. Alternatively, the absence of a decrease in IL-6 could be explained by a continued stimulation of the immune system by remaining trypanosomal products immediately after treatment. The finding of IL-6 in CSF of late stage patients
corroborates with data from *T. b. brucei* infected mice and rat, where the detection of IL-6 in the brains correlates with astrocyte activation (Hunter et al., 1992), and was localised to the choroid plexus (Quan et al., 1999). Astrocyte activation has been shown to occur in late stage *T. b. gambiense* infected patients (Lejon et al., 1999). Furthermore, IL-6 induced B-cell maturation into immunoglobulin-secreting cells might be the origin of intrathecal immunoglobulin synthesis observed in sleeping sickness patients (Bisser et al., 1997; Lejon et al., 1998; Bisser et al., in press).

As far as we know, no reports exist on IL-8 in trypanosomiasis. Major bio-activities of IL-8 are chemotaxis of neutrophils and T cells, and induction of adhesion molecule expression on the cell surface of the latter cells (Spanaus et al., 1997; Lindley, 1998). Similar to IL-6, IL-8 shows neurotrophic activity, induces production of neuronal growth factor and might influence blood-brain barrier integrity (Koßmann et al., 1997).

Our IL-10 findings in serum confirm those of other authors (Rhind et al., 1997). Our CSF findings are however in sharp contrast to the animal model of Quan et al. (1999), who could not detect any IL-10 mRNA in the brains of *T. b. brucei* infected rats. IL-10 might inhibit antigen presentation, resulting in abrogated proliferative responses or cytokine production by the responding T-cells or T cell clones, as was observed in experimental *T. b. brucei* mouse infection where macrophages secreted IL-10 contributing to impairment of T-cell activation (Namangala et al., 2000a). In contrast to its inhibitory activities, IL-10 enhances proliferation, differentiation and immunoglobulin secretion of B-cells, which, in collaboration with IL-6, might result in the large amounts of IgG and IgM observed in sleeping sickness.
Although 19 sleeping sickness patients were co-infected with *Loa loa* or *Dipetalonema perstans*, they were scattered over the 3 patient groups. It seems therefore unlikely that co-infection with microfilaria is responsible for the observed differences in cytokine concentrations. Two malaria patients occurring in group 3 were qua cytokine concentrations comparable to other patients of this group.

Our failure to confirm the high serum TNF concentrations described for late stage *T.b. gambiense* patients (OKOMO-ASSOUMOU et al., 1995; RHIND et al., 1997), or the elevated serum IFN-γ levels as described for *T.b. rhodesiense* patients (MACLEAN et al., 1999) could be due to technical reasons, the detection limit of our assays, the detection of free TNF-α only, interference of anti-IFN-γ antibodies, or, due to sub-optimal conservation of the samples. On the other hand, IL-10 could be responsible for the apparent absence of high IFN-γ and TNF-α concentrations in our study. Low IFN-γ and TNF-α concentrations in combination with high IL-10 concentrations were also detected in plasma of a chronic mouse infection model using attenuated *T.b. brucei* parasites, in contrast to the high IFN-γ and TNF-α and low IL-10 in an acute model (NAMANGALA et al., 2000b).

In conclusion, in *T.b. gambiense* sleeping sickness, IL-6 as well as IL-8 might play a role in neuropathogenesis, functioning as neurotrophic factor and modulator of the blood-brain barrier. IL-6 might act as B-cell differentiator, IL-8 as chemokine for neutrophiles and T cells. IL-10 might play a major role in the later stages as anti-inflammatory protein and B-cell stimulator. Due to its abnormally elevated levels in CSF of late stage patients compared to early stage patients, CSF IL-10 could be an interesting candidate as parameter for stage determination. It might also be used to
monitor treatment success since its concentration drops to near normal levels quickly after treatment.

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References


specific antibodies in serum and cerebrospinal fluid of sleeping sickness patients. 

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Table 1: Minimum, maximum and median cytokine concentrations (pg/ml or IU/ml) detected by ELISA in pre- and post-treatment serum samples of 46 *T.b. gambiense* sleeping sickness patients.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Detection limit</th>
<th>Normal levels</th>
<th>Pre-treatment median (min-max)</th>
<th>Post-treatment median (min-max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/ml)</td>
<td>2.5</td>
<td>&lt;2.5</td>
<td>32.0 (4.77- &gt;1000)</td>
<td>24.5 (8.00-913)</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>5</td>
<td>&lt;5</td>
<td>48.8 (0.21-5138)</td>
<td>34.6 (0-2771)</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>6</td>
<td>&lt;6</td>
<td>107 (8.25-423)</td>
<td>45.6 (13.6-269)</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>5</td>
<td>&lt;5</td>
<td>0 (0-674)</td>
<td>0 (0-34.9)</td>
</tr>
<tr>
<td>IFN-γ (IU/ml)</td>
<td>0.1</td>
<td>&lt;0.1</td>
<td>0 (0-0.32)</td>
<td>0 (0-0.47)</td>
</tr>
</tbody>
</table>
Table 2: Minimum, maximum and median cytokine concentrations (pg/ml or IU/ml) detected by ELISA in pre- and post-treatment CSF samples of 46 *T. b. gambiense* sleeping sickness patients.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Detection limit</th>
<th>Pre-treatment median (min-max)</th>
<th>Post-treatment median (min-max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/ml)</td>
<td>5</td>
<td>0 (0-202)</td>
<td>0 (0-25.7)</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>10</td>
<td>66.2 (19.8-205)</td>
<td>52.0 (8.04-115)</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>6</td>
<td>9.8 (0-349)</td>
<td>1.8 (0-150)</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>5</td>
<td>0 (0-33.3)</td>
<td>0 (0-32.4)</td>
</tr>
<tr>
<td>IFN-γ (IU/ml)</td>
<td>0.2</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
</tr>
</tbody>
</table>
Fig 1: Box and whisker plot of IL-6 concentrations in serum (A) and CSF (B) of 46 *T.b. gambiense* patients before and after treatment, grouped according to disease stage.

……: detection limit of ELISA.

++: Significantly (p<0.05) higher IL-6 concentration in this patient group than in other patient groups.

↓↓: Significant (p<0.05) decrease in IL-6 concentration after treatment.

Fig 2: Box and whisker plot of IL-8 concentrations in serum (A) and CSF (B) of 46 T.b. gambiense patients before and after treatment, grouped according to disease stage.

……: detection limit of ELISA

+ and ++: Significantly (p<0.1 and p<0.05) higher IL-8 concentration in this patient group than in other patient groups.

↓↓: Significant (p<0.05) decrease in IL-8 concentration after treatment.

Fig 3: Box and whisker plot of IL-10 concentrations in serum (A) and CSF (B) of 46 T.b. gambiense patients before and after treatment, grouped according to disease stage.

……: detection limit of ELISA

-- and ++: Significantly (p<0.05) lower and higher IL-10 concentration in this patient group than in other patient groups.

↓ and ↓↓: Significant (p<0.1 and p<0.05) decrease in IL-10 concentration after treatment.
Disease stage
early intermediate late

Concentration IL-6 (pg/ml) in serum

Pre-treatment
Post-treatment

Concentration IL-6 (pg/ml) in CSF

Pre-treatment
Post-treatment

++

↓↓
2A. Disease stage:

- Early
- Intermediate
- Late

Concentration IL-8 (pg/ml) in serum:

- 0
- 1000
- 2000
- 3000
- 4000
- 5000
- 6000

- Before treatment
- After treatment

2B. Disease stage:

- Early
- Intermediate
- Late

Concentration IL-8 (pg/ml) in CSF:

- 0
- 50
- 100
- 150
- 200
- 250

- Before treatment
- After treatment

Symbols:

- +
- ↓↓
- ++
- ↓↓
Disease stage

Concentration IL-10 (pg/ml) in serum

Before treatment
After treatment

early    intermediate    late

Concentration IL-10 (pg/ml) in CSF

Before treatment
After treatment

early    intermediate    late

+ +
- -