Stage determination and therapeutic decision in human African trypanosomiasis: value of polymerase chain reaction and immunoglobulin M quantification on the cerebrospinal fluid of sleeping sickness patients in Côte d’Ivoire

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Summary

In human African trypanosomiasis (HAT), two disease stages are defined: the first, or haemo-lymphatic stage, and the second, or meningo-encephalitic stage. Stage determination forms the basis of therapeutic decision and is of prime importance, as the drug used to cure second-stage patients has considerable side-effects. However, the tests currently used for stage determination have low sensitivity or specificity. Two new tests for stage determination in the cerebrospinal fluid (CSF) were evaluated on 73 patients diagnosed with HAT in Côte d’Ivoire. The polymerase chain reaction (PCR) detecting trypanosome DNA (PCR/CSF) is an indirect test for trypanosome detection whereas the latex agglutination test detecting immunoglobulin M (LATEX/IgM) is an indicator for neuro-inflammation. Both tests were compared with classically used tests, double centrifugation and white blood cell count of the CSF. PCR/CSF appeared to be the most sensitive test (96%), and may be of use to improve stage determination. However, its value for therapeutic decision appears limited, as patients whose CSF was positive with PCR were successfully treated with pentamidine. This result confirms those of previous works that showed that some patients with trypanosomes in the CSF could be treated successfully with pentamidine. LATEX/IgM, which depending on the cut-off, showed lower sensitivity of 76% and 88%, but higher specificity of 83% and 71% for LATEX/IgM 16 and LATEX/IgM 8 respectively, appears more appropriate for therapeutic decision making.

Keywords human African trypanosomiasis, diagnosis, polymerase chain reaction, immunoglobulin M, cerebrospinal fluid, Côte d’Ivoire

Introduction

Human African trypanosomiasis (HAT) remains an important public health problem in sub-Saharan Africa. About 55 million people are daily exposed to the risk of infection and it is estimated that there are about 500 000 infected but untreated people (WHO 1998). The control of the disease heavily relies on the detection and treatment of patients. HAT evolves from a haemo-lymphatic phase (first or early stage) during which the parasite proliferates in the blood and the lymph, to a meningo-encephalitic phase (second, late or advanced stage) corresponding to invasion of the central nervous system (CNS). The disease is lethal if untreated. The nature of treatment classically depends on the stage of the disease. For the chronic form of the disease occurring in West and Central Africa, caused by Trypanosoma brucei gambiense, the drug currently available for treatment of early stage is usually pentamidine, while melarsoprol is used for advanced-stage cases. However, melarsoprol is extremely toxic and is associated with encephalopathy in about 10% of treated patients (WHO 1998). Therefore, reliable diagnosis of this stage is required. A broad clinical diversity is observed in HAT (Jamonneau et al. 2000) and neurological signs and symptoms can be observed during the first stage, becoming more frequent and pronounced in the second stage (WHO 1998). Stage determination is therefore generally based on direct or indirect evidence of CNS invasion, assessed at the
level of the cerebrospinal fluid (CSF) obtained by lumbar puncture. Direct evidence comprises detection of the parasite in the CSF during cell count or after single or double centrifugation (DC) (Cattand et al. 1988; WHO 1998; Miezan et al. 2000). Indirect evidence comes from increased cell counts (>5 cell/µl) or protein concentrations (>37 mg/100 ml) in the CSF, which forms the basis of second-stage determination according to WHO criteria (WHO 1986, 1998). However, these criteria are controversial (Doua et al. 1996; Bisser et al. 1997). Trypanosome detection techniques have low sensitivity, whereas increased cell counts or total protein concentrations in CSF are not necessarily specific for trypanosomiasis (Lejon & Büscher 2001). Moreover, the latter is only rarely determined.

Recently, polymerase chain reaction (PCR) methods have been developed for HAT diagnostic purposes. When performed on blood, high sensitivity and specificity have been reported (Penchenier et al. 1996; Kabiri et al. 1999). Truc et al. (1999) reported the use of PCR on CSF (PCR/CSF) after a simple DNA purification with an anion chelating resin. They observed a good correlation between the PCR and the WHO criteria on a limited number of patients (15), with PCR being more sensitive than parasite detection by DC.

On the other hand, an elevated Immunoglobulin M (IgM) concentration in the CSF, characteristic of the meningo-encephalitic stage of HAT (Mattern 1968), can now be easily detected through a latex agglutination test (LATEX/IgM) which combines sensitivity, specificity and simplicity and which is applicable in the field (Lejon et al. 1998; Lejon et al. 2002).

The aims of this study were first to evaluate on a larger number of patients the value and the contribution of these two new tests in the improvement of the stage determination (discrimination between the first stage P1, and the second stage P2) and second, to evaluate their contribution for therapeutic decision-making. The two new tests were compared with classical parameters (parasite detection and cell count in CSF).

**Materials and methods**

**Field study and collection of samples**

During April and May 2000, active and passive surveillance were carried out in a sleeping sickness focus in Central-West Côte d’Ivoire by the National Control Programme in collaboration with Institut Pierre Richet (IPR), Projet de Recherches Cliniques sur la Trypanosomiase (PRCT) in Daloa and Base de Santé Rurale (BSR) in Bouaflé, respectively. Blood and lymph node aspirates from suspected sleeping-sickness patients were examined for the presence of trypanosomes by the miniature anion-exchange centrifugation technique (mAECT) and wet thin smear, respectively. In order to determine the stage of the disease, a lumbar puncture was performed. In each case, the CSF was examined for the presence of trypanosomes after DC (DC, Cattand et al. 1988), and the cells were counted in a Malassez counting chamber. For each patient, blood and CSF samples were taken prior to treatment, at the end of the treatment, and at 1, 3, 6 and 12 months after treatment. In case of direct evidence of trypanosomes in blood (mAECT) and/or in the CSF (DC) after treatment, the patient was considered as relapsed. Unlike WBC, PCR/CSF, LATEX/IgM 8 and LATEX/IgM 16 were not systematically performed after the beginning of the treatment and corresponding data were not included. In this study, and according to WHO recommendations (WHO 1986), patients with more than 5 cells/µl or with trypanosomes in the CSF were considered in the second stage of the disease. However, according to the prevailing recommendations in Côte d’Ivoire, ‘intermediate stage’ patients with ≤20 cells/µl and no trypanosomes in CSF were not treated with melarsoprol but with the first-stage drug, pentamidine (Doua et al. 1996).

Latex agglutination test (LATEX/IgM) titration of CSF samples was carried out as described by Lejon et al. (2002). Briefly, the lyophilized latex reagent was re-suspended in 1 ml of phosphate buffered saline (PBS, 0.01 M, pH 7.4) and two-fold serial dilutions of CSF were prepared with the same PBS buffer. Latex reagent (20 µl) was then mixed with diluted or undiluted CSF (20 µl) and spread over the white reaction zone (1.5 cm diameter) on the card. The card was rocked at 70 r.p.m. for 5 min on a horizontal rotator and examined for presence of agglutination. The end titre was expressed as the highest dilution of CSF that was positive with the agglutination test. The two cut-off end titres used in this study, that is 1:8 and 1:16 corresponding to LATEX/IgM 8 and LATEX/IgM 16 were considered positive if the CSF end titres were ≥1:8 and ≥1:16, respectively.

Cerebrospinal fluid (2 ml) remaining after WBC count, DC and LATEX/IgM titration was stored at −20 °C for subsequent PCR analysis at IPR. Trypanosome DNA detection in the CSF by PCR was carried out according to Truc et al. (1999).

**Extraction of DNA for PCR**

Briefly, an anion chelating resin was used in order to remove PCR inhibitors from the sample (Walsh et al. 1991). An amount of 500 µl of CSF was transferred to a 0.6 ml Eppendorf conical tube and centrifuged at 13 000 g for...
for 20 min. By means of a pipette, 450 μl of the supernatant was gently removed and discarded. The pellet was resuspended in the remaining liquid and vortexed for 5 min. Subsequently, 100 μl of 1% Chelex solution in sterile purified water (Chelex 100 Resin, Bio-Rad Laboratories, CA, USA) was added to each tube. The tubes were then vortexed for 1 min and incubated at 56 °C for 1 h. This was followed by a second incubation at 95 °C for 30 min. After incubation, the tubes were again centrifuged for 5 min at 13 000 g. The supernatant, now containing the DNA, was used for PCR.

Polymerase chain reaction

Polymerase chain reaction was performed according to the method described by Penchenier et al. (1996) using TBR1–2 primers (TBR1: 5’-CGA-ATG-ATT-AAA-CAA-TGC-GCA-G-3’; TBR2: 5’-AGA-ACC-ATT-TAT-TAG-CTT-TGT-TGC-3’) specific for Trypanosoma brucei sensu lato (Moser et al. 1989). The amplification conditions were as follows: initial denaturation at 94 °C for 3 min, 40 cycles with the denaturation step at 94 °C for 1 min, the annealing step at 56 °C for 1 min and the polymerization step at 72 °C for 1 min. The final elongation was at 72 °C for 5 min. Samples of 10 μl of each reaction product were run in 2% agarose gel with a 0.5 mg/ml solution of ethidium bromide before being visualized under ultraviolet light. A positive control (purified T. brucei DNA), a negative control (without DNA template) and a molecular weight marker (Marker IV, Eurogentec) were run in each PCR test. When the expected 177 bp product was visible, the PCR was considered positive.

Data analysis

The DC allows direct assessment of the presence of trypanosomes in the CSF; it was used as the reference test for the calculation of sensitivity and specificity of PCR/CSF, LATEX/IgM 8, LATEX IgM/16, and WBC count. Then, PCR/CSF, LATEX/IgM 8 and 16 were compared with combined results for DC and WBC count reflecting the current way stage determination is performed. As sample sizes were too small for the approximate confidence interval based on the normal distribution to be reliable, confidence intervals were computed based on the exact theoretical distribution of a proportion, the binomial distribution. To take into account that biological tests were carried out on the same subjects, McNemar chi-square was performed. This procedure yields a P value that quantifies the probability that the difference in the new test response is because of chance, rather than actual difference between the new and the reference test.

Results

Seventy-seven patients were diagnosed during two medical surveys in the area of Bonon, of whom 65 were treated, and subsequently included in this study. The remaining 12 patients did not come to the treatment centre. Twenty-seven other patients presenting themselves spontaneously for examination were also included in the study. For correct testing, haemorragic CSF, i.e. CSF contaminated with red blood cells detected during lumbar puncture, white blood cell count (WBC count) or after DC, was excluded from the tests.

Each of the four techniques (DC, WBC count, PCR/CSF and LATEX/IgM) could only be performed on 73 of the 92 included patients; 31 of these had WBC between 0 and 5 cells/μl and were negative in DC (Table 1). All patients of this first group were treated with pentamidine, and no relapse was observed. Eleven patients had WBC between 6 and 20 cells/μl and were negative in DC. Patients of this second group were also treated with pentamidine, and one relapse was observed 6 months after treatment. Thirty-one patients had >20 cells/μl (n = 25) or were positive in DC (n = 25). Patients of this third group were treated with

<table>
<thead>
<tr>
<th>WBC from 0 to 5 (34)</th>
<th>WBC from 6 to 20 (14)</th>
<th>WBC &gt;20 (25)</th>
<th>Total (n = 73)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>DC+</td>
<td>DC−</td>
<td>DC+</td>
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<tr>
<td>PCR+</td>
<td>2</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>PCR−</td>
<td>1</td>
<td>24</td>
<td>0</td>
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<tr>
<td>LI 16+</td>
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<td>3</td>
<td>29</td>
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<td>LI 8+</td>
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<tr>
<td>LI 8−</td>
<td>1</td>
<td>25</td>
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* The number of observed relapses in the corresponding group.
melarsoprol and two relapses were observed respectively at 2 and 6 months after treatment.

With only the presence of trypanosomes in CSF as the gold standard, PCR/CSF appeared to be the most sensitive test (0.96) with 95% confidence interval (IC95 = 0.80–0.99), whereas LATEX/IgM 16 showed the lowest sensitivity (0.76, IC95 = 0.6–0.92). A negative PCR/CSF had a significantly lower probability to occur in DC positive subjects (4%) than in DC negative ones (75%) (McNemar test = 9.31, df = 1, P < 0.01). On the contrary, the results of LATEX/IgM 16 did not depend on the presence of trypanosome in CSF (McNemar test = 0.28, df = 1, P > 0.5). Sensitivity of LATEX/IgM 8 and of WBC count were 0.88 (IC95 = 0.75–1.00) and 0.88 (IC95 = 0.69–0.97), respectively. Specificity was highest for LATEX/IgM 16 (0.83; IC95 = 0.72–0.94). The other tests presented lower specificity: 0.75 (IC95 = 0.6–0.86) for PCR/CSF; 0.71 (IC95 = 0.58–0.84) for LATEX/IgM 8 and 0.65 (IC95 = 0.49–0.78) for WBC count.

As therapeutic decision is usually based on both presence of trypanosomes and WBC count in the CSF, the results of PCR/CSF, LATEX/IgM 8 and LATEX/IgM 16 were compared, with the combined results of DC and WBC count (Table 1). Within the group of classically first-stage patients (negative in DC and <6 cells/μl in CSF) PCR/CSF was positive for seven of 31 patients. LATEX/IgM 8 and 16 were positive in, respectively, six of 31 and two of 31 patients in the same group. All these patients were treated with pentamidine and no relapses were observed. Among the 11 ‘intermediate-stage’ patients (negative in DC and 6–20 cells/μl), two were positive in both PCR/CSF and LATEX/IgM 16. A third was positive in LATEX/IgM 8 alone. The latter patient relapsed, while all other 10 ‘intermediate-stage’ patients seemed to be treated successfully with pentamidine. Among the 31 late-stage patients with ≥20 cells/μl or positive DC, PCR/CSF was negative in four cases. Within this group, LATEX/IgM 8 and 16 were negative in four and eight of the 31 tested patients, respectively.

Discussion

In most sleeping sickness cases, the presence of trypanosomes is associated with increased WBC numbers in the CSF thus leaving no doubt about disease stage and appropriate treatment. However, in those patients where elevated cell count is not confirmed by detection of the parasite and vice versa, correct stage determination and consequent treatment is not guaranteed. Mechanisms leading to discordance between cell count and trypanosome presence may be of different origins (e.g. low sensitivity of trypanosome detection techniques, limited accuracy of cell counting techniques, very recent CNS invasion by the parasite, low virulent parasite strains not inducing an intrathecal immune response, CNS inflammation without actual trypanosome invasion, CNS inflammation caused by other infections).

Recently, two new techniques have been developed for assessment of trypanosome presence in CSF (Truc et al. 1999) and for assessment of CNS inflammation (Lejon et al. 2002). Their value for stage determination and therapeutic decision making in HAT were evaluated here. When compared with DC, the PCR/CSF appeared the most sensitive test which is in agreement with results from other studies (Truc et al. 1999; Kyambadde et al. 2000). Its specificity seems rather low when compared with DC, which most probably is because of the higher analytical sensitivity of PCR/CSF. On the other hand, false-positive PCR/CSF results cannot be totally excluded, as PCR/CSF does not necessarily detect living trypanosomes in CSF, but rather their DNA. False-positive results might be because of low primer specificity (Solano et al. 2002), or the presence of trypanosome DNA in the CSF without actual presence of the parasite. The latter possibility has been postulated to explain false positivity of PCR performed on blood samples where trypanosome DNA can originate from tsetse bites contaminated with non-human pathogenic trypanosomes (Truc et al. 1998; Garcia et al. 2000). Such phenomenon seems, however, less evident to explain false positivity in CSF. Here, circulating trypanosome DNA may leak from the blood through the blood–brain barrier or alternatively, originate from non-surviving parasites as a consequence of the suboptimal CSF survival environment as already demonstrated by Pentreath and Owolabi (1992).

For IgM detection in CSF by LATEX/IgM, cut-off values of 1:16 and 1:8 were tested. Whatever the cut-off used, some DC-positive samples remained LATEX/IgM negative [24% (six of 25) for LATEX IgM 16 and 12% (three of 25) for LATEX IgM 8] which would lead to some second-stage patients treated with pentamidine thus putting them at risk for relapse when only the result in LATEX/IgM is taken into account for stage determination. The combination DC positive and LATEX/IgM negative might indicate that the presence of trypanosomes in the CSF does not necessarily lead to a host CNS inflammatory reaction, reflected by abnormal number of cells or IgM concentrations in the CSF.

It appears here that the two tests (PCR/CSF and LATEX IgM) have different qualities. The PCR/CSF appears to be a very sensitive test for detection of trypanosome DNA in the CSF. If therapeutic decision in this study had been based on the PCR/CSF result, seven of 31 (23%) of patients with negative DC and with <5 cells/μl would have been treated...
unnecessarily with melarsoprol. Indeed, none of them relapsed after treatment with pentamidine, from which it is known that low amounts cross the blood–brain barrier (Bronner et al. 1991). Moreover, it has been reported that some patients with trypanosomiasis in their CSF were successfully treated with pentamidine (Doua et al. 1996). If therapeutic decisions in this study had been based on LATEX/IgM applying a cut-off of, respectively, 1:16 and 1:8, two of 31 (6.5%) and six of 31 (19%) of patients with negative DC and with <5 cells/μl would have been treated with melarsoprol. For therapeutic decision, these results rather suggest to use LATEX/IgM at 1:16 cut off, combined with WBC count to avoid unnecessary melarsoprol treatment. On the other hand, LATEX/IgM 8 was the only new test which was positive in the relapsing ‘intermediate stage’ patient who had been treated with pentamidine. If therapeutic decision had been based on LATEX/IgM 8 positivity, this patient would have been treated with melarsoprol.

Hence, combining the results of this study with those of previous studies (Bronner et al. 1991; Miezan et al. 1994; Doua et al. 1996), it appears to be increasingly important to make a distinction between stage determination and therapeutic decision: the former should not always condition the latter, especially in view of the severe side-effects of melarsoprol which are not necessarily related to the presence of the trypanosome in the CNS (Blum et al. 2001). Some authors have suggested that immunological reactions within the CNS, against either immune complexes or antigens, could be also involved in such reactions independently of the presence of trypanosome (Pepin & Milord 1994; Bouteille et al. 1998).

In conclusion, this study confirms the complexity of stage determination in sleeping sickness. Sensitive tools to detect the trypanosome within the CSF, such as DC and PCR are not appropriate for making therapeutic decisions in all cases, neither are sensitive tools for assessment of CNS inflammation, such as WBC count and LATEX/IgM.

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