

Human African trypanosomiasis: Quantitative and qualitative assessment of intrathecal immune response.

Veerle Lejon^{1*}, Christian J.M. Sindic², Marie-Paule Van Antwerpen², Felix Doua³, Norbert Djé³, Philippe Solano⁴, Vincent Jamonneau⁴, Ingrid Wouters¹, Philippe Büscher¹

1 Institute of Tropical Medicine, Department of Parasitology, Nationalestraat 155, B-2000 Antwerpen, Belgium.

2 Université Catholique de Louvain, Laboratoire de Neurochimie, 53 Avenue Mounier, B-1200 Brussels, Belgium

3 Projet de Recherches Cliniques sur la Trypanosomiase (PRCT), BP 1425, Daloa, Côte d'Ivoire

4 Institut Pierre Richet (IPR), BP 1500, Bouaké, Côte d'Ivoire

* Corresponding author: Mailing address: Institute of Tropical Medicine, Department of Parasitology, Nationalestraat 155, B-2000 Antwerpen, Belgium. Phone: +32 3 247 63 69, Fax: +32 3 247 63 73, Email: vlejon@itg.be

Running title: Intrathecal immunoglobulin in African trypanosomiasis

Key words: intrathecal humoral immune response, *T.b. gambiense*, human African trypanosomiasis, intrathecal fraction, antibody index, oligoclonal antibody, IgG, IgM.

Funding sources: This work was supported by the Belgian Directorate-General for International Co-operation.

Abstract

Quantitative and qualitative techniques for assessment of the intrathecal humoral immune response in human African trypanosomiasis were compared, and their diagnostic potential for detection of the meningo-encephalitic stage of the disease was evaluated. Total and trypanosome specific IgG and IgM intrathecal synthesis were studied in paired CSF and blood samples of 38 trypanosomiasis patients and in 3 controls using Reiber's formulae. The presence of CSF-specific oligoclonal IgG and of trypanosome specific antibodies was determined using iso-electric focusing followed by immunoblotting and antigen-driven immunoblots. The intrathecal IgG fraction (16% positive) and oligoclonal IgG detection (24% positive) were insensitive for detection of an intrathecal humoral immune response. Trypanosome specific IgG synthesis, reflected by the IgG antibody index (26% positive), was confirmed by the presence of oligoclonal specific IgG (47% positive), but the latter was more sensitive. Although the detection technique failed for oligoclonal IgM, the intrathecal IgM fraction (42% positive) and the IgM antibody index (32% positive) indicated that the meningo-encephalitic stage of the disease is characterised by a dominant intrathecal IgM response, which was higher than the IgG response. The highest combination of diagnostic sensitivity and specificity for the meningo-encephalitic stage of trypanosomiasis was observed for quantitative IgM determinations.

Introduction

Infection with the protozoan parasite *Trypanosoma brucei* (*T.b.*) *gambiense* causes human African trypanosomiasis or sleeping sickness. This lethal disease occurs in sub-Saharan Africa between latitudes 14°N and 29°S, west of the African rift, and is transmitted through the bites of infected tsetse flies (principally *Glossina palpalis*). After their sub-dermal inoculation by the tsetse fly, parasites start to disseminate and the infection passes through two subsequent disease stages. The hemo-lymphatic or first stage of the disease is characterised by parasite spread and proliferation in lymph and blood, and is followed by the meningo-encephalitic stage, also called late or second stage, which implies progressive central nervous system (CNS) involvement and is conventionally defined by the presence of trypanosomes in the CNS (Dumas & Girard, 1978).

The distinction between the two disease stages is an essential step to select the appropriate treatment with minimal risk for the patient. Pentamidine, a relatively save drug used for first stage treatment has limited efficiency in the second stage since it does not sufficiently cross the blood-brain barrier. Melarsoprol, the drug commonly used for second stage treatment is highly toxic (Pépin & Milord, 1994; Van Nieuwenhove, 1999).

Since there are no exclusive clinical signs, nor any clear changes at blood level, indicating the evolution from the hemo-lymphatic to the meningo-encephalitic stage (Bisser *et al.*, 1997), stage determination is performed by examination of the cerebrospinal fluid (CSF). Following the WHO recommendations for stage determination and follow-up (WHO, 1998), the CSF has to be examined for 1°) white cell count; 2°) the presence of trypanosomes and 3°) total protein concentration. If at least one of these parameters is abnormal, a patient is considered in meningo-encephalitic stage and should be treated accordingly. Only the CSF white cell count is widely used for stage determination. The upper limit for normal and the cut-off value for hemo-lymphatic stage have been set at 5 cells/ μ l in CSF (WHO, 1998). This cut-off has been raised to 20 cells/ μ l in some countries, including Côte d'Ivoire and Angola (Doua *et al.*, 1996; Stanghellini & Josenando, 2001). The finding of trypanosomes in CSF classifies a patient into the meningo-encephalitic stage but the techniques for trypanosome detection have limited sensitivity and the meaning of finding trypanosomes in CSF with low cell counts is questioned. Protein quantification on CSF is not current

practice in sleeping sickness control centres due to the need of more or less sophisticated material and the instability of the reagents. Moreover, cut-offs have been set arbitrarily and vary with the method used.

In view of the importance of accurate stage determination in the choice of treatment, alternative markers for diagnosis of CNS involvement are investigated.

Subacute and chronic inflammatory diseases of the CNS are associated with local synthesis of immunoglobulins and antibodies. The demonstration of intrathecal synthesis represents a powerful tool for diagnosis of neuro-inflammatory diseases and requires an accurate discrimination between blood and CNS derived immunoglobulins, either quantitative by calculation of the intrathecal fraction and antibody index, or qualitative by detection of oligoclonal antibodies (Sindic *et al.*, 2001; Reiber & Peter, 2001).

In human African trypanosomiasis, intrathecal total IgG synthesis has been demonstrated using Tourtellotte's formula (Lambert *et al.*, 1981). The quantification of trypanosome specific antibodies by semi-quantitative ELISA allowed calculation of the antibody index (Lejon *et al.*, 1998). Furthermore, the detection of total as well as trypanosome specific IgG and IgM synthesis, using Reiber's formulae, already pointed to the limitations of the traditional parameters for determination of neurological involvement in human African trypanosomiasis (Bisser *et al.*, 2002).

As far as we know, the detection of total IgG and IgM, as well as trypanosome specific IgG and IgM antibodies, by both quantitative and qualitative techniques, has never been performed on the same group of trypanosomiasis patients. Our purpose was 1°) to compare the sensitivity of the intrathecal immunoglobulin fraction, the trypanosome specific antibody index and the detection of oligoclonal immunoglobulins and trypanosome specific antibody for detection of an intrathecal humoral immune response in 38 human African trypanosomiasis patients and 2°) to evaluate the diagnostic sensitivity and specificity of these techniques for stage determination.

Materials and methods

Patients

The patients were identified during a medical survey in April-May 2000, around the town of Bonon in Central West Côte d'Ivoire. Out of 13,900 people screened by CATT (Card agglutination test for trypanosomiasis, (Magnus *et al.*, 1978), 170 serological suspects were parasitologically examined by the mini-anion exchange centrifugation technique (Lumsden *et al.*, 1979) and by direct examination of lymph node aspirate. Trypanosomes were detected in 76 persons which were sent to the "Projet de Recherches Cliniques sur la Trypanosomiase" (PRCT) in Daloa for treatment. At the PRCT, infection was confirmed using the same techniques and CSF was collected for determination of the disease stage. In CSF, the cell number was determined in a KOVA counting chamber (ICL), protein concentration by the Coomassie brilliant blue method and presence of trypanosomes by the modified single centrifugation (Miézan *et al.*, 2000). Remaining serum and CSF was immediately frozen at -30°C . For this study, samples from the first examined 38 patients were used, after exclusion of patients with haemorrhagic CSF. Sixteen patients displayed trypanosomes in the CSF or more than 20 cells/ μl , and were treated with Melarsoprol, the other 22 with Pentamidine following the treatment protocols of the PRCT. The mean age of the patients was 35 years (range 4-64, standard deviation 15), and male/female ratio was 0.5. All underwent a standard clinical and neurological examination before treatment, focusing on signs of CNS invasion in sleeping sickness (Boa *et al.*, 1988). Informed consent was obtained from all patients in the study. The follow-up period (last control visit after treatment) was 24 months (complete follow-up) or more for 5 patients, 17-20 months for 9 patients, 13-14 months for 6 patients, 6-7 months for 3 patients, 2-3 months for 4 patients and 1 month for 9 patients. One patient was lost to follow-up and one patient died during treatment. At these control visits, blood and CSF were examined as described above. During the follow-up period, one patient relapsed (presence of trypanosomes in CSF) 3 months after treatment and died during retreatment, another relapsed 6 months after treatment (Table 1).

Besides patients with confirmed infection, serum and CSF of one CATT seropositive "suspect" and two CATT seronegative persons were available from the PRCT. They served as parasitologically negative controls.

Determination of albumin, IgG and IgM concentrations in serum and CSF

Concentrations of albumin and total IgG were measured by nephelometry (Beckman Array). The total IgM concentration was determined by nephelometry (BN Prospec, Dade Behring), using the N Latex IgM reagent (Dade Behring) for CSF.

Demonstration of trypanosome specific IgG and IgM by ELISA

Trypanosome specific IgG (IgGsp) and IgM (IgMsp) in serum and CSF were detected by a semi-quantitative ELISA (Lejon *et al.*, 1998), which was slightly modified. Briefly, microplates were coated with a mixture of purified variable surface glycoproteins of *T. b. gambiense* LiTat 1.3, LiTat 1.5 and LiTat 1.6 each at a concentration of 0.66 µg/ml, giving a total protein concentration of 2 µg/ml in PBS. Antigen free control wells received PBS. Plates were blocked, treated with a sucrose solution and stored at -70°C until use. For testing, antigen containing and antigen free wells were filled with twofold serial dilutions of serum and CSF, respectively 1:19200 -1:76800 and 1:80-1:320 for IgG determinations, or 1:1200-1:4800 and 1:10-1:40 for IgM determinations. A standard, consisting of a serum with a concentration of 100 arbitrary units in 9 twofold serial dilutions (same starting dilution as the test serum), was run in duplicate on each plate. Plates were shaken for 30 minutes. After 3 washes, the plates were incubated with rabbit-anti-human IgG or IgM (CLB, Amsterdam, The Netherlands) for 1 hour, followed by goat-anti-rabbit IgG (H+L)-peroxidase (Nordic) for 30 minutes. After 5 washes, the ABTS substrate-chromogen solution was incubated for 1 hour. The plate was shaken for 10 seconds and the optical density (OD) read at 415 nm (Multiskan RC Version 6.0, Labsystems). Corrected OD values were obtained by subtracting the OD of the antigen-free control well from the OD of the corresponding antigen containing well. Trypanosome-specific IgGsp and IgMsp antibody concentrations (in arbitrary units) in serum and CSF were interpolated from the standard curve using the 4-parameter algorithm provided with the software (Genesis Lite) of the reader, and their CSF/serum quotient was determined (Q_{IgGsp} and Q_{IgMsp}).

Determination of the Intrathecal Fraction (IF_{IgG} , IF_{IgM}) and Antibody Indices (AI_{IgGsp} , AI_{IgMsp})

For each CSF/serum pair the maximum IgG and IgM quotient (Q_{lim}) in absence of intrathecal immunoglobulin synthesis was calculated (Reiber & Peter, 2001) using the formula $Q_{lim} = (a/b) \times (Q_{alb}^2 + b^2)^{1/2} - c$ (with $Q_{alb} = \text{CSF albumin} / \text{serum albumin}$, with for Q_{limIgG} $a/b = 0.93$, $b^2 = 6 \times 10^{-6}$, $c = 1.7 \times 10^{-3}$ and with for

Q_{limIgM} $a/b = 0.67$, $b^2 = 120 \times 10^{-6}$, $c = 7.1 \times 10^{-3}$). The intrathecally synthesised fraction of IgG or IgM (the proportion of locally produced Ig as % of the total IgG or IgM concentration in CSF) was calculated as $IF_{IgG} = (1 - Q_{limIgG}/Q_{IgG}) \times 100$ and $IF_{IgM} = (1 - Q_{limIgM}/Q_{IgM}) \times 100$ respectively ($Q_{IgG} = \text{CSF IgG} / \text{serum IgG}$, $Q_{IgM} = \text{CSF IgM} / \text{serum IgM}$).

The antibody index for IgGsp was determined as $AI_{IgGsp} = Q_{IgGsp}/Q_{IgG}$ when $Q_{IgG} < Q_{limIgG}$, or $AI_{IgGsp} = Q_{IgGsp}/Q_{limIgG}$ taking into account the local synthesis of immunoglobulins when $Q_{IgG} > Q_{limIgG}$. For IgMsp the same formulae apply, using the respective IgM concentrations. The AI was considered negative (-) if < 1.5 and positive (+) if ≥ 1.5 (Reiber & Peter, 2001).

IgG immunoblots (OC_{IgG})

Detection of CSF-specific oligoclonal IgG bands was performed by iso-electric focusing in agarose gels, followed by immunoblotting onto a polyvinylidene difluoride (PVDF) sheet and IgG immunostaining (Sindic *et al.*, 2001).

Antigen-driven immunoblots for trypanosome specific IgG antibodies (OC_{IgGsp})

Within the same iso-electric focusing run for detecting the total IgG pattern, 10 μl of both undiluted CSF and the corresponding serum diluted to the same IgG concentration were applied to the gel. These were transferred onto a PVDF sheet previously coated overnight by a mixture of purified variable surface glycoproteins of *T.b.gambiense* LiTat 1.3, LiTat 1.5 and LiTat 1.6 each at a concentration of 3.3 $\mu\text{g}/\text{ml}$, giving a total protein concentration of 10 $\mu\text{g}/\text{ml}$ in TBS. The antigen-driven immunoblots and the staining were performed as for IgG immunoblots. The detection of anti-trypanosome oligoclonal IgG antibodies either restricted to the CSF or showing a different pattern in the CSF when compared with the serum, was considered as proof of intrathecal antibody synthesis. In contrast, absence of CSF immunoreactivity, or the occurrence of a similar pattern in both CSF and serum ("mirror pattern") was considered as absence of intrathecal anti-trypanosomes antibody synthesis. Reading of the immunoblots was made without knowledge of the AI results.

Antigen-driven immunoblots for trypanosome specific IgM antibodies

Agarose gel plates were prepared as described above but contained 1 ml ampholine pH 3.5-9.5 and 1 ml ampholine pH 4-6 (Pharmalytes, Pharmacia). CSF and sera were thawed at 37°C for 15 min and vortexed. The undiluted CSF and the corresponding serum were diluted to the same IgM concentration. Five µl of dithiothreitol (DTT, Calbiochem) at a concentration of 0.4 mM in distilled water were added to each 45 µl of sample. After an incubation of 30 min at 37°C, 15 µl paired samples were applied onto the middle of the gel, side by side, and iso-electrically focused for 65 min at 10°C. The gel was blotted onto a nitro-cellulose sheet (BA 85, 0.45µm, Ref 401180 Schleicher Schuell) that had been coated overnight with a mixture of purified variable surface glycoproteins of *T.b.gambiense* LiTat 1.3, LiTat 1.5 and LiTat 1.6, giving a total protein concentration of 25 µg/ml in TBS. The antigen-driven immunoblot was performed under a uniform weight of 1 kg/300cm² for 40 min at 10°C. The immunoblot was then dipped in PBS containing 5% w/vol defatted milk powder and washed in PBS-Tween 0.1% before incubation for 60 min at 20°C with peroxidase-conjugated rabbit anti-human IgM antiserum diluted 400 times in PBS-Tween 0.1%. After five 15-min washings with PBS-Tween, the immunoblot was revealed with Enhanced Chemi Luminescence (ECL kit, RPN 2109, Amersham Pharmacia Biotech) as described by the manufacturer. Exposure time was generally between 30 and 90 seconds.

Statistics

For the 38 trypanosomiasis patients, the number of positive results obtained by the different techniques were compared by Mc Nemar Chi square test with continuity correction, after construction of 2x2 contingency tables. 95 % symmetrical confidence intervals of diagnostic sensitivity and specificity were calculated using JavaStat.

Results

Presence of intrathecal humoral immune response.

Presence of an intrathecal humoral immune response, defined as a detectable pathological value for IF_{IgG} , IF_{IgM} , AI_{IgGsp} or AI_{IgMsp} or, the presence of oligoclonal IgG (OC_{IgG}) or oligoclonal trypanosome specific IgG (OC_{IgGsp}) was observed in 66% of all patients. The sensitivity for detection of a humoral immune response was $OC_{IgGsp} > IF_{IgM} > AI_{IgMsp} > AI_{IgGsp} > OC_{IgG} > IF_{IgG}$. We failed to detect oligoclonal total IgM or oligoclonal trypanosome specific IgM, and these parameters were therefore not useful to detect an intrathecal immune response (see below). The most frequently observed neurological signs were the presence of primitive reflexes (suck, rooting and palmo-mental reflexes, 29%), daytime hypersomnia (21%) and psychiatric problems (18%). These signs were present in 47% of all patients (18/38) and 15 of them had an intrathecal immune response. Details for all 38 *T.b. gambiense* patients are shown in table 1.

Quantitative detection of intrathecal total IgG (IF_{IgG}) and intrathecal total IgM synthesis (IF_{IgM}) was positive in respectively 16% and 42% of all patients. Intrathecal IgM synthesis was observed in significantly more patients ($p=0.004$) than intrathecal IgG synthesis. All IF_{IgG} positive patients were IF_{IgM} positive. In patients with intrathecal synthesis, the IF_{IgG} ranged between 2.7 and 69 % (median 34%), the IF_{IgM} ranged between 7.5 and 98% (median 80%). In all cases, the intrathecal fraction for IgM was higher than for IgG ($IF_{IgM} > IF_{IgG}$). All 3 controls had no intrathecal IgG or IgM synthesis.

Intrathecal trypanosome specific antibody synthesis was observed in 26% of patients for antibodies of the IgG isotype (pathological AI_{IgGsp}) and 32% of patients for antibodies of the IgM isotype (pathological AI_{IgMsp}). The number of positive results with AI_{IgGsp} or AI_{IgMsp} was not significantly different ($p=0.62$). Among all 13 patients with pathological AI_{IgGsp} or AI_{IgMsp} values, the AI_{IgMsp} was higher than AI_{IgGsp} in 12/13 patients (1/13 patient was AI_{IgGsp} positive and AI_{IgMsp} negative). For all 3 controls, AI_{IgGsp} and AI_{IgMsp} were negative.

When the detection of total immunoglobulin synthesis was compared to specific antibody synthesis, no significant differences between the numbers of positive results with IF_{IgG} and AI_{IgGsp} ($p=0.22$), nor between IF_{IgM} and AI_{IgMsp} ($p=0.13$) were observed. All AI_{IgMsp} positive samples were however IF_{IgM} positive.

Qualitative examination of the CSF revealed the presence of oligoclonal total IgG (OC_{IgG}) in 24% of patients and oligoclonal trypanosome specific IgG antibody (OC_{IgGsp} , Fig. 1) in 47% of patients. The number of

positive results with OC_{IgGsp} was significantly higher ($p=0.052$) than with OC_{IgG} . All 3 controls were OC_{IgG} and OC_{IgGsp} negative.

No significant differences in the number of positive samples with IF_{IgG} and AI_{IgGsp} versus OC_{IgG} was observed ($p= 0.45$ and 1 respectively). All cases with positive IF_{IgG} or positive AI_{IgGsp} however displayed oligoclonal trypanosome specific IgG antibodies (OC_{IgGsp} positive), and the latter was detected in significantly more patients than positive IF_{IgG} or positive AI_{IgGsp} ($p=0.001$ and 0.013 respectively).

No attempt was made in the present study to detect oligoclonal IgM. In a previous study on 9 paired samples of trypanosomiasis patients (who all had > 20 cells/ μ l in CSF and positive AI_{IgMsp}), we failed to observe oligoclonal IgM bands restricted to the CSF. Only a diffuse polyclonal staining was present, similar to the one observed in the corresponding serum.

We however looked for trypanosome specific oligoclonal IgM antibodies in all the 38 samples of this series. A positive immunostaining was observed in 10/38 CSF samples, all of them having a high IF_{IgM} ($>67\%$), positive AI_{IgMsp} (>2.7) and a CSF IgM concentration higher than 50 mg/l. No oligoclonal bands were detectable, but a polyclonal background was observed similar to the one in the corresponding serum.

Intrathecal humoral immune response versus stage determination and treatment.

For evaluation of the diagnostic sensitivity and specificity of the different techniques for the meningo-encephalitic stage of the disease (table 2), we assumed 1°) that all patients with more than 20 cells/ μ l in the CSF were late stage patients; 2°) that all patients with trypanosomes in the CSF were late stage patients; 3°) that patients with less than 20 cells and without trypanosomes in the CSF, which were cured by Pentamidine were in first stage and 4°) that patients with less than 20 cells and without trypanosomes in the CSF but which relapsed after Pentamidine treatment were already in late stage prior to treatment.

The detection of intrathecal total IgG synthesis by IF_{IgG} or OC_{IgG} , or trypanosome specific antibody synthesis by AI_{IgGsp} had a sensitivity of respectively 35.3, 29.4 and 52.9% for the meningo-encephalitic stage. A positive IF_{IgG} however had 100% specificity. Detection of trypanosome specific IgG by OC_{IgGsp} resulted in a maximal diagnostic sensitivity of 76.5%. The superior sensitivity of OC_{IgGsp} for detection of an intrathecal immune response however resulted in the lowest diagnostic specificity of 76.2%. The highest combination of diagnostic sensitivity and specificity was observed for the quantitative IgM detecting techniques, with a maximal sensitivity of 76.5% for IF_{IgM} and a 100% specificity of AI_{IgM} . Sensitivities of presence of

neurological signs for the meningo-encephalitic stage were 58.8% for primitive reflexes, 35.3% for daytime hypersomnia and 23.5% for psychiatric problems.

Discussion

Our purpose was to compare the intrathecal fraction, the antibody index and the oligoclonal immunoglobulin and trypanosome specific antibody production for the assessment of the intrathecal humoral immune response in patients infected with *T.b. gambiense* and for stage determination.

Overall, our *quantitative* and clinical findings confirm those of Bisser *et al.* on a group of *T.b. gambiense* patients from Peoples Republic of Congo (Bisser *et al.*, 2002). Using Reiber's formula (Reiber & Peter, 2001), in both studies, a strong, dominant intrathecal IgM synthesis was observed, combined with a weaker IgG synthesis. In patients with a pathological value for the trypanosome specific antibody index, $AI_{IgM_{sp}}$ was higher than $AI_{IgG_{sp}}$. Human African trypanosomiasis due to *T.b. gambiense* thus can be classified among neuro-inflammatory diseases with a dominant IgM immune response pattern in the CNS, like Lyme neuroborreliosis, neurosyphilis or mumps meningoencephalitis (Reiber & Peter, 2001). Abnormal primitive reflexes, sleeping disturbances or psychiatric problems were mainly detected in patients with an intrathecal immune response, but presence of such clinical signs was insufficiently sensitive to diagnose the meningo-encephalitic disease stage.

By iso-electric focusing, oligoclonal total IgG (OC_{IgG}) was detected in 24% of the trypanosomiasis patients. Together with IF_{IgG} , it was the less sensitive technique to detect an intrathecal humoral immune response. This explains the observation of Bisser *et al.*, who observed oligoclonal IgG only in 1.8% of a subgroup of patients without blood-CSF barrier dysfunction, without intrathecal IgG synthesis and without intrathecal IgM synthesis (Bisser *et al.*, 2002). The absence of oligoclonal bands in CSF was also reported for a *T.b. gambiense* patient in meningo-encephalitic stage, who was diagnosed and treated in Canada (Sahlas *et al.*, 2002).

In contrast, oligoclonal trypanosome specific IgG antibodies ($OC_{IgG_{sp}}$) were observed in 47% of the trypanosomiasis patients. All cases with elevated $AI_{IgG_{sp}}$ displayed oligoclonal trypanosome specific IgG but the latter was detected in significantly more patients than IF_{IgG} , OC_{IgG} and $AI_{IgG_{sp}}$. Presence of oligoclonal antibodies, without presence of corresponding oligoclonal IgG, is frequently observed in CNS infections (Sindic *et al.*, 2001, figures 4 and 5). Some $AI_{IgG_{sp}}$ negative samples were $OC_{IgG_{sp}}$ positive, which confirms the higher sensitivity of iso-electric focusing for detecting local synthesis in some infectious diseases (Monteyne *et al.*, 1997).

Iso-electric focusing results for detection of IgM were rather disappointing. Since we failed to observe oligoclonal IgM in a pilot study, no attempt was made in the present study to detect oligoclonal IgM. We therefore decided to look for trypanosome specific oligoclonal IgM antibodies by the more sensitive ECL technique. No bands were detectable, but in samples with a highly elevated IgM concentration, a polyclonal background was observed similar to the one in the corresponding serum. The failure to detect oligoclonal IgM antibodies could be due to the induction of a polyclonal IgM response by trypanosomes, with other specificities including auto-antibodies (Greenwood & Whittle, 1980), or low affinities for the trypanosomal antigens used. This is suggested by the occurrence of 4 positive IF_{IgM} without positive AI_{IgMsp} , whereas all positive AI_{IgMsp} displayed a positive IF_{IgM} . The diffuse "polyclonal" pattern observed on immunoblots could also be due to post-translational modifications. Different glycosylations could specifically be responsible for a microheterogeneity that we did not detect by the present techniques. Similarly, detection of oligoclonal IgA bands and antibodies in most neuro-inflammatory diseases failed (Sindic *et al.*, 1994b) and even if oligoclonal IgM in some cases of neuroborreliosis and neurosyphilis were detected (Sindic *et al.*, 1994a), the antigen-driven immunoblots remained negative for specific IgM antibodies (Sindic, unpublished data). For stage determination of human African trypanosomiasis IF_{IgG} , OC_{IgG} and AI_{IgGsp} had insufficient diagnostic sensitivity, whereas detection of trypanosome specific IgG by OC_{IgGsp} was 76.5 % sensitive. The quantitative IgM detecting techniques offered the best combination of sensitivity and specificity. Among the different techniques used in this study, the highest diagnostic sensitivity for second stage trypanosomiasis was only 76.5%. Patients with CSF trypanosomes were considered in meningo-encephalitic stage, even if cell counts were lower than 20/ μ l. Two out of four of such patients remained negative in all applied tests, thus showed no sign of an intrathecal inflammatory process. One could hypothesise that, based on the presence of trypanosomes in CSF, these two were falsely classified in meningo-encephalitic stage and treated with Melarsoprol. They might have been in the hemo-lymphatic disease stage and might have been cured with Pentamidine. This would be in agreement with reports on successful Pentamidine treatment of patients with CSF trypanosomes and cell counts lower than 20/ μ l (Doua *et al.*, 1996), Legros, personal communication) and with observations on the absence of intrathecal synthesis in a larger group of patients (Lejon *et al.*, 2003).

On the other hand, 11 first stage patients (less than 20 cells/ μ l, no trypanosomes in CSF and cured with Pentamidine) showed an intrathecal humoral immune response by one or more of the applied tests. Four of

them displayed OC_{IgG} without positive AI_{IgGsp} or positive OC_{IgGsp} . The observed CSF-restricted oligoclonal IgG could therefore be unrelated to trypanosomiasis and indicate a concomitant neuro-inflammatory disease, although we have no evidence in this direction. (all patients were HIV negative). More likely, they are due to an earlier CNS infection, e.g. cerebral malaria. Malaria is endemic in Côte d'Ivoire, and corresponding oligoclonal bands may persist for years as an immunological "scar" (Chapel *et al.*, 1987). In contrast, the 4 patients with OC_{IgGsp} , the 2 patients with high IF_{IgM} and the one with both abnormalities could be considered as late stage patients, and misclassified according to the current recommendations for stage determination. They should be considered at high risk of relapse. Perhaps they were still curable with Pentamidine which has been detected in low concentrations in CSF of sleeping sickness patients (Bronner *et al.*, 1991). A less likely explanation is that this persistent humoral response is the witness of a spontaneously healed second stage infection, as has been observed in neuroborreliosis (Sindic *et al.*, 1987) and neurosyphilis (Merritt, 1940). These results could also indicate that OC_{IgGsp} detection is a rather unspecific method for stage determination in sleeping sickness. None of the 4 OC_{IgGsp} positive first stage patients, which had no other signs indicating an intrathecal immune response and were treated with Pentamidine, relapsed within the – relatively long- follow-up time. Methodological problems with OC_{IgGsp} detection are unlikely (e.g. cross-reaction with IgM antibodies), since in these CSF samples relatively normal IgM concentrations and low trypanosome specific IgM levels were present.

In conclusion, we demonstrated here that the sensitivity of IF_{IgG} and OC_{IgG} for detection of neuro-inflammation in sleeping sickness patients is limited. Trypanosome specific antibody synthesis assessed by AI_{IgGsp} was confirmed by OC_{IgGsp} detection, but the latter method was more sensitive. Although we failed to detect oligoclonal IgM, it could be demonstrated by IF_{IgM} and AI_{IgMsp} that the meningo-encephalitic stage of *T.b. gambiense* human African trypanosomiasis is characterised by a dominant intrathecal IgM immune response. This confirms previous results on the importance of detection of intrathecal IgM synthesis for stage determination in trypanosomiasis (Bisser *et al.*, 2002, Lejon *et al.*, 2003).

Acknowledgements

We gratefully acknowledge the mobile team of IPR and the technicians and nurses of PRCT for their dedication to the correct diagnosis and treatment of trypanosomiasis patients.

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Table 1: Cell count (cells/ μ l), presence of trypanosomes (CSF tryp), presence of intrathecal humoral immune response, neurological signs and follow-up period (F.U., in months) in 38 *T.b. gambiense* patients. The intrathecal immune response was detected by 6 different techniques: intrathecal total IgG (IF_{IgG}), intrathecal total IgM (IF_{IgM}), antibody index trypanosome specific IgG (AI_{IgGsp}), antibody index trypanosome specific IgM (AI_{IgMsp}), oligoclonal total IgG (OC_{IgG}), oligoclonal trypanosome specific IgG (OC_{IgGsp}). Major neurological signs were primitive reflexes (Prim reflex), daytime hypersomnia (Sleep) and psychiatric problems (Psych). Patients above the dotted line were treated with Pentamidine, patients below this line were treated with Melarsoprol.

ID	Cells/ μ l	CSF tryps	IF IgG %	IF IgM %	AI IgGsp	AI IgMsp	OC IgG	OC IgGsp	Prim reflex	Sleep	Psych	F.U.
2716	0	-	0	0	0.8	n.d.	-	-	-	-	-	13
2733	0	-	0	0	1	0.8	-	-	-	-	-	26
2737	0	-	0	0	0.7	n.d.	-	-	-	-	-	17
2732	0	-	0	8	0.8	1.1	-	-	-	-	-	3
2741	0	-	0	28	0.7	0.9	-	-	-	-	+	2
2722	0	-	0	0	0.7	n.d.	-	+	-	+	-	18
2708	0	-	0	0	1.4	n.d.	+	-	-	-	-	0
2714	2	-	0	0	0.8	0.7	-	-	-	-	-	3
2709	2	-	0	0	1.2	0.9	-	+	+	-	-	13
2739	2	-	0	0	1.1	0.4	+	-	-	-	-	1
2700	3	-	0	0	1.2	n.d.	-	+	-	-	-	7
2747	3	-	0	0	0.5	n.d.	+	-	-	-	-	1
2718	4	-	0	0	0.7	1.4	-	-	-	-	-	27
2699	4	-	0	0	0.8	0.4	+	-	-	-	-	1
2746	4	-	0	8	2.1	1.4	-	+	-	-	-	1
2712	5	-	0	0	1.0	1.2	-	+	-	-	-	13
2734	6	-	0	0	1.1	1.1	-	-	-	-	-	1

2697	8	-	0	0	0.7	0.8	-	-	-	-	-	14
2702	9	-	0	0	0.7	n.d.	-	-	-	-	+	18
2738	9	-	0	0	0.9	0.5	-	-	-	-	-	1
2729*	10	-	3	83	0.9	22	+	+	-	+	-	13
2710	14	-	0	0	0.2	n.d.	-	-	-	-	+	18
2724	2	+	0	33	1.2	1.6	-	+	+	-	-	24
2706	3	+	0	0	0.7	0.4	-	-	+	-	-	13
2730	10	+	0	78	1.6	2.7	-	+	-	-	-	1
2731	15	+	0	0	1.2	1.0	-	-	-	-	-	25
2727	28	+	0	46	1.0	1.4	-	-	+	-	-	18
2721	30	-	0	0	1.0	0.4	-	-	-	-	-	18
2736	48	+	0	0	1.3	n.d.	-	+	+	-	-	1
2698	64	+	50	87	6.9	30	+	+	-	+	+	18
2713*= 2719	69	+	0	82	2.8	10.8	-	+	+	+	-	3
2719	153	+	51	97	10.6	56	-	+	+	-	-	1
2728	224	+	0	65	1.1	3.7	-	+	+	-	+	18
2735	230	+	0	86	3.4	15	+	+	-	+	-	6
2743= 2703	289	+	69	98	7.4	69	+	+	+	+	-	n.a.
2703	460	+	0	67	3.1	9.2	-	+	+	+	+	24
2745	560	+	3	86	1.7	3.2	-	+	-	-	-	20
2740	1430	+	18	98	5.9	29	+	+	+	+	+	7
Total +		15	6	16	10	12	9	18	11	8	7	

*relapsed after 6 months, *= relapsed after 3 months, died during retreatment, = died during treatment, n.a.

not applicable, nd not detectable (trypanosome specific IgM concentration below detection limit of ELISA)

Table 2: Number of trypanosomiasis patients positive for IF_{IgG}, IF_{IgM}, AI_{IgGsp}, AI_{IgMsp}, OC_{IgG}, OC_{IgGsp} or neuroinflammation (humoral immune response or cell count >20: neuro-infl.) in function of cell number and presence of trypanosomes in CSF. Specificity, sensitivity and exact 95 % confidence intervals (CI) were calculated in function of CSF cell number, presence of trypanosomes in CSF and treatment success.

Abbreviations see legend of table 1.

CSF	n	IF _{IgG} +	IF _{IgM} +	AI _{IgGsp} +	AI _{IgMsp} +	OC _{IgG} +	OC _{IgGsp} +	Neuro-infl.
<u>≤20 cells/μl and</u>								
no trypanosomes:								
-No relapse	21	0	3	1	0	4	5	11
-Relapse	1	1	1	0	1	1	1	1
<u>>20 cells/μl or</u>								
trypanosomes								
% Specificity	21	100	85.7	95.2	100	81.0	76.2	47.6
(95% CI)		(83.9-100)	(63.7-97.0)	(76.2-99.9)	(83.9-100)	(58.1-94.6)	(52.8-91.8)	(25.7-70.2)
% Sensitivity	17	35.3	76.5	52.9	70.6	29.4	76.5	88.2
(95% CI)		(14.2-61.7)	(50.1-93.2)	(27.8-77.0)	(44.0-89.7)	(10.3-56.0)	(50.1-93.2)	(63.6-98.5)

Figure 1: Antigen driven immunoblots of trypanosome specific IgG (OC_{IgGsp}) from unconcentrated CSF (C) and the corresponding serum (S) of 5 patients. The first 3 pairs of samples (patients 2732, 2733 and 2734) display a mirror pattern (identical oligoclonal antibodies at the same iso-electric point and similar immunostaining). The patient in lanes 4 (patient 2735) shows identical bands in CSF as in the corresponding serum, but with stronger immunostaining. Additional oligoclonal antibodies (a double band in CSF at pH 7.6, only 1 band in serum) are observed in CSF. In the last case (lanes 5, patient 2736), the CSF also contains a specific band around pH 7.5, not present in the corresponding serum.

