

Human African trypanosomiasis: quantitative and qualitative assessment of intrathecal immune response

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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 immunoglobulin G (IgG) and IgM intrathecal synthesis were studied in paired cerebrospinal fluid (CSF) and blood samples of 38 trypanosomiasis patients and in three 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 (AI) (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 AI (32% positive) indicated that the meningo-encephalitic stage of the disease is characterized 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.

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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 haemo-lymphatic or first stage of the disease is characterized 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 and 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 safe drug used for first-stage treatment has limited efficiency in the second stage as it does not sufficiently cross the blood-brain barrier. Melarsoprol, the drug commonly used for second stage treatment is highly toxic (Pépin and Milord, 1994; Van Nieuwenhove, 1999).

As there are no exclusive clinical signs, nor any clear changes at blood level, indicating the evolution from the haemo-lymphatic to the meningo-encephalitic stage (Bisser *et al.*, 1997), stage determination is performed by the 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 (i) white cell count; (ii) the presence of trypanosomes; and (iii) total protein concentration. If at least one of these parameters is abnormal, a patient is considered to be 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

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cut-off value for haemo-lymphatic stage have been set at 5 cells/ μl in CSF (WHO, 1998). This cut-off has been raised to 20 cells/ μl in some countries, including Côte d'Ivoire and Angola (Doua *et al.*, 1996; Stanghellini and Josenando, 2001). The presence of trypanosomes in CSF classifies a patient into the meningo-encephalitic stage but the techniques for trypanosome detection have limited sensitivity and the finding of trypanosomes in CSF with low cell counts is questioned. Protein quantification on CSF is not currently in practice in sleeping sickness control centres because of the need for more or less sophisticated material and the instability of the reagents. Moreover, cut-off values 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.

Sub-acute 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 (AI), or qualitative by detection of oligoclonal antibodies (Reiber and Peter, 2001; Sindic *et al.*, 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 enzyme-linked immunosorbent assay (ELISA) allowed calculation of the AI (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 (i) 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 (ii) 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. Of 13 900 people screened by card agglutination test for trypanosomiasis (CATT, Magnus *et al.*, 1978), 170 serological suspects were parasitologically examined by the minianion exchange centrifugation technique (Lumsden *et al.*, 1979) and by direct examination of lymph node aspirate. Seventy-six trypanosome-infected persons 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 38 patients first examined were used, after exclusion of patients with haemorrhagic CSF. Sixteen patients displaying trypanosomes in the CSF or >20 cells/ μl , were treated with melarsoprol, and the other 22 with pentamidine following the treatment protocols of the PRCT. The mean age of the patients was 35 years (range 4–64, SD 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 five patients, 17–20 months for nine patients, 13–14 months for six patients, 6–7 months for three patients, 2–3 months for four patients and 1 month for nine patients. One patient was lost to follow-up and another 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 re-treatment, 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.

Table 1 Cell count (cells/ μ l), presence of trypanosomes (CSF tryp), presence of intrathecal humoral immune response, neurological signs and follow-up period (FU, in months) in 38 *T. b. gambiense* patients. The intrathecal immune response was detected by six 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)

ID	Cells (/ μ l)	CSF tryps	IF IgG (%)	IF IgM (%)	AI IgGsp	AI IgMsp	OC IgG	OC IgGsp	Prim reflex	Sleep	Psych	FU
Treated with pentamidine												
2716	0	-	0	0	0.8	ND	-	-	-	-	-	13
2733	0	-	0	0	1	0.8	-	-	-	-	-	26
2737	0	-	0	0	0.7	ND	-	-	-	-	-	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	ND	-	+	-	+	-	18
2708	0	-	0	0	1.4	ND	+	-	-	-	-	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	ND	-	+	-	-	-	7
2747	3	-	0	0	0.5	ND	+	-	-	-	-	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	ND	-	-	-	-	+	18
2738	9	-	0	0	0.9	0.5	-	-	-	-	-	1
2729*	10	-	3	83	0.9	22	+	+	-	+	-	13
2710	14	-	0	0	0.2	ND	-	-	-	-	+	18
Treated with melarsoprol												
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	ND	-	+	+	-	-	1
2698	64	+	50	87	6.9	30	+	+	-	+	+	18
2713†	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‡	289	+	69	98	7.4	69	+	+	+	+	-	NA
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.

NA, not applicable; ND, not detectable (trypanosome-specific IgM concentration below detection limit of ELISA).

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 3Prospec, 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 phosphate-buffered saline (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, 1:19 200–1:76 800 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 nine twofold serial dilutions (same starting dilution as the test serum), was run in duplicate on each plate. Plates were shaken for 30 min. After three washes, the plates were incubated with rabbit-anti-human IgG or IgM (CLB, Amsterdam, The Netherlands) for 1 h, followed by goat-anti-rabbit IgG (H + L)-peroxidase (Nordic) for 30 min. After five washes, the ABTS substrate-chromogen solution was incubated for 1 h. The plate was shaken for 10 s 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 that 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 four-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 the absence of intrathecal immunoglobulin synthesis was calculated (Reiber and Peter, 2001) using the formula $Q_{lim} = (a/b) \times (Q_{alb}^2 + b^2)^{1/2} - c$ (with Q_{alb} = CSF albumin/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 synthesized fraction of IgG or IgM (the proportion of locally produced Ig as percentage 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} = CSF IgG/serum IgG, Q_{IgM} = CSF IgM/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 and 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 µg/ml, giving a total protein concentration of 10 µg/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 microlitres 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 (0.45 µm, BA 85, 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/300 cm² for 40 min at 10°C. The immunoblot was then dipped in PBS containing 5% w/v 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,

12 Amersham Pharmacia Biotech) as described by the manufacturer. Exposure time was generally between 30 and 90 s.

Statistics

For the 38 trypanosomiasis patients, the number of positive results obtained by the different techniques were compared by Mc Nemar chi-squared test with continuity correction, after construction of 2 × 2 contingency tables. Ninety-five percent symmetrical confidence intervals of diagnostic sensitivity and specificity

13 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 16 and 42% of all patients, respectively. 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 three 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 of 13 patients (1/13 patient was AI_{IgGsp} positive and AI_{IgMsp} negative). For all three controls, AI_{IgGsp} and AI_{IgMsp} were negative.

When the detection of total immunoglobulin synthesis was compared with 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 three 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 nine paired samples of trypanosomiasis patients (who all had > 20 cells/µl 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 of 38 CSF samples, all of them having a high IF_{IgM} (> 67%), positive AI_{IgMsp} (> 2.7) and a CSF IgM concentration > 50 mg/l. No oligoclonal bands were detectable, but a polyclonal background was observed similar to the one in the corresponding serum.

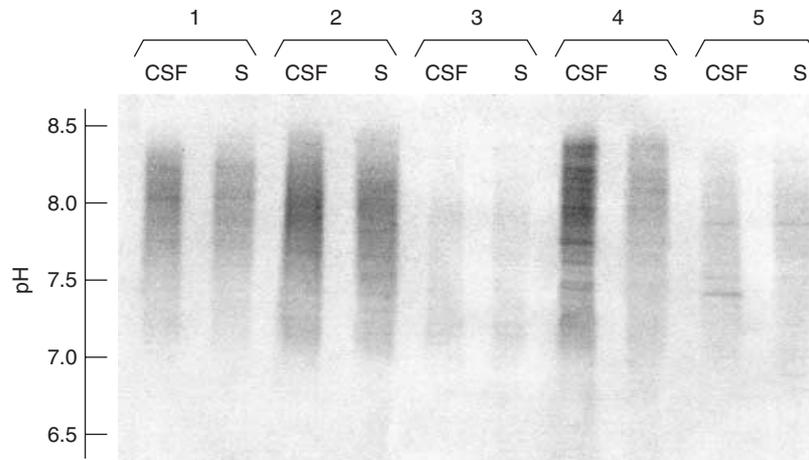


Figure 1 Antigen-driven immunoblots of trypanosome-specific IgG (OC_{IgGsp}) from unconcentrated CSF (C) and the corresponding serum (S) of five patients. The first three pairs of samples (patients 2732, 2733 and 2734) display a 'mirror' pattern (identical oligoclonal antibodies at the same iso-electric point and similar immunostaining). The patient in lane 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 one band in serum) are observed in CSF. In the last case (lane 5, patient 2736), the CSF also contains a specific band around pH 7.5, not present 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 (i) that all patients with > 20 cells/ μ l in the CSF were late stage patients; (ii) that all patients with trypanosomes in the CSF were late stage patients; (iii) that patients with < 20 cells and without trypanosomes in the CSF, which were cured by pentamidine were in first stage; and (iv) that patients with < 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 35.3, 29.4 and 52.9%, respectively 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.* (2002) on a group of *T. b. gambiense* patients from Peoples Republic of Congo. Using Reiber's formula (Reiber and 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_{IgMsp} was higher than AI_{IgGsp} . Human African trypanosomiasis caused by *T. b. gambiense* can thus be classified among neuro-inflammatory diseases with a dominant IgM immune response pattern in the CNS, like Lyme neuroborreliosis, neurosyphilis or mumps meningoencephalitis (Reiber and 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.*

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

CSF	n	IF _{IgG}	IF _{IgM}	AI _{IgGsp}	AI _{IgMsp}	OC _{IgG}	OC _{IgGsp}	Neuro-infl.
≤20 cells/μl and no trypanosomes	21	0	3	1	0	4	5	11
No relapse	1	1	1	0	1	1	1	1
Relapse	16	5	12	9	11	4	12	14
> 20 cells/μl or trypanosomes	21	100 (83.9–100)	85.7 (63.7–97.0)	95.2 (76.2–99.9)	100 (83.9–100)	81.0 (58.1–94.6)	76.2 (52.8–91.8)	47.6
Percentage specificity (95% CI)	17	35.3 (14.2–61.7)	76.5 (50.1–93.2)	52.9 (27.8–77.0)	70.6 (44.0–89.7)	29.4 (10.3–56.0)	76.5 (50.1–93.2)	88.2

For abbreviations see legend of Table 1.

(2002) 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. 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).

On the contrary, oligoclonal trypanosome-specific IgG antibodies (OC_{IgGsp}) were observed in 47% of the trypanosomiasis patients. All cases with elevated AI_{IgGsp} displayed oligoclonal trypanosome-specific IgG but the latter was detected in significantly more patients than IF_{IgG}, OC_{IgG} and AI_{IgGsp}. Presence of oligoclonal antibodies, without presence of corresponding oligoclonal IgG, is frequently observed in CNS infections (Figs 4 and 5 in Sindic *et al.*, 2001). Some AI_{IgGsp} negative samples were OC_{IgGsp} 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. As 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 and Whittle, 1980), or low affinities for the trypanosomal antigens used. This is suggested by the occurrence of four 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 (C. J. M. 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 of four such patients remained negative in all applied tests, showing no signs of an intrathecal inflammatory process. One could hypothesize 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 haemo-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 <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 (<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 four patients with OC_{IgGsp}, the two 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 four 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), as 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 characterized 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).

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