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BIOLOGICAL DATA AND CLINICAL SYMPTOMS AS PREDICTORS OF ASTROGLIOSIS AND NEURODEGENERATION IN PATIENTS WITH SECOND-STAGE *TRYPANOSOMA BRUCEI GAMBIENSE* SLEEPING SICKNESS

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Abstract. Concentrations of glial fibrillary acidic protein (GFAp) and light subunit neurofilament protein (NFL) in cerebrospinal fluid (CSF) were measured in patients with second-stage *Trypanosoma brucei gambiense* sleeping sickness. Correlations between GFAp and NFL in CSF as markers for astrogliosis and neurodegeneration, and clinical and biological data were investigated. Abnormal levels of GFAp and NFL were significantly associated with increasing CSF cell number and protein concentration, and with the absence of lymph nodes or the absence of trypanosomes in lymph node aspirate. A significant association was found between abnormal NFL and presence of trypanosomes in CSF, abnormal limb movements, difficulties in gait and coordination, and low Karnofsky index. By multivariate analysis, it was shown that increasing CSF cell number, increasing CSF protein concentration, and the absence of lymph nodes or the absence of trypanosomes in the lymph node aspirate were the best predictors for astrogliosis and neurodegeneration among the variables tested. These results demonstrate the importance of CSF cell count and protein determination in assessment of the severity of central nervous system involvement and reinforces the importance of laboratory diagnosis to assess the stage of the disease. The clinical symptoms studied were less useful in predicting astrogliosis or neurodegeneration.

INTRODUCTION

Infection with the protozoan parasite *Trypanosoma brucei gambiense* causes sleeping sickness, or human African trypanosomiasis. After the infective tsetse bite, trypanosomes spread into blood and lymphoid tissues, initiating the hemolymphatic disease stage. When trypanosomes invade the central nervous system (CNS), the second, meningoencephalitic stage is initiated. Parasites probably enter the brain via areas with a reduced blood-brain barrier and spread into cerebrospinal fluid (CSF) and stromal spaces via the subarachnoid spaces to the perivascular extensions that pass into the brain.[1–4] This is associated with chronic meningitis that progresses to encephalitis and is accompanied by extensive perivascular cuffing, infiltration and activation of plasma cells, Mott cells, T-cells, and astrocytes, and by neuronal degeneration.[1,5–8]

Changes in the composition of CSF reflect events in the CNS. Increased CSF concentrations of glial fibrillary acidic protein (GFAp) indicate astrogliosis, whereas increased light subunit neurofilament protein (NFL) concentrations in CSF reflect neuronal degeneration.[9,10]

Abnormal concentrations of both GFAp and NFL in the CSF of patients with second-stage *T. b. gambiense* have been reported.[11] Gliosis, neuronal degeneration, or both might explain the clinical symptoms frequently observed in second-stage human African trypanosomiasis, including tremor, fasciculation of the muscles, choreiform, athetoid or oscillatory movements, and increased tonicity or muscular rigidity.[7,12–15] A correlation between GFAp and NFL concentrations in CSF and clinical symptoms has been reported for several neurological diseases, including neuroborreliosis, normal pressure hydrocephalus, and multiple sclerosis.[16–19]

In the current study, we investigated which biological indicators (presence of trypanosomes in the lymph node aspirate, presence of trypanosomes in CSF, CSF cell number, and protein concentration) and which clinical symptoms (coordination, abnormal limb movements, tonus, stance, gait, and Karnofsky index) are the best predictors of astrogliosis and neurodegeneration in patients with second-stage sleeping sickness.

MATERIALS AND METHODS

Patients and CSF samples. One hundred twenty-four parasitologically confirmed patients with second-stage *T. b. gambiense* aged 12–65 years, originating from Arua district, Northern Uganda, were included in the study. Patients were selected at random from a cohort participating in a study on encephalitic reactions that systematically included all second-stage patients visiting the treatment center. Lumbar punctures were performed as part of routine diagnosis and stage determination of patients with human African trypanosomiasis, as prescribed by the World Health Organization (WHO).[20] Patients included in the current study underwent a more extensive clinical examination but received the standard medication: 122 patients were treated with melarsoprol, and 2 patients received eflornithine. The study was approved by the Ugandan Ministry of Health and an oral consent was systematically collected from all patients. Classification of the patients in the second stage, according to WHO guidelines,[20] was based on detection of trypanosomes or the presence of > 5 cells/μL in CSF. Eight of the patients presented themselves for retreatment because they had relapsed after initial treatment.

The following clinical data were collected at admission: abnormal limb movement (absent/present + type of abnormal movement), coordination (normal, difficult, impossible), tonus (normal, increased, decreased), stance (normal, difficult, impossible), gait (normal, difficult or ataxic, impossible), Karnofsky index (a scale quantifying the functional status/performance status ranging from 10% [moribund] to 100% [normal, no evidence of disease]).[21–23]

Trypanosomes were detected in blood by the microhematocrit centrifugation technique or quantitative buffy coat

technique,[24,25] in cervical glands by direct examination of the aspirate (if nodes were present), or in CSF by double centrifugation.[26] Cells in CSF were counted in duplicate in a Nageotte counting chamber. The protein concentration in CSF was determined in duplicate with the bicinchoninic acid protein assay reagent as described by the manufacturer (Pierce, Rockford, IL) using the microtiter plate protocol, with bovine serum albumin as a standard.

Enzyme-linked immunosorbent assay. The GFAp and NFL concentrations in CSF were measured by sandwich enzyme-linked immunosorbent assay.[10,11,27] Briefly, microtiter plates were coated with chicken anti-GFAp or anti-NFL immunoglobulin (Ig) G and reference GFAp or NFL, and duplicate samples of CSF were added; wells were incubated with rabbit anti-GFAp or anti-NFL IgG. Peroxidase-conjugated donkey anti-rabbit IgG (Amersham, Buckinghamshire, UK) was added, and plates were developed with *o*-phenylenediamine and perhydrol. The absorbance was measured at 490 nm. Wells were washed between each step. The GFAp standard curve ranged 16–8,000 ng/L, the NFL standard curve from 62–32,000 ng/L. The concentrations of GFAp and NFL in the samples were interpolated from the standard curves. The detection limits were 16 and 125 ng/L, respectively.

Statistics. The concentration of NFL in CSF was recoded as normal ($\leq$ 200 ng/L) and abnormal ($>$ 200 ng/L). The cutoff for recoding the GFAp concentration as abnormal was adjusted on the age group (based on a white population): $>$ 175 ng/L for 2–19 years, $>$ 500 ng/L for 20–39 years, $>$ 750 ng/L for 40–59 years, and $>$ 1250 ng/L for 60–75 years.

Cell number and protein concentration in CSF were recoded as categorical variables by quartiles. Karnofsky index was recoded in index 100–80% and index $<$ 80%, following the condition subdivisions originally described. Index 80–100% was Condition A (patient able to carry on normal activity and to work, no special care needed); index $<$ 80% was condition B + C (unable to work, but able to care for personal needs; a varying degree of assistance needed, and the patient unable to care for him- or herself). Patients without lymph nodes or without trypanosomes in the lymph node aspirate were considered cervical gland negative. For all clinical and laboratory variables, differences in proportion of abnormal NFL and GFAp concentrations between the groups were assessed by chi-square analysis.

In order to identify the best predictors of astrogliosis and neurodegeneration and to adjust the results on potential confounders, a multivariate analysis was performed by a multiple logistic regression model and the adjusted odds ratio as an estimation of the relative risk. The dependent variables were successively the GFAp and NFL concentration recoded as normal and abnormal. The covariates included both the clinical and the biological variables. Colinearity between covariates was checked by chi-square analysis. When 2 colinear covariates were identified, one was removed from the analysis. All other covariates were included in the initial model and then removed one by one on the basis of the highest P value observed among them. Only the variables with $P < 0.10$ were kept in the final model.

RESULTS

The GFAp concentrations measured in CSF were between 34 and 2672 ng/L, with a median of 664 ng/L and abnormal values in 68% of the patients. The NFL concentrations in CSF ranged 125–9,244 ng/L, with a median of 439 ng/L. An abnormal NFL concentration was observed in 65% of the patients.

The proportion of patients with abnormal GFAp significantly increased with cell number and protein concentration in CSF and was significantly higher for cervical gland negative patients (Table 1). No difference between the subgroups of any of the clinical variables was observed for GFAp.

The percentage of patients abnormal for NFL increased significantly with increasing protein concentration and cell number in CSF and was significantly higher for cervical gland negative patients and patients with trypanosomes in CSF. For the clinical variables, the percentage abnormal for NFL was significantly higher in patients with difficulty in coordination or gait, patients with abnormal limb movements, and patients with Karnofsky indexes $<$ 80 (Table 1).

Colinearity was identified between gait and stance, coordination and stance, tonus and stance, gait and coordination, and tonus and gait. Stance, tonus, and coordination were removed from the multivariate analysis. The other covariates were included in the initial multivariate model (Table 2). In the final regression model, only the number of cells and the protein concentration in CSF, as well as the absence of lymph nodes or the absence of trypanosomes in the lymph node aspirate, remained significantly associated with the risk of presenting abnormal GFAp and NFL levels in CSF (Table 2).

DISCUSSION

The current study confirms the presence of astrogliosis and neurodegeneration, assessed by the CSF markers GFAp and NFL, observed in patients with second-stage sleeping sickness.[11] Compared with the former study,[11] higher concentrations of GFAp and NFL in the CSF of patients with second-stage sleeping sickness and a higher proportion of patients with abnormal GFAp and NFL were observed. As shown in the current study, this can be explained by higher CSF cell numbers, higher CSF protein concentrations, and a higher number of patients with trypanosomes in the CSF within this study compared with the former study.[11]

We found that increased cell number and protein concentration in CSF were associated with abnormal GFAp levels in a higher proportion of patients and were thus predictive for astrogliosis. An increased protein concentration could be the consequence of impairment of the blood-brain barrier, possibly caused by cytokine release from activated astrocytes or microglia.[28] In *T. b. brucei*-infected mice, detection of interleukin (IL)-6 in the CNS correlates with astrocyte activation,[29] and increased IL-6 and IL-8 concentrations have been detected in the CSF of patients with second-stage sleeping sickness (Lejon V and others, unpublished data). Both cytokines can alter the integrity of the blood-brain barrier[30,31] and may contribute to the activation, infiltration, and proliferation of plasma, Mott cells, and T cells, and consequently the increased cell numbers in CSF.[32,33]

Increased cell number and protein concentration in CSF were also associated with a higher proportion of patients having abnormal NFL and were thus predictive for neurodegeneration. These events could be the consequence of gli-

TABLE 1
Proportion of abnormal GFAp and light subunit NFL by group of patients

Variable	*n*	GFAp		NFL	
		Abnormal (%)	*P* value†	Abnormal (%)	*P* value
Abnormal limb movement			0.12		0.05
Absent	74	62.2		58.1	
Present	49	75.5		75.5	
Coordination			0.34		0.008
Normal	111	65.8		61.3	
Difficult or impossible	11	81.8		100.0	
Tonus			0.13		0.09
Normal	108	64.8		62.0	
Increased or decreased	16	87.5		87.5	
Stance			0.50		0.09
Normal	112	66.1		62.5	
Difficult or impossible	11	81.8		90.9	
Gait			0.22		0.03
Normal	110	65.5		61.8	
Difficult or impossible	13	84.6		92.3	
Karnofsky index			0.37		0.01
≥80%	93	65.6		59.1	
<80%	47	74.2		83.9	
Number of treatment			1.0		0.26
First treatment	116	67.2		63.8	
Retreatment	8	75.0		87.5	
Lymph node aspirate			0.007		0.0009
Negative or no nodes	74	77.0		77.0	
Positive	50	54.0		48.0	
Trypanosomes in cerebrospinal fluid			0.13		0.0001
Negative	19	52.6		26.3	
Positive	105	70.5		72.4	
Cells/μL in cerebrospinal fluid			0.0005		<0.0001
5–70	33	39.4		27.3	
71–150	37	73.0		62.2	
151–300	27	77.8		88.9	
>300	27	85.2		92.6	
Total protein in cerebrospinal fluid (mg/L)			<0.0001		<0.0001
200–500	24	41.7		16.7	
501–750	31	45.2		48.4	
751–900	24	95.8		83.3	
>900	45	82.2		93.3	

* GFAp = glial fibrillary acidic protein; NFL = neurofilament protein.
† Differences in proportion of abnormal NFL and GFAp concentrations between the groups were assessed by chi-square analysis.

osis and release of cytotoxic molecules such as NO (Keita M and others, unpublished data)[8,34–37] but could also be linked to the presence of autoantibodies against brain proteins such as myelin basic protein, galactocerebrosides, and neurofilament.[38–40] Evidence exists that production of antibodies to galactocerebroside and neurofilament is induced by trypanosome components with cross-reactive epitopes.[40,41]

In univariate analysis, a significant association was found between abnormal NFL concentrations and the presence of trypanosomes in CSF. This might be explained by the association between the presence of trypanosomes and increased cell numbers in CSF[42] or by the secretion of toxic molecules into the CSF by trypanosomes, adversely affecting neuronal fibers.[8]

A negative lymph node puncture or the absence of lymph nodes as a predictor of CNS pathology might seem surprising. However, a relationship between increasing CSF white blood cell count and absence of trypanosomes in the blood or lymph node aspirate has already been observed, in combination with a higher risk for treatment-induced encephalopathy.[43] It was described in 1906 that glandular enlargements were a constant feature of early stages of trypanosomiasis, and that examination of the lymph node aspirates is indicated for diagnosis of early cases in which all clinical signs are wanting.[44] Ormerod[45] described fibrosis of lymph vessels and shrinking of the lymph glands as characteristic for advanced sleeping sickness. The absence of lymph nodes in patients in the second stage could also indicate an exhausted immune system.

In univariate analysis, a significant association was observed between abnormal neurofilament and abnormal movements of the limbs, coordination, and gait. Because NFL is highly enriched in large myelinated axons, this finding might be compatible with a degeneration of subcortical white matter, cerebellar or spinal tracts such as pyramidal pathways, posterior columns, or cerebellar proprioceptive pathways. This association disappeared in the multivariate model.

We conclude that these clinical symptoms are of less use

TABLE 2
Initial and final multivariate models with GFAp and light subunit NFL as dependent variables*

Variable	GFAp		NFL	
	Initial model OR (95% CI)	Final model OR (95% CI)	Initial model OR (95% CI)	Final model OR (95% CI)
Abnormal limb movement				
Absent	Reference		Reference	
Present	1.2 (0.4–3.2)		1.8 (0.5–6.2)	
Gait				
Normal	Reference		Reference	
Difficult or impossible	2.2 (0.3–15.6)		2.0 (0.1–33.6)	
Karnofsky index				
≥80%	Reference		Reference	
<80%	0.7 (0.2–2.5)		2.5 (0.6–11.4)	
Number of treatment				
First treatment	Reference		Reference	
Retreatment	1.0 (0.1–7.7)		1.4 (0.1–17.6)	
Lymph node aspirate				
Negative or no nodes	Reference	Reference	Reference	Reference
Positive	0.3 (0.1–0.9)	0.4 (0.1–0.9)	0.4 (0.1–1.2)	0.4 (0.1–1.2)
Trypanosomes in CSF				
Absent	Reference		Reference	
Present	0.8 (0.2–3.6)		4.0 (0.7–21.5)	
Cells/μL in CSF				
5–70	Reference	Reference	Reference	Reference
71–150	2.4 (0.6–9.3)	2.2 (0.7–7.2)	0.7 (0.2–3.4)	1.4 (0.4–5.2)
151–300	1.9 (0.4–9.5)	1.8 (0.4–7.3)	3.5 (0.6–20.8)	7.1 (1.4–37.3)
>300	3.7 (0.7–20.6)	3.7 (0.8–16.8)	3.4 (0.4–27.2)	8.4 (1.3–55.2)
Total CSF protein (mg/L)				
200–500	Reference	Reference	Reference	Reference
501–750	0.8 (0.2–2.7)	0.7 (0.2–2.4)	4.3 (0.9–21.5)	3.9 (0.9–16.9)
751–900	24.0 (2.6–223.7)	24.1 (2.6–221.8)	26.3 (3.9–178.8)	21.0 (3.8–115.1)
>900	2.9 (0.7–11.2)	2.9 (0.8–10.8)	40.7 (6.1–273.5)	37.4 (6.3–221.7)

* CI = confidence interval; CSF = cerebrospinal fluid; GFAp = glial fibrillary acidic protein; NFL = neurofilament protein; OR = odds ratio.

to assess CNS pathology compared with the biological data. This finding confirms the minor role played by general clinical symptoms in the diagnosis and staging of patients with human African trypanosomiasis.[15,46,47] The total CSF protein concentration, the CSF cell number, and the absence of lymph nodes or absence of trypanosomes in lymph node aspirate are more reliable predictors of astrogliosis and neurodegeneration and therefore merit careful monitoring. The predictability of astrogliosis and neurodegeneration by the total protein concentration and the cell number in CSF again stresses the importance of these laboratory analyses for determining the CNS pathology of human African trypanosomiasis.

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