African Journal of Biotechnology Vol. 4 (6), pp. 517-521, June 2005
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2005 Academic Journals

Full Length Research Paper

Confirmation of the use of Latex IgM on cerebrospinal fluid for improving stage determination of Human African Trypanosomiasis

P. Truc[1]*, Vincent Jamonneau[1] , Jean-François Guegan[2]

[1]Institut de Recherche pour le Développement, UR177 « Trypanosomoses Africaines », International Campus of Baillarguet, IRD / CIRAD, TA 207 / G 34 398 Montpellier cedex 5 France.
[2]Institut de Recherche pour le Développement, Génétique et Evolution des Maladies Infectieuses, UMR 2724 IRD-CNRS, Equipe "Evolution des Systèmes Symbiotiques", 911 Avenue Agropolis B.P. 64501 34394 Montpellier Cedex 5 France.

Accepted 10 May, 2005

The clinical evolution of the chronic form of Human African Trypanosomiasis starts with the haematolymphatic or first stage (P1). The meningoencephalitic or second stage (P2) begins when trypanosomes reach the cerebrospinal fluid (CSF). The classical stage determination method is based on CSF cell count, CSF protein concentration and/or the presence of trypanosomes detected in CSF. However their cut-off values and the sensitivity of detection of trypanosomes in CSF remains doubtful while the appropriate treatment depends on this determination of disease stage. Thus, the classical stage determination is reconsidered using new serological tests, and results were compared to the clinical data. Thirty-eight patients were classified into 4 clinical groups according to the observed degree of severity of neuropsychiatric signs. Based on multivariate analysis to evaluate the relevance of the new serological tests as compared with clinical groups, we confirm that Latex IgM CSF, cheap and easy to perform under field conditions, may improve stage determination of the disease.

Key words: Human African Trypanosomiasis, stage determination, Latex IgM.

INTRODUCTION

The clinical evolution of the chronic form of Human African Trypanosomiasis (HAT) starts with the haematolymphatic or first stage (P1) with a few typical clinical signs (such as the primary lesion or trypanosomal chancre, or the posterior cervical swollen lymph node). The meningoencephalitic or second stage (P2) begins when trypanosomes reach the cerebrospinal fluid (CSF) with the progressive appearance of neurological disorders. The classical stage determination method is based on CSF cell count (cut-off, 5 cells/µl), CSF protein concentration (cut-off, 37 mg/100 ml by the dye-binding protein assay) and/or the presence of trypanosomes detected by simple or double centrifugation of CSF (WHO, 1998). However the cut-off values and the sensitivity of detection of trypanosomes in CSF remain doubtful (Truc et al., 1999). On the other hand, clinical examination for detection of neurological disorders may contribute to detect a second stage of the disease but is not satisfactory either (Bisser et al., 2000). However, the appropriate treatment requires an accurate determination of disease stage. Indeed, the treatment of patients in stage P1 is usually pentamidine, while melarsoprol is used for stage P2. Because of toxicity and severe,

*Corresponding author. E-mail: truc@ird.fr. Tel: +33 611 888 828.

potentially fatal side effects of melarsoprol and the possibility to cure some P2 patients using pentamidine (Doua et al., 1996), stage determination must be highly accurate.

Recently, several new techniques for parasite detection in the CSF have been discovered. For example, these techniques include specific IgM antibodies (Lejon et al., 1998, 2002 ; Büscher et al., 1999), anti-galactocerebroside antibodies in CSF produced in response to the progressive demyelinating process triggered by the presence of trypanosomes in the cerebral environment (Bisser et al., 2000), or blood-cerebrospinal fluid barrier dysfunction and IgM detection (Bisser et al., 2002).

Thus the classical stage determination might be reconsidered using these new serological techniques, and results could be compared to clinical data. Indeed, patients could be classified into 4 clinical groups according to the observed clinical syndroms. Similar classification was already proposed (Bisser et al., 2000). Based on multivariate analysis to evaluate the relevance of the new serological tests as compared with clinical groups, we confirmed that the Latex IGM could improve stage determination.

PATIENTS AND METHODS

Patients, diagnostic and treatments

The consenting patients were diagnosed between 1996 and 1998 in Côte d'Ivoire either at Daloa (Projet de Recherche sur la Trypanosomiase), Bouaflé (Regional Treatment Centre), or at various sites during several medical surveys conducted by the National Control Program against HAT and the HAT team from the Institut Pierre Richet located in Bouaké.

Diagnosis was established according to the WHO protocol (WHO, 1998), starting with a serological test using whole blood and plasma (Card Agglutination Test for Trypanosomiasis, CATT/T. T.b. *gambiense*; Magnus et al., 1978). In case of positive result, the search for trypanosomes was done either in lymphatic liquid by punction of swollen lymph node, or in whole blood by the mini-anion exchange centrifugation technique (mAECT; Lumsden et al., 1979). Stage determination was done by the classical method recommended by WHO (WHO, 1998): protein content dosage in CSF (P2 if > 37 mg/100 ml of CSF by the dye binding protein assay), the lymphocyte count in CSF (P2 if >5 cells/µl), and by microscopic examination of CSF after double centrifugation for direct observation of trypanosomes. One of these criteria with or without the presence of trypanosomes, forms the basis for second stage of HAT.

Clinical examination

Clinical examination included palpation (search for hepatomegaly, splenomegaly, lateral cervical lymphadenopathy), cardiovascular examination, dermatological examination (chancre of inoculation, trypanids), and search for endocrinological disorders (impotence, facial oedema, amenorrhoea, spontaneous abortion). The neurological disorders were mainly the alteration of mental state (psychiatric symptoms), abnormal reflexes, tone and co-ordination disorders, hyperaesthesia, sleep disturbances (alteration of circadian rythm), and the presence of archaic reflexes.

Patients were asked about other clinical symptoms such as asthenia, anorexia, fever, headache, nausea and pruritus. Several questions were asked also to patients or their family in order to estimate roughly the mode of evolution of the disease.

The presence or absence of each symptom, in particular these relevant to neurological disorders (Jamonneau et al., 2002), allowed the classification of patients into 4 groups, from group 0 corresponding to the absence of neurological signs to group 3 corresponding to the presence of a neurological syndrome. Groups 1 and 2 correspond to intermediate clinical patterns with some neurological disorder noted for patients from the group 2.

Laboratory examination

For all tests, venous samples were obtained by venepuncture, and CSF by lumbar puncture before treatment. Serum and CSF were aliquoted (2 tubes of 1.5 ml for each fluid) and stored in liquid nitrogen. These tubes were shipped to the Institute of Tropical Medicine in Antwerp (Belgium), the Institute of Tropical Neurology in Limoges (France), and the Laboratory of Parasitology, University of Bordeaux II (France). The following dosages were performed: (1) Determination of the end titre (ET) using serum for CATT/*T. b. gambiense* (Magnus et al., 1978). The test is positive if ET>¼. (2) Determination of the end titre (ET) using serum for Latex/*T. b. gambiense* (Büscher et al., 1991). The test is positive if ET>1/16. (3) Determination of the end titre (ET) using CSF for Latex/*T. b. gambiense* (Büscher et al., 1999). The test is positive if ET>1. (4) Determination of the end titre (ET) using CSF for Latex/IgM. The test is positive if ET>1/16 (Lejon et al., 2002). (5) CSF protein concentration using the Kit BCA (Pierce). The cut off-value considered is 750 mg/l to determine a positive result. (6) Dosage of albumin concentration in serum and CSF by nephelometry (Lejon et al., 2002). The functionality of the blood-CSF barrier was evaluated using the CSF/serum albumin ratio, Q_{alb}. The age-related upper reference limits for Q_{alb} are $5x10^{-3}$ (up to 15 years), $6.5x10^{-3}$ (up to 40 years) and $8x10^{-3}$ (up to 60 years). If Q_{alb}>7.4, it might indicate a blood-CSF barrier dysfunction. (7) Dosage of total IgM and IgG concentrations in serum and CSF by nephelometry (Bisser et al., 1997; Lejon et al., 1998). (8) Dosage of specific IgM and IgG concentrations in serum and CSF by ELISA (Lejon et al., 1998) using antibodies against 3 specific antigenic variants LiTat 1.3, 1.5 and 1.6. These dosages allow calculation of the Organism Specific Antibodies Index (OSAI), i.e. IgG OSAI and IgM OSAI. OSAI>1.5 indicates an infection in the central nervous system (CNS; Reiber, 1998). (9) Dosage of anti-cysteine, anti-NO-cysteine, anti-tryptophan and anti-NO-tryptophan-like epitope antibodies in serum by ELISA (Boullerne et al., 1995). (10) Dosage of anti-galactocerebroside antibodies (anti-GalC) in serum and CSF by ELISA (Amevidge et al., 1992). (11) Dosage of anti-neurofilament antibodies in serum and CSF by ELISA (Ayed et al., 1997).

Statistical analysis

First we used a discriminant analysis to assign actual group membership, i.e. the 4 clinical groups as defined above, against predicted membership (Wilkinson et al., 1992). To compute this multivariate model, we used all the available discriminators to predict the 4 groups, and then we selected as much as possible for optimal minimal models using stepwise discriminant analysis. For clarity, we only illustrate the optimal minimal model we obtained for predicting actual group membership.

Table 1. Relevant biological and clinical results for each patient. The results of other laboratory examinations performed (see Patients and Methods) were not statistically significant (p>0.05) between patients assigned to clinical groups.

Patient no.	Age	Stage (WHO)	Clinical group	Cell/µl CSF	Tryps CSF	Protein CSF (mg/l)	Latex IgM CSF (end titre)
611	47	1	0	0	-	487	8
612	11	1	0	0	-	653	2
613	11	1	0	0	-	554	0
614	13	1	0	0	-	507	1
636	29	1	0	4	-	573	64
660	63	1	0	0	-	486	0
661	11	1	0	0	-	537	32
662	21	1	0	0	-	924	64
664	12	1	0	0	-	621	1
669	17	1	0	2	-	650	4
2597	56	1	0	4	-	706	2
2600	47	1	0	4	-	583	1
2604	22	1	0	0	-	522	2
665	55	1	3	0	-	1218	128
2561	14	2	0	28	-	594	8
2584	45	2	0	6	-	665	8
2598	35	2	0	6	-	627	0
2601	56	2	0	10	-	761	4
2603	38	2	0	12	-	514	4
602	23	2	0	45	-	1063	128
2569	48	2	0	304	+	1316	128
2587	65	2	0	87	-	679	32
2499	65	2	1	482	+	1332	128
2570	14	2	1	848	+	974	128
635	55	2	2	240	+	1198	128
648	40	2	2	15	-	757	128
2548	37	2	2	710	+	1179	64
2557	30	2	2	1058	+	1546	128
2582	41	2	2	6	+	774	16
603	15	2	3	140	+	1302	128
610	45	2	3	90	-	739	64
634	31	2	3	48	+	1466	256
638	9	2	3	113	+	776	128
655	38	2	3	120	+	1828	128
656	13	2	3	40	+	1474	256
668	20	2	3	22	-	1358	128
2562	29	2	3	888	+	1532	256
2574	42	2	3	50	+	549	64

Then a generalized linear model (GLIM) was built in order to assess simultaneously which explanatory variables and/or their interaction terms (2-way and 3-way interactions) better explained membership for the 4 clinical groups. Stepwise procedures were used to retain the best models as described in *S-Plus* 2000 guide to statistics (Mathsoft, Inc. 1999). When the order of entry of the different retained predictors altered residual deviances and partial testing, we decided to select variables according to their Akaike criterion (from the lowest to the highest; Crawley, 1993). For further details about statistical methods, see Mc Cullagh & J. A. Nelder. Generalized linear Models. Second edition, Chapman & Hall Ltd. London, UK. 1989. As the response data corresponded to

Table 2. Summary of a Generalized Linear Model (GLIM) using a quasi-likelihood error structure (Phi=1.058) and a logit link function for explaining the 4 HAT clinical groups (see text) with the significant independent parameters retained after a stepwise selection procedure. Final model: Null deviance = 66.36, Residual deviance = 38.10, *df* = 2,36.

***P* (Predictors)**	**df**	**Deviance**	**Res. df**	**Res. Dev.**	**F-value**
Null			38	66.359	
Anti-GalC CSF O.D. 0.052774	1	4.2457	37	62.113	4.0112
Latex IgM CSF E.T. 0.000031	1	24.0077	36	38.106	22.6817

proportions, we first used a Poisson error structure, and then we chose a Quasi-Likelihood estimation with a logit function since residuals tended to be overdispersed (McCullagh and Nelder, 1989). In each case, predictive models based on classification were compared to explanatory models using GLIM.

RESULTS AND DISCUSSION

Thirty-eight patients were included in the present study. The results of laboratory examinations that are useful for stage determination according to WHO, and those of Latex IgM CSF which is the only one new highly significant variable for the new stage determination are summarised in Table 1. The best fit was obtained using a minimal model with lymphocytes/µl, protein in CSF, LatexIgM CSF, IgG OSAI and Q_{alb}, to predict assignment to a given clinical group. According to the multivariate modeling, 18 patients were classified as group 0, and 2 patients perfectly assigned to group 1. In addition, only 3 patients of group 2 were predicted to belong to this group (60%), while 9 of those belonging to group 3 were correctly classified (90%). The classification score, on the average, was relatively high with 84% of patients correctly assigned to their relevant group of clinical symptoms.

Results of GLIM to explain the classification of patients into the 4 clinical groups indicate that the only significant predictor variable was Latex IgM CSF (p=0.000031, Table 2). This finding shows a strong influence of the Latex IgM CSF variable (85% of the total explained deviance) in categorizing the level of pathogenesis in patients affected by sleeping sickness.

Out of the 3 classical tests recommended for stage determination by WHO (WHO, 1998), CSF protein concentration is not usually performed because of lack of reagent necessary for the traditional dye binding protein assay. In the present study, we used also a more sophisticated BCA technique (Pierce). However, routine use of new kit on a large scale in Africa appears to be unrealistic because of the specific and expensive devices and reagents required. Likewise, the low sensitivity of the search for trypanosomes in the CSF by the centrifugation technique is known as compared to PCR-based methods used for parasite DNA detection (Truc et al., 1999). Once again and despite the improvement of its specificity (Truc et al., 2002), the use of PCR based methods for routine parasitological diagnosis appears unrealistic in the actual context in Africa.

Some patients with trypanosomes in CSF were successfully cured using pentamidine in a previous study (Doua et al., 1996), probably because of a small amount of pentamidine passing through the blood-brain barrier (Van Nieuwenhove, 1999). The last classical test for stage determination is the CSF cells (or lymphocytes) count. The cut-off of 5 cells/µl is controversial (Bisser et al., 1997; Doua and Boa, 1994). In addition, even the conditions in which lumbar puncture is performed may influence final cell count. For example, bloody puncture and small volume of CSF may concentrate some cells originating from blood mixed together with CSF (when such a sample is not detectable by eye). Therefore, cell count takes into account all cells whatever their origin, leading to an overestimation of cells in CSF. In the absence of neurological disorders, the relevance of these 3 classical tests remains important because it still leads to the decision of the appropriate treatment, pentamidine for P1 and melarsoprol for P2. The problem is not the reliability of these tests but their doubtful cut-off value and sensitivity. In the present study, statistical analysis confirmed the Latex IgM CSF as major parameter for the determination of CNS involvement (Lejon et al., 2002). This is an important improvement because Latex IgM CSF is a cheap and simple test, easy to perform under field conditions. However, the cut-off value is still controversial (Lejon et al., 2002). The interest of our integrated approach is to consider all clinical and biological results because it has been shown that the cut-off value or sensitivity of only one technique is not sufficient to allow an accurate stage determination. The combination of several parameters appears to be more discriminant. Although the other biological markers were already described as specific of a CNS involvement and most of them are antibodies in serum (anti-cysteine, anti-NO-cysteine, anti-tryptophan, anti-NO-tryptophan), our statistical analysis found also that the combination of only two parameters was much more relevant (Latex IgM CSF and anti-GalC CSF). It has been mentioned that routine use of a very sophisticated technique, such as O.D. determination for anti-GalC CSF under field conditions, appears unrealistic in the actual context of the control against HAT in Africa. Furthermore, the high correlation between IgM and anti-GalC concentrations in the CSF

and the CNS involvement has already been demonstrated (Bisser et al., 2000).

Therefore, we recommend the use of Latex IgM CSF only after a large-scale evaluation (in particular to define an accurate cut-off value). The main advantage is its ease of use under field conditions and its rapidity. However, present results suggest that other biological markers still be of value especially when they can be analysed in serum. Indeed, this may avoid the lumbar puncture which is often a traumatism for the patient. Unfortunatly, lumbar puncture is still in use nowadays.

In the absence of new drug for HAT treatment and because of the actual situation of sleeping sickness in Africa, it is crucial to reconsider the stage determination as soon as possible. Also, further investigations are required to avoid if possible lumbar puncture in the future.

ACKNOWLEDGEMENTS

We thank the Projet de Recherche Clinique sur la Trypanosomiase (PRCT, Daloa, Côte d'Ivoire) and the Regional treatment Center of Bouaflé (Côte d'Ivoire) for the clinical investigations. We thank also P. Vincendeau (Faculty of Medicine, Bordeaux, France), M. Dumas and B. Bouteille (Institut of Tropical Neurology, Limoges, France), V. Lejon (Institut of Tropical Medicine, Antwerp, Belgium) for kindly performing serological and CFS tests. We warmly thank L. Basco and M. Boussinesq (Institut de Recherche pour le Développement, Cameroon) for the valuable discussions and critical reading of the manuscript. This work was supported by Fonds d'Aide à la Coopération, Ministère français des Affaires Etrangères, and by the Institut de Recherche pour le Développement (France).

REFERENCES

Amevidge M, Jauberteau-Marchan MO, Bouteille B, Doua F, Breton JC, Dumas M (1992). Human African trypanosomiasis : presence of antibodies to galactocerebrosides. Am. J. Trop. Med. Hyg. 47: 652-662.

Ayed Z, Brindel I, Bouteille B, Van Merveinne N, Doua F, Houinato D, Dumas M, Jauberteau MO (1997). Detection and characterization of autoantibodies directed against neurofilament proteins in human African trypanosomiasis. Am. J. Trop. Med. Hyg. 57: 1-6.

Bisser S, Ayed Z, Bouteille B, Stanghellini A, Breton JC, Dumas M, Jauberteau MO (2000). Central nervous system involvment in African trypanosomiasis : presence of anti-galactocerebroside antibodies in patients cerebrospinal fluid. Trans. R. Soc. Trop. Med. Hyg. 94: 225-226.

Bisser S, Bouteille B, Sarda J, Stanghellini A, Ricard D, Jauberteau MO, Marchan F, Dumas M, Breton JC (1997). Apport des examens biochimiques dans le diagnostic de la phase nerveuse de la Trypanosomiase Humaine Africaine. Bull. Soc. Path. Ex. 90 (5): 321-326.

Boullerne AI, Petry KG, Meynard M, Geffard M (1995). Indirect evidence for nitric oxide involvment in muliple sclerosis by characterization of circulating antibodies directed against conjugated S-nitrocysteine. J. Neuroimm 60: 117-124.

Büscher P, Draelants E, Magnus E, Vervoort T, Van Meirvenne N (1991). An experimental latex agglutination test for antibody detection in human African trypanosomiasis. Ann. Soc. Belge Méd. Trop. 55: 559-569.

Büscher P, Lejon V, Magnus E, Van Merveinne N (1999). Improved latex agglutination test for detection of antibodies in serum and cerebrospinal fluid of *Trypanosoma brucei gambiense* infected patients. Acta Trop. 73: 11-20.

Crawley MJ (1993). GLIM for Ecologists. Blackwell Science Ltd. Oxford, UK.

Doua F, Boa Y (1994). Actualités thérapeutiques de la trypanosomiase. Bull. Soc. Path. Ex. 87: 337-340.

Doua F, Miézan TW, Sanon Singaro JR, Boa Y, Baltz T (1996). The efficacy of pentamidine in the treatment of earlylate stage *Trypanosoma brucei gambiense* trypanosomiasis. Am. J. Trop. Med. Hyg. 54: 163-168.

Jamonneau V, Garcia A, Ravel S, Cuny G, Oury B, Solano P, N'Dri L, Sanon R, Frézil JL, Truc P (2002). Genetic characterization of *Trypanosoma brucei gambiense* and clinical evolution of human african trypanosomiasis in Côte d'Ivoire. Trop. Med. Int. Health. 7, 7: 610-621.

Lejon V, Büscher P, Sema NH, Magnus E, Van Meirvenne N (1998). Human African trypanosomiasis a latex agglutination field test for quantifying IgM in cerebrospinal. Bull. World Health Organ. 76, 6: 553-558.

Lejon V, Legros D, Richer M, Ruiz JA, Jamonneau V, Truc P, Doua F, Djé N, N'Siesi FX, Bisser S, Magnus E, Wouters I, Konings J, Vervoort T, Sultan F, Büscher P, (2002). IgM quantification in the cerebrospinal fluid of sleeping sickness patients by a latex card agglutination test. Trop. Med. Int. Health. 7, 8: 685-692.

Lumsden WHR, Kimber CD, Evans DA, Doig SJ (1979). *Trypanosoma brucei*: Miniature anion-exchange centrifugation technique for detection of low parasitemias; adaptation for field use. Trans. R. Soc. Trop. Med. Hyg. 73: 312-317.

Magnus E, Vervoort T, Van Meirvenne N (1978). A card-agglutination test with stained trypanosomes (CATT) for the serological diagnosis of *T. gambiense* trypanosomiasis. Ann. Soc. Belge Méd. Trop. 59: 169-176.

McCullagh P, Nelder JA (1989). Generalized linear Models. 2nd edition. Chapman & Hall Ltd. London, UK.

Okomo-Assoumou MC, Geffard M, Daoulouede C, Chaugier C, Lemesre JL, Vincendeau P (1995). Circulating antibodies directed against Tryptophan-like epitopes in sera of patients with Human African Trypanosomiasis. Am. J. Trop. Med. Hyg. 52 (5): 461-7.

Reiber H (1998). Cerebrospinal fluid-physiology, analysis and interpretation of protein patterns for diagnosis of neurological diseases. Mult. Sclerosis. 4: 99-107.

Reiber H, Felgenhauer K (1987). Protein transfer at the blood cerebrospinal fluid barrier and the quantitation of the humoral immune response within the central nervous system. Clin. Chim. Acta. 163: 319-28.

Truc P, Jamonneau V, Cuny G, Frézil JL (1999). Use of polymerase chain reaction in human African trypanosomiasis stage determination and follow-up. Bull. World Health Organ 77, 9: 745-48.

Truc P, Ravel S, Jamonneau V, N'Guessan P, Cuny G (2002). Genetic variability within *Trypanosoma brucei gambiense* and co-infections by different genotypes in Human African Trypanosomiasis patients in Côte d'Ivoire. Trans. R. Soc. Trop. Med. Hyg. 96 (1): 52-55.

Van Nieuwenhove S (1999). Present strategies in the treatment of human African trypanosomiasis. In : "Progress in Human African Trypanosomiasis sleeping sickness". Editors M Dumas, B Bouteille, A Buguet, Springer Publ. Paris. pp. 253-280.

Wilkinson L, Hill M, Vang E (1992). Sysqtat for the Macintosh. Version 5.2. Evanston, IL. USA.

World Health Organization (1998). Control and surveillance of African trypanosomiasis. WHO Technical Series. 881: 113.