J Neurol (2001) 248 : 672–675

ORIGINAL COMMUNICATION

Silver stained isoelectrophoresis of tears and cerebrospinal fluid in multiple sclerosis

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Received: 30 August 2000
Received in revised form:
15 December 2000
Accepted: 19 February 2001

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Abstract Cerebrospinal fluid (CSF) analysis aids in the diagnosis of multiple sclerosis. However, this examination is invasive. The aim of this study was to assess the potentials of a new method of tears isoelectrophoresis (IEF). Silver staining of IEF was used to examined tears and CSF from 123 patients including 60 patients with multiple sclerosis (MS), 50 other neurological patients and 13 patients with inflammatory neurological diseases. Tears were collected on a Shirmer strip placed in one eye, avoiding reflex secretion. This method of IEF with silver staining allowed the detection of oligoclonal bands in tears that were truly immunoglobulin G on immunofixation. The concordance rate between tears and CSF was 83 %, meaning that CSF provided no more information than tears analysis in 83 % of cases. Sensitivity in tears (72 %) and CSF (75 %) was very close as was specificity (respectively 84 % and 86 %). High concordance between tears and CSF is the first step in developing a non invasive test which could replace lumbar puncture, particularly when this procedure is not fearalele or is refused by the patient.

Key words Oligoclonal bands · Multiple Sclerosis · Tears · Cerebrospinal fluid

Introduction

Evidence of oligoclonal bands (OCB) of immunoglobulin G (IgG) in cerebrospinal fluid (CSF) is the most important biological criterion in the diagnosis of multiple sclerosis (MS) [3, 9]. This technique is very sensitive because OCB are found in about 90 % of patients with MS but it requires a lumbar puncture [2, 8, 11]. OCB are defined as discrete populations of IgG with restricted heterogeneity on isoelectrophoresis (IEF). Tears contain a variety of proteins, including locally produced antibodies. Indeed, Coyle revealed the presence of IgG bands in MS tear samples [3]. In addition, tears are an accessible external secretion, which can be collected easily, without the disadvantages of lumbar puncture. However, studies have reported the exceptional presence of IgG bands in tears [3, 4, 6, 7, 8]. This discrepancy could be explained by the different methods used. It seemed of interest to re-evaluate the occurrence of OCB in tears in order to assess the performance of IEF of tears and compared with CSF IEF in MS patients and control patients. The study was carried out in the north of France which is a high frequency area of MS (prevalence: 30 per 100 000) [5]. The concordance rates analysis between tears and CSF in MS patients and control patients is the first necessary condition in the assessment of tears analysis as a possible new diagnostic tool.

Materials and methods

Subject

We examined tears and CSF from 128 selected patients. Three MS patients and 2 patients with systemic disease showing OCB in serum were excluded from the study to avoid uninterpretable tears and CSF

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Tab. 1 Presence of oligoclonal bands (OCB) in tears and CSF according to the aetiologies of the control group. Associated CSF inflammatory signs, including the presence of a pleocytosis and an albumin increase are represented by "+". The patient with the Guillain-Barré syndrome was the only one to display an isolated albumin increase.

Control group n=63	Number	OCB in tears	OCB in CSF	Inflammatory signs in CSF
Brainstem ischaemia	2	–	–	–
Spinal chord ischaemia	2	–	–	–
Meningeal haemorrhage	2	–	–	–
Vascular dementia	2	–	–	–
Alzheimer's disease	3	–	–	–
Cognitive disorder	1	+	–	–
"	1	–	–	–
Parkinson's disease	3	–	–	–
Supranuclear palsy	1	–	–	–
Epilepsy	2	–	–	–
Cervicarthritic myelopathy	2	–	–	–
Facial palsy	2	–	–	–
"	1	–	+	–
Neuropathy	2	–	–	–
"	1	+	–	–
Acute headache	4	–	–	–
"	1	+	–	–
Amyotrophic lateral sclerosis	1	–	–	–
Wernicke's syndrome	3	–	–	–
Psychological disorder	7	–	–	–
Horner's syndrome (no aetiology)	1	+	+	-
Carotid dissection	1	+	–	–
"	1	–	+	–
Normal pressure hydrocephalus	3	–	–	–
"	1	+	+	–
Inflammatory subgroup (n=13)				
Viral meningitis	1	–	–	–
"	1	–	+	+
Herpetic meningoencephalitis	1	+	+	+
"	1	–	–	+
Guillain-Barré syndrome	1	–	–	albumin increase
Encephalitis (no aetiology)	1	+	+	+
Antiphospholipid syndrome	2	–	–	–
Sjögren's disease	1	–	+	+
"	1	–	–	–
"	1	+	–	+
Post-vaccinal encephalopathy	1	–	–	+
Collagen disease with neuropathy	1	–	–	–

IEF. One hundred and twenty three subjects were studied. 60 MS patients and 63 other neurological patients including 13 with inflammatory neurological diseases. There were no oriental patients who have less frequent OCB compared with western patients [14]. The MS group [12] included 37 women and 23 men aged from 18 to 62 years (mean 36.8). The patients had clinically definite MS or clinically probable MS according to the Poser's criteria [12]. The mean disease duration was 3.7 years (range from 1 month to 17 years). Twenty four patients had less than one year of disease. Of the 60 MS patients, 44 had relapsing remitting MS (RR), nine had primary progressive MS (PP) and 7 had secondary progressive (SP) MS. Fifty three patients were in relapse during analysis; 4 early MS and 3 RR MS were in remission. Only four patients were being treated at time of the analysis (2 by interferon ß 1 b and 2 by cyclophosphamide). Tears were collected from the optic neuritis-free eye except for 2 patients with bilateral optic neuritis and 10 patients with a history of optic neuritis. The optic neuritis-free eye status was based on normal clinical examination and visual evoked potentials. The control group was composed of 37 women and 26 men. Aetiologies are summarised in table 1. No patient had hard contact lens [1] or ocular infection or uveitis especially in the inflammatory group. All patients gave their informed consent prior to their inclusion. The study was approved by the local ethics committee.

Tears collection

A one-eye collection of 10 to 25 µL of tears was made on a Shirmer strip filter paper band of Whatman placed in the lower cul de sac of the eye. The collection of tears was completed within one minute to avoid the reflex secretion, (which dilutes the sample) and was then extracted with 50 µl physiological water.

Tears IEF

Proteins in a 30-µL volume of the sample were separated on agarose isoelectric focusing gels (pH 3.5 to 9.5) for 35 minutes at 1200 volts (Resolve-CSF, Isolab Inc) and silver stained. Because of the small sample volume, we chose to deposit a constant volume of 30 µl. OCB, defined by multiple bands (5 to 15) of migration of IgG, that were expected between 8.0 and 9.5 pH [9] (figure 1). IEF was repeated in the tears of 10 patients and 10 controls, randomly chosen, to assess the reproducibility.

Serum and CSF IEF

The CSF volume used for IEF depended on the IgG concentration measured. All MS patients and controls had simultaneously serum, tears and CSF analysis. Serum, CSF and tears of 15 MS patients, randomly chosen, were also analysed with IEF and immunofixation to identify the OCB as Ig. All IEF were read in a blind fashion by the same observer (GF).

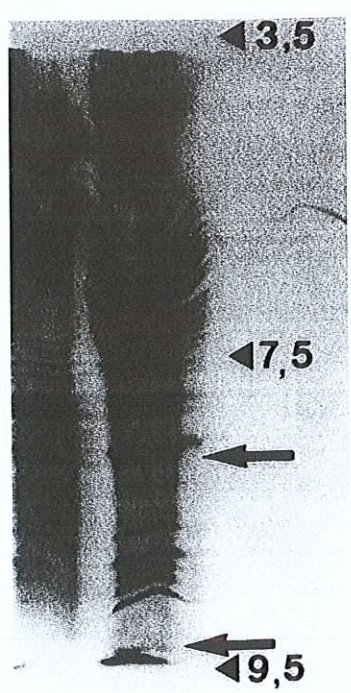

Fig. 1 Typical OCB pattern on IEF in the reading area (between 7.5 and 9.5) in undiluted tears (right column) compare with absence of OCB in diluted (1/100) serum (left column)

Statistics

First we calculated the concordance rate and the chi 2 Mac Nemar systematical bias in the whole population and for each group. Then we assessed sensitivity and specificity. The discriminating capacity of OCB in CSF or tears was calculated from scoring informative capacity for each group [13, 15]. The scoring informative capacity is the performance of a marker indicating the capability of a marker to discriminate between normal and abnormal groups. The numbers of individuals in both groups were not based on a power analysis but were chosen to obtain balanced numbers for comparison. The early MS patients and the MS patients with optic neuritis were compared with other MS patients using χ 2 test.

Results

Out of the 123 patients, 102 had the same pattern of OCB in tears and CSF (48 MS patients and 54 controls) revealing a concordance between tears and CSF of 83 % in the MS group and 86 % in the control group (table 2). The Kappa coefficients were for the MS group and the control group 0.53 and 0.56 respectively. Twenty three MS patients (38 %) displayed pleocytosis (< 50 cells) in the CSF, 25 (41 %) had albumin increased (< 1 g /l) and 42 (70 %) had an increased Delpech ratio. No correlation was found between the presence of inflammatory signs in CSF and the presence of OCB. The control group results are shown in table 1. The systematical bias was not significant in either group. There was no difference between each subgroup of MS (RR, SP, PP). The repetition of IEF in the tears of 10 MS patients and 10 controls displayed the same results. The 15 re-analysed CSF specimens of the randomly chosen MS patients using IEF and immunofixation had revealed that OCB were truly Ig G. Positive OCB were present in the CSF of 75 % of the MS patients and in the tears of 72 % of the MS patients (table 3). The specificity was 84 % in tears and 86 % in CSF (table 3) and respectively 88 % and 92 % when MS group was compared with controls without the 13 patients with inflammatory diseases. The efficiency percentage was 78 % in tears and 80 % in CSF.

There was no significant difference between early and long duration MS. The 2 MS patients with acute optic neuritis had both OCB in CSF and tears from the optic neuritis free eye. There was no significant difference between patients with or without history of optic neuritis.

Tab. 2 Distribution of the presence or absence of oligoclonal bands (OCB or NOCB) in tears and CSF, allowing concordance rates assessment in MS patients and controls.

MS	OCB	NOCB
Tears	43	17
CSF	45	15
Controls	**OCB**	**NOCB**
Tears	10	53
CSF	9	54

Tab. 3 Mean percentages and standard deviations of sensitivity and specificity of IEF in tears and CSF.

	Tears	CSF
Sensitivity	72 % (± 4.7)	75 % (± 4.6)
Specificity	84 % (± 3.4)	86 % (± 3)
Scoring information capacity	75	91

Discussion

We demonstrated that the detection of OCB in tears is of possible interest. OCB can be detected in tears in more than 70 % of MS patients. This result is similar to the previous study of Coyle [3], which revealed the presence of OCB in tears with IEF and silver staining, but contradicts Mavra's study and Liedtke's study, which reported no OCB in MS patient's tears, analysed by IEF with immunoperoxidase staining. Our results were very clear in 123 patients, which indicated good reliability of our IEF method with silver staining. This method was reproducible in tears and the immunostaining revealed that it was truly Ig G. The negativity of some studies [6, 8] could be explained by the higher volume of tears, which were collected by reflex secretion, diluting the sample. Samples were from 25 to 250 µl in Mavra'study and from 30 to 700 µl in Liedtke'study while our samples did not exceed 30 µl. These studies [6, 8] to collected tears in the same way, using capillary tubes. Other studies have reported that OCB were occasionally detected in tears but besides the different methods of IEF, the samples were small including only 7 patients [4], and 18 patients [7]. Our patients had a tear collection with a Shirmer strip. The Shirmer strip seemed to be more practical than capillary tubes. With a collection of one minute, it did not lead to reflex secretion. Besides the volume of collection, controversial results about detection of OCB in tears [3, 4, 6–9] could also be explained by the different methods of IEF used. The staining by silver or immunoperoxidase could explain a difference of sensitivity, possibly higher with immunoperoxidase staining [8]. We used a 30-µL volume of the sample which was separated on agarose isoelectric focusing gels (pH 3.5 to 9.5) for 35 minutes at a high voltage of 1200 volts but we did not assess the methods which lead to a negative result to emphasize the points of discrepancy. Besides possible technical discrepancies of the different IEF and staining, the type of immunoglobulins G detected might be different according to the biological fluid tested. Indeed it was shown in a MS study assessing biological markers, such as interleukin-2, OCB of Ig G, light Kappa and lambda chains

and basic myelin protein that the biological activity was different according to the fluid tested (CSF, tears, plasma, saliva and urine) [10].

The detection of OCB in 72 % of MS tears raises questions about the source of Ig in MS tears. Evidence of local production of Ig G bands has been provided within normal tears. Indeed it has been suggested that activated T-cells can cross the blood tears barrier like the blood brain barrier [3]. But this supposes a local reactivation of T-cells and the presence of the specific auto-antigen in the lachrymal glands [3]. It should be remembered that the eye and nervous system tissues have the same embryological origin.

We demonstrated the presence of OCB in tears from the beginning of the disease with or without a history of optic neuritis. The high concordance of 83 % between tears and CSF suggest that in 83 % of cases we obtained no more diagnostic information by performing a lumbar puncture. The scoring informative capacity was also close between tears and CSF. A good performance and a high concordance are first necessary conditions in the assessment of tears analysis in the diagnosis of MS. No significant systematical bias was displayed, suggesting a tendency of independence or complementarity between both analyses. A concordance of 86 % in controls and a tears analysis specificity close to 90 % could suggest that CSF analysis might be avoided if OCB were present in tears. However, lower sensitivity could required a CSF analysis if no OCB were displayed in the tears analysis. Before establishing a diagnosis strategy further independent studies are required. Sensitivity has to be improved to avoid missing the diagnosis as was shown in 7 MS patients having OCB in CSF and none in tears. So also for the specificity, with 3 patients of the non inflammatory group displaying OCB in tears and none in CSF. On the other hand tears analysis could avoid some false positive results of CSF analysis, such as the control patient with facial palsy and some false negative results, such as the five MS patients with a negative CSF analysis.

CSF sensitivity in our study was not as in high as previously reported studies [2, 11]. In order to assess the concordance between tears and CSF, patients with clinically definite and probable MS were both studied which could lead to a lower sensitivity of tears and CSF analyses in MS group. Indeed sensitivity is lower in probable MS (75 % [2]; 80 % [11]) than definite MS (95 % [2]; 91.7 % [11]). Moreover silver staining might be less sensitive than immunostaining [8]. In our study, the 15 tears and CSF analyses with immunostaining in MS patients disclosed OCB with both techniques of silver staining and immunostaining.

High concordance between tears and CSF could be the first step toward a new diagnosis strategy in MS. Tears analysis, less invasive with a Shirmer strip, could be at least helpful when lumbar puncture is not recommended or is refused by the patient.

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