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Detection of oligoclonal IgG bands in cerebrospinal fluid and serum: comparison between commercial immunofixation method and home-made affinity immunoblotting method and evaluation of interobserver agreement

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SUMMARY

Background: We compared two different agarose isoelectric focusing methods for detection of oligoclonal IgG bands in cerebrospinal fluid and serum: commercial method with immunofixation (Sebia) and home-made method using Multiphor II apparatus followed by affinity immunoblotting. Interobserver agreement for both methods was tested concerning the presence of intrathecal IgG synthesis, the detailed isoelectric focusing pattern type, the number of CSF-restricted oligoclonal IgG bands, and the number of oligoclonal IgG bands in CSF and in serum.

Findings: Using kappa statistics for evaluation of agreement, we found there was very good agreement concerning the presence of intrathecal IgG synthesis (kappa 0.870 to 1.000 between methods, and 0.947 and 0.920 between observers, respectively, representing 0 to 5 out of 114 samples classified differently). The agreement was less pronounced when international consensus classification of isoelectric focusing patterns into 5 different types was taken into account (kappa 0.389 to 0.596 between methods); using home-made method, the interobserver agreement regarding pattern type was worse (kappa 0.478) than using commercial Sebia method (kappa 0.791). There was moderate agreement on the number of CSF-restricted oligoclonal IgG bands, and mostly poor agreement on the number of oligoclonal IgG bands in CSF and serum.

Conclusions: Both methods were capable to detect oligoclonal IgG reliably, and neither method could be evaluated as superior to the other. However, better interobserver agreement regarding to the pattern type was obtained using commercial Sebia immunofixation method. Rather poor reproducibility of oligoclonal IgG bands numbering should be known to clinicians, since it entails the risk of potentially misleading interpretations.

Keywords: oligoclonal IgG bands, different agarose isoelectric focusing methods, cerebrospinal fluid

SOUHRN

Nováčková L., Zeman D.: Detekce oligoklonálních IgG pásů v likvoru a séru: srovnání komerčně dostupné imunofixační metody s „home-made“ metodou s afinitním imunoblottingem a posouzení shody mezi hodnotícími

Úvod: Byly srovnány dvě různé metody izoelektrické fokusace v agarosovém gelu pro detekci oligoklonálních IgG pásů v likvoru a séru: komerční metoda s imunofixací (Sebia) a „home-made“ metoda s použitím přístroje Multiphor II následovaná afinitním imunoblottingem. Byla posouzena shoda mezi metodami i hodnotícími, pokud jde o přítomnost intrathékální syntézy IgG, zařazení nálezu do jednoho z pěti typů podle mezinárodní klasifikace, počet intrathékálně syntezovaných IgG pásů a počet pásů v likvoru a v séru.

Výsledky: Pro posouzení shody jsme použili statistiku kappa. Pokud jde o přítomnost intrathékální syntézy IgG, byla shoda velmi dobrá (kappa 0,870 až 1,000 mezi metodami a 0,947 a 0,920 mezi hodnotícími, což představuje 0 až 5 neshodně hodnocených nálezů z celkového počtu 114). V zařazení nálezu do jednoho z pěti typů podle mezinárodní klasifikace byla shoda méně vyjádřená (kappa 0,389 až 0,596 mezi metodami); při použití „home-made“ metody byla shoda mezi hodnotícími horší (kappa 0,478) než v případě komerční imunofixační metody (kappa 0,791). Shoda v počtu intrathékálně syntezovaných IgG pásů byla jen mírná, shoda v počtu oligoklonálních IgG pásů v likvoru a v séru byla slabá.

Závěry: Oběma metodami lze spolehlivě detekovat oligoklonální IgG a metody lze považovat za rovnocenné. Pro klasifikaci typu nálezu však byla shoda mezi hodnotícími lepší při použití komerční imunofixační metody. Poměrně špatná reprodukovatelnost počítání oligoklonálních IgG pásů by měla být známa klinickým lékařům, protože porovnávání počtu pásů s sebou přináší potenciální riziko zavádějících interpretací.

Klíčová slova: oligoklonální pásy IgG, různé metody izoelektrické fokusace na agaróze, mozkomíšní mok

Introduction

Intrathecal synthesis of IgG, revealed preferably by detection of oligoclonal IgG bands (IgG OCB) in cerebrospinal fluid not present in the corresponding serum using isoelectric focusing (IEF), is an important diagnostic marker in multiple sclerosis (MS) as well as other inflammatory CNS diseases. There is a consensus that IEF followed by specific IgG detection should be used [1-4]; nevertheless, there are many ways how to perform the test, including commercial in vitro diagnostic kits (Sebia or Helena) as well as home-made methods. It is not clear whether the results obtained depend more on the method or on the evaluation [5]. While the demon-stration of intrathecal IgG synthesis has well--proven clinical significance [3, 4, 6], reliability and diagnostic significance of the detailed IEF pattern type [5, 7] or the number of bands in CSF and serum [5, 8] have been less well examined. In a similar previous study [7], the authors compared nearly identical IEF methods for

IgG OCB detection. We wished to compare two different agarose IEF methods, with which we had previous experience: commercial agarose IEF with subsequent immunofixation and home-made agarose IEF on Multiphor II apparatus, followed by affinity immunoblotting. For both methods, we also assessed interobserver agreement on the evaluation of results.

Material and methods

114 consecutive paired CSF and serum samples sent to our laboratory for oligoclonal IgG detection by „routine" Sebia immunofixation method (Sebia/IF) were examined in parallel by home-made agarose IEF on Multiphor II followed by affinity immunoblotting (Multiphor/AIB). Albumin and IgG concentrations were measured on Beckmann Coulter Immage nephelometer. Intrathecal IgG synthesis was estimated quantitatively by Reiber´s hyperbolic formula [9]. IgG index was also calculated as Q-IgG/Q-Albumin, values > 0.7 believed to reflect intrathecal IgG synthesis. The same amounts of IgG in diluted paired CSF and serum samples were applied, side by side, on the gel (300 ng IgG per position for Sebia/IF and 30 ng IgG per position for Multiphor/AIB).

Sebia Hydragel 9 CSF isofocusing (Ref. 4353) was performed according to manufacturer´s instructions (except the amount of sample applied was 15 µl) by experienced laboratory technician.

Agarose IEF on Multiphor II with subsequent „affinity" immunoblotting was performed according to our unpublished modification based on the methods of Knisley and Rodkey [10], Keir et al. [11], Kaiser [12], and Sindic and Laterre [13]. Briefly, nitrocellulose (NC) membrane was covered with anti-IgG (H+L) antibody (AbD Serotec) diluted in Tris-buffered saline (TBS) pH 7.5 to 20 mg/l and incubated for 5-8 hours at room temperature. IEF was performed on home-made agarose gels (1.1 % agarose IEF, 12 % D-sorbitol, 6.2 % of Pharmalyte 3-10, 1.7 % of Pharmalyte 8-10.5) using Multiphor II apparatus (limit values: 1700 V, 150 mA, and 10 W; interelectrode distance: 8.5 cm; cathodic solution: 0.5M NaOH, anodic solution: 0.04M H_2SO_4). After starting the IEF run, the NC membrane was rinsed in TBS and blocked by 3% bovine serum albumin (BSA) in TBS for 45-60 minutes, and rinsed in TBS again just before the end of the IEF run. After IEF, the gel was briefly touched by filter paper moistened in TBS before the membrane was applied, followed by 1 sheet of moistened filter paper, 6-8 sheets of dry filter paper, a glass plate, and a weight. After 50 minutes, the membranes were rinsed in TBS, re-blocked in 0.3 % BSA in TBS, and again rinsed in TBS, incubated with anti-human IgG (Fc) antibody labelled with alkaline phosphatase (AbD Serotec, diluted 1:800) for 75 minutes, washed 3 times in TBS containing 0.05 % Tween 20 (Serva) and 2 times in TBS. Finally, BCIP/NBT detection solution (Vector Laboratories) was mixed and applied for 15-20 minutes, the membrane was rinsed in TBS and deionized water, and left to dry.

Results were read independently by both of us in a blinded fashion. Positivity of intrathecal IgG synthesis, IEF type (1-5) according to international classification, number of bands in CSF and in serum, and number of CSF-restricted bands (i. e., those present only in CSF or those that were considerably stronger in CSF compared to serum) were recorded. According to recommendations of the German society of CSF diagnostics and clinical neurochemistry [14], the presence of 1 CSF-restricted IgG OCB only was evaluated as type 1. Cases with recognazible migrational microheterogeneity of IgG (discussed below in the text) were classified as type 4 if CSF and serum pattern was the same, but as type 2 if this pattern was seen in CSF only.

Statistical analysis was performed using MedCalc software version 11.4 (Frank Schoonjans, Belgium). Kappa statistics was used for the evaluation of agreement.

Results

There was good agreement with respect to the presence of intrathecal oligoclonal IgG synthesis, defined as ≥ 2 bands in CSF absent (or clearly less pronounced) in the corresponding serum, both between methods and between observers (0 to 5 out of 114 samples classified differently) (Table 1). Analysis of five disagreements on the presence of intrathecal oligoclonal IgG synthesis revealed a maximum interobserver difference of 3 intrathecally synthesized oligoclonal bands (Table 2).

With respect to IEF pattern type according to consensus classification [1], interobserver agreement was good for Sebia/IF method but moderate only for Multiphor/AIB method. Likewise, agreement between methods was only moderate in this respect (Table 1).

There was moderate agreement in counting of „CSF-restricted" IgG OCB, although differences were mostly small, as reflected by substantially higher weighted kappa values (Table 1). The agreement in counting CSF IgG OCB or serum IgG OCB was rather poor (interobserver agreement using Sebia/IF: kappa 0.412 for CSF OCB and 0.419 for serum OCB; for interobserver agreement using Multiphor/AIB as well as for agreement between methods, all kappa values were < 0.30). Again, better agreement was achieved using Sebia/IF method.

Quantitative measures of intrathecal IgG synthesis correlated with OCB positivity. Taken Sebia/IF evaluated by observer 1 as a „gold standard", diagnostic sensitivities of the IgG index (cut-off 0.7), Reiber formula, and Reiber formula using 10% intrathecal fraction as cut-off were 60.9 %, 60.9 %, and 43.5 %, respectively; diagnostic specificities were 94.5 %, 93.4 %, and 100 %, respectively.

Discussion

Concerning the presence or absence of intrathecal IgG synthesis, agreement between methods as well as between observers was fully satisfactory and, perhaps except for 1 case (case 5 in Table 3, case 2 in Figure 1),

Table 1: Agreement on the presence of intrathecal oligoclonal IgG synthesis, IEF pattern type, and number of CSF-restricted IgG OCB

	Presence of intrathecal IgG synthesis	IEF pattern type	Number of CSF-restricted IgG OCB	
	Kappa (95% CI)			Weighted kappa (95% CI)
Sebia/IF, inter- observer	0.947 (0.875 to 1.000)	0.791 (0.694 to 0.888)	0.519 (0.414-0.624)	0.923 (0.894-0.952)
Multiphor/AIB, inter-observer	0.920 (0.830 to 1.000)	0.478 (0.359 to 0.597)	0.493 (0.399-0.587)	0.853 (0.798-0.908)
Sebia/IF vs. Multiphor/AIB, same observer	1.000 (1.000 to 1.000)	0.596 (0.462 to 0.729)	0.475 (0.406-0.545)	0.824 (0.775-0.874)
	0.870 (0.759 to 0.981)	0.389 (0.256 to 0.523)	0.408 (0.330-0.486)	0.794 (0.738-0.851)
Sebia/IF vs. Multiphor/AIB, different observers	0.920 (0.830 to 1.000)	0.411 (0.283 to 0.539)	0.413 (0.348-0.477)	0.779 (0.716-0.842)
	0.947 (0.875 to 1.000)	0.499 (0.366 to 0.633)	0.423 (0.346-0.501)	0.804 (0.750-0.859)

Table 2: Analysis of disagreements concerning the presence of intrathecal oligoclonal IgG synthesis

Case	Sebia/IF				Multiphor/AIB			
	Observer 1		Observer 2		Observer 1		Observer 2	
	type	ith OCB	type	ith OCB	type	ith OCB	type	ith OCB
1.	1	1	2	3	1	0	4	0
2.	1	0	2	2	1	0	1	0
3.	1	0	1	1	1	0	2	2
4.	1	0	1	0	1	0	3	2
5.	3	8	3	5	3	4	4	1

ith OCB, „intrathecal“, i.e. CSF-restricted, oligoclonal IgG bands (not present in serum or clearly more pronounced in CSF than in serum)
Please note that numbering of cases does not match numbering of cases in Figure 1 (case 5 in Table 2 corresponds to case 2 in Figure 1)

disagreement was present only in clearly borderline findings (2-3 weak CSF bands) that may be of uncertain significance [15-18]. The oppinion of Wurster [17] that only ≥ 4 bands should be regarded as definitely positive whether 2-3 bands might be better classified as „borderline“ seems reasonable, although the intensity of bands should be also taken into account (see Figure 1, case 3). Although Multiphor/AIB method seems to be more sensitive analytically (10 times less IgG applied compared to Sebia/IF method for optimal staining intensity); this did not result in better oligoclonal IgG detection.

Agreement concerning IEF pattern type was less satisfactory. Namely, the differentiation between „type 1“ and „type 4“, as well as between „type 2“ and „type 3“, was difficult in some cases (Table 3). Significance of serum IgG OCB is much less clear than that of intrathecally synthesized IgG OCB. Serum IgG OCB detection is believed to reflect systemic immune activation [19]; however, this can occur in many instances and does not provide any definitive diagnostic information. IEF is a very sensitive method and „polyclonal IgG“ consists of a large number of monoclonal immunoglobulins [20] and does not represent exactly equal amounts of undefinite number of IgG molecules with infinitesimal differences in their isoelectric points. Every individual is confronted with different antigens during her/his life and has her/his own pattern of „polyclonal“ IgG, where some clones may be overrepresented to some extent.

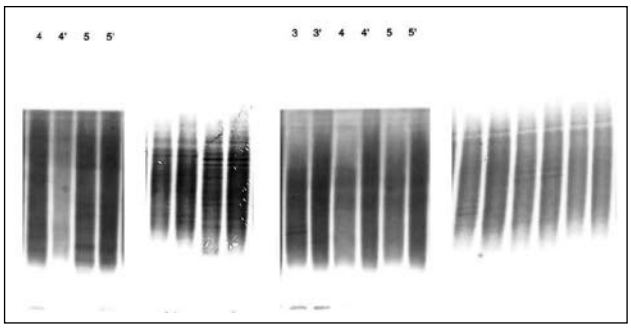

Figure 1. Comparison among several Sebia/IF (with numbers denoting positions in Sebia gels) and corresponding Multiphor/AIB results.

Two paired CSF and serum samples on the left: The first (case 1) was evaluated as type 4 by both observers and both methods; the second (case 2) was evaluated as type 3 on Sebia/IF by both observers, but (apparently mistakenly) as type 4 on Multiphor/AIB by observer 2; observer 1 evaluated this Multiphor/AIB pattern (correctly) as type 3.

Three paired CSF and serum samples on the right: The first (case 3) was evaluated as type 2 by both observers on Multiphor/AIB, and as type 2 by observer 1 and as type 3 by observer 2 on Sebia/IF; nevertheless, intrathecal IgG synthesis could be clearly detected by both methods, although only a few bands are visible. The second sample (case 4) was evaluated as type 4 by both observers on Multiphor/AIB, but as type 1 by observer 1 and as type 4 by observer 2 on Sebia/IF. The third sample (case 5) was evaluated as type 1 by both observers using either method.

Anode is at the top.

Table 3. Interobserver agreement on IEF pattern type

Sebia/IF						
Observer 2: IEF pattern – type	Observer 1: IEF pattern – type					
	1	2	3	4	5	
1	56	0	0	0	0	56 (49.1 %)
2	2	12	0	0	0	14 (12.3 %)
3	0	3	8	0	0	11 (9.6 %)
4	10	0	0	22	0	32 (28.1 %)
5	0	0	0	0	1	1 (0.9 %)
	68 (59.6 %)	15 (13.2 %)	8 (7.0 %)	22 (19.3 %)	1 (0.9 %)	114
Multiphor/AIB						
Observer 2: IEF pattern – type	Observer 1: IEF pattern – type					
	1	2	3	4	5	
1	42	0	0	3	0	45 (39.5 %)
2	1	8	0	0	0	9 (7.9 %)
3	1	11	3	0	0	15 (13.2 %)
4	23	0	1	20	0	44 (38.6 %)
5	0	0	0	0	1	1 (0.9 %)
	67 (58.8 %)	19 (16.7 %)	4 (3.5 %)	23 (20.2 %)	1 (0.9 %)	114

We believe that decision about the presence of IgG OCB is rather arbitrarily made in cases where the non-homogeneity of the polyclonal background exceed a certain level, namely, when sharp, clearly visible bands are present. It is known that artefactual bands may occur, and these could be recognized by their occurrence in more neighbouring positions of the same run. However, possible presence of minimal „physiological" IgG microheterogeneity is not appreciated enough. The term „migrational microheterogeneity of IgG" is used in our laboratory to describe the presence of weak and/or less sharp bands than „typical" oligoclonal IgG bands; these bands are not counted. Usually, this pattern is the same in CSF and serum and is classified as type 4; however, in 1 case in our series this pattern was visible in CSF only, which means intrathecal synthesis.

Our results indicating poor agreement in counting IgG OCBs are in accordance with those of Franciotta et al. [5, 8]. Further, in our study counting of CSF-restricted IgG OCB was more reliable than counting of CSF and serum IgG OCB. Poor agreement in counting CSF and serum bands suggest it may be unreliable and even misleading to directly compare the number of these bands among pooled patient populations in multicenter studies, as well as in cases of repeated lumbar puncture, especially if CSF analyses were performed in different laboratories. If sequential CSF analyses are performed in our laboratory, we use direct visual comparison of both IEF patterns and report whether there is any significant difference.

Finally, quantitative measures of intrathecal IgG synthesis were relatively insensitive compared to the „gold standard" of IgG OCB detection; false-positive quantitative results were also observed, except for Reiber´s formula with a „cut-off" > 10 % intrathecal fraction that had exclusive specificity for intrathecal IgG synthesis.

Conclusions

The most reliable finding was the presence or absence of intrathecal oligoclonal IgG synthesis, which is also most significant clinically. The discrimination among 5 types of IEF patterns was more problematic. Both methods of agarose IEF (Sebia/IF and Multiphor/AIB) gave similar results; it was not possible to claim superiority of one method over the other concerning detection of intrathecal IgG synthesis, but better inter-rater agreement concerning IEF pattern type was obtained using Sebia/IF than with Multiphor/AIB method. There was only moderate agreement in counting CSF-restricted OCB, and rather poor agreement in counting OCB in CSF and serum. This may suggest that number of OCB should not be compared in studies pooling results from different laboratories. Likewise, in cases of repeated lumbar puncture, number of OCB should not be compared by the clinician; rather, direct visual inspection of both IEF patterns should be preferred.

List of abbreviations

AIB – affinity immunoblotting
BCIP/NBT – bromo-chloro-indolylphosphate/nitroblue-tetrazolium
BSA – bovine serum albumin
CI – confidence interval
CSF – cerebrospinal fluid
IEF – isoelectric focusing
IF – immunofixation
IgG – immunoglobulin G
NC – nitrocellulose
OCB – oligoclonal band(s)
Q-Albumin – albumin quotient (Albumin-CSF/Albumin--Serum)
Q-IgG – IgG quotient (IgG-CSF/IgG-Serum)
TBS – tris-buffered saline

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