The New Reference Material CRM 470 for Standardization of Immunoassays for Plasma Proteins*

Das neue Referenzmaterial CRM 470 für die Standardisierung von Immunoassays für Plasmaproteine*

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Keywords: Plasma Proteins/cerebrospinal fluid: Calibration: Cerebrospinal Fluid; Reference Standards.

Schlüsselwörter: Kalibration; Liquor cerebrospinalis; Plasmaproteine/Liquor cerebrospinalis; Referenzmaterial.

In the last years many different automated systems for the measurement of proteins in serum, urine and cerebrospinal fluid have been introduced by various manufacturers. The methods which were mainly based on immunonephelometric and immunoturbidimetric techniques gave rather different results, especially because the reagents, protein standards and controls used in the assays were not properly standardized [1]. International reference materials for plasma proteins are "theoretically" available through the World Health Organization, but the calibrants produced by diagnostic manufacturers differed significantly in their assigned values [2].

Recently, the situation concerning the measurement of plasma proteins and the possibility of a better clinical use has improved substantially because of the preparation of a new reference material for plasma proteins by the Committee on Plasma Protein Standardization of the International Federation of Clinical Chemistry (IFCC) [3].

After the release in 1993 in Europe by the Community Bureau of Reference of the European Commission (BCR) of a "Certified Reference Material for Immunochemical Measurements of 14 Human Serum Proteins" (CRM 470) and in 1994 in USA by the College of American Pathologists (CAP) of the same material as "Reference Preparation for Proteins in Human Serum" (RPPHS Lot No. 5) an optically clear reference material for 14 proteins in serum is now available for world-wide use by manufacturers, professional organizations and laboratories [4, 5, 6]. In the meantime, Japanese and Australian institutions have also accepted this material as reference preparation for their respective countries. CRM 470 is intended to be used as a matrix-based reference material for 14 proteins (α1-acid-glycoprotein, albumin, α1-antitrypsin, C3, C4, C-reactive protein, coeruloplasmin, haptoglobin, IgA, IgG, IgM, α2-macroglobulin, prealbumin and transferrin) for transfer of the certified values to tertiary materials (calibrants and controls) for immunoassays of proteins in various body fluids via a well-defined protocol [4].

The serum used for the reference materials was obtained by pooling blood of healthy donors from 5 different countries. The processing of the serum pool (preservation, stabilization, depilidization, filling and lyophilization) was carried out by Behringwerke AG in its manufacturing facilities in Marburg/Germany [7]. Protocols for value transfer for 14 proteins were established for immunonephelometry on the Behring Nephelometer Analyzer (BNA) and for immunoturbidimetry on the Cobas-Bio/Cobas-Fara and Hitachi 704/717 by different reference laboratories in Europe and USA using international reference preparations as well as purified proteins. Because a reference method is lacking, the radial immunodiffusion has been used as "consensus method" since it is less influenced by the matrix and by certain interferring substances.

Preliminary consensus reference ranges for 14 proteins in serum on the basis of the standardization against the IFCC/BCR/CAP Reference Material (CRM 470) have been in the meantime established and accepted by several professional societies and diagnostic companies [8, 9].

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Nonstandard abbreviations: BCR, Community Bureau of Reference of the European Commission; CAP, College of American Pathologists; CRM, certified reference material; IFCC, International Federation of Clinical Chemistry; RPPHS, Reference Preparation for Proteins in Human Serum.

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Quality Assurance and Sample Handling in Cerebrospinal Fluid Investigation*

Qualitätssicherung und Probenhandhabung bei Liquoruntersuchungen*

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Keywords: Cerebrospinal Fluid; Quality Control; Sample Handling.

Schlüsselwörter: Liquor cerebrospinalis; Probenhandhabung; Qualitätskontrolle.

A satisfactory quality assurance system is essential in all analytical methods, including CSF analysis. Quality must be maintained through the whole series of events, from the decision to do a lumbar puncture to the evaluation of the result. Not only a high analytical quality should be maintained but a professional approach to quality assurance in all these events.

Sampling

A good sampling technique is important for maintaining a high quality. Especially for CSF-IgM, contamination with serum may yield erroneous results. Erythrocyte count has commonly been used as an indicator of blood contamination but recent findings question this statement. Most proteins have a concentration gradient within the spinal canal and therefore a defined amount of CSF (e. g. 10 ml) should be collected.

Storage and transport

CSF should be collected and stored in polypropylene or siliconized glass tubes in order to avoid adsorption of proteins to the surface. Cells (i.e. erythrocytes and leukocytes) rapidly change their shapes after sampling, and they should be counted not later than 2 h after sampling. Malignant cells are more sturdy and can be sent to special laboratories.

At temperatures below 30 °C IgA and IgM may form aggregates that can sedimentate and may react dissimilarly in the assay than free protein. Freezing and thawing may damage proteins and should therefore be performed rapidly.

Analytical procedure

This part of quality assurance, called *Quality Control*, should be applied to all quantitative and qualitative methods. Every run should contain at least one internal control. External control samples should be analyzed periodically.

Cytological methods. Because of rapid change in cell morphology after sampling, no satisfactory quality control method has been developed so far. Efforts should be made to establish such a program.

Qualitative methods. A qualitative method (e. g. determination of oligoclonal IgG bands) has only two possible results: "presence" and "absence". Each run needs two internal controls, one with a known oligoclonal pattern (positive control) and one containing only polyclonal IgG (negative control).

Quantitative methods. Quality control should detect both systematic and random errors. Quantitative methods should be calibrated against a reference method, which in turn is calibrated against a definitive method. For proteins this means that they should be tracable to international standards such as CRM 470. Any systematic deviation from this standard should be known.

Formulae. Formulae are equations where the results of two or more CSF and serum analyses are combined. The analytical errors add to each other in a way that can be deduced mathematically. Different approaches should be considered if the formula includes: (1) transformations and constants, (2) differences, (3) quotients, or (4) more than two analytical results. Such additive effects of analytical errors should be considered when evaluating quality control for formulae.

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Standardization of Immunoassays for Measuring Cerebrospinal Fluid (CSF) Proteins with CRM 470: Consequences to Formulae for Calculation of Intrathecal IgG, IgA, and IgM Production*

Standardisierung von Immunoassays zur quantitativen Bestimmung von Liquor-Proteinen mit CRM 470: Konsequenzen für Formeln zur Berechnung einer intrathekalen IgG-, IgA- und IgM- Produktion*

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Keywords: Calibration; Cerebrospinal Fluid; Cerebrospinal Fluid Proteins/analysis; Immunoglobulins/cerebrospinal fluid; Models, Neurological; Reference Standards.

Schlüsselwörter: Kalibration; Liquor cerebrospinalis; Liquorproteine/Analytik; Immunglobuline/Liquor cerebrospinalis; Modelle, neurologische; Referenzmaterial.

Mechanized systems for the measurement of immunoglobulin concentrations in cerebrospinal fluid (CSF) have been introduced by several companies, however most of them yield varying values of IgG, IgA, and IgM in CSF and serum samples. Therefore, different results are expected when an intrathecal immunoglobulin production is calculated by formulae. One main cause is that diagnostic manufacturers used so far calibrants differing significantly in their assigned values (1,2).

Here exemplary results are compared which have been obtained by two immunonephelometric systems:
i. Array Protein System (Beckman Instruments GmbH München) measuring the rate of increase in light scatter using Beckman reagents and control samples;
ii. Laser Nephelometer BN 100 (Behringwerke AG, Marburg) measuring its forward-scattered light in a fixed time mode with reagents (latex-particle-amplified for IgA and IgM) and control samples from Behringwerke.

When comparing the results in CSF samples according to the procedure of Passing & Bablok [3] no concordance was found despite of low inaccuracy (< 9%): Compared with BN 100 data Beckman Array yielded in average higher IgG values (≈ 8%), but albumin, IgA, and IgM ones were lower (≈ 7%, ≈ 31%, ≈ 45%, respectively).

Therefore, both systems were standardized with the new calibrant CRM 470. 112 paired CSF/serum samples were analysed: With BN 100 IgG, IgA, IgM (but not albumin) contents decreased between 15 and 34%; with Beckman Array the values deviated around 1 to 6%. The variations of immunoglobulin and albumin values corresponded to the magnitude of assigned conversion factors for BN 100 and Beckman Array (data in parentheses): IgG: 0.85 (0.96); IgA: 0.83 (0.99); IgM: 0.67 (0.95); albumin: 1.00 (1.04).

Comparing the CRM 470 corrected values obtained with both systems in CSF samples, those for albumin, IgG and IgA varied around ±10% as can be expected from the CVs of the two methods. In the corresponding serum samples, however, differences were greater ranging between 10 and 20%. IgM concentrations in the same CSF samples measured by Beckman Array were distinctly higher than the BN 100 values (>40%), also in the corresponding serum samples up to 20%. This points to methodical differences.

Intrathecal immunglobulin (Ig) production was calculated by two common formulae with non-corrected and CRM 470 corrected Ig and albumin values of the 112 paired CSF and serum samples: the linear "index" formula (CSF-Ig/serum-Ig)/(CSF-albumin/serum-albumin) [4, 5] and *Reiber's* hyperbolic formula [6] corrected in 1994 [7, 8]: As expected, index values measured by both systems were similar with non-corrected and CRM 470 corrected values of IgG, IgA, IgM; but with the hyperbolic formula [7, 8] results with CRM

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470 corrected values of BN 100 decreased in the magnitude of assigned conversion factors indicated above when compared with those obtained with non-corrected values.

For comparison between BN 100 and Beckman Array, the fraction of positive results (intrathecal Ig production) of both formulae, only calculated with CRM 470 corrected values of the 112 paired CSF/serum samples, are presented here:
IgG index > 0.6: 5 for BN 100; 10 for Beckman Array;
IgG index > 0.7: 2 for BN 100; 2 for Beckman Array;
IgA index > 0.4: 34 for BN 100; 17 for Beckman Array;
IgM index > 0.2: 7 for BN 100; 69 for Beckman Array.
(Index cut-off values according to [9]).
With the hyperbolic formula [7, 8]:
for IgG > 0 : 2 for BN 100; 1 for Beckman Array;
for IgA > 0 : 19 for BN 100; 15 for Beckman Array;
for IgM > 0 : 3 for BN 100; 56 for Beckman Array.

The data indicate distinct differences in the number of positive results calculated with data from BN 100 and Beckman Array, especially for IgA and IgM. Range of differences between the 112 BN 100 results and Beckman Array results calculated by the hyperbolic formula [7, 8] varied from 66.7 to 0.0 mg/l (median: 5.2) for IgG, from 65.1 to 0.0 mg/l (median: 2.4) for IgA, from 14.6 to 0.0 mg/l (median: 1.5) for IgM.

Our data indicate different results for the hyperbolic formula [7, 8] with BN 100 and Beckman Array probably caused by methodical variations which could not be eliminated with the new calibrant CRM 470.

Moreover, CSF/serum quotient diagrams also demonstrate some differences between Q_{IgA}, Q_{IgM} and Q_{IgG} values calculated with CRM 470 corrected values from BN 100 and Beckman Array which were greater than $Q_{Albumin}$ values (for example, see Fig. 1). Our data obtained by two standardized systems with different methods do not confirm that CSF/serum quotients yield method-independent values [11]. Rather they imply the importance of well defined cut-off values for both formulae for the decision of an positive intrathecal immunglobulin production as has been discussed earlier [12].

In conclusion, the observed differences between immunoglobulin and albumin values in CSF and serum obtained with the Beckman Array system or with the Behring BN 100 system cannot be completely eliminated by calibration of the immunoassays with the new reference material CRM 470. They may be caused by methodical variations. Different data lead to variations in the statistical establishment of cut-off values for formulae and CSF/serum quotient diagrams to estimate an intrathecal immunoglobulin production. Moreover, uncertainty may increase by imprecisions of the immunoglobulin and albumin measurements (CV < 10%) which add to each other in both formulae to CV ≤ 25% which are higher than reported [10]. There-

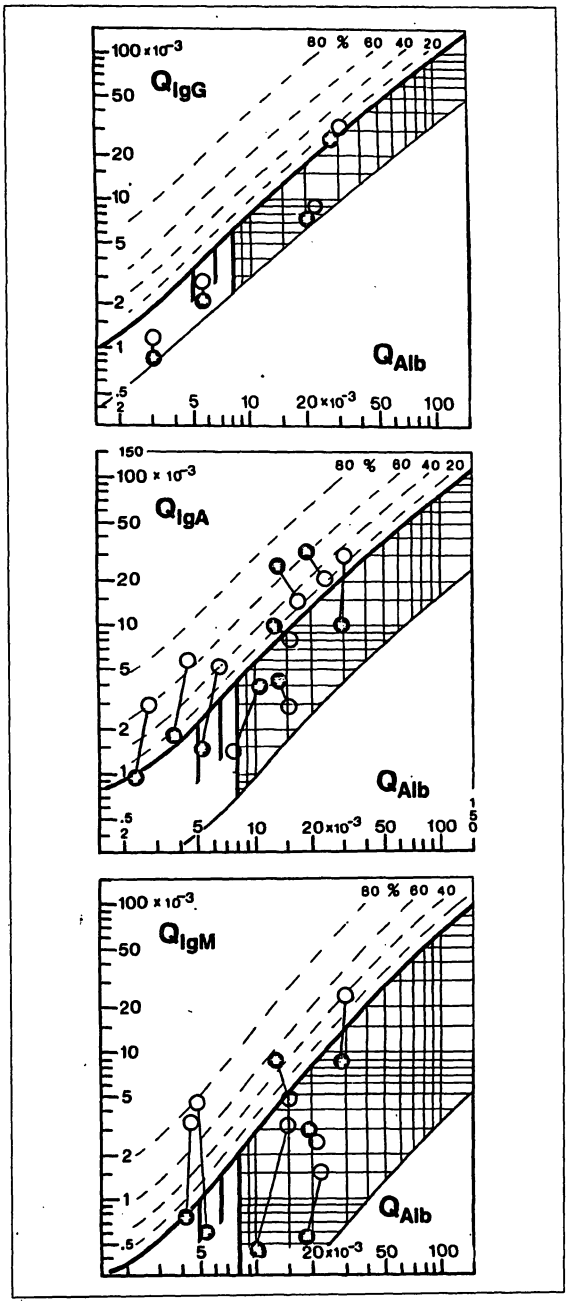

Fig 1 CSF/serum quotient diagrams with hyperbolic functions [7, 8] for the ratios between Q_{IgG}, Q_{IgA}, Q_{IgM}, and Q_{Alb} calculated with immunoglobulin (Ig) and albumin (Alb) contents of CSF and serum samples measured by the Array Protein System Beckman (o) and the BN 100 system Behringwerke (•) after calibration with CRM 470. Although no analytical errors are indicated (cf. [10] Fig. 1, area 5) Q_{IgA}, Q_{IgM} (and Q_{IgG}) values differed between both systems to a higher extent than Q_{Alb} ones probably caused by methodical variations. The deviations cannot be compensated by cut-off values of an intrathecal Ig fraction ≤10% [10]. Thus CSF/serum quotient diagrams, statistically established [6, 7], do not yield method-independent result.

Nonstandard abbreviations: CSF, cerebrospinal fluid; CRM, certified reference material.

fore, new reference values for IgG, IgA, IgM, and albumin have to be established for CSF (as in serum [13]) as well as new cut-off values for the formulae and CSF/serum quotient diagrams when CRM 470 is used as the calibrant.

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Qualitative versus Quantitative Analysis in the Detection of Intrathecal Synthesis of Immunoglobulins*

Qualitative versus quantitative Analyse bei der Bestimmung einer intrathekalen Immunglobulinsynthese*

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Keywords: Antibody Affinity; Cerebrospinal Fluid Proteins/analysis; Immunoglobulins/cerebrospinal fluid; Nervous System Diseases/cerebrospinal fluid.

Schlüsselwörter: Antikörperaffinität; Liquorproteine/Analytik; Immunglobuline/Liquor cerebrospinalis; Neurologische Erkrankungen/Liquor cerebrospinalis.

There is now a clear international consensus that isoelectric focusing followed by immunofixation for IgG is the preferred method for detection of local synthesis and thus aid the diagnosis of multiple sclerosis [1]. Qualitative analysis of CSF and serum IgG/albumin is more relevant in following the effects of various therapies or in studies of prognosis. Various mathematical formulations have been proposed but the best expressions are clearly non-linear. This is especially true for the larger molecules of IgA and mostly IgM. Some investigators have fractionated dimeric IgA, electrophoresed IgM and/or sieved free light chains to derive additional information about their local synthesis.

This mini-review will deal more with the principles which should guide comparisons, rather than indulge in the various disagreements which have divided this area. The comparison of qualitative versus quantitative analysis of CSF is analogous to the comparison of diagnosis versus follow-up of various neurological diseases.

The qualitative analysis of the IgG patterns revealed after separation by isoelectric focusing and visualisation by immunostaining of the heavy chains with IgG, has revealed a consensus of 5 patterns.

The ability to recognise the systemic immune response has important relevance to the question of differential diagnosis, especially in the case of the "greater than" pattern, which can be a useful indicator for infection, although it is also seen in patients with multiple sclerosis (where it may be related as a "trigger" phenomenon and/or intercurrent infection). On the other hand, the "mirror" pattern has a much wider distribution, as seen in Table 1 [2].

There is an important area in which the combination of qualitative analysis and quantitative analysis can once again provide initial information and that is in the question of affinity of antigens or their antibodies, namely is it specific or non-specific [3]. This technique involves the use of the chaotropic ion sodium thiocyanate in increasing concentrations, which effectively dissociates antigen bonds in ELISA plates. We have also applied the thiocyanate technique to nitrocellulose antigen blots. Although it has been well described that patients with multiple sclerosis have antibodies against measles and rubella, herpes zoster and other antigens (e.g. mumps and herpes simplex), we found that these antibodies are easily dissociated by low concentrations of sodium thiocyanate, and thus represent non-specific, low-affinity binding. In patients with infections of the nervous system, on the other hand, the

Table 1 Isoelectric focusing patterns among patients with various neurological diseases: Percentage of "mirror" (*) and "greater than" (>) patterns.

*	Diagnosis	>
14	Infection	29
18	Autoimmune	9
16	Neoplastic	2
5	Paraneoplastic	4
16	Guillain-Barré Syndrome	0
18	Peripheral Neuropathy	0
2	MS	57
5	Vascular	0
5	Degenerative	0

* = same no. of bands in CSF and serum;
> = more bands in CSF than in serum.

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specific antibodies to the infective agent in question require much higher concentrations of sodium thiocyanate to be dissociated, and indeed some antibodies persist even in the highest concentration of 5 mol/l sodium thiocyanate. It is also relevant that the antibodies against an unrelated antigen, (e.g. measles antibody in the case of herpes simplex encephalitis) are also of low affinity, as in the multiple sclerosis patients. This may thus reflect part of the primitive "anamnestic" response, which is initially displayed when a foreign antigen invades the body.

In conclusion, qualitative analysis and quantitative analysis can be complementary, but it is important not to confuse the purpose for which they are applied, nor indeed the mathematical manner in which they have been manipulated.

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Topic III: Poster Abstracts*

Comparison of Two New Techniques for Increasing Sensitivity in CSF-IgG Detection by Isoelectric Focusing and Chemiluminescent Specific Antibody Staining

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Isoelectric focusing (IEF) and IgG-specific antibody staining is the most sensitive test for cerebrospinal fluid (CSF) IgG oligoclonal bands detection. Unfortunately, sometimes very weak bands appear and results are doubtful. Light chain immunostaining increases sensitivity reducing background. Two different techniques have been proposed to make the procedure easier: (A) multiple PVDF blotting on the same agarose gel; (B) different antibody staining on the same blotted PVDF using chemiluminescence. We tested 30 IEF runs (about 300 CSF and serum samples) with both techniques and compared the results. Multiple blotting consisted in two consecutive 3 and 20 min blottings and IgG staining on the first paper and κ or λ staining on the second one. Multiple staining on 1 h blotted paper consisted in a first IgG peroxidase-chemiluminescent staining on X-ray film and, after enzyme inactivation, a second κ or λ chemiluminescent staining. Our results point out that multiple blotting give changeable outcome as sometimes one of two papers appear "foggy" or difficult to stain, probably due to incomplete PVDF binding. Multiple staining, warranted by 1 h blotting, give results and absence of carry over. In our experience mainly IgG-λ bands are hidden by IgG-κ background. So we suggest in case of doubtful routine IgG results a second λ immunostaining.

Cerebrospinal Fluid (CSF) Protein, Immunoglobulin and Cellular Abnormalities in Acquired Immune Deficiency Syndrome (AIDS)

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In this study we performed a CSF analysis in 44 HIV-1 infected patients (29 males, 15 females) belonging to Group IV of the Center for Disease Control Classification of Atlanta: Group IVA: 1; Group IVB: 10; Group IVC1: 31; Group IVC2: 2. CSF cell pleocytosis was found in 25% of the patients, increase of CSF protein and IgG concentrations in 55% resp. 82%, blood-brain barrier damage in 64% (induced by opportunistic infections), and a high IgG index in 43%. CSF oligoclonal bands (OB) were detected by agarose isoelectric focusing (IEF) with silver staining or with IgG specific immunoblotting (IB) and peroxidase staining in 41%, resp. 73% of the patients, while serum OB was demonstrated in 16%, resp. 41%. A "mirror" pattern was found in 7%, resp. 20% of the patients and a "local synthesis" plus "mixed" patterns in 32%, resp. 48%. The data indicate IEF with immunoblotting to be more sensitive (and specific) than IEF with silver stainig. Our results regard only HIV-1 infected patients at the late state of the disease mainly with primary and secondary neurological manifestations and a decline of the systemic humoral response; they are in accordance with the literature data.

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Identische oligoklonale Banden im Liquor und Serum: Bezug zur Blut-Liquor-Schrankenfunktion und intrathekalen IgG-Produktion

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Nach isoelektrischer Fokussierung in Polyacrylamidgelen und Silberfärbung wurden 2151 Serum/Liquor-Paare auf oligoklonale Banden untersucht. Es fanden sich (n=714) Banden nur im Liquor, (n=43) mehr Banden im Liquor, (n=63) gleiche Anzahl von Serum- und Liquorbanden, mehr Banden im Serum (n=4) und nur Serumbanden (n=2). Eine Korrelation von Banden im Serum mit einer bestimmten Krankheit konnte nicht nachgewiesen werden. Bei gleich vielen Banden im Liquor und Serum fand sich nur ein Patient mit Multipler Sklerose, bei mehr Banden im Liquor handelte es sich in 63% der Fälle um eine Multiple Sklerose (MS). – MS-Patienten mit hauptsächlich Liquorbanden hatten meistens eine intrathekale IgG-Produktion bei intakter Schrankenfunktion. Bei Patienten mit gleich vielen Banden im Liquor und Serum war fast nie eine intrathekale IgG-Produktion nachweisbar und die Schrankenfunktion in der Hälfte der Fälle normal. - Bei der MS ist zu vermuten, daß eine hohe intrathekale IgG-Produktion mit dem Übertritt von Banden ins Blutserum einhergeht. Diese sind möglicherweise mit verbesserten immunologischen Färbungen häufiger nachweisbar als bisher. Wenn gleich viele Banden im Liquor und Serum auftreten, handelt es sich um andere Krankheiten als die MS; auch der Bandenursprung scheint ein anderer zu sein, zumal fast nie eine intrathekale IgG-Produktion nachweisbar und häufig die Schrankenfunktion nicht gestört ist.

Microimmunoblotting applied to PhastSystem™ Increases Sensitivity of Detection of Oligoclonal Bands in Cerebrospinal Fluid (CSF)

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The demonstration of the presence of intrathecal synthesis of immunoglobulins as oligoclonal bands (OB) in cerebrospinal fluid (CSF) is considered to be important as an aid to diagnose multiple sclerosis (MS) and other neurological diseases. We used the PhastSystem™ equipment for detection of OB in the CSF. Separation was performed by isoelectric focusing (IEF) on polyacrylamide gels, pH 3-9. After separation proteins were visualized with silver staining according to the manufacturer's instructions or transferred electrophoretically (25 mA, 100V, 1h) to nitrocellulose membranes (pore size 0.45 µm) with PhastTransfer equipment. After immobilisation of the proteins and blocking with Tween 20, membranes were incubated with antibody against β-chains of immunoglobulins conjugated with horseradish peroxidase and detected with DAB (diaminobenzidine). For silver staining we used concentrated CSF (15 mg/l IgG) and unconcentrated CSF for immunoblotting. We tested CSF of patients with clinically defined MS (n=29) according to the Poser criteria. In all cases IgG index was normal (<0.7). OB were present in 24 patients (83%) with silver staining and in 27 patients (93%) when we used the immunoblotting technique.
Conclusions:
i. Detection of OB in the CSF PhastSystem is easily performed in routine diagnosis process of CSF.
ii. Immunoblotting method with PhastTransfer equipment increases sensitivity of detection of OB and allows the characterization of antigens as well as antibodies.
iii. Phast Transfer is a microtechnique which needs small amounts of CSF, antibodies, and other chemicals and takes relatively shorter time for complete analyses than conventional methods.