J. Clin. Chem. Clin. Biochem.
Vol. 27, 1989, pp. 211–216

Detection of IgG Oligoclonal Bands in Unconcentrated CSF by Isoelectric Focusing in Ultrathin Polyacrylamide Gel, Direct Antiserum Immunofixation and Silver Nitrate Staining

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(Received September 21, 1988)

Summary: Isoelectric focusing of proteins in ultrathin polyacrylamide gel (0.4 mm), followed by direct immunofixation with monospecific antisera and silver nitrate staining, is a highly specific, sensitive and simple method for the detection of oligoclonal IgG in unconcentrated CSF samples. The ultrathin polyacrylamide gels have several advantages, i.e. significantly smaller amounts of reagents are required, and thinner gel can be more efficiently cooled, resulting in higher resolution and shorter running, washing, staining and destaining times. Direct immunofixation in the gel, a time-saving and simple step, increases the sensitivity and specificity of the method. We reduced the samples to 5–10 µl. For the present method, the optimal concentration of IgG was 0.025–0.030 g/l. It is possible to detect oligoclonal IgG bands at an IgG concentration corresponding to the applied amount of 80–100 ng. In our testing of this method, oligoclonal bands in CSF specimens were clearly demonstrated in 33 (97%) out of 34 patients with definite multiple sclerosis, in 16 (42%) out of 38 patients with infectious diseases of the central nervous system and in 11 (18%) out of 58 patients with other neurological disorders. The method appears to be a useful alternative for the demonstration of oligoclonal IgG bands in unconcentrated CSF samples, and can be recommended for use in the CSF laboratory routine.

Introduction

Intrathecal synthesis of immunoglobulins is the expression of a local pathologic immune reaction within central nervous system (CNS). Several authors have devised formulae for calculating the rate of intrathecal IgG synthesis (1–3). Immunoglobulins produced in the CNS tend to have a restricted heterogeneity and appear as discrete bands in the gammaglobulin region, i.e. "oligoclonal bands". The incidence of oligoclonal IgG bands in some neurological

diseases, e.g. multiple sclerosis, subacute sclerosing panencephalitis, is quite high (70–100%), but is also influenced by the method of electrophoresis (4–8). New methods, such as isoelectric focusing (IEF) in polyacrylamide gel (PAG) or agarose, followed by immunofixation and silver staining of proteins, allow the oligoclonal IgG bands in CSF to be demonstrated without previous concentration of the fluid (9–12). The introduction of ultrathin gels is a step forward in the use of PAG IEF in routine CSF analysis. The method saves time and reagents. *Mehta & Patrick* (13) were the first to describe the use of ultrathin PAG IEF with direct immunofixation for the demonstration of CSF oligoclonal bands.

In the present study, a method involving separation of unconcentrated CSF proteins by ultrathin PAG IEF, followed by direct antiserum immunofixation and silver staining of precipitated IgG, was employed. The method is very sensitive, specific, and simple. It was tested for the demonstration of oligoclonal IgG bands in CSF and serum from 130 patients with various neurological diseases.

Materials and Methods

Patients and controls

The CSF and sera of 34 patients with clinically manifest multiple sclerosis, 38 patients with infectious diseases of the CNS, and 58 patients suffering from other neurological diseases were examined. Samples from 15 patients with tension headache or psychoneurosis were used as controls. Data on the major diagnostic groups are presented in table 1.

Routine CSF and serum studies

After cell counting, CSF samples were centrifuged within 30 minutes after lumbar puncture. Concentrations of albumin and IgG in CSF and serum were determined by laser immunonephelometry (Behring Laser Nephelometer).

The functional state of the blood-brain barrier was determined by the evaluation graph according to *Reiber* (2), enabling the calculation of the IgG fraction in CSF originating from the CNS. The IgG concentrations in CSF and serum were adjusted to 0.025 g/l by dilution with an appropriate volume of distilled water.

Home-made ultrathin PAG casting (0.4 mm) and IEF

An ultrathin PAG with dimensions 120 × 120 × 0.4 mm was prepared according to the following procedure: the mould was readily made from two glass plates, using adhesive tapes of known thickness as a gasket. The supporting glass plate was covered with GelBond PAG Film.

The normal acrylamide/Bis mixture was used (T = 5%, C = 3%). The amount required was reduced to 6 milliliters: 3 ml of stock acrylamide/Bis solution (T = 10%, C = 3%), 0.8 ml glycerol (870 g/l) 0.38 ml Bio-Lyte 3/10 and 1.82 ml distilled water. The solution was subjected to degassing for 10 min, and 35 µl of riboflavin-5-phosphate solution (1.0 g/l) and 2.5 µl of ammonium persulphate solution (400 g/l) were added. This polymerization mixture was poured in the mould using a pipette. A thin spatula was introduced on the opposite side between the GelBond PAG Film and upper glass plate to control the rate of pouring of the solution and to avoid the entrapment of air bubbles. Polymerization of acrylamide monomers was carried out by exposure to uniform light for 60 min.

The anode-EF solution was 0.15 mol/l orthophosphoric acid, and the cathode-EF solution was 0.3 mol/l ethylene-diamine. The sample (5–10 µl) was applied on a piece of paper. The IEF was first carried out at 16 °C and a constant power of 4 W for 20 min. The paper application pieces were then removed, and a constant voltage of 1900 V was applied. Focusing was completed in about 50–60 min. Immediately after focusing, the proteins were fixed for 20 min in a trichloroacetic acid solution (1.2 mol/l), or a direct immunoprecipitation with antiserum to human IgG was performed.

Immunofixation

Direct immunofixation was employed to identify oligoclonal bands. A thin filter paper strip was soaked in goat anti-human IgG serum (Institute of Immunology, Zagreb) diluted 1:10 with saline, and applied on the gel. Incubation time was 20 min. The unprecipitated proteins were washed out for at least 3–4 hours, or better overnight.

Silver staining and recycling

Silver staining was performed using a slightly modified procedure of *Merril* (14). Prior to staining, the gel was rinsed in several changes of distilled water, then washed three times for 10 min each with a solution containing methanol/acetic acid/water (10 + 5 + 85). After the washing steps, the gel was soaked for 7 min in a solution of potassium dichromate/nitric acid, followed by silver nitrate solution for 20 min. After a single rinse with distilled water, the image was developed in a solution of sodium carbonate/formaldehyde. This step requires three changes of the solution. When a slightly greyish background appears (2–3 min), development of the image is usually stopped by washing with solution of acetic acid 10 ml/l.

The silver staining procedure is repeated (recycling) using *Farmer*'s Reducer in order to improve the sensitivity of the silver staining method. This was performed by the procedure of *Heukeshoven* et al. (15).

Gel drying

After immersing the gel in a stopping solution of acetic acid for 3–5 min and one-step rinsing with distilled water, the gel was directly dried with a hair drier.

Results

Ultrathin PAG IEF, followed by silver staining, is a very sensitive method for the detection of oligoclonal bands from unconcentrated CSF specimens (fig. 1). The amounts of reagents are significantly reduced, i.e. by 60% as compared to the traditional 1 mm-thick gels. It is time-saving with regard to fixing, washing and gel staining. In the method described here, the gel casting and polymerization, IEF and silver staining can be completed within 5 hours. Sensitivity and specificity of the method are improved by including IgG immunoprecipitation. Only IgG, the

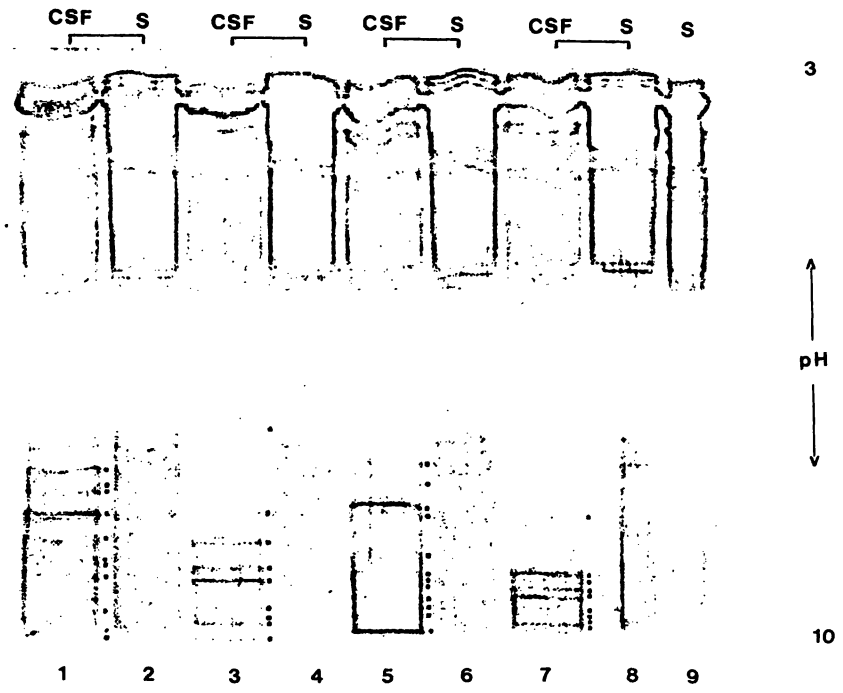

Fig. 1. Patterns from ultrathin PAG IEF (0.4 mm) and silver nitrate staining of unconcentrated CSF and diluted serum (S) from patients with multiple sclerosis (MS).
No. (9), diluted serum as negative control.
Samples (15 µl) with an IgG concentration of 0.04—0.05 g/l were applied. In all CSF specimens in the pH region exceeding 7, narrow, intensely staining bands were demonstrated. These "oligoclonal bands" are indicated by dots.

required protein, was detected (fig. 2). The optimal concentration of CSF IgG was 0.025—0.030 g/l, corresponding to the applied amount of IgG of 125—150 ng. It is possible to detect oligoclonal bands at a concentration of 0.01 g/l, which corresponds to the applied amount of 80—110 ng, although less intensive bands may disappear from the band pattern (figs. 2, 3).

The oligoclonal IgG bands in CSF usually appear in the pH region exceeding pH 7 as narrow, intensely staining bands not seen in serum, or they may be more intense in CSF than in serum. In our study of this method, oligoclonal IgG in CSF were clearly demonstrated in 33 (97%) out of 34 patients with clinically definite multiple sclerosis (tab. 1). In most CSF samples from patients with multiple sclerosis, "high-alkaline" bands between pH 8.5 and pH 9.5 were present. Fifteen (44%) of the multiple sclerosis patients also displayed abnormal bands in serum, usually fewer and less intense than in the corresponding CSF. Intrathecally synthesized IgG fraction was mathematically demonstrated in 27 (79%) patients with multiple sclerosis. Among 38 patients with infectious diseases of the CNS, 16 (42%) patients displayed oligoclonal bands in CSF, whereas an IgG fraction originating from the CNS was calculated to be present in 13 (34%) of them. Oligoclonal bands were also demonstrated in CSF of 11 (18%) out of 58 patients with other neurological diseases; a pathological intrathecal immune response was calculated in 6 specimens (10%).

Discussion

Ultrathin gels can be more efficiently cooled. This allows a higher power setting to be used without overheating. For focusing at 1900 V, a cooling temperature of 15—17 °C (tap water) was satisfactory. The IEF at higher voltage results in shorter running time and sharper bands, especially for proteins in low concentration in the alkaline region, like oligoclonal IgG. A combination of various ampholytes to increase the resolution in the alkaline pH region, as reported by *Mehta* et al. (13), was not necessary. Ultrathin PAGs are prone to disturbances in iso-pH lines influenced by high amounts of salts and proteins. This problem was overcome by the use of 5—10 µl of unconcentrated CSF samples, rendering prefocusing unnecessary. A small amount of CSF sample (1—5 µl) with a higher protein concentration can be applied directly to the PAG without using sample application paper pieces (not shown). In this case, a constant high power supply (25—30 W) can be immediately used. Focusing is completed in about 60 min.

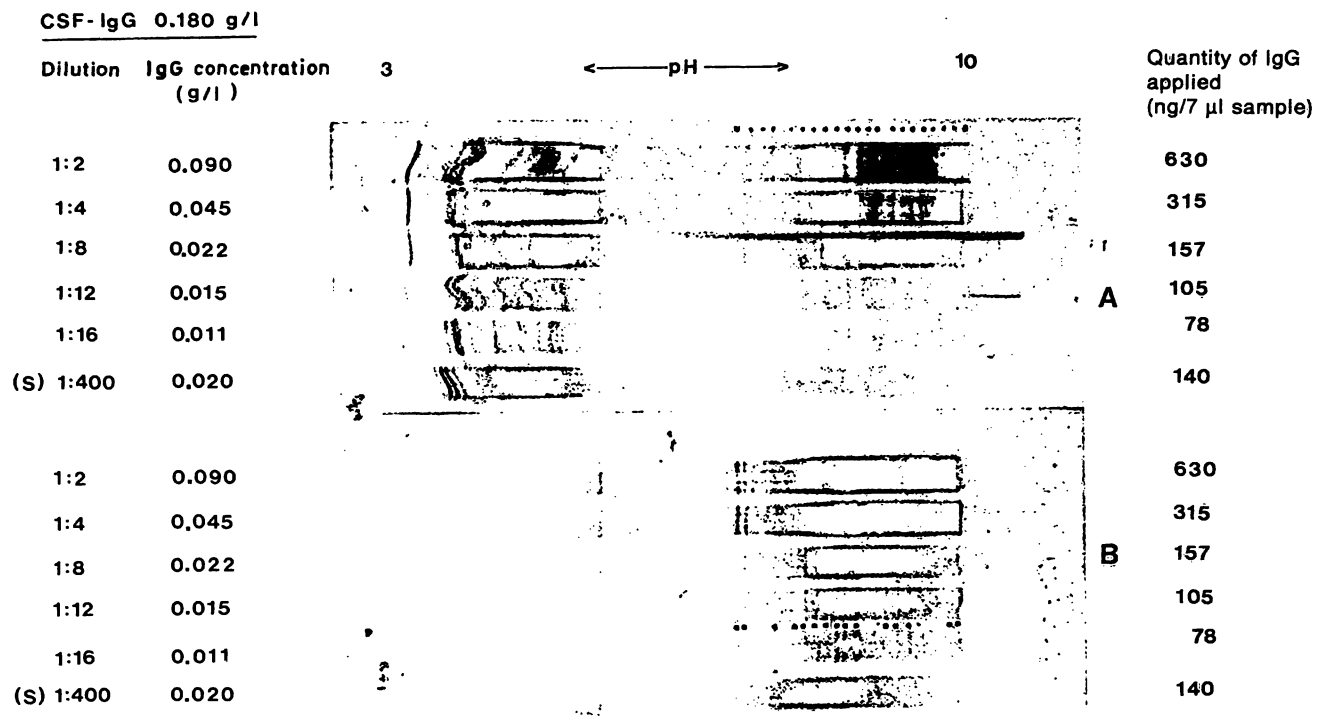

Fig. 2. Patterns from ultrathin PAG IEF (A) and immunofixation after IEF (B) of serially diluted CSF sample of a patient with multiple sclerosis (MS), with an IgG concentration of 0.180 g/l.
(S) = Serum
Silver nitrate staining. Goat antiserum against human IgG γ-chains was used.
Immunofixation with monospecific antiserum increases the PAG IEF method sensitivity and specificity. Note that oligoclonal bands were demonstrated in a sample with an IgG concentration of 0.011 g/l (corresponding to 78 ng of IgG applied). Dots denote oligoclonal IgG bands.

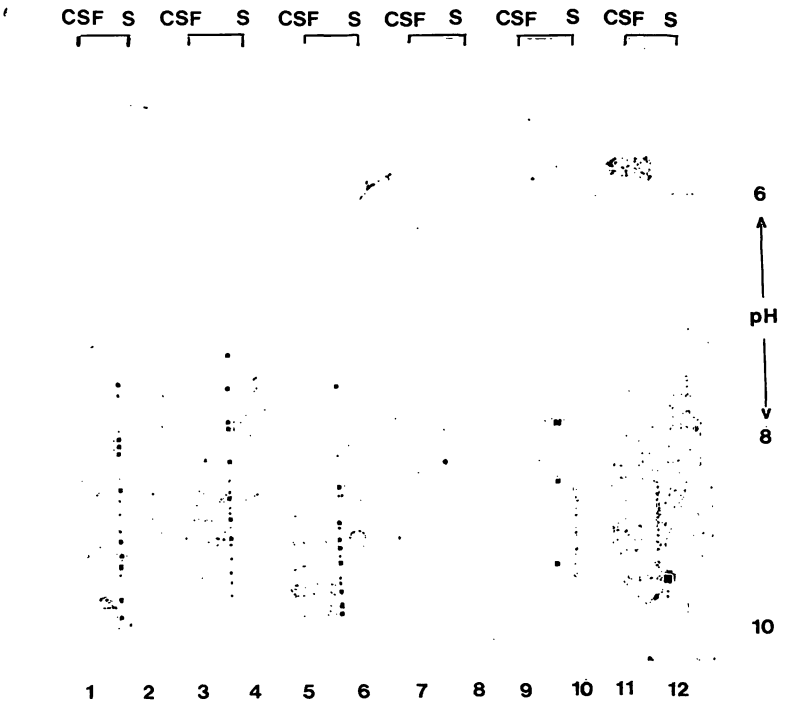

Fig. 3. Patterns from ultrathin PAG IEF and immunofixation of diluted serum (S) and unconcentrated CSF in various neurological diseases. Silver nitrate staining.
Goat antiserum against human IgG γ-chains was used.
Samples (7 µl) were loaded on the sample application paper pieces, corresponding to 120–180 ng of applied IgG.
No. (1–2), samples from a patient with *Guillain-Barré* syndrome.
No. (3–4), samples from a patient with chronic aseptic meningoencephalitis.
No. (5–6), samples from a patient with multiple sclerosis.
No. (7–8), samples from a patient with amyotrophic lateral sclerosis.
No. (9–10), samples from a patient with postvaccinal poliomyelitis.
No. (11–12), samples from a patient with cryptococcal meningitis.
Dots denote oligoclonal IgG bands.

Tab. 1. Frequencies of oligoclonal bands* on ultrathin PAG IEF and the intrathecally synthesized IgG in subjects suffering from various neurological diseases

Diagnosis	No. of patients	No. (%) showing oligoclonal IgG bands in CSF only		No. (%) showing oligoclonal IgG bands in both the CSF and in the abnormal serum pattern		No. (%) showing intrathecally synthesized IgG	
1. Multiple sclerosis (definite)	34	33	(97)	15	(44)	27	(79)
2. Nervous system infections	38	16	(42)	4	(10)	13	(34)
Purulent meningitis	5	0		0		0	
Tuberculous meningitis	6	4	(67)	2	(33)	3	(50)
Cryptococcal meningitis	1	1	(100)	0		1	(100)
Aseptic meningitis meningoencephalitis and encephalitis	25	10	(40)	2	(8)	8	(32)
Postvaccinal poliomyelitis	1	1	(100)	0		1	(100)
3. Other neurological diseases	58	11	(18)	4	(7)	6	(10)
Cerebrovascular diseases	23	4	(17)	1	(4)	2	(9)
Transient ischemic attacks	5	0		0		0	
Nervous system tumours	6	1	(16)	0		0	
Polyneuropathy	9	2	(22)	2	(22)	1	(11)
Mononeuropathy	8	1	(13)	0		0	
Amyotrophic lateral sclerosis	4	1	(25)	0		1	(25)
Guillain-Barré syndrome	3	2	(66)	1	(33)	2	(66)
4. Tension headache and psychoneurosis	15	1	(6)	0		0	

* Two or more bands presented in CSF only, or intensive staining in CSF as compared to serum.

Direct immunofixation on the gel is a simple and time-saving step. PAGs were shown to be less suitable for direct immunofixation, because the remaining unprecipitated proteins were difficult to wash out of the thick gel. Using ultrathin PAG and diluted samples, the washing time can be significantly reduced to 3–4 hours, although an overnight washing step appears to be preferable.

A compromise must be made between gel thickness, sample volume and detection limit. The thinner the gel, the shorter the washing and staining time, but a smaller volume of sample is required, so that oligoclonal bands in CSF specimens with low IgG concentration cannot be detected.

Our results are in agreement with those of *Mehta* et al. (13), presenting the ultrathin PAG IEF as a simple, sensitive and rapid method for the demonstration of oligoclonal IgG bands in unconcentrated CSF. We used a higher voltage, shorter fixing (immunofixation) time and recycling silver staining step, which resulted in shorter experimental time and improved sensitivity. The gel preparation, IEF, immunofixation with a 3-hour washing step, followed by silver staining can be completed within an 8-hour working-day.

Some investigators have analysed the CSF oligoclonal bands, employing very sensitive methods, combining IEF, protein transfer to nitro-cellulose membrane, double antibody avidin-biotin peroxidase staining or radioimmunofixation (16, 17). These methods are a useful research tool but are time-consuming and not available at a routine laboratory. Agarose is widely used as a supporting medium for IEF, because it is not neurotoxic, but generally the oligoclonal bands detected by high voltage PAG IEF were usually more numerous and easily discerned. When using a high resolution PAG IEF method, CSF samples, patient sera and negative controls should always be examined in parallel, and the amount of CSF and serum IgG applied on the gel standardized.

The frequencies of oligoclonal IgG bands in CSF from patients with clinically definite multiple sclerosis (97%), infectious diseases of the CNS (42%) and other neurological diseases were similar to those reported earlier, using high resolution IEF methods.

The present study also confirms the previous reports, indicating that the demonstration of oligoclonal IgG in CSF is a more sensitive evidence of local immune response in the CNS than the quantification of intrathecally produced IgG.

In conclusion, ultrathin PAG IEF appears to be a useful alternative for the demonstration of oligoclonal IgG bands in unconcentrated CSF, and it can be recommended for use in the CSF laboratory routine. The method is highly specific and sensitive, economic of reagents and time, and very simple.

Acknowledgement

This study was financially supported by the Scientific Fund of Croatia.

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