Original Article *Ann Clin Biochem* 1990; **27**: 436–443

Isoelectric focusing of cerebrospinal fluid immunoglobulin G: an annotated update

G Keir, R W Luxton and E J Thompson
From the Department of Special Chemical Pathology, National Hospital for Nervous Diseases, Queen Square, London WC1, UK

SUMMARY. A revised agarose isoelectric focusing method for detecting oligoclonal IgG in unconcentrated cerebrospinal fluid is presented. The technique is shown to be robust and reproducible and suitable for the detection of intrathecal IgG synthesis.

Additional key phrases: oligoclonal banding; multiple sclerosis

In 1983 we published a method for studying cerebrospinal fluid (CSF) immunoglobulin G (IgG) heterogeneity.[1] This utilized isoelectric focusing (IEF) in agarose followed by passive protein transfer onto nitrocellulose membrane (NCM) with immunodetection of IgG by double antibody, using horseradish peroxidase as a visualizing agent.

In the intervening period widespread interest has been shown in this approach. Also, as a consequence of our experience in applying this technique to over 40 000 CSF samples, significant changes have been made to all stages of the method. The present manuscript outlines these improvements.

METHOD

Preparation of gel

Dissolve 3·6 g d-Sorbitol (Sigma Chemical Co., Poole, UK) in 27 mL 10% v/v aqueous glycerol (BDH, Dagenham, UK). Add 0·3 g Agarose IEF (Pharmacia, Milton Keynes, UK) and dissolve in a boiling water bath. Equilibrate the liquid gel in a water bath at 65°C and add 2 mL Pharmalyte 3–10 and 0·5 mL Pharmalyte 8–10·5 (both Pharmacia).

Pour into a level perspex casting frame (220 mm × 110 mm, Pharmacia) on a pre-warmed sheet of Gelbond film (ICN Biomedicals Ltd, High Wycombe, UK) sealed to a glass plate with 50% aqueous methanol.

Allow to set then place in a damp chamber overnight at 4°C.

Correspondence: Dr G Keir.

IEF run

Allow the gel to equilibrate to room temperature in the damp chamber.

Remove frame and trim casting meniscus by cutting the outermost 2 mm from the gel using a surgical blade.

Seal the gel on to the cooling platen of the IEF apparatus using 50% aqueous methanol.

Remove surface fluid from the gel using a sheet of dry NCM as follows: place the NCM carefully on the gel starting at one of the short ends and allow the membrane to fold gently onto the surface. Avoid trapping air underneath the membrane. When using Sartorius NCM apply the shinier side of the membrane to the gel surface (see Discussion on NCM), remove the membrane and discard.

Position sample application foil (Pharmacia) such that the outermost edge is 2·5 cm in from the anodic (+) edge of the gel.

Load samples (see Discussion) using a 25 μL Hamilton microsyringe with a blunt needle.

Soak filter paper wicks (see Discussion) in electrolytes: 0·05 M sulphuric acid for the anode and 1 M sodium hydroxide for the cathode. Position the electrode wicks to give an approximate interelectrode distance of 7 cm. Position paper towel soakaways (see Fig. 1).

If the Pharmacia apparatus is being used then set limit voltage to 1200 V and power to 20 W. Integrate voltage over time to give a total run interval of 1000 volt hours (Vh). Conditions at the start of a run are typicaly 520 V, 40 mA at 20 W. The run time for a gel is usually about 1 h.

Remove sample application foil after 300 Vh, or 20 min.

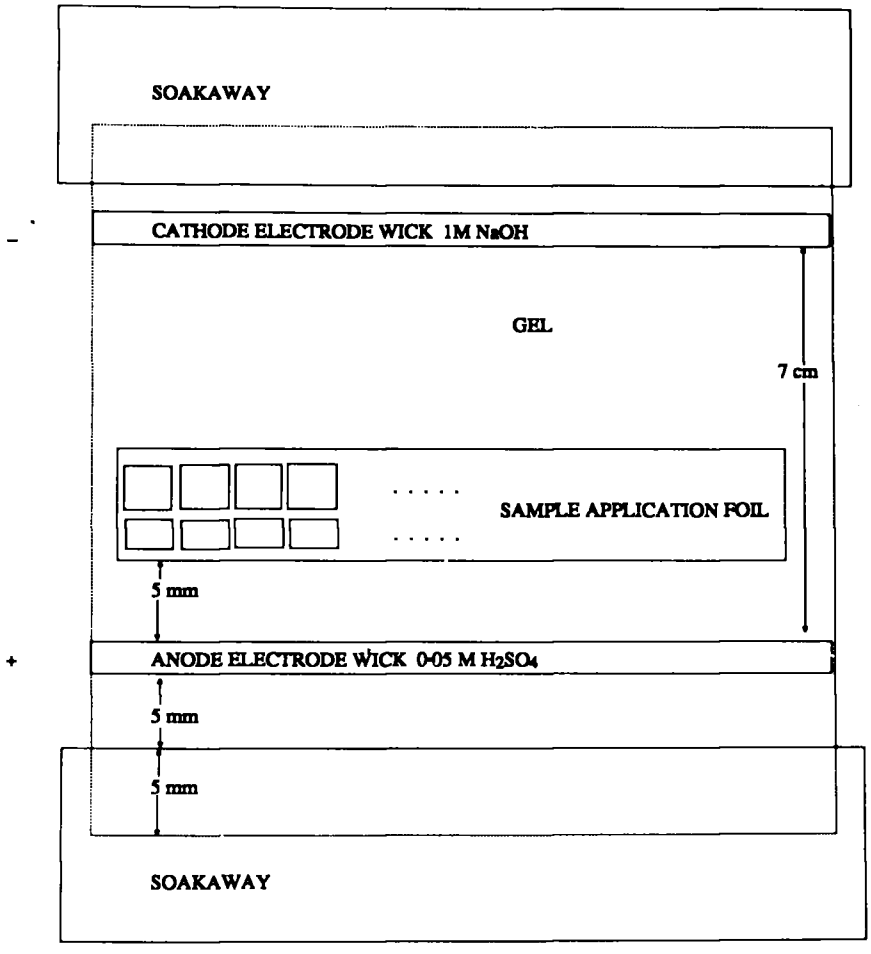

FIGURE 1. *Plan showing the layout of the plate at the start of IEF. The diagram is not to scale.*

Blotting

It is advisable to wear gloves when handling nitrocellulose membranes.

At the end of the run use the soakaways to dry excess fluid from the electrode wicks. Remove and discard wicks.

Pre-blot the gel surface using a sheet of dry NCM for 10 s, remove and discard.

Transfer proteins using a second sheet of dry NCM followed by a sheet of fine grain filter paper, such as Whatman Type 50, and several layers of blotter such as Phoprinto (Postlip Mills, Cheltenham, UK) or Whatman 3MM, under a glass plate and 3 kg weight, for 30 min.

Remove and discard blotters and place NCM 'protein side up' in 20 g/L dried, skimmed milk ('Marvel') in saline for 30 min. Rinse in tap water and replace with a solution of 50 mL 2 g/L dried milk in saline containing 50 μL goat anti-human IgG Fc (Atlantic Antibodies, Winnersh, UK) for 30 min. Wash in several changes of tap water and one wash in saline over 5 min. Replace with 50 mL 2 g/L milk in saline containing 50 μL horseradish peroxidase conjugated rabbit anti-goat serum (Dako, High Wycombe, UK) for 30 min. Wash as above. Rinse in distilled water then develop with a solution of 20 mg ethylamino carbazole (Sigma) dissolved in 20 mL methanol and added to 100 mL 0·02 M acetate buffer pH 5·1 containing 100 μL hydrogen peroxide (100 vol, 30%, BDH). Develop colour for approximately 20 min then wash in several changes of tap water, rinse in distilled water then dry using a stream of warm air from a hair dryer. Store developed pattern out of direct sunlight.

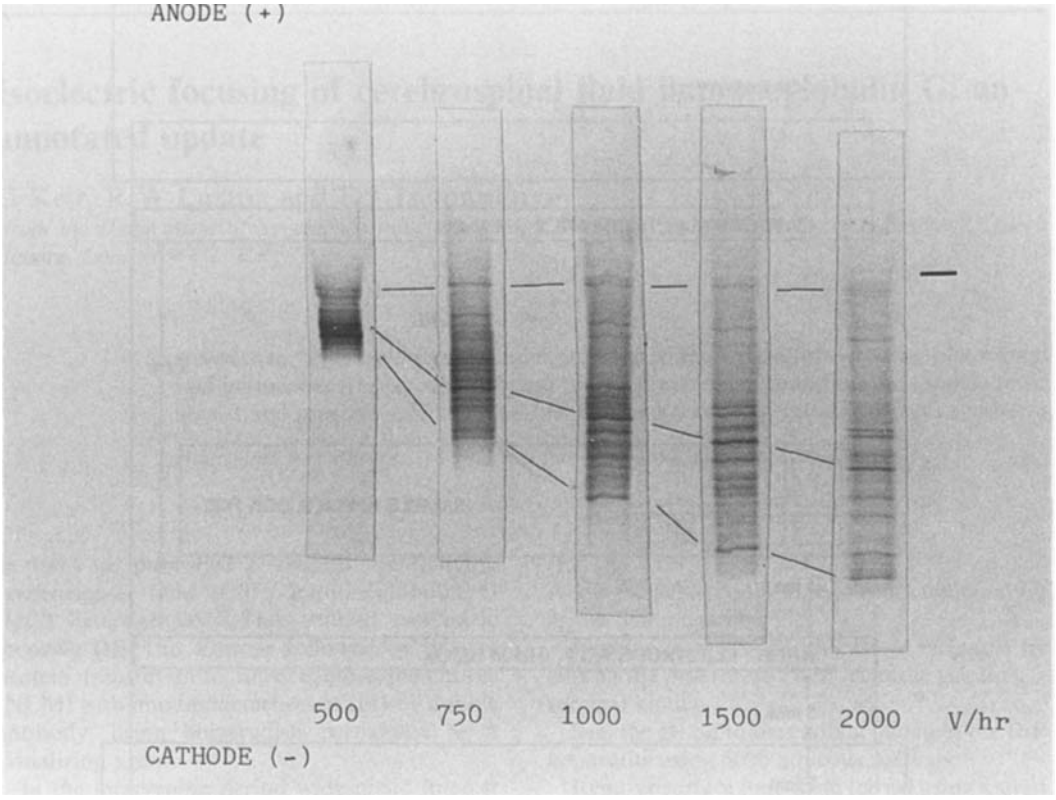

FIGURE 2. *Progress of IEF shown by terminating the run after various time intervals and staining for IgG. The individual lanes are lined up with reference to the thick mark. The migration paths of three readily identifiable components are shown by thin lines.*

DISCUSSION

The gel

Gels are routinely prepared in the afternoon for use the following day. Once poured, gels are stable at 4°C for up to 10 days. Gels can be used after 30 min at 4°C although, as agarose gels exhibit hysteresis, they continue shrinking for some time and exude buffer from the liquid phase of the gel. This is why it is necessary to dry the surface of gel prior to applying the samples. Most of the gel shrinkage occurs within the first few hours.

IEF

This technique relies upon the formation of a buffering pH gradient which is formed and maintained within the gel by synthetic ampholytes. Proteins placed within the gradient will migrate electrophoretically until they reach their isolectric point, whereupon mobility will cease and the proteins will be focused. IEF is therefore an ideal method for concentrating small amounts of proteins into tight bands.

When used in the fashion outlined here IEF is not an equilibrium technique. This is not important, however, as the aim of the technique is to identify any inherent homogeneity of the IgG (i.e. oligoclonal bands). IEF is used here in a stacking mode more akin to isotachophoresis, by allowing the pH gradient and the proteins to migrate simultaneously, which means that the ampholytes and proteins stack according to their pI values without necessarily reaching equilibrium. This can be seen in Fig. 2 in which the same samples are shown after 'focusing' for periods between 500 and 1500 Vh. It is seen that by 500 Vh the oligoclonal bands are formed, although adequate separation has not yet occurred. Thus the immunoglobulins are stacked according to their charge values but have not yet migrated to their isoelectric points. That stacking

has occurred is further suggested by the observation that 500 Vh is the point where the conductivity of the gel (measured as current passing through the gel) starts to fall. Any attempt by the stacked components to separate will result in a zone of pure water which is non-conductive. The falling current through the gel reflects the increased amount of time which the components spend in a partially separated state. By 1000 Vh the pH gradient is fully developed and the proteins are migrating to their isolelectric points. By 1500 Vh the system has achieved pseudo-equilibrium in that both ampholytes and proteins are now focused, but by this time cathodic drift and residual electroendosmosis are affecting the stability of the system and there is a loss of some bands into the catholyte (see Fig. 2).

Restricting the run time to 1000 Vh has the benefits of reducing the overall analysis time whilst minimizing the loss of cathodic components. Although the power/conductivity characteristics vary between gels due to changes in total fluid loading on gels and to slight differences in gel due to drying and storage, overall reproducibility of patterns is acceptable. We do not routinely use pI markers to determine the pI values for the focused bands as this provides no additional clinical information over simple detection of the bands. The use of agarose as a support medium ensures unrestricted migration of protein through the matrix and maximum transfer of focused proteins frrom the gel to the NCM.

Serum samples are diluted in water to reduce salt effects. CSF samples are used undiluted. The

TABLE 1. *Sample volume to be used for IEF based upon CSF total protein*

CSF total protein (mg/L)	Volume to be applied (μL)
120– 150	12
150– 180	11
180– 200	10
200– 220	9
220– 240	8
240– 280	7
280– 320	6
320– 380	5
380– 500	4
500– 700	3
700–1000	2

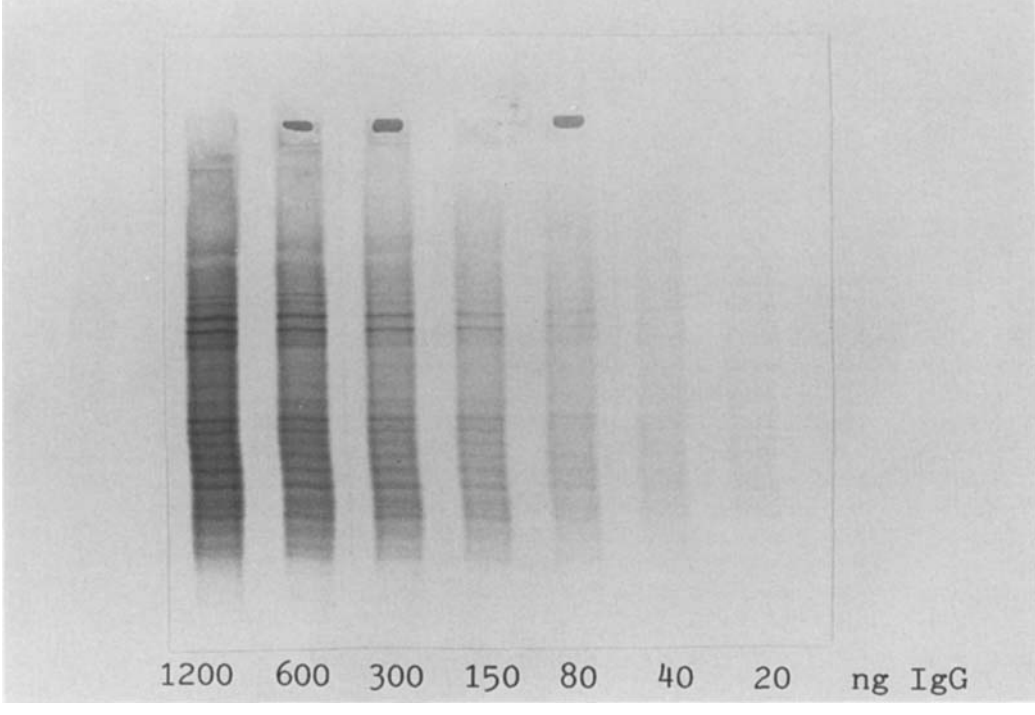

FIGURE 3. *A titration covering the concentrations 20 to 1200 ng of total IgG applied per track. It is apparent that the method will tolerate at least a 50-fold range of application amounts yet yield interpretable results. The anode is at the top.*

volume of CSF sample applied depends upon the total protein. The higher the CSF total protein, the less sample is applied. This is ideally achieved by quantitating both CSF and serum IgG and diluting as appropriate to achieve balanced amounts. The aim is to load approximately 150 ng of total IgG. This has been reduced from 500 ng in the original method,[1] to allow loading of smaller CSF volumes. This helps overcome problems when carrying out IEF of fluids with low levels of IgG, such as ventricular CSF, which may result in distorted lanes as a consequence of the increased volume and salts applied. The technique is very tolerant and we find that the amount of IgG can be varied from 20 to 1200 μg with no detriment to the pattern (see Fig. 3). Because of this the method is simplifed by loading 5 μL of serum diluted 1 in 400 irrespective of total IgG, and by assuming that IgG constitutes 10% of the total CSF protein. Table 1 outlines the volume of CSF to be applied for a given total protein value. This system works sufficiently well for routine use, although for more critical applications quantitative balancing may still be required.

Electrolyte wicks are prepared from hardened filter paper ('Phoprinto' paper) by cutting 1 cm wide by 20·5 cm long strips from the sheet using a guillotine. Three such strips stacked together are sufficient as wicks. Using such thin wicks decreased the amount of electrolyte used to create the pH gradient. This may help reduce drift caused by the diffusion of terminal ampholytes into the electrode wick and their dilution in the electrolyte by convection. A consequence of this phenomenon is a flattening of the pH gradient which, although theoretically enhancing resolution, can result in the loss of basic immunoglobulins as discussed above (see also Fig. 2). Such terminal drift is only a problem in extended runs and has not proved to be a problem in the short run times used in this method. However, such phenomena in conjunction with residual electroendosmotic flow result

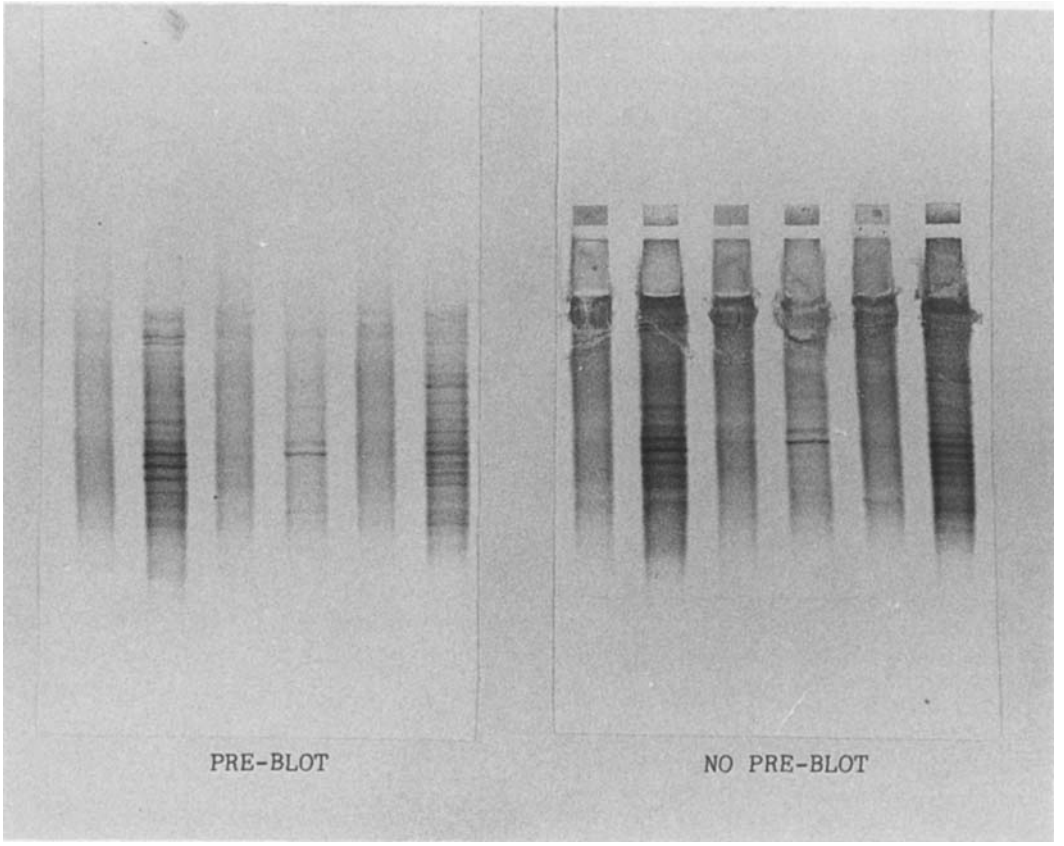

FIGURE 4. *The influence of the pre-blot on the patterns is shown. The absence of a pre-blot results in obscuring of the sample tracks particularly in the anodic regions. The anode is at the top.*

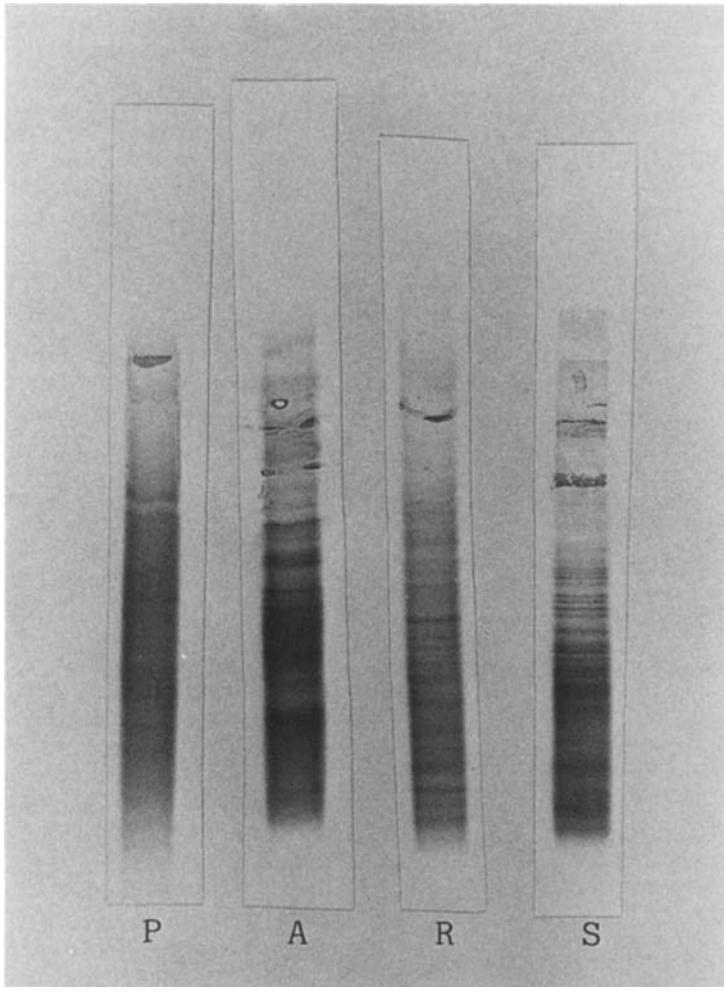

FIGURE 5. *Patterns of pooled normal serum IgG using four different commercially available ampholytes are shown. Pharmalyte is shown on the extreme left and can be seen to be relatively artefact-free when compared to the alternatives. The anode is at the top.*

in the accumulation of fluid at both electrodes during the run, hence the need for the paper soakaways which effectively drain the fluid away from the electrode wicks. Failure to incorporate soakaways results in excessive dilution of terminal ampholytes and proteins with inadequate buffering and loss of resolution.

The appearance of ridges in the gel parallel to the two electrode wicks are an essential sign that focusing is occurring. Failure of ridges to appear within 10–15 min imply there is a serious failure in either electric power getting to the gel or through the gel, and corrective steps should be taken. Where the facility of volt-hour integration is not available the run can be monitored using a coloured protein, such as haemoglobin or myoglobin. If a strong solution of myoglobin is spotted beside the anode and cathode wicks the spots will be observed to flatten into bands and move through the gel as the focusing takes place. When the two sets of bands meet then the myoglobin has focused and the run can be terminated. Using this approach the pH gradient has fully formed so there is the risk of cathodic drift with loss of bands.

Nitrocellulose membranes

Proteins stick to NCM by non-covalent forces. They can be washed off using high salt concentra-

TABLE 2. *Troubleshooting guide*

1. No staining of sample lanes

(a) Background nitrocellulose unstained

No samples loaded
Primary (anti IgG Fc) or secondary (HRP-conjugated) antiserum omitted
Incorrect primary or secondary antiserum used (e.g. rabbit primary followed by anti-sheep secondary)
No hydrogen peroxide added to ethyl-amino car basole solution

(b) Background nitrocellulose heavily stained

Background staining even:

Blocking step omitted
Inadequate washing before addition of either second antibody or substrate solution

Background staining uneven:

Omitted or inadequate pre-blot (cleaning stage)

2. Sample tracks stained but faint

Inadequate sample
Insufficient development time
Old or contaminated hydrogen peroxide
Decay of peroxidase-conjugated antibody
pH of acetate buffer incorrect (should be between pH 4 and 8 and preferably pH 5·0–5·2)
Ethyl-amino carbazole oxidized
Shorting due to fluid accumulation under the sample application foil

3. Sample tracks too intense

Excessive sample
Overdevelopment (rare)

4. Sample tracks distorted

Inadequate cooling during IEF
Condensation on gel surface during run
Sample volume > 15 μL may cause 'ballooning' into adjacent tracks
Infected samples (may cause ballooning)
Saline used instead of water to prepare gel (current high, gel distortion during run)

5. No separation—all staining restricted to sample application zone

No power
Supply not connected
Lid not connected
Broken electrode connections

No ampholytes in gel (current low)

tions and by certain detergents. Up to 50% of the protein initially bound to NCM may wash off during the subsequent incubation steps. For some proteins, other than IgG, it may be necessary to increase sensitivity incorporating additional steps to fix the chemicals prior to probing, e.g. using glutaraldehyde.[2] Such steps are not required in the present method as sensitivity is not a problem.

We have wide experience with NCM from two manufacturers Schliecher and Schuell (available through Anderman and Co., Kingston-upon-Thames, UK) and Sartorius (Epsom, UK). It is important to note that the Sartorius membranes have a 'dull' and a 'shiny' side. When laying on the Sartorius membranes it is important that the 'shiny' side is placed on the gel surface. Placing the dull side down leads to uneven wetting and trapping of small air pockets under the membrane. Schleicher and Schuell membranes do not show this property and both sides are equivalent. We find no difference in the performance or binding characteristics of these membranes.

The pre-blot is a most important step in the technique for this step removes non-specific cross-reacting materials which can obscure the patterns. The chemical nature of these non-specific materials has not been determined but they probably comprise high molecular weight aggregates, cell fragments and lipoproteinaceous materials. Figure 4 shows the results of omitting the pre-blot stage. It can be seen that the pre-blot removes some protein, including IgG, from the gel. However, it is most unlikely that oligoclonal bands would be selectively removed by the pre-blot to give a false-negative interpretation of the final blot. The pre-blot is an effective step in cleaning up the final blot and improving the pattern.

Artefacts

IEF is often criticized as a technique prone to artefacts, which may conveniently be defined as bands inappropriately generated by the method. Our experience however, is that artefacts are largely a consequence of inappropriate technique and as such can be avoided or at least minimized. The chemistry of ampholyte synthesis[3] shows that, on a theoretical basis at least, Pharmalytes should contain a larger number of constituent ampholytes per pH unit of gradient than other commercial products. This is important in that, if we assume an even distribution, the larger the number of ampholyte species per pH unit the more uniform the gradient. When studying

homogeneous proteins, such as α_1- antitrypsin phenotypes or isoenzymes, uneveness in the gradient is not a major problem and all commercial systems are equally applicable. When dealing with polydisperse proteins such as immunoglobulins however, it is important to have as even a gradient as possible. Figure 5 shows the patterns obtained by four commercial ampholyte mixtures. It is obvious that some mixtures give rise to banded distributions for sera which are known to have polyclonal IgG.

Problems

The technique suffers from few problems and Table 2 outlines the major symptoms, causes and cures for errors which we have experienced.

Working definition of oligoclonal and local synthesis

Originally the term oligoclonal was defined in terms of bands observed on low resolution techniques, such as agar, agarose or cellulose acetate electrophoresis. Different workers used different criteria, with as few as one band having been used to define oligoclonal when this was demonstrated to be restricted to the CNS. It was assumed that each individual band in the oligoclonal response was the product of a unique clone of lymphocytes within the brain.

This use of bands to define oligoclonal however, poses a problem when applied to a high resolution technique such as isoelectric focusing. The realization that monoclonal immunoglobulins can give rise to a variable number of bands makes it difficult to define an arbitrary number above which the term oligoclonal can be applied. Furthermore, it cannot be assumed that the intrathecal synthesis of a solitary monoclonal immunoglobulin represents an oligoclonal response, as the same pattern can be found in cases of subdural plasmacytomas and meningeal lymphomas. It is, however, correct to think of intrathecal synthesis in terms of lymphocyte clones and our definition of 'oligoclonal' using isoelectric focusing is that it is necessary to demonstrate identifiable products of at least two clones of lymphocytes within the CSF which are absent from serum. This last point is important as it is possible to have oligoclonal bands without having local synthesis. Local synthesis can only be defined when bands are present in CSF but absent from plasma. The different types of patterns which can be seen after IEF and the clinical implications are discussed in recent papers.[4,5]

Conclusion

It is hoped that this paper has demonstrated the robust nature of IEF and nitrocellulose immunoblotting as techniques for the study of CSF IgG. We feel that these approaches have many applications in routine clinical chemistry which have yet to be realized.

REFERENCES

1 Walker RWH, Keir G, Johnson MH, Thompson EJ. A rapid method for detecting oligoclonal IgG in unconcentrated CSF, by agarose isoelectric focusing, transfer to cellulose nitrate and immunoperoxidase staining. *J Neuroimmunol* 1983; **4**: 141–8

2 Sharief MK, Keir G, Thompson EJ. Glutaraldehyde-enhanced immunofixation: A sensitive new technique for detecting oligoclonal immunoglobulin M. *J Neuroimmunol* 1989; **23**: 149–56

3. Righetti PR. *Isoelectric Focusing: Theory, Methodology and Applications*. Amsterdam: Elsevier Biomedical, 1983; 31–9

4. Thompson EJ, Keir G. The laboratory investigation of cerebrospinal fluid proteins. *Ann Clin Biochem* 1990; **27**: 425–435

5. McLean B, Luxton RW, Thompson EJ. A study of immunoglobulin G in the cerebrospinal fluid of 1007 patients with suspected neurological disease using isoelectric focusing and the log IgG-index: A comparison and diagnostic applications. *Brain*, in press

Accepted for publication 22 March 1990