Detection and identification of serum monoclonal immunoglobulin by immunoisoelectric focusing. Limits of sensitivity and use during relapse of multiple myeloma

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SUMMARY The limits of detection of four classes of monoclonal immunoglobulin and free light chain in serum by isoelectric focusing and immunoisoelectric focusing have been determined and the sensitivity of these techniques compared with that obtained using immunoelectrophoresis and zonal electrophoresis with immunofixation. Immunoisoelectric focusing was 10–40 times more sensitive than immunoelectrophoresis and could be used to detect concentrations of monoclonal immunoglobulin that were undetectable by zonal electrophoresis with immunofixation. The relevance of this work in monitoring multiple myeloma during treatment and relapse is discussed.

An important factor in the diagnosis of multiple myeloma and other B cell malignancies is the detection of a paraprotein in the patient's serum. We have shown that isoelectric focusing and immunoisoelectric focusing are superior to immunoelectrophoresis and zonal electrophoresis for the detection of monoclonal immunoglobulin when it is present at concentrations below 5 mg/ml or is masked by polyclonal immunoglobulin.¹ A potentially important clinical application of these techniques is the detection of early relapse in patients with B cell neoplasia who are in clinical and serological remission. In this paper we show how isoelectric focusing and immunoisoelectric focusing may be used to monitor monoclonal immunoglobulin at an early stage in the relapse and we have compared the limits of sensitivity of isoelectric focusing and immunoisoelectric focusing for the detection of paraproteins with more conventional techniques.

Material and methods

Immunoelectrophoresis, isoelectric focusing, and immunoisoelectric focusing were performed as previously described.¹ Zonal electrophoresis with

immunofixation was performed as described by Ritchie and Smith.² Patients' sera were referred for investigation of serum paraproteinaemia either as a clinical assessment of effectiveness of treatment or for detecting monoclonal immunoglobulin after treatment.

In order to determine the limits of detection of monoclonal immunoglobulin, sera from patients with multiple myeloma or Waldenström's macroglobulinaemia were diluted with pooled normal human serum and analysed by zonal electrophoresis, immunoelectrophoresis, isoelectric focusing, and immunoisoelectric focusing. Sera containing monoclonal IgA or IgM were treated with 2-mercaptoethanol and focused in a narrow range pH gradient to facilitate focusing of these molecules. Interpretation of the immunoelectrophoretic pattern of IgM is made easier by the use of 2-mercaptoethanol.¹

Results

Fig. 1a shows the isoelectric focusing pattern of serum from a patient with multiple myeloma (IgG (κ) isotype) with a total IgG concentration of 54.0 mg/ml (measured by radial immunodiffusion). This serum was serially diluted in pooled normal human serum to determine the limits of detection of monoclonal immunoglobulin. Monoclonal immunoglobulin was still clearly detectable at a dilution of 1/200

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Fig. 1 Comparison of the sensitivity of detection of monoclonal IgG in serum by isoelectric focusing, immunoelectrophoresis, and immunoelectrophoresis. Troughs containing anti-IgG and anti- λ light chain are indicated at the right hand side of the immunoelectrophoresis plate. Dilutions of the myeloma serum in normal human serum (NHS) are indicated at each track. pH gradient for isoelectric focusing and immunoelectrophoresis = 3–10.

and very faint bands were visible at 1/1000, although these could have been missed in a routine specimen. Immunofixation with anti-IgG (immunoelectrophoresis) gave similar results, the monoclonal IgG again being detectable down to a dilution of at least 1/200.

If we assume that most of the IgG is monoclonal immunoglobulin, this represents a detection limit below 0.27 mg/ml. This figure is a maximum value since polyclonal IgG will also be present in the serum of the myeloma patient, albeit in low concentration because of the suppression of normal immunoglobulin production often associated with multiple myeloma.

Fig. 1b shows the immunoelectrophoresis of the same IgG dilutions. Monoclonal IgG could be identified with certainty, at best, at concentrations down to 2.7 mg/ml. Thus isoelectric focusing was at least 10 times more sensitive than immunoelectrophoresis in detecting monoclonal IgG in this case.

Fig. 2a shows the isoelectric focusing and immunoelectrophoresis patterns of a serum containing monoclonal IgA (λ) (original IgA concentra-

tion = 81 mg/ml). The monoclonal IgA could be detected by isoelectric focusing at a concentration of 1.6 mg/ml (1/50) and by immunoelectrophoresis at the highest dilution—that is, 0.16 mg/ml; this figure, as before, represents a maximum value. With immunoelectrophoresis (Fig. 2b) monoclonal IgA was detectable with certainty at concentrations down to only 4.05 mg/ml (1/20), although the dilution containing 1.62 mg/ml (1/50) was abnormal but equivocal. This represents an improvement of between 10 and 25 fold for immunoelectrophoresis compared with immunoelectrophoresis.

Monoclonal IgM (original concentration 17.9 mg/ml) could be detected at concentrations down to 0.45 mg/ml (1/40) by isoelectric focusing and less than 0.11 mg/ml (1/160) by immunoelectrophoresis (Fig. 3a), while immunoelectrophoresis (Fig. 3b) could confirm monoclonal IgM, at best, at concentrations down to 3.6 mg/ml (1/5). This represents at least a 32 fold increase in sensitivity by immunoelectrophoresis compared with immunoelectrophoresis.

Monoclonal IgD (original concentration 54

Fig. 2 Comparison of the sensitivity of detection of monoclonal IgA in serum by the three methods described in Fig. 1.
pH gradient for isoelectric focusing and immunoelectrophoresis = 5–8.

mg/ml), a less common paraprotein, could be detected at a concentration of 1 mg/ml (1/50) by isoelectric focusing and less than 0.05 mg/ml (1/1000) by immunoelectrophoresis (Fig. 4a) compared with a detection limit of 2 mg/ml (1/25) by immunoelectrophoresis (Fig. 4b). This represents a 40 fold increase in sensitivity by immunoelectrophoresis focusing.

Fig. 5 compares immunoelectrophoresis, isoelectric focusing, and immunoelectrophoresis in the detection of λ light chains in serum. Free light chain is visible at dilutions down to 1/20 by immunoelectrophoresis, but down to 1/320 by immunoelectrophoresis, representing a 16 fold improvement in sensitivity.

To show the value of isoelectric focusing and immunoelectrophoresis in an individual case, Fig. 6 shows a comparison of zonal electrophoresis, immunoelectrophoresis, and immunoelectrophoresis focusing for the analysis of serum from a patient with a solitary plasmacytoma and rising IgG concentrations but with no paraproteins as shown by conventional techniques. No paraprotein was found by zonal electrophoresis of the serum, but immunofixation of the strip with antikappa serum showed a faint band of restricted mobility.

Immunoelectrophoresis showed only a slight abnormality at the anodic end of the kappa precipitin arc. Isoelectric focusing of the serum showed a single abnormal band which was identified as kappa light chain by immunoelectrophoresis. There was no detectable monoclonal IgG, a paraprotein which had been suspected owing to the rising IgG concentration; nor was monoclonal immunoglobulin of any other class found. Isoelectric focusing of concentrated urine also showed a single band at the same isoelectric point as the "serum band," and this was identified as free kappa light chain by immunoelectrophoresis focusing.

Table 1 shows the results of using the four techniques on sera from four patients with paraproteins of different classes. In each of these cases, except patient 4, only immunoelectrophoresis gave positive identification of a monoclonal immunoglobulin. Such examples of sera containing low concentrations of monoclonal immunoglobulin detectable by immunoelectrophoresis but not by zonal or immunoelectrophoresis have often been encountered in our laboratory.

Table 2 shows the results from 27 patients who had undergone treatment for multiple myeloma, now in clinical and serological remission, and whose

Fig. 3 Comparison of the sensitivity of detection of monoclonal IgM in serum by the three methods described in Fig. 1.
pH gradient for isoelectric focusing and immunoisoelectric focusing = 5–8.

Fig. 4 Comparison of the sensitivity of detection of monoclonal IgD in serum by the three methods described in Fig. 1.
pH gradient for isoelectric focusing and immunoisoelectric focusing = 3–10.

Fig. 5 Comparison of the sensitivity of detection of monoclonal λ light chains in serum by the three methods described in Fig. 1.
pH gradient for isoelectric focusing and immunoelectrophoretic focusing = 3–10.

Fig. 6 Comparison of (a) zonal electrophoresis and immunoelectrophoresis with (b) isoelectric focusing and immunoelectrophoretic focusing. Antisera in troughs are indicated at the right hand side of the immunoelectrophoresis pattern: numbers over the isoelectric focusing and immunoelectrophoretic focusing tracks indicate the antisera used for overlay. Isoelectric focusing was performed at pH 3–10.
TCA = trichloroacetic acid.

Table 1 Comparison of zonal electrophoresis, zonal electrophoresis with immunofixation, immunoelectrophoresis and immunoelectrophoresis focusing on sera from four patients with putative paraproteins

Patient	ZEP	IEP	ZEPI	IIEF
1	No paraprotein	IgM, λ arcs distorted	No paraprotein	Monoclonal IgG λ
2	No paraprotein	IgA arc equivocal	No paraprotein	Monoclonal IgA λ
3	No paraprotein	IgM, κ and λ arcs distorted	No paraprotein	Monoclonal IgM κ
4	No paraprotein	κ arc distorted	Monoclonal κ	Monoclonal κ

ZEP = zonal electrophoresis.

IEP = immunoelectrophoresis.

ZEPI = zonal electrophoresis with immunofixation.

IIEF = immunoelectrophoresis focusing.

sera showed no qualitative abnormality of the immunoglobulin profile by zonal and immunoelectrophoresis. Of these 27 patients, 16 had monoclonal immunoglobulin still detectable using immunoelectrophoresis, whereas only seven of these could be detected by zonal electrophoresis with immunofixation. Patient 19 was originally thought to have two paraproteins (IgG and IgM), the presence of which was confirmed by immunoelectrophoresis, whereas the monoclonal IgM was not detected by zonal electrophoresis with immunofixation. An IgA paraprotein was detected by zonal electrophoresis with immunofixation in the serum of patient 8, but the light chain typing was negative.

Discussion

Study of the immunoelectrophoresis patterns in Fig. 1–5 shows that we have been generous in our estimates of the sensitivity of immunoelectrophoresis for the detection of monoclonal immunoglobulin. Had some of the immunoelectrophoresis patterns occurred in a routine investigation, they could easily have been missed.

For each of the four immunoglobulin classes studied, immunoelectrophoresis has been shown to be the most sensitive for detecting small concentrations of monoclonal immunoglobulin. Since the dilutions were made in normal serum, whereas polyclonal immunoglobulin is usually suppressed in

Table 2 Identification of monoclonal immunoglobulin in sera of patients in remission

Patient	Pretreatment paraprotein (identified by IEP)	Findings by ZEPI during remission	Findings by IIEF during remission
1	NQAD	NQAD	Monoclonal IgG lambda
2	NQAD	NQAD	NQAD
3	IgM kappa	NQAD	Monoclonal lambda
4	IgG kappa	NQAD	NQAD
5	IgG kappa	NQAD	NQAD
6	NQAD	NQAD	NQAD
7	IgG kappa + IgG kappa	Monoclonal IgG kappa	Monoclonal IgG kappa
8	IgA lambda	IgA +ve, -ve	NQAD
9	Monoclonal lambda	Monoclonal lambda	Monoclonal lambda
10	NQAD	NQAD	NQAD
11	Monoclonal kappa	Monoclonal kappa	Monoclonal kappa
12	Monoclonal kappa	NQAD	NQAD
13	IgG lambda	NQAD	Monoclonal IgG lambda
14	IgG lambda	NQAD	NQAD
15	IgA kappa	NQAD	NQAD
16	Monoclonal lambda	NQAD	Monoclonal lambda
17	IgG kappa + free lambda	NQAD	Monoclonal kappa
18	NQAD	NQAD	NQAD
19	IgG kappa + ? IgM lambda	Monoclonal IgG kappa	Monoclonal IgG kappa + Monoclonal IgM lambda
20	Monoclonal kappa	NQAD	Monoclonal kappa
21	IgG kappa + free kappa	Monoclonal IgG kappa	Monoclonal IgG kappa
22	Monoclonal lambda	Monoclonal lambda	Monoclonal lambda
23	IgG kappa + free kappa	NQAD	Monoclonal IgG kappa
24	IgG kappa	NQAD	Monoclonal IgG kappa
25	IgA kappa	NQAD	NQAD
26	IgG kappa	Monoclonal IgG kappa	Monoclonal IgG kappa
27	IgG kappa	NQAD	Monoclonal IgG kappa

NQAD = no qualitative abnormality detected.

IEP = immunoelectrophoresis.

ZEPI = zonal immunoelectrophoresis with immunofixation.

IIEF = immunoelectrophoresis focusing.

multiple myeloma, our estimates of the limits of sensitivity of detection of IgG, IgM, and IgA should be regarded as maximum values. Furthermore, the ease of interpretation of immunoelectrophoresis bears little comparison with that of immunoelectrophoresis. The immunoelectrophoresis and immunoelectrophoresis patterns of monoclonal IgM dilutions illustrate this well (Fig. 3). The immunoelectrophoretic arc of IgM is notoriously difficult to interpret, mainly because of its small size and position. Although abnormalities are seen, monoclonality is often in doubt with immunoelectrophoresis.³ This problem is greatly obviated by the use of immunoelectrophoresis. Monoclonal IgG is probably the easiest to interpret by isoelectric focusing (Fig. 1) because of the spreading of polyclonal IgG. Immunoelectrophoresis gives similar sensitivity. Antihuman IgG precipitates the polyclonal IgG and, as the monoclonal IgG concentration drops, so differences in staining become less apparent. This is not a problem in the case of IgD, however, because of its low concentration in normal serum. In this case, using immunoelectrophoresis, the normal polyclonal IgD does not mask the monoclonal IgD pattern until a high dilution is reached, whereas only a thickening of the IgD arc is seen by immunoelectrophoresis and one has to rely on an abnormality of the light chain arc restricted to kappa or lambda for evidence of monoclonality. Isoelectric focusing of monoclonal IgD dilutions is relatively less sensitive than immunoelectrophoresis because of the tendency of IgD to focus at the anodic end of the gel, with many of the other serum proteins. In a previous paper¹ we showed that precipitation of the γ globulins by washing the gel with 18% wt/vol Na_2SO_4 or 50% saturated $(\text{NH}_4)_2\text{SO}_4$ before fixing with trichloroacetic acid improves this situation by removing most of the interfering proteins. Sensitivity can be increased further by using a restricted pH gradient, which provides better separation of the monoclonal immunoglobulin from other serum proteins and provides greater spreading of the polyclonal immunoglobulin. We have used gels of this type in isoelectric and immunoelectrophoresis of monoclonal IgA and IgM (Fig. 2 and 3) and have obtained extremely convincing results with immunoelectrophoresis in the detection of low concentrations of these two immunoglobulins.

The case illustrated in Fig. 6 and the data in Table 1 show the value of immunoelectrophoresis in the identification of paraproteins. With regard to Fig. 6, patients with malignancies involving abnormalities of light chains only have poorer prognoses and shorter remission times than those with malignancies involving complete immunoglobulin

molecules.⁴ If we had followed our original laboratory procedure of reviewing the patient at intervals of three months until the abnormality disappeared or could be characterised by immunoelectrophoresis, then this would have delayed confirmation of light chain abnormality for an unacceptably long time. This case also emphasises that the examination of urine for free light chains is a mandatory procedure in evaluating patients for suspected B cell neoplasia, even if the serum does not show a paraprotein.

Table 1 shows that the major immunoglobulin classes concerned in B cell neoplasia can be characterised at a concentration not possible in all cases by immunoelectrophoresis or immunofixation of the zonal electrophoretic strip.

Hobbs⁴ estimated that a paraprotein in serum could first be detected (presumably by zonal and immunoelectrophoresis) when about 20 g of tumour tissue is present in a 70 kg patient; this figure corresponds to around 9×10^6 tumour cells. Salmon⁵ suggested that minor M components were undetectable by electrophoresis unless the tumour mass was at least 40 g. Since the concentration at which monoclonal immunoglobulin is first detectable by conventional methods can easily vary by a factor of two between samples, Salmon's estimate is in broad agreement with that of Hobbs. One of the main problems in detecting low concentrations of monoclonal immunoglobulin by these methods is the masking of the paraprotein by polyclonal immunoglobulin. This can make identification of a paraprotein on the basis of light chain isotype restriction and distortion of the heavy chain arc difficult, thus limiting sensitivity. In this paper, we have shown that these problems are minimised by immunoelectrophoresis focusing owing to the spreading of polyclonal immunoglobulin and the concentration of the monoclonal immunoglobulin into a limited number of bands, thereby increasing the sensitivity of detection by a factor of between 10, in the case of IgG, and 40 in the case of IgD. Free serum light chain can also be detected with comparably increased sensitivity.

If, as Hobbs suggests, the concentration of monoclonal immunoglobulin is an indication of the tumour mass, use of immunoelectrophoresis focusing would make it possible to detect a primary monoclonal gammopathy and the regrowth of a myeloma in a patient in relapse at a much earlier time. In a previously undiagnosed case, the presence of serum monoclonal immunoglobulin does not necessarily indicate a B cell malignancy. Confirmation of multiple myeloma requires several other criteria to be satisfied.⁶ Kyle⁷ noted that monoclonal gammopathy of undetermined significance (MGUS) is a relatively

common clinical problem and that long term follow up of these patients is essential as some patients go on to develop multiple myeloma and macroglobulinaemia. Kyle found that the only reliable way of distinguishing between MGUS and myeloma or macroglobulinaemia was serial measurement of monoclonal protein, where increasing monoclonal immunoglobulin concentration often indicates a malignancy. Other methods which attempt this distinction are reported regularly, most notable being a plasma cell labelling index,⁸ measurement of serum β_2 microglobulin concentrations,⁹ and enumeration of immunoglobulin secreting cells in peripheral blood.^{10,11} The results reported here show that monoclonal immunoglobulin can be detected when the tumour load is presumably much smaller. The early detection of MGUS by immunoelectrophoresis focusing will influence the long term management of patients at risk. Furthermore, it should be possible to quantitate the paraprotein by scanning the stained immunoelectrophoresis focusing gel, allowing monitoring of changes in paraprotein concentrations during the course of the disease. Work on this is in progress.

The detection of monoclonal immunoglobulin in the relapse stage of multiple myeloma poses a different problem from that encountered in cases of MGUS. In most relapsing patients, the isotype of the monoclonal immunoglobulin is not in doubt, and it may be detectable by conventional methods throughout remission. In some patients, however, the monoclonal immunoglobulin, as assessed by conventional methods, actually disappears.¹² In these cases, a more sensitive method of detection of monoclonal immunoglobulin is obviously desirable and may be a useful indicator of early relapse, as we have shown in 27 patients studied here (Table 2).

Zonal electrophoresis on cellulose acetate membranes, immunoelectrophoresis,^{13,14} and, more recently, high resolution agarose electrophoresis followed by immunofixation¹⁵ have been recommended for detecting low concentrations of monoclonal immunoglobulin. We have shown that

dependable, and at least as rapid as the more conventional techniques.

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