Immunofixation. III. Application to the Study of Monoclonal Proteins

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We have shown how immunofixation can be successfully used to study proteins exhibiting electrophoretic polymorphism. An analogous situation is seen in the disorders of the immune system that result in the production of variable quantities of homogeneous immunoglobulins. Immunoelectrophoresis has been heavily used—sometimes unsuccessfully because of poor resolution—to study these materials. The appearance of a monoclonal immunoglobulin band is identical in both immunofixation and standard gel electrophoresis, a feature that allows unambiguous identification of a protein as to its heavy-chain class and light-chain type. Because antigen and antibody are placed in contact almost immediately after electrophoresis, interfering diffusion does not occur and even extremely small bands can be demonstrated and characterized.

Additional Keyphrases: immunoelectrophoresis • electrophoresis • immunoglobulins • plasma proteins • myeloma • "M" component • diagnosis and prognosis

Many disorders of the immune system result in the production of various amounts of homogeneous immunoglobulins. Since the advent of microimmuno-electrophoresis and an understanding that the immunoglobulins occur in five distinct classes, each subdivided into two major types by virtue of their heavy- and light-chain components, identification of these proteins by use of monospecific antisera has become widespread. Nowhere has immunoelectrophoresis been used more extensively, in both clinical and research laboratories, than in the study of monoclonal proteins. Although immunoelectrophoresis generally fails to identify a monoclonal protein and may produce ambiguous results, even when the abnormal fraction is in high concentration, this essentially unimproved test continues to increase in popularity.

As electrophoresis technology and resolution have improved, the number of homogeneous immunoglobulins of low concentration being identified in clinical samples has increased, as exemplified by the eight samples shown in Figure 1. Because these small bands (whether of real or only suspected immediate significance) may represent forerunners of future overt monoclonal proteinemia, further study of them is of more than academic importance. Here, we describe the application of immunofixation (1) to the study of human monoclonal proteins.

Methods and Materials

Apparatus: the water-cooled chamber and method described by Laurell (2) and the Panagel system (Millpore Biomedical, Acton, Mass. 01720) have been used interchangeably and without modification as described previously (3).

Samples: We used serum, plasma, isolated protein, urine concentrates, and column fractions, adjusting immunoglobulin concentrations to about 2 g/liter

Fig. 1. Standard protein electrophoresis (Laurell) patterns of six serums (on left) and two urines (on right) containing monoclonal proteins of the type difficult to study by traditional immunoelectrophoresis:

From left to right, the monoclonal protein bands are: IgA (beneath transferrin band†), multibanded IgG, IgM (kappa), double IgA (lambda), IgM (lambda, cryoprotein), double IgG (kappa), two bands (kappa light chain, IgG kappa whole myeloma protein), urinary kappa light chain. (In all these figures, the anode is at the top)
Fig. 2. Pattern for serum and urine from a patient whose disease was first suspected from the appearance of a standard serum protein electrophoresis. Immunofixation clearly identifies two fine lambda light-chain bands in the serum, confirmed by the barely perceptible bands on the Fab strip (IgG and IgE values were normal). Large amounts of free lambda-type light chain are seen to be present in the urine. Note that in both serum and urine, antibody to the heavy-chain portion of the IgG molecule fails to show bands. Also note that the urine study demonstrates three additional bands not seen in the serum study.

Fig. 3. Four monoclonal protein bands of IgG kappa type found in a single patient's sample. The original electrophoresis is shown in Figure 1 (second from the left). The additional band present in Figure 1 is due to precipitate at the origin not associated with immunoglobulin, therefore not demonstrated by immunofixation. Standard immunoelectrophoresis showed only an above-normal concentration of IgG kappa. No suggestion of multiple bands was seen, owing to the distribution and intensity of the monoclonal proteins paralleling normal IgG.

When testing serum or plasma and to 100 to 200 mg/liter for urinary light chains. Depending on the potency of the antibody used and the number of immunologically related bands present in the sample, resolution can be improved by changing the concentration of either or both reactants. Samples with much precipitate that will not dissolve on warming to 37 °C should be either centrifuged or allowed to settle before the clear supernatant fluid is applied to the gel surface.

Antiserum: The potency and specificity of antisera used for immunofixation is important; however, as with most other applications, widely accepted quality-control standards are not available and will not be addressed here (4). For the purpose of this paper it is assumed that the antiserum being used is potent and monospecific.

Electrophoresis: The procedure is the same as for standard protein electrophoresis at pH 8.6. In the separation of closely apposed fractions, particularly those present in urine, prolonged migration may enhance resolution markedly. In general, cool water (15–20 °C) is circulated through the Laurell-type chamber; however, if a serum contains a cryoprotein, electrophoresis at higher temperatures may be necessary before these sensitive proteins migrate satisfactorily.

Immunofixation: Cellulose acetate strips, 1 cm wide and 3 cm long, are dipped into undiluted nephelometric-grade antiserum (Atlantic Antibodies, Westbrook, Me. 04094), drained briefly, and carefully applied to the appropriate zone on the surface of the agarose film immediately after electrophoresis. The strips are left in contact with the gel for about 1 h, in a moist chamber.

With rare exception, a monoclonal protein band in serum, is located cathodal to the α2 zone. However, monoclonal proteins in urine may migrate close to albumin and so be missed unless the antiserum-containing strips include the α1 area as well.

Washing, fixation, and staining are as previously described (3).

Results

Even at very low concentration, discrete bands visible in standard agarose electrophoresis are always visible by immunofixation, whether the protein is an entire immunoglobulin or an immunoglobulin subunit (Figure 2). Because electrophoresis for immunofixation is done exactly as for routine staining, bands made visible by both methods are compared easily. In instances where there are several closely apposed bands, they remain resolved on immunofixation (Figure 3), but are completely lost, through diffusion, on immunoelectrophoresis. Light-chains, even of IgA and IgM monoclonal proteins, are often impossible to identify by immunoelectrophoresis (5), but are clearly apparent by immunofixation (Figure 4).

In instances where a monoclonal protein is either too highly concentrated for the amount of antisera applied or it is antigenically relatively unfamiliar, a clear zone of antigen excess without precipitation is flanked by two well-defined zones where an appropriate precipitation reaction occurs (Figures 5 and 6). This is seen most often in light-chain typing of IgA myeloma proteins. Nevertheless the study yields appropriate data corroborated by the absence of a band in the other
light-chain immunofixation strip (Figures 2 and 4). Repeat studies with lower antigen concentrations are unnecessary unless desired for aesthetic reasons.

Samples from patients with heavy-chain disease, on the other hand, would show an abnormal distribution of precipitate in the strip processed with the respective anti-heavy-chain antiserum, but not in the strip incubated with any of the various anti-light-chain reagents. Samples that contain free alpha chain must be interpreted cautiously. Such sera usually show a broad distribution of the abnormal protein. Occasionally, IgA myeloma proteins will also show a poorly resolved wide band, even when electrophoresed at low concentrations. IgA myeloma proteins of kappa light-chain type appear to have light chains of an antigenic make-up that is readily recognized by most available anti-kappa antisera. On the other hand, IgA myeloma proteins of lambda type are highly variable in light-chain antigenicity and may have only miniscule reaction with commercially available anti-lambda antisera. Such a sample could present a broad precipitation with anti-alpha-chain antisera and apparently none with anti-light-chain reagents. By reducing the amount of protein electrophoresed and loading the antiserum strip heavily, the lambda precipitate can be shown in most cases. Furthermore, use of an anti-Fab antiserum has proven helpful for cross checking.

The normal immunoglobulins can be seen clearly after immunofixation, allowing estimation of monoclonal protein immunoglobulin concentrations. Generally, this is difficult when IgA and IgM are studied by immunoelectrophoresis, and often IgG as well.

A single patient’s immunoglobulins can be completely surveyed on a single plate. Figure 6 illustrates a normal serum studied with antisera to four immunoglobulin heavy chains and four antisera to different types of light-chain antigens. Samples were applied at different dilutions because of the wide normal variation in concentrations. Figure 2 shows a different type of survey, where both serum and urinary proteins were studied simultaneously, yielding unambiguous results.

Serum monoclonal proteins are sometimes sensitive to temperature changes. Because electrophoresis may be done at temperatures of +4 to +20 °C, this is an important consideration. In the Laurell water-cooled chamber, temperatures can be adjusted easily to allow cold-sensitive proteins to migrate properly (Figure 7).

Samples with high titers of rheumatoid factor or other immune complexes also may leave a precipitate at the origin, even though the sample has been centrifuged. In these instances immunofixation will give a reaction, not only with antisera to IgM and Fab as expected, but in addition with antisera to IgG, kappa, and lambda and perhaps complement as well. The rheumatoid factor, perhaps monoclonal itself, will react indiscriminately with IgG of both light chain types. In these instances,
2-mercaptoethanol should be used, or the protein isolated, for proper interpretation.

Antiserum monospecificity is required in immunofixation, but an antiserum's failure to react appropriately with a monoclonal protein may be misleading. Here again, immunofixation has a valuable asset, as shown in Figures 5 and 6, where antigen excess may occur even with very satisfactory reagents. Antiserum lacking antibody to a particular protein variant may show a pale zone where no precipitation has occurred, flanked by a normal amount of precipitate.

Discussion

With the improvements in electrophoretic resolution attained in the last few years, more samples are found to contain small monoclonal protein bands that are of concern to the clinician and investigator. Whether they are of clinical significance remains to be ascertained. Nevertheless, many studies are being done by immunoelectrophoresis in an effort to unambiguously describe these proteins, which are believed to be abnormal homogeneous immunoglobulins. Because of the poor resolution of immunoelectrophoresis, identification of these small oligoclonal bands may not be possible. Furthermore, if the worker is concerned with further characterization of the light chain of an IgM or a small IgA monoclonal band, the "umbrella effect" (5) of the IgG molecules of the same light-chain type often inhibits identification. Immunofixation is generally unaffected by these problems, as illustrated in Figure 7.

Immunofixation reduces reagent consumption and cuts performance time while requiring no equipment beyond that needed for standard serum protein electrophoresis in agarose gels.

The simplicity and economy of the method and the clarity of final results suggest that immunofixation should be considered as a possible successor to clinical immunoelectrophoresis for the study of monoclonal proteins.

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References