

Immunoglobulins and paraproteins

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Abstract

Measurement of immunoglobulins (IgG, IgA and IgM) is a commonly requested investigation in both clinical biochemistry and immunology laboratories. Whilst abnormal concentrations of these proteins are found in many conditions, only in the investigation of immune deficiency and B cell malignancy is there clear clinical justification for their measurement. Methods used for immunoglobulin measurements should be accurate, sensitive and precise. It is recommended that a protein electrophoretic separation should also be done as part of the investigation of immunoglobulins as this is the only reliable way of assessing the clonality of the immunoglobulins and so detecting paraproteins. Paraproteins are the earliest described tumour markers and remain an essential part of the investigation, diagnosis and monitoring of patients with B cell dyscrasias. Electrophoresis methods should be optimised so the protein is not too heavily applied to the support medium and to enable good separation of the various zones (particularly within the beta-gamma region). Automated gel electrophoresis methods and automated capillary zone electrophoresis are now becoming available. Samples showing paraproteins or samples with raised IgA and IgM concentrations that cannot be confirmed as polyclonal by the electrophoresis pattern, should be investigated by immunofixation to confirm or exclude the presence of a paraprotein. Immunofixation remains the method of choice for paraprotein typing because it is fast, specific, flexible and easy to interpret. Immunofixation is more sensitive than electrophoresis and may detect paraprotein bands that are not visible on routine electrophoresis.

In the laboratory investigation of B cell malignancy, both serum and urine investigations are essential. Monoclonal free light chain, Bence Jones protein, in urine is suggestive of B cell malignancy and may be the only tumour marker present. A trace of albumin should be visible in the electrophoretic separation of the urine sample to indicate adequate sensitivity.

Immunochemical quantification of paraproteins (in both serum and urine) is unreliable. The recommended method of is densitometric estimation from the protein separation and calculation of the paraprotein concentration relative to the serum or urine total protein or (in certain situations) to the globulin concentration i.e. total protein minus albumin concentration.

The measurement of serum immunoglobulin concentrations is readily available but with budgets that seem to increase more slowly than the workload, we must assess whether we are doing the tests to the best of our abilities and whether all the requests are justified.

Introduction

Immunoglobulins, high molecular weight proteins with antibody activity were identified in 1959.¹ During the 1970s and 1980s, reliable, easy and rapid methods to measure the concentration of these proteins in clinical laboratories became available. Immunoglobulin quantification became a common part of the investigation of patients with a whole variety of diseases. It is interesting to consider whether we would be doing so many immunoglobulin assays had they been discovered more recently, and had to complete a rigorous evidence-based evaluation before their introduction into routine laboratory practice.

Immunoglobulins - basic structure and function

In 1959, Heremans used the term 'immunoglobulins' for the 5 groups of proteins (designated IgG, IgA, IgM, IgD and IgE) within the gamma globulins. The immunoglobulins have a common basic monomeric unit of a Y shaped molecule consisting of two identical heavy chains (α , γ , μ , δ or ϵ) and two identical light chains (κ or λ). The monomeric structure can be joined into dimers (the predominant form of IgA in secretions) or into pentamers (IgM). Both heavy and light chains are divided into two regions, a constant region, which confers function e.g. macrophage binding or complement binding, and a variable region where antigen binding occurs. Antigen binding is dependent upon a three dimensional shape that fits the antigen and is determined by the amino acid sequence of the heavy and light chain. As the name suggests, the variable region shows diversity enabling the recognition of an enormous range of antigens. There are various estimates of the potential number ranging from 10^6 to 10^{10} .

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Paraproteins

Factors influencing serum concentration

The serum concentration of the immunoglobulins, like that of other proteins, is a result of the balance between synthesis and catabolism (and/or loss) and the volume in which the proteins are distributed. The rate at which IgG is catabolised is proportional to the plasma concentration. The normal catabolic rate for IgG is approximately 7% of the plasma pool per day. This can increase to 16-18% with raised IgG concentrations (e.g. myeloma, chronic infection or inflammation) or fall as low as 2% in hypogammaglobulinaemia. The catabolic rate of IgA and IgM are independent of serum concentration and both have half-lives of approx. 5 days in contrast to the much longer half life (19-21 days) of IgG.

Patterns in disease

Disease states may cause either an increase or a decrease in the production of any one or combinations of the immunoglobulins. The paper describing immunoglobulin patterns and disease associations published by Hobbs in 1971 remains a seminal publication.² For example, most generalised infections result in increased concentrations of IgG, IgA and IgM, whereas if the challenge is mainly directed at mucosal surfaces, IgA may predominate, and with viral infections or when the challenge is predominantly in the bloodstream, IgM may predominate. These are non-specific responses and of negligible clinical value other than to show that the patient has "something going on". In liver disease, a predominant (and significant) increase in IgM is often associated with primary biliary cirrhosis (this may be a useful finding and we always check for mitochondrial antibodies in these patients). Predominant increases of IgA are seen with 'toxic' liver damage e.g. from alcohol or iron, and predominant increases of IgG are seen with chronic aggressive hepatitis. Again, these patterns are, at best, associations and the measurement of immunoglobulin concentrations will add little to the diagnosis or management of the patients. It is worthwhile noting that once a patient has had abnormal results, the investigations tend to be repeated at every visit! This seems particularly true for patients with autoimmune rheumatic diseases where polyclonally raised IgG and IgA concentrations are commonly seen. There is no indication for repeated measurement of IgG, IgA and IgM in patients who have polyclonally raised immunoglobulin concentrations.

Indications for measurement

There are two clear indications for the measurement of serum immunoglobulin concentrations. These are (i) the investigation of immune deficiency - either primary or secondary and (ii) the investigation of B cell malignancy.^{3,4} In both cases, the serum IgG, IgA and IgM measurements should satisfy the following criteria:

- Be sensitive (suggested lower limits of sensitivity for IgG of 1g/L, IgA of 0.1g/L and IgM of 0.1 g/L (lower limits for IgA and IgM in paediatric samples)

- Be accurate (calibrated to the International Reference Preparation CRM470)
- Be precise (coefficient of variation <5% at low, normal and high concentrations)
- Use an antigen excess check
- Be validated by adequate performance in an external QA programme.
- Be accompanied by serum and, if investigating for B cell malignancy, urine protein electrophoresis

The investigation of immune deficiency has been well described in many documents so the remainder of this review will concentrate on the detection and typing of paraproteins in the investigation of suspected B cell malignancy.

Serum protein electrophoresis

In 1937, Tiselius separated serum globulins into three parts, which he called the alpha-, beta and gamma globulins. The term paraprotein was introduced by Apitz in 1940 to describe the abnormal proteins in blood, urine and tissues that are produced by myeloma cells.

Immunoglobulins are the product of B lymphocytes. Proliferation of a single B cell produces a 'clone' producing immunoglobulin with identical heavy and light chains, exactly the same amino acid sequence and therefore electrophoretic mobility. This will show as an abnormal band on the electrophoretic separation as a paraprotein.

The B cells usually produce a slight excess of light chains, these are polyclonal, of low molecular weight and are cleared through the kidney. A malignant (or non-malignant) transformation of a B cells may lead to clonal expansion, secretion of a monoclonal immunoglobulin and secretion of excess monoclonal free light chains or Bence Jones protein.

Reasons why the investigation of serum and/or urine for a paraprotein is useful include:

- the presence of a paraprotein in serum and/or urine is a diagnostic criteria in some B cell malignancies
- in B cell malignancy, the paraprotein is a sensitive tumour marker
- immunoglobulin fragments (particularly Bence Jones protein) are rarely found in non-malignant conditions
- the serum and/or urine gives an 'overview' of the B cells products while B cell disease may be patchily distributed
- some symptoms may be directly related to the presence of a paraprotein e.g. hyperviscosity with high concentrations of IgM paraproteins

Electrophoresis is the only reliable method for the detection of paraproteins in serum and or urine. Automated quantification of kappa and lambda light chains and calculation of the ratio has been proposed as an alternative. In practice, this method has many limitations; it is poor at identifying serum paraproteins at low concentrations (below 5g/L) and identifying situations where there are two distinct paraprotein types. It is also poor at detecting Bence Jones protein in addition to an intact immunoglobulin paraprotein and identifying paraproteins where a polyclonal increase in immunoglobulins is dominated by one light chain type. This system does not lend itself to typing IgD or IgE paraproteins simply because these antisera are rarely available for nephelometric analyses.

Detection of paraproteins

Agarose electrophoresis is the most common method currently in use in the U.K. for the detection of paraproteins in serum and urine.⁵ There are still a few users of cellulose acetate as a support medium. There has been a trend towards automation of electrophoresis with automated agarose gel systems, suitable for both serum and urine electrophoresis, now available. Automated capillary zone electrophoresis systems are also available but to date, only suitable for serum electrophoresis.

Serum electrophoresis

The electrophoresis is the initial screening procedure and should therefore have sufficient resolution to do this adequately. Ideally agarose separations should be long enough (3 – 4 cm) to allow good separation of zones and show a good spread of the beta-gamma zone, which is achieved by properties of the agarose gel and buffer system that produce high endosmotic flow. The majority of serum paraproteins will be found in the region from the start of the beta to the end of the gamma zones. Occasionally paraprotein bands appear in the alpha-zones and in the post gamma.

Paraprotein bands may be 'missed' if they are at low serum concentration (<5.0 g/L) or where their mobility coincides with other bands such as beta globulins. It is also possible to overlook a paraprotein where there is no suppression of normal immunoglobulin concentrations. In our laboratory, serum electrophoresis and immunoglobulin quantification are always done together - it is impossible to comment upon the clonality of immunoglobulins without serum electrophoresis and impossible to comment upon IgA and IgM concentrations from electrophoresis alone. We will do immunofixation on samples when no obvious paraprotein band is detected but where there is raised IgA or IgM without the increased staining of the beta-gamma region that is associated with polyclonally raised IgA or IgM. In our experience capillary zone electrophoresis more readily detects such polyclonally raised IgA and IgM than does agarose gel. IgD paraproteins and free heavy chains are susceptible to post-synthetic degradation, which results in diffuse paraprotein bands on electrophoresis. These may be missed if present at low concentrations or if there is an expectation of seeing a clearly defined band.

There are a number of situations where a band is seen in a serum electrophoretic separation that is not monoclonal immunoglobulin, these are shown in the following list:

- additional bands in the alpha-1 region due to allotypic variation in alpha-1 antitrypsin
- split alpha-2 zone due to the different mobility of the haptoglobin-haemoglobin complex (most often formed from haemolysis during collection of the blood sample)
- an additional band in the beta-gamma due to high concentrations of C-reactive protein
- additional bands in the fast gamma due to the presence of fibrinogen.

It is also worth noting that some paraproteins precipitate at temperatures below 37°C - so called cryoproteins. Samples where cryoprotein is being considered must be collected, transported and separated at 37°C. Failure to do this may result in the precipitation of the cryoprotein and its subsequent discard with the cell pellet.

Low concentration paraproteins:

The chance finding, either on electrophoresis or revealed by immunofixation, of a low concentration paraprotein, in the absence of any clinical indicators of myeloma or related malignant diseases, should be considered as monoclonal gammopathy of unknown significance (MGUS). This is not rare, occurring in 1% of the population over the age of 50 and 3% over the age of 70 years.⁶ If the serum paraprotein concentration is less than 15 g/L the recommendation is that electrophoresis should be repeated annually.⁷ Unless there is progressive increase in paraprotein concentration or emerging symptoms, additional studies including bone marrow biopsy are not indicated.

Urine electrophoresis

Urine analysis is an essential component in the investigation of patients with paraproteinaemia or with suspected B cell malignancies. An important feature used to help distinguish malignant from non-malignant conditions is the finding of immunoglobulin fragments produced by tumour cells. The fragments, which may not be detectable on serum electrophoresis separations, are usually of lower molecular weight than intact immunoglobulin molecules, pass readily through the kidney, and may be clearly visible in the urine due to the concentration effect. The finding of Bence-Jones protein (BJP) therefore provides a high index of suspicion for malignancy, although it does occur in apparently benign conditions. International guidelines for the detection of Bence Jones protein have recently been published⁸ A mid-stream early morning urine (usually the most concentrated of the day) should be used for the detection of Bence Jones protein. A 24hr collection is required if the disease is being monitored by Bence Jones protein excretion.

Paraproteins

Even low concentrations (10mg/L) may be significant so that high sensitivity electrophoresis of urine is essential. This degree of sensitivity can be achieved by using agarose electrophoresis of non-concentrated urine and a sensitive stain. Alternatively, concentrated urine (at least 100-fold concentration of an early-morning urine preferable) may be run in the serum systems. Whatever system is used, a trace of albumin must be visible in all urine samples, if this is not the case, the sample should be re-run after being further concentrated. Where a BJP is present in significant concentrations (>100mg/L) with no accompanying glomerular or tubular proteinuria, detection is straightforward and the immunofixation identification step is unequivocal. However, the frequent renal damage associated with BJ proteinuria, results in complex, non-standard patterns requiring immunofixation to resolve the possible presence of BJP. A low concentration of BJP may also accompany significant glomerular proteinuria in patients with light chain renal amyloidosis, the urine of any such patient should be investigated by immunofixation even in the absence of a band suggestive of BJP. Patients with serum paraproteins may show a "leak" of the serum paraprotein into the urine. This may occur with or without Bence Jones protein and immunofixation is essential to distinguish this.

A number of other proteins may appear as discrete bands on urine electrophoretic separations, particularly where there is an element of tubular proteinuria. These include:

- the alpha- and beta-microglobulins,
- lysozyme (migrating in the slow gamma region)
- degraded fragments of glomerular origin
- rarely seminal fluid proteins
- occasionally the beta-2 microglobulin will be present in high concentrations and give a very prominent band.

Bence Jones protein associated with IgM paraproteins

Waldenström's macroglobulinaemia is demonstrated by the infiltration of the bone marrow by a lymphoplasmacytic cell population, By definition a serum monoclonal IgM is present. Although low concentrations of Bence Jones protein are found in up to 70% of patients, it exceeds 1g/24hrs in only 3% of patients⁹ and adds little to the diagnostic profile of tests and clinical findings. However, Waldenström's macroglobulinaemia is not the only condition in which a monoclonal IgM may be found; others include chronic lymphocytic leukaemia, diffuse large B-cell lymphoma, follicular lymphoma, and mantle-cell lymphoma. Patients with asymptomatic monoclonal IgM without bone marrow infiltrate of less than 20% may be classified as IgM-monoclonal gammopathy of unknown significance.

Due to the range of conditions in which monoclonal IgM may be found the finding of significant Bence Jones protein serves a similar function to that in myeloma of increasing the suspicion of malignancy.

Identification of paraproteins

The detection of a paraprotein band must always be followed up with the specific typing of the band. It is important that the heavy and light chain components are identified because this confirms monoclonality and the paraprotein type may give the clinician additional information about the underlying tumour and prognosis. A patient's electrophoresis pattern may change during the course of their disease or treatment so the initial investigations can serve as a point of reference. Complete disappearance of the paraprotein is rare but is occurring increasingly with treatment regimens using high dose chemotherapy and bone marrow or stem cell transplantation. An oligoclonal-banding pattern is sometimes seen in samples from patients post bone marrow transplantation and it is important to distinguish this from the original paraproteinaemia. Oligoclonal banding is associated with the engraftment process whereas re-appearance of the original paraprotein will indicate residual disease.

Agarose gel immunofixation

Immunofixation on agarose gels has been the method of choice for the detection of paraproteins for many years.¹⁰ It is quick, flexible and simple to interpret. In our laboratory, initially immunofixation is done to test for the alpha, gamma or mu heavy chains and the kappa and lambda light chains. Additionally we test for the delta and epsilon heavy chains where a serum shows monoclonal light chains without a corresponding alpha, gamma or mu heavy chain. Occasionally some IgA or IgD paraproteins will not react with light chain antiserum due to their conformational arrangement. Also some large aggregated IgM paraproteins may precipitate into the gel on application and do not separate electrophoretically; these samples show a reaction in all antiserum lanes. In both these situations the true identity can be revealed by mild sulphhydryl reduction using dithiothreitol prior to electrophoresis and immunofixation. The immunofixation is very flexible and by using appropriate antiserum, a variety of other proteins may be identified. The most likely proteins to exclude when investigating samples for monoclonal gammopathy are fibrinogen, C reactive protein, beta-2 microglobulin and complement components.

In recent years, the suggested follow-up protocol for patients post haematopoietic stem cell transplantation for myeloma has included the detection of monoclonal immunoglobulin to establish whether the paraprotein has disappeared and to detect minimal residual disease. It is recommended that the samples be immunofixed even if there is no paraprotein band visible in the electrophoretic separation. Immunofixation is more sensitive and specific than electrophoresis for the identification of paraproteins in these circumstances and has been shown to have

comparable sensitivity to the detection of monoclonal immunoglobulin gene rearrangement.¹¹

Immunofixation for Bence Jones protein is done with the same antibodies used in the serum system. The anti-lambda and anti-kappa antibodies are unable to distinguish between free light chains and those incorporated into an immunoglobulin molecule but the presence of free light chains is assumed if no heavy chain reaction is present. Antibodies reacting only with free light chains are commercially available but these are generally of low titre and react poorly in immunofixation systems.

The major difficulty in the interpretation of urine electrophoresis and immunofixation patterns is the distinction between monoclonal light chains and light chain fragments generated from normal immunoglobulin catabolism. The light chain fragments typically show a "ladder-like" pattern, particularly in the immunofixation reaction with antiserum to kappa light chains.^{12,13}

Paraprotein identification in CZE

The immunofixation technique, where antigen-antibody complexes are precipitated into a gel matrix, is not applicable to the automated CZE instruments. Instead a technique termed immunosubtraction is used. The sample is pre-reacted with sepharose beads coated with antiserum to the various heavy and light chains, large macromolecular antigen-antibody complexes form on the coated beads and these sediment leaving a supernatant that should not contain the antigen corresponding to the antibody on the bead. The supernatants are aspirated into the system and sequential electrophoretic separations are generated i.e. one showing the pattern after IgG has been 'subtracted', another after IgA has been 'subtracted' and so on. The interpretation of this technique relies on the disappearance of a whole peak, part of a zone or a change in shape of a zone. Unlike immunofixation, immunosubtraction does not amplify the signal that is read and it is entirely dependent upon a paraprotein band being clearly visible in order for its disappearance to be noticeable. Immunosubtraction does work well for the typing of large paraproteins superimposed on a gamma zone of reduced intensity. However, in our experience, it is not satisfactory for typing small paraproteins particularly with gamma zones of normal intensity and for distinguishing polyclonal increases of the gamma where one of the light chain types predominates. To date, immunosubtraction methods show neither the flexibility nor the sensitivity of immunofixation.

Quantification of serum paraproteins

Immunochemical quantification of paraproteins is unreliable.^{14,15} The densitometric estimation of electrophoretic separations is recommended for measurement of paraprotein concentration. In agarose systems it is important to be aware that there is a differential dye binding between albumin and the globulins, therefore the most precise estimation of paraprotein is derived from the percentage of relative dye-binding of the paraprotein band compared with the total globulin fraction rather than the total protein. It is also important to note that there is a non-linear relationship between dye-binding and protein concentration at high paraprotein concentrations. The CZE system directly reads the protein concentration at the 'exit' end of the capillaries by UV absorption and therefore is not influenced by the problems related to the staining stage of the electrophoretic technique.

Measurement of serum total protein and albumin are generally reliable and it can be useful to use these two concentrations as a 'rough check' of the paraprotein quantification. The albumin concentration added to the paraprotein concentration cannot exceed the total protein concentration and (accepting that there may be differential albumin to globulin binding) bands of similar areas should ultimately be of similar concentrations.

Quantification of Bence-Jones protein

Quantification of BJP is being recommended as a criterion for response, progression of relapse of multiple myeloma treated by high-dose therapy and stem cell transplantation.¹¹ We recommend that this is done, like serum paraprotein quantification, by densitometry of the urine electrophoresis and calculation of the paraprotein band with respect to the urine total protein (either random or 24 hour).

Conclusion

It is important that laboratories offering immunoglobulins and protein electrophoresis are aware of the most appropriate reasons for doing these tests and that these tests are done in the most appropriate way.

Reviews

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