Laboratory investigation of paraproteinaemia

The term "paraprotein" describes an abnormal discrete band seen on electrophoresis of serum or urine, comprising intact or fragmented homogeneous immunoglobulin molecules derived from a single clone of B cells. Bence-Jones protein describes free monoclonal light chain. This expansion of a B cell clone can be progressive and malignant or self-limiting and benign. A paraprotein is often associated with malignant neoplasms such as multiple myeloma and Waldenström's macroglobulinaemia (in which it is a sensitive tumour marker) but the occurrence of paraproteinaemia in benign conditions limits its specificity. Identification, typing, and quantitation of paraproteins is used in the diagnosis and management of patients with B cell tumours but several other laboratory tests can also provide information helpful in distinguishing malignant from benign conditions. Some tests can be regarded as essential; others are indicated only in special circumstances (table).

The methods provided in this broadsheet are those which have given consistent results in our hands.^{1,2}

Collection and storage of samples

SERUM

Blood is collected into a plain tube with no anticoagulant. Serum is preferred to plasma as fibrin-

ogen forms a band in the fast gamma region on electrophoresis. Haemolysis should be avoided as the haemoglobin-haptoglobin complex forms a band between the α -2 and β region, and free haemoglobin forms a band in the β region. *These are all zones of the electrophoretic separation in which paraproteins can be found.* Serum is stored at 4°C for investigations needing immediate attention; sodium azide (1 mg/ml) may be added to the serum as a preservative. Long term storage should be at -40 to -70°C; freezing and thawing of serum is undesirable and rare samples (monoclonal IgD or IgE, or free heavy chains) which make valuable reference materials or controls, should be stored frozen in small aliquots for convenient use.

URINE

An early morning urine sample is required for the detection of Bence-Jones protein. *A urine sample should accompany any serum referred for paraprotein studies.* In the absence of albuminuria dip-stick testing may be negative even when Bence-Jones protein is present. *Chemical screening tests such as Bradshaw's are insensitive or non-specific; all samples require concentration and electrophoresis.* Quantitation of Bence-Jones protein ideally requires a careful 24 hour collection. Samples can be preserved in a similar way to serum by the addition of sodium azide (0.1 mg/ml). Alternatively, for spot samples, urine can be pressed through a prefilter and then a millipore filter of 0.2 μ M pore size. Such filtering will remove bacteria which otherwise would be concentrated with the urine and cause protein degradation.

Concentration of urine

Urine samples with a total protein concentration of 1g/l or more should be examined directly. All other samples should be concentrated up to 300-fold as necessary. Adequate concentration is achieved when at least some protein is visible on electrophoresis. Concentration techniques based on ultrafiltration are recommended. Semipermeable membranes should have a molecular exclusion below 20 kilodaltons. We prefer cellulose nitrate collodion thimbles (SM 132 00, Sartorius, Belmont, Surrey) as there is high recovery (>85%) of Bence-Jones protein monomer. The urine

Table 1 *Laboratory investigation of paraproteinaemia*

Essential investigations:

Electrophoresis of serum and urine (concentrated as appropriate)

Immunofixation to identify and confirm serum or urine paraprotein

Quantitation in serum of:

Total protein

Albumin

Paraprotein

Immunoglobulins

β -2-microglobulin

(Calcium, urea or creatinine, haemoglobin, bone marrow biopsy)

Quantitation of urine total protein

Specialised investigations:

Aggregates or high molecular weight complexes:

Cryoglobulins

Hyperviscosity

Paraproteins with antibody activity:

Rheumatoid factor

Cold agglutinin

Fig 1 Illustration of the use of a Collodion thimble to concentrate prefiltered urine.

is placed into the thimble, around which is assembled a glass flask previously filled with 2.7% w/v solution of sodium chloride, with a side arm connected to a water pump (fig 1). Samples are rapidly concentrated and dialysed at the same time by such a vacuum. If thimbles are handled carefully and not allowed to dry out they may be washed and reused until they become so "clogged" as to be too slow for use (after about 12 times). Centriflo membrane cones or Mincon B15 concentrators (Amicon, Woking, Surrey) are more convenient systems but we are unable to provide data on recovery of Bence-Jones protein monomer.

Electrophoresis

Good quality electrophoresis is essential for two reasons: firstly, it is most often used as a screening test, and only those serum or urine samples showing an abnormal band will be selected for further investigations; secondly, the recommended method for quantitation of paraprotein is densitometry, and precision will be reduced by poor quality separations.

Cellulose acetate membranes (CAM) or agarose gels are the support media used in most laboratories for electrophoresis. Many "in house" and commercially available systems perform adequately. The most important requirements are: (i) for separations of sufficient length, to readily distinguish a monoclonal from a polyclonal immunoglobulin increase (at least 4 cm from the front of the albumin zone to the back of

the gamma zone is recommended); and (ii) for several samples to be run in parallel so that test samples can be compared simultaneously with a reference sample, and serum and urine samples from an individual patient can be applied side by side. Microzone electrophoresis definitely cannot be recommended.

The review by Kohn⁴ remains a valuable source of information regarding the technical considerations of CAM electrophoresis.

Equipment required is as follows: a multiapplicator; a power source; an electrophoresis tank. Suitable equipment can be obtained from Shandon (Shandon Southern Products Ltd., Runcorn, Cheshire); Vokam power supply, 600 Electrophoresis chamber; but other equipment may be used as long as the bridge width on the chamber permits the recommended CAM separation distance of 4–5 cm.

REAGENTS AND MATERIALS

Unless otherwise stated all reagents are Analytical Reagent grade and obtained from BDH, Poole, Dorset.

1 0.06 M barbitone buffer (pH 8.6) comprising 206 g sodium barbitone and 36.8 g diethylbarbituric acid (barbitone). The reagents are made up to 20 litres with distilled water. For long term storage, 100 ml propan-2-ol and 5.0 g finely crushed thymol should be added as a paste. When the solution is clear, which may require gentle warming, add 8.0 g calcium lactate. Finally the pH is checked (8.6) but should not require adjustment.

2 Cellulose acetate membranes (Sartorius, Belmont, Surrey) 78 × 150 mm sheets.

3 3MM chromatography paper (Whatman, Maidstone, Kent).

4 Bromo-phenol blue-saline diluent comprising saturated bromo-phenol blue prepared in 0.9% w/v sodium chloride solution and 30 drops added to 30 ml of 0.9% w/v sodium chloride solution.

5 Naphthalene black stain comprising 2.5 g naphthalene black 12B (TS Gurr), 500 ml distilled water, 500 ml methanol, 30 ml glacial acetic acid. Filter before use.

6 Destain solution comprising 5% v/v glacial acetic acid in industrial methanol.

7 Industrial methanol.

8 Distilled water.

METHOD

1 Serum samples are diluted 9/1 with bromo-phenol blue-saline diluent. The dye forms a complex with albumin and allows visual assessment of migration to standardise the length of the electrophoresis separation. Urine samples (concentrated as necessary) are not diluted.

2 The CAM strips are presoaked in a small bath of electrophoresis buffer by carefully layering them on to

the liquid surface and then submerging for a few minutes. White areas on the soaked strips are caused by trapping of air and indicate incomplete wetting; such strips are not suitable for electrophoresis.

3 Excess moisture is removed from the strip by blotting between sheets of chromatography paper. The strip is then placed on a clean dry piece of chromatography paper, on a flat surface, for sample application.

4 Up to 10 samples are applied across the centre of the strip using a multiapplicator. This must be done quickly to prevent the strip drying out as this will give uneven electrophoresis.

5 The strip is then placed into the electrophoresis tank. Contact between buffer and CAM is made with "wicks" of two layers of 3MM chromatography paper. One hundred and fifty volts are applied with the power source held on "constant voltage". With the buffer described this should generate about 10 amps per strip and adequate migration distance in 30 to 40 minutes. The migration distance is best monitored on each run by observing the blue dye-albumin front.

6 After electrophoresis, strips are stained in naphthalene black for 10 minutes placed into destain solution for five minutes, then into a second destain solution for another five minutes. The strip should not be removed from this destain until the background is completely clear. Destain solution must be renewed often to ensure background clarity. The strip is then transferred for five minutes to methanol which removes the acetic acid, and finally to distilled water for 10 minutes to rehydrate.

7 The strip is finally dried between two sheets of chromatography paper under a weighted glass sheet. This prevents shrinkage of the strip on drying, which can be a problem with strips that have been subjected to solvents.

NOTES

1 Electrophoresis buffer can be reused but the polarity should be reversed after each run. Alternatively, the buffer can be taken from the tank, mixed, and then replaced.

2 Some multiapplicators allow up to 16 sample applications, but the resulting zones are too narrow and interpretation is more difficult. A maximum of 10 is recommended.

3 Different grades of CAM influence the protein separation. Changes in relative mobilities of zones are generally acceptable but an essential requirement is that there should be sufficient spreading of the gamma zone (at least one third of the total separation length).

4 Ponceau S is widely used as an alternative stain for CAM. Sera should be diluted 1/1 with buffer when this stain is used, or else non-linear dye binding occurs for albumin. Ponceau S (0.2% w/v) in 3% (w/v) tri-

chloracetic acid (TCA) is prepared as follows: 12 g TCA; 400 ml distilled water; 0.8 g Ponceau S. Dissolve and filter before use. CAM strips are destained in successive washes of 5% acetic acid in water and finally air dried. There is no shrinkage after this staining procedure so pressing of the membranes is not required.

5 Higher resolution protein separations result from electrophoresis in agarose gels at high voltage. Similar separations can be achieved on CAM with a simple modification to the methods described above.⁵ A 25% stock solution of Tween 20 is prepared by adding 10 ml of Tween 20 to 30 ml of electrophoresis buffer (as Tween 20 is very viscous this should be done using a measuring cylinder). This stock is then used to make the CAM soaking buffer by adding 3.2 ml to another 200 ml of electrophoresis buffer. The stock Tween solution can be stored at room temperature.

Agarose gel electrophoresis

Our preferred method for agarose gel electrophoresis has been published⁶ with complete details of techniques and the preparation of buffer, fixative, and stains. The following details are given mainly to indicate more convenient United Kingdom suppliers of reagents or equipment than the American companies referred to in the selected method, or minor variations in methodology.

Equipment required is as follows: glass plates (205 × 110 mm); U-frame for casting gel (1 mm thick) obtainable from Biorad Laboratories Ltd, Watford, Hertfordshire; electrophoresis chamber with water cooled platform; high voltage power supply (minimum 300 volts).

REAGENTS AND MATERIALS

1 0.06M barbitone buffer (as for CAM).

2 1% w/v Litex HSA agarose (Park Scientific Ltd, Northampton) boiled in electrophoresis buffer until dissolved.

3 Gelbond (Miles Laboratories, Slough, Buckinghamshire)

4 Sample application foil (LKB, Croydon, Surrey or Pharmacia, Uxbridge, Middlesex)

5 3MM chromatography paper

6 Saturated solution of bromo-phenol blue in 0.9% w/v sodium chloride

7 Fixative comprising 11.7 g picric acid, 833 ml distilled water. Solution is heated gently at 60°C (best done in a water bath), filtered through Whatman No 1 filter paper, and cooled. To this solution is added 167 ml glacial acetic acid.

8 Amido black 10B stain comprising 1.25 g amido black 10B, 250 ml destaining solution (see below).

9 Destaining solution comprising 2250 ml ethanol, 2250 ml distilled water, 500 ml glacial acetic acid.

METHOD

1 To prepare the gel, a sheet of Gelbond is placed, hydrophilic side uppermost, on a glass plate wetted with 95% v/v methanol. The methanol helps to exclude air bubbles from between the glass and the film, which would otherwise lead to an uneven gel layer. The U-frame is placed on top of the film and then a second glass plate makes a "sandwich" which is held together with small paper clips. The molten agarose, cooled to about 70°C, is then poured into the mould; this is easier if the two glass plates are slightly staggered so as to form a lip at the top. The agarose is allowed to set for at least 30 minutes. The top glass plate and U-frame are removed and the gel is ready for use. Leaving the bottom plate in position until after sample application helps in handling the gel.

2 The surface of the gel is blotted with a sheet of cellulose acetate and then the sample foil, which is a thin plastic sheet with pre-cut slits, is layered on to the surface of the gel, at about one third of the distance along the short axis. Care should be taken to exclude all air bubbles so that samples cannot run under the foil. Undiluted sample is either applied as a fixed volume (3–5 μ l) to each of the slits and allowed to diffuse in completely (five minutes), or a larger volume is flooded over the slit and excess removed by blotting after five minutes. Bromophenol blue may be used to mark the albumin zone: 20 μ l of the saturated solution is added to 0.5 ml of serum.

3 The gel is then placed on to the cooling platform with the sample application nearest the cathode. Cooling with running tap water is adequate. Contact between buffer and gel is with "wicks" made of lint or three layers of Whatman 3MM filter paper. Electrophoresis time is 30 to 40 minutes at 20 volts/cm. This is the actual, not applied, voltage and will correspond to a power pack setting of 250–300 volts. It is desirable that the actual voltage should be checked on the agarose surface using a voltmeter.

4 After electrophoresis the gel is immediately fixed for 15 minutes, pressed under several layers of filter paper for 10 minutes, dried, stained for 10 minutes, destained until the background is clear and finally dried again.

NOTES

1 Agarose is available with defined electroendosmosis (– Mr). Many brands are suitable for electrophoresis, the general requirement being that the – Mr value should be between 0.13 and 0.19.

2 PAGE blue 83 or Coomassie brilliant blue are alternative stains, the latter being a more sensitive protein stain.

3 Application foils may be washed and reused many times.

4 Prepared agarose gels save time but are expensive.

INTERPRETATION

The presence of a paraprotein is assessed from visual inspection of the stained electrophoretic separation, using diffuse light for agarose and a direct light for CAM, which does not need to be cleared. Paraproteins appear as discrete bands and may occur anywhere within the separation, most often in the β or γ region. In malignant paraproteinaemia there is usually severe immune suppression and in such cases the band will be prominent against a pale background. Immune suppression is only seen in some 10% of benign paraproteinaemia and rarely affects the background immunoglobulins so that the band appears less prominent due to the background staining. Monoclonal IgD and free heavy chains may result in small diffuse bands which can easily be missed. The normal bands of the β and α -2 regions can mask serum paraproteins, particularly small bands of monoclonal IgA or free light chains. Monoclonal bands seen in urine are most often due to Bence-Jones protein which may occur either in addition to a serum paraprotein (from which it often has a different mobility) or in the absence of a serum paraprotein (in 20% of myeloma cases). Tubular proteinuria, which may accompany Bence-Jones proteinuria, results in the appearance of small bands in the α and β region and these can be mistaken for Bence-Jones protein. If there is glomerular proteinuria then a serum paraprotein may "leak" into the urine. A strong urine band (more intense than the albumin) is almost certainly Bence-Jones protein but less prominent bands require further investigation. Occasionally, more than one monoclonal band will be seen in the separation of some serum and urine samples.

Immunofixation

Immunofixation is the recommended method for further investigation of abnormal bands detected by electrophoresis or samples from patients with suspected B cell malignancies. The technique requires initial electrophoretic separation of samples; either CAM or agarose can be used as the support medium but it is preferable to use the same medium for immunofixation as that used for the routine electrophoresis.

REAGENTS

1 Antisera to IgG, A, M, D, E and κ and λ light chains. Antisera to IgG, A, and M from Kallestad (Brill, Buckinghamshire), Atlantic Antibodies (ATAB (UK) Ltd, Winnersh, Berkshire), and Dako (High Wycombe, Buckinghamshire) all perform well. Antisera to IgD, E, κ and λ light chains are obtained from Dako.

2 Antiserum diluent comprising 6 g polyethylene

Fig 2 Stained electrophoresis pattern of serum and urine sample from patient with multiple myeloma, together with identification of paraproteins by immunofixation.

glycol 6000, 100 ml 0.06 l/l barbitone buffer.

3 Saline wash comprising 9.0 g sodium chloride, 1 l distilled water.

METHOD

CAM technique

1 Serum is diluted to give a paraprotein concentration between 0.1–0.5 g/l—that is, a sample with a paraprotein of 40 g/l would be diluted 1/300. Urine is diluted to give a Bence-Jones protein concentration of 0.002–0.1 g/l.

2 Antisera are diluted with an equal volume of diluent. Although more dilution would be possible with some high titre antisera, it is generally advisable to work in conditions of antibody excess which is assumed by this 1 in 2 dilution of all antibodies.

3 CAM is divided into five equal segments across the width of the sheet by lines drawn with an indelible ink pen (laundry markers are ideal), permitting two

application zones in each segment. Diluted serum samples from two patients or the serum and urine from one patient are then applied on to the same position in each segment; thus each sample is applied five times. This pattern can be seen in fig 2, which shows the completed immunofixation. The serum shown in the stained pattern was diluted 1/200 and applied to each tract labelled A on the CAM. Urine was diluted 1/100 and applied to the tracts labelled B. After electrophoresis, antiserum, as indicated, was overlaid as described in the immunofixation method. Reaction of the serum with anti-IgG and λ light chains identifies the serum paraprotein as an IgG λ and reaction of the urine with anti- λ only indicates Bence Jones λ light chains. Close examination of the fixation with anti- λ shows a reaction in the serum, at the same position as the Bence-Jones protein in the urine, and represents a small amount of retained Bence Jones protein in the serum.

4 Electrophoresis is then performed as described in the previous section.

5 After electrophoresis the CAM is left in position on the tank with the power pack turned off. Diluted antiserum to IgG is gently "painted" on to the surface of the first zone using a soft camel-hair brush. Using a different brush each time, anti-IgA is applied to the second segment, IgM to the third, anti- κ and anti- λ light chains, respectively, to the fourth and fifth segments.

6 The power pack is switched back on and electrophoresis continued for five minutes more. This prevents diffusion and results in a "sharp" immunofixation zone.

7 The CAM is rinsed under running tap water for 30 seconds and given two 10 minutes washes in saline before staining as described for electrophoresis.

Agarose technique

1 Serum is diluted to give a paraprotein concentration of 0.5–2 g/l. Concentrated urine can usually be used without dilution.

2 Antisera are diluted with an equal volume of diluent (see point 2 under CAM technique).

3 Samples are applied and electrophoresis done as described.

4 After electrophoresis narrow strips of CAM, impregnated with the individual diluted antisera, are layered on to the surface of the agarose. The CAM strips are cut so as to cover one or more application zones, depending on the pattern chosen, and a scheme similar to that described for CAM can be used.

5 The gel with the applied CAM strips is incubated in a moist chamber for two hours at room temperature.

6 The CAM strips are removed and the gel squashed under a wad of filter paper and a 1 kg weight.

7 The gel is placed for one hour in saline wash,

pressed again, and dried in a stream of warm air.

8 The gel is stained as described for electrophoresis.

NOTES

1 Many brands of antiserum to human IgG, A, and M have been found to be satisfactory. The general requirement is for specific, high titre antibodies.

2 Requirements for antisera to light chains are more stringent and, particularly for CAM, only Dako (Dakopatts, High Wycombe, Buckinghamshire) give consistently satisfactory results at present.

3 Antisera to κ and λ should be to "bound" light chains so that these can be fixed when in whole immunoglobulins. Antisera to free light chains can fail to fix such "bound" light chains.

4 Absence of reaction of a serum paraprotein with antiserum to IgG, A, or M but a reaction with light chain antiserum requires investigation of possible serum Bence-Jones protein or an IgD or IgE paraprotein. Apart from the use of different specific antisera, the technique of detecting IgD or IgE paraproteins is exactly as described.

INTERPRETATION

The washing step that follows application of the specific antiserum removes all protein except the insoluble immune complex formed by the antiserum reacting with the paraprotein. Hence a stained band is formed—for example, a sharp band in the region where antiserum to IgG was applied indicates the presence of an IgG paraprotein. A corresponding reaction with antiserum to κ but not λ light chains indicates an IgG κ paraprotein. To be meaningful, these reaction bands have to be in the same position as the visible band on the electrophoretic separation (fig 2). Polyclonal immunoglobulin will give diffuse reactions with antisera to IgG, and both κ and λ light chains; the polyclonal reactions with antisera to IgA and IgM may or may not be seen, depending on the dilution factor but if present they are again diffuse. The increased sensitivity of immunofixation may show small additional bands which were not detected by electrophoresis. These can also occur if the paraprotein has affinity for other proteins such as rheumatoid factor activity against other immunoglobulins, anti-lipoprotein, anti-albumin, etc.

Bence-Jones protein is present when there is reaction with one light chain antiserum but no corresponding heavy chain reaction with anti -G, -A, -M, -D, or -E. Bence-Jones protein may be seen in the serum, particularly if there is renal impairment and may have the same mobility as the urine Bence-Jones protein band or bands. The rare occurrence of Bence-Jones protein in the serum and absence from urine indicates either polymerisation of the Bence-Jones protein (tetramer) or its affinity for another serum protein (α_2 -

antitrypsin, transferrin etc.) Reactions of urine bands with both a heavy and a light chain antiserum indicates a "leak" of the serum paraprotein and in these cases both serum and urine bands will have the same relative mobility.

Reaction with a heavy chain antiserum but no corresponding light chain reaction may indicate free heavy chains. Heavy chain disease is very rare and great caution must be exercised as some paraproteins (particularly IgA and IgD) may have "hidden" or unreactive light chains. Unless a laboratory has expertise in investigating free heavy chains, we recommend that such samples should be referred to a specialist centre for confirmatory studies.

The techniques described so far enable consistent detection, and sensitive and reliable confirmatory typing of paraproteins, and today we rarely use immunoelectrophoresis. This is of importance in myeloma, where paraprotein detection, the finding of abnormal plasma cells in the bone marrow, and lytic bone lesions form a triad of diagnostic features. Qualitative investigations are accompanied by several quantitative tests that either help in distinguishing malignant from benign conditions or are useful for assessing disease progression and determining management of the patient. Serum calcium (interpreted in the light of an accurate albumin estimation), urea (or creatinine), haemoglobin and C-reactive protein are all of value in management and prognosis, but will not be discussed here.

Quantitative investigations

SERUM TOTAL PROTEIN

In most laboratories serum total protein will be estimated in an automated system and this is adequate for screening. Hyperviscosity and cryoprecipitation, which may accompany paraproteinaemia, will cause problems in automated systems. In the estimation of paraprotein concentration, a manual method is recommended for measurement of total protein, approximating as far as possible to the candidate reference method.⁷ Liquid bovine albumin standard, quoted in the reference method, is now difficult to obtain. We have found that a bovine albumin monomer standard solution (ICN Biomedicals, High Wycombe, Buckinghamshire) is acceptable in practice.

Interpretation

High concentrations of paraprotein will raise the serum total protein (reference range 55–79 g/l) but normal total protein values can be seen in early myeloma, IgD myeloma, and heavy chain diseases. In Bence-Jones protein myeloma the total protein may be normal or even low if there is severe immunosuppression.

SERUM ALBUMIN

Most laboratories will have an automated method for determination of albumin, which will usually be vulnerable to viscosity errors. Detailed methodology will not be considered but an essential requirement is that the method used should accurately determine low values. Immunochemical techniques such as immunoturbidimetry and immunonephelometry, which are also readily automated, offer the most reliable methods.

Interpretations

The promise of serum (normally < 40 mg/l) and urine albumin (reference range 27–42 g/l) concentrations as prognostic indicators in the earlier Medical Research Council myeloma trials (1964–1971) was based on immunochemical determinations against a human albumin standard of the quality finally recommended by the International Federation of Clinical Chemists in 1979.⁸ These results could not be confirmed in later Medical Research Council trials using other methods for albumin but have been fully vindicated in America using the proper methods. Determination of serum albumin is still a useful measurement: low values are generally associated with more advanced malignancy^{9,10} and values are usually normal in benign conditions, provided other disease is absent. The albumin concentration can also be subtracted from the total protein value to permit more precise estimation of paraprotein. Because dye-binding often differs between albumin and globulins this subtraction also removes that source of error, and only the less important differences between the paraprotein and the lower level of remaining globulins persist.

PARAPROTEIN

Paraprotein quantitation has as its most important function the establishment of a baseline level for future follow up studies but may contribute also to distinguishing benign from malignant conditions. Scanning densitometry of stained electrophoretic separations remains the accepted reference method for estimation of paraprotein concentration. Most scanning densitometers work on the principle of absorbance of transmitted light by the dye-protein complex. It is essential when setting up the method to work within the linearity range of the stain—that is, where the absorbance is directly proportional to the amount of dye-protein complex, which in turn is directly proportional to the paraprotein concentration. Even when a densitometer is being used according to a manufacturer's instructions we suggest that linearity is checked by scanning stained separations of a series of dilutions of a serum with a high concentration of paraprotein. Many machines give satisfactory results and no particular one is recommended.

Equipment required is as follows: as for electrophoresis; a scanning densitometer.

Reagents and materials required are as follows: as for electrophoresis; clearing solution (for CAM only): Dekalin (decahydronaphthalene) used as supplied (BDH).

METHOD

- 1 Samples electrophoresed on CAM or agarose gel as described.
- 2 Dried agarose strips are scanned directly. CAM strips are temporarily "cleared" by immersing in Dekalin before scanning.
- 3 After scanning CAM strips are blotted to remove excess Dekalin then air dried.
- 4 The serum paraprotein or Bence-Jones protein concentration can be calculated from the total protein by relating the dye-binding of the band to the total dye-binding. Most stains have disproportionately higher binding to albumin which results in overestimation of its concentration; for this reason we recommend that the albumin concentration is subtracted from the total protein so that the dye-binding of the paraprotein band can be related only to the dye-binding of the globulins. There can be problems in this approach with some pre-programmed scanners.

Notes

- 1 Background staining resulting from insufficient washing in the electrophoresis procedure can cause gross errors.
- 2 Small paraprotein bands, particularly those in the same position as normal serum components, are difficult to quantitate accurately. If they are cut out of a cellulose acetate strip with a similar width from the adjacent normal γ -globulin the dye can be eluted from each and the latter subtracted from the former. The percentage of the total globulin dye can then be used to derive the paraprotein contribution. Alternatively, cuts can be taken from a scan and weighed.

Interpretation

In 90% of patients with benign paraproteinaemia the average paraprotein concentration at presentation is less than 10 g/l, while in malignant conditions values tend to be higher. At best, however, these values can only be considered as probability factors in distinguishing the two conditions. More importantly, the paraprotein concentration in benign conditions remains stable over long periods of time; in untreated myeloma there is a progressive increase (average doubling time is six months).

The paraprotein is a tumour product and although

there are variations in the amounts generated by various tumour masses in different patients, increasing or decreasing concentrations in a patient will generally indicate progression or regression, respectively; thus the paraprotein concentration can be used to monitor treatment, except in rare (<2%) cases where the tumour loses its paraprotein production.

IMMUNOGLOBULINS

There are many satisfactory laboratory methods for the quantitation of serum immunoglobulins, mainly based on the techniques of radial immunodiffusion, immunonephelometry, and immunoturbidimetry, and as there is a national quality assurance programme, detailed methods will not be considered in this paper. Suffice to say that laboratories measuring immunoglobulins should participate in the quality assurance programme and perform adequately within their method related group. The technique of "rocket" immunoelectrophoresis can no longer be recommended.

Interpretation

These ranges (table 2) relate to caucasians, using a standard SPS01 (PRU, Department of Immunology, Royal Hallamshire Hospital, Sheffield) with its assigned immunoglobulin content. Concentrations of immunoglobulin classes not associated with the paraprotein (normal polyclonal or background immunoglobulin) are suppressed more often in malignant myeloma (98%) than in benign conditions (10%). As with the paraprotein concentration, immune suppression is not pathognomonic in distinguishing benign from malignant disease: patients with myeloma may not show suppression, and values may be low in benign conditions. Very low values can be useful in anticipating risk of infection in patients with malignant B cell tumours. Being of one subclass and one idiotype only, paraproteins should not be measured by routine immunochemical assays (radial immunodiffusion, nephelometry), because the polyclonal antisera and standards used routinely are chosen to represent the usual variations in polyclonal IgG, IgA, etc.⁹ Serious errors can occur due to non-parallelism in the calibration curves between normal and paraprotein immunoglobulins.^{10,11} The preferred method is the percentage of the total globulin by dye-binding, but if the original paraprotein sample has

been kept in aliquots (each thawed once only for use for a standard curve) and aggregates are not a serious problem (like most IgG paraproteins) it is possible to determine by nephelometry subsequent values as a percentage of the original, always using the same anti-IgG.

Under no circumstances should any attempt be made to estimate the relative concentrations of paraprotein and non-paraprotein immunoglobulin of the same class by subtracting the paraprotein concentration derived by densitometry from the total concentration measured immunochemically. These methods are not interchangeable; for this reason we advocate that in reporting paraprotein studies the non-paraprotein immunoglobulin concentrations should be given together with only one estimation of paraprotein concentration (either densitometric or immunochemical), but never both. A written report should then indicate whether the paraprotein occurs with immune suppression or against a normal background concentration of immunoglobulin, as judged from visual inspection of the stained electrophoretic separation.

BETA-2-MICROGLOBULIN (β -2-M)

Beta-2-M is a low molecular weight protein forming part of the HLA system on cell surfaces. HLA is expressed heavily on myeloid and lymphoid cells, and normally serum β -2-M is largely derived from cell turnover. As this small protein is readily filtered through the renal glomerulus and absorbed and catabolised by the tubules, serum concentrations will also reflect renal function. Serum concentrations are raised in several diseases and malignancies, particularly those entailing lymphoproliferation.

In myeloma the serum β -2-M concentration has important prognostic implications and can also be used to monitor the disease. As most laboratories prefer to use commercial radioimmunoassay or enzyme linked immunosorbent assay kits for β -2-M determinations, detailed methodology will not be given.

Interpretation

Serum concentrations below 4 mg/l (reference range 1.0–3.0 mg/l) at presentation of myeloma indicate the best prognosis; higher values are associated with poorer prognosis, with values above 20 mg/l having the worst prognosis. Values are usually low in benign conditions but as with so many other laboratory measurements, values cannot distinguish completely benign from malignant conditions. Tumour mass and renal function in myeloma are important to prognosis and as both affect serum concentrations of β -2-M, this probably accounts for β -2-M being the single best prognostic indicator in myelomatosis.¹² In patients

Table 2 Reference ranges of immunoglobulins

Reference age (years)	Reference ranges (g/l)		
	IgG	IgA	IgM
15–45	5.4–16.1	0.9–3.4	0.5–2.0
>45	5.4–16.5	0.8–4.0	0.5–2.0

responding to chemotherapy and in "plateau" phase, serum concentrations of β -2-M stabilise. Increasing concentrations indicate failure of response and hence β -2-M can be used to monitor chemotherapy. In monitoring disease, however, the marker should reflect tumour mass only and any increases in serum concentrations due to changes in renal function have to be taken into consideration.

When used to monitor disease, serum β -2-M concentrations are corrected for serum creatinine concentrations. The predicted serum β -2-M concentration is calculated using the equation¹²:

$$\log_{10}\beta\text{-2-M} = -1.77 + 1.15\log_{10}\text{creatinine}$$

The calculated value is then subtracted from the observed value. In some patients this results in a negative index, as only the excess β -2-M is being determined, but this is acceptable as only the trend is important in monitoring treatment.

URINE TOTAL PROTEIN

Many of the methods used for quantitation of urine total protein are not satisfactory and there is a great need for collaborative studies to attempt to establish a reference method. Two approaches seem to offer the best of the available methods at present. The first of them uses biuret to quantitate urine proteins precipitated with TCA,¹³ and the second uses the binding of dyes such as Coomassie blue (Protein Assay Kit, Bio-Rad Laboratories, Watford, Hertfordshire) or Ponceau S.¹⁴

Interpretation

Bence-Jones proteinuria (reference range below 150 mg/day) occurs in more than 80% of myeloma and 70% of patients with Waldenström's macroglobulinaemia. Many sources quote a lower incidence for Waldenström's macroglobulinaemia but this is due to lower concentrations of Bence-Jones protein than in myeloma,¹⁰ coupled with inadequate concentration of urine. Glomerular or tubular proteinuria may accompany Bence-Jones proteinuria but total protein concentration is unhelpful in distinguishing the type of proteinuria, which should be assessed from the electrophoretic pattern. Bence-Jones proteinuria can occur in benign paraproteinaemia¹⁵ but concentrations tend to be very low (less than 10 mg/l).

The 24 hour excretion of Bence-Jones protein is used by some workers to monitor Bence-Jones myeloma, but this can be unreliable in view of unsatisfactory methodology and faulty urine collection. As most people are creatures of habit, a fresh urine sample at the same time of day (such as 10:00 am) can give consistent results. After estimation of total protein, the Bence-Jones protein band may be measured by densitometry (as for serum paraproteins). As about one third of patients who

relapse show disproportionate increases in Bence-Jones protein excretion, any rise in urine total protein indicates the need for a repeat electrophoretic search for new mutant Bence-Jones proteins. Serum β -2-microglobulin offers an acceptable alternative for monitoring Bence-Jones protein myeloma.

Specialised investigations

The tests described so far constitute the main group for investigating paraproteinaemia. Additional testing may be necessary with some specimens. Tests that show the effects of paraproteins (table) are worthwhile as plasmapheresis may be very effective in reducing the paraprotein concentration and hence its clinical effects. As viscosity determinations and rheumatoid factor tests are established methods and widely applied they will not be discussed further.

Samples for the investigation of cryoglobulins and cold agglutinins are often unnecessarily referred to a specialist centre. Apart from attention to collection and storage temperatures the methods required are essentially the same as those used for routine paraprotein investigation.

CRYOGLOBULIN INVESTIGATIONS

Cryoglobulins are proteins that form a gel or precipitate on cooling and redissolve when warmed to 37°C. Paraproteins may complex with themselves (monoclonal cryoglobulins) or with other proteins such as polyclonal immunoglobulin (mixed cryoglobulins).

Collection and storage of samples

It is essential to collect blood into syringes and tubes pre-warmed to 37°C (we keep a few syringes, needles, and tubes in a small thermos flask in the 37°C incubator ready to be taken to a patient bleeding area or ward) and to separate it at 37°C. Serum aliquots are then incubated, one each, at 4°C, 20°C, and 37°C and visually inspected after 24 hours for gelling or precipitation in the 4°C and 20°C samples. The gel or precipitate should be shown to redissolve at 37°C.

Quantitation of cryoglobulins

The simplest approach is to perform electrophoresis, densitometry, and measure immunoglobulin on the sample stored at 37°C and the supernatant from the 4°C sample (if necessary, centrifuged at 4°C) and to quantitate the cryoglobulin by difference.

Isotype of cryoglobulin

When a narrow band is visible after electrophoresis (if necessary, at 37°C) it can be typed by immunofixation. Sometimes, to avoid confusion between monoclonal rheumatoid factors and polyclonal immunoglobulin

targets it is necessary to dissociate the complex and separate the monoclonal component under acid conditions; this is a job for experts.

Interpretation of results

About 2% of paraproteins produce symptoms of cryoglobulinaemia and in most cases these are of IgM class and present in high concentrations (greater than 25 g/l). The laboratory demonstration of a cryoglobulin does not invariably mean that a patient will show related symptoms.

Proteins that do not precipitate until the temperature is below 20°C seldom cause symptoms and precipitates that form very slowly correlate less well with symptoms than those forming rapidly in the first hours of cooling. When cryoprecipitates form, however, it is essential to redissolve them at 37°C to assess accurately the paraprotein concentration in follow up studies.

COLD AGGLUTININS

Cold agglutinins are IgM autoantibodies that bind to antigenic determinants on the surface of red blood cells; binding is strong at 4°C and weak or absent at 37°C, but cooling to only 34°C in a centrifuge can result in no supernatant detection, all the cold agglutinin having spun down on the red cells. Rewarming the sediment and carefully respinning at 37°C can often elute the cold agglutinin.

Collection of samples

Serum is collected and separated as described for cryoglobulins. Once separated from the red cells the serum does not need to be stored at 37°C.

CRYOFIBRINOGEN

This is mentioned because it can cause confusion in plasma samples. It is precipitated by heparin, so is best identified by finding a cryoprecipitate present only in cooled plasma (by oxalate, edetic acid, or citrate but not heparin) and not in serum off blood collected at the same time, and allowed to clot at 37°C.

Investigations

Samples are investigated by electrophoresis and immunofixation as described.

Interpretation

Cold agglutinins can occur secondary to infections or inflammatory conditions and then are usually polyclonal. A monoclonal paraprotein is seen in primary cold agglutinin disease and is typically an IgM κ ; concentrations are often quite low (less than 10 g/l). Apart from the cold agglutinating properties the paraprotein is generally benign and therefore associated Bence-Jones protein is found in less than 10% of such patients.

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