Electron microscopy and immunocytochemistry in the assessment of renal biopsy specimens: actual and optimal practice

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Abstract

***Aims*—To determine the variation in practice of British renal histopathologists in the use of immunohistochemistry and electron microscopy in the investigation of renal biopsy specimens; to attempt to identify a consensus on what acceptable practice should be; and to satisfy requests from laboratories which have found immunoperoxidase methods unreliable on renal biopsy specimens, by disseminating methods from laboratories which have had success.**

***Methods*—A questionnaire was sent to all 58 laboratories which participate in the UK National Renal Pathology External Quality Assessment Scheme.**

***Results*—A response rate of 88% was achieved. Most laboratories use immunocytochemistry and electron microscopy to investigate most renal biopsy specimens, but a few use these methods only rarely and one, never. There is a widespread wish to switch from immunofluorescence to immunoperoxidase, but this is frustrated by the unreliability of the method. This seems to be mainly because of the need to tailor the time of enzyme pretreatment to each biopsy specimen.**

***Conclusions*—The majority view is that electron microscopy and immunocytochemistry are necessary in the investigation of most native renal biopsy specimens, and the few pathologists who report renal biopsy specimens without these methods risk accusations of negligence. Difficulty in using fixed renal tissues for immunocytochemistry stem largely from variations in the requirement for enzyme pre-digestion. Even where immunoperoxidase methods are usually successful, the occasional use of immunofluorescence in parallel to check that false negatives are not occurring is advocated. In all cases it is wise to keep some frozen cortex in reserve in case there is an unexpected or inconsistent immunoperoxidase result.**

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Soon after the introduction in 1951 of the percutaneous renal biopsy as a new method by which to investigate renal disease,[1] it was appreciated that examination of the biopsy specimen by light microscopy alone did not extract all the available information. The relatively new technique of immunofluorescence was used to distinguish the presence or absence of immunoglobulins and complement.[2] Electron microscopy could delineate many ultrastructural abnormalities of the glomerular microfilter, including extracellular "electron dense deposits", the structural analogue of the immunoglobulins shown by immunofluorescence.

Since then, if the major textbooks are to be believed, the importance to renal pathology of electron microscopy and immunohistochemistry has not changed, despite fluctuating fashions in other diagnostic areas. However, the establishment of a National External Quality Assessment (EQA) Scheme in Renal Pathology within the UK has made it obvious that large variations in practice exist. In that scheme, participants are asked in rotation to contribute cases for circulation. They are asked to provide all the information which was available at the time of the original diagnosis. A significant proportion of cases are submitted in the absence of information on electron microscopy, immunohistochemistry, or both; often it is explicit that these studies were not done. Some EQA participants find this acceptable; others complain that renal biopsy specimens should never be reported under these conditions.

We therefore sent a simple questionnaire to all the participating laboratories in the National Renal Pathology EQA Scheme, asking for details of current practice and what tests the participants felt should be carried out on diagnostic renal biopsy specimens in an ideal world. The results are presented here.

One of the most frequent comments expressed was a wish to use immunoperoxidase rather than immunofluorescence to detect immunoglobulins and complement in glomeruli. Many laboratories use immunoperoxidase (or some other permanent immunohistochemical method) for everything except renal biopsy specimens. A few laboratories, however, do use immunoperoxidase successfully, so we requested details of the approaches used by these laboratories and we present a description of these methods and a discussion of the problems involved.

Methods

Questionnaires were sent to all the participants in the UK National EQA Scheme in Renal

Pathology, a total of 59 laboratories. In the few cases where two participants work in one laboratory, only one response was recorded.

The questionnaire was intentionally brief to maximise the response rate. It asked the following questions:

Actual practice

- What proportion of renal biopsy specimens do you investigate by electron microscopy?
- What proportion of renal biopsy specimens do you view the electron microscopy grid on the microscope yourself?
- What proportion of biopsy specimens do you investigate by immunofluorescence?
- What proportion of renal biopsy specimens do you investigate by immunoperoxidase?
- What proportion of renal biopsy specimens would you investigate by immunoperoxidase and immunofluorescence?

In an ideal world (that is, without constraints on time or budget)

- What proportion of renal biopsy specimens would you investigate by electron microscopy?
- What proportion of renal biopsy specimens would you view the electron microscopy grid on the microscope yourself?
- What proportion of biopsy specimens would you investigate by immunofluorescence?
- What proportion of renal biopsy specimens would you investigate by immunoperoxidase?
- What proportion of renal biopsy specimens would you investigate by immunoperoxidase and immunofluorescence?

Space was provided for comments. A request was also made to the same group of pathologists to submit detailed practical protocols of any immunohistochemical methods for immunoglobulins and complement which are regularly and successfully applied to fixed, paraffin wax embedded renal biopsy specimens.

Results

Of the 59 participating laboratories, responses were received from 52, a response rate of 88%.

ELECTRON MICROSCOPY

Only 3·8% (two pathologists) never request electron microscopy for renal biopsy specimens; 59·6% request electron microscopy on all. The remainder request electron microscopy selectively, the rates quoted varying from 2 to 90%. If the average rate can be regarded as a consensus, 74% of renal biopsy specimens are investigated by electron microscopy. These figures showed relatively little change in the ideal world; 19% of pathologists would request electron microscopy more often, but 8% would request less electron microscopy, for reasons which were not stated.

The difference between the optimum and reality is much greater when the question is direct viewing of the electron microscopy sections. Only 23% of pathologists do this for all biopsy specimens; 25% never view the grids. This is often due to geographical separation of the pathologist from the electron microscopy unit. Forty two per cent of pathologists said they would like to view the grids more often; this is more than half of the 77% who do not already view all the sections directly. Several comments indicated that this discrepancy is the result of pressure of work.

IMMUNOHISTOCHEMISTRY

Immunofluorescence remains the most popular method of detecting immunoglobulins and complement deposits, with 83% of laboratories using the method. Of the nine laboratories which never use immunofluorescence, eight use immunoperoxidase on every biopsy specimen. The ninth uses no immunohistochemistry at all. Thirty four per cent never attempt to use immunoperoxidase, even if the frozen sample for immunofluorescence contains no glomeruli. Only 7·7% of laboratories routinely attempt both methods on all biopsy specimens.

Considering the responses relating to the ideal world, it is obvious that many would switch from immunofluorescence to immunoperoxidase, if only the method was reliable. Eight laboratories volunteered a wish to abandon completely their use of immunofluorescence (15%). This is undoubtedly an underestimate, as several respondents made it clear that even in an ideal world, they did not believe that immunoperoxidase methods would work on renal biopsy specimens! This is matched by 30% who would like to use immunoperoxidase more, and 23% who would like to use both methods.

Tables 1 to 3 summarise the results. It is obvious that a switch to the use of immunoperoxidase in renal pathology is widely desired, but is being inhibited by the technical difficulty of the method. Our responses from those laboratories which have some success with the method are therefore likely to be of considerable interest.

CHARACTERISTICS OF SUCCESSFUL IMMUNOPEROXIDASE METHODS

Methodologies were submitted from nine laboratories claiming to use immunoperoxidase techniques successfully on renal biopsy specimens. The amount of detail given is variable, but it is still possible to extract some common characteristics.

All nine laboratories are using the three-step peroxidase-antiperoxidase method for the demonstration of immunoglobulins and complement components, with one describing a slight variation of a two stage immunoperoxidase technique for C3. All seek IgG, IgA, IgM, and C3. Various other complement components and immunoglobulin subclasses are studied by a minority of laboratories. The majority closely follow the technique originally described in the review by MacIver and Mepham.[3]

Where mentioned, it is agreed that adequate and standardised fixation is of prime importance. Most departments use neutral buffered

Table 1 Actual practice. Number of laboratories (% = to nearest 1%)

Proportion of biopsy specimens	Request electron microscopy	View grid	Immunofluorescence	Immunoperoxidase	Both
0%	2 (4%)	13 (25%)	9 (17%)	18 (35%)	29 (56%)
1 to 99%	19 (36%)	27 (52%)	6 (12%)	18 (35%)	19 (36%)
100%	31 (60%)	12 (23%)	37 (21%)	16 (31%)	4 (8%)

Table 2 Ideal practice. Number of laboratories (% = to nearest 1%)

Proportion of biopsy specimens	Request electron microscopy	View grid	Immunofluorescence	Immunoperoxidase	Both
0%	2 (4%)	6 (11%)	12 (23%)	15 (29%)	29 (56%)
1 to 99%	19 (37%)	28 (54%)	8 (15%)	10 (19%)	13 (25%)
100%	31 (60%)	18 (35%)	32 (62%)	27 (52%)	10 (19%)

Table 3 Desired changes. Number of laboratories (% = to nearest 1%)

Proportion of biopsy specimens	Request electron microscopy	View grid	Immunofluorescence	Immunoperoxidase	Both
Decrease	4 (8%)	4 (8%)	8 (15%)	6 (12%)	9 (17%)
No change	38 (73%)	26 (50%)	39 (75%)	31 (60%)	31 (60%)
Some increase	10 (19%)	19 (36%)	2 (4%)	8 (15%)	6 (12%)
0 to 100% increase	0 (0%)	3 (6%)	3 (6%)	7 (14%)	6 (12%)

formalin for a minimum of 18, but preferably for 24, hours. Howie *et al* advise, however, that urgent cases can be successfully processed more rapidly by immersing the biopsy specimen immediately in boiling formalin.[4] One other laboratory fixes urgent biopsy specimens in minutes, by microwaving in formalin. Care is obviously needed to avoid the toxic effects of formalin vapour.

Proteolytic enzyme digestion of the sections is carried out by all. The purpose of this is twofold: to uncover the antigenic sites in the glomeruli, and to reduce background staining, especially of plasma which has been fixed in the capillary loops. Trypsinisation is the usual method, although a few use protease. The procedure often causes problems and needs to be carefully controlled, with one laboratory recommending that fresh reagent is made up for each batch of slides. The optimum digestion time varies enormously, depending on the length and method of fixation, the section thickness; in some places it also depends on the antigen to be demonstrated. One laboratory comments that two biopsy specimens illustrating the same disease, taken at the same time and processed in parallel, have been observed to require more than a twofold difference in trypsinisation time. This laboratory first stains a set of slides for IgG only with a range of trypsinisation times, then selects the time which has just resulted in elimination of staining of plasma IgG from glomerular capillary loops before using the other reagents. Howie *et al* measure the time more directly; they have found that the best results are achieved by observing the digestion process microscopically, making the cut off when all plasma has been removed from glomerular capillary loops.[4] This can take anything from five to 60 minutes. Anecdotal evidence suggests that other laboratories have found this method difficult, perhaps due to lack of practice.

Commercially manufactured polyclonal primary and secondary antibodies are then applied to the sections at set dilutions and for set incubation times. These are standardised in each method, but vary considerably from one centre to another and obviously depend on many factors. As with routine immunoperoxidase staining, the use of the highest possible dilution of primary antibody will reduce background staining. Finally, the PAP label is applied and, where detailed, the sections are counterstained with haematoxylin.

It is difficult to draw any conclusions about the running of controls, as there is little mention of this in the submitted methods. It is assumed that all laboratories run a negative control by omitting the primary antibody. Positive controls are obviously desirable but more difficult to obtain. Some use albumin as the primary antibody, and one department only documents that they always run a known positive IgG with each batch of slides.

Discussion

ELECTRON MICROSCOPY

While the popularity of electron microscopy in other areas of diagnostic histopathology has waxed and waned, the results of this survey confirm that in renal pathology it has remained an important part of diagnosis. Numerous reasons for this are evident. Some important diagnoses in renal pathology can only be made at ultrastructural level—for example, Alport's disease, thin membrane nephropathy, and determination of the type of mesangiocapillary glomerulonephritis. Rather more numerous (and commoner) diagnoses require electron microscopy for confirmation. This is especially true if other special investigations are not available or do not work. This often occurs; immunoperoxidase is unreliable in most laboratories and immunofluorescence requires fresh frozen tissue. The unfixed sample often contains no glomeruli. In this situation ultrastructural examination can detect and localise electron dense deposits. Thus, the

Table 4 Advantages and disadvantages of immunofluorescence and immunoperoxidase, as applied to renal biopsy specimens

Immunofluorescence	*Immunoperoxidase*
Reliable (if glomeruli available)	Unreliable, capricious technique
Frozen tissue required	Paraffin sections—comparison with other stains possible
Poor morphology	Good morphology
Technically easy	Technically difficult
Consumes skilled laboratory staff time	Consumes skilled laboratory staff time
Not permanent	Permanent
Rapid	Slow
Needs special equipment	Often misses linear staining of Goodpasture's disease

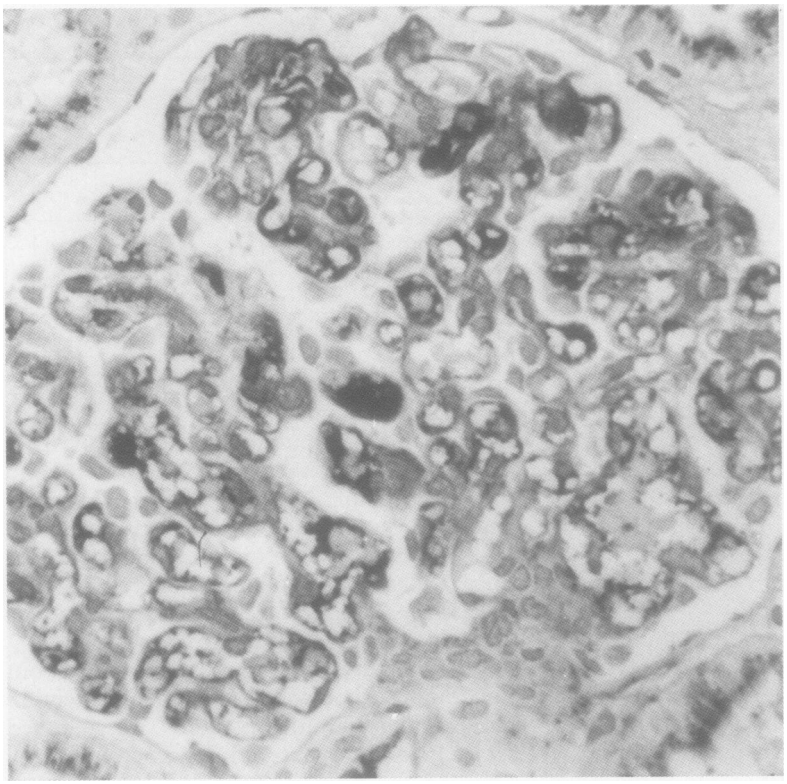

Figure 1 A case of minimal change nephropathy; immunoperoxidase preparation for IgG. Immunofluorescence was negative and electron dense deposits were not evident on electron microscopy. The staining in the illustration is of normal plasma in capillary loops, an artefact due to insufficient trypsinisation.

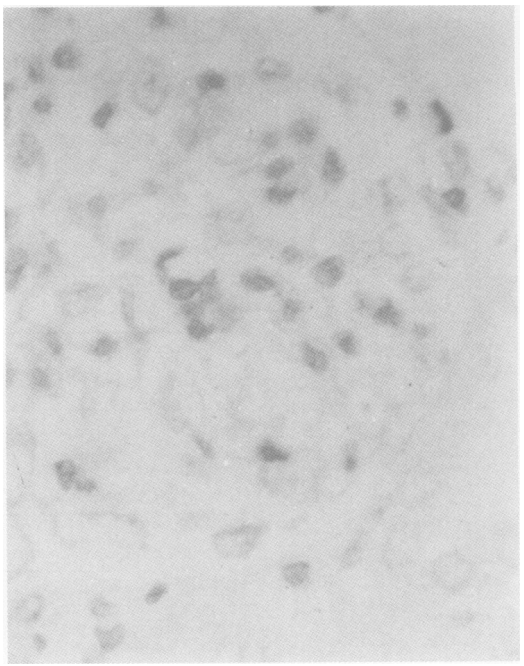

Figure 2 A case of membranous nephropathy; immunoperoxidase preparation for IgG. Immunofluorescence showed finely granular capillary loop IgG and electron microscopy showed subepithelial electron dense deposits. The absence of staining in this immunoperoxidase preparation is due to excessive trypsinisation.

distinction between minimal change nephropathy and "early" membranous glomerulonephritis (without subepithelial "spikes") can be made. In the absence of immunocytochemistry, characteristically well defined deposits, confined to the mesangium, in an appropriate clinical setting, can permit a reasonably confident diagnosis of IgA nephropathy. Even if immunohistochemistry is available, the absence of electron dense deposits provides useful confirmation that "negative immunohistochemistry" is not a technical failure. In light chain nephropathy, the ultrastructural appearances are specific but immunohistochemistry for κ light chains often fails.[5] By electron microscopy alone, we have made a diagnosis of light chain nephropathy months before a paraprotein became detectable in the plasma. One could continue; electron microscopy is more sensitive than Congo Red in the detection of amyloid; thickened basement membranes reflect diabetes, sometimes in the pre-diabetic state.[6]

Of the UK laboratories, 19 (36%) are selective about the use of electron microscopy. The proportion of cases studied (from 2 to 90%) indicates that some omit the investigation only when there is an obvious and clinically acceptable diagnosis by light microscopy, whereas others only resort to electron microscopy when a diagnostic problem is clearly evident. We find the latter approach a little worrying, as it seems likely that some important diagnoses may be missed.

It is, however, cause for rather greater concern that some laboratories report renal biopsy specimens without any recourse to electron microscopy, a position which several of our respondents spontaneously described as "unacceptable" or "negligent". One of our respondents defended vigorously a choice never to use electron microscopy, but we contend (for reasons given above) that one cannot offer an adequate service without it. This survey suggests that not using electron microscopy is usually not due to lack of resources, but reflects the genuinely held opinions of the few pathologists concerned. Although the response rate to this survey was remarkably high, it was not 100%, and only pathologists with a sufficient interest in renal work to join the National Renal Pathology EQA scheme were consulted. A failure to use electron microscopy may therefore be more widespread than our data suggest.

Electron microscopy need never be "unavailable". To set up an ultrastructure unit is very expensive, but many exist and are willing to compete to provide a service by post, providing photomicrographs and (if required) a local pathologist's opinion. Samples are small and can easily be posted, with suitable packing. The delay incurred in postage is not great when compared with a typical processing schedule. Even where a sample has not been fixed spe-

cifically for electron microscopy, or contains no glomeruli, material from the paraffin wax block can be reprocessed (and is easier to transport). Detail of cell structure is lost by this approach, but extracellular matrix and immune complex deposits are usually adequately preserved.

IMMUNOHISTOCHEMISTRY

The value of immunohistochemical studies for immunoglobulins (at least G, A and M) and complement (at least C3) was supported by all but one of our respondents. The responses, however, confirm that immunohistochemistry has special problems when applied to renal biopsy specimens. These stem from two factors: (1) the importance of a negative result; and (2) the fact that in seeking immunoglobulin deposition, one is trying to detect small amounts of proteins which are abundant in normal plasma.

There is a widespread desire to use immunoperoxidase rather than immunofluorescence. Reasons for this are evident from the comparisons summarised in table 4.

Immunofluorescence is a more reliable technique, so control material is less important. It is, however, easier to provide; immunoglobulins are relatively stable if stored in liquid nitrogen, but in our hands become very difficult to detect after a few months in a paraffin wax block.

The elimination of plasma immunoglobulins from frozen sections is simple. They are not fixed, they are soluble, so they wash out leaving tissue bound immune complexes behind. In paraffin wax sections the plasma proteins are fixed and must be removed by enzymatic digestion (fig 1). Unfortunately, too much digestion removes the immune complexes too, resulting in a false negative result (fig 2) and may destroy tissue integrity. Furthermore, digestion conditions are difficult to standardise; times required vary with degrees of fixation and section thickness. These may be controlled, but unfortunately, and for reasons that are not understood, even biopsy specimens which are taken at the same time and are handled together in an identical manner may have very different digestion requirements. Requirements for a single biopsy specimen may vary considerably if the block is left for a few weeks. These problems are reflected in the methods which were provided to us by laboratories which consider their immunoperoxidase methods to be successful.

The three-step peroxidase-antiperoxidase technique for renal biopsy specimens was carried out by all these laboratories, and most followed variations of the technique described by MacIver in 1982. The important points seem to be: (1) standardised fixation times, which need to be at least 18 hours; (2) carefully controlled enzyme digestion, probably tailored for each biopsy specimen; (3) set dilutions and incubation times for primary and secondary antibodies; and (4) aim to run a known positive control with each batch of slides.

We conclude that the majority of British renal pathologists believe that electron microscopy and immunocytochemistry are necessary in the investigation of most native renal biopsy specimens. We agree with the views expressed that to attempt to report renal biopsy specimens without access to both methods will result in missed diagnoses, and risks being described as negligent. Although desirable, using fixed renal tissues for immunocytochemistry is fraught with difficulty and should not be attempted without a period where both methods are used in parallel, to check their concurrence. Even where immunoperoxidase methods are found to be successful, we would advocate occasionally using immunofluorescence in parallel to check that false negative results are not occurring.

We are grateful to all the pathologists who took their time to respond to our questionnaire.

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