Immunoperoxidase staining of formalin-fixed, paraffin-embedded, human renal biopsies with a comparison of the peroxidase-antiperoxidase (PAP) and indirect methods

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SUMMARY The results of immunoperoxidase staining of 33 routinely processed renal biopsies correlated with the results of direct immunofluorescence in 79% of tests performed. Most of the discrepant results were due to positive immunoperoxidase staining, possibly reflecting greater sensitivity of the method. A comparison of two immunoperoxidase methods showed that the indirect method produced less intense staining than the peroxidase-antiperoxidase (PAP) method when equivalent primary antibody titres were used. Lowering the primary antibody titres for the indirect technique resulted in equivalent staining by the two methods. Predigestion by protease VII effectively revealed the antigens under study without causing tissue damage or section loss.

In localisation of tissue antigens, immunoenzymatic examination of paraffin sections offers several advantages over immunofluorescence performed on fresh tissue. These include the use of a microscope with a conventional light source, the simultaneous visualisation of tissue antigens and tissue structure, the possibility of retrospective examination, and permanency of the preparations. A number of successful applications of immunoenzymatic methods to fresh frozen renal biopsies¹⁻³ were followed by a report from MacIver, Giddings, and Mepharm,⁴ who applied the immunoperoxidase method to formalin-fixed paraffin-embedded material. These authors, who utilised the peroxidase-antiperoxidase (PAP) method of Sternberger *et al.*,⁵ reported that immunoperoxidase staining of trypsin-digested paraffin sections provides a reliable and sensitive technique for demonstrating immune deposits. In the present study we have sought to confirm the value of the PAP method in paraffin-embedded renal biopsies and have compared its sensitivity with an indirect enzyme-labelled antibody method. In routine use the latter technique has inherent advantages over the PAP method since it involves fewer steps.

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Material and methods

Thirty-three renal biopsies (28 needle biopsies and 5 wedge biopsies) were examined. These had been fixed in neutral buffered formol saline for 6-18 h and, after routine processing, had been embedded in paraffin and stored for periods ranging from 2 wk to 7 yr. Both the PAP and indirect methods were performed on each case, using, with minor modifications, the techniques outlined by Burns.⁶

PAP METHOD

1 Cut thin paraffin sections (microtome setting 2-3 μ m), mount on uncoated glass slides, dry overnight at 37°C, dewax in xylene, and hydrate.

2 Digest prewarmed sections at 37°C with 0.05% protease type VII (Sigma Cat No P5255) in phosphate-buffered saline (PBS), pH 7.3 for 20 min.

3 Terminate digestion in cold running tap water for 10 min and transfer to two changes of PBS over 10 min.

4 Expose sections to normal swine serum, diluted 1/5 with PBS, for 10 min. Drain off excess swine serum before stage 5.

5 Incubate sections with rabbit antihuman sera for 30 min. Wash in four changes of PBS over 20 min.

6 Block endogenous peroxidase activity with a fresh 0.5% solution of hydrogen peroxide in methanol for 10 min then wash in PBS for 10 min.

7 Incubate sections with unlabelled swine anti-

rabbit IgG for 30 min and then wash in four changes of PBS over 20 min.

8 Incubate sections with rabbit PAP soluble complexes for 30 min and then wash in four changes of PBS over 20 min.

9 Reveal the end-product with a fresh solution of 0.05% 3,3'-diaminobenzidine (DAB) in 0.01% hydrogen peroxide in wash buffer for 5 min. Handle DAB with care because of potential carcinogenicity.

10 Wash in running tap water, counterstain with Harris' haematoxylin, dehydrate, clear and mount in DPX.

INDIRECT METHOD

Proceed as for the PAP method but replace steps 7 and 8 by incubation with peroxidase labelled swine antirabbit IgG and then wash in four changes of PBS over 20 min. Perform all incubations in a moist chamber at room temperature.

ENZYME DIGESTION

The optimum enzyme digestion time was determined in preliminary studies by digesting known positive sections for periods ranging from 5 min to 1 h. Staining of serum proteins within glomerular capillary lumina, which obscured the staining of the antigens under study, regularly occurred with digestion times of less than 15 min and staining of immune complexes progressively diminished following more than 25 min digestion. Although no section adhesive was used, only very occasional sections were lost after digestion, and histological evidence of tissue disintegration was not seen.

REAGENTS AND DILUTIONS

All antisera and peroxidase-antiperoxidase complexes were obtained from Dakopatts A/S through Mercia Brocades Ltd (UK). Optimal primary antisera dilutions were determined by titration against a number of known positive sections. Although a positive reaction could often be obtained at high dilution—for example, 1/6400, lower dilutions were selected for use with the aim of detecting even small amounts of antigen. The dilutions used in the PAP method were as follows: anti-IgG 1/800; anti-IgM 1/200; anti-IgA 1/400; anti-C3 1/100; anti-C4 1/200; anti-C1q 1/200; anti-fibrinogen 1/1600; unconjugated swine antirabbit immunoglobulin 1/20; PAP complexes 1/50. Occasionally, individual cases required increased titres—for example, anti-IgG 1/1600, to reduce background staining. In 17 of the biopsies studied the titres of primary antibody for the PAP and indirect method were identical. This allowed a direct comparison of the sensitivity of the two methods. In 22 cases, however, the titres for the indirect method were decreased

fourfold—that is, from 1/800 to 1/200, in an attempt to compensate for an observed relative insensitivity of this technique. Blocking of endogenous peroxidase activity was performed after, rather than before, the application of the primary antibodies. This was done in order to avoid the possibility that antigen reactivity or specificity might be altered by exposure to methanol and hydrogen peroxide. The dilution of peroxidase conjugated swine antirabbit IgG used in the indirect method was 1/30. All dilutions were performed in 1% ovalbumin in PBS. Fluorescein-conjugated antisera to IgG, IgM, IgA, complement components C3, C4, and C1q, and fibrinogen, were obtained from Dakopatts A/S, Hoechst Pharmaceuticals, and Wellcome Reagents Ltd.

Some of the positive immunoperoxidase preparations were counterstained by the periodic acid-Schiff (PAS) method.

CONTROLS

Current fluorescein conjugates and all primary antisera used in the immunoperoxidase methods, with the exception of the anti-C1q sera, showed single precipitin lines against human plasma or serum on immunoelectrophoresis. The animal antisera, PAP complexes, and anti-C1q sera showed no reaction. Other controls included the omission of the primary antibody for the immunoperoxidase methods, the observation of negative reactions with inappropriate primary antibodies and the presence of staining patterns consistent with the morphological diagnosis. In addition, as recommended by Heyderman,⁷ antibody absorptions with purified antigens (IgG, IgM, IgA) were performed. Elimination of antibody activity as shown by negative immunoperoxidase staining was achieved with each antibody and there was no cross reactivity with inappropriate antigens.

CASES STUDIED

The cases examined included: membranous glomerulonephritis 11; mesangiocapillary glomerulonephritis 8; focal proliferative glomerulonephritis 5; diffuse proliferative glomerulonephritis 3; Berger's disease 2; crescentic glomerulonephritis 2; and one case each of microscopic polyarteritis nodosa and gold nephropathy.

The results obtained from the immunoperoxidase preparations were scored on a zero to ++++ scale and then compared with the results of direct immunofluorescence which had been performed on frozen sections at the time of biopsy.

Results

Both the PAP and indirect methods were effective in demonstrating proteins in glomeruli, arterioles,

peritubular capillaries, tubular lumina and tubular cytoplasm. Within glomeruli, characteristic patterns of localisation of immune deposits were readily recognised within capillary walls, in the mesangium and at the periphery of glomerular lobules (Figs. 1-5). Localisation of deposits was confused rather than clarified by PAS counterstaining of the glomerular basement membrane. Fibrin or fibrinogen was readily stained in glomerular tufts and Bowman's space. Good results with the quoted enzyme digestion time depended on the use of consistently thin paraffin sections. Thicker sections—that is, cut at a microtome setting of 5 μ m, resulted in markedly increased background staining and staining of serum proteins within glomerular capillary lumina. Intraluminal staining occasionally occurred even with thin sections. Prolonging the digestion time in this situation diminished, but usually did not completely remove, the staining.

Background staining was kept to a minimum by enzyme digestion, the use of thin sections, the application of normal swine serum and, particularly, by washing of the sections for at least 15 min after exposure to antibody. Washing for 30 min or more produced some loss of staining intensity. Endogenous peroxidase activity was never detectable after methanol-hydrogen peroxide inhibition.

COMPARISON OF IMMUNOFLUORESCENCE WITH PAP

Comparison of the immunofluorescence results with

immunoperoxidase staining by the PAP method (Table) showed agreement in 79% of 200 tests performed. Of the discordant results, the immunoperoxidase preparations were negative only in relation to six instances of minor fluorescence positivity. On the other hand there was positive peroxidase staining in 37 tests where the immunofluorescence had been negative, and of these, 15 were examples of moderate (++) or strong (+++) staining reactions. The discrepant results did not involve particular antigens or particular histological diagnoses and in these biopsies there was always positive immunofluorescence for one or more antigens and electron-dense deposits were present.

There was generally good correlation of the intensity of staining of individual antigens between the immunofluorescence and the two immunoperoxidase methods. The exception was staining for the C3 component of complement where in six of 33 cases immunoperoxidase staining was inexplicably weak. This weak staining was not improved by reduction of the enzyme digestion time.

COMPARISON OF PAP WITH INDIRECT METHOD

There was good correlation between the results of the PAP method and both the high and low titre indirect methods. With high titres the indirect method produced six divergent results in 104 tests and with lower titres there were six divergent results in 119 tests. With both high and low titres there were four

Fig. 1 *Mesangiocapillary glomerulonephritis. Peripheral lobular staining of C1q. Indirect method $\times$ 360.*

Fig. 2 *Membranous glomerulonephritis. Finely granular staining of IgG. PAP method $\times 360$.*

Fig. 3 *Detail of Fig. 2. Discrete extramembranous deposits of IgG. PAP method $\times 900$.*

negative and two positive results in relation to the PAP method and all of these involved only minimal ($\pm$) staining reactions.

When reaction intensity rather than positivity was considered, the indirect method at high titres was apparently less sensitive than the PAP method. This

was obvious in individual biopsies, and by semi-quantifying individual results—for example, ++ = 2 points, +++ = 3 points, and comparing the totals from parallel tests performed on 16 cases, a difference in staining intensity was seen (PAP method 132 points, indirect method, 94 points).

Fig. 4 *Diffuse proliferative glomerulonephritis. Diffuse granular staining of C3. PAP method $\times 360$.*

Fig. 5 *IgA nephropathy. Mesangial staining of IgA. Indirect method $\times 360$.*

However, when a similar comparison was made between the PAP method and the indirect method performed at low titres, there was no loss in staining intensity (PAP method 207 points, indirect method, 209 points, from parallel tests on 20 biopsies).

Discussion

This study has shown that immunoperoxidase staining of proteolytically-digested paraffin sections is at least as reliable and sensitive as direct immuno-

Comparative results of immunofluorescence (IF) and immunoperoxidase (IP) staining (PAP method) on 33 renal biopsies

Antigen	Number examined	Concordant		Discordant	
		IF+ IP+	IF- IP-	IF+ IP-	IF- IP+
IgG	33	29	4	—	—
IgM	29	14	5	2	8
IgA	30	10	13	—	7
C3	33	33	—	—	—
C4	27	8	12	2	5
C1q	22	8	6	—	8
Fibrinogen	26	1	14	2	9
Total	200	103	54	6	37

fluorescence on fresh tissue in detecting immune deposits in renal biopsies. Our results showed an overall concordance with immunofluorescence of 79%, very similar to that reported by MacIver *et al.* (81%).⁴ However, we found fewer negative and more positive discrepancies. The positive discrepancies were fairly numerous and when present they were a consistent finding by both immunoperoxidase techniques. Moderate or strong staining reactions were often involved and the results may reflect greater sensitivity of immunoperoxidase methods compared with direct immunofluorescence. However, a definitive statement on relative sensitivities is not possible because the immunofluorescence and immunoperoxidase studies were not performed prospectively by a single individual.

Proteolytic digestion, usually by trypsin, is a vital step in immunoenzymatic staining of paraffin embedded renal tissue. We have shown that protease VII, when used at a dilution of 0.05%, effectively reveals a variety of antigens. The results were achieved after a conveniently brief period of proteolysis and without the effects of overdigestion which have been described with both trypsin⁴ and protease VII.⁸ A recent comparative study⁹ has shown protease to be more effective than trypsin in antigen revelation. The relatively high cost of protease VII is a disadvantage, particularly if large numbers of tests are being performed.

The relative sensitivities of the PAP and indirect immunoperoxidase methods have been the subject of conflicting reports.¹⁰⁻¹² In our hands the PAP method was slightly more sensitive than the indirect method. The relative insensitivity of the indirect method was fully compensated for by a decrease in primary antibody titres. Since the indirect method is quicker to perform than the PAP method, it may be preferred for routine immunoperoxidase staining. When choosing an immunohistological method for routine use, the advantages of the immunoperoxidase technique must be weighed against its disad-

vantages,¹³ particularly its time-consuming nature and the relatively large number of steps which require standardisation for consistent results to be obtained. Simplifying the procedure—for example, eliminating the need for proteolytic digestion by the use of fixatives other than formol saline,^{13 14} such as Bouin's, Susa's, would make the method more attractive. At this stage, most pathologists will probably continue to use immunofluorescence for renal immunohistology. However, immunoperoxidase methods certainly have a role, at least in retrospective studies and for those cases, up to 1 in 4,¹⁵ where needle biopsy produces insufficient material for fluorescence examination.

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