Technical methods

Demonstration of immunoproteins in araldite-embedded tissues

JUNE GIDDINGS, RL GRIFFIN, AG MACIVER *Department of Pathology, South Pathology Block, Southampton General Hospital, Southampton*

Immunoperoxidase methods are widely used to demonstrate protein antigens in fixed tissues, for the diagnosis and classification of tumours¹ and for the detection of immune reactants in glomerular disease.² A further development lies in the use of similar techniques on resin-embedded tissues, to give clearer localisation of antigens within cells and membranes. Almost all reported methods employ an immunoperoxidase method performed on fresh or partially-fixed tissue which is subsequently embedded in resin³⁻⁸ and, as a result, there is poor localisation of antigen due to inadequate preservation of cellular structure. Despite the suggestion that direct immunohistochemical examination of resin-embedded tissues was unreliable⁹ it is now apparent that neither fixation nor tissue processing causes permanent destruction of antigen. Trypsin digestion is able to unmask the antigenicity of formalin-fixed proteins¹⁰⁻¹³ and partial removal of methacrylate or epoxyresins enables them to be detected within sections.^{14 15}

We now describe an immunoperoxidase method which will consistently detect immunoglobulin heavy and light chains as well as complement components (C3, C4 and C1q) within cells or basement membranes of araldite-embedded tissues. Sodium ethoxide is used for resin removal and trypsin digestion for re-exposure of antigenicity.

Material and methods

TISSUES

Small slices, maximum thickness of 1 mm, of human lymph node, tonsil, and kidney are fixed in adequate amounts of neutral buffered formalin for periods ranging from two days to several months. They are then processed in the following manner.

Dehydration and embedding schedule

- 1 Wash fixed tissues in 0.033 M cacodylate-sucrose buffer, pH 7.3 overnight (18 h).

- 2 Rinse in distilled water for 5 min.
- 3 Rinse in freshly prepared 1.5% uranyl acetate in distilled water for 30 min, and wash in distilled water.
- 4 Dehydrate either through acetone or alcohol as follows:
- 5 Impregnate in araldite, 4 h.
- 6 Embed in fresh araldite and polymerise at 60°C for 16 h.

Acetone dehydration		Alcohol dehydration	
1 70%	acetone 2 × 5 min	1 70%	alcohol 2 × 5 min
2 90%	acetone 2 × 10 min	2 90%	alcohol 2 × 10 min
3 Absolute acetone	2 × 15 min	3 Absolute alcohol	2 × 15 min
4 Equal parts acetone and araldite	60 min	4 Epoxyp propane	30 min
		5 Equal parts epoxyp propane and araldite	60 min

REAGENTS

Sodium ethoxide (2.5%)¹⁶

Carefully dissolve 2.5 g elemental sodium (divided into small pieces) in 50 ml absolute ethanol.

Add 50 ml benzene and store solution in dark bottle. Allow to mature for at least 2 days, until it achieves a yellow-brown colour.

Trypsin solution 0.05% trypsin (Code T8128 Sigma) is prepared in 0.05% calcium chloride. The solution is adjusted to pH 7.8 with 0.1 N NaOH and warmed to 37°C for use. The solution is used within one hour of preparation.

Diaminobenzidine solution¹⁷ 3,3' diaminobenzidine tetrahydrochloride (5 mg) is dissolved in 10 ml 0.2 M Tris/HCl buffer (pH 7.6). 0.1 ml 1% H₂O₂ is added immediately before use.

CUTTING AND ETCHING SCHEDULE

- 1 Cut sections at 1 μm.
- 2 Dry sections on hotplate for 10 min.
- 3 Treat sections with 2.5% sodium ethoxide in benzene.
- 4 Wash sections in absolute alcohol four times at 2 min each.
- 5 Rinse sections in 70% alcohol.

TRYPsin DIGESTION AND IMMUNOPEROXIDASE METHOD

- 1 Inhibit endogenous peroxidase with 0.5% hydro-

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- gen peroxide in methanol for 10 min.
- 2 Rinse in 70% alcohol. Wash well in running water.
- 3 Prewarm in distilled water to 37°C.
- 4 Incubate in prewarmed 0.05% trypsin solution for 5-40 min.
- 5 Stop digestion in cold, running water, 5 min.
- 6 Transfer to moist chamber at room temperature and wash twice in Tris-HCl buffer, pH 7.6, diluted 1/10 with saline (TBS), 10 min each.
- 7 Apply normal swine serum (Dakopatts AS, Denmark) diluted 1/5 in TBS, 30 min.
- 8 Treat with rabbit antisera raised against human proteins (Behringwerke AG, Germany), diluted in TBS, 30 min.
- 9 Wash in TBS three times, 10 min each.
- 10 Apply swine antirabbit serum (Behringwerke AG, Germany) diluted in TBS, 30 min.
- 11 Wash in TBS three times, 10 min each.
- 12 Apply peroxidase-rabbit antiperoxidase (PAP) (Behringwerke AG, Germany) diluted in TBS, 30 min.
- 13 Wash in TBS three times, 10 min each.
- 14 Demonstrate peroxidase with freshly prepared diaminobenzidine/hydrogen peroxide solution.
- 15 Wash in TBS and then in running tap water 5 min.
- 16 Counterstain nuclei in Harris' haematoxylin 10 min.
- 17 Rapidly differentiate in 0.25% acid alcohol and blue in tap water, 10 min.
- 18 Dehydrate through graded alcohols, clear in xylol and mount in DPX.

The optimal dilution of all antisera is established by chessboard titration, so that the lowest concentration of primary antisera was employed which would produce strong positive staining of tissues known to contain the appropriate antigen.

CONTROLS

Endogenous peroxidase was removed completely by treatment with hydrogen peroxide in methanol.

Each antiserum to human protein was confirmed as monospecific by standard immunodiffusion and immunoelectrophoretic methods. In addition, further immunodiffusion tests showed that there was no reaction between human proteins and either swine-antirabbit serum or peroxidase-rabbit antiperoxidase complexes.

All positive reactions were confirmed on further blocks of formalin-fixed tissues processed through wax and examined by the standard immunoperoxidase methods.^{12 13} Immunological specificity was confirmed by the blocking (inhibition) technique¹⁸ using excess goat antiserum, raised against each of the human proteins, mixed with each rabbit antiserum used in step 8 and the technique performed

as described above. This produced abolition of all positive staining.

Results and discussion

Preliminary studies had already demonstrated that fixation in 2% glutaraldehyde and processing through 2% osmium tetroxide would produce changes which inhibit immunoreactivity by this technique. Both these agents were therefore avoided and fixation was performed in neutral buffered formalin. Inclusion of uranyl acetate prolongs the necessary exposure to trypsin and can also be avoided. Further experiments demonstrated that short times of infiltration with liquid resin were advantageous, although conventional araldite impregnation could be used if sections were exposed to trypsin for a longer period. Times for dehydration, impregnation, and resin polymerisation were therefore carefully controlled so that tissues processed on different days would be comparable. Examination of sections prepared from each of the detailed processing schedules revealed that both gave satisfactory resin impregnation but that the subsequent incubation time in trypsin was shorter when dehydration was performed in alcohol-epoxypropane (Table 1).

Table 1 *Effect of dehydration using graded acetone or alcohol-epoxypropane on the demonstration of light chain in B lymphocytes by trypsin-immunoperoxidase technique*

Trypsin incubation time (min)	Acetone dehydration	Alcohol-epoxypropane dehydration
10	—	+++
15	—	+++
20	+++	—
25	+++	—
30	+++	—
35	+++	—
40	++	—

Resin etching times are unaffected, 20 min.

Resin removal (etching) is produced by a matured brown solution of sodium ethoxide in benzene. The variation in results following different amounts of etching is shown in Table 2. A minimum period of 20 min at room temperature is necessary to achieve satisfactory results with 1 µm sections processed to araldite. In all subsequent reactions, this time was used with no significant day-to-day variation in results.

Table 3 shows the effect of varying the time of trypsin digestion on tonsillar and renal tissues which were subsequently examined for the presence of IgG. There is a difference in the amount of trypsin

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Table 2 *Effect of varying times of treatment with 2.5% sodium ethoxide on the demonstration of IgG in plasma cells and glomerular deposits*

Time (min)	Tonsillar B lymphocytes	Glomerular deposits in rejecting renal allograft
10	±	±
15	+	+
20	+++	+++
25	+++	+++
30	+++	+++

Sections graded: ± trace, + weak positive, +++ strong positive result.

Table 3 *Effect of varying times of trypsin incubation at 37°C on the demonstration of IgG in tonsillar and renal tissues*

Time (min)	Cytoplasm of tonsillar cells		Basement membrane deposits in renal glomeruli	
	Follicle centre cells	Plasma cells	Membranous glomerulonephritis	Rejecting renal allograft
5	++	+	—	—
10	++	++	—	—
15	++	+++	—	—
20	+++	+++	—	—
30	+++	+++	+++	+
40	+++	+++	+++	+++
50	+++	+++	+++	+++
60	+	+	++	++

Sections graded: + weak positive, ++ moderate positive, +++ strong positive result.

incubation required for the demonstration of intra-cellular and extracellular protein antigens, perhaps because of the different penetration of fixative to proteins in these different positions. We have made similar observations when examining paraffin wax-embedded tissues. Based on these times for sodium ethoxide and trypsin treatment, a number of different protein antigens have been satisfactorily demonstrated in araldite-embedded tonsillar and renal tissues (Figs. 1 & 2, Tables 4 & 5).

Although the addition of resin etching and trypsin incubation adds significantly to the time necessary to perform a three-stage immunoperoxidase method, all steps are necessary to achieve satisfactory results. In particular, the PAP technique provides such increased sensitivity that immunoglobulin can be demonstrated in tissues which apparently contained none when examined using a single step (direct) immunoperoxidase method. This improved sensitivity has been noted on a number of occasions.⁹

The advantages of a technique which can be used on well fixed resin-embedded tissues are evident. Clear and accurate localisation of protein deposits in or adjacent to basement membranes is essential for the diagnosis and classification of glomerular

Fig. 1 *Plasma cells in araldite-embedded human tonsil. Cytoplasmic γ heavy chain, PAP technique, counterstained with haematoxylin $\times 1000$.*

Fig. 2 *Basement membrane deposits of γ heavy chain in araldite-embedded renal tissue. Membranous glomerulonephritis, PAP technique, counterstained with haematoxylin $\times 800$.*

Table 4 *Demonstration of proteins in the cytoplasm of normal and reactive B lymphocytes*

Immunoglobulin fragments	Tonsil		Rejecting renal allograft
	Follicle centre cells	Plasma cells	Plasma cells
γ heavy chain	+	+	+
α	+	+	+
μ	+	+	+
κ light chain	+	+	+
λ	+	+	+

disease.² Also, the recognition of different lymphoid cells in resin-embedded tissues will allow a more satisfactory assessment of tumours of the lymphoid and macrophage systems and enable the morphological and functional characteristics of each cell to

Table 5 Demonstration of proteins in glomerular deposits

Immunoglobulin	Membranous glomerulonephritis	Mesangial IgA-IgG disease	Membranoproliferative glomerulonephritis, type I	Rejecting renal allograft
γ heavy chain	+	+	+	+
α	+	+	+	+
μ	—	+	—	—
Complement				
C3	+	—	+	+
C4	—	—	+	+
C1q	—	—	+	—

be directly identified and compared.

The demonstration that immunoreactivity is maintained when tissues are embedded in araldite suggests that further work for its use at the electron-microscope level may now be expected.

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Requests for reprints to: Dr AG MacIver, Faculty of Medicine, Level E, South Laboratory/Pathology Block, Southampton General Hospital, Tremona Road, Southampton SO9 4XY, England.

A rapid and accurate differential centrifugation method for platelet counts

S COUSINS, SM LEWIS *Department of Haematology, Royal Postgraduate Medical School, Du Cane Road, London W12 0HS*

Various methods have been proposed for preparing platelet suspensions for platelet counting by electronic counters. With some systems the platelets can be counted in the presence of red blood cells, either by the use of hydrodynamic focusing to avoid coincidence and masking of platelets by erythrocytes,^{1,2} or by computational techniques for estimating platelet counts from extrapolated volume distribution curves.³ Other counting systems require lysis of erythrocytes, leaving intact platelets, which

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