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Letters to the Editors

Immunoperoxidase techniques and controls

As we have considerable experience of immunoperoxidase techniques we were most interested to read Dr Heyderman's article in your journal (*J Clin Pathol* 1979;**32**:971-8). We think, however, that many of her comments regarding immunohistochemical techniques are appropriate only for laboratories that are able to prepare affinity-purified labelled antibodies. Most clinical histopathology laboratories would prefer a sensitive, reliable, and convenient immunoperoxidase method which does not entail any special immunological purification procedures.

Such a method, the peroxidase-antiperoxidase (PAP) technique, used after treatment of formalin-fixed sections with proteolytic enzymes such as trypsin[1 2] and pronase,[3] has been dismissed lightly by Dr Heyderman. Pre-treatment with trypsin ensures reliable detection of protein antigen[4] while the PAP method provides a high degree of sensitivity.[5-9] Although the amount of treatment with trypsin may vary a little with the degree of fixation, surgical biopsies, which have been fixed for similar periods, will require the same amount of treatment with trypsin to ensure consistent results.[10 11] An important feature of this particular immunoperoxidase method is that the primary antibody may be used at considerably higher dilutions than those required for either the indirect or the direct methods, thus reducing the possibility of staining due to cross-reactions.

Dr Heyderman has made some important comments on the use of immunological controls, and there is no doubt that absorption methods are the most satisfactory[12] but, as many laboratories do not have access to purified antigens, blocking controls are more readily performed and will ensure immunological specificity.[13] The use of antisera raised in differing animal species and obtained from different commercial sources overcomes the likelihood of significant contaminating antibodies occurring in both test and blocking antisera.

The technique (Table) is now well established for the routine assessment of intracellular protein components in lym-

Trypsin-immunoperoxidase (PAP) technique

1	Deparaffinise sections in xylol and take to alcohol	10 min × 2
2	Inhibit endogenous peroxidase by treating with freshly prepared 0·5% H_2O_2 in methanol	10 min
3	Wash well in tap water	
4	Equilibrate temperature of slides in distilled water at 37°C	10 min
5(*a*)	*For intracellular immunoglobulins and other protein antigens*	
	Treat with 0·1% trypsin in 0·1% $CaCl_2$ (adjust to pH 7·8 with N/10 NaOH) at 37°C	15-30 min* (usually 20 min)
(*b*)	*For extracellular immunoglobulins and other protein antigens*	
	0·05% trypsin solution in 0·05% $CaCl_2$ (adjust to pH 7·8 with N/10 NaOH) at 37°C	30-60 min* (usually 40 min)
6	Rinse in cold distilled water with agitation and transfer to moist chamber	2-3 min
7	Wash in Tris buffered saline (TBS) (0·5 M Tris/HCl buffer, pH 7·6, diluted 1/10 with saline)	10 min × 2
8	Normal swine serum, diluted 1/5 with TBS	10 min, drain off
9	Rabbit anti-human Ig sera (usually diluted 1/1000 in TBS)†	30 min
10	TBS wash	10 min × 3
11	Swine anti-rabbit IgG (usually diluted 1/100 in TBS)†	30 min
12	TBS wash	10 min × 3
13	PAP (peroxidase/rabbit antiperoxidase), usually diluted 1/200†	30 min
14	TBS wash	10 min × 3
15	Demonstrate peroxidase with 5 mg 3,3′ diaminobenzidine tetra-HCl dissolved in 10 ml 0·2 M Tris/HCl buffer (pH 7·6), to which 0·1 ml fresh 1% H_2O_2 has been added immediately before use[14]	10 min
16	Wash in TBS followed by a wash in running tap water	5 min
17	Counterstain with haematoxylin, differentiate, blue, dehydrate, clear, and mount in DPX	

*Time may vary with batch of trypsin and degree of fixation.
†Determined by titration.

phoproliferative disorders[15–21] and in human renal disease.[11]

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Dr Heyderman and her colleagues reply as follows:

We are pleased to have the opportunity to reply to the letter by Dr MacIver and Mr Mepham.

We agree that we would all prefer a sensitive, reliable, and convenient immunoperoxidase method which does not entail any special immunological purification procedures, but unfortunately this is not possible until affinity purified or monoclonal antisera are generally available, or manufacturers offer reliable absorbed reagents as controls.

Absorption controls

Although validation of the reagents is time-consuming and demanding, it is essential and should precede any investigation. The need for careful screening and controls was recently pointed out by Dr Walker in her paper on the localisation of carcinoembryonic antigen (CEA) in breast carcinoma.[1] Work previously published using the same antiserum to CEA without absorption with normal cross-reacting antigen (NCA;CEX) now needs to be re-evaluated.

'Blocking' controls

These were suggested by Goldman[2] to determine whether conjugation with FITC changes specificity of an unlabelled antibody. He does not recommend the method for antisera raised in two different species.

It has been shown[3] that antisera raised in different species may recognise different antigenic determinants on the CEA molecule. Some of the antisera are directed against determinants on the carbohydrate moiety and some against the protein determinants. All are anti-CEA, but since they recognise different parts of the molecule one may not block the others. It has also been shown that when rabbits are immunised with a haptenised synthetic trisaccharide, antisera to one, two, or three of the sugars may result (Fouron, personal communication). These antisera may or may not block each other; all would react with the parent antigen. Finally, spleen cells from a single immunised mouse when fused to myeloma cells give rise to clones which each recognise a different determinant on the original immunogen.[4]

We have carried out three series of experiments to investigate the use of antisera to an antigen raised in two species using one as a blocking control for the other and have found the method unsuccessful.

1 We were unable to block goat anti-CEA binding by pretreatment with rabbit anti-CEA (Dr Darcy).

2 We used antibodies to human placental lactogen (HPL) raised in rabbits by Dr Rosen and Ms Calvert and in swine (Orion) on sections of placenta and a secondary choriocarcinoma. Application of one failed to block binding of the other when applied sequentially, though the specificity of both antisera to HPL was established by absorption experiments.

A second pair of antibodies used was rabbit anti-IgG (Dakopatts) and goat anti-IgG (Miles) applied to tonsil sections. Again we failed to achieve complete blocking.

3 We first demonstrated that we could localise HPL in the placenta using a goat anti-rabbit alkaline phosphatase conjugate