

phoproliferative disorders<sup>15-21</sup> and in human renal disease.<sup>11</sup>

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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.<sup>1</sup> 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<sup>2</sup> 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<sup>3</sup> 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.<sup>4</sup>

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

instead of a peroxidase conjugate. We then used double labelling<sup>5</sup> to show that swine anti-HPL (peroxidase indirect conjugate) did not block all the sites recognised by the rabbit anti-HPL (visualised by the alkaline phosphatase conjugate).

Similar double labelling experiments with anti-immunoglobulins were difficult to interpret because both antisera stained the stroma. However, our impression was that blocking was incomplete. Even if blocking had been successful and both antisera recognised the same determinant, the specificity of one of the antisera would still require to be shown by loss of activity after absorption with IgG.

#### PAP method

Both the indirect and PAP methods are sensitive, reliable, and convenient. We use the indirect method because it produces excellent results, and satisfactory commercial peroxidase conjugates are available. Using the PAP technique we have failed to show a consistent increase in sensitivity; an extra reagent is involved so the number of variables is increased; it takes 45-60 minutes longer and is more expensive.

There is no theoretical reason why the PAP method should increase specificity. All second and subsequent antibodies are merely disclosing solutions, attaching only to the immunoglobulin of the first antibody, and should have no antihuman activity. The second antibody cannot (and should not) distinguish between the specific and the contaminating antibodies which cause so-called 'background staining'. If sensitivity is increased it must be to all the first antibodies.

Previous comparisons of the PAP and indirect methods have not used the same second antibodies. A valid comparison would be a quantitative experiment comparing the PAP method with a conjugate made with the same second (unlabelled) antibody in similar antibody concentrations.

#### Trypsinisation

We regularly use antisera to many different antigens, including CEA, alphafetoprotein, placental and pituitary hormones, Hb<sub>s</sub>Ag, and epithelial membrane antigen, at dilutions of 1:50 to 1:2000 and are able to demonstrate them satisfactorily without enzyme pre-digestion.<sup>6</sup> Improvement in staining for immunoglobulin after tryptic

sinisation has been reported by several groups<sup>7</sup> (references in MacIver and Mepham letter) but its use may cause false-negative results and increased collagen staining as well as loss of sections.<sup>8</sup> The question whether collagen staining is due to the presence of immunoglobulin or some other antigen remains to be resolved. Not all antisera stain collagen. It is possible that an anti 'collagen' is a common contaminating antibody.

#### Non-immune serum

Dr MacIver and Mr Mepham still include incubation with non-immune serum in their schedule to avoid 'background staining'. We have not found that leaving out this reagent impairs our results, and we use several non-affinity purified antisera which do not stain stroma or tissues in which the antigen is absent. Although very low levels of non-specific protein binding can be detected by RIA, the immunoperoxidase method is too insensitive to show this binding. We do not recognise the concept of 'background' or 'non-specific' staining and think that such staining is specific and is generally the result of the presence of contaminating antibodies to irrelevant antigens. Only absorption controls can elucidate this problem.

#### CONCLUSION

Whether the indirect or PAP method is preferred or whether trypsinisation is included or omitted remain matters for investigation and debate. In spite of the problems of antigen purity and availability, absorption is the most reliable control at the present time. Although we prefer affinity purified antisera, we use several that are not so treated but produce good clean results, and all the techniques we use are within the capacity of a routine department. In immunocytochemistry, as in histopathology in general, there is a need for quality control; the validation of positive results is as important as their achievement.

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#### Discrepancies in the weight of plastic vials

When using single-pan analytical balances, discrepancies of several milligrammes in the weight of plastic objects have been observed. Occasionally a gradual drift in the apparent weight was found to occur.

We found that weighing discrepancies were worst with plastic vials or plastic material when they were handled with latex or vinyl disposable gloves. Even minimal handling of the vial, such as that involved in unscrewing and replacing the cap while wearing gloves, was found to lead to errors of the order of 1-5 mg. This is illustrated in Table 1.

Of the various attempts to overcome this problem, including spraying the inside of the balance with antistatic fluid, using earthed forceps to handle the vials, placing earthed conducting foil over the base of the balance immediately below the balance pan, and using an antistatic gun to remove the charge on the vials, only the last was found to give a measure of success (Table 1). The discrepancies in weight between columns I and III, particularly in the case of the more sensitive five-place balance—the Sartorius