Technical method

A digestion technique for the reduction of background staining in the immunoperoxidase method

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A major limitation in the application of the immunoperoxidase technique to the identification of antigens in paraffin tissue sections is non-specific background staining of connective tissue. This problem was highlighted in this laboratory when an attempt was made to count immunoglobulin containing cells in the bronchial wall using a standard immunoperoxidase procedure. In these preparations the background is often so dark and diffuse as almost to obscure any positively staining cells. Rectal biopsies are also troublesome to interpret because of similar heavy brown background staining (Piris and Whitehead, 1974). Dilution of antisera and the use of non-immune swine serum before application of the first immune sera and incorporation of normal swine serum into the dilutions of immune sera are all helpful in suppressing non-specific background staining (Burns, 1975). In the bronchus, however, even these methods are insufficient to reduce background staining to an acceptable level. This paper describes a technique which abolishes background connective tissue staining without significantly affecting the capacity of the immunoperoxidase technique to detect tissue immunoglobulins.

Material and methods

Rabbit antisera to human kappa and human lambda and to human IgG and IgA, and rabbit antiperoxidase peroxidase conjugates were obtained from Dakopatts A/C (Denmark). All antisera and normal serum were diluted in 0.05M Tris (pH 7.6) containing 0.15M sodium chloride (Tris-saline); normal swine serum was added routinely to these antisera dilutions in a ratio of 1:5 (vol/vol) before use. Pepsin (P-7012) was purchased from Sigma Chemical Company.

Immunoglobulin containing cells were identified in paraffin embedded bronchial tissue before and after pepsin digestion by an immunoperoxidase procedure (Burns, 1975). In brief, the technique is

as follows: Sections used were dried at 50°C overnight; longer drying periods reduced staining intensity. Sections were dewaxed in xylol and taken down to water. They were then incubated for 20 to 30 minutes at 37°C in a solution of pepsin (4 mg/ml) in 0.01 normal hydrochloric acid. Control sections were not exposed to pepsin.

Endogenous peroxidase activity was blocked with a fresh 0.5% solution of hydrogen peroxide in methanol for 30 minutes. Sections were again washed in three changes of Tris-saline. To reduce non-specific background staining still further, sections were treated with normal non-immune swine serum diluted 1:5 for 15 minutes. Excess serum was drained off and the sections were treated with antibody to immunoglobulin for 30 minutes; in this study antihuman kappa and antihuman lambda diluted 1:100 and anti IgG and anti IgA diluted 1:50 were used. After being washed in three changes of Tris-saline for 15 minutes the sections were processed through the rest of the peroxidase anti-peroxidase method (PAP method) for immunohistochemical detection of tissue antigens as described by Burns (1975).

Results and discussion

The identification of IgG containing cells in the bronchial wall by the standard immunoperoxidase PAP method is obscured by background connective tissue staining as illustrated in figure 1. By contrast, such cells are readily detected by this procedure if the sections are first digested by pepsin before application of the antibody (fig 2). Similar results were obtained in sections treated with antisera to human IgA, and to kappa and lambda chains, ie, immunoglobulin containing cells were much more readily seen in pepsin treated tissue compared to undigested controls.

To determine whether pepsin digestion destroyed the antigenicity of immunoglobulins in bronchial wall the following experiment was done. Sections were processed through the immunoperoxidase procedure for the identification of immunoglobulins with and without prior pepsin digestion. Immunoglobulin containing cells in pepsin and non-pepsin treated sections were then counted in 20 high-power fields. As shown in the table, pepsin treatment not only did not reduce the number of immunoglobulin positive cells but in all instances increased the number of cells detectable. This increase in immuno-

Fig 1 Section of bronchial wall showing IgG positive cells without pepsin digestion. $\times 95$.

Fig 2 Section of bronchial wall showing IgG positive cells after pepsin digestion. $\times 95$.

Table Immunoglobulin positive cells in undigested and pepsin digested sections of bronchial wall

Immunoglobulin	Number of immunoglobulin containing cells	
	Before pepsin	After pepsin
IgG	79	524
IgA	41	452
Lambda	51	479
Kappa	63	292
Total	234	1747

globulin containing cells may be more apparent than real and is due probably to the ease with which such cells are seen in these preparations.

It is clear from these results that the use of pepsin digestion before the application of the immunoperoxidase technique provides a relatively simple solution to the problem of non-specific background staining in sections of bronchial tissues. We have also shown that this technique is applicable to rectal and prostatic tissue.

The mechanism whereby pepsin abolishes the background staining of connective tissue has not been investigated but it is probably related to the removal of ground substance protein molecules rather than to an effect on collagen fibres *per se* because bacterial collagenase is ineffective in abolishing background staining.

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Letters to the Editor

2-Hydroxyethyl methacrylate for sectioning bone marrow

Dancey *et al.* (1976), in their paper entitled 'Section preparation of human bone marrow for light microscopy', seem to me to have been unfortunate in the difficulties encountered when using hydroxyethyl methacrylate for this purpose. Perhaps they did not read my paper on the subject (Green, 1970). We have used the method given therein for about eight years with good results, without trouble, and without the use of special equipment. I agree with their finding that Ruddell's (1971) embedding mixture containing pyridine as an activator instead of N,N-dimethyl aniline used previously (Ruddell 1967a, 1967b) is unreliable. In fact in our own trials we could not make it work. However, the N,N-dimethyl aniline mixture is entirely satisfactory for aspirated marrow particles and also for large pieces of tissue. We regularly section blocks, measuring up to 2 × 2 cm, of a wide range of tissues including decalcified (by EDTA) bone marrow slabs. The

polymerisation failures experienced by Dancey *et al.* do not occur.

The most important technical hints appear to be the use of a cooling water-bath during polymerisation (which occurs within 30 min), and the fact that impregnation of large tissue blocks by monomer can be prolonged more or less indefinitely at 4°C without partial polymerisation, so ensuring complete impregnation. In sections undue staining of the plastic matrix with basic dyes can be reduced by treating the monomer with basic ion exchange resin beforehand (North, 1971).

Many valuable and relevant points about this easy-to-use plastic embedding medium for 'ordinary' hospital laboratories are given by Sims (1974), who used steel knives for sectioning, and Bennett *et al.* (1976), who present some original and stimulating information on the use of large glass knives and ordinary (rather than ultra-) microtomes. Although we use a base sledge microtome, Murgatroyd (1976) has reported good results with a rotary microtome. The great improvement in histological morphology in

hydroxyethyl methacrylate sections compared with paraffin sections makes its use for selected tissues well worth while.

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