PRO EXPERIMENTIS

The unmasking of antigens in paraffin sections of tissue by trypsin

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Summary. Paraffin sections of optimally fixed tissues often fail to react immunohistochemically. Short (15–30 min) exposure of the sections to 0.1% trypsin solution proves to be a very effective method of 'unmasking' the antigens.

The unlabelled antibody peroxidase-antiperoxidase (PAP) sequence has proved to be a sensitive method for demonstrating antigens in paraffin sections of tissue. However the results tend to be unpredictable and are often unexpectedly negative, as in a recent study of the distribution of immunoglobulin (Ig)-containing cells in human tonsil[1]. It was found that the PAP sequence failed to demonstrate Ig-containing cells in tissues fixed in solutions which gave excellent preservation of histological structure, whereas the same procedure revealed large numbers of Ig-containing cells in the same tissues when they were fixed in relatively 'crude' fixatives such as 4% formaldehyde in saline or water. The reagents in the PAP sequence are large protein (antibody) molecules which have to gain access to the target antigen within the tissues, and it was suggested[1] that the more refined fixatives 'masked' the antigen by preserving the integrity of the cells and tissues and thereby reducing their permeability. Conversely, the cruder fixatives succeeded immunohistochemically because they were less effective at preserving cell and tissue structure. On this assumption, and in the knowledge that trypsin digestion has been used to 'improve' immunofluorescent staining in paraffin sections[2], an attempt was made to unmask the antigen (Ig) by use of this enzyme.

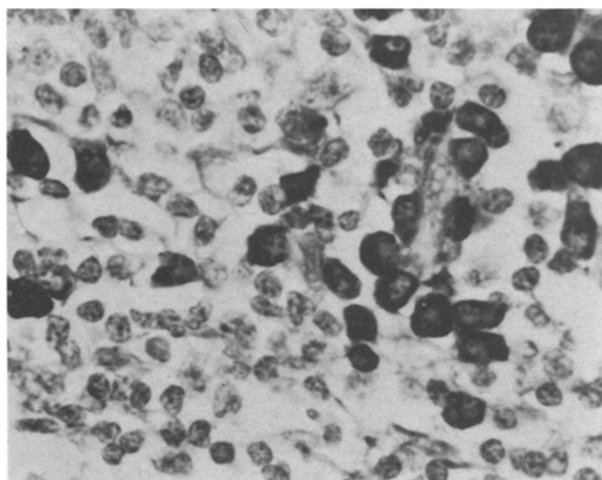

Fig. 1. Tonsil, fixed for 24 h in glutaraldehyde/formalin/calcium acetate solution. Paraffin section of crypt epithelium showing IgG-containing cells (black). Section treated with trypsin prior to the PAP sequence. Counterstained haematoxylin. ×680.

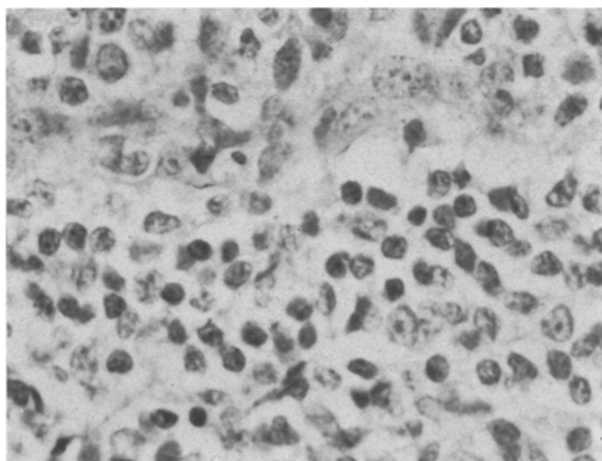

Fig. 2. Same site in adjacent section, without trypsin treatment. No IgG-containing cells visible. PAP sequence. Counterstained haematoxylin. ×680.

Materials and methods. 3 specimens of human tonsil and 10 of lymphomatous lymphoid tissue were fixed for 24 h at 20 °C in buffered 4% formaldehyde solution prepared from 'paraformaldehyde'[3], to which 3% sucrose was sometimes added, or in glutaraldehyde/formalin/calcium acetate solution[4] for 2 or 24 h at 20 °C. 'Control' tissues were fixed for 24 h at 20 °C in 4% formaldehyde in water or physiological (0.85%) saline. The tissues were then processed to paraffin wax. Sections 5 μm thick were cut and incubated at 37 °C with 0.1% trypsin solution (pH 7.8)[2] for periods ranging from 1 min to 3 h. They were then subjected to the PAP sequence[1], in which the sections were incubated at 20 °C for 30 min with each of the following reagents: rabbit antihuman Ig antiserum[5] at various dilutions; swine anti-rabbit Ig serum[5] 1 in 80; PAP complex[5] 1 in 40; followed by 0.05% diaminobenzidine-peroxide solution for 5 min to give a brown reaction product.

Results. It was found that after trypsin treatment Ig-containing cells could be demonstrated in large numbers in tissues which were previously unreactive (figures 1 and 2). The optimum time for trypsin treatment was generally about 15 min for tissues fixed in glutaraldehyde/formalin/calcium acetate solution and around 30 min for those fixed in formaldehyde, but incubation for only 1 min often had a readily detectable effect. When sections of control tissues, in which Ig-containing cells were demonstrable without prior enzyme treatment, were incubated with trypsin, the number of Ig-containing cells increased markedly and the quantity of reaction product within each cell was much greater than in non-trypsinized sections. The optimum titre for the first stage of the immunohistochemical sequence (rabbit antihuman Ig) was also much higher, often 1 in 8000 (0.3 μg ml^{-1}) or

1 R. C. Curran and E. L. Jones, Clin. exp. Immun. *28*, 103 (1977).
2 S. Huang, H. Minassian and J. D. More, Lab. Invest. *35*, 383 (1976).
3 A. R. Maunsbach, J. ultrastruct. Res. *15*, 242 (1966).
4 A. J. Garvin, S. S. Spicer and P. E. McKeever, J. exp. Med. *139*, 457 (1974).
5 Dakopatts (Denmark) Code No. 10-090, 21-090 and Z113.+ DIFCO, 1:250.

more. Perhaps as a result of this increase in sensitivity, nonspecific reactions were weaker. Washing in buffer for 12–18 h at 4°C also diminished nonspecific reactions. Initially various 'glues', including albumen, gelatin and araldite, were used to prevent the sections floating off the slides during the period of treatment with trypsin but the short incubation times in the enzyme solution made their use unnecessary. When enzyme treatment was optimal, the excellent preservation of the histological structure was unaffected and the sections reacted to high titres with the PAP sequence with very little non-specific staining. The results obtained with trypsin-treated tissues fixed in glutaraldehyde/formalin/calcium acetate solution were particularly impressive.

Discussion. In immunohistochemistry, prior fixation helps to stop antigens diffusing away from their sites within the tissues and by preserving the structural integrity of the tissue facilitates detailed histological study. Nevertheless it is well-known that fixation often renders a tissue immunologically unreactive and it tends to be assumed that the fixative has either destroyed the antigen or allowed it to diffuse away. The ease with which trypsin unmasks antigen suggests that when carefully-fixed tissue fails to react immunologically, the cause is more likely to be fixative-induced impermeability of the tissue rather than actual loss or destruction of the antigen by the fixative. Trypsin presumably acts by causing some disruption of tissue structure but this appears to be at the ultrastructural or molecular level since it is not apparent in the light microscope in sections optimally exposed to the enzyme. It is difficult to postulate a specific site of action for trypsin, particularly in view of the fact that other experiments have shown that sialidase (100 U ml^{-1} at pH 5.5) and streptokinase (100 IU ml^{-1} at pH 7.8) can be used instead of trypsin but with rather less effect. Whatever the action of the enzymes on the tissues, it is undoubtedly facilitated by the fact that the reactions in the PAP sequence take place on the surface of the tissue section, as can be shown by cutting a section of a section which has been treated in this way (unpublished observations). The enzyme would therefore only have to 'etch' the surface of the section of tissue in order to influence its responsiveness, and the short incubation times tend to confirm this. The other experiments also showed that trypsin can unmask antigens other than Ig, including carcino-embryonic antigen in carcinomas of breast and colon and the α subunit of human chorionic gonadotrophin in breast carcinoma. The results confirm that trypsin treatment is so efficient at unmasking antigens in tissue sections that it should be employed routinely when fixed tissues are used in immunohistochemical procedures.

A simple enzymatic method for isolation of hepatocytes

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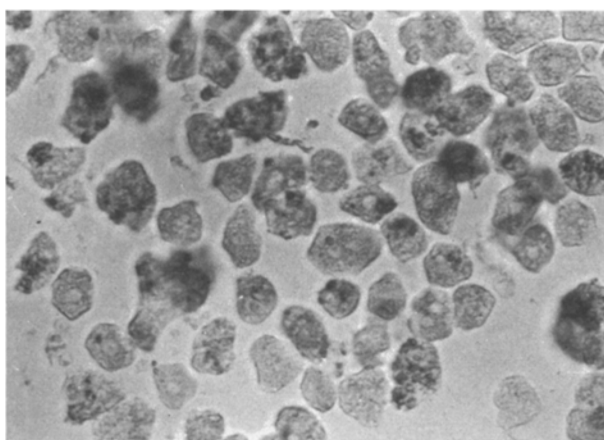
Summary. A new method is described for isolating hepatocytes by an enzymatic procedure: 0.05% bacterial collagenase in calcium-free Hanks' solution is injected into the liver tissue which is removed aseptically. The time required to prepare culture and risk of bacterial and fungal contamination is greatly reduced.

A new technique for the isolation of hepatocytes was developed. For the isolation of hepatocytes, enzymatic digestion with trypsin, bacterial collagenase or hyaluronidase has commonly been used[1] and recently in order to obtain high yield of cells, various perfusion techniques have been employed[2–4]. However, these perfusion techniques require a considerable degree of skill and take a long time. Furthermore, because of the long time required for the procedures, cultures are exposed to increased risk of bacterial and fungal contamination. Our new technique is very simple and the time required is reduced to $^1/_2$ to $^1/_3$ compared to that required by hepatic perfusion methods of enzymatic solution.



Isolated hepatocytes suspended in Eagle's minimum essential medium containing 20% of fetal calf serum.

Materials and method. Liver was removed aseptically as a whole from rats of Wistar strain, weighing 100–150 g, and washed 2–3 times with physiological saline; 0.05% bacterial collagenase (cl. histolyticum, purity grade 11, Boehringer, Mannheim) in calcium-free Hanks' solution, warmed to 37°C in advance, was injected into the liver tissue at the speed of 10 ml/min through a tuberculin needle equipped to a sterile syringe. With infiltration of the enzyme solution into the tissue, a diffuse whitish colour change of the whole liver was observed. If the colour change was only local around the injected site, the enzyme solution was injected at several different sites. After the injection of about 20 ml of collagenase solution, the liver was washed once in calcium-free Hanks' solution and then torn into small pieces with 2 forceps in a Petri dish containing 10–20 ml of calcium-free Hanks' solution. Hepatocytes were liberated during this procedure. Cal-

1 P. E. Kruse and M. K. Patterson, in: Tissue Culture. Method and Application, p. 3. Academic Press, New York and London 1973.
2 G. E. Moretimore, Am. J. Physiol. *200*, 1315 (1961).
3 R. J. Bonney, J. E. Becker and V. R. Potter, In Vitro *9*, 399 (1974).
4 M. N. Berry and D. S. Friend, J. cell. Biol. *43*, 506 (1969).