MICROSCOPY HANDBOOKS 11

An introduction to immunocytochemistry: current techniques and problems

Julia M. Polak and Susan Van Noorden

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An introduction to immunocytochemistry: current techniques and problems

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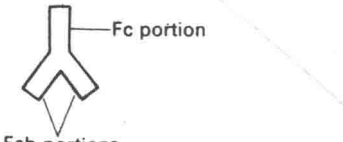
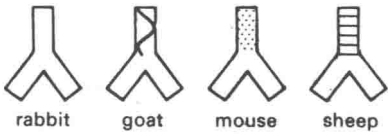
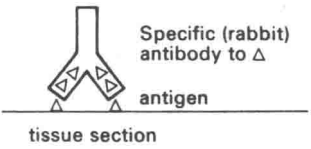
Key to symbols



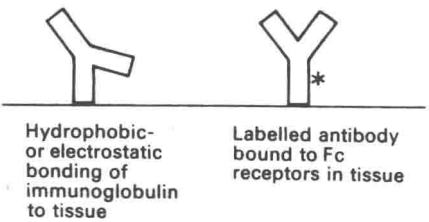
Immunoglobulin (antibody) molecule



Species in which antibody is raised is indicated by the symbol in the Fc portion of the antibody



Antigen specificity is indicated by the symbol in the Fab portion of the antibody and the substrate to which it binds.



Non-specific reactions

Key to symbols

Labels


Fluorophore (or other label)


Peroxidase


Insoluble end-product of peroxidase reaction (with H_2O_2 and DAB or other chromogen)


Alkaline phosphatase


End-product of alkaline phosphatase reaction (e.g. with naphthol phosphate and diazonium salt)


Hapten (as label)


Colloidal gold particles


Silver grains

Contents

Figs. 7, 11 and 18 and Table 2 are adapted from Figs. 2.1, 2.2, 2.3, and Table 2.1 in Polak and Van Noorden (1983).

Introduction

1.1. Definition

Immunocytochemistry is the use of labelled antibodies as specific reagents for the localization of tissue constituents (antigens) *in situ*.

1.2. History and development

The practice of immunocytochemistry originated with Albert H. Coons and his colleagues (Coons *et al.* 1941, 1955; Coons and Kaplan 1950) who were the first to label an antibody with a fluorescent dye, and use it to identify an antigen in tissue sections. As a result of this work, much of the uncertainty has now been removed from some aspects of histopathology, which were previously entirely dependent on special stains with interpretation sometimes precariously based on intuition and deduction. Because an antigen–antibody reaction is absolutely specific, positive identification of tissue constituents can now be achieved, although there are still problems, as will become apparent in the following pages.

The first fluorescent dye to be attached to an antibody was fluorescein isocyanate, but fluorescein isothiocyanate soon became the label of choice because the molecule was much easier to conjugate to the antibody. Fluorescein compounds emit a bright apple-green fluorescence when excited at a wavelength of 490 nm.

Following the early work and as better antibodies to more substances became available, the technique has been enormously expanded and developed. New labels have been introduced, for example, rhodamine isothiocyanate as an alternative, red, fluorescent dye and enzyme labels, visualized by established 'histochemical' techniques. The first enzyme to be used was peroxidase (Nakane and Pierce 1966; Avrameas and Uriel 1966). Other enzymes include alkaline phosphatase (Mason and Sammons 1978) and glucose oxidase (Suffin *et al.* 1979). The end-product of some of these enzyme reactions can be made electron-dense, but other, intrinsically electron-dense labels such as ferritin (Singer and Schick 1961) and colloidal gold (Faulk and Taylor 1971) have also been used to identify immunocytochemical reactions at the ultrastructural level. Antibodies have been labelled with radioactive elements, and the immunoreaction visualized by autoradiography, and some labels (e.g. latex particles) can even be used in scanning electron microscopy.

Among the techniques, the first modification of the original, directly-labelled antibody was the introduction of the indirect method (§ 4.2). This was followed by unlabelled antibody–enzyme methods which entirely avoided conjugation of a

label to an antibody and the damage to its reactivity which that entailed. Other methods involved the use of a second antigen (hapten) as a label, visualized by a second antibody, the exploitation of the strong attraction between avidin and biotin, and numerous ways of improving the specificity and intensity of the final reaction and of carrying out multiple staining.

Some of these methods are described here, and the appendix gives practical details of the basic techniques. However, the subject is too vast to be covered completely in this handbook, which aims only to introduce the concept, and the reader is referred to several other useful publications which provide more detail on selected aspects (Sternberger 1979; Bullock and Petrusz 1982, 1983; Wick *et al.* 1982; Polak and Van Noorden 1983).

Production of antibodies

2.1. Immunization

Antibodies, which are mainly γ-globulins, are raised by immunizing rabbits (or guinea pigs, etc.) with antigen. The antigen must be completely pure or (preferably) synthetic to ensure as specific an antibody as possible. Despite this, the resulting antiserum will not be directed specifically and solely to the injected antigen. The antibodies produced will be directed to various parts of the antigen molecule and to the carrier protein, or parts of it. The donor animal serum will also contain many natural antibodies, which may react with tissue components. A positive-appearing immunoreaction cannot, therefore, be assumed to be due to the specific, desired antigen–antibody reaction unless stringent controls are carried out. It may be necessary to immunize many animals in order to end up with even one usable antiserum because little is known about what makes an animal react to a foreign protein and the production of antibodies is still a matter of chance.

If the antigen is large, for example an immunoglobulin, it can be used by itself to immunize an animal. If it is as small as many peptides or if the molecule is not itself antigenic, it must be combined with a larger one for immunization. The small molecule (hapten) is chemically coupled (e.g. by glutaraldehyde or carbodiimide) to a larger 'carrier' protein molecule (e.g. limpet haemocyanin, thyroglobulin, or bovine serum albumin). The larger complex is a better stimulant of antibody formation than the small molecule alone. The donor animal's serum will contain a mixture of antibodies, reactive with different amino-acid sequences of the hapten and the carrier molecules, but the antibodies to the carrier molecule will either not react with the tissue to be stained (unless it were, for example, limpet tissue) or can be absorbed out, if necessary, by addition of the carrier protein, e.g. bovine albumin, to the antiserum prior to use. At an appropriate interval after the primary injection (subcutaneous), the rabbit (or guinea pig) is given a booster injection. Subsequently, blood is taken from it for testing for antibodies. No standard time course can be given for antibody raising, which is a highly individual procedure. The blood is then centrifuged to remove red blood cells. Although the antibody is contained in plasma which is not strictly serum since the fibrin has not been removed, the working solution is illogically known as an antiserum.

2.2. Testing

The next step is to test for the presence of antibody. The antiserum may be tested by the Enzyme-Linked ImmunoSorbent Assay (ELISA) against the pure antigen,

or by radioimmunoassay (RIA) if there is one available (see p. 37 for these methods) but by far the most satisfactory way of testing for an antibody to be used in immunocytochemical staining is by immunocytochemistry on known positive tissue. This is because in the two *in vitro* methods mentioned above, pure antigen only is offered to the potential antiserum, and thus any interfering unwanted reactions due to the other constituents of the serum are not detected. By immunocytochemistry the antiserum may be evaluated for the quality of the specific staining against the 'background' and, if the background staining is unacceptably high and cannot be eliminated, the antiserum must be abandoned. Another reason for preferring an immunocytochemical test concerns the 'avidity' or 'stickiness' of the antibody (see p. 6). A useful antibody for immunocytochemistry must combine strongly with its antigen so as not to be washed off the tissue during the staining procedure.

In radioimmunoassay, competition takes place between radiolabelled and unlabelled antigen for binding to the antibody, leading to an equilibrium between the two, depending on the proportion of each available. However, a 'good' antibody for radioimmunoassay is often not 'good' for immunocytochemistry, and vice versa. The ELISA technique resembles the immunocytochemical technique more closely.

2.3. Region-specific antisera

The various couplers used react preferentially with different functional groups of the hapten molecule. Glutaraldehyde, for example, attaches primarily to amino groups. The free portion of the hapten molecule, distant from the combining site, is most likely to stimulate antibody formation; thus if the only free amino group in the hapten molecule is the $-NH_2$ terminal, then immunization with that hapten after coupling with glutaraldehyde is likely to produce antibodies to the 'free' C-terminal. Carbodiimide, on the other hand, will react with free amino or carboxyl groups; thus immunization with carbodiimide-coupled hapten, having these groups only at either end of the molecule, is likely to produce a mixture of antibodies directed to the C- and the N-terminals. A knowledge of the structure of the hapten is thus essential if the coupler is to be chosen intelligently so that the likely region-specificity of the resulting antibodies may be foreseen (Szelke 1983).

In the case of a peptide, it is often advantageous to have an antibody which is specific for only a certain area of the molecule; for instance, in cases where two peptides to be identified have amino-acid sequences in common and the antibody is required to distinguish between them (e.g. gastrin and cholecystokinin which share a C-terminal pentapeptide sequence). Most antibodies will only recognize sequences of four to eight amino acids. It is usually only by chance that a serum will contain antibodies to the desired part of the molecule. Attempts to characterize an antibody by assaying or absorbing against fragments of the antigen are not necessarily reliable because there is a danger that a small fragment of an antigen molecule in solution may lose the particular molecular configuration which

gave it antigenicity when it was part of a whole molecule. Antigenic sites need not be straight-chain sequences of amino acids, but could be merely spatially adjacent sequences created by folding of the amino-acid chain. It is occasionally possible to use selected synthetic fragments of the molecule for immunization. With the proviso noted above, the resulting antiserum is then more likely to be specific for those fragments. Unfortunately, the smaller the amino-acid sequence used for immunization, the less antigenic it is, so that the chances of obtaining a good antibody to a peptide fragment are slim. Another problem is that the shorter the amino-acid sequence, the more likely it is to be common to several different peptides. Thus, in peptide immunocytochemistry it is often essential to stain sections with antibodies to several different regions of the molecule, for example, to the N-terminal, mid-portion, and C-terminal amino acid sequences. Results of staining with such antibodies can confirm that the peptide being localized is the genuine molecule or suggest that a related, but not identical peptide is being identified (Larsson 1980).

2.4. Monoclonal antibodies

A method of obtaining 'pure' antibodies, even after immunization with a whole molecule, has recently been proposed by Milstein. (For a clear account of the development of the concept of monoclonal antibodies and their potential uses, see Milstein *et al.* 1979; Milstein 1980; McMichael and Fabre 1982.)

Antibodies are produced in mice, and lymphocytes from the spleen, source of the antibodies, are fused with mouse myeloma cells in culture. The fusion allows the hybrid cells to continue to grow and divide in culture and also to produce antibodies. One cell produces only one type of antibody and the cultured hybrid myeloma cells are gradually cloned into cell lines producing one antibody only. The procedure consists of screening the culture fluid from the various clones for antibody by radioimmunoassay, ELISA, or immunocytochemistry. Antibodies specific to fragments of molecules could be produced in this way and, as the culture can be stored until further production is required, the method allows for a continuous supply of standard antibodies. A 'cocktail' of monoclonal antibodies to different areas of the antigen molecule should provide a reliable immunostain.

In addition to the use of monoclonal antibodies as pure antibodies to known antigens, monoclonal antibodies may be raised to unknown antigens and used as markers for particular cell types or cytoplasmic constituents. By immunizing mice with human thymocytes and cloning the antibodies produced, several series of monoclonal antibodies to human T lymphocytes have been produced which can be used to separate the cell types immunocytochemically (Kung *et al.* 1979). The antibodies act against constituents of membranes of the cells, but the chemical nature of these antigens is not yet known (Fig. 1).

Similarly, monoclonal antibodies separated from the antibodies resulting from immunization of mice with rat-brain homogenates are being used to study the relationships between different classes of brain cell (Sterberger 1983).

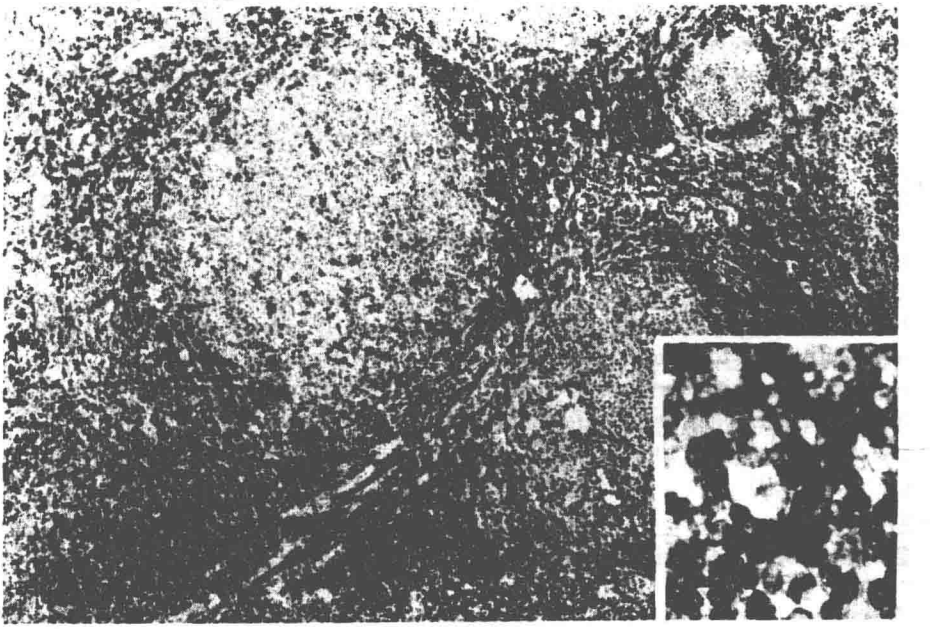


Fig. 1. Human tonsil immunostained by the indirect immunoperoxidase method using a monoclonal antibody to T-lymphocyte cell membranes and a peroxidase-conjugated rabbit anti-mouse IgG. Preparation counterstained with haematoxylin. Note the distribution of the T cells in the paracortex with a few cells only in the follicle centre. The membrane localization is demonstrated in the inset. Tissue preparation: Cryostat section (4 μm) thoroughly air-dried, fixed for 10 minutes in 100 per cent ethanol at 4°C, and then transferred to phosphate-buffered saline without further drying.

Monoclonal antibodies have truly revolutionized immunocytochemistry and will surely lead to major discoveries in the field of cell biology.

2.5. Characteristics of a 'good' antibody

The main requirement for a good antibody is that it shall be of high affinity for its antigen, in other words that its binding sites fit well with the antigenic sites on its specific antigen and do not attach to other antigens. The avidity, or binding strength, is a connected property depending on the number of fitting sites between the antigen and antibody (Roitt 1980). Immunocytochemistry requires antibodies of high avidity (stickiness) so that they are not washed off the section during the staining process.

It is useful to find that the unwanted antibodies in an antiserum are less avid than the antibody to the specific antigen and are, therefore, fortunately washed off the section during the staining procedure. However, it should be emphasized that great care must be taken when dealing with histological sections of abnormal

or damaged tissue such as come to a routine histopathology department and with whole cells in smears, because these preparations seem to attract and hold non-specific antibodies. Very thorough controls are essential for these, as for all, preparations.

The titre or concentration of the antibody is also very important. A high titre allows a high dilution which, in immunocytochemistry, means that the population of unwanted antibodies which might react with tissue components is diluted out. In radioimmunoassay, where the dilutions used are generally about 10 000 times higher than for immunocytochemistry, specific antigen only is offered to the antibody, so that unwanted reactions are ruled out. Nevertheless, a high dilution allows for removal of contaminating proteins and great economy in the use of labelled antigen which helps to eliminate 'noise' due to excess radioactivity. High dilutions are also advantageous in that they allow for the fullest use of the available quantity of good antibodies, which are expensive to produce. Unfortunately, the factors leading to antibodies of high avidity and titre are unknown.

If monoclonal antibodies are being used, the dilution factor becomes less important as unwanted reactions are absent and there is theoretically an unlimited supply of antibody.

3

Requirements for immunocytochemistry

The essential conditions for immunocytochemistry are summarized in Table 1.

Table 1. *Essential conditions for immunocytochemistry*

1. Preservation of antigen (see Table 2, p. 9)
2. Specific staining (see Table 4, p. 36)
3. Well characterized antibody
4. Easily visible label

3.1. Insoluble and available antigen

Successful immunostaining requires tissue antigens to be made insoluble and yet their antigenic sites must be available to the applied antibody without great alteration of their tertiary structure. In addition, the tissue architecture must be preserved (fixed) so that the immunoreactive cell or organelle may be identified in context. It used to be thought that good tissue fixation meant poor antigen availability, due to the strong cross-linking of tissue proteins by the conventional aldehyde fixatives, but recent work indicates that such fixatives can be used, even, in some cases, with osmium tetroxide post-fixation for electron microscopy, provided that the correct pre-treatment is used. This advance is probably the result of generally improved antibodies and more rigorously controlled technique. Good preservation for most of the peptide hormones and neuropeptides can be achieved, without the need for further treatment, by freeze-drying the tissue and exposing it to the vapour of weakly cross-linking reagents such as formaldehyde, p-benzoquinone, or diethylpyrocarbonate (Pearse and Polak 1975) but this method does not provide good morphological preservation for electron microscopy. The embedding process, with impregnation of the tissue in hot wax or epoxy resin, may also damage the antigenicity of some peptides. Cryostat sections of frozen tissue, pre-fixed by immersion or perfusion in buffered formaldehyde or p-benzoquinone, are, therefore, preferred by some authors for light microscopical investigations, paticularly for peptide-containing nerves (Elde *et al.* 1976; Bishop *et al.* 1978). Methods of fixation and their uses are summarized in Table 2.

A recent report suggests that fixation of peptides with p-benzoquinone solution may be improved by using the fixative at a higher pH and temperature, e.g. pH 8.0 and 37°C, to improve its cross-linking reactivity (Bu'Lock *et al.* 1982).

Fixation with formaldehyde may also be improved by altering the pH of the

Table 2. *Fixation*

Tissue preparation	Use
Smears or impressions of fresh tissue or cryostat sections of fresh-frozen tissue, unfixed or post-fixed in acetone, alcohol, etc.	Identification of autoimmune sera, cell surface antigens, extracellular antigens (e.g. immune deposits in glomerular basement membrane), tumour markers in cytological preparations
Cryostat sections or whole-mount preparations of tissue pre-fixed in para-benzoquinone or paraformaldehyde	Particularly useful for tracing antigens in nerves (e.g. peptides and amines)
Freeze-dried tissue, fixed in formal-dehyde or parabenzoquinone vapour and embedded in paraffin	Intracellular water-soluble antigens (e.g. peptides in endocrine cells)
Routine formaldehyde-containing fixatives; paraffin sections (preferably dried at 37°C). 'Over-fixed' antigenic sites may be revealed by pre-treatment of the sections with a proteolytic enzyme	Histopathological diagnosis (tumour markers, intracellular immunoglobulins, peptide hormones, etc.)
Periodate–lysine–paraformaldehyde; glutaraldehyde-based fixatives; resin-embedded, frozen, or vibratome sections.	Electron microscopical immunocytochemistry and light microscopical immunocyctochemistry on semi-thin sections.

solution. In the method reported by Berod *et al.* (1981) for localization of a diffusible antigen, tyrosine hydroxylase, formaldehyde was perfused at pH 6.5 for rapid penetration, although it fixes poorly at this pH. When the fixative was widely distributed, its pH was abruptly raised to 11 to increase the cross-linking reaction.

Pre-fixed cryostat sections, organ- or cell-culture preparations, smears, or whole-mount preparations which, unlike paraffin sections, have not been subjected to solvents during processing, often need to have the lipid components of the cell membranes broken down to improve penetration of the antibodies. This break-down may be achieved by soaking the preparations in a buffer containing a detergent such as Triton X-100 (0.2 per cent) or saponin before immunostaining. Larsson (1981) recommends this procedure for paraffin and resin sections as well, but in our experience, this is not necessary; however, addition of detergent to the rinsing buffer may help to prevent non-specific attachment of protein to the section.

An alternative method is to subject the preparations to dehydration through graded alcohols to xylene and then to rehydrate them before staining. The disadvantage of this method is that solvent-labile antigens may be leached out.

If the tissue to be processed contains a large amount of proteolytic enzymes (e.g. gut or pancreas), it may be found useful to include a proteinase-inhibitor such as trasylol in the fixative solution and the buffer rinse. The morphology of the tissue will probably be better and some labile peptides such as vasoactive intestinal polypeptide will stand a better chance of preservation.

Diethylpyrocarbonate and p-benzoquinone have not yet been tried to any extent as fixatives for cell surface or extracellular antigens, for which fresh-frozen cryostat sections are usually used, or for intracellular immunoglobulins, which can generally be shown in formalin-fixed material.

For electron microscopy, pre-embedding and post-embedding methods are available. Vibratome or cryostat sections of pre-fixed material may be immunostained, the reaction product being made electron-dense before the material is embedded in resin for the electron microscope. This method has the advantage that antigens are not exposed to solvents before immunostaining and that the immunostained area may be selected by light microscopy before embedding, but the disadvantage that adjacent thin sections cannot easily be stained by different antibodies. As mentioned above, some method of lipid breakdown should be incorporated when immunostaining these sections, but it need not be used for post-embedding staining of ultra-thin sections, in which the cell contents are exposed to the antibody.

The latest technology for electron microscopical immunocytochemistry provides for the preparation of ultra-thin sections of frozen, pre-fixed tissue (Tokuyasu 1980, 1983). These sections may be immunostained on grids without processing through solvents or resins and are particularly useful for fine structural localization of labile antigens.

Other fixatives have included a periodate/lysine/paraformaldehyde mixture (McLean and Nakane 1974) particularly for glycoproteins and a carbodiimide/glutaraldehyde mixture (Willingham 1980) for fibroblast constituents localized by ferritin. Fresh-frozen cryostat sections fixed in alcohol or acetone are useful for surface immunoglobulin antigens of lymphocytes (see Fig. 1). These methods have not been extensively used for peptides.

The method of choice depends on the antigen–antibody reaction to be carried out and the expected localization of the antigen. It is of great importance that the tissue be processed as freshly as possible, whether fixation is by immersion in solution or by vapour fixation after freeze-drying. In the latter case, once the tissue has been frozen, it may be stored at $-70^{\circ}C$ for an indefinite period before drying.

Protease treatment

A little understood, but practical way of revealing strongly cross-linked peptides in conventionally formalin-fixed and paraffin-embedded material is to treat the sections with a protease such as trypsin or pronase (Huang *et al.* 1976) before immunostaining. It is thought that the protease treatment breaks the cross-linking bonds of the fixative with the protein to reveal the antigenic sites. Some antigens may be destroyed by the enzyme incubation and there is also a possibility that large protein molecules (e.g. precursors of bioactive peptides) may be cleaved to smaller molecules by the enzyme. If these molecules display antigenic sites that were not available on the precursor, a false positive stain may be achieved. However,

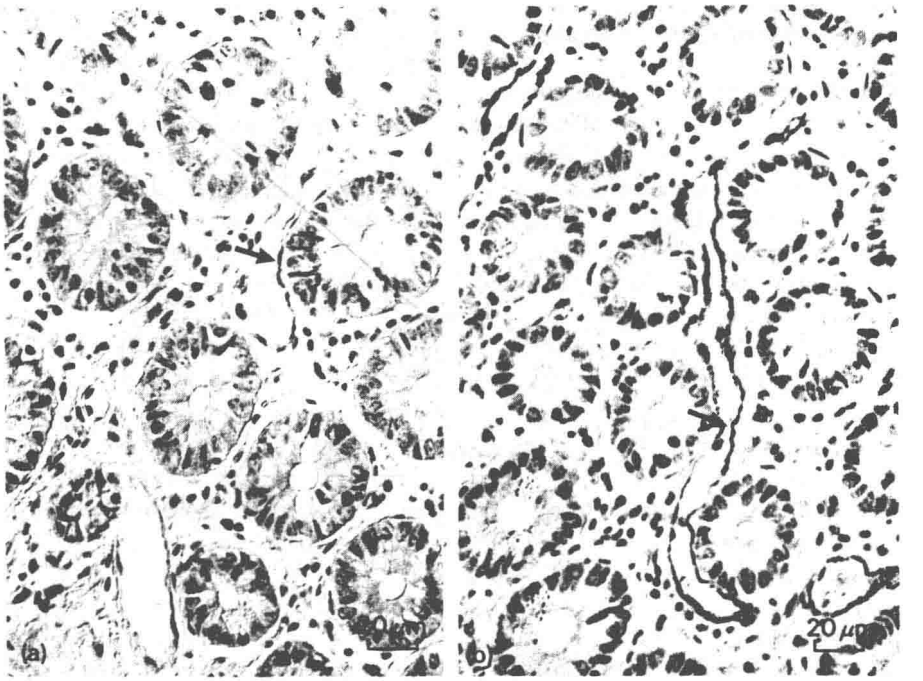


Fig. 2. Human duodenum immunostained for Factor VIII-related protein, a marker for endothelial cells, by the peroxidase anti-peroxidase method using a rabbit antibody to Factor VIII followed by unconjugated goat anti-rabbit IgG, then rabbit PAP complex. The section shown in (a) was stained without trypsin pretreatment, the adjacent section in (b) was stained after 'digestion' for 30 minutes at 37°C in 0.1 per cent trypsin in 0.1 per cent calcium chloride at pH 7.8. Note the very slight reaction in the endothelial cells of the vessel wall in (a) compared with the intense reaction in (b) (arrows). Counterstained with haematoxylin. Tissue preparation: tissue fixed in neutral phosphate-buffered formalin and embedded in paraffin; 4 μm section. Photographed with Nomarski interference-contrast optics.

bearing in mind these possibilities, protease pre-treatment should be tried routinely whenever preliminary immunostaining is found to be inadequate (Fig. 2). A useful discussion is that of Finley and Petrusz (1982). For practical details see § A5.

Oxidation treatment

Even conventional glutaraldehyde or paraformaldehyde–glutaraldehyde mixtures have now been successfully used for a combination of immunocytochemistry and electron microscopy. Ultra-thin sections of glutaraldehyde/osmium-fixed material may also be used after oxidation with hydrogen peroxide (Baskin *et al.* 1979; Beauvillain and Tramu 1980) or sodium metaperiodate (Bendayan and Zollinger 1982, 1983).