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Demonstration of immunoglobulin in cryostat and paraffin sections of human tonsil by immunofluorescence and immunoperoxidase techniques

Effects of processing on immunohistochemical performance of tissues and on the use of proteolytic enzymes to unmask antigens in sections

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SUMMARY A fluorescein isothiocyanate (FITC) technique and one based on peroxidase-antiperoxidase (PAP) were used to study the distribution of immunoglobulin (Ig) in cryostat and paraffin sections of human tonsil. Trypsin and other proteolytic enzymes were used to 'unmask' the antigen in paraffin sections. The effects of processing, and particularly of fixation, on the immunohistochemical response of tissues were studied.

The FITC and PAP methods detected Ig in paraffin and cryostat sections equally well. The distribution of the antigen was the same with both methods but the PAP method was the more informative. Formaldehyde-sucrose solution proved more suitable for fixing tissues for immunohistochemistry than glutaraldehyde. Trypsin revealed antigen in paraffin sections more efficiently than pepsin, papain, or pronase. Surface Ig (s-Ig) could be demonstrated in trypsinised paraffin sections but less effectively than in cryostat sections. Trypsinised paraffin sections were, however, more suitable for intracellular Ig (c-Ig) than cryostat sections although the performance of cryostat sections could be improved by prior fixation with a coagulative fixative.

It is well recognised that peroxidase has a number of advantages over fluorescein as a 'label' in immunohistochemical techniques. A major one for histopathologists is the production of permanent preparations, which can be studied by conventional light microscopy. For this reason, and also because of the realisation that antigens, including immunoglobulin, frequently survive formaldehyde fixation and embedding in paraffin wax (Burns *et al.*, 1974), many histopathologists are applying peroxidase-based methods to paraffin sections. The results have been unpredictable, however, and it is clear that much remains to be discovered about the precise effects that the various steps in the processing of tissue have on its immunohistochemical response. The present study was undertaken to determine some of these effects and particularly that of fixation. The relative merits of cryostat and paraffin sections were also assessed; and at the same time the ability of trypsin and other proteolytic enzymes to unmask antigens in paraffin sections (Curran and Gregory, 1977) was investigated. In order to determine whether the 'label' itself had a significant influence, a fluorescein isothiocyanate (FITC) technique and one based on peroxidase-antiperoxidase (PAP) complex were applied routinely to pairs of sections and the results were compared. The antigen studied was immunoglobulin (Ig) and the tissue was human palatine tonsil.

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Material and methods

Cryostat and paraffin sections were prepared from 15 palatine tonsils collected immediately after surgical removal.

FIXATIVES

The following solutions were used: (1) formaldehyde-sucrose containing 4% formaldehyde (prepared from paraformaldehyde) and 2·5-8% sucrose in

0·135 M phosphate buffer pH 7·4 (Maunsbach, 1966). Occasionally 4% formaldehyde in physiological (0·85%) saline (formol-saline) was also used, mainly for fixing cryostat sections; (2) glutaraldehyde/formalin/calcium acetate solution (G/F/C) (Garvin *et al.*, 1974); (3) methanol; or (4) ether-ethanol (40:60) (Leduc *et al.*, 1969). A 50:50 mixture was also used occasionally.

CRYOSTAT SECTIONS

1 Unfixed tissues
Slices of fresh unfixed tonsil (approximately 3 mm thick), surrounded by OCT compound (AMES), were quenched in liquid nitrogen. From these, cryostat sections, 6 μm thick, were cut and subjected to the immunohistochemical procedures without fixation. In order to determine the effects of fixation, occasional sections were fixed at 20°C before step 1, 3, 5, 7 or 9 of the immunohistochemical procedure (see below) for either 18 hours in formol-saline; 1-5 minutes in G/F/C; 10 minutes in methanol; or 20-60 minutes in ether-ethanol. It is important to wash sections fixed in this way thoroughly with ethanol to remove the ether before the immunohistochemical method.

2 Presoaked tissues
Cryostat sections were also prepared from blocks of unfixed tissue (3 mm thick), which had been previously soaked at 4°C for two, three or four days in phosphate buffer (pH 7·4) before quenching. Sometimes the buffer contained sucrose ($2\frac{1}{2}$-8%).

3 Previously fixed tissues
Cryostat sections were also prepared from tissues previously fixed for 24 hours at 20°C in formaldehyde-sucrose or for 2 to 24 hours in G/F/C at the same temperature. The sections did not adhere readily to the glass slides and were usually processed as free-floating sections.

PARAFFIN SECTIONS

1 Tissues fixed by immersion or perfusion-immersion
Slices of tissue, 3 mm thick, were fixed in formaldehyde-sucrose for 24 hours at 20°C or for 48-72 hours at 4°C. In addition, whole tonsils were fixed by continuous perfusion (2·5 ml/h) with fixative through two needles (no. 26G × $\frac{3}{8}$ in) for 24-72 hours at 4°C and occasionally slices of tonsil perfused in this way were placed in formaldehyde-sucrose for a further 24 hours at 20°C. Blocks of tonsil were also fixed in G/F/C for 2 to 24 hours at 20°C or 4°C. The tissues were processed to paraffin in an Auto-Technicon Ultra, and 5 μm sections were cut.

2 Pretreated tissues
Paraffin sections were also prepared from tissues treated in one of two ways before fixation in formaldehyde-sucrose or G/F/C: (*a*) from blocks of tissue that had been quenched in liquid nitrogen and immediately thawed; or (*b*) from blocks of tissue that had been previously soaked at 4°C for two, three, or four days in buffer or buffer-sucrose solution (see 2 above).

TRYPSINISATION
Trypsinisation was used to increase the response of the paraffin sections to the immunohistochemical procedures (Curran and Gregory, 1977). After treatment with xylene and alcohol, the sections were placed in Tris-saline buffer[1] (pH 7·8) containing 2·5% sucrose at 20°C. After a further rinse at 37°C, they were incubated at 37°C for 5 to 30 minutes in Tris-saline buffer (pH 7·8) containing 0·1% trypsin[2] and 0·1% calcium chloride. The solution was stirred vigorously to circulate it freely between the slides, which were held with their long axis vertically and at least 5 mm apart. Enzyme treatment was followed by washing at 20°C with several changes in Tris-saline buffer (pH 7·8) containing 2·5% sucrose. Occasional sections were incubated for 5 to 120 minutes at 37°C with pepsin[3] (4 mg/ml in 0·01 normal hydrochloric acid) (Reading, 1977); 0·1% pronase[4] (in 0·05 M Tris-saline buffer pH 7·5) (Denk *et al.*, 1977); or 0·1% papain[5] (in 0·05 M Tris-saline buffer pH 7·5). A small number of cryostat sections, which had been fixed in formaldehyde or G/F/C, were also trypsinised.

BLOCKING OF ENDOGENOUS PEROXIDASE
After treatment with trypsin, the paraffin sections which were to be subjected to the PAP and FITC procedures were immersed in hydrochloric acid (HCl)-methanol solution (Mazurkiewicz and Nakane, 1972) for 30 minutes at 20°C to block endogenous peroxidase and then washed for some minutes in several changes of Tris-saline-sucrose solution. The HCl-methanol has a fixing effect on the cryostat sections and was rarely applied to them.

IMMUNOHISTOCHEMICAL PROCEDURES
Pairs of sections prepared in the same way were subjected simultaneously to the PAP and FITC methods. Tests were made with the PAP and FITC procedures for γ, α, μ, and δ heavy chains and for

[1]Tris 0·2M in distilled water 10 parts; 0·1 N HCl 13 parts; and physiological saline (0·85%) 17 parts (pH7·8)
[2]Difco 1: 250
[3]BDH product no. 39032
[4]BDH product no. 39052
[5]BDH product no. 39030

κ and λ light chains[1]. All antisera were diluted in Tris-saline buffer (pH 7·6)[2] and the Tris-saline buffer for the washes contained 2·5% sucrose.

PAP method

1 Normal swine serum (1 in 2 parts of Tris-saline buffer): 10 minutes.
2 Tip off excess.
3 Rabbit anti-human Ig antiserum 1 in 500: 45 minutes. The optimum titre varies, and with trypsinised paraffin sections may reach 1 in 6000 or more. If the titre is too low, a false-negative result may occur.
4 Wash in Tris-saline-sucrose solution: 20 minutes.
5 Swine anti-rabbit immunoglobulin 1 in 100: 45 minutes.
6 Wash as 4.
7 PAP complex 1 in 40: 45 minutes. The solution was always spun for 5 minutes at approximately 500 *g* before use to remove insoluble aggregates of PAP. Failure to do this results in the formation of black deposit on the slides after treatment with DAB.
8 Wash as 4.
9 5mg 3,3-diaminobenzidine tetrahydrochloride (DAB) in 10 ml Tris-saline solution + 1 drop of 100 vol. hydrogen peroxide: 5 minutes. The ethylcarbazole method (Graham *et al.*, 1965) proved an effective alternative to DAB, the sections however being mounted in glycerol jelly.
10 Mayer's haemalum: 20 seconds.
11 Dehydrate, clear, and mount in Canada balsam.

FITC method

Steps 1 to 4 are the same as in the PAP sequence but are followed by the application of FITC-labelled swine anti-rabbit serum IgG (1 in 30) for 45 minutes. The sections are mounted in glycerol-phosphate buffer-saline and examined, using incident UV light, in a Leitz SM-LUX microscope.

Specificity

Non-specific reactions were minimised by the use of the optimum (highest) titre of the specific antibody in step 3. Tests for specificity included the substitution of normal rabbit serum for the specific antiserum in step 3; the omission of step 3, 5, or 7 of the PAP method, and of step 3 of the FITC method. The use of six different specific antibodies for step 3 provided a form of intrinsic control, in that a different distribution of antigen was obtained with each antibody, which differed from those provided by the other five. Each of the specific antisera against γ, α, and μ heavy chains was also blocked by the addition, 45 minutes before using it, of an equal volume of a range of dilutions (1 in 40 to 1 in 2560) of pure IgG (10·5 mg/ml), IgA, or IgM (1·5 mg/ml).[1] The reaction for each heavy chain was blocked by the appropriate Ig even at the highest dilution. In addition to the specific antibodies against human Ig, antisera against two other antigens lacking in human tonsil were also used for step 3, viz, against carcinoembryonic antigen (CEA) and secretory component of IgA.

[1]The reagents used were: Dakopatts 10-090, 10-MAT, 10-091, 10-9K2, 10-9L2, Z113, P2190 (obtained from Mercia Brocades Ltd, Watford); and Behring OTND 05 and OTNP 05 (from Hoechst Pharmaceuticals).

[2]Tris 0·2 M in distilled water 10 parts; 0·1 N HCl 15 parts; and physiological saline (0·85%) 15 parts (pH 7·6).

Results

CRYOSTAT SECTIONS

1 Unfixed tissues

In FITC and PAP preparations of cryostat sections, c-Ig (γ, α, δ, and μ heavy chains; κ and λ light chains) is detectable in many plasmocytes within and beneath the crypt epithelium (Figs 1 and 2). The reaction is moderately strong. The reaction product is not sharply defined, forming a kind of 'corona' round each cell, and where plasmocytes are numerous, for example, in and beneath the crypt epithelium, a weak zone of reactivity is often detectable. Approximately twice as many cells contain γ heavy chains as contain α chains, the numbers with δ and μ heavy chains being comparatively small. Cells containing κ or λ light chains are present in approximately equal numbers, and the number of cells containing light chains is approximately the same as those containing heavy chains. s-Ig (μ and δ heavy chains; κ and λ light chains) is detectable on the small lymphocytes surrounding the lymphoid follicles and also on individual lymphocytes around the follicles and in the crypt epithelium (Figs 1 and 2). The number and distribution of cells containing Ig and the distribution pattern of s-Ig are identical in FITC and PAP preparations. It is slightly easier, however, to detect individual s-Ig positive lymphocytes with the FITC method. In both types of preparation, extracellular Ig (γ, α, and μ heavy chains; κ and λ light chains) is abundant between the cells of the germinal centres of the lymphoid follicles. Some collagenous tissues react strongly for γ and weakly for α and μ heavy chains.

When the cryostat sections are fixed in ether-ethanol or methanol before the immunohistochemical

[1]Kindly supplied by the Immunodiagnostic Research and Development Laboratory of the Department of Immunology, University of Birmingham

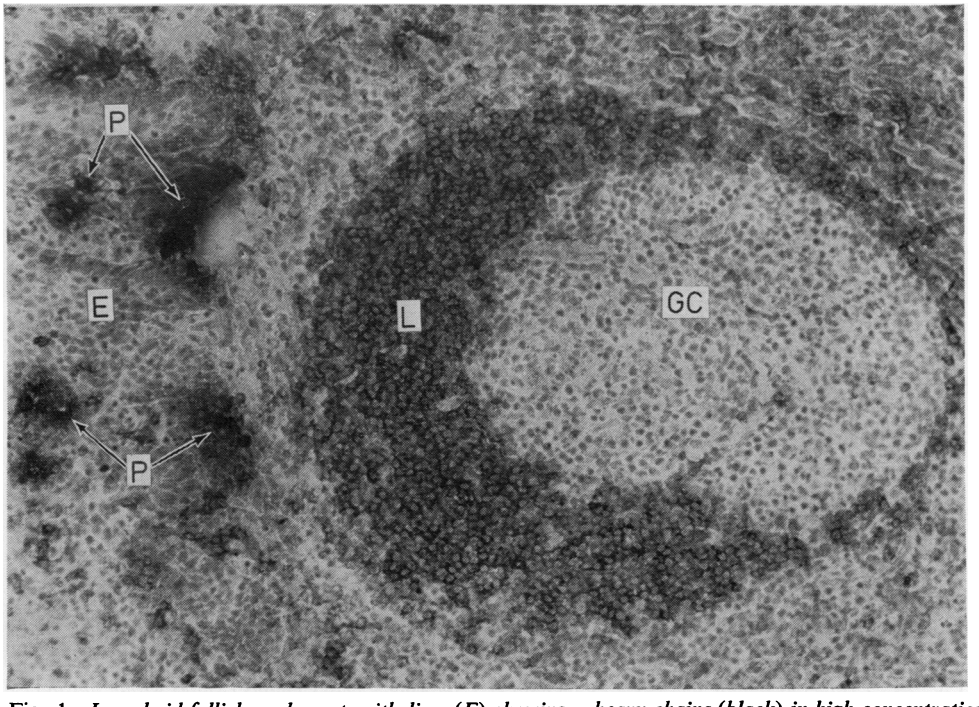

Fig. 1 *Lymphoid follicle and crypt epithelium (E) showing γ heavy chains (black) in high concentration within plasma cells (P) in and beneath the epithelium. The reaction product is not sharply defined (compare with Figs 3 and 4). Lower concentrations of γ heavy chains are present on the surface of the lymphocytes (L) surrounding the germinal centre (GC). Cryostat section, fixed in ether-ethanol. PAP method. × 150.*

method, the distribution of Ig is unchanged but the reactions for c-Ig are strengthened. If the period of fixation is short (20-30 minutes), the reactions for s-Ig are also strengthened, but if it is prolonged they may be weakened or abolished. Prior fixation in formaldehyde improves the histological appearances but generally weakens the reactions for both c-Ig and s-Ig, the latter being particularly susceptible. After prolonged (18 hours) fixation in formol-saline, however, c-Ig and extracellular Ig (but not s-Ig) are sometimes well demonstrated, and the result is comparable with a trypsinised paraffin section (see below). G/F/C is much more destructive than formaldehyde, and immersion for 30 seconds in G/F/C can render a section completely unreactive. As a rule fixation in formaldehyde after step 3 of the immunohistochemical procedure has less effect than fixation before it, but G/F/C quickly destroys the reactions for s-Ig whenever applied.

2 Presoaked tissues

In cryostat sections prepared from tissues soaked in buffer for two days or more at 4°C, the histological structure seems to be 'looser'. The reactions for s-Ig (μ and δ heavy chains; κ and λ light chains) on the lymphocytes are comparable in strength to those obtained with tissues not previously soaked, as are the reactions for c-Ig, including the corona of reaction product around each plasma cell. Diffusion artefact is detectable, however, in the form of false-positive reactions (for both heavy chains and light chains) on the surfaces of epithelial cells, histiocytes, and lymphocytes, and on the nuclei of many of these cells. Diffusion artefact is less marked in tissues soaked in buffer containing the higher concentrations of sucrose.

3 Previously fixed tissues

Cryostat sections prepared from fixed tissues react poorly immunohistochemically, although trypsinisation improves their performance.

The general conclusion is that the distribution of Ig (heavy and light chains) in cryostat sections, however prepared, is identical with the FITC and PAP methods.

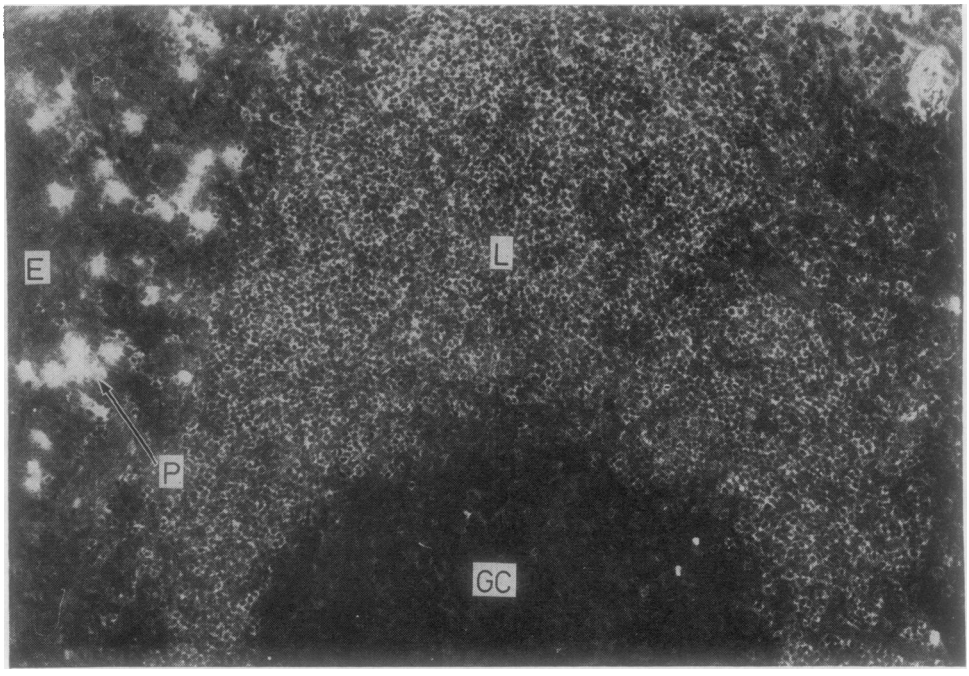

Fig. 2a *Lymphoid follicle and crypt epithelium (E) showing γ heavy chains (white) within plasma cells (P) in the crypt epithelium. As in Fig. 1, the positive sites are not sharply defined, with a 'corona' around each cell. γ heavy chains are also present on the surface of the lymphocytes (L) surrounding the germinal centre (GC). Cryostat section, fixed in ether-ethanol. FITC method. × 155.*

PARAFFIN SECTIONS

1 Tissues fixed by immersion or perfusion-immersion
Histological structure is well preserved in tissues fixed in G/F/C/ or in solutions of formaldehyde containing 2·5% or 3% sucrose, shrinkage of cells being detectable after fixation in formaldehyde solutions containing the higher concentrations of sucrose. The paraffin sections react unpredictably with the FITC and PAP procedures and are often virtually negative, particularly those which show optimal preservation of histological structure. After trypsinisation, however, the sections invariably react consistently and at much higher titres of the specific antiserum (step 3). Trypsinised sections of tissues fixed in formaldehyde-sucrose are much more satisfactory than similar sections from tissues fixed in G/F/C, even when the period of fixation in the G/F/C has been only a few hours. Twenty-four hours at 20°C appears to be the optimum time in formaldehyde-sucrose, longer fixation times tending to be deleterious. Formaldehyde fixes very slowly at 4°C, and even after 72 hours the tissues are only lightly fixed. Of all the fixation procedures tested, that which gives the most consistent results is perfusion of the whole tonsil by needle for 24 hours at 4°C with formaldehyde-sucrose followed by slicing and soaking of the slices in the same fixative for 24 hours at 20°C. In trypsinised paraffin sections of tissue fixed in this way, the PAP and FITC techniques both reveal many Ig-containing plasmocytes in and beneath the crypt epithelium (Figs 3 and 4). In sections treated with trypsin for 10 minutes or less, the c-Ig is located in the peripheral cytoplasm of each plasmocyte, but as the period of incubation is extended to 30 minutes, the amount of c-Ig increases until it fills the cell. The reaction product is much more densely staining, and its location within each cell is more sharply defined than in cryostat sections. However, the numbers of cells containing light and heavy chains, and their relative numbers, are the same in paraffin and cryostat sections.

s-Ig (μ heavy chains; κ and λ light chains) is also detectable on the lymphocytes of the 'mantle' in sections of tissues fixed in formaldehyde-sucrose and most consistently in those fixed by perfusion at 4°C (Fig. 4). Trypsinisation for 5 to 10 minutes is often sufficient, s-Ig tending to be lost from sections treated for longer periods. However δ chains are usually not detectable on lymphocytes, even with

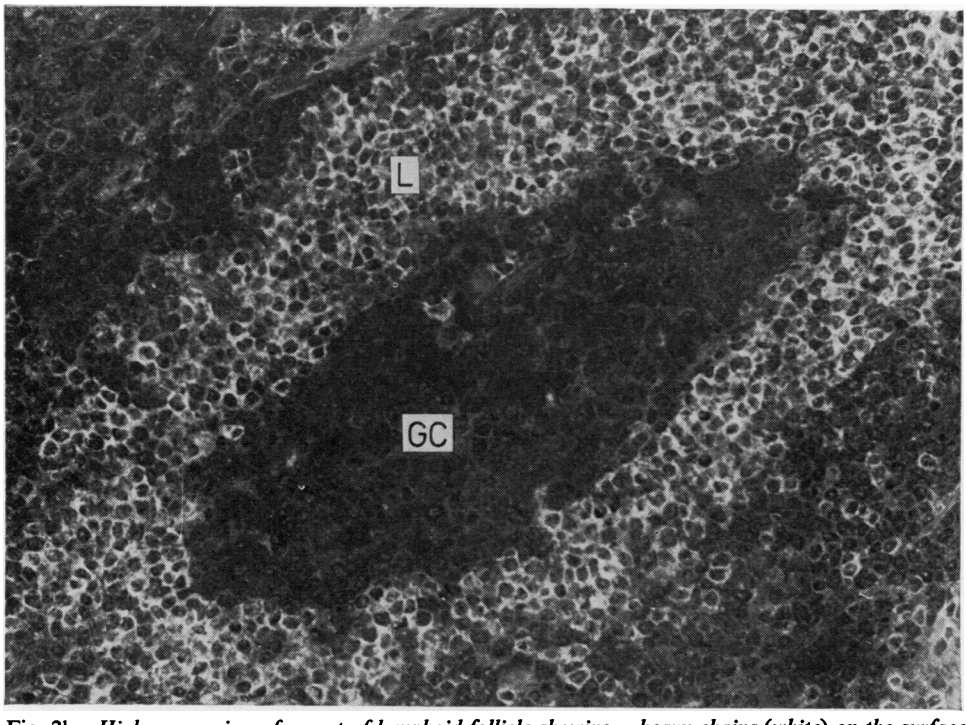

Fig. 2b *High-power view of a part of lymphoid follicle showing γ heavy chains (white) on the surface of the lymphocytes (L) surrounding the germinal centre (GC). Cryostat section, fixed in ether-ethanol. FITC method.* × *350.*

optimal fixation and trypsinisation.

Extracellular Ig (γ, μ, and α heavy chains; κ and λ light chains) is readily demonstrated in the centres of the lymphoid follicles (Figs 3 and 4). Some collagenous tissues also react, mainly for γ heavy chains. Intracellular and extracellular Ig (but not s-Ig) is detectable readily in sections pretreated with pepsin, papain, or pronase and has the same distribution as in trypsin-treated sections. The results are inferior to those obtained with trypsin. Optimum incubation time for the sections is one to two hours with pepsin and papain, but five minutes' treatment with pronase is sufficient.

2 Pretreated tissues

(*a*) Paraffin sections (untrypsinised) prepared from tissues frozen and immediately thawed before fixation in formaldehyde or G/F/C show reasonably good preservation of histological structure but are comparatively unreactive, except for an unusually strong reaction for μ heavy chains, intracellular and extracellular, in the follicle centres.

(*b*) In paraffin sections (untrypsinised) prepared from tissues which have been soaked for two days and then fixed in formaldehyde or G/F/C, diffusion artefact is very obvious. However, the reactions for c-Ig are strong; and when the fixative is formaldehyde, the number of Ig-containing cells is large.

The general conclusion is that, as with cryostat sections, the FITC and PAP procedures demonstrate Ig (c-Ig, s-Ig, and extracellular Ig) in paraffin sections at the same sites and with approximately the same degree of sensitivity.

NON-SPECIFIC REACTIONS

Non-specific reactions are given by necrotic cells, epithelial cell tonofibrils, and desquamated epithelial cells lying in the crypts (Fig. 3). They are equally evident in the FITC and PAP preparations and in cryostat and paraffin sections, and are typically present irrespective of which specific antiserum is used in step 3. They are comparatively insensitive to the method of fixation but are weaker in tissues first soaked in buffer for two days.

ENDOGENOUS PEROXIDASE

The HCl-methanol method usually blocks the endogenous peroxidase (in red cells and leucocytes) completely, without affecting adversely the immunohistochemical response of the sections. Blocking may

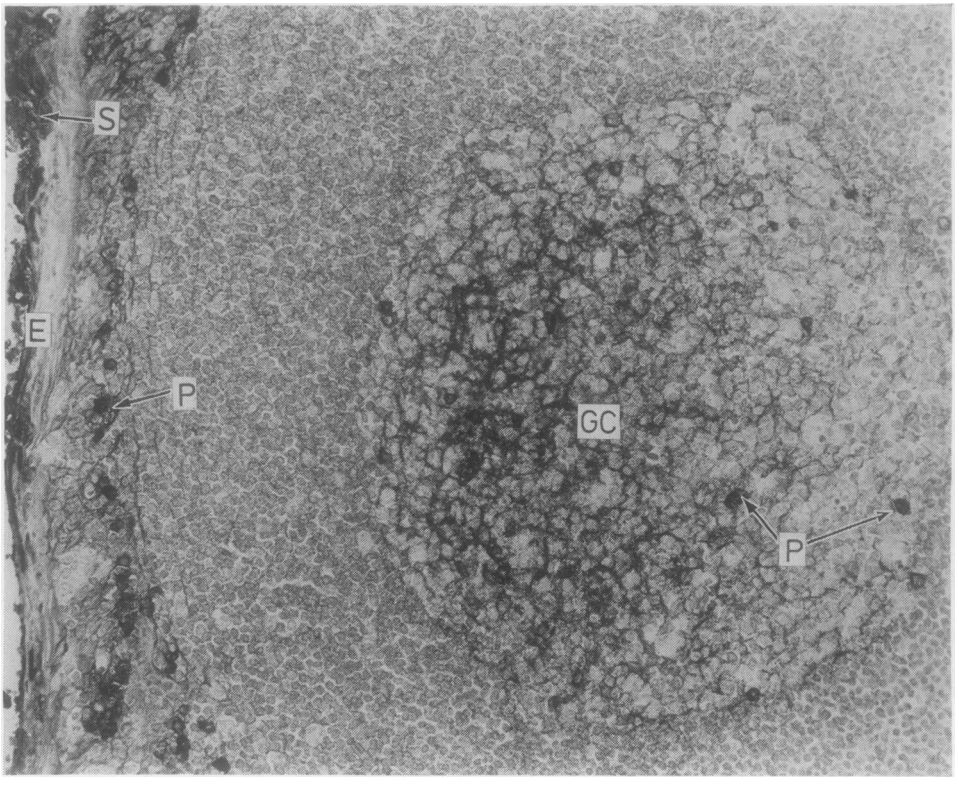

Fig. 3 *Lymphoid follicle and crypt epithelium (E) showing α heavy chains (black) within plasma cells (P) in and beneath the crypt epithelium and in the follicle centre (GC). α chains are also present between the cells in the follicle centre. Squames (S) in the lumen of the crypt react non-specifically. Trypsinised paraffin section. PAP method. × 220.*

even improve the response, presumably by a 'secondary fixation' effect.

AUTOFLUORESCENCE

This is seen in FITC preparations in red cells, collagen, and nuclei. The colours are unlike that of fluorescein and readily distinguished from those of the specific reactions.

Discussion

PARAFFIN SECTIONS

The difficulties involved in getting the large molecules used in immunohistochemical techniques to penetrate blocks of tissue are well recognised by electron microscopists (Kuhlmann *et al.*, 1974), but it seems often to be assumed that in sections intended for light microscopy reagents should have free access to the interior of the cells since these are cut open when the section is prepared. Consequently, when a section fails to react immunohistochemically, it is usually attributed to destruction of antigen by fixation and/or processing. The present study showed that such an assumption is unjustified, since even low concentrations of antigen (Ig) could be revealed in paraffin sections, previously unreactive, by treating them with trypsin. Fixation in the cross-linking fixatives, formaldehyde and glutaraldehyde, had therefore not destroyed the antigen but, possibly by preserving the cells' fine structure, had made the antigen inaccessible to the large molecules used in the immunohistochemical methods. Trypsin's ability to 'unmask' antigen could therefore depend on its increasing cell and tissue permeability. There is support for this view in the way in which the amount of antigen (Ig) detectable in, for example, plasma cells, increased as the time of exposure of the section to trypsin was extended, until it appeared to fill the cells. The enzyme could also act directly on the antigen to free cross-linked molecules. In earlier studies, made without the use of trypsin (Curran and Jones, 1977), we found that Ig was often localised

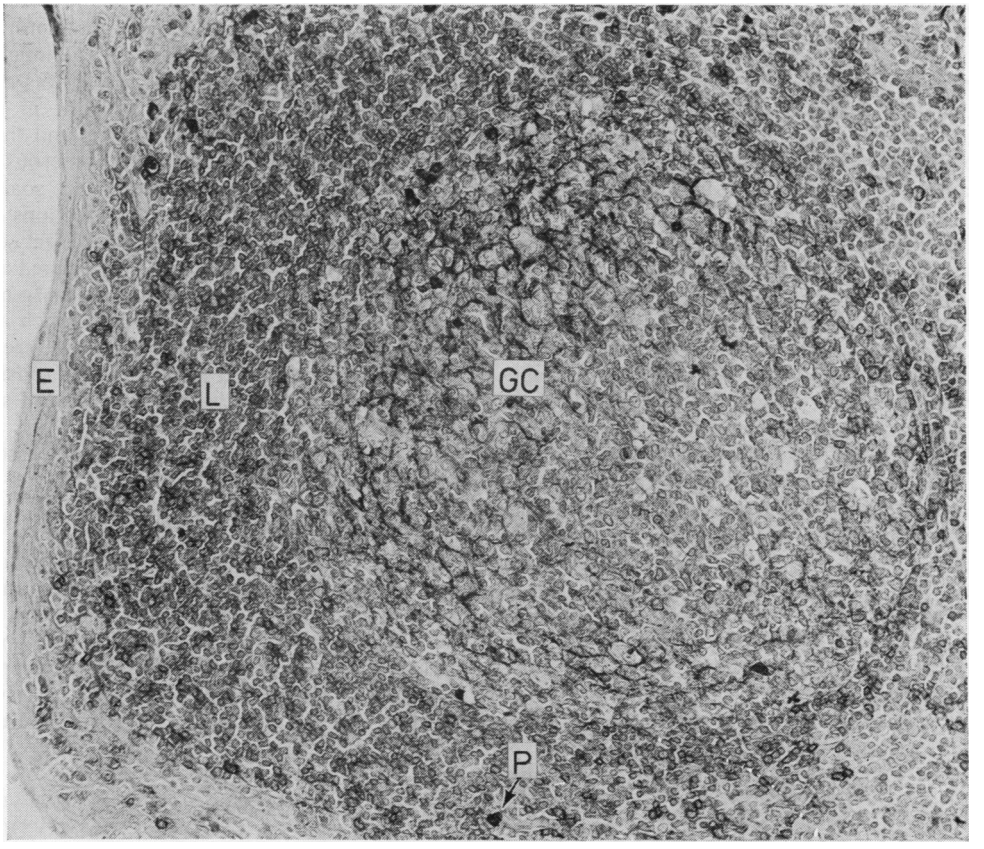

Fig. 4 *Lymphoid follicle and crypt epithelium (E) showing μ heavy chains (black) on the surface of the lymphocytes (L) surrounding the germinal centre (GC). μ heavy chains are also present in plasma cells (P) and extracellularly in the germinal centre. Trypsinised paraffin section. PAP method. × 230.*

to one part of a cell which we postulated might be the Golgi zone. The present results suggest, however, that it may be only that part of the cell not rendered unreactive by fixation. It is difficult to reconcile this idea with the fact that the large reagent molecules are able to penetrate the better fixed and unreactive peripheral part of the cell to reach the deeper reactive zone.

The increase in reactivity of paraffin sections after trypsin treatment was such that the FITC and PAP methods were able to reveal not only intracellular Ig (including δ heavy chains) more effectively in them than in cryostat sections but also μ heavy chains on the surface of the lymphocytes. However, δ heavy chains could not be demonstrated at this site, whereas in cryostat sections (unfixed or fixed in ether-ethanol), both μ and δ heavy chains were detectable as s-Ig on lymphocytes. The failure to show low concentrations of δ chains in the trypsinised paraffin sections is presumably the result of IgD's susceptibility to proteolysis (Jefferis and Matthews, 1977).

In a previous study (Curran and Jones, 1977), we showed that tissues fixed in unbuffered formaldehyde generally gave better results with the PAP method than those treated with fixatives suitable for electron microscopy, and postulated that the 'cruder' fixatives were more effective immunohistochemically because they preserved the cells' structure less well than the more refined fixatives and accordingly left them more permeable to large molecules. However, optimum preservation of the fine structure of cells and tissues by fixation is the ideal, and the present results show that by using trypsin to 'unmask' the antigens in well-fixed tissues, the opposing demands of histology and immunohistochemistry can be reconciled to a considerable degree. Pepsin, papain, and pronase did not give results comparable with

trypsin, and trypsin would appear to be the enzyme of choice for this purpose. The best and most reproducible results were obtained when the tissues were fixed in buffered 4% formaldehyde solution containing 2·5% sucrose; and tonsil being a delicate and highly cellular tissue, perfusion at 4°C by needle prior to slicing and immersion for 24 hours at 20°C in the same solution proved a useful additional step.

CRYOSTAT SECTIONS

Although we previously recommended fixation of unfixed cryostat sections before the DAB stage (Curran and Jones, 1977), it is possible to process well-cut cryostat sections through the PAP sequence without fixation at any stage and still retain reasonably good morphology. Fixation in formaldehyde, however, improves the histological appearances, and its application after step 3, when it has relatively little effect on the immunohistochemical response of the section, is generally worthwhile. If applied before step 3, it quickly abolished the reaction for s-Ig but prolonged fixation in formol-saline frequently enhanced the response for c-Ig. Glutaraldehyde is not recommended since it quickly rendered sections unreactive at any stage, except when applied before the DAB-peroxide stage.

The coagulative (precipitating) fixatives, ether-ethanol and methanol, had markedly different effects on cryostat sections from the cross-linking fixatives in that brief exposure to either of them generally strengthened the reactions for c-Ig and often those for s-Ig. Nevertheless the reactions for c-Ig remained weaker and less well-defined than in trypsinised paraffin sections. Presumably as with unbuffered formaldehyde's action on tissues (see above), these relatively 'crude' fixatives rendered the tissues and cells more permeable to large molecules and so more responsive immunohistochemically; and certainly the action of ether-ethanol and methanol was more marked when the antigen was intracellular than when it was on cell surfaces or extracellular.

EXTRACELLULAR Ig

Comparatively little information is available concerning 'extracellular' Ig. There appear to be considerable quantities of IgG in the wall of the intestine, and IgA and IgM are also abundant in inflamed tissues (Brandtzaeg, 1974). Much of this Ig is 'diffusible' and can be removed by soaking unfixed tissues in buffer at 4°C, thereby allowing Ig-containing cells to be counted more easily (Brandtzaeg *et al.*, 1974). Our results suggested that Ig, mostly IgG, is present in collagenous tissues, and the reduced strength of reaction in tissues that had been soaked in buffer before fixation supported this concept. Soaking also had the reverse effect of strengthening reactions for c-Ig, presumably by 'opening-up' the cells in some way. Unfortunately this beneficial effect was often accompanied by diffusion artefact, even when sucrose was added to the buffer. The 'corona' of Ig seen in cryostat sections around mature plasma cells and the 'zone' of reaction where these cells were numerous did not appear to consist of diffusible Ig, since soaking did not abolish either reaction. Both reactions were so consistently present that diffusion artefact seemed a much less likely cause than Ig associated in some way with the plasma cells. Extracellular Ig has been noted in lymphoid follicles in tissues such as colon (Baklien and Brandtzaeg, 1975), and was seen in the centres of the lymphoid follicles of the tonsil, being equally prominent in cryostat and paraffin sections. It too resisted prolonged soaking. This, and its pattern of distribution, suggest a close association with the surfaces of the cells there.

FREEZING-THAWING

Freezing followed by immediate thawing has been shown to make tissues more permeable and more reactive immunohistochemically (Nakane, 1975), but our results were disappointing and markedly inferior to those obtained with sections prepared in other ways. The explanation may be that whereas Nakane used fragments, our reagents were whole antibody molecules.

COMPARISON OF CRYOSTAT AND PARAFFIN SECTIONS AND OF FITC AND PAP METHODS

The present study showed that although the distribution of antigen (Ig) was broadly similar in cryostat and paraffin sections, paraffin sections demonstrated intracellular Ig more effectively than cryostat sections and conversely for surface Ig, μ and δ heavy chains being detectable on cells in cryostat sections but only μ chains in paraffin sections. The FITC and PAP procedures were equally effective in revealing antigen in cryostat and paraffin sections, and the distribution of antigen in each type of section was identical with both techniques. The difference in the molecular weights of the fluorescein and peroxidase molecules was therefore of no significance, the large size of the antibody molecule to which each is attached presumably being the dominant factor. Our results are in general accord with previous studies (Vladutiu *et al.*, 1973; Burns *et al.*, 1974; Pich *et al.*, 1976) and contrary to the view held by some histopathologists that FITC and peroxidase-based techniques differ in some way immunohistochemically. This view probably stems from the disparity in the sizes of the two 'labels' and from the fact that fluorescein-based techniques have more often been applied to unfixed cryostat sections and

peroxidase methods to paraffin sections of fixed tissue.

At comparable titres of the specific antibody (step 3), it was slightly easier to detect very low concentrations of Ig with the FITC method than with the PAP technique, for example, s-Ig on individual lymphocytes. The FITC method is less time-consuming, though no less expensive, than the PAP technique and proved useful as a screening method, positive results obtained with it being studied subsequently in more detail with the PAP technique, which was histologically much more informative. It certainly served this purpose better than a similar two-stage technique based on a peroxidase-labelled second stage. The latter was decidedly less sensitive than either of the two methods employed in this study and also had a consistently higher 'background' than the PAP method.

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