Technical method

Influence of decalcification on immunohistochemical staining of formalin-fixed paraffin-embedded tissue

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The pathologist often faces the situation where specimens are fixed and processed before it is known that immunohistochemical analysis may be required. It is now accepted that the adverse effects of routine formalin fixation and processing on the immunoreactivity of a variety of antigens can be overcome by digestion of tissue sections with proteases prior to immunostaining.[1-6] However some specimens containing hard tissue components must be decalcified, usually with dilute solutions of mineral or organic acids, prior to processing and sectioning. The effects of decalcification on the immunoreactivity of formalin fixed tissue has received little attention[7] even though compound fixatives containing acetic acid seem to be effective in preserving tissue antigens in a form detectable without enzyme treatment.[7-11] This paper describes a study to determine the influence of formic acid and acetic acid, two commonly used decalcifying agents, on the immunoreactivity of formalin-fixed paraffin-embedded tissue.

Materials and methods

Slices of palatine tonsil 2–3 mm thick were fixed in neutral buffered formalin for 18 h at room temperature. Fixed tissue was then either processed via ethanol (8 h) and xylene (8 h) to Ralwax (RA Lamb; 3 h) or "decalcified" prior to processing. Specimens were decalcified with 10% aqueous formic acid (6–120 h), 10% aqueous acetic acid (6–120 h), DECAL (Decal Chemical Corp, Pomona, NY; 18 and 42 h) or Cal-Ex (Fisher Scientific Co, Fair Lawn, New Jersey; 18 and 42 h) at room temperature with gentle agitation. After washing in running tap water (6 h) the treated specimens were processed to Ralwax.

Five micron sections were mounted on clean, adhesive-free slides and dried at 56°C for 45 min. Prior to immunostaining sections were dewaxed in xylene (3 x 2 min) and rehydrated through 740P ethanol (3 x 2 min) and 640P ethanol (1 x 2 min) to 0·05 *M* Tris-HCl buffered saline, pH 7·6 (TBS), which was used for trypsinisation and reagent dilution.

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The unlabelled antibody peroxidase-antiperoxidase (PAP) complex method was performed with and without prior trypsinisation of sections as described previously.[6,12]. The standard dilutions and times for each antiserum layer were as follows: rabbit antihuman IgG, IgA and IgM (1/1000), antihuman lysozyme (1/400) 60 min; swine antirabbit Ig (1/50) 30 min; PAP complex (1/50) 30 min. Occasionally the diaminobenzidine (DAB) reaction was performed in the presence of 0·01 *M* imidazole to enhance the colour reaction.[13] Controls included omission of the primary and secondary layers, replacement of the primary antiserum with normal rabbit serum and in some cases (IgG, IgA, IgM) specific inhibition by preincubation of antihuman protein antiserum with purified antigen. All antisera were obtained from Dakopatts As, Denmark.

Subjective assessment of the numbers of positive cells in follicles and extrafollicular areas together with preservation of histological structure were performed without prior knowledge of tissue and section treatment.

Results

The staining results for IgG obtained with formic acid treated tissue are summarised in the Table. Similar results were obtained for the other antigens tested (IgA, IgM, lysozyme) and do not require separate discussion. Formic acid-treated tissue showed a variable increase in the number of positive cells when stained without prior trypsinisation. As with non-acid treated tissue optimal staining with respect to numbers of positive cells and reproducibility of results was only obtained after trypsin digestion. However the formic acid-treated tissue was more susceptible to trypsin and required shorter periods of digestion to give maximal immunoreactivity with acceptable histology. All tissues, after optimal digestion, gave equivalent results in respect of numbers of positive cells, intensity of staining, detection of extracellular antigen, background staining of collagen and reticulin and, in the case of IgM, apparent cell surface staining of the lymphocytes forming the cap of each lymphoid follicle. Inclusion of imidazole in the DAB reaction mixture generally resulted in a more intense staining reaction but did not appear to increase the number of positive cells detectable in any staining run.

Acetic acid caused essentially similar changes in tissue immunoreactivity although susceptibility to trypsin digestion was less than that associated with formic acid.

Non-trypsinised sections of tissue treated with the commerical decalcifying fluids, DECAL and Cal-Ex, stained poorly. However tissue immunoreactivity could be recovered in a similar manner to that of formic acid treated specimens even though these agents, as tested, did not allow normal blue nuclear staining with Mayer's haematoxylin.

Influence of formic acid decalcification of PAP staining for IgG and preservation of histological structure with and without trypsin pretreatment of sections

Decalcification time (h)	**Trypsin time (min)*	*†Number of Ig containing cells: Extrafollicular*	*Follicle centre*	*‡Preservation of histological structure*
0	0	±	−	+++
	5	+	+	+++
	10	++	++	+++
	15	++	++	+++
	30	+++	+++	+++
6	0	+	+	+++
	5	++	++	+++
	10	+++	+++	+++
	15	+++	+++	+++
	30	+++	+++	++
12	0	+	+	+++
	5	++	++	+++
	10	+++	+++	+++
	15	+++	+++	+++
	30	+++	+++	++
24	0	+	+	+++
	5	+++	+++	+++
	10	+++	++	++
	15	++	+	++
	30	+	−	+
48	0	++	+	+++
	5	+++	++	+++
	10	+++	+++	++
	15	++	+++	++
	30	+	−	+
72	0	++	++	+++
	5	+++	+++	+++
	10	++	++	++
	15	+	−	+
	30	−	−	−
120	0	+	+	+++
	5	+++	+++	++
	10	++	+	+
	15	+	−	+
	30	−	−	−

*Trypsin digestion at room temperature.
† − no positive cells; + few, ++ moderate and +++ large numbers of positive cells.
‡ +++ no digestion of tissue; ++ less than 50% of cells digested; + greater than 50% of cells lost; − no cells remaining

Discussion

The results indicate that decalcification of tissues with 10% aqueous solutions of acetic and formic acids for periods up to five days after fixation in neutral formalin does not significantly alter immunoreactivity. This finding is in agreement with our experiences of staining routine specimens containing mineralised components (bone and tooth) which have been decalcified in 10% formic acid.[12,14] Indeed these results are not surprising as proteins such as immunoglobulin heavy and light chains can withstand treatment with 70% formic acid without greatly affecting their antigenicity.[15,16]

It is interesting to note that the adverse effects of fixation with neutral formalin were reversed, to a limited extent, by teatment with formic and acetic acid. Thus tissue immunoreactivity was marginally improved in the absence of enzyme digestion and maximal reactivity obtained with less "unmasking" by trypsin. However the full protective effect of acetic acid (low pH) on tissue antigens would seem to depend upon its presence during primary fixation[7–11] or post-fixation following a short period (4–8 h) in neutral formalin.[7,8]

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References

[1] Brozman M, Brozmanova E. Immunocytochemical methods to localise hormones in the human adenohypophysis. *Commun Czechoslov Soc Histochem Cytochem* 1966;**1**:25–30.

[2] Huang SH. Immunohistochemical demonstration of hepatitis B core and surface antigens in paraffin sections. *Lab Invest* 1975;**33**:88–95.

[3] Denk H, Radaszkiewicz T, Weirlich E. Pronase pretreatment of tissue sections enhances sensitivity of the unlabelled antibody enzyme (PAP) technique. *J Immunol Methods* 1977;**15**:163–7.

[4] Curran RC, Gregory J. The unmasking of antigens in paraffin sections of tissue by trypsin. *Experientia* 1977;**33**;1400–1.

[5] Mepham BL, Frater W, Mitchell BS. The use of proteolytic enzymes to

improve immunoglobulin staining by the PAP technique. *Histochem J* 1979;**11**:345–57.
[6] Matthews JB. Infuence of clearing agent on immunohistochemical staining of paraffin-embedded tissue. *J Clin Pathol* 1981;**34**:103–5.
[7] Banks PM. Diagnostic use of an immunoperoxidase method in hematopathology. *J Histochem Cytochem* 1979;**27**:1192–4.
[8] Caron BL, Banks PM. Acetic acid-formalin postfixation for routine tissue processing. *Lab Invest* 1979;**40**:244.
[9] Heyderman E. Immunoperoxidase techniques in histopathology: applications, methods and controls. *J Clin Pathol* 1979;**32**:971–8.
[10] Curran RC, Gregory J. Effects of fixation and processing on immunohistochemical demonstration of immunoglobulin in paraffin sections of tonsil and bone marrow. *J Clin Pathol* 1980;**33**:1047–57.
[11] Leatham A, Atkins N. Fixation and immunohistochemistry of lymphoid tissue. *J Clin Pathol* 1980;**33**:1010–2.
[12] Matthews JB, Basu MK. Plasma cell lesions within the oral tissues: Immunoperoxidase staining of routinely fixed and processed tissue. *Oral Surg* (in press).
[13] Straus W. Imidazole increases the sensitivity of the cytochemical reaction for peroxidase with diaminobenzidine at neutral pH. *J Histochem Cytochem* 1982;**30**:491–3.
[14] Matthews JB, Mason GI. Immunoglobulin producing cells in human periapical granulomas. *Br J Oral Surg* (in press).
[15] Matthews JB, Jefferis R. The effects of chemical modification on the antigenicity of a human kappa Bence Jones protein. *Immunochemistry* 1977;**14**:799–807.
[16] Jefferis R, Matthews JB, Bayley PM. Studies of human IgD myeloma proteins. Conformational changes induced in the Fc fragment on heating or exposure to acid pH. *Immunochemistry* 1978;**15**:19–25.

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Letters to the Editor

TPHA compared with cardiolipin tests for serological detection of early primary syphilis

The requirement for syphilis screening of blood before it is transfused continues to stimulate debate and was the subject of a review in *Vox Sanguinis* in 1981.[1] In this "International Forum" the advantages of *Treponema pallidum* haemagglutination (TPHA) over cardiolipin reagents were noted by several participants.

At the North London Transfusion Centre we have been using TPHA routinely for over two years. We use a modification which is both economical and sensitive.[2] Diluted reagents (Fujizoki, Diamed Diagnostics, Liverpool) are added to serum dilutions in microtitre plates. After centrifugation the plates are inclined at 70°. Positive samples form "buttons" of cells whereas negative samples form "streaks". Other laboratories have employed economical modifications in Terasaki plates.[3]

One criticism of TPHA tests has been that they may be less sensitive than cardiolipin reagents for detecting early primary syphilis, though they detect infections from many years ago; the latter are presumably less significant in the context of transmission by transfusion.

Serum samples from 15 patients with early primary syphilis were therefore collected for us by Dr Johnston at the Venereal Diseases Reference Laboratory, Whitechapel. Also serum from each of 10 rabbits inoculated with a treponemal suspension was obtained at 3, 7, 10, 14 and 21 days after inoculation. The rabbits were inoculated to provide TPI reagents and had been pre-tested to avoid any false-positive reactions due to rabbit syphilis, caused by *T cuniculi*.

The sera were tested by a cardiolipin test (RPR, Becton and Dickinson, Wembley) and by Fujizoki TPHA. TPHA titrations on the human sera were by standard as well as modified methods.

The three-day serum samples from all 10 rabbits were negative with cardiolipin and modified TPHA tests. However, by seven days (when orchitis is just beginning) and thereafter, all 10 were positive with both cardiolipin and modified TPHA tests. There was sufficient serum for standard Fujizoki TPHA testing from only two of the rabbits. The sera of both these rabbits were TPHA positive using the standard method by day 7.

Nine of the 15 human sera were positive by both cardiolipin and Fujizoki tests (standard or modified). The patterns of reaction for the remaining six sera are shown in the Table.

As a result of these studies (both in experimental animals and using human sera) we feel that the standard TPHA method has similar success in detecting early primary syphilis as the cardiolipin test. Furthermore, the modified TPHA is, if anything, slightly better for detecting early primary syphilis than the cardiolipin test and is certainly no worse.

We gratefully acknowledge the generous help of Dr NA Johnston of the Venereal Diseases

Pattern of reactions in six sera using cardiolipin and TPHA

Serum No	*Cardiolipin result*	*Standard Fujizoki TPHA*	*Modified Fujizoki TPHA*
1	±	−	±
2	±	−	±
3	−	−	±
4	±	−	+
5	±	+	+
6	−	+	+

± = weakly positive reaction.