Effect of decalcification agents on immunoreactivity of cellular antigens

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SUMMARY The effects of several strong acids, weak acids, a proprietary decalcifier, and edetic acid on the immunohistochemical staining of cryostat and fixed paraffin embedded sections of tissue from a variety of normal and pathological calcified and uncalcified specimens were studied. Even decalcification in strong acids (HCl, HNO_3 , 5% trichloroacetic acid, HCl-edetic acid did not diminish the reactivity of many useful antigens (including leucocyte common antigen, intermediate filaments, S100 protein and epithelial membrane antigen). Weaker acids (formic acid, acetic acid) and edetic acid decalcified more slowly and generally showed greater preservation of antigenic reactivity with better morphology and staining quality. Trichloroacetic acid was also useful as a quick one step fixation and decalcifying agent for both cryostat and routinely processed sections. Knowledge of the preservation of antigenic reactivity in decalcified tissue will be useful in the diagnosis of tumours of uncertain histogenesis and origin which affect calcified tissues.

Immunohistochemical analysis of surgical specimens using monoclonal antibodies has assumed an important role in histopathological diagnosis. Although frozen material and cryostat sections are optimal for the preservation of the reactivity of many antigens, routine formalin fixation and paraffin embedding, often associated with protease digestion of tissue sections prior to immunostaining, has been found to interfere only partially with the localisation of many cellular and tissue antigens useful in tissue diagnosis.¹⁻³ With calcified tissues, the situation is more complex as fixation is generally followed by a decalcification procedure. Although there are several decalcifying agents in common use, their effect on the immunoreactivity of the various cellular and tissue antigens has been little studied.^{4,5} This is often of particular importance where panels of monoclonal antibodies are used in the histopathological diagnosis of tumours of uncertain origin.⁶

In this study we examined the effect of several decalcifying agents, including strong acids, weak acids, and chelating agents on immunohistochemical staining for several diagnostically useful cellular and tissue antigens in both calcified and uncalcified surgical specimens.

Material and methods

Surgical specimens included normal appendix, thyroid, skin, breast, prostate, bone, and tonsil, as well as cases of carcinoma (lung, kidney), myeloma, and lymphoma affecting bone. Table 1 shows the antigenic determinants sought in each of these tissues.

The tissues were either snap frozen in liquid nitrogen for cryostat sectioning or fixed in 10% formol saline at room temperature for a minimum of 24 hours (range 24 hours-14 days). Slices of the fixed tissues were then routinely processed to paraffin wax. A parallel fixed slice was also placed in one of several decalcifying agents for 24 hours and three days. The agents studied were 5% aqueous nitric acid (HNO_3), 5% aqueous hydrochloric acid (HCl), 10% RD0 (Bethlehem, Gotheberg), 5% trichloroacetic acid (TCA), 10% aqueous formic acid (FA), 10% aqueous acetic acid (AA), and 10% HCl-edetic acid (ethylene diaminetetracetic acid) mixture. Specimens were also decalcified in edetic acid alone (24 hours and seven days), as well as being solely fixed and decalcified in TCA for 24 hours. After washing in running tap water for four to six hours the treated specimens were processed via ethanol (six hours) and xylene (three hours) to paraffin wax. Five micron sections were mounted on Multislide slides (Hendley, Essex) and dried at 60°C for 60 minutes. Before immunostaining, all paraffin sections were dewaxed in xylene and

Table 1 Antigenic determinants sought in surgical specimens examined

Specimen	Antigenic determinants sought
Appendix	Leucocyte common antigen (LCA), HLA-DR Light and heavy immunoglobulin chains (Ig's) Vimentin, S100 protein, desmin Factor VIII related protein Cytokeratin intermediate filaments Carcinoembryonic antigen (CEA) Epithelial membrane antigen (EMA) Ig's LCA Melanoma associated antigen (MAA), cytokeratins, S100
Tonsil	Thyroglobulin (Tgb)
Skin normal, compound naevus	EMA, cytokeratins
Thyroid	Prostatic acid phosphatase (PAP)
Breast	Prostate specific antigen (PSA)
Prostate	LCA, Ig's
Bone marrow	LCA, Ig's
osteoarthritic femoral head }	
Lymphoma } affecting bone	LCA, Ig's
Myeloma }	
Squamous cell carcinoma lung } affecting bone	EMA, cytokeratins
Adenocarcinoma lung }	
Adenocarcinoma kidney }	

rehydrated through graded alcohols to water. Parallel slices of appendix were also processed to cryostat sections after fixation and decalcification (24 hours) in the various agents, as noted above, to assess if specimens could be rapidly fixed and decalcified without destroying antigen reactivity.

Table 2 gives details of monoclonal antibodies used in this study. Polyclonal rabbit antibodies against human immunoglobulin heavy and light chains (Ig), thyroglobulin, and prostate specific antigen (PSA) were obtained from Dako (UK) and antiprostatic acid phosphatase (PAP) from Miles Scientific Ltd. Immunohistochemistry was performed using an indirect immunoperoxidase or alkaline phosphatase antialkaline phosphatase (APAAP) procedure, as previously described.¹⁷ Both control and decalcified sections of fixed tissue were digested by 0.1% trypsin (Sigma T: 8128) in 0.1% calcium chloride at pH 7.8 before immunostaining with the following antibodies: CR3/43, anti-light chain Ig, anti-heavy chain Ig, anti-carcinoembryonic antigen, anti-PSA, anti-PAP, anti-Tgb, and anti-factor VIII related antigen (30 minutes); CAM 5.2 (15 minutes); DER-11 (20 minutes). The histological quality, degree, and specificity of immunohistochemical staining was scored relative to that of fixed undecalcified control sections.

Results

Table 3 summarises the effect on the immunoreactivity of cellular antigens after treatment with the various decalcifying agents (figs 1 and 2). Prolonged (three day) decalcification produced no noticeable differences in terms of antigen reactivity or antibody specificity but had an effect on morphology and stain-

Table 2 Monoclonal and polyclonal* antibodies used for analysis of routinely processed decalcified tissues

Antibody	Antigenic determinant
PD7/26	Leucocyte common antigen ⁷
2B11	Leucocyte common antigen ⁷
CR3/43	HLA-DR ⁸
Anti-κ	Light and heavy immunological chains*
Anti-λ	
Anti IgG, IgM	
E29	Epithelial membrane antigen ⁹
CEA	Carcinoembryonic antigen ¹⁰
KL1	Cytokeratin intermediate filaments ¹¹
CAM 5.2	Cytokeratin intermediate filaments ¹²
S1-61	S100 protein ¹³
DER.11	Desmin ¹⁴
V9	Vimentin ¹⁵
NK1/C3	Melanoma associated antigen ¹⁶
Anti-PSA	Prostate specific antigen*
Anti-PAP	Prostatic acid phosphatase*
Anti-thyroglobulin	Thyroglobulin*
Anti-F VIII	Factor VIII related antigen ¹⁷

*Indicates polyclonal antibodies.

ing quality. Undecalcified controls that were processed to cryostat sections of formalin fixed paraffin embedded sections were all strongly positive with the antibodies tested.

Treatment with the strong acids HCl and HNO₃ and the proprietary decalcifier RD0 for 24 hours and three days preserved immunoreactivity of all antigens tested with the exception of HLA-DR and epithelial membrane antigen (EMA). Tissue morphology and staining quality was adversely affected, particularly after prolonged (three day) decalcification, but immunostaining was still interpretable. HCl-edetic acid treatment was associated with better morphology and weak EMA reactivity was retained. TCA treatment (both with and without prior formalin

Table 3 *Effect of decalcifying agents on immunoreactivity of tissue antigens**

Antigenic determinant	Undecalcified control	HCl	HN ₃	RD0	HCl-edetic acid	TCA†	AA	FA	Edetic acid‡
LCA	++	++	++	++	++	++	++	++	++
HLA-DR	++	—	—	—	++	++	++	++	++
Light Ig chain	++	+	+	+	++	++	++	++	++
Heavy Ig chain	++	+	+	+	++	++	++	++	++
Cytokeratins	++	++	++	++	++	++	++	++	++
CEA	++	—	—	—	—	—	++	++	++
Vimentin	++	+	+	+	++	++	++	++	++
S100 protein	++	+	+	+	++	++	++	++	++
FVIII	++	+	+	+	++	++	++	++	++
MAA	++	+	+	+	++	++	++	++	++
PAP	++	++	++	++	++	++	++	++	++
PAA	++	++	++	++	++	++	++	++	++
Tgb	++	++	++	++	++	++	++	++	++
Desmin	++	++	++	+	++	++	++	++	++
EMA	++	—	—	—	+	—	++	++	++

*Each tissue was decalcified for 24 hours and 3 days: the results were similar for both time periods.

++ = strong staining; + = weak staining.

†With and without formalin pre-fixation. ‡Also seven days of edetic acid decalcification.

Fig 1 *Squamous cell carcinoma of lung infiltrating bone: tumour cells are stained for cytokeratin intermediate filaments (CAM 5.2). (Immunoperoxidase, decalcification HCl-edetic acid 24 hours).*

Fig 2 *Lymphoma infiltrating bone (right) with tumour cells showing membrane staining for leucocyte common antigen (PD7/26). (Immunoperoxidase, decalcification TCA 24 hours).*

fixation) also showed good staining quality and preservation of both morphology and antigenic reactivity. No particular advantage in terms of immunohistochemistry was found in using the proprietary decalcifier (RD0) tested. It should also be noted that, although both anti-leucocyte common antigen antibodies tested, PD7/26 and 2B11, reacted with leucocytes after 24 hours in all decalcifying agents, 2B11 did not react with leucocytes after three days of decalcification in HCl, HNO_3 , RD0 and TCA.

Treatment with weak acids (FA, AA) and edetic for 24 hours and three days showed generally better staining quality and morphology in tissue sections, with preservation of the reactivity of most antigens tested. Decalcification of the bone specimens affected by tumour, however, was rarely complete after 24 hours and often still incomplete after three days.

To determine if tissues could be rapidly fixed and decalcified without destroying antigen reactivity and cell morphology cryostat sections of parallel slices of appendix were cut after fixation and decalcification for 24 hours in the agents listed (edetic acid, a slow decalcifying agent, excepted). Antigenic reactivity in cryostat sections was similar to that seen in the correspondingly decalcified routinely processed paraffin embedded sections. In addition, cryostat sections of TCA fixed and decalcified specimens of bone and appendix showed a similar pattern of antibody staining to that seen in material embedded in paraffin and previously treated with TCA. This indicates that TCA could be useful as a rapid one step fixation and decalcification agent for both cryostat and routinely processed sections. In immunoperoxidase stained cryostat sections background staining of red cells was strong with all decalcifying agents, even after blocking with methanol- H_2O_2 , and could potentially cause difficulty in interpretation. Background staining of red cells was also seen in immunoperoxidase stained paraffin sections but was not as pronounced. This effect was avoided by use of the APAAP technique.

Discussion

The selection of an appropriate decalcifying agent is largely governed by two factors: the amount of mineralised tissue present in the specimen; and the urgency of the specimen requiring decalcification. Subsequent staining and investigative procedures, which may need to be carried out on the decalcified specimen, are also important, however, and it is this last factor that we examined with respect to immunohistochemistry.

Even the most common rapid decalcification procedure in general use—that is, strong acids such as HCl, HNO_3 —did not lessen the reactivity of many diagnostically useful antigenic determinants. Cytological detail and staining quality were adversely

affected, particularly after prolonged decalcification in strong acids. Interpretation of immunohistochemical staining, however, was still clear, and most specimen blocks of bone should be decalcified within 24 hours in the above solutions. HCl-edetic acid also provided rapid decalcification with excellent morphology and good preservation of antigenic reactivity.

Treatment with TCA was useful as it provided a rapid one step fixation and decalcification procedure that led to retention of many useful antigenic components, as well as excellent morphology and staining quality. TCA decalcification was also suitable for use with cryostat and paraffin sections, allowing a quick tissue diagnosis to be obtained. Treatment with weak acids and edetic acid undoubtedly resulted in better morphological detail and staining quality, with greater retention of antigenic reactivity. These agents, however, are not suitable for urgent or larger specimens as decalcification of the bone specimens was often not complete within 24 hours.

Recognition of antigenic determinants in the decalcified specimens by the polyclonal and monoclonal antibodies studied should aid in the histological diagnosis of tumours of uncertain histogenesis and origin that affect calcified tissues.⁴ Antigenic determinants for tumours of lymphoid or haemopoietic (leucocyte common antigen, light and heavy immunoglobulin chain), epithelial (cytokeratins, epithelial membrane antigen), mesenchymal (vimentin, desmin, factor VIII related antigen), or other (MAA, S100) differentiation can be distinguished, even in specimens decalcified in strong acid solutions. In addition, antigenic components of tumours likely to metastasise to bone such as PSA, PAP-prostate,^{16 17} Tgb-thyroid,¹⁸ Carcinoembryonic antigen large bowel cancer¹⁰ can be recognised in decalcified tissue if the appropriate decalcifying agent is used. Knowledge of the effects of the various decalcifying agents on the immunoreactivity of cellular antigens should rationalise prospective and retrospective immunohistochemical analysis of tumours in calcified tissue.

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