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Tissue preparation for immunocytochemistry

J H Williams, B L Mepham, D H Wright

Abstract

***Aims*—To investigate the effect of tissue preparation on immunostaining and to establish whether there is a standard tissue preparation schedule that allows optimal demonstration of all antigens.**

***Methods*—Blocks of tonsil were subjected to variations to a standard fixation, processing, and section preparation schedule. The sections were stained with five antibodies—L26 (CD20), UCHL1 (CD45RO), CD3, vimentin, and anti-kappa light chain—using the streptavidin-biotin immunostaining technique. When further investigation was necessary, other tissues and antibodies were used and where weak immunostaining was obtained the use of microwave pretreatment to improve staining was tested.**

***Results*—Several factors involved in fixation were found to affect immunoreactivity. These included the duration, pH, and type of fixative used. In tissue processing only temperature and the duration of the dehydration and wax infiltration steps affected immunoreactivity. Of all the factors investigated, the temperature and duration of the section drying had the greatest effect. In contrast, long term storage of cut sections before immunostaining had no effect on the reactivity of the antibodies tested. Antibodies were found to be affected by alterations to tissue preparation by varying degrees, UCHL1 and vimentin being the most susceptible to changes in fixation and L26 to changes in processing. Where weak staining occurred, microwave pretreatment was generally found to eliminate the problem.**

***Conclusions*—There is no standard tissue preparation schedule for the optimal demonstration of all antigens. Factors involved in all aspects of tissue preparation can affect immunoreactivity, so it is important that precise details of the preparation schedule are given when reporting immunocytochemical studies, rather than using the general term "routinely fixed and processed".**

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University Department of Pathology, Southampton General Hospital, Southampton SO16 6YD, United Kingdom
J H Williams
B L Mepham
D H Wright

Correspondence to:
Mrs J H Williams.

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The most common cause of poor immunostaining in paraffin wax embedded tissue is not the technique itself but the alteration of the antigenic sites by the initial process of tissue preparation[1] which incorporates fixation, processing, and the preparation of the tissue sections. The establishment of a tissue preparation schedule for optimal immunostaining of all antigens has been a problem since immunocytochemical staining of paraffin embedded tissue was first developed.[2] Since that time several studies have been undertaken to investigate this problem, although these have mainly been concerned with fixation rather than with tissue processing or section preparation.

Tissue fixation has a significant influence on immunostaining as most antigens are altered during the process. Fixation involves a number of variables including the type, concentration, pH, and osmolarity of the fixative, and the duration and temperature of the fixation process; all of which can affect immunocytochemistry. Other factors reported to affect fixation and therefore possibly immunoreactivity are the volume of fixative,[3] delayed fixation,[4–8] or secondary fixation by alcohol—either during fixation by methanol which is added as a preservative to commercially prepared formalin[9] or in alcohol during processing if fixation is incomplete.[10–13]

In contrast to the number of studies on the effect of fixation on immunoreactivity, investigations into the effect of tissue processing have been limited; weak immunostaining because of poor tissue preparation being blamed on inadequate fixation rather than on other aspects of tissue preparation. It was reported in the late 1980s, shortly after the development of the enclosed tissue processor, that the use of this system could cause problems with both tissue and antigen preservation. This was either through carryover of reagents from one step to the next, in particular xylene from the wax cleansing cycle at the end of the programme into the first step of the cycle,[14–16] or through the use of inferior quality xylene during processing.[15] The type of reagent and the timing and temperature used for each step of processing (dehydration, clearing, and wax infiltration) have also been reported to affect immunoreactivity.[17–22] Other factors which can affect processing and therefore possibly immunoreactivity are the use of xylene as a substitute for water to make up graded alcohols,[23] and the application of vacuum during processing.[24]

No studies have exclusively investigated the effect on immunoreactivity of section preparation, which includes wax embedding, section cutting and drying, and subsequent section storage. It has been suggested that the use of lower drying temperatures or shorter times[25–29] and the avoidance of long term storage of cut sections can help preserve immunoreactivity.[30–32]

The introduction of antigen retrieval by heating tissue sections in a microwave[33] or pressure cooker[34 35] before immunostaining has been a major breakthrough in improving weak immunoreactivity, particularly in suboptimally prepared tissue.[12 34]

The aim of this study was to investigate all aspects of tissue fixation, processing, and section preparation and their effect on subsequent immunostaining, to establish a standard tissue preparation schedule which would allow optimal staining of all antigens. This was carried out using primary antibodies which from experience were known to be fixation or processing dependent to varying degrees.

Methods

TISSUE PREPARATION

Tonsils were obtained fresh from the operating theatre, cut into 2 mm slices, and subjected to a number of variations to a standard fixation, processing, and section preparation protocol. Each block was subjected to variation of only one of the three components of tissue preparation, the other two remaining standard. The standard tissue preparation protocol comprised fixation for 24 hours in 10% neutral buffered formalin (NBF) at room temperature in 50 ml of fixative, followed by processing on a 16 hour overnight schedule at ambient temperature on a Shandon Hypercenter 2 (Life Sciences International, Basingstoke, UK) (table 1). Paraffin sections were cut at 4 mm, mounted on 3-aminopropyltriethoxysilane coated slides, and dried at 37°C overnight.

Variations to fixation protocol

(1) Different types of fixative:
  (a) 10% NBF[36];
  (b) 10% formalin in tap water[36];
  (c) 10% formal saline[36];
  (d) 10% neutral buffered formalin with saline (NBFS);
  (e) 10% formal acetic[37];
  (f) 10% zinc formalin[38];
  (g) Carson's fixative[39];
  (h) Bouin's fixative[36];
  (i) B5.[40]
(2) Period of fixation: < 5, 5, 12, 24, 36, and 48 hours.
(3) Temperature of fixation: 18°C, 27°C, and 37°C.
(4) pH of 10% formalin: pH 3.0, 5.0, 7.0, 9.0, and 11.0.
(5) Delay before fixation: 0, 1, 2, 4, and 8 hours.
(6) Different volumes of formalin: ratio of tissue:formalin of 1:1 to 1:20.
(7) Addition of methanol to formalin: 0, 0.25, 0.5, 1, 1.5, 2, and 5%.

Variations to processing protocol

(1) Comparison between the carousel type and the enclosed vacuum processor.
(2) Comparison of different clearing agents with xylene:
  (a) chloroform;
  (b) Clearene (Surgipath, St Neots, UK);
  (c) xylene substitute (Shandon).
(3) Comparison of different paraffin waxes:
  (a) non-polymer (Ames Tissue Tek II, Bayer, Newbury, UK);
  (b) polymer (Ames Tissue Tek III, Bayer);
  (c) polymer + DMSO ("Paramat extra", Merck, Poole, UK);
  (d) microcrystalline (Fibrowax, Merck).
(4) Reduced and extended processing times for dehydration, clearing and wax infiltration.
(5) Temperature of processing: ambient and 45°C.
(6) Use of vacuum during all processing steps.
(7) Number of blocks and position of tissue in the carrier during processing.
(8) Xylene contamination of formalin.
(9) Comparison between repeated use of high and lower quality xylene for clearing.

Variations to section preparation protocol

(1) Dried at either 37°C overnight in a section drying oven, 60°C for 1, 4, or 8 hours and overnight in an incubator, or face down on a section drying hotplate at approximately 70°C for 0.5, 2, and 8 hours.
(2) Dried at 37°C overnight and stored for 1 day, 1 week, 1, 3, and 6 months at room temperature and 4°C before immunostain-

Table 1 Standard processing schedule

Station	*Reagent*	*Temperature*	*Vacuum*	*Time (h)*
2	70% alcohol	A	Y	1
3	90% alcohol	A	Y	1
4	100% alcohol	A	Y	0.5
5	100% alcohol	A	Y	1
6	100% alcohol	A	Y	2
7	100% alcohol	A	Y	2
8	Xylene:alcohol (50:50)	A	Y	1
9	Xylene	A	Y	1.5
10	Xylene	A	Y	1.5
11	Lambwax (RA Lamb, London, UK)	60°C	Y	1.5
12	Lambwax	60°C	Y	3
	Total processing time			16

A, ambient temperature.

Table 2 Main panel of primary antibodies used

Antibody	*CD No*	*Supplier*	*Cat No*	*Dilution*	*Pre-treatment*
L26	CD20	Dako	M755	1/1000	NT
UCHL1	CD45RO	Dako	M742	1/100	NT
CD3	CD3	Dako	A452	1/200	+T
Vimentin (3B4)	–	Dako	M7020	1/1000	+T
Kappa	–	Dako	A191	1/500	+T

NT, no pretreatment; +T, trypsin pretreatment.

Table 3 Additional antibodies used for section preparation experiments

Antibody	*Supplier*	*Cat No*	*Dilution*	*Pretreatment*
MIB1	Binding Site*	0505	1/1000	+MW
PC10	Gift†	–	1/800	+T
1D5	Dako	M7047	1/500	+MW
BCL-2	Dako	M887	1/150	+MW
αSMA	Dako	M851	1/2000	+MW
CEA	Dako	M7072	1/400	+T
Vimentin (V9)	Dako	M725	1/2000	+MW
Desmin	Dako	M760	1/500	NT

NT, no pretreatment; +T, trypsin pretreatment; +MW, microwave pretreatment.
*Birmingham, UK; †Kindly donated by David Lane, University of Dundee.

Table 4 Summary of immunostaining results after increasing fixation time in formalin

Antibody	<5 h*	5 h	12 h	24 h	36 h	48 h
L26	+++	+++	+++	+++	+++	+++
UCHL1	+++	+++	+++	++	++	+
CD3	++	++	+++	+++	+++	+++
Vimentin	+	+++	+++	+++	++	++
Kappa	-	++	+++	+++	+++	+++

*No trypsin digestion was used on sections fixed for < 5 hours as even very short pretreatment times of less than 30 seconds severely disrupted the tissue.

ing. The section cutting was staggered from six months to one day so that all the sections could be stained at the same time.

IMMUNOSTAINING

Sections from each experiment were stained with a panel of five primary antibodies (table 2). For the section preparation experiments additional tissues and antibodies were included (table 3). The primary antibodies were demonstrated using the StreptABC peroxidase (HRP) method as follows: sections were dewaxed and taken through to 70% alcohol before treating with 0.5% hydrogen peroxide for 10 minutes to inhibit endogenous peroxidase. Sections were then pretreated as required for each antibody (tables 2 and 3) and the trypsin time was adjusted for each section to take into account the type and period of fixation used. Sections were rinsed in Tris buffered saline (TBS) and incubated in primary antibody for the appropriate time, that is, polyclonal immunoglobulins, 30 minutes at room temperature; monoclonal and other polyclonal antibodies, overnight at 4°C. After a further rinse in TBS, sections were incubated in the relevant biotinylated second stage (Dako, High Wycombe, UK) for 30 minutes at room temperature, followed by incubation in StreptABC HRP complex (Dako) for the same period of time. The HRP was visualised by reacting with diaminobenzidine/H_2O_2 (Sigma Fast, Sigma, Poole, UK) for 10 minutes. After washing, the sections were counterstained in Harris haematoxylin, blued in tap water, dehydrated through alcohol to xylene, and mounted in DPX.

ASSESSMENT OF STAINING

The stained sections were examined independently by two of the authors (BLM and JHW) without prior knowledge of the section treatment. The two sets of results were then compared and any which differed by one grade or more were re-examined by the two assessors together. If there were any further discrepancies the slides were re-assessed by the third author (DHW). The grades for recording

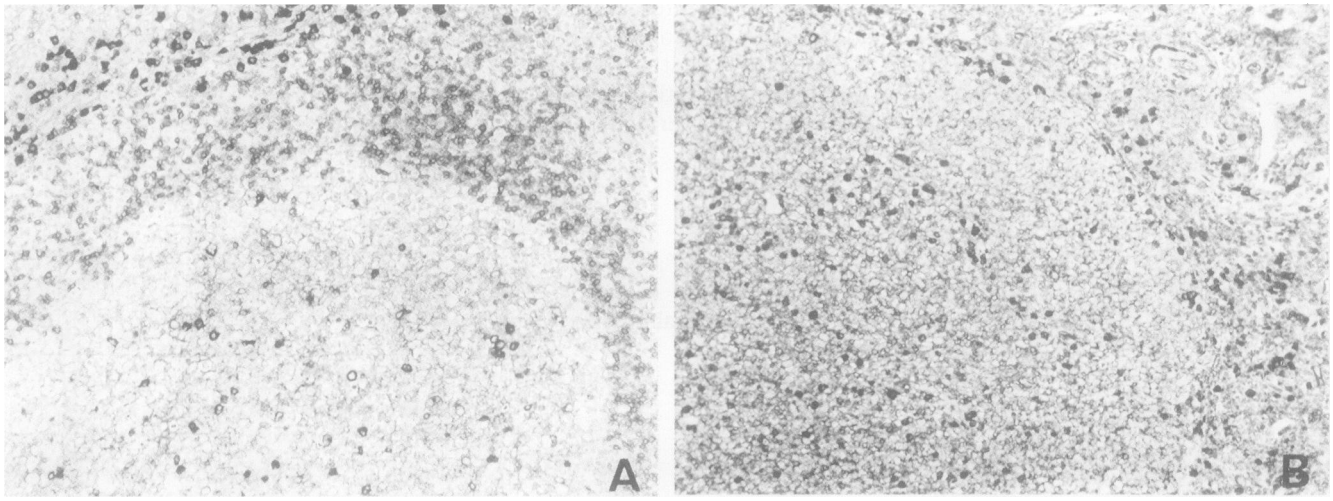

Figure 1 Sections of tonsil fixed in 10% NBF (A) and 10% formal acetic (B) stained for kappa light chain. Tissue fixed in 10% NBF shows staining of follicle centre cells, mantle cells, and plasma cells. Mantle cell staining is not seen in (B).

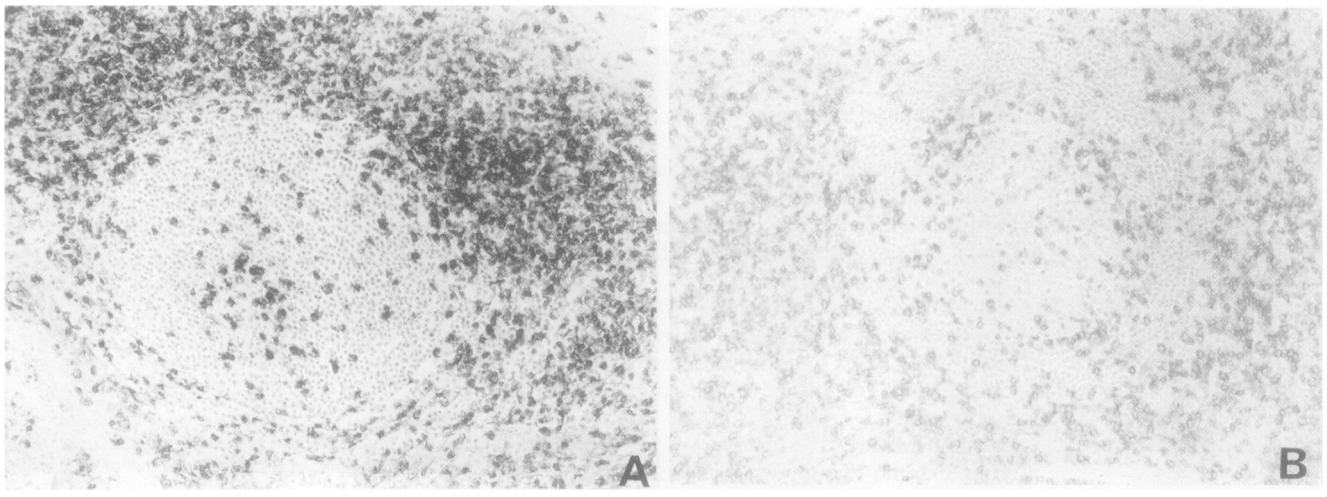

Figure 2 Sections of tonsil fixed for < 5 hours (A) and 48 hours (B) in 10% NBF and stained with UCHL1. Much stronger staining is seen with the shorter period of fixation.

Table 5 Summary of immunostaining results after varying the pH of formalin

Antibody	*pH 3.0*	*pH 5.0*	*pH 7.0*	*pH 9.0*	*pH 11.0*
L26	+++	+++	+++	+++	+++
UCHL1	+++	++	++	+	+
CD3	++	+++	+++	+++	++
Vimentin	+++	++	++	+	+
Kappa	+++	+++	+++	+++	++

intensity of specific staining were as follows: > +++, excellent; +++, very good; ++, moderate; +, weak; +/−, very weak; −, no staining.

USE OF MICROWAVE PRETREATMENT

Where weak staining was obtained with antibodies not usually requiring heat retrieval, the staining was repeated using microwave pretreatment.

Results

FIXATION

Several of the factors involved in the process of fixation were found to affect immunoreactivity of some of the antibodies tested. These included the type, duration, and pH of the fixative. Of the types of fixatives tested, 10% formal saline, 10% NBF (except UCHL1), and 10% zinc formalin (except CD3) gave the most consistent results overall and showed excellent antigen preservation. Of those recommended for immunocytochemistry, 10% formal acetic (fig 1), B5, and Bouin's fixative all showed poor antigen preservation. The use of B5 fixative resulted in high levels of background staining. This was not improved by the use of microwave pretreatment. Staining after Bouin's fixation was weak, although the use of microwaving did enhance staining with L26 and UCHL1 but not with CD3 and vimentin. Only weak immunostaining was obtained after using both the traditionally used fixatives—10% NBFS and 10% formalin in tap water—and the less commonly used Carson's fixative.

The period of fixation (table 4; figs 2 and 3) and the pH of formalin (table 5; fig 4) also significantly affected the immunoreactivity of some of the antigens. UCHL1 and vimentin were particularly susceptible to both longer fixation times and higher pH. In contrast, the demonstration of both kappa and CD3 improved with increasing fixation times but was less affected by changing the pH.

The other fixation factors tested—delay before fixation, volume of fixative, and the

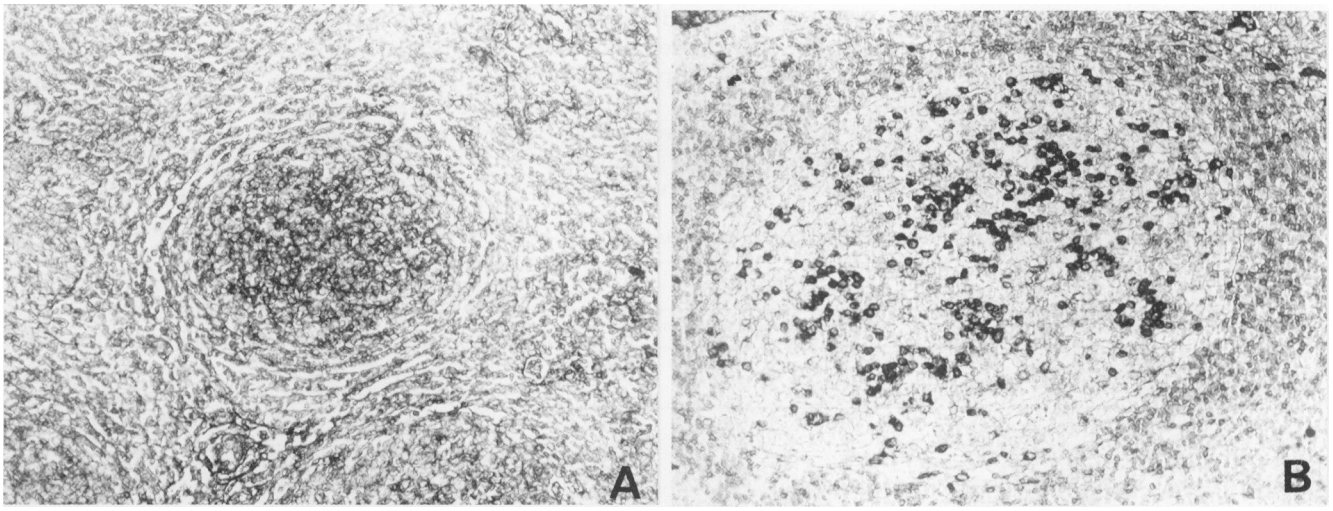

Figure 3 Sections of tonsil fixed for < 5 hours (A) and 48 hours (B) in 10% NBF and stained with kappa light chain. Tissue fixed for < 5 hours shows poor staining and appears overdigested, even though no trypsin pretreatment was used; 48 hours fixation results in clear staining of follicle centre cells, mantle cells, and plasma cells.

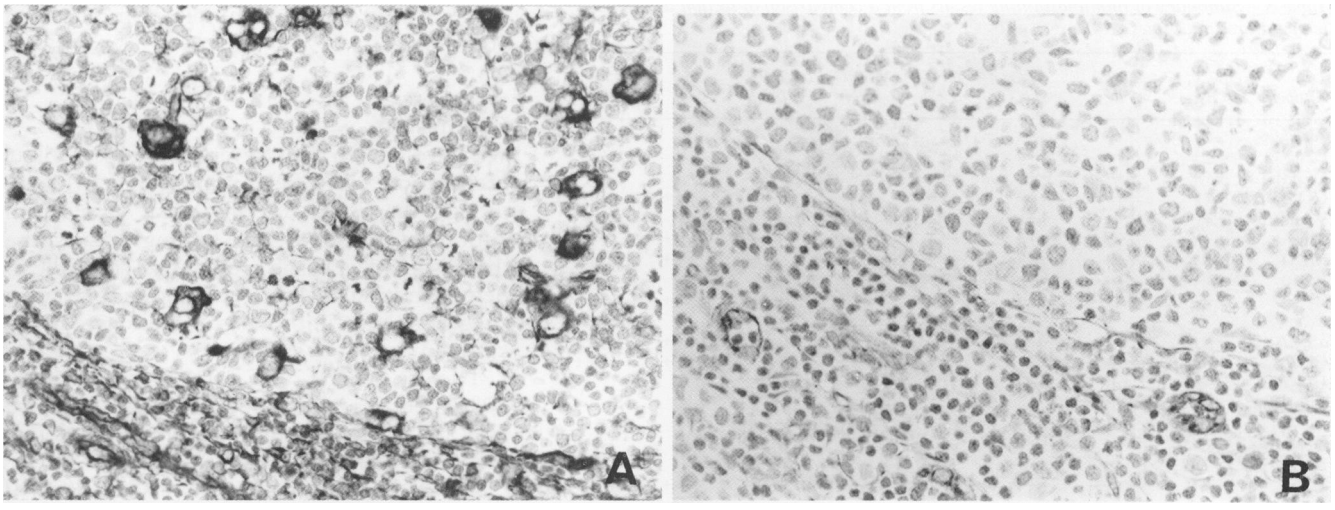

Figure 4 Sections of tonsil fixed in 10% formalin at pH 3.0 (A) and pH 11.0 (B) and stained with vimentin. The staining in (B) is clearly much weaker than in (A).

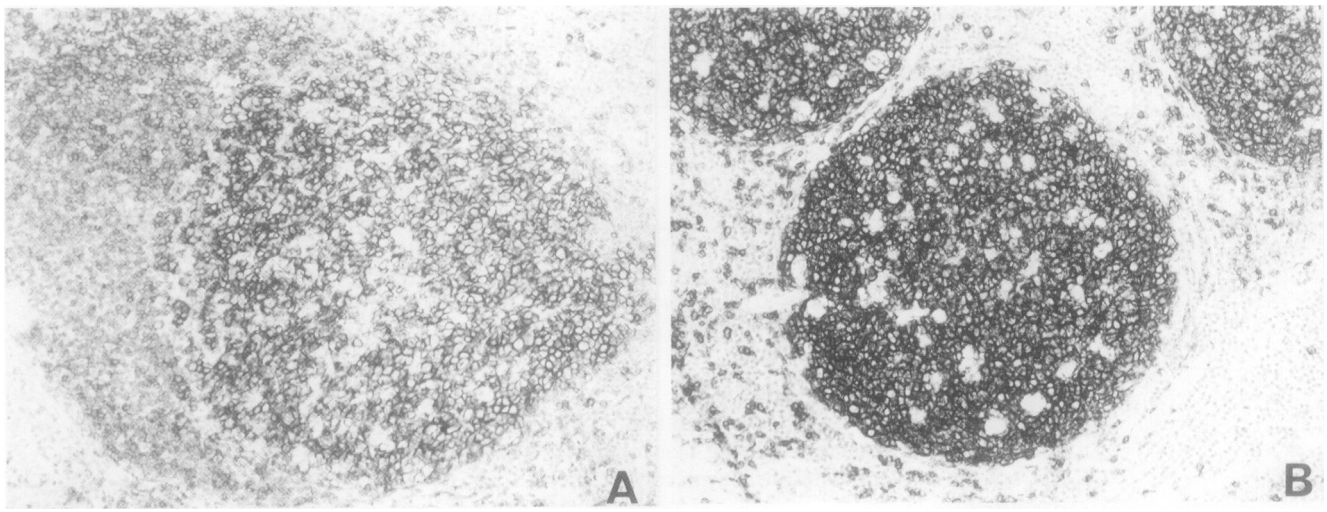

Figure 5 Sections of tonsil processed at ambient temperature (A) and at 45°C (B) and stained with L26. The higher processing temperature gives stronger staining of B cells.

Table 6 Summary of immunostaining results from comparison of processing temperatures

Antibody	*Ambient*	*45°C*
L26	+++	>+++
UCHL1	+++	>+++
CD3	+++	>+++
Vimentin	++	+++
Kappa	++	++

addition of methanol to formalin—had no effect. Increasing the temperature of fixation was generally found to produce the same results as increasing the time in fixative.

PROCESSING

Increasing the processing temperature from ambient to 45°C for all stages up to the wax infiltration step (table 6; fig 5) and increasing the duration of both the dehydration and wax infiltration stages (table 7) improved the immunostaining of most of the antibodies tested. None of the other factors investigated—the type of processor, type and quality of reagent, use of vacuum, xylene contamination of formalin, length of clearing stage, and the number and position of the tissue in the carrier—had any effect.

Table 7 Summary of immunostaining from comparison of different processing times

	Dehydration			*Wax infiltration*		
	Total dehydration times			*Total wax infiltration times*		
Antibody	*Reduced (2 h)*	*Standard (7.5 h)*	*Extended (10 h)*	*Reduced (2 h)*	*Standard (4 h)*	*Extended (8 h)*
L26	++	+++	>+++	+	++	+++
UCHL1	+++	+++	+++	++	++	+++
CD3	+++	+++	+++	++	++	++
Vimentin	+++	+++	+++	++	++	++
Kappa	++	+++	+++	++	++	++

Table 8 Summary of immunostaining results from a comparison of section drying temperatures and times

Antibody	*37°C ON*	*60°C 1 h*	*60°C 4 h*	*60°C 8 h*	*60°C ON*	*HP 0.5 h*	*HP 2 h*	*HP 8 h*
Tonsil								
L26	+++	++	++	++	+	+	+	+
UCHL1	+++	+++	++	++	++	+/–	+/–	–
CD3*	+++	+++	+++	+++	+++	+++	+++	+++
Vimentin	++	++	++	++	++	++	++	++
Kappa	+++	+++	+++	++	++	+++	+++	+++
MIB1	+++	+++	+++	+++	+++	+++	+++	+++
PC10	+++	+++	+++	+++	++	+	+	–
BCL-2	+++	+++	+++	+++	+++	+++	+++	+++
Colon								
αSMA	+++	+++	+++	+++	+++	+++	+++	++
CEA	+++	+++	+++	+++	+++	+++	+++	+++
Uterus								
Vimentin (V9)	+++	+++	+++	+++	+++	+++	++	+
Desmin	+++	+++	++	++	+/–	+	+	–
Breast								
1D5	+++	+++	+++	+++	+++	+++	+++	+

ON, overnight; HP, hotplate at 70°C.
*Background staining increased with increasing temperature/time.

SECTION PREPARATION

The temperature and duration of section drying was found to affect immunoreactivity of several of the antibodies tested (table 8; figs 6 and 7). In contrast with section drying it was found that, with the exception of desmin, the immunoreactivity of all antigens was unaffected in sections stored at room temperature or at +4°C for up to six months after cutting.

USE OF MICROWAVE PRETREATMENT

The use of microwave pretreatment was found to reduce or eliminate most of the weak staining occurring in this study: it improved immunostaining of L26 and UCHL1 in Bouin's fixed tissue; it improved CD3 staining in tissue fixed for less than five hours; and it significantly improved immunostaining with UCHL1 and L26 in sections dried at higher temperatures and for longer periods.

The only exceptions were: immunostaining of all antibodies in B5 fixed tissue; CD3 and vimentin staining in Bouin's fixed tissue; vimentin staining in tissue fixed for less than five hours; and staining of most antibodies if sections were dried on a hotplate for more than 30 minutes.

Discussion

We have shown that several factors in tissue preparation can affect immunoreactivity, although the extent of this effect varies from antibody to antibody. The findings suggest that there is no ideal tissue preparation schedule for demonstrating all antigens, although various suggestions for improving immunocytochemical staining can be made.

The use of 10% NBF, 10% zinc formalin, or 10% formal saline is recommended, particularly for demonstrating kappa light chains on mantle and follicle centre cells.

A fixation time of 12 hours in 10% NBF showed optimum staining of all antigens, but as such a defined time does not allow for variation

of tissue type and composition and for the day to day working of the laboratory, a fixation time of between 12 and 24 hours is recommended.

The pH of formalin affected different antigens to varying degrees. A highly acidic formalin of pH 3.0 produced the best immunostaining but in our experience at the expense of morphology. The use of formalin at pH 5.0 is therefore recommended giving good morphology and immunoreactivity.

Generally the other factors involved in fixation had little effect on the intensity of immunostaining provided that the recommended type, time, and pH of fixative were used.

Of the nine tissue processing factors investigated, only two had any significant effect on immunoreactivity. Increasing the temperature of processing from ambient to 45°C, and longer processing times for dehydration and wax infiltration were both found to improve immunostaining. It is therefore recommended that wherever possible longer processing times at higher temperatures should be used, and the use of a short daytime schedule should be avoided for immunocytochemical studies. We found no effect of varying the other tissue processing factors, including the type of processor, type and quality of reagents, time in clearing agent, and use of vacuum, most of which have been suggested as possible causes of poor processing.[14–17 19]

The conditions of section preparation have mainly been ignored, but we have shown that one of the fundamental steps of section preparation—section drying—can have a significant effect on immunoreactivity. It is recommended that sections, regardless of the antigen to be demonstrated, should be dried overnight at 37°C. If they are required more rapidly, then drying at 60°C for up to four hours is acceptable, particularly if followed by microwave pretreatment. The use of a hotplate (70°C) for section drying should be avoided as the staining with several of the antibodies tested was reduced and could not be improved by microwave pretreatment. In contrast to the findings of others,[30–32] this study has shown that with the exception of desmin (clone D33) all antibodies stained strongly in sections cut and stored for up to six months at either room temperature or +4°C.

It has been stated that heat pretreatment enhances immunoreactivity and evens out irregular staining due to inadequate tissue fixation or processing,[12 34] and this is supported by the findings of this study. It should be noted that microwave pretreatment does not always

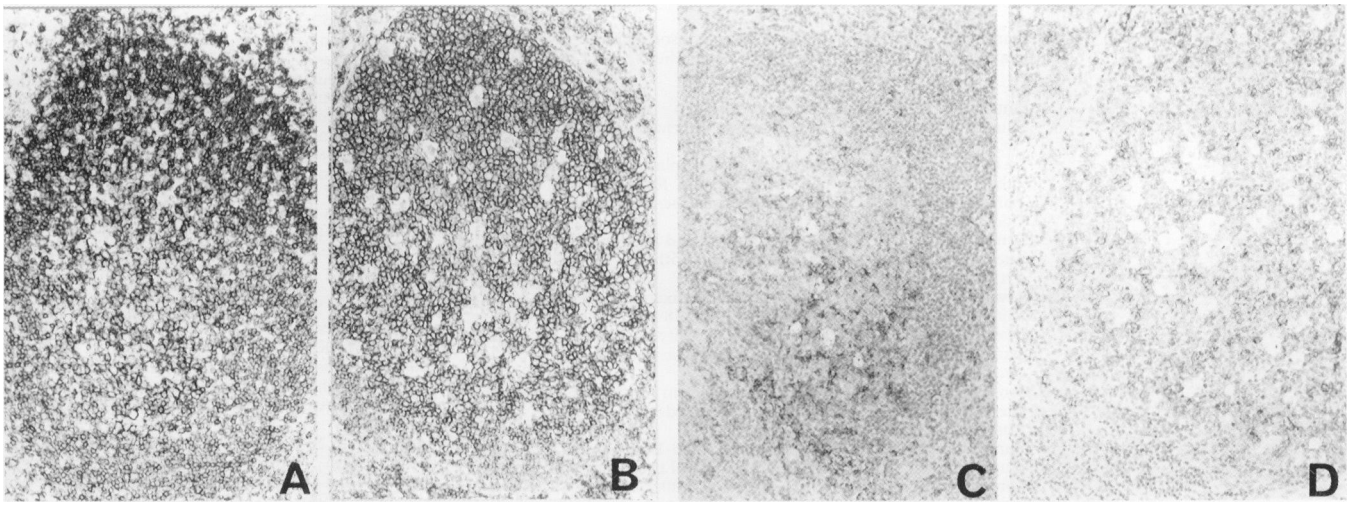

Figure 6 Sections of tonsil stained with L26 following section drying at 37°C overnight (A), 60°C for 4 hours (B), 60°C overnight (C), and on a hotplate for 30 minutes (D). The results show the adverse effect of drying sections at high temperatures on a hotplate or for long periods at 60°C.

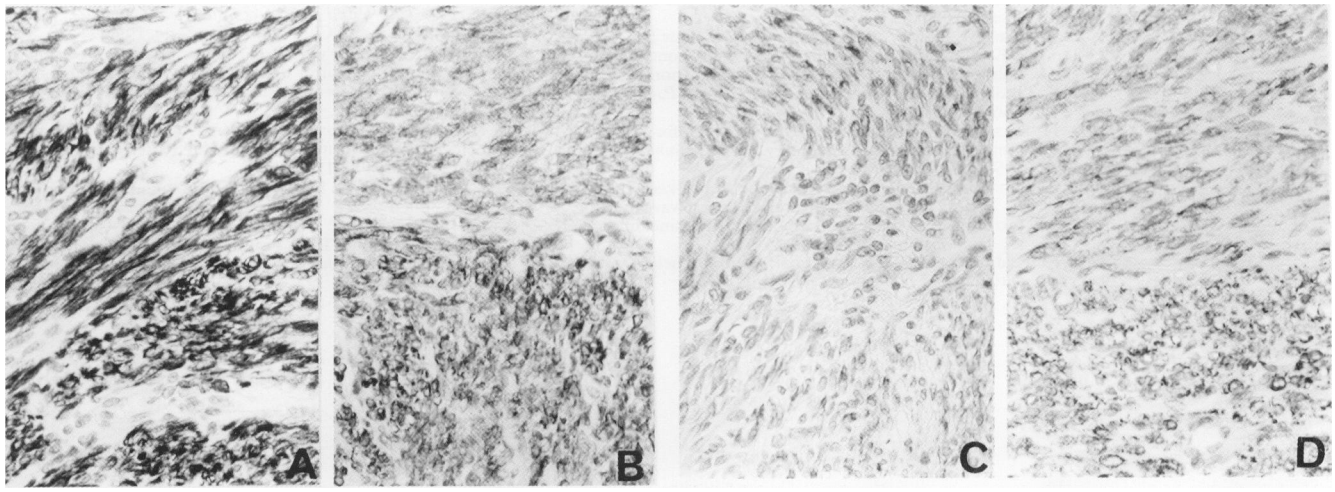

Figure 7 Sections of myometrium stained for desmin following section drying at 37°C overnight (A), 60°C for 4 hours (B), 60°C overnight (C), and on a hotplate for 30 minutes (D). The adverse effect of high section drying temperatures is clearly shown.

overcome the problem–for example, after the use of high section drying temperatures. It is therefore suggested that "prevention is better than a cure", in that it is preferable to minimise the chance of weak staining by using an optimised fixation and processing schedule rather than producing sections which have to be heat pretreated for immunostaining to be achieved.

Several of our findings are contrary to those reported by others and this can be explained by the variable response of different antigens to the effects of tissue preparation. We therefore conclude that there is no standard universal tissue preparation schedule for the optimal demonstration of *all* antigens, although the immunostaining of the majority of antigens can be optimised by following the recommendations given in this report.

The results of this study have shown the importance of resisting the pressure for shorter preparation times for tissue requiring immunocytochemistry, as increased processing and section drying times enhances immunoreactivity. The results have also emphasised the importance of including precise details of tissue preparation when reporting immunocytochemical findings rather than using the commonly used term "routinely fixed and processed."

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