Intracellular immunoglobulins

A comparative study on three standard tissue processing methods using horseradish peroxidase and fluorochrome conjugates

J. BURNS, MARIA HAMBRIDGE, AND C. R. TAYLOR

From the Gibson Laboratories, Radcliffe Infirmary, Oxford

SYNOPSIS Formalin fixation followed by routine paraffin embedding allows the demonstration of intracellular immunoglobulin by a sandwich technique using either peroxidase or fluorescein isothiocyanate labelled antibody conjugates. The results compared favourably with those obtained using either the fresh frozen or the Sainte-Marie method for preserving tissue immunoglobulins. The formalin-paraffin method with peroxidase-labelled antibody has advantages for routine use. Morphology is excellent, preparations are permanent, and retrospective studies of stored paraffin-embedded tissue are possible.

Some of the problems of labelled antibody studies are discussed.

Immunological studies on tissue sections are usually performed on fresh frozen unfixed tissue or cold alcohol-fixed, paraffin-embedded material (Sainte-Marie, 1962; Heron, 1970).

Routine surgical material, however, is almost invariably received in formalin or has been previously fixed and embedded in paraffin wax. In these circumstances the requirement for an immunological study only becomes apparent after microscopic examination of the paraffin sections, at which time fresh unfixed tissue is not available.

A procedure to demonstrate the presence of intracellular immunoglobulins in routine formalin-fixed and paraffin-embedded tissues has recently been described (Taylor and Burns, 1974). The present study is an extension of this work and its main purpose is to compare the formalin-paraffin method with established procedures such as the Sainte-Marie and cryostat methods. These latter two techniques involve the use of fluorescein isothiocyanate (FITC)-labelled antibodies to demonstrate the intra-

cellular immunoglobulins. A direct comparison of the specificity and sensitivity of FITC and peroxidase-labelled antibody preparations is included in the design of the study.

Materials and Methods

Four surgical specimens of fresh spleen and one of fresh lymph node (table I), were sliced and three adjacent blocks from each case were processed as follows: (1) quenched in liquid nitrogen, sectioned by cryostat and stored in 2-octanol (Maxwell, Ward, and Nairn, 1966); (2) fixed in cold absolute alcohol and paraffin embedded according to the method of Sainte-Marie (1962); (3) fixed in formalin and paraffin embedded using the routine laboratory procedure (see Taylor and Burns, 1974).

Serial 5 μ sections were taken from each block and examined for the presence of intracellular immunoglobulins by a sandwich technique using peroxidase- or FITC-labelled antibody. The experimental design is summarized in table II.

<i>Radcliffe Infirmary Surgical Number</i>	<i>Diagnosis</i>	<i>Tissue Embedded</i>
4748/72	Hodgkin's disease	Spleen, histologically involved
6404/72	Hodgkin's disease	Spleen, reactive changes only
6406/72	Hodgkin's disease	Spleen, reactive changes only
441/73	Lymphosarcoma	Spleen, histologically involved
10063/72	Hodgkin's disease	Lymph node, histologically involved

Table I *Details of the five surgical specimens from which blocks were taken for the comparative study*

Preparation	Consecutive Sections	
	Pretreatment	RaHuman Immunoglobulin ¹
Cryostat	a None	K ²
	b α -N-pyronin ⁴	L 1/10, 1/0, 1/40
	c Methanol-H ₂ O ₂ ⁴	M
Sainte-Marie	a None	K
	b α -N-pyronin ⁴	L 1/10
	c Methanol-H ₂ O ₂ ⁴	
Formalin-paraffin	a None	M
	b α -N-pyronin ⁴	
	c Methanol-H ₂ O ₂ ⁴	

Table II Summary of study design and procedure in each of the five cases (see text)

¹Appropriate saline controls (Taylor and Burns, 1974)

²Swine antirabbit IgG antibody conjugated to peroxidase (SWAR/P) plus diaminobenzidine reaction followed by haematoxylin counterstain and examined by orthodox light microscopy.

³Swine antirabbit IgG antibody conjugated to fluorescein isothiocyanate (SWAR/F), examined for fluorescence (see text).

⁴Blocking sequence not necessary with SWAR/F preparations.

⁵Kappa (K); Lambda (L); M (μ heavy chain).

Formalin-fixed sections were stained for intracellular immunoglobulin by the basic procedure of Taylor and Burns (1974), namely, sections were first treated with specific rabbit antihuman immunoglobulin antibodies at a dilution of 1/10 for one hour. (Anti-Kappa (K), anti-Lambda (L), and anti-M were chosen to demonstrate different proportions of plasma cells in parallel sections, the low proportion staining with anti-M also serving as one of the control preparations. Anti-G and anti-A also produced satisfactory results in pilot studies, but were not included in the main comparative study in order to restrict the number of slides to manageable proportions.)

After washing in phosphate-buffered saline (PBS) the sites of specific antibody binding were identified using a peroxidase-conjugated swine antibody directed against rabbit IgG (SWAR/P), followed by washing in PBS and staining with the diaminobenzidine (DAB) reaction. In the first series (a in table II) endogenous peroxidase activity was not blocked by pretreatment. In a second series (b in table II) granulocyte peroxidase was 'blocked' using α -naphthol pyronin (α -N-pyronin) (Taylor and Burns, 1974) and in the third series (c in table II) all endogenous peroxidase activity was blocked using the methanol-H₂O₂ procedure advocated by Streefkerk (1972).

Cryostat and Sainte-Marie sections were treated with the same antibody preparations in parallel with the formalin-paraffin sections. All peroxidase sections were counterstained with a modified Harris haematoxylin (Lie, Holley, Kampa, and Titus, 1971), mounted in DPX, and examined by orthodox light microscopy. The contrast of the positive peroxidase reaction could be enhanced by viewing the sections using a Tri-blue filter or a Leitz KP 490 FITC interference filter.

A further series of sections was treated in an identical way, but sandwiched with swine antirabbit immunoglobulin labelled with FITC (SWAR/F). In this series blocking reactions were not routinely employed. All FITC preparations were mounted in 80% buffered glycerol and examined for specific fluorescence using a Leitz Ortholux quartz iodine fluorescence microscope system incorporating a Tiyoda superwide dark field oil substage condenser with a KP 490 FITC filter and a K 530 suppression filter. A BG 38 red suppression filter was added to the system for photomicrographs which were taken

on GAF Anscochrome (Daylight ASA 200) 24 Din colour film.

Fluorescein-isothiocyanate and peroxidase preparations were scored independently by two observers on an arbitrary scale (+ occasional strongly positive cell, + + + numerous) to provide some assessment of the relative sensitivity and specificity of the different methods.

Swine antirabbit serum IgG conjugated with horseradish peroxidase (SWAR/P) was obtained from Dakopatts (Mercia Ltd, Sandown Road, Watford, England). Fluorescein-isothiocyanate-conjugated swine antirabbit immunoglobulin (SWAR/F) was obtained from Nordic Pharmaceuticals (Tilburg, Netherlands). Unconjugated rabbit antihuman kappa (K) and lambda (L) light chains (Bence Jones) were also obtained from Dakopatts, and rabbit antihuman IgM (heavy chain) from Behringwerke AG, Germany. Extensive control preparations were used as previously reported (Taylor and Burns, 1974).

Black and white photomicrographs were obtained using a Tri-blue filter to reduce the intensity of exposure of the haematoxylin counter stain so enhancing contrast for the purposes of photography. This, however, obscured the fine morphological detail visible on microscopy.

Results

These are summarized in table III.

PEROXIDASE-LABELLED ANTIBODY (SWAR/P)

Formalin-paraffin (figs 1, 2, 3, and 4)

Plasma cells were most clearly and precisely identified in the formalin-paraffin processed material. The final reaction was intense and provided a sharp contrast with surrounding negative cells. The simultaneous development of endogenous peroxidase in red cells and granulocytes (fig 4) presented some problems in rapid assessment of plasma cell numbers.

All photomicrographs are from one case (4748/72) and have been selected to illustrate the results summarized in table III.

Fig 1

Fig 2

Fig 1¹ Formalin-paraffin section. Methanol-H₂O₂ blocked. Anti-K. SWAR/P. Strongly positive cells with Hodgkin giant cell (arrowed). Morphology excellent in haematoxylin counterstained preparations. × 551.

Fig 2¹ Formalin-paraffin section. Methanol-H₂O₂ blocked. Anti-K. SWAR/P. Strongly positive Hodgkin giant cell. × 551.

¹The fine morphological detail of the formalin-paraffin peroxidase preparations is obscured by the Tri-blue filter used in preparing these black and white photomicrographs, therefore see colour plate in Taylor and Burns (1974).

Preparation	FITC-labelled Antibody Method	Peroxidase-labelled Antibody Method
Cryostat	Moderate variable intensity, poor definition (fig 9) ¹ Not prominent ²	Moderate intensity, poor definition (fig 6) ¹ Granulocytes strong, blocked by methanol-H ₂ O ₂ and by α-N-pyronin. Red cells weaker ²
	Collagen variable, diffuse background (reduced at 1/40 see text); Poor, unstained cells not classifiable ⁴	Collagen variable, diffuse background (little improvement at 1/40—see text) ³ Poor for positive and negative cells ⁴
Sainte-Marie	Strong intensity, moderate definition (fig 8) ¹ Not prominent. Eosinophils autofluorescent ²	Moderate intensity and definition (fig 5) ¹ Granulocytes strong, blocked by methanol-H ₂ O ₂ and by α-N-pyronin. Red cells weak ²
	Collagen variable, reticulin strong intensity ³ Poor, unstained cells not classifiable ⁴	Collagen variable, reticulin strong intensity ³ Moderate. Identification uncertain for some cell types ⁴
Formalin-paraffin	Strong intensity, moderate definition (fig 7) ¹ Not prominent ²	Strong intensity, good definition (figs 1-4) ¹ Strong intensity (fig 4), both blocked by methanol-H ₂ O ₂ (figs 1 & 2) but only granulocytes blocked by α-N-pyronin (fig 3) ²
	Collagen variable low intensity ³ Poor, unstained cells not classifiable ⁴	Collagen variable low intensity ³ Excellent. Ready cell identification ^{4,5}

Table III Summary of principal findings

¹Plasma cells

²Red blood cells and granulocytes

³Collagen, reticulin, and background

⁴General morphology

⁵See colour plate (fig 1) in Taylor and Burns (1974)

Fig 3

Fig 3¹ Formalin-paraffin section. α -N-pyronin blocked. Anti-K. SWAR/P. Red cells show weak positive reaction (arrowed). $\times 551$.

Fig 4

Fig 4¹ Formalin-paraffin section. Not blocked. Anti-K. SWAR/P. Red cells strongly positive, also granulocytes. Plasma cell arrowed. $\times 551$.

¹The fine morphological detail of the formalin-paraffin peroxidase preparations is obscured by the Tri-blue filter used in preparing these black and white photomicrographs, therefore see colour plate in Taylor and Burns (1974).

The prior use of α -N-pyronin to counterstain granulocytes (fig 3) was of considerable value (Taylor and Burns, 1974) and the methanol-H₂O₂ sequence (Streefkerk, 1972) almost completely blocked endogenous peroxidase activity in red cells and granulocytes, allowing the most precise identification and enumeration of immunoglobulin-containing cells (figs 1 and 2).

The morphology of the cells in preparations counterstained with haematoxylin was excellent, and the identification of both positive and negative cell types was on a par with orthodox haematoxylin and eosin preparations.

Collagenous elements showed non-specific uptake (brown discolouration) of very variable degree which was entirely absent in control preparations treated with SWAR/P and DAB only. This collagen stain-

ing was attributed to non-specific linkage of anti-K, L, and M to the charged collagenous elements and generally did not interfere with interpretation of results. Dilution of anti-K beyond 1/20 reduced this background staining further but was accompanied by a reduction of intensity of positive cells.

Sainte-Marie (fig 5)

Plasma cells, although staining intensely, could only be easily identified in the methanol-H₂O₂ blocked preparations (fig 5). In untreated preparations (a table II) the generalized background staining and numerous granulocytes and red cells made identification difficult. α -N-pyronin counterstained the granulocytes a clear pink colour and thus improved identification slightly. Intensity of red cell peroxidase staining was less than in the corresponding

Fig 5

Fig 5 *Sainte-Marie section. Methanol-H₂O₂ blocked. Anti-K. SWAR/P. Positive cells present, but partly obscured by reticulin staining. × 551.*

Fig 6

Fig 6 *Cryostat section. Methanol-H₂O₂ blocked. Anti-K. SWAR/P. Positive cell, poorly defined with indistinct background. × 551.*

formalin-paraffin sections. The dense brown staining of reticulin, and to a lesser extent collagen, provided the major problem. Overall morphology was reasonable but nuclear detail was not good.

Cryostat (fig 6)

Morphology was extremely poor and it was only possible to identify positive plasma cells with any degree of certainty in methanol H₂O₂ blocked sections. Those plasma cells which could be recognized showed diffusion of positive stain from the cytoplasm thus producing indistinct cell boundaries. In all sections variable diffuse background staining rendered identification very uncertain. On reducing the concentration of anti-K, L, and M slight improvement was observed.

FLUORESCCEIN ISOTHYOCYANATE-LABELLED ANTIBODY (SWAR/F)

Formalin-paraffin (fig 7)

Plasma cells were clearly and precisely identifiable

with strong intensity and contrast. Collagenous tissue showed variable non-specific fluorescence but this was less of a problem than we had anticipated, and could be minimized by the use of appropriate filters (vide supra). Red cells were birefringent but not prominent and eosinophils showed weak auto-fluorescence.

The morphology of positive cells was satisfactory, but it was not possible to recognize and classify individual cell types in the negative cell population. This appeared to be a major disadvantage in comparison with the peroxidase method.

Sainte-Marie (fig 8)

Some plasma cells were clearly positive but again individual negative cells could not be classified. The reticulin network stained strongly, and this was a significant disadvantage. Red cells and eosinophils were not prominent.

Cryostat (fig 9)

With antisera to K, L, and M at a concentration of

Fig 7

Fig 8

Fig 7 Formalin-paraffin section. Anti-K. SWAR/F. Strongly positive cells, with negative indistinct background. $\times 551$.

Fig 8 Sainte-Marie section. Anti-K. SWAR/F. Strongly-positive cells present, but reticulin also fluoresces. $\times 551$.

1:10 the sections were unreadable because of heavy diffuse background fluorescence over the whole section, comparable to that described in the corresponding SWAR/P preparations. However, dilution of anti-K, L, and M to 1:20 or 1:40 produced a marked diminution of the background staining and positive plasma cells were then clearly recognizable. The intensity of the positive fluorescence was variable, with many cells of strong intensity and others grading down imperceptibly into the background of negative cells. This lack of clear demarcation was not apparent in the formalin-paraffin and Sainte-Marie series.

Morphology was poor, the overall architecture was difficult to define, and the recognition and classification of individual cells was not possible.

Sensitivity

COMPARISON OF PEROXIDASE WITH FLUORESCENCE

Serial sections of each case were used to compare the

number and intensity of positive cells in anti-K, L, and M preparations. With the scoring system used there was no significant variation in plasma cell numbers between corresponding sections sandwiched with SWAR/P and SWAR/F for each of the formalin-paraffin and Sainte-Marie processes. With the cryostat preparations SWAR/F at a dilution of 1:20 or 1:40 was clearly superior to SWAR/P, probably due to the diffuse background obscuring specific positive cells in the peroxidase preparations.

COMPARISON OF FORMALIN-PARAFFIN WITH SAINTE-MARIE AND CRYOSTAT SECTIONS

Precise comparative counts were not possible due to errors inherent in selecting three separate blocks for processing by the three different methods. The scoring system used showed close similarities for corresponding sections of formalin-paraffin and Sainte-Marie, with matching proportion of K, L, and M positive cells for each of the five cases examined by the peroxidase or FITC procedures. As pre-

Fig 9 Cryostat section. Anti-K. SWAR/F. Strongly positive cells present, but morphology is poor. $\times 551$.

viously stated, the cryostat sections stained by the fluorescence method showed positive cells of variable intensity and a clear distinction from the negative cell population was difficult for the low intensity cells. We formed a subjective impression that the strongly staining cells were equivalent to those observed in formal-paraffin and Sainte-Marie sections, but that the cells staining with low intensity were more prominent in the cryostat preparations and generally had been discounted as part of the negative population in the other preparations. These low intensity cells may be below the threshold of sensitivity attainable with the formalin-paraffin or the Sainte-Marie method. This would indicate a greater sensitivity for the cryostat method.

SPECIFICITY

Some assessment of specificity was obtained by comparing the type and number of individual positive cells in serial sections exposed to K, L, or M antisera. Only with the peroxidase method applied to

methanol- H_2O_2 blocked formalin-paraffin sections, was the morphology of individual cells made clear and the varying proportions of positive and negative plasma cells were readily seen with the different antihuman immunoglobulin antibodies. The proportion of positive cells with anti-K or anti-L showed a reciprocal relation, with K-positive cells comprising a variable but small majority in all five cases examined. M-positive cells scored between 1% and 10% of morphological plasma cells. Other preliminary findings have been reported by Taylor and Burns (1974) and the specificity has been confirmed in these laboratories by a study of 30 cases of myeloma in which the immunoglobulin types of the abnormal plasma cells have closely corresponded to the serum paraprotein types reviewed independently (in preparation).

GIANT CELLS

Reed-Sternberg type cells, variant Hodgkin giant cells, and characteristic abnormal reticulum cells were present in two cases (nos. 4748/72 and 10063/72). In the peroxidase preparation a proportion of these cells showed positive staining for immunoglobulin (figs 1 and 2) albeit at a lower intensity than seen in adjacent plasma cells. Similar positive staining was present, but less obviously, in the fluorescence preparations. This was judged to represent a specific reaction, indicating the presence of immunoglobulin components in some of the atypical reticulum cells of Hodgkin's disease.

Discussion

Since its introduction by Coons, Creech, and Jones (1941) the immunofluorescence technique has been the method of choice for most studies of immunoglobulins in tissues. The method, however, possesses several inherent disadvantages.

The final product is impermanent and fades rapidly on storage and efforts made to overcome this fault (Sander, 1969; Bienenstock and Dolezel, 1970) have met with limited success. As a result direct visual quantitative or comparative studies are not readily conducted on sections using FITC-labelled antibody techniques. The final product may also be masked by non-immunological binding of sera (Mayersbach, 1967) and the method is inapplicable to electron microscopy.

Some of the problems of the immunofluorescence method have been overcome by the advent of the peroxidase antibody conjugate method (Nakane and Pierce, 1966; Avrameas, 1969, 1972). Both the sensitivity and specificity of peroxidase antibody conjugates are now well established (Nakane and Pierce, 1966; Benson and Cohen, 1970; Davey and Busch,

1970; Dorling, Johnson, Webb, and Smith, 1971; Petts and Roitt, 1971; Seligman, Shannon, Hoshino, and Plapinger, 1973) and endogenous peroxidase activity in red cells and granulocytes which sometimes provided problems in interpretation has now largely been resolved (Straus, 1972; Streefkerk, 1972; Taylor and Burns, 1974). In the present study the methanol H_2O_2 procedure of Streefkerk (1972) for blocking endogenous peroxidase compared very favourably with the α -N-pyronin procedure (Taylor and Burns, 1974) but unlike the latter it also partially blocked red cell haemoglobin peroxidase in addition to granulocyte peroxidase. These blocking procedures had no apparent effect on the subsequent demonstration of immunoglobulin-containing cells or on non-specific background staining.

The background staining of labelled antibody preparations, which can at times present difficulties in the interpretation of results, is mainly due to non-immunological binding of sera onto basically charged tissue components and this property may be enhanced during the staining process (Gervais, 1972; Moriarty, 1973). With peroxidase conjugates some background staining may also be due to non-specific binding of basic isoenzymes of peroxidase present in commercial preparations (Poole and Barrett, 1970, as cited by Dingle, 1973). This effect was not evident in this study.

Cryostat sections of fresh unfixed tissue are preferred for most studies of tissue immunoglobulins by immunofluorescence methods. That freezing and thawing facilitates penetration of antibody conjugates (Leduc, Scott, and Avrameas, 1969) into fixed tissue suggests that there may be diffusion and loss of intracellular immunoglobulins during the sectioning, mounting, drying, and staining of unfixed cryostat sections (Thomsen, 1971). Thus the intrinsically poor morphology of cryostat sections may be compounded by loss or diffusion of antibody. Also this requirement for fresh unfixed tissue has been a major factor limiting the useful application of immunofluorescence methods by the surgical pathologist, because the majority of specimens received in a routine laboratory are formalin fixed. Under these circumstances a need for labelled antibody studies often arises. However, it is generally believed that the immunofluorescence technique is unsuited to formaldehyde-fixed, paraffin-embedded material due to enhancement of intrinsic tissue autofluorescence (Goldman, 1968) and, more importantly 'because . . . antigen and antibody activity is inactivated by conventional histological methods' (Sainte-Marie, 1962). Also dilute alcohols denature immunoglobulins (Ahlqvist, 1972) and clearing agents have an extracting quality (Zeitoun and Lehy, 1970). The combination of those two factors in the standard

wax embedding process may have a deleterious effect on immunoglobulins.

Nonetheless in 1962 Sainte-Marie reported satisfactory results on paraffin-embedded tissues previously fixed by a variety of methods including 5% formalin in 95% alcohol, and finally recommended the method of embedding in wax after cold 95% ethanol fixation, which produced some improvement in morphology but added inherent disadvantages of its own, viz, special fixation, processing, and storage. Good general morphology, and in particular, clear cellular detail, were still lacking with fluorescence methods. These findings were confirmed by our results, although the staining of reticulin, be it specific or otherwise, greatly interfered with the assessment of positive plasma cells in FITC and peroxidase preparations (figs 5 and 8).

With regard to conventional histological methods the routine formalin fixation-paraffin embedding procedure has not been investigated in relation to immunological study of immunoglobulins but in 1972 Miller wrote that 'the possibility of using formalin fixation and cold paraffin embedding techniques has not yet been explored'. However, in related ultrastructural studies Kuhlmann and Miller (1971) and Miller (1972) examined the effects of fixation upon intracellular immunoglobulins in animals immunized with horseradish peroxidase, and found that tissues fixed as long as one week in 4% formaldehyde showed no diminution of their reaction with the peroxidase antigen. Of course, the peroxidase molecule (mol wt 40 000) by itself may be more accessible to its specific antibody than a peroxidase conjugate (mol wt 200 000 plus) to its parent antigen under the same experimental conditions. Also, with the anti-enzyme method the observed results depend upon the preservation of the antibody specificity site of the immunoglobulin molecule (variable sequence N-terminal end), whereas with the peroxidase conjugate method the specific results observed are dependent upon the integrity of the antigenic determinants of the immunoglobulin molecule (C-terminal end). The two techniques are therefore not strictly comparable and indeed there are suggestions that while aldehyde fixatives may not denature immunoglobulins they may render the antigenic determinants inaccessible to antibody (Avrameas and Ternynck, 1971; Kraehenbuhl, De Grandi, and Campiche, 1971; Webb and Dorling, 1973).

The effects of fixation on antibody activity were also examined by Yokota (1973) and in an acrylamide gel assay of anti-horseradish peroxidase he demonstrated a loss of 45% activity of antibody after 15 minutes' fixation in ice cold ethanol (90%) or absolute acetone. A 45% loss of antibody was recorded after exposure to 2.5% glutaraldehyde for 90 min-

utes at 4°C and a 35% loss in 4% formalin for the same period. A similar loss of enzyme activity was also noted.

In the face of the conflicting evidence from the literature the present study not only confirms the finding of Taylor and Burns (1974) that intracellular immunoglobulins are demonstrable in formalin-fixed, paraffin-embedded material by peroxidase conjugates but also that they may be similarly demonstrated by immunofluorescence methods (table II, figs 1, 5-9). The position with regard to extracellular immunoglobulins is less clear. This aspect has not been studied by us, but pilot studies by others in these laboratories have not produced results comparable to those obtained by fluorescence methods on cryostat sections.

In conclusion, formalin fixation followed by orthodox embedding in paraffin wax certainly preserves some intracellular immunoglobulins and these may be demonstrated using either peroxidase or FITC labelled antibody by a sandwich technique. The evidence presented indicates that there is some loss of sensitivity in comparison with fresh cryostat material examined by immunofluorescence. Against this the formalin paraffin method with peroxidase-labelled antibody possesses several clear advantages. Preparations are permanent, are readily counterstained with routine histological stains, and are easily examined with the ordinary light microscope. Morphology is excellent and both positive and negative cells can be accurately classified and counted. These facilities are helped by less background staining of formalin-paraffin preparations than in Sainte-Marie or cryostat preparations. Also specialized processing is not required and valuable retrospective studies can be performed on almost any routine paraffin block¹ even after several years storage. We have noted in a small number of cases that Bouin's fixative also preserves some intracellular immunoglobulin, but satisfactory results have not been obtained with Zenker formalin.

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¹Populations of abnormal cells containing monoclonal immunoglobulin have been demonstrated in necropsy material from several cases of multiple myeloma.

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