

donors screened, similar to the proportions of IgA deficient blood donors reported in other British studies.<sup>2,4</sup> None of the 239 sera agglutinated uncoated latex, but 228 agglutinated IgA coated latex directly in the absence of added anti-IgA (including seven of the 18 confirmed IgA deficient sera). With ethical committee approval, follow up serum samples were obtained from the 18 IgA deficient blood donors six months after the initial samples had been taken: 14 of these still showed IgA deficiency (IgA < 0.5 g/l) by laser nephelometry while four had returned to normal serum IgA values. One IgA deficient donor had mild but clinically important recurrent infections (sinusitis, otitis media), two had mild allergies, and two had possible autoimmune disorders. No other important clinical abnormalities were elicited at interview.

These results show that the manual indirect latex agglutination test can provide a simple and satisfactory preliminary screen for IgA deficient blood donors, reducing the requirement for quantitative IgA testing by more sophisticated and expensive methods. It is more rapid than the indirect passive haemagglutination test described by Hunt *et al*<sup>4</sup> which uses a similar preincubation step; it is free of interference from any anti-red cell antibodies; and IgA coated latex has been found to be stable for at least two months, unlike IgA coated red cells which must be prepared about every week. The relatively simple ammonium-sulphate precipitation for enrichment of IgA myeloma immunoglobulin for coating renders coated latex highly sensitive to agglutination by anti-IgA and enables accurate adjustment of cut off to desired values to be made. With this degree of sensitivity the test does not distinguish between IgA deficient donors and donors with autologous anti-IgA. The latter group would tend not to be distinguishable from donors with anti-red cell antibodies in passive haemagglutination techniques. The importance of finding donors with normal serum IgA values and anti-IgA antibodies is not clear and may merit further studies.

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## PLP fixation for combined routine histology and immunocytochemistry of liver biopsies

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The development of monoclonal antibodies has facilitated both histopathological diagnosis and research by allowing the presence of various cytoplasmic and cell surface antigens to be shown on lymphoid and other cells.<sup>1,2</sup>

Showing the presence in liver biopsy specimens of surface markers for lymphocytes, accessory immune cells, and epithelial cells by using monoclonal antibodies has largely been limited to frozen tissue<sup>3-8</sup>; reliable and reproducible results have not been obtained in paraffin embedded tissue. This creates several problems. Frozen sections are inferior in morphological detail to paraffin sections and are less easily stored. Dividing the biopsy specimen into two for frozen and paraffin sectioning increases the possibility of sampling error, and frozen sectioning introduces a risk of infection for laboratory staff, especially when material from patients with viral hepatitis is handled.<sup>9</sup>

Recently Collings *et al*<sup>10</sup> reported the successful use of several monoclonal antibodies using paraffin embedding after fixation of tissues in periodate-lysine-paraformaldehyde (PLP).<sup>11</sup> In this study we applied this technique to liver biopsy specimens to assess its suitability for routine diagnostic purposes.

#### Material and methods

The study was carried out in two parts. The first part was designed principally to test "routine" staining methods after PLP fixation and paraffin embedding;

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20 liver biopsy specimens (16 percutaneous needle and four wedge biopsies), together with two needle post-mortem examination specimens were divided into two parts and fixed in 10% neutral buffered formalin and PLP, respectively, before processing, paraffin embedding, and section cutting at 3  $\mu$ m. Sixteen of the specimens were from patients with hepatitis B virus related chronic liver disease: one showed alcoholic cirrhosis and one was histologically normal. Four were from patients with  $\beta$  thalassaemia major. Four surgically removed spleens from these patients, as well as surgically removed tonsils were used as positive control material for immunocytochemical staining with the monoclonal antibodies given in Table 1. Both sets of sections were stained with haematoxylin and eosin, chromotrope aniline blue, diastase-periodic acid Schiff, Perls Prussian blue for iron, Shikata's orcein, rhodanine for copper, and by Gordon and Sweets' method for reticulin. Spleens and tonsils were only stained with haematoxylin and eosin and for reticulin. Staining methods used were those given by Scheuer<sup>12</sup> with minor modifications.

The second smaller study was designed to compare the results of PLP fixation and paraffin embedding with those obtained in frozen sections. Each of five specimens was divided into three parts immediately after it had been obtained from the patient: one was snap frozen with isopentane in liquid nitrogen while the other two parts were fixed in PLP and 10% neutral buffered formalin, respectively, before embedding. Frozen sections 6–7  $\mu$ m thick were cut on a cryostat from the snap frozen material. Frozen and paraffin sections were stained with haematoxylin and eosin and with the same panel of antibodies used in the first experiment (Table 1). Final diagnoses in the five patients were fatty liver, fibrosis due to alcoholism, cirrhosis of uncertain cause, mild chronic active hepatitis, and lymphomatous infiltration.

#### FIXATION, EMBEDDING, AND STORAGE

PLP fixative was freshly prepared for each specimen from two stock solutions:

1 *Lysine-phosphate buffer* 0.2M lysine-hydrochloric acid was adjusted to pH 7.4 with 0.1M disodium hydrogen orthophosphate, then diluted with 0.1M phosphate buffer (pH 7.4) to a final concentration of 0.1M lysine.

2 *Paraformaldehyde solution* an 8% w/v solution of paraformaldehyde was prepared by continuous stirring while heating at 60°C.

Stock solutions were renewed after 10 days. Just before use the two stock solutions were mixed, using three parts of the lysine-phosphate buffer to one part of paraformaldehyde. Sodium metaperiodate was then added to a final concentration of 0.05M.

The tissues were fixed in PLP at 4°C for three hours, four hours, overnight, or for three days, with roughly equal numbers of specimens in each group. They were then dehydrated through graded alcohols and cleared in xylene at 4°C, using 30 minute steps. Once cleared they were infiltrated with three changes of paraffin wax at 56°C and embedded. The paraffin blocks were stored at 4°C until use.

Formalin fixed tissues were processed routinely at room temperature and embedded at 56°C.

#### IMMUNOCYTOCHEMICAL STAINING

Table 1 lists the monoclonal antibodies used in the study: RFB4, RFB6, RFT2, and RFDR1 were kindly provided by Dr LW Poulter, department of immunology, Royal Free Hospital School of Medicine.

Sections fixed in PLP were air dried overnight at 4°C, while sections fixed in formalin were dried at 56°C. Sections were dewaxed in xylene (two changes) for five minutes at room temperature and taken to absolute ethanol. Endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol for 20 minutes at room temperature; after this sections were washed for five minutes in tap water. They were next treated with 0.025% protease (type XIV, Sigma) prepared in Tris buffered saline (TBS, 0.05M, pH 7.6) for nine minutes at 37°C. The reaction was stopped by washing in cold tap water for five minutes. The sections were taken to TBS (three changes of two minutes

Table 1 *Monoclonal antibodies used in this study*

Antibody	Specificity	Source	Dilutions	
			Frozen	Paraffin
Dako-LC	Leucocyte common antigen	Dakopatts	1/50	1/1 and 1/10
Pan B	Pan B cell	Miles	1/300	1/300
RFB4	B cells	Immunology, Royal Free Hospital	1/5	1/1 and 1/5
RFB6	B cells, follicular dendritic cells	Immunology, Royal Free Hospital	1/10	1/1 and 1/10
RFT2	T blasts	Immunology, Royal Free Hospital	1/2	1/1 and 1/2
OKT4	T cells (helper/inducer)	Ortho Pharmaceuticals	1/5	1/2 and 1/5
HB2	T cells (helper/inducer)	ATCC	1/1	1/1
Dako-T8	T cells (suppressor/cytotoxic)	Dakopatts	1/1	1/1
Dako-HLA-DR	HLA-DR antigens	Dakopatts	1/10	1/50
RFDR1	HLA-DR antigens	Immunology, Royal Free Hospital	1/10	1/5
Dako-DRC1	Dendritic reticulum cells	Dakopatts	1/10	1/10 and 1/50

each), incubated with normal swine serum (diluted 1/20 in TBS) for 15 minutes, drained, and incubated with appropriately diluted monoclonal antibody for 90 minutes. Working dilutions (Table 1) were determined using spleen and tonsils and varied according to the age of the antibody. After a further three washes in TBS sections were incubated for 30 minutes with peroxidase conjugated polyclonal rabbit antibody to mouse immunoglobulin (Dakopatts) for 30 minutes, washed three times in TBS, incubated for 30 minutes with peroxidase conjugated swine antirabbit serum (Dakopatts), and again washed three times. Peroxidase activity was developed with 0.01% diaminobenzidine (Sigma). Sections were lightly counterstained with Carazzi's haematoxylin. Negative controls were obtained by substituting the primary antiserum with normal swine serum or TBS.

The frozen sections used in the second experiment were air dried for 60 minutes, fixed in acetone for 10 minutes, and stained by a similar method to the above, but with 60 minutes of incubation in primary antisera.

**Results**

Routinely stained sections were equally suitable for diagnosis after fixing in formalin or PLP; there were no detectable morphological changes with different fixation times. In sections stained with haematoxylin and eosin cell membranes were somewhat better defined in tissue fixed in PLP than that fixed in formalin. Reticulin-diasatase periodic acid Schiff, and chromotrope aniline blue sections showed no detectable differences between PLP and formalin, whereas iron stained with greater intensity by Perls's method in tissue fixed in PLP. In orcein preparations elastic fibres stained well after PLP, but hepatitis B surface antigen (HBsAg) was very pale and copper associated protein negative. Due to the presence of the oxidising agent sodium metaperiodate in the PLP fixative, it was thought that there might be over-oxidation of the tissue; oxidation time with potassium permanganate in the orcein method was therefore reduced from 15 to five minutes. The results of orcein

staining were then similar using both fixatives, with good staining of HBsAg and copper associated protein.

Immunostaining gave positive results with all antibodies in frozen sections of positive control tissues. Different PLP fixation times did not substantially affect subsequent staining with monoclonal antibodies, with the exception of Dako-DRC1 which was positive in spleen after three hours and overnight fixation but negative after fixation for three days. Other antibodies gave only slightly weaker results after three days' fixation. Of the 11 monoclonal antibodies tested, 10 gave positive results after PLP fixation and embedding, whereas all were negative in formalin fixed material (Table 2). Only Dako-T8 gave negative results in spleen and tonsil, fixed in PLP, as well as in liver tissue; RFB6 and Dako-DRC1 were positive in spleen and tonsil but negative in the liver biopsy specimens tested. All the other antibodies gave positive results in spleen, tonsil, and a variable proportion of the liver biopsy specimens. Where the relevant cells were present in liver biopsies the strength of the reaction was somewhat weaker in PLP sections than in frozen sections. The quality of the PLP sections, however, was greatly superior with respect to tissue detail (Figure).

**Discussion**

Many surface markers for human T and B lymphocytes, accessory cells of the immune system, and epithelial cells are denatured by routine fixatives used in histopathology laboratories, including formalin, formalin sublimate (B5), and glutaraldehyde.<sup>13-15</sup> Attempts to apply monoclonal antibodies to fixed tissues have been restricted to a small range of antibodies.<sup>15-16</sup> PLP has the advantage over routine fixatives that it does not interact with proteins but stabilises the carbohydrate moieties, so that antigenic sites are preserved.<sup>11</sup> Subsequent paraffin embedding does not prevent the demonstration of surface antigens by means of monoclonal antibodies, as shown by Collings *et al*<sup>10</sup> using human tonsil and skin. We confirmed their results using liver biopsy specimens

Table 2 Results of immunocytochemical staining

Tissue	Sections	Antibodies										
		Dako-LC	Pan B	RFB4	RFB6	RFT2	OKT4	HB2	Dako-T8	Dako-HLA-DR	RFDR1	Dako-DRC1
Liver	Frozen	+	+	+	-	+	+	+	+	+	+	-
	Formalin	-	-	-	-	-	-	-	-	-	-	-
	PLP	+	+	+	-	+	+	+	-	+	+	-
Spleen, tonsil	Frozen	+	+	+	+	+	+	+	+	+	+	+
	Formalin	-	-	-	-	-	-	-	-	-	-	-
	PLP	+	+	+	+	+	+	+	-	+	+	+

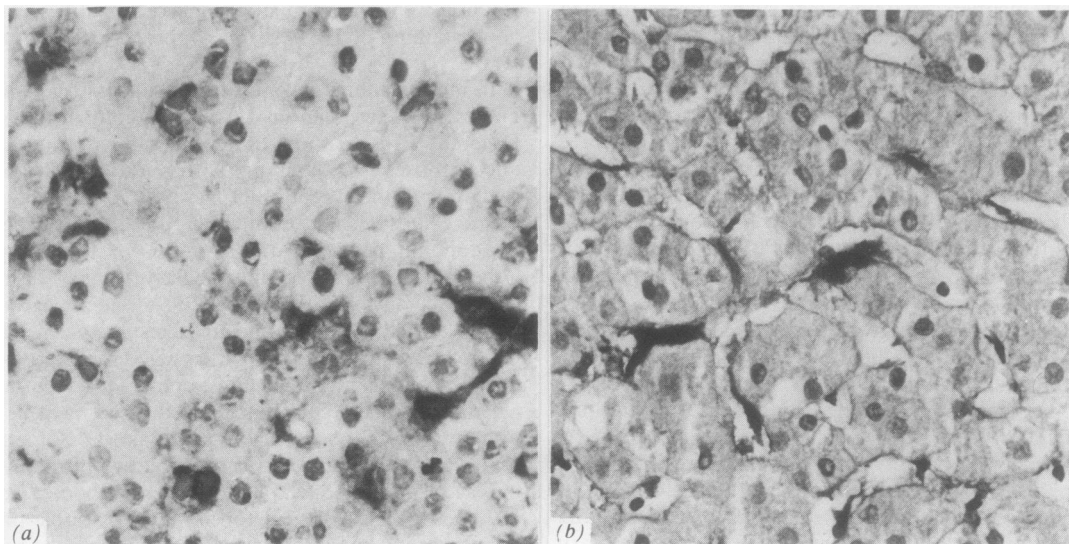


Figure Part of liver biopsy specimen from patient with fatty change and fibrosis attributed to alcoholism. 1a) frozen section; 1b) paraffin section after PLP; note better tissue definition in Fig. 1b. Both show sinusoidal cells positive with monoclonal antibody Dako-HLA-DR. Formalin fixed material from the same specimen gave a negative result.  $\times 400$ .

and have further shown that the method is suitable for routine staining methods with only minor modification in the case of the orcein stain.

PLP fixation followed by paraffin embedding is thus suitable for handling liver biopsy specimens taken for diagnostic purposes and permits more extensive investigation of the material. Several of the monoclonal antibodies tested have previously been shown to work after PLP fixation and paraffin embedding.<sup>10</sup> In addition, we tested Dako-T8, Dako-LC, Ortho OKT4, and Miles pan B; the first of these was negative in all tissues fixed in PLP, including positive controls, while the other three were positive in liver biopsy specimens. RFB6 and Dako-DRC1, which recognise follicle centre B cells and dendritic reticulum cells, respectively, were negative in all liver biopsies tested but positive in control material fixed in PLP and embedded in paraffin. These negative results with RFB and Dako-DRC1, as well as the failure to stain cells in all liver biopsies with each antibody, we attribute tentatively to absence of the appropriate cells from the liver tissue. This requires confirmation.

The PLP processing technique used in the study is very similar to that routinely used in histopathology laboratories and overcomes problems of handling, preservation, and storage inherent in frozen section techniques. The immunocytochemical method used is a simple three layer technique, which is convenient with respect to time and use of reagents. Safe handling of fixed tissues is particularly important when liver

biopsies are taken from patients with various forms of hepatitis. Such biopsies provide important research material for the study of lymphocytes and accessory cells and their part in the pathogenesis of liver disease.

Fixation times were not critical, and it was possible to obtain good results even with overnight fixation. One potential disadvantage of PLP, however, is that very long fixation (over a weekend or during transportation of a specimen) is not advisable because of probable loss of antigenicity, which might interfere with results obtained with some antibodies. The fixative has to be prepared fresh for each specimen, and specimens are dehydrated and cleared manually at 4°C rather than in a tissue processor. The method is therefore more time consuming for laboratory staff than formalin fixation. Nevertheless, PLP fixation followed by paraffin embedding should prove helpful for specimens in which morphological diagnosis needs to be combined with immunocytochemical identification of surface markers.

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Letters to the Editor

*Pseudomonas maltophilia*

The article by Wheat, Winstanley, and Spencer<sup>1</sup> discusses discrepant results for susceptibility testing of *Pseudomonas maltophilia* with aminoglycosides and polymyxin.

In 1981, in the May 30 edition of the *Medical Journal of Australia*, Guinness and Levey described a widespread epidemic of infection caused by *Pseudomonas cepacia*.<sup>2</sup> In this article it was noted that *Ps cepacia* appeared to be fully sensitive to kanamycin by disc testing, although quantitative testing showed this result to be false. A variation was also noted with carbenicillin.

It was suggested that a false zone of sensitivity with kanamycin may have been a combination of factors. Included among the suggested causes were the slower growing nature of the organism compared with enteric Gram negative bacilli. Another important factor could well be that *Ps cepacia* would produce alkaline by-products of metabolism, which would diffuse into the test medium and strongly enhance the activity of kanamycin.

With respect, I feel that the main point to be made is that disc sensitivity testing has been standardised using particular media, taking into account the commonly encountered pathogens. When more unusual pathogens with special growth requirements are found the disc method may not be the definitive method of choice, and a more conservative approach to the problem may be required.

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Dr Spencer and colleagues reply as follows:

With respect, the main point of our paper was not that discrepant results occurred between disc sensitivity and minimum inhibitory concentrations, but that the difference in results occurred due to difference in temperature. In our study strains of *Ps maltophilia* had identical susceptibility results whether they were measured by disc or minimum inhibitory concentrations at the same temperature. Dr Levey discusses the problems encountered with *Ps cepacia*, an organism that we have not investigated, although we were unable to show this temperature phenomenon with *Ps aeruginosa*. Although it may be true that disc susceptibility testing has been standardised with common organisms and certain media, we have never suggested that the disc technique is the method of choice, especially when dealing with unusual pathogens.

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