Technical methods

Effect of formalin pigment removal on peroxidase-antiperoxidase immunoperoxidase technique

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In immunoperoxidase preparations formalin pigment (acid formaldehyde haematein) may be difficult to distinguish from the brown staining of the 3,3'-diaminobenzidine (DAB) reaction product. The pigment, produced when formalin is in contact with blood rich tissues, takes the form of dark brown microcrystals that have the ability to rotate the plane of polarised light.¹ Even though formalin pigment can be differentiated by this means, the technique may be cumbersome and tedious.

Several methods for the removal of formalin pigment have been described. These have been performed in the current study, both before and after treatment in immunoperoxidase sequences.

Material and methods

TISSUES AND FORMALIN PIGMENT REMOVAL

Specimens of normal splenic tissue were sampled from necropsies. The tissue was fixed in 10% formol saline and then routinely processed and embedded in paraffin wax. Sections were then cut at 2-3 μ m. The following techniques for the removal of formalin pigment were carried out, both before and after the immunoperoxidase staining sequence:

- 1 Saturated alcoholic picric acid followed by 30 minutes running tap water (15 minutes).
- 2 One per cent sodium hydroxide in 70% ethanol followed by five minutes running tap water (10 minutes).
- 3 Ten volumes hydrogen peroxidase followed by five minutes running tap water (five hours).
- 4 Concentrated nitric acid followed by 15 minutes running tap water (one hour).
- 5 Five per cent chromium trioxide followed by 15 minutes running tap water (30 minutes).
- 6 Five per cent potassium permanganate (two minutes), followed by 5% oxalic acid, (two minutes),

followed by five minutes running tap water. These methods are standard techniques.¹

IMMUNOPEROXIDASE METHOD

After treatment with 0.1% trypsin in calcium chloride solution for 20 minutes the endogenous peroxidase activity was blocked using a solution of 0.3% hydrogen peroxidase in methanol. Non-specific background staining was minimised by the application of normal swine serum at a titre of 1/5. After 10 minutes the excess normal swine serum was discarded and optimally diluted antisera directed against κ and λ immunoglobulin light chains were overlaid for 20 minutes. (The antisera used were obtained from Dako, United Kingdom). The sections were washed for five minutes, after which swine-antirabbit serum was applied for 20 minutes at a titre of 1/50. After a further wash rabbit peroxidase antiperoxidase (PAP) complex (1:50) was overlaid. The peroxidase was demonstrated by a 3,3'-diaminobenzidine reaction.

To diminish the possibility that our results were true only for sequences for light chains sections were also stained for the following molecules: cathepsin G, cathepsin B, and factor VIII related antigen.

In addition, the immunoperoxidase sequence only was applied in the absence of methods for formalin pigment removal.

Fig. 1 Section of spleen stained for κ immunoglobulin light chain. Numerous κ positive plasma cells are present but there is also copious, finely and coarsely granular formalin pigment. (PAP reaction for κ Ig light chain; haemalum counterstain. No other treatment.) $\times$ 350.

Evaluation of immunoperoxidase staining in combination with formalin pigment removal

<i>Treatment</i>	<i>Pretreatment</i>	<i>Post-treatment</i>
Saturated alcoholic picric acid	+++	++++
Sodium hydroxide (1%) in 70% ethanol	++	+/-
Ten volumes hydrogen peroxide	++	+++
Concentrated nitric acid	-	-
	(Most of the tissue was detached from slide)	
Chromium trioxide (5%)	+/-	+
Potassium permanganate (5%)	-	-
Oxalic acid (5%) (Mallory bleach)	-	-

++++ = optimal result; +++ = clearly interpretable result; ++ = appreciably diminished staining; + = severely diminished staining; +/- = severely diminished or absent staining; - = negative staining.

Results

In all untreated sections copious formalin pigment was present (Fig. 1).

The post treatments using picric acid (Fig. 2) and hydrogen peroxide gave optimal results, being equal in intensity to those that did not receive treatment. Pretreatments using these techniques also produced good results. The other techniques gave much less satisfactory results (Table) (Fig. 3).

Discussion

In immunoperoxidase preparations formalin pigment may easily be confused with the brown reaction product of 3,3'-diaminobenzidine. We have shown that several of the formalin pigment removal techniques

may be used with the immunoperoxidase sequence and that post treatments with picric acid or hydrogen peroxide produce the best results. The harsher treatments—nitric acid, for example—remove not only the formalin pigment but also deleteriously affect the antigenic sites and tissue.

The use of labels or developing agents, giving differently coloured reaction products, could allow them to be distinguished from formalin pigment.² Their use, however, is generally not as widespread or as satisfactory as peroxidase reactions using DAB.

Other pigments, such as haemosiderin, may also be difficult to distinguish from the DAB and peroxidase reaction product. In this instance simple procedures may be performed following the immunoperoxidase sequence—namely, the Perls's stain^{3,4}—to differentiate the reaction product from haemosiderin.

Fig. 2 Section of spleen stained for κ immunoglobulin light chain. κ positive plasma cells and vascular endothelium are intensely stained but there is no formalin pigment in the background. (PAP reaction for κ Ig light chain; haemalum counterstain. Post treatment with saturated alcoholic picric acid.) $\times 240$.

Fig. 3 Section of spleen stained for κ immunoglobulin light chain. No immunohistochemical staining is present. (PAP reaction for κ Ig light chain; haemalum counterstain. Post treatment with 5% potassium permanganate and 5% oxalic acid.) $\times 220$.

Apart from the κ and λ immunoglobulin light chains, the other antigens studied—namely, cathepsin G, cathepsin B, and factor VIII related antigen—are structurally quite distinct. It seems unlikely, therefore, that our results will not be applicable, in peroxidase sequence, to other antigens.

References

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Laboratory diagnosis of peritonitis in continuous ambulatory peritoneal dialysis by lysis and centrifugation

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Continuous ambulatory peritoneal dialysis (CAPD) has become accepted as an important form of dialysis treatment for patients with end stage renal disease.¹ Advantages include greater patient mobility and independence, lack of requirement for vascular access, and no fluid restriction. The most important infective complication of CAPD, as with any form of peritoneal dialysis, is undoubtedly peritonitis.² Conventional methods of culture of peritoneal dialysate from these patients has often proved negative, despite the presence of pus cells in the peritoneal fluid and

clinical evidence of infection.³ Various techniques have been investigated in an attempt to improve the rate of recovery.⁴ In this study we evaluated the use of a lysis-centrifugation (Isolator) blood culture system⁵ compared with standard laboratory techniques in the diagnosis of peritonitis in CAPD.

Material and methods

The following methods were used on 100 consecutive CAPD specimens, irrespective of the patients' clinical condition. Peritonitis was diagnosed if the following criteria were fulfilled: pain or discomfort in the abdomen, associated with a "cloudy bag" that had numbers of white blood cells in excess of 100 cell mm⁻³.²

A count of white cells was performed on the cloudy dialysate specimens in a Fuchs-Rosenthal counting chamber. One ml of dialysate was mixed with 10 ml of molten nutrient agar and poured into a sterile Petri dish.⁶ A 20 ml aliquot of dialysate was centrifuged at 3000 rpm for five minutes and 0.01 ml of centrifuged deposit inoculated on blood agar (incubated aerobically), blood agar (incubated anaerobically), and MacConkey agar. The lysis-centrifugation system used consisted of a double stoppered tube containing 0.3 ml of high density hydrophilic fluorinert, overlaid by 0.5 ml of aqueous lysing solution.⁷ Dialysate (10 ml) was added aseptically, mixed, and centrifuged at 3000 g for 30 minutes in a 35° fixed angle rotor centrifuge. After lysis and centrifugation 0.01 ml of the concentrated sediment was inoculated on to two blood and one MacConkey agar plates. All plates were incubated for a total of 72 hours at 37°C. Cultures were read at 24, 48, and 72 hours.

Results

Of the 100 specimens, 90 fulfilled the criteria of being obtained from a patient with peritonitis and 25 (27%) of these had antibacterial activity present. Of the remaining 65 samples, the detection rates of potential pathogens were pour plates 48 (74%), centrifuged deposit 51 (78%), and lysis-centrifugation 56 (86%). For the 25 samples with antibacterial activity present, figures were seven (28%), six (24%), and 13 (52%), respectively. The Table shows the overall isolation

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Isolation rates in 90 dialysate samples by various techniques under study

Technique	Organisms		
	Gram positive (n = 42)	Gram negative (n = 29)	Total (%) (n = 71)
Pour plate	38	17	55 (77)
Centrifuged deposit	36	21	57 (80)
Lysis-centrifugation	41	28	69 (97)