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An immunohistological study of human lymphoma

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SUMMARY

In this study the problems encountered in staining immunoglobulin (Ig) in sections of paraffin-embedded human lymphoma samples have been investigated. It was found that the 'masking' of cytoplasmic Ig, which occurs when tissues are fixed in formol saline (the fixative employed in most previous studies), can be avoided by the use of mercury-based fixatives. When non-Hodgkin's lymphoma samples fixed in this way were studied it was found that cytoplasmic Ig labelling of both lymphoid and histiocytic cells is often attributable to non-specific uptake of serum proteins. This phenomenon probably accounts for a number of published anomalous immunoperoxidase staining results in human lymphoma (e.g. the presence of both kappa and lambda chains in the same neoplastic cell). Double immunoenzymatic labelling (using alkaline phosphatase and peroxidase) proved valuable in the elucidation of this phenomenon. When staining due to absorbed Ig was discounted it was possible to demonstrate monoclonal Ig labelling in seven out of sixteen cases of non-Hodgkin's lymphoma. In each case IgM was found in association with a single light chain type and these results were in agreement with those obtained by direct immunofluorescent labelling of cryostat sections. In a further case μ chains without associated light chains were demonstrated by immunoperoxidase staining.

Seven cases of Hodgkin's disease were studied by immunoenzymatic techniques. Although IgG was frequently found in Reed–Sternberg and Hodgkin's cells its presence was not attributable to non-specific uptake of serum protein since albumin was absent or only present in small amounts. These findings are in support of the macrophage origin of these cells.

INTRODUCTION

The independent reports by Taylor (1974) and by Garvin *et al.* (1974) that immunoglobulin (Ig) can be demonstrated by immunoperoxidase staining of paraffin-embedded formalin-fixed lymphoma tissue sections prompted a number of groups to adopt this potentially valuable approach in the study of these diseases (Table 1). However, several of these studies have yielded unexpected results which suggest that these methods may be subject to a variety of technical problems.

Frequency of Ig detection. Ig-positive neoplastic cells have been detected in some studies in more than 50% of non-Hodgkin's lymphoma samples (Davey *et al.*, 1978; Li & Harrison, 1978; Taylor, 1978; Zulman, Jaffe & Talal, 1978). The facility with which positive labelling of routine surgical histological samples was achieved in these series contrasts with the rarity of positive staining in other studies (Garvin, Spicer & McKeever, 1976; Warnke *et al.*, 1978) and with reports that

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conventional fixation and embedding procedures frequently render cellular Ig unreactive (Garvin *et al.*, 1976; Curran & Jones, 1978a; Mepham, Frater & Mitchell, 1979).

Heavy and light chain types. Human B cell lymphomas produce IgM more frequently than IgG, whether this is assessed by analysis of lymph node extracts (Stein, 1978), immunofluorescent labelling of membrane Ig on cell suspensions or cryostat sections (Stein, 1978; Warnke & Levy, 1978) or by typing serum paraproteins (Alexanian, 1975). It is therefore surprising that in each of the three reports in which heavy chain data are given the immunoglobulin detected in neoplastic non-Hodgkin's lymphoma cells was frequently IgG (Garvin *et al.*, 1976; Halliday, Davey & Marucci, 1978; Davey *et al.*, 1978; Isaacson & Wright, 1978).

Unexpected findings have also been reported when staining human lymphoma sections for light chain in that both kappa and lambda light chains have been found within the same cells (Taylor, 1976, 1978; Landaas, Godal & Halvorsen, 1977; Curran & Jones, 1978c; Isaacson & Wright, 1978; Warnke *et al.*, 1978). When this phenomenon has been encountered in cells of undisputed B cell origin (e.g. myeloma) it has been interpreted as evidence that the malignant cells are synthesizing either aberrant Ig or both light chain types (Taylor, Russell & Chandor, 1978). The presence of both kappa and lambda chains within Reed–Sternberg and Hodgkin's cells (Taylor, 1976; Landaas *et al.*, 1977; Curran & Jones 1978c), since it is usually associated with cytoplasmic staining for gamma chains, has been taken by several groups as evidence of Fc receptor-mediated uptake of IgG (Kadin *et al.*, 1978). Poppema, Elema & Halie (1978), however, have reported that other serum proteins (albumin and α_1-anti-trypsin) accompany IgG in Reed–Sternberg and Hodgkin's cells and favour non-specific leakage of serum into these cells as an explanation of their immunohistological reactions for IgG.

In the present paper we have explored the causes of these discrepant immunoperoxidase reactions of human paraffin-embedded lymphoma samples and propose ways in which these problems can be avoided.

MATERIALS AND METHODS

Tissues. Tissue samples were either collected prospectively and fixed for 2·5 to 72 hr in formol sublimate, B5, B4, 10% formalin in 0·9% saline, Zenker's or Bouin's fixatives (Drury & Wallington, 1967; Lillie, 1965), or alternatively obtained from the surgical histology files of the Pathology Department, Radcliffe Infirmary, Oxford. The latter tissues had been fixed for undetermined periods in formol saline.

Subsequent dehydration and paraffin embedding was by conventional techniques.

Antisera. All antisera were obtained from Dako Immunoglobulins A/S with the exception of goat antisera to human albumin, IgG and κ chains, donkey anti-goat IgG and goat PAP, were obtained from Miles Laboratories.

Dako anti-Ig antisera have been used extensively in this laboratory (Taylor & Mason, 1974; Taylor, 1976) and by other workers (Curran & Jones, 1977; Landaas *et al.*, 1977; Isaacson & Wright, 1978; Taylor, 1978; Zulman *et al.*, 1978) for the immunoenzymatic staining of paraffin-embedded human lymphoid tissue, and their specificity has been established by blocking and haemagglutination techniques (Taylor & Burns, 1974; Mason, 1975; Zulman *et al.*, 1978).

Immunoenzymatic staining. This was performed as described previously using either the PAP procedure (Sternberger *et al.*, 1970) or the labelled antigen technique (Mason & Sammons, 1979). The latter technique is a highly specific method which involves sequential application to sections of unlabelled antiserum (e.g. anti-human IgG) followed by peroxidase-conjugated antigen (e.g. human IgG : peroxidase). The antibody acts bivalently, linking antigenic sites in the tissue section to the conjugated antigen. Lymphoma sections were stained initially with anti-κ and anti-λ antisera, supplemented as required by staining for IgM, IgG and/or albumin. In some experiments the primary antibody was applied to the sections overnight at 4°C.

Trypsinization of formol saline-fixed material (Curran & Gregory, 1977) was performed by incubating de-waxed sections for 10–20 min in 0·1% trypsin (Sigma, Grade II) dissolved in distilled water containing 0·1% $CaCl_2$ and adjusted to pH 7·8 with NaOH.

Double immunoenzymatic staining (Mason & Sammons, 1978) for κ and λ chains or for IgG and albumin was performed by applying the following 'sandwiches':

κ and λ chains

(1) Rabbit anti-λ chains.
(2) Swine anti-rabbit IgG.
(3) Rabbit anti-alkaline phosphatase plus goat anti-κ chains.
(4) Alkaline phosphatase plus peroxidase-conjugated κ chains.

IgG and albumin

(1) Rabbit anti-IgG.
(2) Swine anti-rabbit IgG.
(3) Rabbit anti-alkaline phosphatase plus goat anti-albumin.
(4) Alkaline phosphatase plus peroxidase-conjugated albumin.

In some experiments double κ/λ staining was achieved using goat PAP in conjunction with an alkaline phosphatase:anti-alkaline phosphatase sandwich as previously described (Mason & Sammons, 1978).

The two enzymatic cytochemical reactions are then developed sequentially (Mason & Sammons, 1978) yielding blue staining for alkaline phosphatase and brown staining for peroxidase. Cells containing both antigens stain a characteristic intermediate purple colour.

Control slides, in which the primary anti-Ig and anti-albumin antisera were omitted from the immunoenzymatic staining procedure, gave negative reactions. The specificity of staining for individual Ig chains and for albumin was assessed by comparing the type and frequency of cells which stained with each antiserum. Furthermore, extensive use of the double immunoenzymatic staining technique for light chains has failed to reveal reactive plasma cells (with the artefactual exceptions detailed in the Results section) which stain for more than a single class of light chain, indicating the monospecificity of the anti-light chain antisera. Additional proof of the specificity of the immunoenzymatic reactions came from the use of the labelled antigen technique, which is inherently of high specificity.

The specificity of staining after trypsinization of sections was assessed in the same way as the reaction of non-trypsinized samples. Trypsin treatment enhances the strength of immunoenzymatic staining (with the consequence that the primary antisera have usually to be used at higher dilutions than are optimal for non-trypsinized sections) but no evidence was obtained to suggest that proteolytic digestion reduced the specificity of staining.

Immunofluorescent staining. Unfixed cryosections were preincubated for 10 min in PBS containing 5% foetal calf serum (PBS–FCS) prior to incubation with antisera. Appropriate dilutions (1:5 to 1:10) of FITC–F(ab′)$_2$ fragments of anti-IgM, anti-IgD, anti-IgA, anti-κ and anti-λ (Kallested Laboratories, Stockholm) were used. FITC–F(ab′)$_2$ anti-IgG was obtained from the National Bacteriological Laboratory, Stockholm. The antisera were tested on bone marrow smears from myeloma patients and lymphoblastoid cell lines and proved monospecific at the dilutions used. The sections were incubated with antibodies at room temperature for 1 hr and subsequently washed three times in PBS–FCS before mounting in buffered glycerol. The slides were examined in a Zeiss microscope under epi-illumination at × 100 and × 40 magnification.

RESULTS

Fixation

When samples of human tonsil were fixed in a variety of fixatives (see Materials and Methods section) for periods of 2·5, 6, 22 and 72 hr and stained with a range of dilutions of anti-κ and anti-λ light chain antisera (1/1,000–1/16,000) by the PAP procedure, cytoplasmic Ig was found to be well preserved in formol sublimate, Zenker- and Bouin-fixed material. The period of fixation had little effect on the intensity of staining. Bouin-fixed material showed a higher degree of background staining, probably representing extracellular Ig. Cellular detail (particularly in the nuclei) was optimal in sublimate-fixed samples. Trypsinization did not enhance staining of sublimate or Bouin-fixed material, although it reduced the background staining associated with Bouin's fixation. Enzymatic digestion diminished the reactivity of Zenker-fixed samples.

Table 1. Immunoperoxidase staining of paraffin-embedded human lymphoma samples

Authors	No. of cases	Fixation	Result	Comments
Non-Hodgkin's Lymphoma				
Taylor, 1974, 1976, 1978	358	Formalin, Zenker's B-5 or unknown	Definite monoclonal staining in 107 cases. Anomalous staining (i.e. for more than one heavy or light chain) in 40 cases	Only limited data given on heavy and light chain staining patterns
Garvin *et al.*, 1974, 1976	33	Buffered formalin or glutaraldehyde/formalin/ calcium acetate	Positive staining for IgG in 3 cases	No positive IgM or IgA staining. Anti-light chain sera not used. Routine formalin-fixed material stained poorly
Davey *et al.*, 1978 Halliday *et al.*, 1978	22	Bouin's or buffered formalin	Positive staining for IgM in 6 cases and for IgG in 6 cases	Anti-light chain sera not used
Isaacson & Wright,* 1978	10	No data	Staining for γ, α, μ, κ & λ chains observed in (?) all cases. Lysozyme also present	All tumours were intestinal lymphomas. Results taken as evidence of histiocytic origin of tumours
Li & Harrison, 1978	74	No data	37 cases Ig-positive	Anti-heavy chain sera not used. No data on κ/λ patterns
Warnke *et al.*, 1978	11	Bouin's and neutral formalin	2 cases showed monoclonal staining. 1 case showed polyclonal (κ & λ) staining	Anti-heavy chain sera not used. 10 cases shown to be monoclonal by immunofluorescent staining of cryostat sections
Zulman *et al.*, 1978	9	No data	6 cases stained for IgMκ	All cases were of lymphoma supervening on Sjögren's syndrome

Hodgkin's disease				
Taylor, 1974, 1976, 1978	35	Formalin, Zenker's B-5 or unknown	Both κ and λ chains found in R–S cells. Occasional case showed monoclonal pattern	No heavy chain data
Garvin *et al.*, 1974, 1976	50	Buffered formalin or glutaraldehyde/formalin/ calcium acetate	IgG present in H & R–S cells in 25 cases. IgM infrequently present	Positive R–S cells found in 10/10 optimally fixed samples. 15/40 routine formalin-fixed samples
Landaas *et al.*, 1977	42	Formalin	IgG in H & R–S cells in 15 cases and IgM in 1/15. IgA-, IgD- and IgE-negative. Both κ and λ chains demonstrated in same cells	
Curran & Jones,* 1978b and c	17	Formalin, formalin/ paraformaldehyde or glutaraldehyde/formalin/ calcium acetate	IgG in H & R–S cells in 12 cases. Both κ and λ found in same cells	Concluded that R–S and H cells are follicle centre dendritic macrophages
Papadimitriou *et al.*, 1978	103	Formalin	H & R–S cells contained IgG (75 cases), IgD (28 cases) IgM (9 cases) and IgA (6 cases). Albumin and α_1-anti-chymotrypsin found in 12 cases	5 cases showed monoclonal Ig in H & R–S cells. Concluded that data provided 'evidence for both a lymphatic and a macrophage origin' of R–S cells
Poppema *et al.*, 1978	42	Bouin's, Zenker's or formalin	IgG, serum albumin and α_1-anti-trypsin found in R–S cells. Both κ and λ chains found in same cell	Concluded that Ig enters R–S cells by non-specific diffusion

H cell = Hodgkin's cell; R–S cell = Reed–Sternberg cell.

* Sections required digestion with trypsin for satisfactory immunoperoxidase staining.

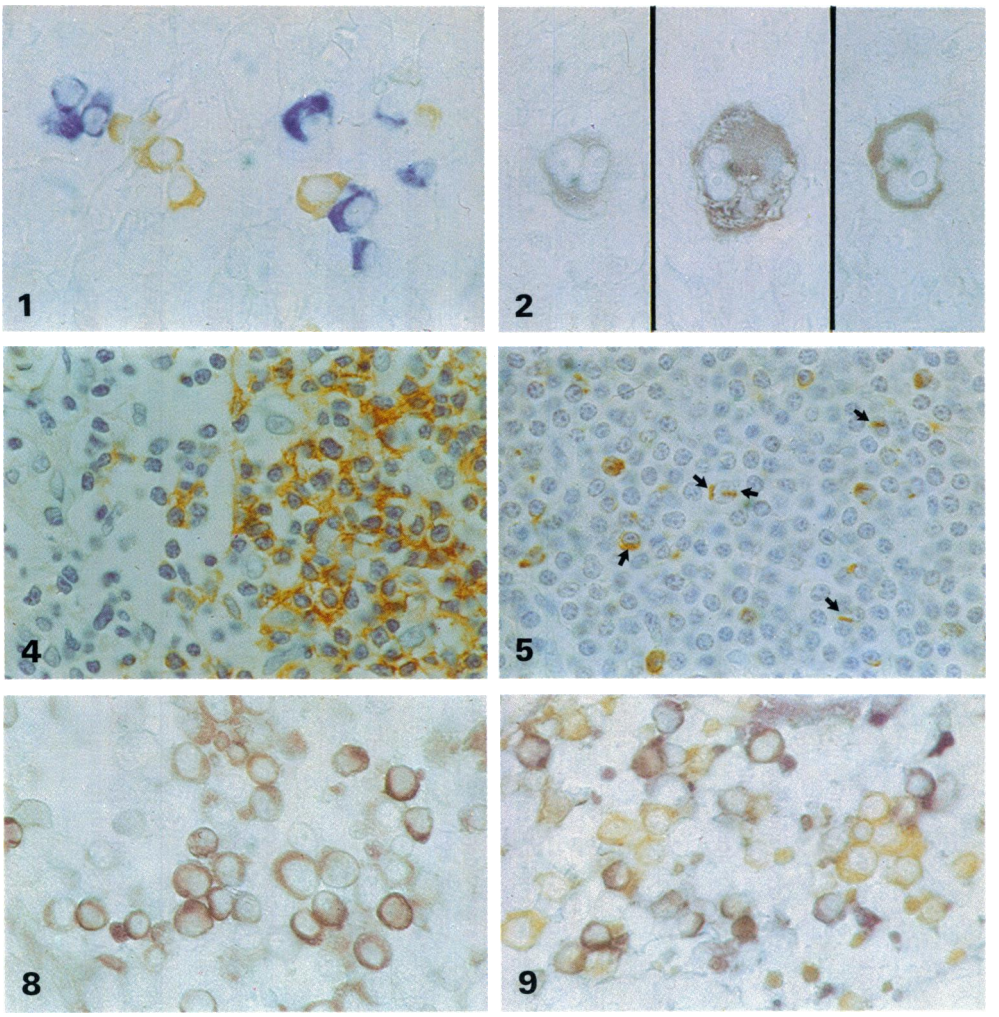

Fig. 1. Double staining of plasma cells in a Hodgkin's lymph node section (trypsinized before staining) for κ chains (brown) and λ chains (blue).

Fig. 2. Hodgkin's cells from the same section as Fig. 1 showing mixed staining indicating the presence of both light chains.

Fig. 4. High power view of the edge of a neoplastic nodule from the case of lymphoma illustrated in Fig. 3. Note the strong staining for extracellular μ chains within the nodule, contrasting with negative-staining of adjacent non-involved tissue. (N.B. Both Figs 4 and 5 show immunoperoxidase staining for a *single* antigen).

Fig. 5. Immunoperoxidase staining of a non-Hodgkin's lymphoma (Case 2). Small rod shaped IgMλ inclusions are present in several cells.

Fig. 8. Double labelling of a non-Hodgkin's lymphoma sample (Case 9—see Fig. 7) for κ chains (blue) and λ chains (brown). Positive cells stain for both light chain classes with a mixed colour reaction. (Compare with reactive plasma cells in Fig. 1 which show the pure blue and brown colour reactions.)

Fig. 9. Double labelling of same case for IgG (blue) and albumin (brown). The majority of positive cells stain for both proteins or for albumin alone.

Plasma cells in formol saline-fixed tissue gave inconsistent results, but often contained no detectable Ig or small clumps of Ig lying close to the nucleus. The poor results in formol saline-fixed material compared to formol sublimate were not due to the difference in pH between these two fixatives since adjustment of the pH of formol saline to that of formol sublimate (pH 3·0) did not improve cellular Ig reactivity. It was possible consistently to reverse the negative or incomplete staining pattern observed in formol saline-fixed sections by trypsinization prior to staining.

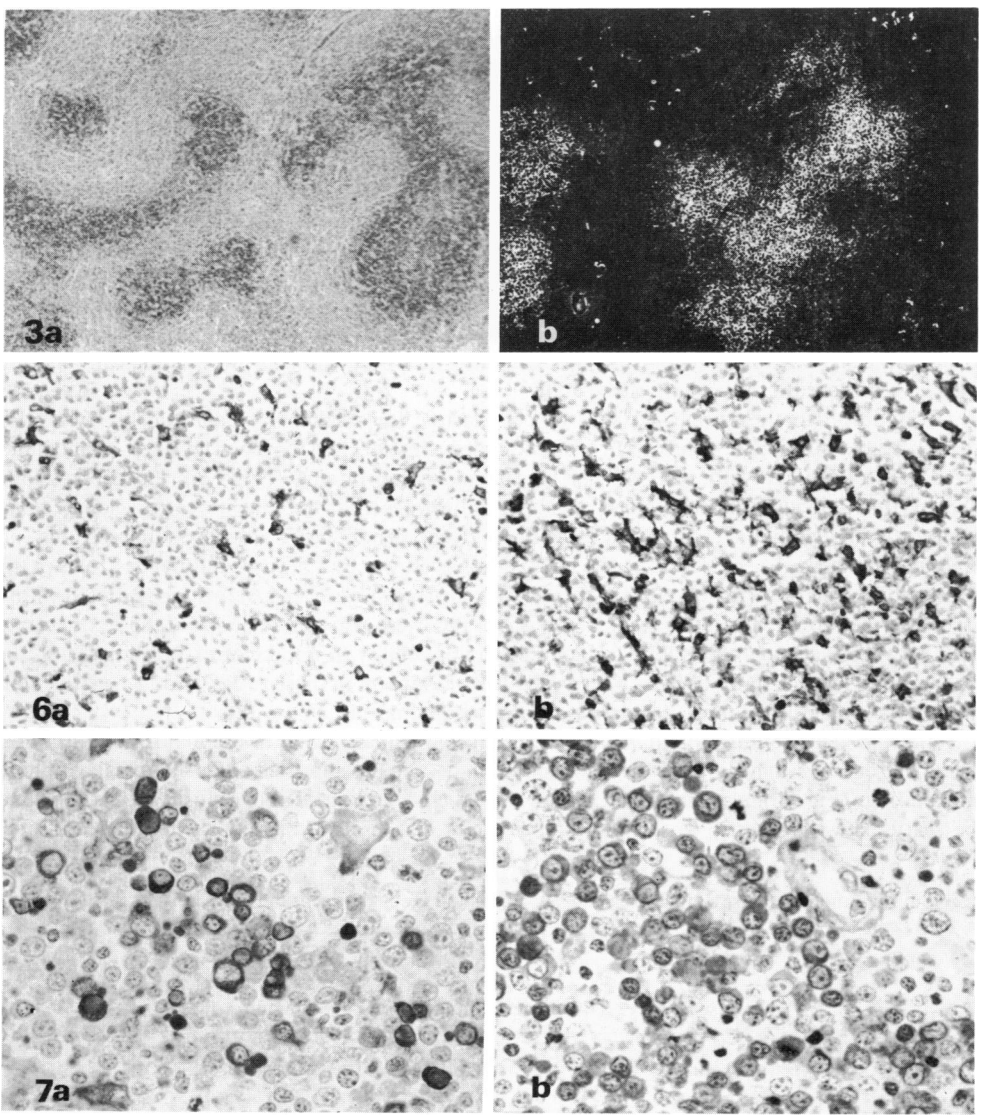

Fig. 3. Staining of μ chains in (a) a non-Hodgkin's lymphoma sample (Case 1) by the immunoperoxidase technique, and in (b) a cryostat section from the same node by direct immunofluorescence. Note the similar nodular staining pattern in both figures. IgMκ detected by immunoperoxidase staining was predominantly extracellular (see Fig. 4).

Fig. 6. Immunoperoxidase staining for IgG (a) and for albumin (b) in a non-Hodgkin's lymphoma sample (Case 10) showing scattered strongly positive cells, mostly of histiocytic morphology, against a negative background.

Fig. 7. Immunoperoxidase staining of a non-Hodgkin's lymphoma sample (Case 9) stained for IgG (a) and for albumin (b). Scattered positive cells of lymphoid morphology are present.

As a result of these studies formol sublimate and B5 (formol sublimate buffered with sodium acetate) were adopted as the fixatives of choice for this study and the majority of the results reported below were obtained using untrypsinized sections of tissue fixed in this way.

Immunoenzymatic staining patterns

Immunoglobulin. This was readily demonstrated, as expected from previous work, in the cytoplasm of normal plasma cells and in occasional normal follicle centre cells. IgG was also demonstrated in Reed–Sternberg and Hodgkin's cells (Table 2) and double immunoenzymatic staining for κ and λ chains (Figs 1 & 2) confirmed the polyclonal nature of this immunoglobulin (Taylor, 1976; Landaas *et al.*, 1977; Curran & Jones, 1978c). In a case of angioimmunoblastic lymphadenopathy large IgG-positive cells resembling Reed–Sternberg cells were plentiful and these also showed a mixed staining pattern for κ and λ light chains.

Seven cases of non-Hodgkin's lymphoma (Table 3) stained for IgM of a single light chain class, whilst in one further case (No. 5) μ chains unassociated with light chains were demonstrated. This staining showed a number of different patterns. In one case (No. 1) IgM was present extracellularly lying in strands between the cells of neoplastic follicles (Figs 3 & 4). In the remaining seven cases Ig labelling was localized in the cytoplasm. In one of these cases (No. 2) many cells contained rod-shaped inclusions of IgMλ (Fig. 5) while cells in another case showed coarse granular cytoplasmic inclusions. In a further case (No. 3) IgMκ was present in the cytoplasm of clearly neoplastic lymphoid cells, but the same pattern was seen also in a population of morphologically mature plasma cells beneath the capsule of the node.

In addition to these expected sites of cytoplasmic Ig labelling, cytoplasmic IgG was also demonstrated in some sections within typical dendritic histiocytes (Fig. 6a) and small- to medium-sized cells of typical lymphoid (but not plasmacytoid) morphology (Fig. 7a). Double labelling showed that individual cells contained both light chain types (Fig. 8). Labelling of polyclonal IgG in histiocytes and lymphoid cells was a very variable phenomenon, being absent in some sections and frequent in others. A very characteristic finding, however, was that positive cells tended to lie in the vicinity of vessels or shrinkage clefts or in poorly fixed areas of tissue and were often scattered within circumscribed zones. Small extracellular clumps of IgG were also characteristic of these zones (Fig. 7a). However, although the staining suggested the possibility of uptake of extracellular IgG, the background level of stainable IgG was usually weak or absent in these zones, and Ig-negative cells were always plentiful among the positive-staining cells (Fig. 7a).

Albumin. This serum protein was detectable intracytoplasmically in the majority of sections, being present in both histiocytes (Fig. 6b) and in small- and medium-sized cells of typical lymphoid morphology (Fig. 7b). As with the polyclonal IgG labelling described above, positive cells tended to be scattered within zones close to shrinkage clefts or vessels, or in poorly fixed areas, often

Table 2. Immunoperoxidase staining of Reed–Sternberg and Hodgkin's cells in Hodgkin's biopsies

		Staining reactions	
Case no.	Histological type	IgG	Albumin
1	Lymphocyte-predominant	Pos.	Neg.
2	Lymphocyte-predominant	Pos.	Neg.
3	Lymphocyte-predominant	Pos.	Neg.
4	Granulomatous	Pos.	Neg.
5	Nodular sclerosing	Pos.	Pos.
6	Mixed cellular	Pos.	Weakly pos.
7	Mixed cellular	Pos.	Weakly pos.

Samples 1–6 were of formol saline-fixed material and sections were trypsinized prior to staining. Sample 7 was formol sublimate-fixed and trypsinization was not necessary.

Table 3. Immunohistological staining of non-Hodgkin's lymphoma samples

	Immunofluorescent staining		Immunoperoxidase staining*		Histological diagnosis	
Case no.	Ig class	Pattern	Ig class	Pattern	Rappaport	Kiel
Immunoperoxidase-positive cases						
1	μ, δ, κ	N, MA	μ, κ	N, extracellular	Mixed lymphocytic 'histiocytic', D	Centrocytic
2	μ, δ, λ	D, MA	μ, λ	S, Cyto., rod-shaped inclusions	Lymphocytic, D	Lymphoplasmacytoid
3	μ, κ	D, MA	μ, κ	S, Cyto.	'Histiocytic', D+N	Centrocytic/blastic
4	μ, λ	D, MA+Cyto.	μ, λ	S, Cyto., coarsely granular	Poorly differentiated lymphocytic, D	Centrocytic
5	μ, λ	D, MA	μ	S, Cyto., perinuclear	Well differentiated lymphocytic, D	Centrocytic/blastic
6	μ, κ	D, MA+Cyto.	μ, κ	C, Cyto.	Poorly differentiated lymphocytic, D	Centrocytic/blastic
7	μ, κ	N, MA	μ, κ	C, Cyto.	Well differentiated lymphocytic, D	Centrocytic
8		n.t.	μ, λ	N, Cyto.+?extracellular	'Histiocytic', D+N	Centroblastic
Immunoperoxidase-negative cases						
9		n.t.		Neg.	Mixed lymphocytic 'histiocytic', D	Centrocytic/blastic
10	μ, δ, λ	D, MA		Neg.	'Histiocytic', N+D	Centrocytic/blastic
11	μ, δ, κ	D, MA		Neg.	'Histiocytic', N+D	Centrocytic
12	γ, κ	M+D, MA+?Cyto.		Neg.	'Histiocytic', D	Centroblastic
13	μ, κ	N+D, MA		Neg.	Well differentiated lymphocytic, D	Centrocytic
14	$\mu (\kappa)$	N+D, MA+?Cyto.		Neg.	Poorly differentiated lymphocytic, D	Centrocytic
15	μ, κ	D, MA		Neg.	Well differentiated lymphocytic, D	Centrocytic/blastic
16	μ, κ	D+N, MA		Neg.	Mixed lymphocytic 'histiocytic', N	Lymphoepithelioid

C=clusters of positive cells; Cyto.=cytoplasmic; D=diffuse; MA=membrane-associated; N=nodular; n.t.=not tested; S=scattered positive cells.

* Staining results listed in this column refers to presumed endogenous lymphoma cell Ig. Labelling due to absorbed Ig (see text) is not included. All samples were fixed in B5 fixative or formol sublimate. Note that anti-IgD antiserum was not used for immunoperoxidase staining.

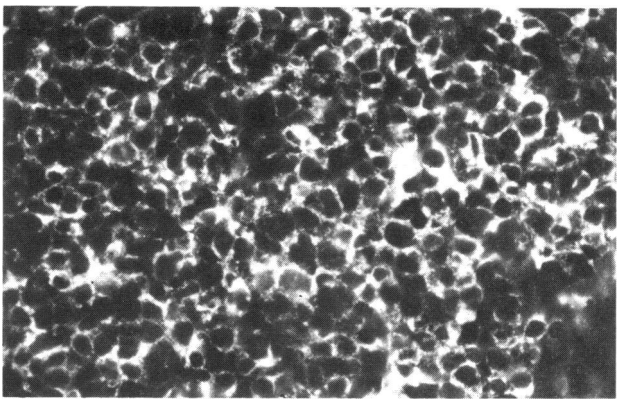

Fig. 10. Cryostat section of non-Hodgkin's lymphoma sample (Case No. 11) stained by direct immunofluorescence for μ chains showing a diffuse honeycomb pattern.

associated with small clumps of extracellular albumin. Double labelling of these cells for IgG and albumin showed that the majority of IgG-positive non-plasmacytoid cells in these areas also contained albumin (Fig. 9). There was usually in addition to the mixed cell component a considerable number of cells staining for albumin alone. Very few non-plasmacytoid cells in these regions stained for IgG and not for albumin.

In contrast to the frequency with which polyclonal IgG-containing lymphoid cells and histiocytes stained for albumin, IgG-positive Reed–Sternberg cells and Hodgkin's cell rarely contained albumin (Table 2). The same pattern was seen in the case of angioimmunoblastic lymphadenopathy described above in which Reed–Sternberg-like cells containing IgG but not albumin were plentiful.

Immunofluorescent staining patterns

Immunofluorescent staining of cryostat sections revealed a positive reaction for a single light chain type in each of fourteen cases tested (Table 3). The predominant heavy chain type was μ (13/14 cases) associated in four instances with δ chains. A single case showed γ chain staining. The $\kappa:\lambda$ ratio was 8:5. Ig appeared to be membrane-associated, giving a honeycomb or lace-like pattern in all positive cases (Fig. 10). In four cases a variable number of cells were also present which appeared to be cytoplasmically labelled for monoclonal Ig.

DISCUSSION

This immunoenzymatic study of paraffin-embedded tissue from sixteen cases of non-Hodgkin's lymphoma revealed the presence of monoclonal immunoglobulin in half the samples. In each case the Ig was IgM in class, a finding in keeping with the fact that IgM is the first Ig to be produced as immature B cells differentiate towards plasma cells. In seven cases a single class of light chain was detected, and these results were in agreement with the light chain classes as determined by direct immunofluorescent staining of cryostat sections. The fact that immunoperoxidase staining was not observed in seven cases which showed monoclonal immunofluorescent labelling is presumably due to the fact that fixation and paraffin embedding tends to denature or mask membrane Ig, so that usually only cases in which cytoplasmic Ig is present can be stained by immunoperoxidase techniques.

The immunoperoxidase-positive cases showed a variety of staining patterns, an aspect which has received little attention in published reports. Although in most cases positive cells were scattered through the section, two samples showed clusters of monoclonal cells. Small cytoplasmic inclusions, granular in one case and rod-shaped in another, were found in two samples. Such inclusions differed from the larger inclusions (e.g. Russell and Dutcher bodies) reported previously in immunoperoxidase studies of B cell lymphomas (van den Tweel *et al.*, 1978), although crystalline rod-shaped

inclusions have been demonstrated in cell smears from cases of chronic lymphocytic leukaemia (Cawley *et al.*, 1976; Feremans, Neve & Caudron, 1978). A further case differed from previously reported patterns in that the IgM was apparently extracellular. One case showed IgM of the same light chain type in both immature neoplastic cells and in mature plasma cells, a phenomenon which has been described as an occasional occurrence in follicle centre neoplasms by Taylor (1978), but has not been noted by other authors.

In addition to revealing clearly monoclonal staining reactions for IgM in eight cases of non-Hodgkin's lymphoma, this study also elucidated two important causes of artefactual staining which are probably relevant to the conflicting and unexpected results reported in published immunoperoxidase studies of human lymphoma (Table 1).

Fixation artefact

It is apparent that the fixative most commonly used in previous studies (formol saline—Garvin *et al.*, 1976; Taylor, 1976, 1978; Landaas *et al.*, 1977; Davey *et al.*, 1978; Halliday *et al.*, 1978; Isaacson & Wright, 1978; Li & Harrison, 1978; Papadimitriou, Stein & Lennert, 1978; Warnke *et al.*, 1978) gives unreliable results. Plasma cell immunoglobulin is frequently unstainable after this fixation; when reactivity survives it is frequently limited to a small area of cytoplasm close to the nucleus. This latter phenomenon, initially described by Curran & Jones (1977), is reversed by trypsin digestion of the section, whereupon Ig in more peripheral parts of the cell cytoplasm is revealed. This 'masking' phenomon is avoided by the use of fixatives such as formol sublimate or B5. The superiority of formol sublimate has been reported independently in an immunoperoxidase study of human intestinal biopsies by Bosman *et al.* (1977).

Serum protein uptake

A second potential problem in the interpretation of lymphoma cell staining was revealed when sections were stained for serum albumin. This protein was shown to be present in many cells (both lymphoid and histiocytic) in which polyclonal IgG had been demonstrated. In addition, albumin was also found in IgG-negative cells. The most likely explanation for these findings is that serum proteins are non-specifically absorbed by these cells, either before or during fixation, and then remain within the cytoplasm during subsequent dehydration and embedding. In support of this interpretation is the more ubiquitous pattern of albumin staining relative to IgG, in keeping with its higher serum concentration and lower molecular weight, both of which features would enhance its cellular penetration. Furthermore, the tendency of positive cells to lie in zones close to vessels or to clefts in tissue suggests that extravasation of serum and its penetration through poorly fixed tissue allows cytoplasmic absorption to take place. The clumps of extracellular albumin and IgG seen in these regions suggest the presence of free extracellular protein which precipitates during fixation. Identification of these characteristic histological features of cellular staining due to absorbed serum IgG was found to be of value in recognizing the phenomenon.

Reassessment of previous immunoperoxidase studies

The absorption of serum proteins by non-Hodgkin's lymphoma cells as a cause of misleading immunohistological staining has not previously been reported. In retrospect, however, this phenomenon may offer an explanation for reports of neoplastic (Taylor, 1978), as well as normal (Hartmann & Liacopoulos, 1977), lymphoid cells containing both kappa and lambda light chains, and also for descriptions of IgG within lymphoma cells (Garvin *et al.*, 1976; Davey *et al.*, 1978; Halliday *et al.*, 1978; Isaacson & Wright, 1978). It should be noted that in many published reports (Table 1) details of the heavy and light chain staining patterns have been incomplete or even absent (Li & Harrison, 1978) making it difficult to assess the significance of the results. Since non-Hodgkin's lymphoma cell Ig is usually IgM in type (see above) immunohistological staining of IgG in lymphoma cells should always raise the suspicion that it is of exogenous origin and this possibility should be investigated by double staining for kappa and lambda light chains and for IgG and albumin (Figs 1, 8 & 9).

It may be noted that strong immunohistological staining for absorbed intracellular serum proteins is not confined to lymphoid tissue, since similar phenomena have been observed in

squamous (Coruh & Mason, 1980) and columnar epithelium (Bell, Piris & Mason, 1979), in cells in the central nervous system (Esiri, Taylor & Mason, 1976) and in hepatocytes (Guillouzo *et al.*, 1978). A striking fact in all these instances is that positive cells are not associated with generalized background staining for extracellular proteins (which would give a clue as to the origin of the cytoplasmic proteins). We attribute this to the fact that extracellular proteins are eluted, denatured or masked during fixation and embedding. In contrast those proteins which have penetrated within the cell cytoplasm are relatively protected from this process. Consequently the positive cells stand out against an artificially negative background. When less harsh fixation methods are employed (e.g. Sainte Marie's cold ethanol technique) extracellular IgG in inflamed tissues is well preserved, and indeed positive plasma cells may be difficult to identify against this background (Brandtzaeg, 1974). We have also noted that trypsin treatment may unmask extracellular IgG in the vicinity of IgG-positive squamous epithelial cells (Coruh & Mason, 1980).

Reed–Sternberg cell IgG

Cells in lymphoma sections which stain for IgG because of non-specific uptake of serum proteins may be contrasted with Reed–Sternberg and Hodgkin's cells (as well as the similar cells seen in the case of angioimmunoblastic lymphadenopathy) which were found to contain polyclonal IgG associated with little or no albumin. This IgG may be acquired by a process of selective Fc receptor-mediated uptake (Kadin *et al.*, 1978) rather than by passive non-specific diffusion of serum proteins into the cell cytoplasm. Our results are in accord with those of Papadimitriou *et al.* (1978) and conflict with the findings of Poppema *et al.* (1978), who observed serum proteins (albumin and α_1-anti-trypsin) in IgG-positive Reed–Sternberg and Hodgkin's cells. We favour the interpretation that Hodgkin's cells are predominantly albumin-free *in vivo*, but that variable artefactual uptake of serum proteins may occur in some cases during tissue processing, its extent presumably being governed by factors such as tissue cellularity, concentration of extravascular proteins, histological type, etc. It is of interest that the case of Hodgkin's disease in the present study in which albumin was most clearly demonstrable in Reed–Sternberg cells was of the nodular sclerosing variety. In the study cited above by Poppema *et al.* (1978), in which albumin-positive Reed–Sternberg cells were frequent, this class of Hodgkin's disease predominated.

CONCLUSIONS

The present study has demonstrated the importance of three aspects of immunoenzymatic labelling of paraffin-embedded human lymphoma biopsies. Firstly, 'masking' of lymphoma cell Ig (and the necessity for trypsin digestion of sections before staining) can be avoided by the use of fixatives such as formol sublimate. Secondly, positive staining due to exogenous IgG uptake is a common phenomenon which can be recognized by staining for other serum proteins such as albumin and/or by double immunoenzymatic labelling. Finally, immunoenzymatic labelling of paraffin-embedded sections should be supplemented when possible by immunofluorescent labelling for membrane Ig on cryostat sections. In keeping with the findings of Warnke & Levy (1978) the latter technique was found to provide a rapid method for demonstrating the monoclonal B cell origin of many non-Hodgkin's lymphomas with a higher positivity rate than immunoperoxidase staining (Warnke *et al.*, 1978). Only by the combined use of both immunohistological techniques can the membrane and cytoplasmic Ig of human lymphoma cells be adequately characterized.

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