

Demonstration of light chain monotypia in B cell non-Hodgkin's lymphomas using unfixed freeze-dried and formalin-fixed trypsinised paraffin sections

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SUMMARY Immunohistological light chain analysis was carried out in 55 patients with non-Hodgkin's lymphoma of B cell origin. Unfixed freeze-dried paraffin sections were used to detect surface Ig and formaldehyde-fixed trypsinised sections to detect cytoplasmic Ig. Cytoplasmic Ig was seen inconstantly in freeze-dried paraffin sections. There was complete agreement with regard to the type of light chain between freeze-dried and formaldehyde-fixed trypsinised sections. Immunohistology showed the monotypic immunoglobulin light chains in 83% of cases when unfixed freeze-dried paraffin sections were used for sIg demonstration while only in 45% of cases when formaldehyde-fixed material was used for cIg detection. The efficiency of sIg demonstration in unfixed freeze-dried paraffin sections was comparable with that based on unfixed cryostat sections or on cell suspensions when the lymphocytic lymphoma/CLL group was excluded from the evaluation. The relatively low frequency of monotypic sIg positivity obtained in this group (12/19) is due to the low density of surface Ig in CLL lymphocytes.

Since the majority of non-Hodgkin's lymphomas are of B cell lineage, a variety of techniques have been developed to document light chain monotypia in these neoplasms.

Cell suspension techniques often yield spurious results mostly due to non-representative aliquots of the neoplasm and contamination with residual non-neoplastic or reactive cells. Cell suspension studies have been reported to be unable to establish light chain monotypia in nearly half the cases of B cell lymphomas in which immunohistological analysis was capable of establishing the monoclonal nature of the proliferation.¹

A more useful method is to study sections of tissue. This allows assessment of the topographical relation between the neoplastic and non-neoplastic components. Immunohistological techniques after proteolytic digestion of routinely fixed paraffin section seem to be the most sensitive and reproducible methods for detecting cytoplasmic immunoglobulins (cIg) and other cytoplasmic antigens in tissue^{2,3} while surface immunoglobulins (sIg) are not detected by this method.^{4–6} Surface immunoglobulins have been reliably detected only on cryostat sections of fresh tissue,^{7–10} on cryostat sections of tissue

preserved in transport medium^{11,12} or on unfixed freeze-dried paraffin sections.¹³ The latter method yields improved tissue morphology compared to cryostat sections.

In this report, we present the results of immunohistological light chain analysis in a series of patients with non-Hodgkin's lymphomas of B cell origin. We were interested in knowing: (i) the percentage of cases with monotypic sIg on unfixed freeze-dried paraffin sections; (ii) the percentage of patients with monotypic cIg on formaldehyde-fixed paraffin sections following standardised trypsinisation and (iii) the agreement between the two methods with regard to the type of light chains

Material and methods

Fresh lymph node biopsies, spleens and palatine tonsils have been studied from 55 patients with non-Hodgkin's lymphomas of B cell origin. The histological classification was performed according to the Kiel classification system.¹⁴ The biopsies have been split into two parts: one part used for freeze-drying and the other fixed in 4% phosphate-buffered formaldehyde (prepared from paraformaldehyde).

FORMALDEHYDE-FIXED TISSUES

Blocks of tissue (approx 5 mm × 5 mm × 3 mm) were fixed in formaldehyde for 6 h at room temperature. The tissues were washed overnight in physiological saline and processed to paraffin as follows: 50% acetone (30 min), 100% acetone (60 min × 3), xylene (15 min × 3). Vacuum paraffin embedding was performed for 30 min at 55–56°C. Paraffin sections (5 μm) were cut, deparaffinised in xylene followed by acetone. These sections were used for staining methods (haematoxylin and eosin and Giemsa), and also for the immunohistological detection of cIg.

UNFIXED FREEZE-DRIED TISSUES

Blocks of tissue (approx 5 mm × 5 mm × 3 mm) were snap frozen in dry ice isopentane mixture and freeze-dried for 36 h in an Edwards-Pearse EP D2 tissue dryer (–45°C, 10⁻² Torr, in the presence of P₂O₅). The freeze-dried tissue was slowly warmed to 20°C and brought to atmospheric pressure prior to embedding. Vacuum paraffin embedding was performed for 30 min at 55–56°C. Paraffin sections (5 μm) were cut, deparaffinised in xylene followed by acetone and washed in Tris-saline for 5 min prior to immunohistology.

TRYPsinISATION

Formaldehyde-fixed sections were incubated at 37°C for 15 min in Tris-HCl buffer (pH 7.8) containing 0.1% trypsin (Difco 1/250) and 0.1% CaCl₂.

INDIRECT IMMUNOFLOUORESCENCE (IF) TECHNIQUE

Sections were washed in 0.05 M Tris-HCl buffer (pH 7.4) and incubated for 30 min at room temperature with specific antisera diluted in Tris-HCl buffer containing 1% bovine serum albumin. Rabbit anti-human antisera were used in dilutions 1/100 (freeze-dried material) and 1/400 (formaldehyde-fixed material). After 20 min washing in Tris-HCl buffer, the sections were incubated for 45 min with FITC-labelled polyvalent swine antirabbit Ig diluted 1/50 in Tris HCl buffer. The sections were mounted in glycerol-Tris-saline. Immunofluorescence was viewed in a Leitz Orthoplan microscope equipped with Ploemopak epi-illuminator and narrow band filter system. Control sections were regularly examined with normal rabbit serum instead of specific antisera.

PEROXIDASE-ANTIPEROXIDASE (PAP) TECHNIQUE

For inhibition of endogenous peroxidase activity sections were treated for 30 min with a mixture of methanol and H₂O₂ at 22°C (99 ml methanol + 1 ml

30% H₂O₂). The sections were rinsed in Tris-HCl buffer and subsequently incubated for 30 min at room temperature with specific antisera diluted in Tris-HCl buffer containing 1% bovine serum albumin. Rabbit antihuman antisera were used in dilutions 1/100 (freeze-dried material) and 1/400 (formaldehyde-fixed material). After 20 min washing in Tris-HCl buffer the sections were incubated in a 1/40 diluted polyvalent swine antirabbit Ig for 45 min. This step was succeeded by 20 min washing in Tris-HCl buffer and an incubation in peroxidase-antiperoxidase complex diluted 1/100 in Tris-HCl buffer for 45 min. Sections were subsequently washed in Tris-HCl buffer. The peroxidase activity was demonstrated with 3,3'-diaminobenzidine reaction.¹⁵ Control sections were routinely examined with normal rabbit serum instead of specific antisera.

ANTISERA AND REAGENTS

Rabbit antisera specific to human kappa and lambda light chains, polyvalent swine antirabbit Ig, polyvalent swine antirabbit Ig FITC-labelled, normal rabbit serum and rabbit PAP complex were obtained from DAKO-immunoglobulins Ltd (Copenhagen, Denmark). 3,3'-diaminobenzidine free base was purchased from Sigma Chemical Co. (St Louis, Mo, USA).

INTERPRETATION OF IMMUNOHISTOLOGICAL RESULTS

A case was regarded as sIg-positive when the majority of cells showed sIg positivity. For a case to be classified as cIg positive, a minimum number of 5% positive cells was required.

Results

The distribution of cases between the different histological groups of the Kiel classification system and the results of light chain analysis obtained by IF on unfixed freeze-dried sections and those obtained by IF or PAP technique on formaldehyde-fixed trypsinised sections, is shown in Table 1.

In all surgical biopsies and necropsy cases giving a monotypic light chain reaction there was complete agreement with regard to the type of light chain between freeze-dried and formaldehyde-fixed trypsinised sections.

The immunohistological demonstration of sIg was successful in freeze-dried sections but that of cIg was unreliable. Distinction between sIg and cIg positivity was difficult when the cytoplasmic rim of the cell was narrow. Furthermore, it was often felt that the penetration of antisera into the cytoplasm of unfixed freeze-dried cells was restricted to the periphery of

Table 1 Monotypic (M) and polytypic (P) light chain reactivity of sIg (unfixed freeze-dried paraffin sections) and cIg (formaldehyde-fixed trypsinised sections) in relation to histological groups

Diagnosis	sIg	cIg	No of cases	No of necropsy cases
ML lymphocytic/CLL	M	M	4/19	1
	M	—	6/19	
	M	P	2/19	
	P	P	2/19	
	—*	P	2/19	
	—*	—	3/19	
ML immunocytic, lymphoplasmacytoid	M	M	6/7	2
	—	M	1/7	
ML immunocytic, lymphoplasmacytic	M	M	1/1	1
ML immunocytic, polymorphic	M	M	2/4	
	M	P	1/4	
	—	P	1/4	1
ML centrocytic	M	—	3/3	
ML centroblastic-centrocytic	M	M	4/7	1
	M	—	3/7	
ML centroblastic	M	M	3/4	
	M	—	1/4	
ML immunoblastic	M	M	4/5	1
	M	—	1/5	
Burkitt's lymphoma	M	—	3/3	
Hairy cell leukaemia	M	—	2/2	

*In the peripheral blood, lymphocytes were sIg-positive by direct immunofluorescence utilising F(ab')₂ fragments of rabbit antihuman immunoglobulin antiserum.

the cytoplasm, giving a marginal staining pattern instead of diffuse cytoplasmic staining (Fig. 9a). Thus, the sIg reactivity indicated in Table 1 may overlap cIg staining. In formaldehyde-fixed trypsinised sections, however, IF or PAP techniques demonstrated only cIg reactivity.

Immunohistological results of light chain analysis in various histological groups were as follows:

Malignant lymphoma lymphocytic/CLL

As shown in Table 1, 12 of 19 patients gave monotypic staining for sIg in unfixed freeze-dried sections (Figs. 1a and b). Kappa chain was detected in nine cases and lambda chain in three cases. In addition to monotypic sIg reactivity, seven cases revealed moderate cIg staining of the same light chain type in plasmacytoid lymphocytes. Such cells occurred focally and usually did not exceed 2% of the population. Polytypic sIg was observed in two cases. Polytypic cIg, usually in intensely stained mature plasma cells, was seen in four cases. In formaldehyde-fixed trypsinised sections, monotypic cIg (exceeding 5% of the population) was demonstrated in four patients, polytypic cIg in six patients with IF or PAP methods (Fig. 2).

Malignant lymphoma immunocytic, lymphoplasmacytoid

All cases had monotypic cIg positivity in formaldehyde-fixed trypsinised sections with IF or PAP methods (Figs. 3a and b). Kappa chain was detected in five cases, lambda chain in two cases.

The earliest site of Ig accumulation is in the perinuclear space. Further accumulation at this site may indent the nucleus giving rise to intranuclear inclusions (Dutcher bodies)(Figs. 4a and b). If the endoplasmic reticulum is the site of Ig accumulation, the staining pattern is often granular. This is seen in most extreme form as intracytoplasmic inclusions (Russell bodies). Six of seven cases showed monotypic sIg as well as cIg in unfixed freeze-dried material.

Malignant lymphoma immunocytic, lymphoplasmacytic

There was monotypic sIg staining (lambda) in lymphocytes and a moderate cIg staining in plasma cells in unfixed freeze-dried material (Fig. 5). The plasma cells showed lambda chain positivity in formaldehyde-fixed trypsinised sections.

Malignant lymphoma immunocytic, polymorphic

Three out of four patients showed monotypic sIg positivity in unfixed freeze-dried sections. Kappa chain was demonstrated in two, lambda chain in one case. Monotypic cIg positivity was detected in one biopsy material (lambda) and polytypic cIg positivity in a necropsy case. In formaldehyde-fixed trypsinised sections cIg positivity was evident in all cases but monotypic light chain composition was found only in two cases (Figs. 6a and b)

Malignant lymphoma centrocytic

This group consists of biopsies from three patients. They all contained monotypic sIg in unfixed freeze-

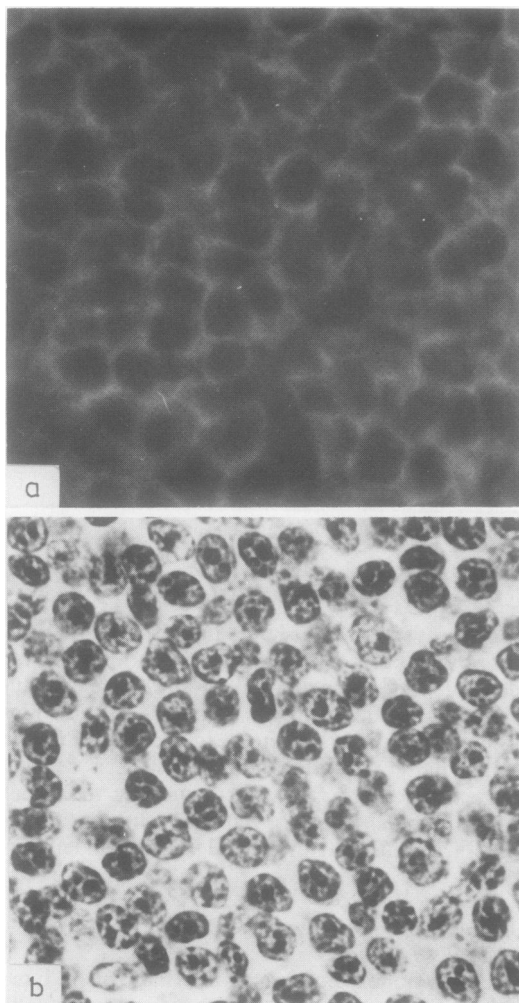


Fig. 1 (a) *Malignant lymphoma lymphocytic/CLL. Kappa chain detection by IF on freeze-dried paraffin section. Weak positivity on the surface of cells. $\times 800$* (b) *Formaldehyde-fixed section stained by haematoxylin and eosin. Small lymphocytes have regular round nuclei with coarse granular chromatin. $\times 800$*

dried sections (kappa). No cIg positivity was observed either in unfixed freeze-dried or formaldehyde-fixed trypsinised sections (Figs. 7a, b and c).

Malignant lymphoma centroblastic-centrocytic

All of the seven cases showed monotypic sIg positivity whereas true cIg positivity was not observed in unfixed freeze-dried paraffin sections. Kappa chain was found in four, lambda chain in three cases. In the nodular type, the monotypic sIg pattern was

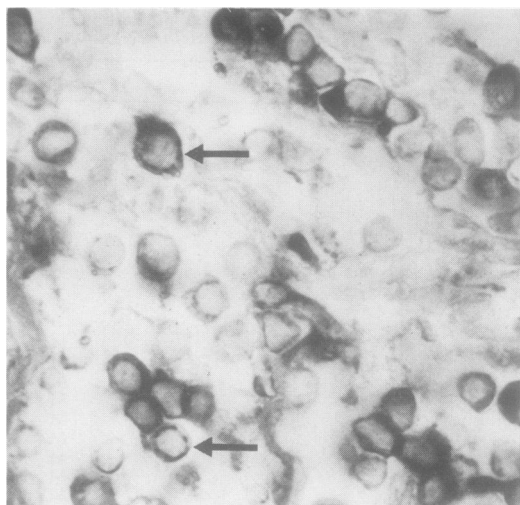


Fig. 2 *Malignant lymphoma lymphocytic/CLL. Kappa chain detection by PAP on formaldehyde-fixed trypsinised section. A number of cytoplasmic lymphocytes show cIg positivity. The earliest site of Ig accumulation is the perinuclear space (arrows) $\times 800$*

restricted to the nodules (Figs. 8a and b). In formaldehyde-fixed trypsinised sections monotypic cIg positivity was established in four cases.

Malignant lymphoma centroblastic

All of the four cases showed monotypic sIg positivity (Figs. 9a and b). Kappa chain was found in two cases and lambda chain in two cases. Unequivocal cIg positivity was observed only in scattered plasma cells in two cases in freeze-dried material. In formaldehyde-fixed trypsinised sections, three of the four cases gave monotypic cIg positivity in more than 5% of the cell population. The majority of these cells were large blast cells, plasma cells were inconspicuous.

Malignant lymphoma immunoblastic

In unfixed freeze-dried sections, all of the five cases showed monotypic sIg positivity in immunoblasts. Kappa chain was detected in four, lambda chain in one case. True cIg positivity was not observed in these cells. In formaldehyde-fixed trypsinised sections, however, the majority of immunoblasts gave monotypic cIg positivity in four of the five cases studied (Figs. 10a and b).

Malignant lymphoma Burkitt type

All of the three cases revealed monotypic sIg staining in unfixed freeze-dried sections. Kappa chain was detected in one, lambda chain in two cases. No cIg positivity was observed either in unfixed freeze-dried or formaldehyde-fixed trypsinised sections.

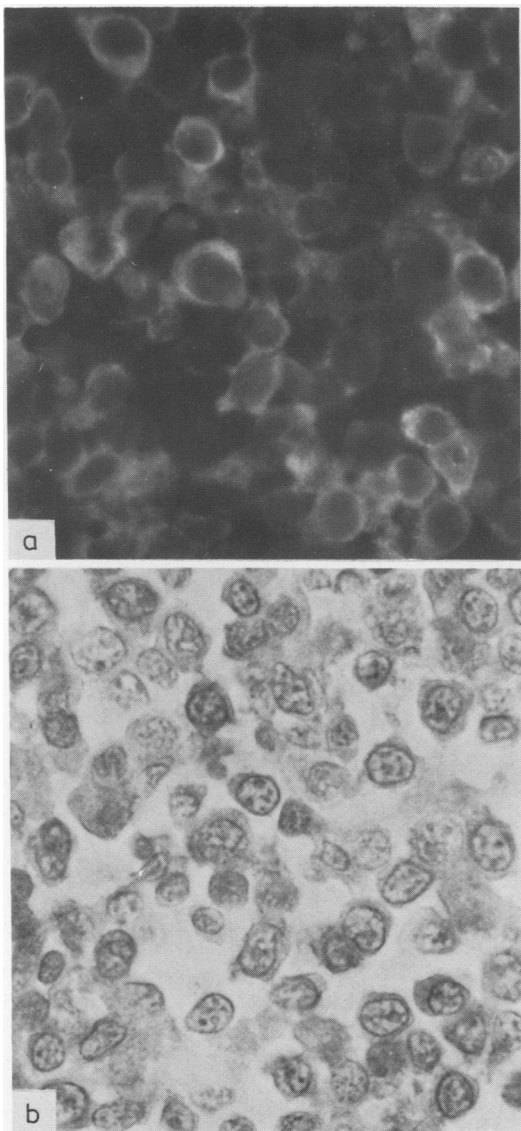


Fig. 3 (a) Malignant lymphoma immunocytic, lymphoplasmacytoid. Lambda chain detection by IF on formaldehyde-fixed trypsinised section. Plasmacytoid lymphocytes show various amounts of cIg. Higher amounts of cIg appear granular. $\times 800$ (b) Identical section slightly post-stained by haematoxylin and eosin. Monotonous picture of plasmacytoid lymphocytes. $\times 800$

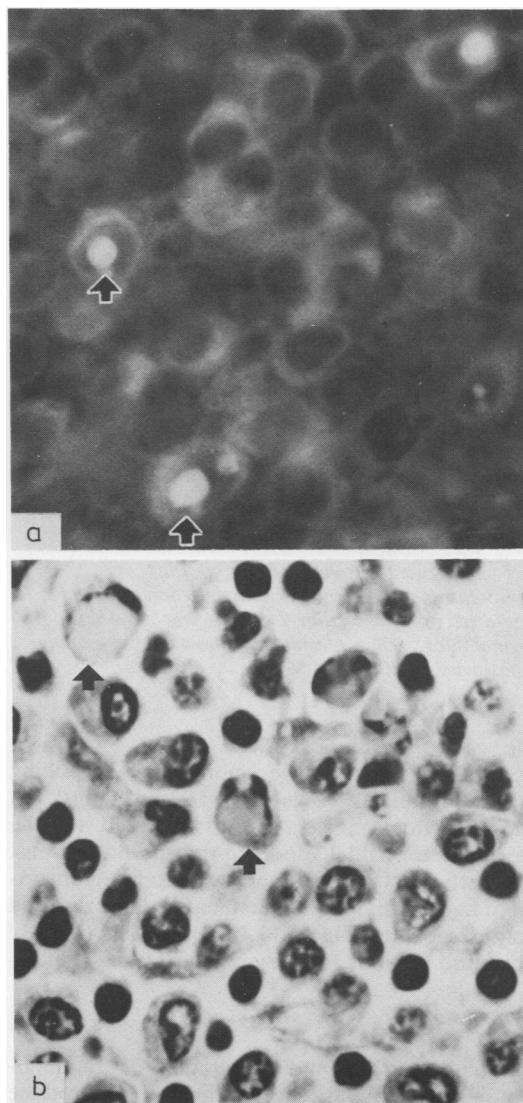


Fig. 4 (a) Malignant lymphoma immunocytic, lymphoplasmacytoid. Kappa chain detection by IF on formaldehyde-fixed trypsinised section. Plasmacytoid cells contain moderate amounts of cIg and occasional intensely fluorescent intranuclear inclusions (arrows). $\times 800$ (b) Adjacent section stained by haematoxylin and eosin. The plasmacytoid lymphocytes have ample cytoplasm, eccentric nuclei with a cartwheel chromatin pattern. Some of the cells show nuclear inclusions (Dutcher bodies) indicated by arrows. $\times 800$

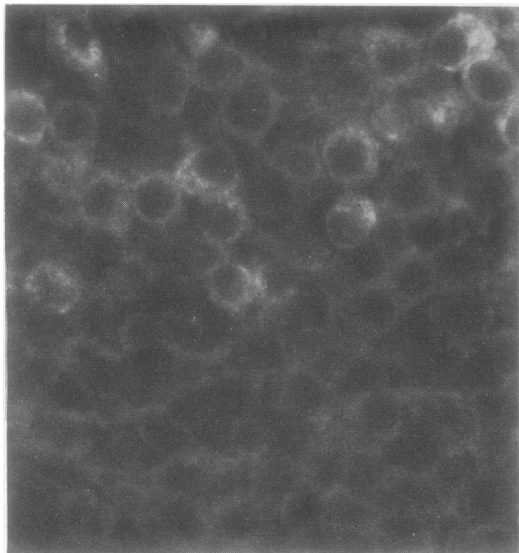


Fig. 5 Malignant lymphoma immunocytic, lymphoplasmacytic. Lambda chain detection by IF on freeze-dried paraffin section. Lymphocytes show weak marginal fluorescence, plasma cells show intense granular cIg positivity. $\times 800$

Hairy cell leukaemia

All of the two cases showed monotypic sIg staining (kappa) in unfixed freeze-dried sections (Figs. 11a and b). Cytoplasmic Ig positivity could not be detected in formaldehyde-fixed trypsinised sections.

The efficiency of immunohistological demonstration of light chain monotypia in unfixed freeze-dried paraffin sections (sIg) and in formaldehyde-fixed trypsinised sections (cIg) is summarised in Table 2.

Discussion

Monotypic surface and/or cytoplasmic immunoglobulins in lymphoid cells have been considered the criterion by which the B cell nature of neoplastic lymphoid cell populations is established. Immunohistological methods on fixed paraffin embedded tissues, although preferable in terms of convenience and morphology, are limited to the detection of cytoplasmic constituents, including cIg,⁴⁻⁶ while surface immunoglobulins are reliably detected in unfixed tissue.^{7-10 13}

Table 2 Occurrence of light chain monotypia in non-Hodgkin's lymphomas of B cell lineage

sIg positive	46/55	(83%)
cIg positive	25/55	(45%)
Total positive (sIg and/or cIg)	47/55	(85%)

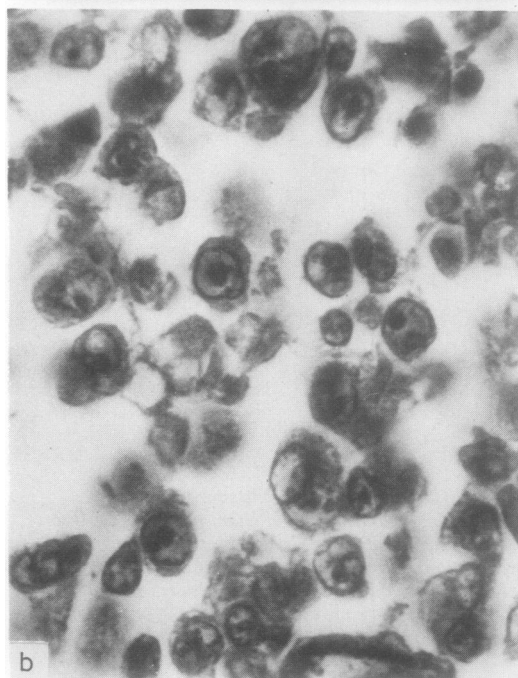
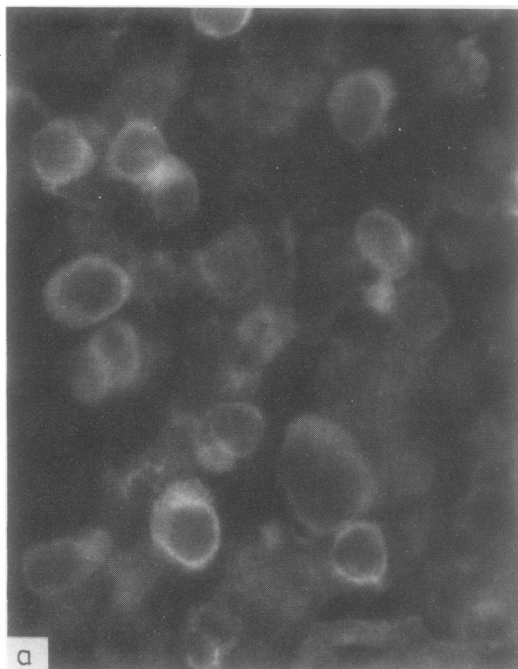


Fig. 6 (a) Malignant lymphoma immunocytic, polymorphic. Kappa chain detection by IF on formaldehyde-fixed trypsinised section. Immunocytes have stronger, large blasts weak cIg positivity. $\times 800$ (b) Identical section slightly post-stained by haematoxylin and eosin. Immunocytes, centrocytes and large blasts with irregular nuclei (centroblasts). $\times 800$

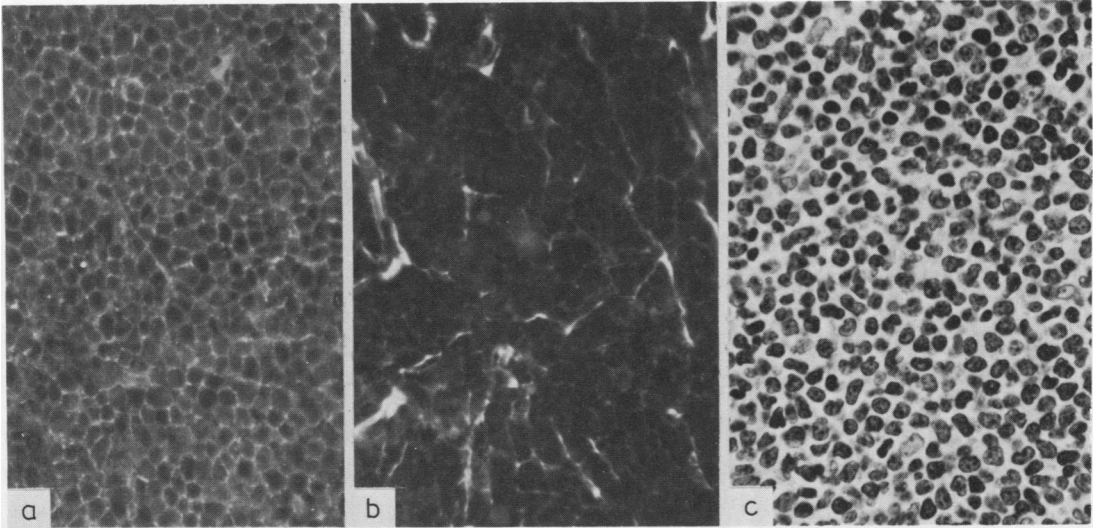


Fig. 7 (a) Malignant lymphoma centrocytic. Kappa chain detection by IF on freeze-dried paraffin section. Strong sIg positivity on all cells. $\times 200$ (b) Adjacent section stained by IF for lambda chains. Only the connective tissue framework is stained. $\times 200$ (c) Formaldehyde-fixed section stained by haematoxylin and eosin. Monotonous field of centrocytes with nuclear irregularities and dense granular chromatin structure. $\times 200$

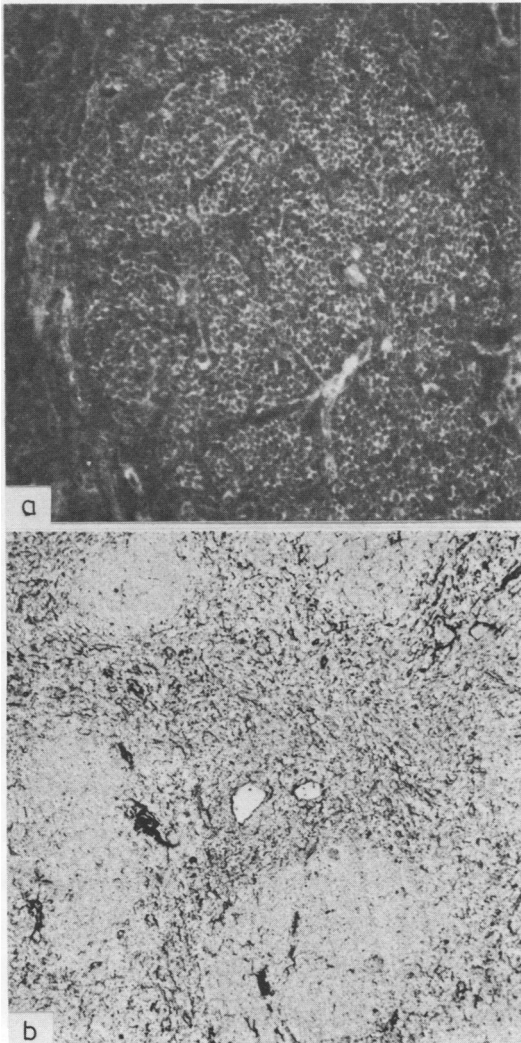


Fig. 8 (a) Malignant lymphoma centroblastic-centrocytic, nodular. Kappa chain detection by IF on freeze-dried paraffin section. Intense sIg positivity restricted to the nodule. $\times 130$ (b) Formaldehyde-fixed section stained with Gomori's reticulin technique. The nodular architecture is clearly indicated. $\times 50$

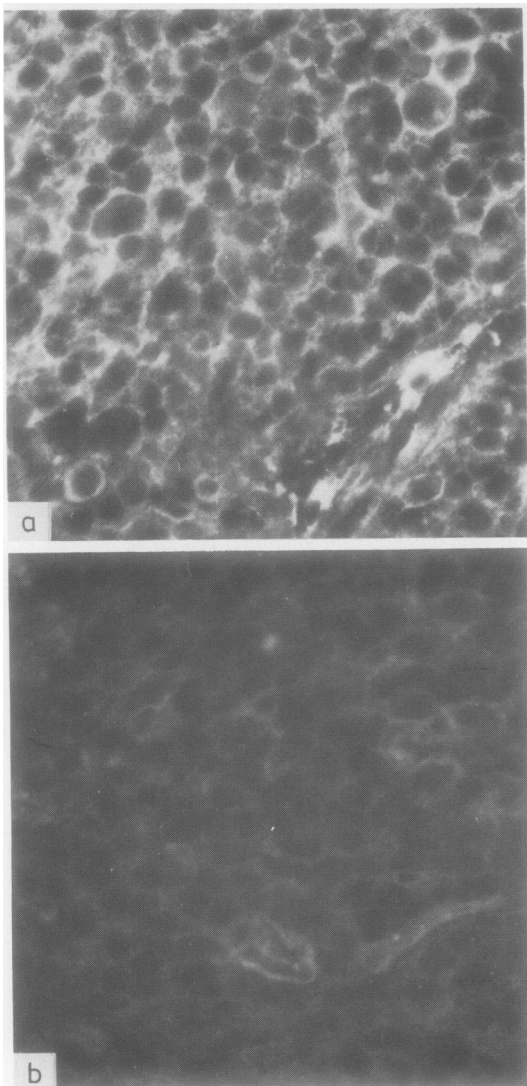


Fig. 9 (a) Malignant lymphoma centroblastic. Lambda chain detection by IF on freeze-dried paraffin section. Intense marginal staining of large and smaller cells. $\times 400$ (b) Adjacent section stained by IF for kappa chain. Slight background staining. Small vessels are moderately stained $\times 400$

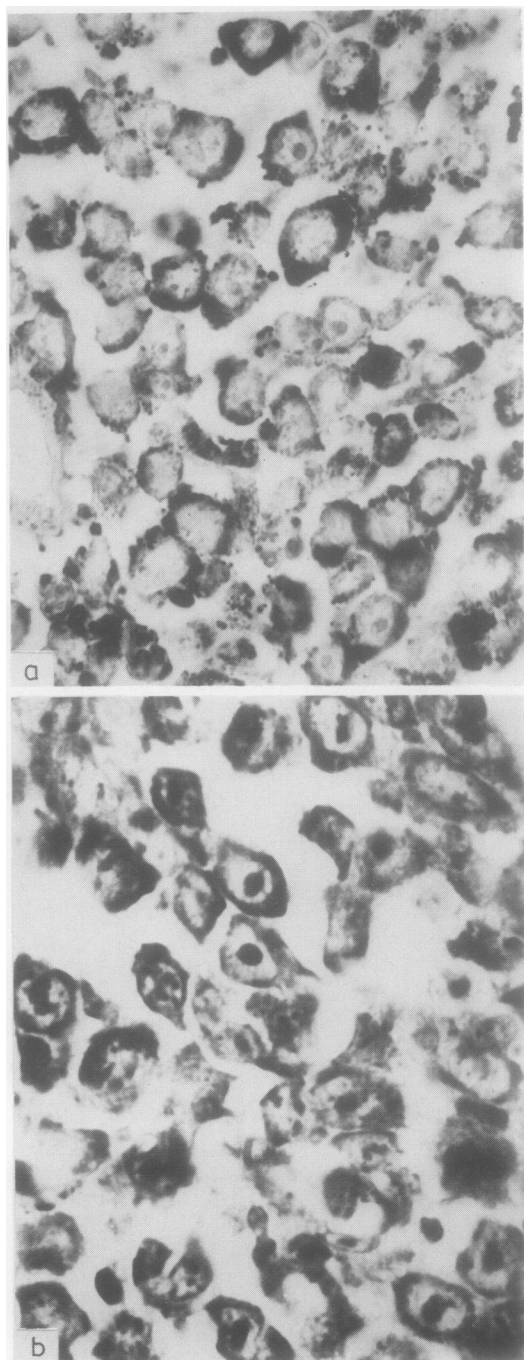


Fig. 10 (a) Malignant lymphoma immunoblastic. Kappa chain detection by PAP on formaldehyde-fixed trypsinised section. Various amounts of granular cIg in immunoblasts. $\times 800$ (b) Adjacent section stained by Giemsa. Immunoblasts have strongly basophilic cytoplasm, vesicular nuclei with prominent central nucleoli. $\times 800$

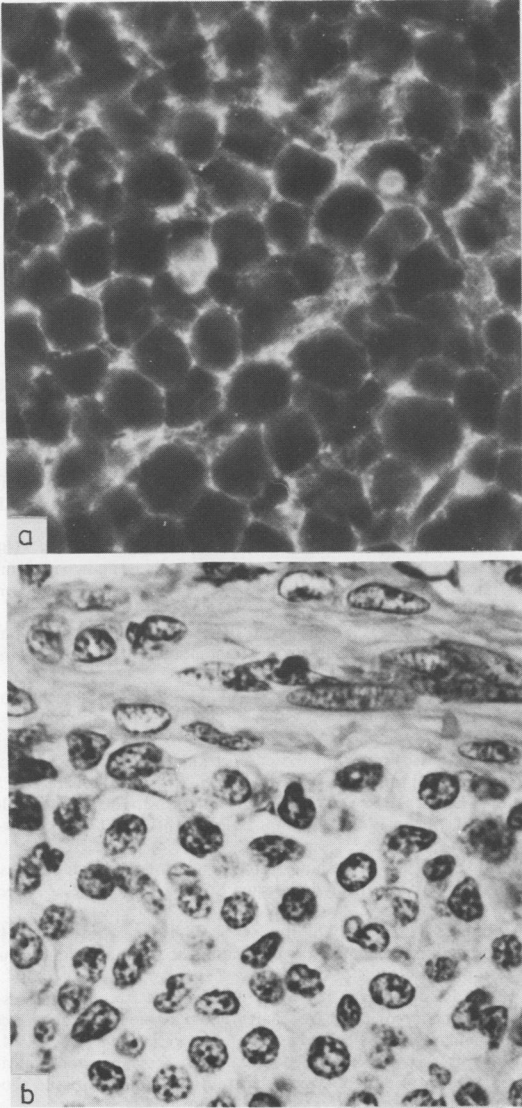


Fig. 11 (a) Hairy cell leukaemic infiltration in the spleen. Kappa chain detection by IF on freeze-dried paraffin section. Intense sIg positivity on all cells. $\times 800$ (b) Formaldehyde-fixed paraffin section stained by haematoxylin and eosin. Uniform small cells with granular nuclear chromatin and indistinct cytoplasm surround a splenic arteriole (top) $\times 800$

Most of the immunohistological studies on the relative frequency of cIg positive cases in B cell non-Hodgkin's lymphomas rely on undigested paraffin sections.^{5,16-19} The immunohistological staining of formalin-fixed tissue without proteolytic digestion is insensitive and capricious.^{3,20} Comparative immunohistological studies employing efficient methods for detecting the monotypic light chain composition of sIg and cIg in B cell non-Hodgkin's lymphomas are lacking. The present study is based on unfixed freeze-dried paraffin sections to detect sIg¹³ and on formaldehyde-fixed trypsinised sections to detect cIg.²

Out of 55 B cell lymphomas 46 (83%) revealed monotypic sIg positivity by IF in unfixed freeze-dried sections. IF has been found slightly superior to PAP to detect sIg on cryostat sections²¹ or on unfixed freeze-dried paraffin sections.¹³ The efficiency of the method can only be determined in comparison with studies based on a collection of cases with a similar distribution of histological types. Using cell suspensions, 56/66 (85%) revealed monotypic sIg staining in a collection of B cell non-Hodgkin's lymphomas similar to ours with regard to the distribution of histological types.²² Another study, based on the direct immunoperoxidase technique and unfixed cryostat sections yielded 29/30 (96%) cases with monotypic staining pattern.¹⁰ The ratio of lymphocytic lymphoma/CLL group to other histological types cannot be determined from this publication. If such cases are excluded from our material 34/36 (94%) shows monotypic sIg positivity which is superior to the efficiency of direct immunofluorescence and cell suspension employed on a similarly corrected material²²: 50/60 (83%).

The relatively low proportion of cases with monotypic sIg positivity in the lymphocytic lymphoma/CLL group (12/19) is due to the constant low density of sIg in CLL (tenfold decrease when compared to normal lymphocytes²³). This group has well defined cytomorphological features and it is easily distinguished from immunocytomas by histological staining methods. Immunohistology, however, reveals scattered plasmacytoid lymphocytes in 7 of 12 sIg positive cases showing cIg of the same light chain type as the sIg on the majority of lymphocytes in unfixed freeze-dried sections. The proportion of such cells exceeded 5% of the population in formaldehyde-fixed trypsinised sections only in four cases. Such cases are classified as LP immunocytomas by the Lennert group.^{24,20}

We did not reclassify these cases as immunocytomas on the basis of immunohistology. These four cases can be taken as borderline cases between lymphocytic lymphoma/CLL and lympho-

plasmacytoid immunocytoma. The relatively high frequency of monotypic sIg positive cases with occasional cIg positive cells (7/12) is a characteristic feature of the lymphocytic lymphoma/CLL group. In most patients with CLL, the leukaemic B lymphocytes are "frozen" at a relatively early stage of maturation. In some of the patients, however, some leukaemic lymphocytes are able to escape the block and to mature into secreting plasma cells.²⁵⁻²⁷ It is hard to explain why cIg positive cells are so efficiently detected in unfixed freeze-dried material in this group while cIg in other histological types of non-Hodgkin's lymphoma tends to give sIg-like marginal staining in unfixed freeze-dried sections. Therefore the presence of cIg positive cells can only be reliably detected in formaldehyde-fixed trypsinised sections.

The constant monotypic cIg positivity in LP immunocytomas (lymphoplasmacytoid and lymphoplasmacytic) is in agreement with the findings of others using the same technique.^{3,22} Malignant lymphoma immunocytic, polymorphic cannot be compared with other studies since these cases are probably included in the follicle centre cell lymphomas²⁸ or if they are composed predominantly of large cells they are termed as immunoblastic lymphomas.

Malignant lymphoma centrocytic is characterised by a strong monotypic sIg positivity and lack of cIg. The same pattern is found in Burkitt's lymphoma and in hairy cell leukaemia.

Malignant lymphoma centroblastic-centrocytic showed constant monotypic sIg positivity and cIg positivity in four out of seven patients. This ratio of cIg positive cases is less than 81% found in follicular centroblastic-centrocytic lymphomas by Stein *et al*²⁰ or 68% found in follicle centre cell lymphomas by Isaacson *et al*³ but is higher than that found by Landaas *et al*²²: 26% of follicular and 33% of diffuse centroblastic-centrocytic lymphomas.

Malignant lymphoma centroblastic revealed constant monotypic sIg positivity and three of four cases with cIg positivity. The latter ratio is similar to the 68% found in centroblastic lymphomas by Stein *et al*²⁰ or to the 68% found in follicle centre cell lymphomas by Isaacson *et al*³ but exceeds the 40% obtained in centroblastic lymphomas by Landaas *et al*.²²

Malignant lymphoma immunoblastic yielded constant monotypic sIg positivity and cIg positivity in four of five cases. The latter ratio is among the best in the literature: 15/30 found by Stein *et al*,²⁰ 11/16 found by Landaas *et al*²² and 4/4 found by Isaacson *et al*.³

It is noteworthy that immunohistology by itself provided confirmatory evidence for the diagnosis of B cell lymphoma in 83% of cases when unfixed

freeze-dried paraffin sections were used, while only in 45% of cases when an improved technique for cIg demonstration was used on formaldehyde-fixed material. The sIg demonstration in unfixed freeze-dried paraffin sections has special diagnostic advantage in the following diagnostic groups: malignant lymphoma centrocytic, Burkitt's lymphoma and hairy cell leukaemia.

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