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. Immunohistological showed the monotypic immunoglobulin light chains in 83% of cases when unfixed freeze-dried paraffin sections were used for slg demonstration while only in 45% of cases when formaldehyde-fixed material was used for clg detection. The efficiency of slg 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 slg 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.

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 (clg) and other cytoplasmic antigens in tissue while surface immunoglobulins (slg) are not detected by this method. Surface immunoglobulins have been reliably detected only on cryostat sections of fresh tissue, on cryostat sections of tissue preserved in transport medium or on unfixed freeze-dried paraffin sections. 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 slg on unfixed freeze-dried paraffin sections; (ii) the percentage of patients with monotypic clg 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. 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 clg.

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 IMMUNOFLUORESCENCE (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 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. 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 slg-positive when the majority of cells showed slg positivity. For a case to be classified as slg 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 slg was successful in freeze-dried sections but that of clg was unreliable. Distinction between slg and clg 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
Demonstration of light chain monotypia in B-cell non-Hodgkin’s lymphomas

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

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>slg</th>
<th>clg</th>
<th>No of cases</th>
<th>No of necropsy cases</th>
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<tbody>
<tr>
<td>ML lymphocytic/CLL</td>
<td>M</td>
<td>M</td>
<td>4/19</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>—</td>
<td>6/19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>P</td>
<td>2/19</td>
<td></td>
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<td></td>
<td>P</td>
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<td>2/19</td>
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<td>P</td>
<td>2/19</td>
<td></td>
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<tr>
<td></td>
<td>—</td>
<td>—</td>
<td>3/19</td>
<td>1</td>
</tr>
<tr>
<td>ML immunocytic, lymphoplasmacytoid</td>
<td>M</td>
<td>M</td>
<td>6/7</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>—</td>
<td>1/7</td>
<td>1</td>
</tr>
<tr>
<td>ML immunocytic, lymphoplasmacytoid</td>
<td>M</td>
<td>M</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>ML immunocytic, polymorphic</td>
<td>M</td>
<td>M</td>
<td>2/4</td>
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<td>M</td>
<td>M</td>
<td>4/7</td>
<td>1</td>
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<tr>
<td>ML centroblastic</td>
<td>M</td>
<td>M</td>
<td>3/7</td>
<td></td>
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<tr>
<td>ML immunoblastic</td>
<td>M</td>
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<td>3/4</td>
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<tr>
<td></td>
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<td>—</td>
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<tr>
<td>Burkitt’s lymphoma</td>
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<td>3/3</td>
<td></td>
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<tr>
<td>Hairy cell leukaemia</td>
<td>M</td>
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<td>2/2</td>
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*In the peripheral blood, lymphocytes were slg-positive by direct immunofluorescence utilising F(ab’), fragments of rabbit antihuman immunoglobulin antiserum.

the cytoplasm, giving a marginal staining pattern instead of diffuse cytoplasmic staining (Fig. 9a). Thus, the slg reactivity indicated in Table 1 may overlap clg staining. In formaldehyde-fixed trypsinised sections, however, IF or PAP techniques demonstrated only clg 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 slg 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 slg reactivity, seven cases revealed moderate clg staining of the same light chain type in plasmacytoid lymphocytes. Such cells occurred focally and usually did not exceed 2% of the population. Polytypic slg was observed in two cases. Polytypic clg, usually in intensely stained mature plasma cells, was seen in four cases. In formaldehyde-fixed trypsinised sections, monotypic clg (exceeding 5% of the population) was demonstrated in four patients, polytypic clg in six patients with IF or PAP methods (Fig. 2).

**Malignant lymphoma immunocytic, lymphoplasmacytoid**

All cases had monotypic clg 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 (Dutch 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 slg as well as clg in unfixed freeze-dried material.

**Malignant lymphoma immunocytic, lymphoplasmacytoid**

There was monotypic slg staining (lambda) in lymphocytes and a moderate clg 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 slg positivity in unfixed freeze-dried sections. Kappa chain was demonstrated in two, lambda chain in one case. Monotypic clg positivity was detected in one biopsy material (lambda) and polytypic clg positivity in a necropsy case. In formaldehyde-fixed trypsinised sections clg 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 slg in unfixed freeze-
Malignant lymphoma lymphocytic/CLL
Kappa chain detection by IF on freeze-dried paraffin section. Weak positivity on the surface of cells. × 800
Formaldehyde-fixed section stained by haematoxylin and eosin. Small lymphocytes have regular round nuclei with coarse granular chromatin. × 800

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 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.
Demonstration of light chain monotypia in B-cell non-Hodgkin's lymphomas

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. × 800 (b) Identical section slightly post-stained by haematoxylin and eosin. Monotonous picture of plasmacytoid lymphocytes. × 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). × 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. × 800
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. × 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, while surface immunoglobulins are reliably detected in unfixed tissue.

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

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<td>sIg positive</td>
<td>46/55</td>
<td>(83%)</td>
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<tr>
<td>cIg positive</td>
<td>25/55</td>
<td>(45%)</td>
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<tr>
<td>Total positive</td>
<td>47/55</td>
<td>(85%)</td>
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(Oral and/or cIg)

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(Oral and/or cIg)
Fig. 7 (a) Malignant lymphoma centrocytic. Kappa chain detection by IF on freeze-dried paraffin section. Strong sIg positivity on all cells. × 200 (b) Adjacent section stained by IF for lambda chains. Only the connective tissue framework is stained. × 200 (c) Formaldehyde-fixed section stained by haematoxylin and eosin. Monotonous field of centrocytes with nuclear irregularities and dense granular chromatin structure. × 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. ×130 (b) Formaldehyde-fixed section stained with Gomori's reticulin technique. The nodular architecture is clearly indicated. × 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. × 400
(b) Adjacent section stained by IF for kappa chain. Slight background staining. Small vessels are moderately stained × 400

Fig. 10  (a) Malignant lymphoma immunoblastic. Kappa chain detection by PAP on formaldehyde-fixed trypsinised section. Various amounts of granular clg in immunoblasts. × 800 (b) Adjacent section stained by Giemsa. Immunoblasts have strongly basophilic cytoplasm, vesicular nuclei with prominent central nucleoli. × 800
Demonstration of light chain monotypia in B-cell non-Hodgkin's lymphomas

Most of the immunohistological studies on the relative frequency of clg positive cases in B cell non-Hodgkin's lymphomas rely on undigested paraffin sections.\textsuperscript{5,16-19} The immunohistological staining of formalin-fixed tissue without proteolytic digestion is insensitive and capricious.\textsuperscript{3,20} Comparative immunohistological studies employing efficient methods for detecting the monotypic light chain composition of slg and clg in B cell non-Hodgkin's lymphomas are lacking. The present study is based on unfixed freeze-dried paraffin sections to detect slg\textsuperscript{13} and on formaldehyde-fixed trypsinised sections to detect clg.\textsuperscript{2}

Out of 55 B cell lymphomas 46 (83%) revealed monotypic slg positivity by IF in unfixed freeze-dried sections. IF has been found slightly superior to PAP to detect slg on cryostat sections\textsuperscript{21} or on unfixed freeze-dried paraffin sections.\textsuperscript{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 slg staining in a collection of B cell non-Hodgkin's lymphomas similar to ours with regard to the distribution of histological types.\textsuperscript{32} Another study, based on the direct immunoperoxidase technique and unfixed cryostat sections yielded 29/30 (96%) cases with monotypic staining pattern.\textsuperscript{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 slg positivity which is superior to the efficiency of direct immunofluorescence and cell suspension employed on a similarly corrected material: 50/60 (83%).

The relatively low proportion of cases with monotypic slg positivity in the lymphocytic lymphoma/CLL group (12/19) is due to the constant low density of slg in CLL (tenfold decrease when compared to normal lymphocytes\textsuperscript{23}). 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 slg positive cases showing clg of the same light chain type as the slg 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.\textsuperscript{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-

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Fig. 11 (a) Hairy cell leukaemic infiltration in the spleen. Kappa chain detection by IF on freeze-dried paraffin section. Intense slg 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
plasmacytoid immunocytoma. The relatively high frequency of monotypic sIg positive cases with occasional clg 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 clg positive cells are so efficiently detected in unfixed freeze-dried material in this group while clg 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 clg positive cells can only be reliably detected in formaldehyde-fixed trypsinised sections.

The constant monotypic clg 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 clg. The same pattern is found in Burkitt’s lymphoma and in hairy cell leukaemia.

Malignant lymphoma centroblastic-centrocytic showed constant monotypic sIg positivity and clg positivity in four out of seven patients. This ratio of clg positive cases is less than 81% found in follicular centroblastic-centrocytic lymphomas by Stein et al20 or 68% found in follicle centre cell lymphomas by Isaacson et al3 but is higher than that found by Landaas et al22: 26% of follicular and 33% of diffuse centroblastic-centrocytic lymphomas.

Malignant lymphoma centroblastic revealed constant monotypic sIg positivity and three of four cases with clg positivity. The latter ratio is similar to the 68% found in centroblastic lymphomas by Stein et al20 or to the 68% found in follicle centre cell lymphomas by Isaacson et al3 but exceeds the 40% obtained in centroblastic lymphomas by Landaas et al.22

Malignant lymphoma immunoblastic yielded constant monotypic sIg positivity and clg 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 al22 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 clg 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.

We are grateful to Mrs Gabriella Nagy for her excellent technical assistance.

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