

## THE MONOCLONALITY OF HUMAN B-CELL LYMPHOMAS\*

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A malignant tumor is thought to represent the proliferation of a clone of cells arising from a single transformed cell (1). Evidence for this concept has come from three different approaches: karyotype analysis, isoenzyme patterns, and immunoglobulin expression. All of these approaches have limitations, but when they have been employed, they have generally supported the concept of malignant growth being monoclonal. The only exceptions to this rule have been the tumors arising as a result of a dominant genetic trait, such as neurofibromatosis or trichoepitheliomata, where it is thought that multiple primary tumors exist side by side within the same tumor nodule (2, 3). Karyotype analyses of tumor cells have occasionally disclosed a stable chromosome marker that can serve to identify a particular clone of cells (4, 5). Isoenzyme patterns of tumor cells have been examined in patients who are female and heterozygous for an X-linked enzyme, such as glucose-6-phosphate dehydrogenase. Since, according to the Lyon hypothesis (6), each somatic cell expresses only one X chromosome, normal tissues of heterozygotes are composed of mosaics of cells expressing alternate forms of the enzyme. The finding of uniform expression of a single isoenzyme within a tumor cell population indicates that the entire population is derived from a single cell (1). Similarly, the immunoglobulin type of lymphoid malignancies can be examined for its uniformity among the tumor cell population (7-12). The immunoglobulin producing lymphoid neoplasms provide a special opportunity to test the concept of monoclonality. All of these tumors are suitable for analysis, not just the ones that have a definable chromosome marker or that occur in females who happen to be heterozygous for an X-linked enzyme.

Individual differentiated lymphoid cells are restricted in their potential for immunoglobulin expression and these restrictions are stable within their clonal descendants (13). These restrictions are somewhat more relaxed than was once believed to be the case. For instance, a single cell can give rise to cells that produce immunoglobulin of different heavy chain classes (14). However, the Ig products of the same clone all contain a single  $V_H$  and  $V_L$  region and a single light chain of either  $\kappa$  or  $\lambda$  type (13-19). Thus, the monoclonality of a population of malignant lymphoid cells can be inferred from its uniform restriction to a single light chain type.

In the example of Ig-secreting lymphoid tumors, such as multiple myeloma and Waldenström's macroglobulinemia, monoclonality is easily established by an examination of the secreted product. However, it has now become clear that a number of other types of lymphoid neoplasms produce, but do not necessarily secrete, Ig. The best-known example of the latter is chronic lymphocytic leukemia (CLL).<sup>1</sup> Here a number of careful

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<sup>1</sup> Abbreviations used in this paper: CLL, chronic lymphocytic leukemia; FCS, fetal calf serum;

studies have established that the Ig found on the surface of the CLL cell is produced by that cell and is restricted to a single light chain type (8, 9, 17-19). For example, in a given patient with CLL, all the leukemic cells will have either  $\kappa$  or  $\lambda$ -type Ig on their cell surface, whereas normal human lymphoid populations consist of a mosaic of  $\kappa$ -containing and  $\lambda$ -containing cells in a ratio of approximately 2:1. Interestingly, the same CLL cell may have cell surface Ig of both IgM and IgD, but both the IgM and the IgD share a common idiotype, indicating that a single heavy chain variable region can be joined to different constant regions (19). Hence, the monoclonality of CLL is well established.

In addition to CLL a number of other human lymphoproliferative disorders are characterized by the production, but rarely the secretion, of Ig. This growing list includes lymphosarcoma cell leukemia, hairy cell leukemia, well-differentiated lymphocytic lymphomas, nodular poorly differentiated lymphocytic lymphoma, and some cases of "histiocytic" lymphoma (9-12, 20). In some of these diseases there is evidence of monoclonality based on  $\kappa$  or  $\lambda$  uniformity of the tumor cells. In the cases of lymphoma (i.e., solid lymphoid tumors) the evidence comes from studies on cell suspensions derived from involved tissues (9-12, 20); however, when cell suspensions are prepared, one loses the information contained in the details of tissue architecture. As a consequence, only tissues that are massively replaced by tumor cells are suitable for such analysis, since in order to be apparent, the monoclonal population must perturb the normal  $\kappa/\lambda$  ratio. This same limitation applies to isoenzyme analyses, also usually performed in tissue homogenates.

In the present study we have applied techniques for immunofluorescent staining of lymphoma cells in tissue sections. The ability to examine the clonality of human lymphomas *in situ* should provide a more sensitive approach to the investigation of the immunobiology of these diseases.

## Materials and Methods

All chemicals were reagent grade. Pepsin was obtained from Worthington Biochemical Corp., Freehold, N. J. DEAE cellulose (DE52) was obtained from Whatman, Inc., Clifton, N. J. Acrylamide, bisacrylamide, TEMED (NNN'N' tetramethylethylenediamine) and CNBr were obtained from Eastman Kodak Co., Rochester, N. Y. Sepharose 4B and Sephadex were from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.

**Immunoabsorbents.** Immunoabsorbents were prepared by reacting proteins with CNBr-activated Sepharose 4B (21). 5-20 mg of protein were coupled to 1 g packed wet Sepharose. Antibodies were eluted from the absorbents with 0.1 N  $\text{NH}_4\text{OH}$ .

**Polyacrylamide Gel Electrophoresis.** Polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol and sodium dodecyl sulfate was performed in gradient slab cells as described by Laemmli (22).

### Immunoglobulin Reagents

All immunofluorescence studies were performed with  $\text{F}(\text{ab}')_2$  fragments of purified antibodies. These reagents were prepared as follows:

**IMMUNOGENS.** Human  $\kappa$  and  $\lambda$  Bence Jones proteins were isolated from the urine of patients (one each) with multiple myeloma by precipitation with  $(\text{NH}_4)_2\text{SO}_4$  at 55% saturation, followed by gel filtration on Sephadex G-75 superfine. These purified light chain preparations, when analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE), migrated in a single sharp band even on overloaded gels.  $\text{F}(\text{ab}')_2$  fragments of human  $\kappa$  and of human  $\lambda$  IgG myeloma proteins were prepared from a pool of seven sera each (obtained from Dr. M. Lahav, Sick Fund Organization, Central Laboratory, Tel Aviv). Their globulin fractions were obtained by precipitation with  $(\text{NH}_4)_2\text{SO}_4$  at 35% saturation. IgG was isolated by ion exchange chromatography on

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FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline (0.14 M NaCl, 0.01 M  $\text{KH}_2\text{PO}_4$ , pH 7.4); RITC, rhodamine isothiocyanate; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

DEAE cellulose (23) and the  $F(ab')_2$  fragments were prepared by pepsin (24) digestion with a ratio of 2% enzyme:substrate by weight and a 24-h incubation at 37°C in 0.1 M sodium acetate, pH 4.5. The digestion was stopped by the addition of saturated Tris buffer to bring the pH to 8.0 and the reactants and products were separated by gel filtration on Sephadex G-200. The final products gave no reaction by Ouchterlony analysis (25) with a goat antiserum specific for the Fc fragment of human  $\gamma$  chains. They likewise showed no heavy chain contamination by SDS-PAGE analysis.

**ANTISERA, ANTIBODIES, AND  $F(ab')_2$  FRAGMENTS.** Anti- $\kappa$  and anti- $\lambda$  antisera were prepared in goats by repeated intradermal injections of antigen emulsified in complete Freund's adjuvant. Each immunization consisted of 1 mg of antigen divided into multiple intradermal sites. After a course of four monthly immunizations with purified Bence Jones proteins, the antisera, while specific for  $\kappa$  or  $\lambda$ , reacted primarily with free light chains and very poorly with whole Ig molecules. Accordingly, the animals were boosted with a series of three biweekly injections of the  $F(ab')_2$  fragments of appropriate light chain type, prepared as described above.

The IgG fraction from the serum of the goat immunized with the  $\kappa$  Bence Jones protein and the  $F(ab')_2$  fragments of  $\kappa$  myeloma proteins was passed over an IgG- $\lambda$  myeloma-Sepharose immunoabsorbent to remove anti-Fd activities. Anti- $\kappa$  antibodies were then purified from the material that passed through the absorbent, on an IgG- $\kappa$  myeloma-Sepharose column. Serum from the goat immunized with the  $\lambda$  Bence Jones protein and  $F(ab')_2$  fragments of  $\lambda$  myeloma proteins had a low titer of anti- $\lambda$  antibodies. Thus, it was first applied to an IgG- $\lambda$  myeloma-Sepharose column to concentrate the antibodies, which were then eluted and rendered specific by absorption on IgG- $\kappa$  myeloma-Sepharose. Both the purified anti- $\kappa$  and anti- $\lambda$  antibodies reacted strongly by Ouchterlony analysis with myeloma proteins of their respective types and with normal Ig, and they were specific for  $\kappa$  and  $\lambda$ , respectively. Their specificity was further characterized by radioimmunoassay (26). Briefly, purified myeloma proteins of either  $\kappa$  or  $\lambda$  type were labeled with  $^{125}\text{I}$  (27) and the binding of  $^{125}\text{I}$ -Ig to the goat antibodies was determined by indirect immunoprecipitation. Solutions containing 0.5  $\mu\text{l}$  of normal goat serum and various amounts of antibody were mixed with  $^{125}\text{I}$ -labeled myeloma protein (2–10 ng; 10,000 cpm) and incubated for 2 h at 24°C. Rabbit anti-goat IgG serum was added to the reaction mixtures and incubation was extended another 15–20 h at 4°C. Radioactivity was then determined in the washed precipitates. The rabbit anti-goat Ig and the normal goat serum used for these assays were first absorbed on a normal human serum-Sepharose immunoabsorbent. By this assay, the purified anti- $\kappa$  and anti- $\lambda$  antibodies were found to have specificity ratios of greater than  $10^3$ , i.e., maximal binding of homologous Ig type occurred at an antibody concentration less than 1/1,000 of that necessary to see any binding of Ig of the opposite light chain type.

$F(ab')_2$  fragments of the purified goat antibodies were prepared by pepsin digestion. Optimal conditions for pepsin digestion of goat IgG were found to be different from those for human IgG. The intention here was to achieve complete digestion while minimizing loss of antibody activity. The final conditions employed included an enzyme-to-substrate ratio of 4%, a solution 0.2 M in acetate, a pH of 4.25, and an incubation time of 15 h at 24°C.

### *Immunofluorescence*

Conjugated  $F(ab')_2$  fragments of purified anti- $\kappa$  and anti- $\lambda$  antibodies were used for direct immunofluorescence. They were conjugated with tetramethylrhodamine isothiocyanate (RITC) or fluorescein isothiocyanate (FITC) as described by Cebra and Goldstein (28). The conjugates used had dye:protein ratios of 2–3 mol/mol.

### *Cell Suspensions*

Human lymphoblastoid cell lines MOLT, a T-cell line (29), and SB, a B-cell line (30), were obtained from Dr. Ivor Royston and grown in RPMI 1640 containing 10% fetal calf serum. Peripheral blood lymphocytes were isolated from normal human donors or from patients with leukemia by density sedimentation in Ficoll-Hypaque (31). Cells were washed in phosphate-buffered saline (PBS) and then incubated 18–24 h in Eagle's minimum essential medium containing 5% fetal calf serum (FCS) to allow the shedding of passively absorbed serum Ig.  $10^6$  cells were incubated with appropriate dilutions of  $F(ab')_2$  fragments of anti- $\kappa$  and anti- $\lambda$  for 30 min at 4°C. The cells were then washed by centrifugation through a layer of de complemented FCS, smeared on glass slides, fixed in 100% ethanol, and mounted with cover slips under a drop of glycerol.

### *Tissue Sections*

**SOURCE AND PREPARATION OF TISSUE.** Human lymphoid tissues were obtained immediately after surgical removal. Adipose tissue was carefully trimmed from lymph nodes. Representative blocks were embedded in mounting medium (OCT compound, Tissue Tek II; Lab-Tek Products, Div. Miles Laboratories Inc., Naperville, Ill.) and frozen in a mixture of isopentane and dry ice. The blocks were frozen and stored in air-tight plastic capsules at  $-70^{\circ}\text{C}$  before being sectioned. Frozen sections were prepared for immunofluorescence essentially by the method described by Gutman and Weissman (32). The sections were cut at  $4-6\text{ }\mu\text{m}$  and fixed immediately in acetone for less than 5 s.

**STAINING OF SECTIONS.** Tissue sections on glass slides were washed for 5 min in PBS with 5% fetal calf serum, pH 7.4 (PBS + 5% FCS). Excess wash solution was wiped from the slides, which were then incubated with fluorescent antibody fragments for 10 min at  $37^{\circ}\text{C}$ . The slides were then washed for 5 min in PBS + 5% FCS and subsequently with normal saline. The slides were then mounted in Tris-buffered glycerol, pH 9.2.

### *Immunofluorescence Microscopy and Photomicrography*

Sections and smears were examined in a Zeiss microscope equipped with vertical illumination (Carl Zeiss, Inc., New York) with an HBO 50 mercury vapor lamp and exciter barrier filter combinations for fluorescein 440-490-nm excitation or rhodamine 546.1-nm excitation. Black-and-white photographs were taken with a Polaroid camera (Polaroid Corp., Cambridge, Mass.). Color slides were taken with a Zeiss 35-mm camera on high speed Ektachrome (Eastman Kodak Co.), with ESP-1 processing (ASA-400).

## Results

**Cell Suspensions.** Viable cell suspensions from human lymphoblastoid cell lines or from peripheral blood lymphocytes were examined by direct immunofluorescence for cell surface Ig of  $\kappa$  or  $\lambda$  type, as described in Materials and Methods.

Staining was performed with a mixed reagent consisting of  $\text{F(ab')}_2$  fragments of fluorescein-labeled anti- $\kappa$  and rhodamine-labeled anti- $\lambda$ . Each cell could then be individually examined for  $\kappa$  and for  $\lambda$ . We found that for peripheral blood lymphocytes a critical step was an incubation of the cells in a medium free of human serum proteins before staining (Table I) (33).<sup>2</sup> Thus, when staining was performed on freshly isolated cells, the total percentage of cells showing surface Ig was approximately 35%; however, the majority of these cells were positive both of  $\kappa$  and for  $\lambda$ . This staining occurred in a beaded membrane pattern with *individual beads* showing staining for both  $\kappa$  and  $\lambda$ . After incubation at  $37^{\circ}\text{C}$  in tissue culture medium free of human serum, which presumably allowed the shedding of passively absorbed serum Ig (33),<sup>2</sup> the total percentage of Ig-positive cells fell to less than 10% with no double-staining cells. No significant loss of cell viability occurred during incubation. The shedding process was somewhat dependent upon temperature since incubation at  $4^{\circ}\text{C}$  resulted in a residual population of 4% double-stained cells. Incubation in the presence of autologous plasma at  $4^{\circ}\text{C}$  resulted in an *increase* in double-staining cells to as high as 60%. All subsequent results with peripheral blood lymphocytes were, therefore, derived from experiments in which the cells were incubated overnight at  $37^{\circ}\text{C}$  in tissue culture medium before staining.

The results of  $\kappa$ - $\lambda$  staining of cells from a variety of sources are shown in Table

<sup>2</sup> Hofman, F., A. Cohen, D. Zipori, R. Burstein, Z. Bentwick, and J. Haimovich. 1976. Membrane Ig and auto-antibodies to lymphocyte membrane. Manuscript submitted for publication.

TABLE I  
*Effects of Various Incubations*

Incubation	Cells Staining		
	$\kappa$	$\lambda$	Double
	%	%	%
None	36	29	24
4°C, 18 h in autologous plasma	63	66	59
4°C, 18 h in RPMI + 5% FCS	8	6	4
37°C, 18 h in RPMI + 5% FCS	4	3	0

Normal peripheral blood lymphocytes were isolated by the Ficoll-Hypaque technique and washed in PBS. After incubation,  $10^6$  cells were stained at 4°C with a mixed fluorescein-labeled anti- $\kappa$  and rhodamine-labeled anti- $\lambda$ . After washing and fixing on slides, the cells were examined for both fluorescein and rhodamine fluorescence with a Zeiss microscope equipped with incidence illumination.

II. SB, a human B lymphoblastoid cell line, gave exclusive staining for  $\kappa$ , while MOLT, a T lymphoblastoid line, gave staining for neither. Likewise, the peripheral blood cells from a patient with acute lymphoblastic leukemia were uniformly negative for Ig. 14-cases of CLL were examined, with 13 showing a monoclonal staining pattern (8 $\kappa$  and 5 $\lambda$ ) and 1 negative for Ig. Cells from three patients with lymphosarcoma cell leukemia and one patient with hairy cell leukemia gave monoclonal Ig staining. These results on viable cell suspensions confirmed other reports (8, 9, 33-35) and served to validate the immunofluorescent reagents.

**Tissue Sections.** Cryostat sections of normal lymph nodes were stained with the mixed anti- $\kappa$  and anti- $\lambda$  antibody fragments. Lymphoid follicles (Fig. 1) were evident not only by their Ig positivity but by their pattern of staining. Toward the center of the secondary follicles there was a variable amount of densely staining Ig distributed in a lacy pattern. This material always stained positive, both for  $\kappa$  and for  $\lambda$  and, therefore, represented extracellular protein. The cells of the mantle, most of which were small lymphocytes, were composed of a mixture of  $\kappa$ -staining and  $\lambda$ -staining cells. Most of the cells could easily be distinguished as staining either for  $\kappa$  or for  $\lambda$  but not for both (Figs. 1 and 2). Because of the scant amount of cytoplasm in these cells it was not usually possible to determine whether Ig staining was limited to the membrane or was also intracellular. However, in the case of plasma cells, where cytoplasm could easily be seen, there was bright intracellular staining which once again was restricted either to  $\kappa$  or to  $\lambda$  in individual cells. In normal lymphoid tissues the number of  $\kappa$ -staining cells was always greater than the number of  $\lambda$ -staining cells.

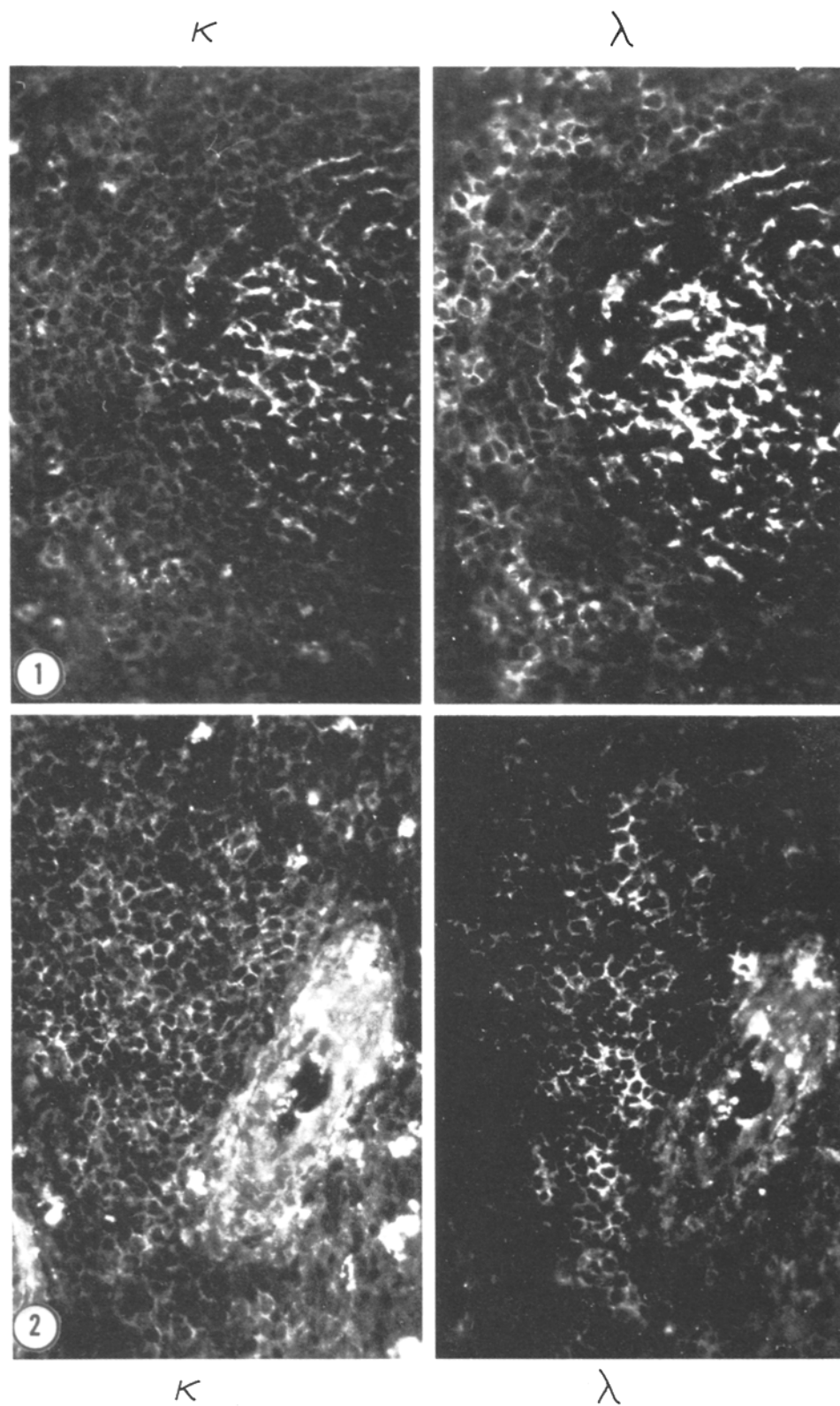
A totally different pattern of staining was found in lymphoid tissues involved by lymphoma. In Fig. 3 is shown an example of a lymph node from a case of diffuse, well-differentiated lymphocytic lymphoma. As can be seen, the entire tissue was composed of small lymphoid cells that stained uniformly for  $\kappa$ ; no  $\lambda$ -staining cells were seen. This finding of exclusive  $\kappa$  staining was confirmed when the fluorescent labels were reversed, i.e., employing fluorescein-labeled anti- $\lambda$  and rhodamine-labeled anti- $\kappa$ . In a second example (Fig. 4), a spleen from

TABLE II  
Results of  $\kappa$ - $\lambda$  Staining on a Variety of Cells

Source of cells	Cells staining		
	$\kappa$	$\lambda$	Neither
	%	%	%
Normal peripheral blood lymphocytes	4-6	3-4	90-93
Lymphoblastoid cell line SB	86	0	14
Lymphoblastoid cell line MOLT	0	0	100
Acute lymphoblastic leukemia	0	0	100
Number of cases			
Cases with >85% of cells staining	Exclusively $\kappa$	Exclusively $\lambda$	Neither
Chronic lymphocytic leukemia	8	5	1
Lymphosarcoma cell leukemia	1	2	0
Hairy cell leukemia	0	1	0

a case of hairy cell leukemia, the converse pattern of staining was noted. Here, no  $\kappa$ -staining cells were seen, but instead the tissue was diffusely infiltrated by cells staining exclusively for  $\lambda$ . In Fig. 5 is shown an example of a lymph node from a case of nodular poorly differentiated lymphocytic lymphoma. Here, the nodule of lymphoma was composed of cells staining exclusively for  $\kappa$ . It should be noted that in this case not all of the cells within the lymphoma nodule contained Ig. Another example of nodular poorly differentiated lymphocytic lymphoma is shown in Fig. 6. Here, the connective tissue, which shows autofluorescence, accentuated the boundaries of the nodules. Once again, there was exclusive light chain staining of the cells, this time with  $\kappa$ . In this example the node was totally replaced by lymphoma and no residual polyclonal staining normal tissue was evident outside the nodules.

In addition to the examples illustrated here, a total of 12 consecutive lymphoma patients have been examined, including 10 cases of lymphocytic lymphoma and 2 cases of "histiocytic" lymphoma. None of the patients in this group had an M-component in the serum to confuse the analysis of tissue Ig. All of these lymphomas were composed of immunoglobulin-bearing cells restricted in light chain type, with a total of seven cases of  $\kappa$  type and five cases of  $\lambda$  type. In each instance the assignment of light chain type was confirmed by reversing the color of the reagents as described above. Furthermore, when multiple focal areas of lymphoma were present within a given tissue or when different tissue sites were examined from a given patient, they were always composed of cells of a consistent light chain type; hence all of the disease in a given patient was presumably derived from the same clone. A polyclonal staining pattern, i.e., a mixture of  $\kappa$ - and  $\lambda$ -staining cells, was found in the tissues of two cases of Hodgkin's disease and one case of angioimmunoblastic lymphadenopathy. One case of mycosis fungoides, a T cell disease (36, 37), and several cases of other malignancies, including malignant melanoma and adenocarcinoma, showed replacement of lymph nodes by Ig-negative cells. Details of our findings in specific disease entities will be reported separately.



### Discussion

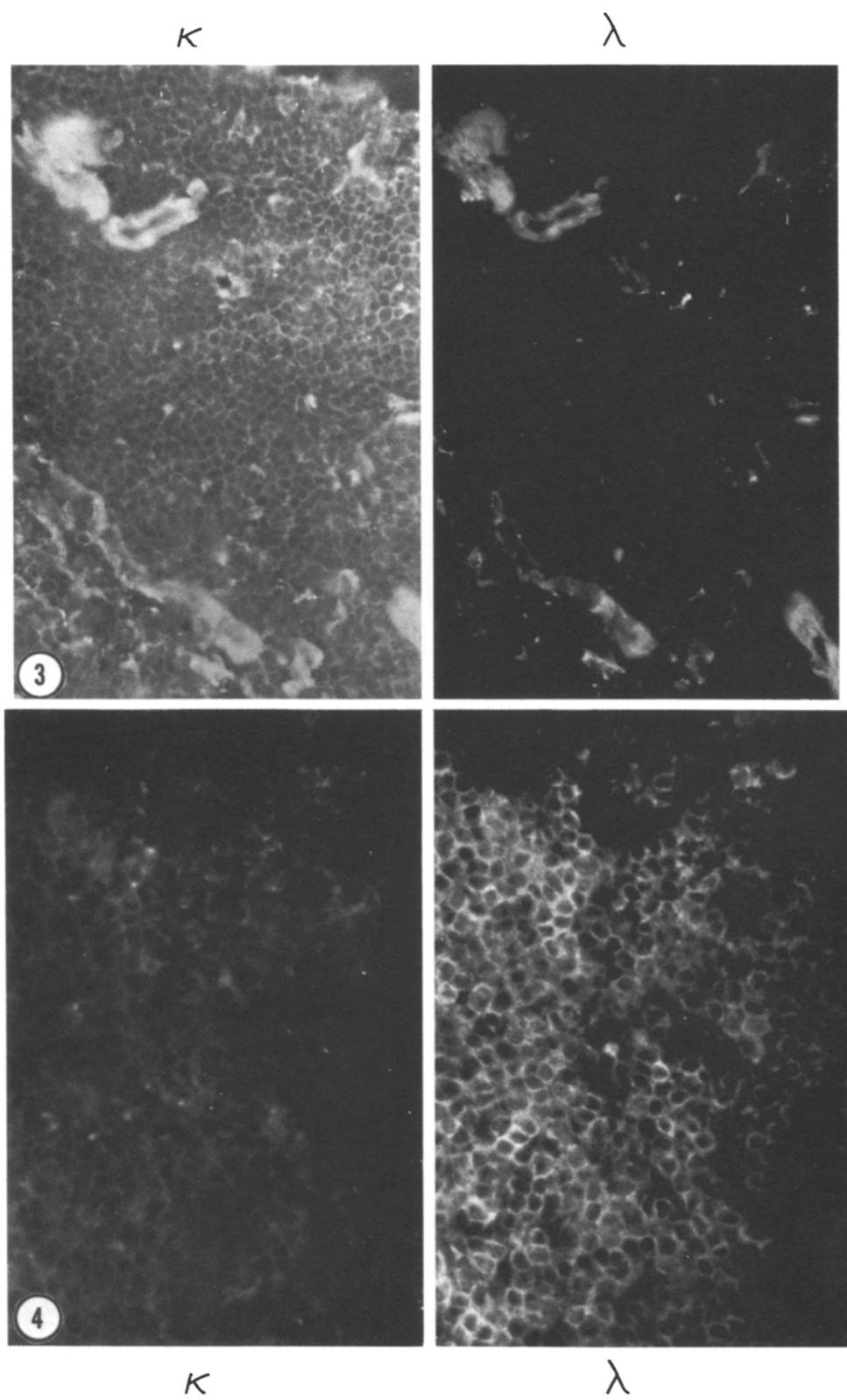
A single clone of B lymphoid cells is committed to the expression of a particular immunoglobulin sequence of  $V_H$  and  $V_L$ , which together form a particular antibody combining site and impart the binding specificity of the Ig molecule (13-15, 19). A formal proof of monoclonality within a population of B cells would, therefore, require the demonstration that all the cells were restricted to the production of a single combining site, for instance, by the use of an anti-idiotypic reagent (19, 38). This goal has been accomplished for human lymphoproliferative diseases in which the immunoglobulin product is secreted, such as multiple myeloma and Waldenstrom's macroglobulinemia (38, 39). In these instances, the abundance of the monoclonal Ig made possible the production of anti-idiotypic reagents that could be used to identify the malignant clone.

Since many lymphoid malignancies are associated with the production but not the secretion of Ig, the use of anti-idiotypic reagents, while not impossible (40), may not be applicable as a general approach for the diagnosis of monoclonality. For this reason we and many other investigators (8-12, 35) have employed light chain typing as an indicator of monoclonality. Here, the argument is a statistical one and is based on the restriction within a clone to the production of Ig light chains of either  $\kappa$  or  $\lambda$  type. The basis for this restriction is not known, and indeed it may not be absolute (41, 42). Nevertheless, the finding of a uniform light chain type within a malignant lymphoid population, while not a proof of monoclonality, is taken as strong presumptive evidence. Of course, the demonstration of monoclonality does not in and of itself make the diagnosis of malignancy. There are numerous examples of benign monoclonal lymphoproliferation (43). Moreover, it is possible that malignant transformation could occur in a cell that is a member of a B cell clone previously expanded by an antigenic stimulus. In that case not all the cells bearing the particular Ig marker would be malignant, even though they would have arisen from the same original B cell. The concept of monoclonality within human B lymphoid malignancies should, therefore, be regarded as a useful working model and not an absolute criterion of malignancy.

The unique contribution of the present study lies in its confirmation of monoclonality within human B cell lymphomas by  $\kappa/\lambda$  restriction of the cells when examined *in situ*. Numerous previous reports have appeared in which suspensions of cells derived from tissues involved by lymphoma were studied (10-12, 35). Here the imperfection of the  $\kappa/\lambda$  statistical argument is compounded by the presence of residual population of normal lymphoid cells, some of which share the light chain type of the lymphoma cells. Nevertheless, in massively

FIG. 1. Frozen section of normal human lymph node, reactive follicle. Immunofluorescent staining was performed with a mixture of fluorescein-labeled  $F(ab')_2$  anti- $\kappa$  and rhodamine-labeled  $F(ab')_2$  anti- $\lambda$ . The same field was photographed for fluorescein ( $\kappa$ ), left; and for rhodamine ( $\lambda$ ), right. There is a dense lacy network of double-staining material at the center of the follicle surrounded by a mantle of cells staining either for  $\kappa$  or for  $\lambda$ . Magnification 400.

FIG. 2. Frozen section of normal human spleen follicle. Immunofluorescent staining for  $\kappa$  and  $\lambda$  as in Fig. 1. Bright autofluorescence of collagen in blood vessel serves as a landmark for orientation. Surrounding cells are composed of a mosaic of  $\kappa$ -staining and  $\lambda$ -staining cells. Magnification 400.



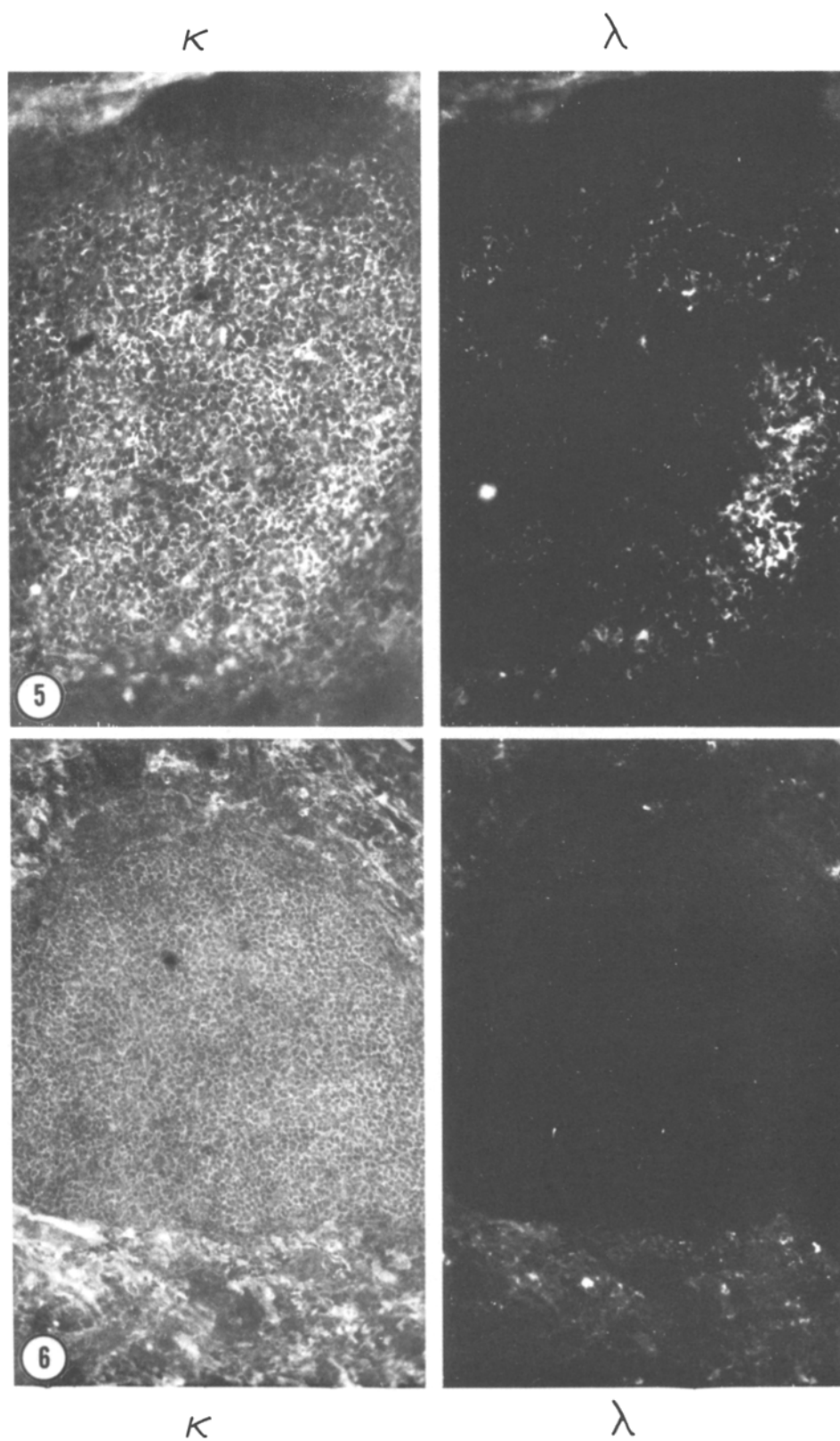
involved tissues, clear demonstrations of  $\kappa/\lambda$  restrictions have been possible in these studies of cell suspensions. However, our results do not confirm those of Leech et al. (11) who suggested, on the basis of cell suspension studies, that nodular lymphomas tend to be of  $\kappa$  type and diffuse lymphomas tend to be of  $\lambda$  type.

Tissue sections have been examined by Jaffe et al. (44), using an *in situ* C'3 rosette technique, an approach applicable only to nodular lymphomas and which serves to identify the nodules as being derived from germinal centers. But their method does not indicate the monoclonality of the lymphoma and does not distinguish between nodular lymphoma and benign lymphoid hyperplasia. Taylor and others have reported the application of an immunoperoxidase technique on sections of fixed, paraffin-embedded lymphoma tissues (45, 46). While advantageous in that it can be used on previously fixed tissues, the immunoperoxidase technique does not allow a double-label approach, which we have found adds an additional dimension to the immunofluorescence methods. Serial sections cannot convincingly demonstrate the mosaicism of normal lymphoid tissues with respect to  $\kappa$ - and  $\lambda$ -producing cells, which is readily apparent from double-label fluorescence applied to individual sections (Figs. 1 and 2). More importantly, our results do not agree with those of Taylor, who reported that in nodular lymphoma the nodules were composed of Ig-negative cells and the internodular areas were composed of monoclonal populations. Most likely these discrepancies arise from problems related to technique, i.e., the specificity of reagents and fixation of tissues. In our study great care was taken in the preparation and characterization of the anti- $\kappa$  and anti- $\lambda$  reagents. The difficulty of preparing a reliable anti-human  $\lambda$  reagent is not apparent from the literature. In addition, the use of  $F(ab')_2$  fragments of purified antibodies for direct immunofluorescence eliminated many of the technical difficulties previously associated with the technique (34). The ability to distinguish polyclonal from monoclonal staining on a cell-by-cell basis provided an extra measure of confidence in the results. It is clear that many of the reports in the literature of 20–40% Ig-bearing cells in normal peripheral blood (47) are overestimates due to the artifact of passive absorption of serum Ig to the surface of cells (Table I) (33).<sup>2</sup> In addition, it is clear that the dense, lacy distribution of Ig in the center of reactive lymphoid follicles described by Braylan and Rappaport (48) and in Fig. 1 is likewise a deposit of extracellular protein since it stains for both  $\kappa$  and  $\lambda$ .

The approach described here should be useful in the diagnosis of lymphoma, helping to differentiate a lymphoma from reactive hyperplasia. In addition, the classification of lymphomas, currently a subject of controversy (49–53), should be aided by techniques, such as the one described here, which will allow the *in situ* identification of B cell neoplasms among the diffuse lymphomas as well as the nodular lymphomas. A growing consensus (20, 51) suggests that many "histiocy-

FIG. 3. Frozen section of lymph node from a patient with diffuse, well-differentiated lymphocytic lymphoma. Immunofluorescence staining as in Fig. 1. The tissue is diffusely infiltrated with cells staining only for  $\kappa$ . No  $\lambda$ -staining cells can be seen. Magnification 400.

FIG. 4. Frozen section of spleen from a patient with hairy cell leukemia. Immunofluorescence staining as above. The tissue is diffusely infiltrated with cells staining for  $\lambda$ . No  $\kappa$ -staining cells are seen. Magnification 400.



tic" lymphomas are, in fact, neoplasms of B lymphoid cells. Furthermore, the question of clonal stability can now be raised in situations of recurrent lymphomas and composite lymphomas. When treated lymphoma recurs, does this represent reappearance of the same clone or the appearance of a new malignant clone? Evidence accumulated for Burkitt's lymphoma seems to imply that relapse occurs in the same clone for early recurrences and that new clonal disease accounts for late recurrences (1).

Approximately 10–20% of human lymphoma patients have what appear to be two different histologic types of lymphoma occurring simultaneously in different sites (54). Are these lesions all derived from the same malignant clone or do they represent the simultaneous appearance of two different neoplasms? The approach described here should provide an answer to this question. Finally, the ability to examine and immunologically identify the monoclonal population *in situ* should provide further insight into the pattern of involvement and spread of human lymphomas within the lymphoid architecture.

### Summary

Human tissues involved with lymphoma have been examined in frozen sections for immunoglobulin-bearing cells by a technique involving double-label immunofluorescence with mixed anti- $\kappa$  and anti- $\lambda$  antibodies. F(ab')<sub>2</sub> fragments of purified antibodies were employed to avoid any binding via Fc receptors. B cell lymphomas were shown to be composed of monoclonal populations of Ig-bearing cells, whereas normal or reactive lymphoid follicles contained a mosaic of Ig-bearing cells derived from multiple clones. Nodules of lymphoma were often surrounded by normal polyclonal B cell populations. We anticipate that the approach described here will be useful in the diagnosis of lymphoma, differentiating it from reactive lymphoid hyperplasia by the demonstration of monoclonality. In addition, it should provide a sensitive and reliable tool for investigating the immunobiology of human lymphoma.

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FIG. 5. Frozen section of lymph node from a patient with a nodular poorly differentiated lymphocytic lymphoma. Immunofluorescence staining for  $\kappa$  and  $\lambda$  as in Fig. 1. The nodule of lymphoma is composed exclusively of  $\kappa$ -staining cells, but a rim composed of a mixture of  $\kappa$ - and  $\lambda$ -staining cells can be seen. Magnification 250.

FIG. 6. Frozen section of lymph node from another patient with a nodular poorly differentiated lymphocytic lymphoma. Here, the nodule of lymphoma is surrounded by connective tissue, seen as autofluorescence. Again, the nodule is composed exclusively of  $\kappa$ -staining cells. But here, even the cells outside the nodule are exclusively stained for  $\kappa$ . Magnification 250.

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