American Journal of Pathology, Vol. 135, No. 4, October 1989

The Role of Cell Cycle Activity in the Generation of Morphologic Heterogeneity in Non-Hodgkin's Lymphoma

David S. Weinberg
From the Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts

Cellular and nuclear size heterogeneity are frequently observed in non-Hodgkin's lymphomas, yet the biological basis of this morphologic variability is not well understood. The possible relationship between cell size and cell cycle activity in malignant lymphomas was investigated using multiparameter flow cytometry and digital image analysis. Flow cytometric analysis of ten cases of B cell lymphoma revealed that, regardless of the diagnosis or the proportion of large cells present, large neoplastic cells showed a much greater proportion of S + G2/M phase activity (mean, 35.2%; range, 14.6% to 70.5%) than did small cells (mean, 4.4%; range, 0.5% to 18.4%). Further studies aimed at distinguishing the features of cycling and noncycling cells were performed using quantitative image analysis of nuclear staining by Ki-67 antibody. In both benign and malignant lymphoid infiltrates, resting (Ki-67-negative) nuclei were uniformly smaller than Ki-67-positive nuclei and exhibited a restricted size distribution, whereas the Ki-67-positive nuclei had a broad size distribution and included the vast majority of large forms. Thus, the actively cycling component of the tumor consisted of cells with large nuclei. These studies support the hypothesis that cellular and nuclear size heterogeneity in malignant lymphomas is related to cell cycle phase. The combined influences of cell differentiation and proliferation on cellular morphology can explain many of the histologic features observed in non-Hodgkin's lymphomas. (Am J Pathol 1989, 135:759–770)

Morphologic heterogeneity is a commonly observed feature in non-Hodgkin's lymphomas, in that most histologic types consist of variable mixtures of small, intermediate, and large lymphoid cells. Indeed, the relative proportion of small and large lymphoid cells is a major diagnostic criterion used in histologic classification.[1–4] Although benign T cells are a significant proportion of the small lymphoid cells seen in B cell neoplasms[5] and may contribute to the heterogeneous appearance of these tumors, we and others[6,7] showed that in most cases the malignant B cells are included among both large and small lymphoid forms. Despite the presence of intratumor morphologic heterogeneity, most lymphomas are monoclonal by DNA hybridization studies[8] and are phenotypically uniform despite the presence of significant intratumor morphologic heterogeneity.[9,10] Therefore, tumor multiclonality or intratumor differentiation cannot explain the range of morphologies observed within lymphomatous infiltrates.

Certain aspects of the normal lymphocyte cell cycle may provide a better explanation for the morphologic variability seen in malignant lymphomas. It has long been known that the induction of proliferative activity in normal lymphocytes is accompanied by marked morphologic changes, including increased cell and nuclear size.[11] It is reasonable to assume, therefore, that malignant lymphoid cells undergo similar size changes with cell cycle activity. Asynchronous growth in a lymphoma, therefore, would be expected to result in a morphologically heterogeneous population of cells. Although it has often been assumed or suggested that large malignant lymphoid cells represent "transformed" or actively proliferating cells,[2,12,13] there has been no direct proof of this assertion. The studies described in this article were undertaken to provide direct evidence for a relationship between morphology and cell cycle activity in malignant lymphomas. Multiparameter flow cytometric and quantitative digital imaging techniques demonstrated a definite correlation between cell

This work was supported in part by grants AI123360 and CA40357 from the National Institutes of Health.

Accepted for publication June 15, 1989.

This article was presented in part at the 76th Annual Meeting of the International Academy of Pathology, United States–Canadian Division, March 1987.

Address reprint requests to David S. Weinberg, MD, PhD, Department of Pathology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.

size and cell cycle activity in non-Hodgkin's lymphomas, as well as in benign reactive states. We hope these findings will provide a biological basis for understanding much of the morphologic heterogeneity and diversity seen in the non-Hodgkin's lymphomas.

Materials and Methods

Tissues

Fresh human tissues were obtained from lymph node biopsies from adult patients suspected of having malignant lymphoma, and were prepared for study without knowledge of diagnosis. Studies performed on cases not confirmed as either non-Hodgkin's lymphoma or benign reactive hyperplasia were later excluded from the study. The diagnoses were confirmed by standard histology, employing the Rappaport system for classification of the non-Hodgkin's lymphomas.[1,4] Routine phenotyping was performed on cryostat sections of snap-frozen tissue by standard immunocytochemical methods (see below). All tissues were obtained with approval of the Human Subjects Committee of Brigham and Women's Hospital.

Specimen Preparation

Single cell suspensions were prepared by teasing tissues with forceps in Hanks' balanced salt solution (HBSS; Gibco Laboratories, Grand Island, NY) containing 0.2% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO). Viable cells were obtained after centrifugation over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), suspended in tissue culture medium consisting of RPMI 1640, 10% fetal calf serum, HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, and 50 U/ml streptomycin (Gibco) at 2×10^6 cells/ml, and subjected to 1 hour of incubation in tissue culture flasks (75 cm^2, Corning Glass Works, Corning, NY) at 37 C in an atmosphere of 5% CO_2. The nonadherent cells were decanted and kept at 4 C until further preparation.

In a separate series of cases in which cell suspensions were not prepared, cytologic smears were obtained from fresh lymph node biopsy specimens by scraping the freshly cut surface with the edge of a glass slide and smearing the tissue on a second clean glass slide in a manner similar to that used in the preparation of peripheral blood smears. The smears were air dried and stored for subsequent immunocytochemical studies. One slide was routinely fixed in methanol and stained with Wright-Giemsa to confirm the cellular composition of the smear and its adequacy for study. Again, as for cases studied in suspension, the final diagnosis and phenotype were confirmed by standard histologic and immunocytochemical methods.

Cell Labeling in Suspension

Nonadherent lymphoid cells were studied on the same day to determine the basic phenotype of the tumor (B or T cell), as well as the light chain type. Staining was performed by adding 5 μl of either heteroantiserum or monoclonal antibody to a pellet of 0.5 to 1.0×10^6 cells followed by washing in ice-cold phosphate-buffered saline and incubation with 5-μl fluoresceinated secondary staining reagent.[14] After several washes, the cells were kept at 4 C and immediately studied by flow cytometry (see below). Controls consisted of cells stained with the secondary reagents alone. T and B cells were quantitated using anti-Leu-4 (CD3, Becton Dickinson, Mountain View, CA) and B1 antibodies (CD20; Coulter Immunology, Hialeah, FL), respectively. Light chains were labeled with $(Fab')_2$ fragments of rabbit antibodies to human kappa and lambda light chains (Accurate Chemical and Scientific Corp., San Diego, CA). Secondary staining was achieved using fluoresceinated goat antibody to mouse immunoglobulin (Cappel Laboratories, West Chester, PA) and fluoresceinated goat antibody to rabbit IgG (Cappel Laboratories, West Chester, PA) for mouse monoclonal antibodies and rabbit antisera, respectively.

Double labeling for DNA content and surface markers was performed by a modification of the method describe by Braylan et al,[15] as previously described.[14] Briefly, cells that had been surface labeled as described above were fixed in 50% ice-cold ethanol (by slowly adding an equal volume of absolute ethanol to the cell suspension) for 1 hour at 4 C, washed three times with HBSS containing 0.2% BSA, and treated with ribonuclease A (500 U/ml, Sigma) for 30 minutes at room temperature. This was followed by staining with 50 μl/ml propidium iodide (Sigma) for at least 30 minutes at room temperature before analysis.

Flow Cytometric Analysis

Analysis of suspended cells was performed on a FACS Analyzer equipped with Consort 30 software (Becton Dickinson). This flow cytometer, which employs a mercury arc lamp as the light source, was used with appropriate combinations of filters to simultaneously measure emission from fluorescein (525/20 bandpass) and propidium iodide (570 longpass). Cell size was determined by electronic volume. The freshly stained, unfixed surface-labeled cells were first immediately studied using single-parameter flow cytometric analysis for the presence of

surface light chain, as well as for staining with B1 and anti-Leu-4 antibodies. Cases were selected for further study only if a definite monotypic staining pattern for one light chain type (kappa or lambda) was observed, or if most cells stained with B1 antibody (to identify surface light chain-negative malignant B cells or benign polyclonal B cells in reactive lymph nodes). Dual color analysis was performed on cells subsequently double labeled for surface marker and DNA content by collecting list mode data on a minimum of 10,000 cells, with volume gates set to include both large and small lymphocytes, excluding debris and cell clumps. List mode data was analyzed using Consort 30 software, at which time it was possible to correlate any two parameters under a variety of gating conditions. Two-parameter correlated data were displayed as contour plots, with DNA content shown as red fluorescence in the Y axis (linear scale), and either cell volume or green fluorescence in the X axis (log scale). Initial gates were selected to exclude light chain-negative cells or non-B cells, and volume gates were selected from a single-parameter display of the volume distribution to selectively examine the DNA content of small and large cells. A single-parameter DNA histogram of the total cell population was displayed to set markers to allow the quantitation of G0, S, and G2/M phases of the cell cycle. These phases were used to quantitate subsequently the cell cycle phases of populations of gated cells selected on the basis of size. Because ethanol-fixed cells displayed a lower electronic volume than cells fixed in paraformaldehyde, the volume distribution of nonadherent peripheral blood mononuclear cells was used as a standard to distinguish large and small lymphocytes. Volume gates were set so that small cells corresponded to cell sizes that included at least 95% of peripheral blood lymphocytes. The coefficients of variation (CVs) of the G0/G1 peaks of large and small cells did not differ significantly in any of the cases (mean CVs, 6.5% and 6.2%, respectively). Also, there were no differences with regard to the modal value of the G0/G1 peaks of large and small cells in individual cases.

Immunocytochemistry

Air-dried smears from lymph nodes were fixed in ice-cold 1% paraformaldehyde (in phosphate-buffered saline) for 6 minutes and washed in 0.05 M Tris buffer (pH 7.6) containing 4% swine serum (Gibco) at 4 C. Ki-67 antibody (Dako Corp., Santa Barbara, CA) was prepared as a 1:15 dilution in 0.10 M Tris buffer (pH 7.6), containing 4% swine serum, and added to the slide surface. After incubating for 1 hour in a moist chamber at room temperature, the slides were rinsed for at least 15 minutes in several changes of Tris buffer containing 2% swine serum. Slides were then incubated with peroxidase-conjugated goat antibody to mouse immunoglobulin (Tago, Burlingame, CA) for 1 hour, washed in Tris buffer, and incubated with peroxidase-conjugated swine antibody to goat immunoglobulin (Tago) for 1 hour. After washing in Tris buffer, staining was achieved using 3-3′-diaminobenzidine tetrahydrochloride (Aldrich Chemical Co., Milwaukee, WI) as chromagen. Sections were counter-stained with methyl green and mounted. Controls consisted of smears incubated in buffer not containing the primary antibody. Staining was performed on air-dried smears stored no longer than 48 hours, because longer storage resulted in markedly reduced staining intensity. Immunophenotyping of cryostat sections of lymph nodes was performed by standard immunoperoxidase methods.[16]

Quantitative Image Analysis of Ki-67 Staining

Quantitative image analysis was performed using the CAS 100 System (Cell Analysis Systems, Lombard, IL). This microscope-based image analyzer employs a solid state video camera mounted on a light microscope, with image data transmitted to and stored in an IBM AT computer equipped with a digital imaging board.[17] Analog video signals were digitalized and stored as 256 × 256 pixels containing 8 bits of intensity values, sufficient for 256 levels of grey determination. Digitalized light intensity values were converted to optical density by means of a calibrated input look-up table. Thus, the image memory contained values for optical density for each pixel in the field. Smears on glass slides stained with Ki-67 antibody were viewed using a green (540/10 nm bandpass) filter, which markedly increased the contrast between positively stained (brown) and negative nuclei (green). Microscopic fields containing nonoverlapping cells with uniform distribution were selected for quantitation. All measurements were performed using the Cell Measurement Program provided with the CAS 100 System. After capturing an image of a 400× microscopic field (40/0.63 objective, 10× ocular), the object detection threshold was set to include both positive and negative nuclei. As the system automatically located nuclei, the operator (DW) assigned nuclei to one of two groups, either Ki-67 positive or negative. Quantitative measurements were automatically performed on assignment of each nucleus, including nuclear area (μ^2) and average nuclear optical density. In both groups 100 to 200 nuclei were measured in each case. On completion of the measurements, a histogram comparing the average optical densities of the Ki-67 negative and positive groups was displayed to insure adequate operator distinction between the groups and to detect possible errors in assignment. Histograms of nuclear area distribution were then displayed and recorded. Statistical mea-

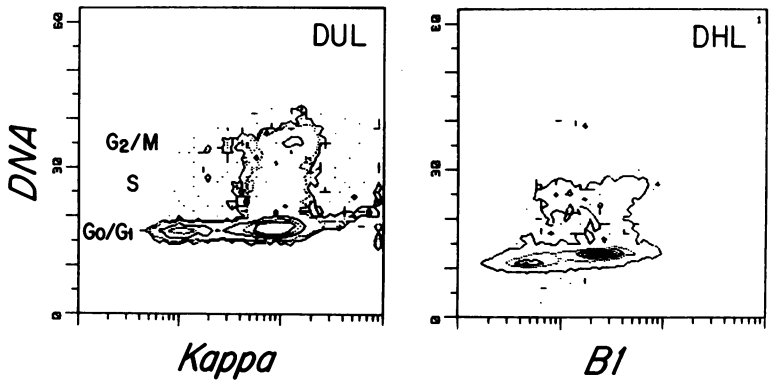


Figure 1. *Dual label flow cytometry. Two cases of non-Hodgkin's lymphoma are shown, dual labeled for DNA content and a cell surface marker. DNA content is displayed on the vertical axis (red fluorescence, linear scale) and the surface immunofluorescence is shown on the horizontal axis (green fluorescence, log scale). On the left, a case of diffuse undifferentiated lymphoma (DUL), known to be monotypic for kappa light chain; all of the S + G2/M phase DNA content is associated with the kappa-positive cells. The background cells (low green fluorescence) consist of diploid cells with negligible S + G2/M phase activity. Separate studies revealed these background cells to consist mainly of T cells (not shown). On the right is a case of diffuse "histiocytic" (large cell) lymphoma, with the tumor cells identified by virtue of their staining for B1 antigen (CD20). The B1-positive tumor cells have aneuploid (hyperdiploid) DNA content, revealed by the position of the G0/G1 peak of the B1-positive cells at a slightly higher level of red fluorescence (DNA content) compared with that of the B1-negative (nontumor) cells. Note that the S + G2/M phase activity is associated with both B1-positive and negative populations in this case.*

surements of nuclear size distribution were performed using Statgraphics (Plus*Ware, STSC Inc., Rockville, MD) on data files downloaded in DIF format to an IBM AT computer.

Results

Flow Cytometric Studies

To study the relationship between cell size and cell cycle activity in non-Hodgkin's lymphoma, multiparameter flow cytometry was used to specifically identify the cell population of interest and to examine the cell cycle activity of small and large lymphoid cells within that specific cell population. The suspended cells were first labeled with a fluoresceinated antibody against a surface marker that would identify the cells in the population of interest: light chain-positive cells (kappa or lambda) in cases of B cell lymphoma, or B1(CD20)-positive cells in B cell lymphomas lacking light chain or polyclonal B cells in reactive nodes (see Materials and Methods). The cells were additionally labeled with propidium iodide to allow quantitation of the DNA content of each cell. The primary goal of this double labeling technique was to exclude irrelevant cells (mostly benign T cells) from the analysis. Because only cases of B cell lymphoma were studied, B cells from reactive lymph nodes appeared to be the most appropriate control cell population to study.

Examples of double labeled cells from two cases of non-Hodgkin's lymphoma are shown in Figure 1. Green fluorescence is shown in the X axis (surface label), DNA content is displayed in the Y axis (linear scale), and the isocontours indicate cell number (Z axis). In the first case, that of a diffuse undifferentiated lymphoma known to be positive for kappa light chain, the lymphoma cells were identified by kappa-positive staining. The background (non-B) cells in this and most other cases consisted predominantly of CD4-positive T cells (not shown). Figure 1 shows that virtually all the cell cycle (S and G2/M phase) activity was associated with the lymphoma cells. The background cells (kappa-negative cells) contributed little to the overall proliferative content of the lymphoid infiltrate. Note also that the tumor appears euploid, in that the background (T) cells and the lymphoma cells had identical G0/G1 DNA content. In the second case, a large cell lymphoma, the tumor cells lacked surface light chain, but could be identified by staining with B1 antibody (con-

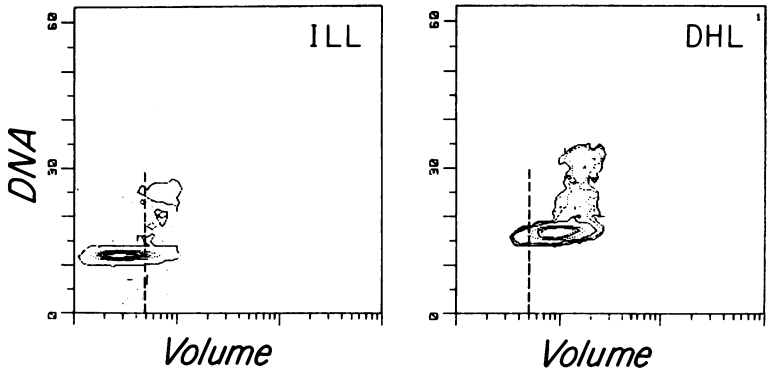


Figure 2. *Dual parameter correlated histograms: DNA content* vs. *cell volume. Two cases are shown in which the lymphoma cells are specifically examined by selective gating. The vertical dotted line in each histogram indicates the separation of small and large cell subsets. The two cases represent lymphomas of predominantly small lymphocytes (ILL, intermediate lymphocytic lymphoma) and large lymphocytes (DHL, diffuse histiocytic lymphoma). Note that, although the proportion of large cells is markedly different in each case, the S + G2/M phase activity in both cases is associated with the large cell fraction (to the right of the dotted line).*

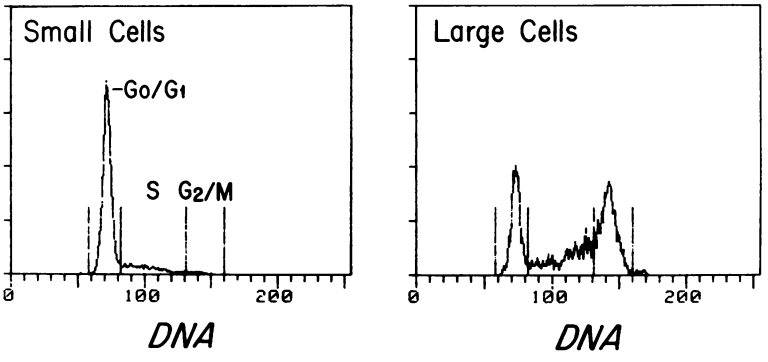


Figure 3. *DNA histograms of small and large lymphoma cells from a case of diffuse undifferentiated lymphoma. Vertical markers show the separation between the cell cycle phases. Note that the large cell population shows a much greater proportion of cells in the S and G2/M phases of the cell cycle.*

firmed by immunocytochemical studies). The lymphoma cells were aneuploid, having a slightly greater DNA content than the background cells, which confirmed the identification of the malignant cells in the B1-positive population. In this case, proliferative activity was present among both the tumor cells and the background cells.

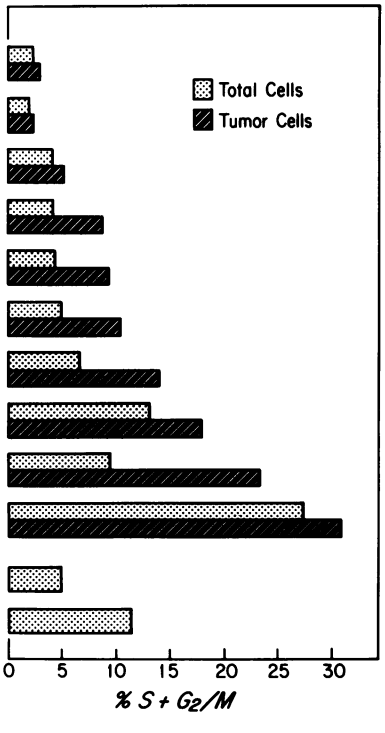
Using the above method to identify the tumor cells, selective gating on these cells was applied to examine the relationship between cell size and cell cycle activity. Two-parameter correlated histograms of DNA content and cell size are shown for two cases in Figure 2. Cells having G0/G1 phase DNA content were included among both the small and large lymphoma cells. Although these cases differed markedly in the proportion of large lymphoma cells present, the proliferative (S and G2/M phase) activity in both cases was associated with the large cell fraction. Figure 3 shows the DNA histograms of small and large cells from a single case, a highly proliferative undifferentiated lymphoma. In this case, the larger cell population was comprised of cells predominantly in the S or G2/M phases of the cycle (70.5%), whereas small cells were mainly in the G0/G1 phase (81.6%).

The cases studied by flow cytometry are listed in Figure 4, including ten cases of non-Hodgkin's lymphoma and two benign reactive lymph nodes (follicular hyperplasia). The diagnostic classification and phenotype of each case are shown. The lymphomas were of various histologic types, including low-, intermediate, and high-grade tumors, and are listed in order of grade. All the lymphoma cases studied were of B cell type, with tumor cells identified either by light chain staining or B1 positivity. For each case, the percentage of large cells among the lymphoma cells is indicated, as well as the proliferative activity (S + G2/M phase content) of the total cell population and

Figure 4. *Cases studied by flow cytometry. WDLL, well differentiated lymphocytic lymphoma; PDLL, poorly differentiated lymphocytic lymphoma; ILL, intermediate lymphocytic lymphoma; MLH, mixed lymphocytic-histioctic lymphoma; HL, histiocytic lymphoma; DUL, diffuse undifferentiated lymphoma; N, nodular; D, diffuse. Cases are listed in approximate order of grade, from low (WDLL) to high (DUL).*

No.	Diagnosis	% Tumor Cells	% Large Tumor Cells
1	WDLL	79.0	18.4
2	PDLL (N+D)	88.2	13.4
3	ILL	75.3	30.9
4	ILL	56.2	29.3
5	MLH (N+D)	43.3	16.8
6	MLH (N+D)	50.9*	18.8
7	HL (N)	41.0	29.6
8	HL (D)	60.9*	34.3
9	HL (D)	60.0*	93.2
10	DUL	75.0	30.9
11	Reactive	—	14.7
12	Reactive	—	26.3

*Determined by DNA Ploidy



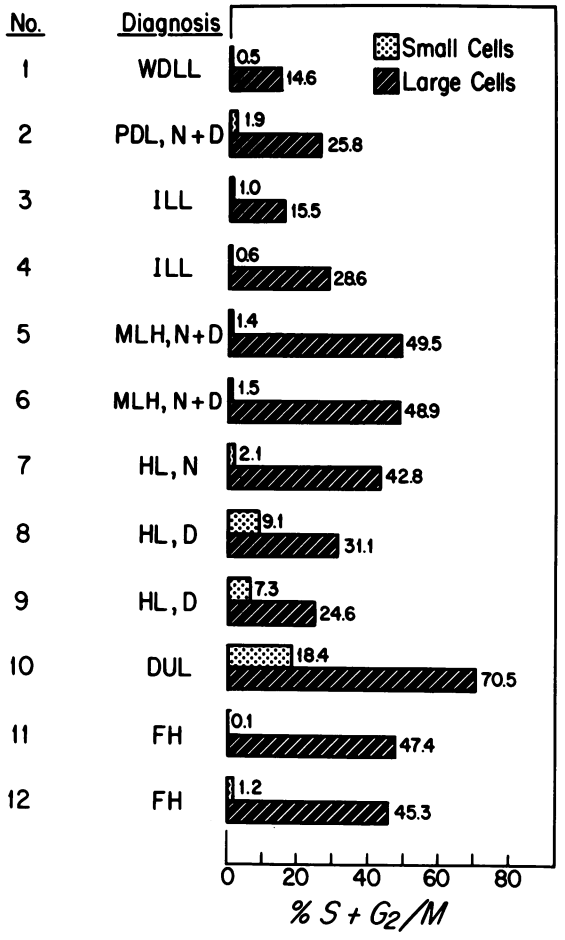


Figure 5. *S + G2/M phase DNA content of large and small cell populations. In each case, the lymphoma cells were selectively gated, using either light chain type or the presence of an aneuploid population in addition to a B cell marker (CD20). In the two reactive cases (FH, follicular hyperplasia), B cells (CD20-positive) were specifically examined. These cases correspond to those shown in Figure 4.*

the tumor cells. Note that there is great variation among the cases with regard to the percentage of total cells identified as tumor cells, which ranged from 41.0% to 88.2%. In general, the more aggressive histologic types had a greater proportion of large tumor cells and a greater proliferative component. It should also be noted that some tumors contained similar proportions of large cells (eg, cases 1 and 6), yet differed significantly with regard to proliferative content. It can be seen that the overall (total cell) proliferation was slightly lower than that measured specifically among the tumor cells, as the nontumor cells demonstrated little or no proliferation. The benign B cells in follicular hyperplasia showed significant proliferation, as expected. Figure 5 compares the S + G2/M phase content of the small and large tumor cells (or reactive B cells) in each case. In every case, large cells showed a much higher proportion of S + G2/M phase activity (mean, 35.2%; range, 14.6% to 70.5%) than did small lymphoma cells (mean, 4.4%; range, 0.5% to 18.4%).

Quantitative Image Analysis of Ki-67 Staining

Although the previous analysis showed that S + G2/M phase activity is relatively restricted to the large lymphoid cells, both large and small cell fractions exhibited a significant proportion of cells in the G0/G1 phase. Because G0 phase cells are in the resting state, whereas G1 phase cells represent a cycling population, it is important to distinguish these stages of the cell cycle. If the original hypothesis were true, ie, that cell and nuclear size an directly related to cell cycle activity, then the small lymphoid cells should exist mainly in the resting (G0) state and the large cells in G1 phase and beyond. Resting and cycling cells can be distinguished on the basis of nuclear staining with the monoclonal antibody Ki-67. This antibody binds to a nuclear antigen that is expressed during G1 phase and is expressed throughout the cycle, but is absent in noncycling (resting) cell nuclei.[18] Preliminary studies showed that Ki-67 staining was best demonstrated by immunoperoxidase staining of tissue smear preparations (Figure 6). This preparative technique was ideally suited to quantitative digital microscopy, which was employed to correlate Ki-67 staining with nuclear size.

A separate series of cases was collected prospectively for the purpose of image analysis of nuclear staining with K-67 antibody, including non-Hodgkin's lymphomas and benign reactive nodes (follicular hyperplasia). As shown in Figure 7, it was relatively easy for the operator to distinguish stained and unstained nuclei on the video screen of the image analyzer (CAS 100 System). As nuclei were assigned to Ki-67-positive and negative groups, the size (nuclear area) and average optical density of the nuclei were automatically recorded, and could be displayed as frequency histograms. The accuracy of group assignments was validated by viewing histograms of the nuclear optical densities (not shown). As shown in Figure 8, a characteristic pattern in the histograms of nuclear size distributions emerged that was independent of the diagnosis. Ki-67-negative nuclei in each case were relatively small and included a narrow range of sizes. Ki-67-positive nuclei, on the other hand, included a broad range of nuclear sizes, usually larger than Ki-67-negative nuclei, but including a minor population of nuclei of relatively small size. Large nuclei were uniformly Ki-67-positive. The data for all the cases studied (15 cases of B cell lymphoma and five cases of benign follicular hyperplasia) are shown in Figure 9. On average, Ki-67-positive nuclei were 1.95 times the size (area) of Ki-67-negative nuclei, and included a far greater range of nuclear sizes (as indicated by greater standard deviations). The one exception is the first case, a well-differentiated lymphocytic lymphoma that contained negligible large cells in the infiltrate. Thus, in all cases, the resting (G0 phase) lymphocytes were restricted to a population having small nuclei, whereas ac-

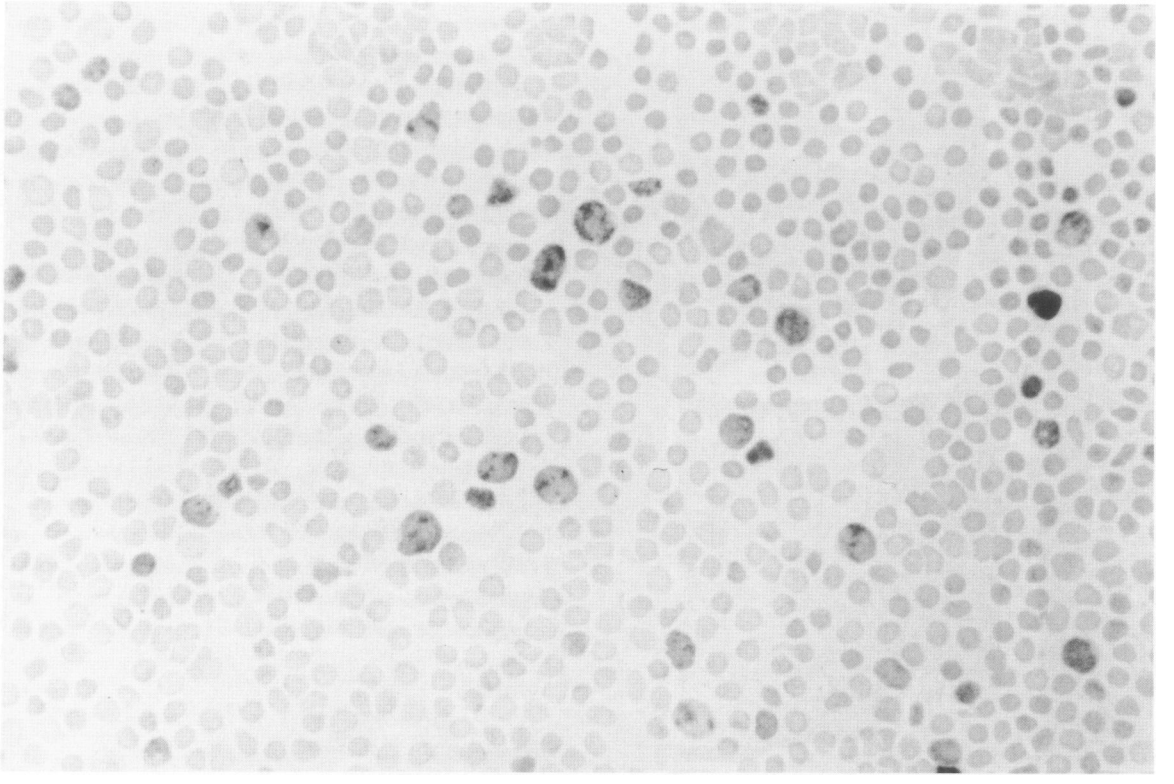


Figure 6. *Immunoperoxidase staining of a cell smear preparation with Ki-67 antibody. Ki-67-positive nuclei from a reactive lymph node are identified by the presence of diffuse or globular staining. Note the variable sizes of the Ki-67-positive nuclei (Methyl green counterstain, ×400).*

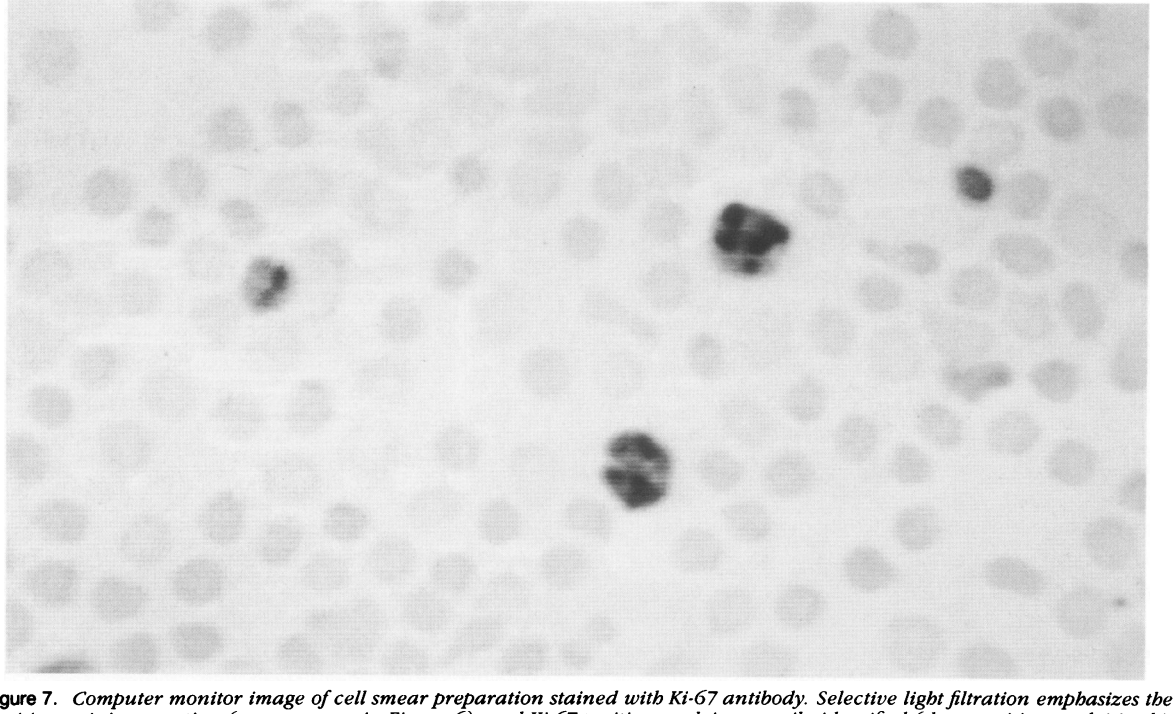


Figure 7. *Computer monitor image of cell smear preparation stained with Ki-67 antibody. Selective light filtration emphasizes the positive staining reaction (same case as in Figure 6), and Ki-67-positive nuclei are easily identified (three positive nuclei in this field). (Methyl green counterstain, ×400 microscopic magnification, CAS-100 System captured image).*

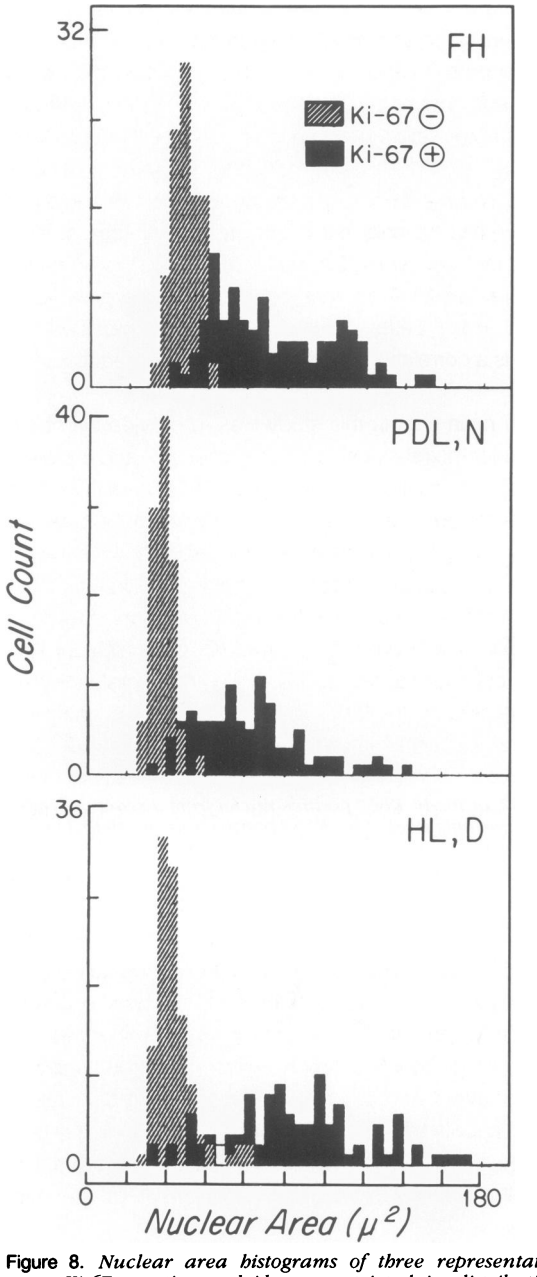


Figure 8. *Nuclear area histograms of three representative cases. Ki-67-negative nuclei have a restricted size distribution, with a relatively small nuclear area. The Ki-67-positive nuclei exhibit a broad size distribution, most being larger than K-67-negative nuclei, but including a small fraction having a relatively small nuclear area. Note that regardless of the diagnosis, the histograms appear similar (FH, follicular hyperplasia; PDL, poorly differentiated lymphoma; N, nodular; HL, histiocytic lymphoma; D, diffuse.*

tively cycling cells (Ki-67 positive) demonstrated a spectrum of nuclear sizes; a minor population had small nuclei and almost all forms had large nuclei.

Discussion

Morphologic heterogeneity, particularly nuclear and cell size variation, is commonly observed in non-Hodgkin's lymphomas, and forms the basis for most currently used classification systems.[1-4] Of the lymphomas believed to be of follicular center cell origin, the main difference among the histologic subtypes is the relative proportions of large and small lymphoid cells.[1,2] Although it has been frequently stated that the large malignant lymphoid cells represent a "blastic" or "transformed" component of the neoplasm, there exists surprisingly little direct evidence to support this assertion. In both the Kiel classification system and that of Lukes and Collins, it was hypothesized that the morphologic features of the malignant cells paralleled the transformation events seen among normal lymphoid cells *in situ*.[2,3] This idea was supported by the discovery that the neoplastic cells in most large cell lymphomas are of lymphoid, rather than true histiocytic, origin.[19]

There is a great deal of indirect evidence that cellular size, nuclear size, or both are related to proliferative activity in non-Hodgkin's lymphomas. The morphologic features of the large cells (ie, enlarged nuclei, diffuse chromatin, and prominent nucleoli) are features long known to be associated with blastic transformation of small lympho-

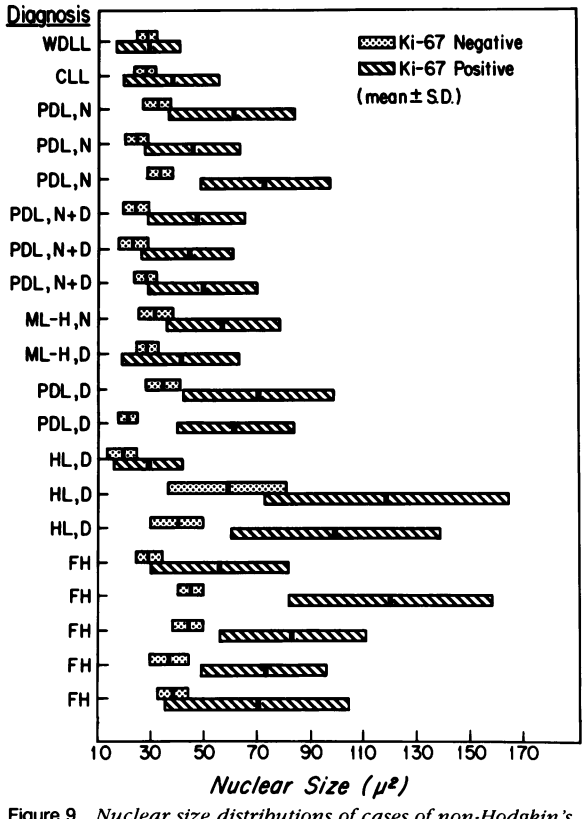


Figure 9. *Nuclear size distributions of cases of non-Hodgkin's lymphoma and follicular hyperplasia (FH). The mean and 1 SD of the nuclear size (area) distributions of Ki-67-positive and negative nuclei in each case. Note that in each case (except the first) the positive nuclei have a greater mean size and show a broader distribution than Ki-67-negative nuclei. WDLL, well differentiated lymphocytic lymphoma; CLL, chronic lymphocytic leukemia; PDL, poorly differentiated lymphoma; N, nodular; D, diffuse; ML-H, mixed lymphocytic-histiocytic lymphoma, FH, follicular hyperplasia.*

cytes in response to plant lectins.[11] Within normal germinal centers, the proliferative (or "dark") zone contains predominantly larger centroblasts and is the site of most of the proliferative activity in the follicle, whereas the "light" zone contains predominantly nonproliferating small centrocytes.[20,21] In well-differentiated lymphocytic lymphoma and tissues involved by chronic lymphocytic leukemia, scattered proliferative zones are often seen, which are conspicuous by virtue of the presence of large transformed lymphocytes.[3,22] In follicular center cell lymphomas, morphologic transformation to large cell types is accompanied by more rapid tumor growth, greater mitotic activity, and a worsened clinical course.[23,24]

These kinds of observations led Taylor to propose a decade ago that morphologic classification of lymphomas is mainly related to the extent of cell cycle activity among the neoplastic cells.[25] However, in more recent years a large body of knowledge has accumulated concerning the phenotypic features of both benign and malignant lymphocytes. The concept has been developed that malignant lymphomas represent clonal proliferations of lymphocytes that are "frozen" at certain levels of differentiation,[26] and attempts have been made to correlate lymphoma classification with states of normal lymphocyte maturation.[10] This approach has provided great insight into both normal and neoplastic lymphocyte differentiation, as illustrated by our improved understanding of the relationship between the diversity of phenotypes seen in acute lymphoblastic leukemia and stages of early B cell development.[27] However, in neoplasms of more mature B and T cells, phenotypic abnormalities are not uncommon, and it is not always possible to correlate the lymphoma phenotype with known normal lymphoid subsets.[9,28,29] Also, this attempt to relate lymphoma classification with specific stages of lymphocyte differentiation largely ignores other aspects of lymphocyte biology, such as activation and proliferation.

Cellular and nuclear size changes during the cell cycle are characteristic of normal lymphocyte proliferation. *In vitro* studies showed that increased cell size is one of the earliest features seen after stimulation of small resting B cells.[30,31] Size increase appears to be a marker for entry of cells into the G1 phase of the cycle from the resting (G0) state, and lymphoid cells continue to increase in size throughout the cycle before mitosis.[32] In general, the attainment of a critical cellular mass is believed to be an important factor for commitment of mammalian cells to DNA replication.[33] Using flow cytometric analysis, several earlier studies have noted an association between increased cell/nuclear size and the greater S phase activity in non-Hodgkin's lymphomas.[7,34] Shakney et al[35] found that increased S phase activity is associated with lymphomas of large cell type, and the authors proposed that the small cell size of indolent lymphomas reflects their slow proliferation rate. In further flow cytometric studies,[36] they showed that in individual cases of lymphoma, large neoplastic cells have a higher S phase fraction than small cells, and suggested that the large neoplastic cells represent a more rapidly proliferating population of tumor cells. Others[7,37,38] noted the relationship between histologic grade and the extent of S phase activity, with large cell lymphomas typically exhibiting greater S phase content than small cell types. Braylan et al[39] noted the presence of increased DNA content (aneuploidy) among the large cells in many cases of lymphomas, and suggested that there is a correlation between cell size and genomic content.

My main goal in this study was to provide direct evidence for the relationship between cell size and cell cycle activity in non-Hodgkin's lymphoma. Multiparameter flow cytometry provides a means for correlating multiple features among large numbers of cells, and provides the advantage of selective "gating" of cell populations based on specific markers or other measurements. Initially, it was particularly desirable to exclude nonmalignant lymphoid cells from analysis, which was accomplished by selecting cells on the basis of light chain type or the presence of a B cells marker (CD20). As Braylan et al[39] and Kruth et al[40] have previously shown, gated analysis of the malignant cells provides a more accurate measurement of S phase activity in B cell lymphomas, as nonproliferating T cells may be a significant portion of the total cell population. Cell size gates were additionally chosen to divide the selected cells into two groups ("large" and "small") based on the size distribution of normal lymphocytes, although cell size distribution is not clearly bimodal in most lymphomas.[6,7,34] In each case examined, as well as in benign reactive lymph nodes, the large lymphoid cells showed a much higher proportion of S + G2/M phase activity than did the small cells. This finding is consistent with the idea that the large lymphoid cells represent the actively proliferating component of the neoplasm.

In some cases, such as that illustrated in Figure 3, some S phase activity is seen among the small lymphoid cells. Such a finding was probably due to imprecise selection of cell size gates, as well as to the continuous nature of cellular changes that occur as cells progress through the cell cycle. Thus, the gates should probably be thought of as "enriching" for large or small cells, rather than as representing a precise selection of cell populations. More important, although the flow cytometric studies revealed a significant content of G0/G1 phase cells among both the large and small cell populations, it was not possible to distinguish resting (G0 phase) and cycling (G1 phase) cells by this method. One would predict that the large lymphoid cells with diploid DNA represent cells in G1 phase, whereas small diploid cells are in the resting phase. Other

parameters, such as RNA and total protein content, have been used to distinguish G0 and G1 phases by flow cytometry[41,42]; however, preliminary experiments failed to provide satisfactory separation of these phases, and a different approach became necessary.

Ki-67 mouse monoclonal antibody recognizes a nuclear antigen expressed in human cells during G1, S, G2, and M phases of the cell cycle, but not in the resting (G0) phase.[18,43] Thus, this antigen may serve as a marker that distinguishes resting cells from those that have entered the cell cycle. Preliminary experiments determined that nuclear staining with Ki-67 antibody was ideally suited to quantitative image analysis using the CAS 100 System.[17] Although nuclear area rather than total cell size was evaluated by this method, such a study appeared relevant to the issues raised by the flow cytometry studies for two reasons: (1) The distribution of nuclear sizes plays an important role in lymphoma classification, and (2) Nuclear size, as well as total cell size, is known to increase during the cell cycle.[42,44,45] Indeed, studies on mitogen-stimulated lymphocytes showed that there is a marked increase in nuclear volume that precedes the onset of S phase, occurring predominantly in the G1 phase.[46] Due to variation in the preparation of smears, nuclear size distributions of Ki-67-positive and negative nuclei were compared in individual cases; data from all cases were not grouped. One disadvantage of the method used in this study is that benign and malignant cells within the infiltrate cannot be distinguished. However, the flow cytometric studies indicated that benign and malignant lymphoid cells show similar relationships between cell size and cell cycle activity. In addition, benign control cases were studied as well as lymphomas, and differences in the features of benign and malignant cell proliferation would have become apparent. The results for benign lymphoid hyperplasias and for each case of malignant lymphoma, regardless of histologic type, were identical: Ki-67-negative cells made up a population having small nuclei with a restricted size distribution, whereas Ki-67-positive nuclei were approximately twice as large (in average area) and included a wide spectrum of sizes. It can be concluded that lymphoid cells with large nuclei represent cycling cells that have at least entered the G1 phase of the cell cycle. Thus, the large dipoid cells observed in the flow cytometry studies most likely represent G1 phase cells.

Unexpectedly, a minor proportion of cells having small nuclei were Ki-67-positive. It is possible that a minor fraction of small cells were immediately postmitotic, and briefly retained the Ki-67 antigen while entering the resting state. However, *in vitro* studies on fetal lung cells showed that Ki-67 staining disappears shortly after metaphase,[47] and is not present in resting cells. Studies of lymphocyte proliferation are typically based on *in vitro* methods, and it is possible that features of *in vivo* lymphocyte proliferation are somewhat different. Dardick et al[48] showed that *in vivo* stimulation of mouse splenic lymphocytes introduced into the peritoneal cavity fails to produce cells having morphologic features of germinal center cells, indicating that some aspects of proliferation may be microenvironment specific. The significance of the presence of cells having small Ki-67-positive nuclei cannot be easily explained until more is known about the molecular biology of the Ki-67 antigen and the process of lymphocyte proliferation *in situ*.

Taylor[25] stated that the ". . . observed differences in morphologic features between the various lymphomas are dependent not so much on their being composed of cells of different origin, for they are not, but rather on the cell cycle phase of the predominant cell." Although cellular differentiation certainly plays a role in determining the morphology of malignant lymphoma cells, cell proliferation undoubtedly is an important determinant of tumor morphology, classification, and prognosis. These studies demonstrate the intimate relationship between morphology and cell cycle activity in malignant lymphomas, and provide a biologic explanation for many of the histologic features observed in these neoplasms.

References

1. Rappaport H: Tumors of the hematopoietic system, Atlas of Tumor Pathology, Section 3, Fascicle 8. Washington, US Armed Forces Institute of Pathology 1966
2. Lukes RJ, Collins RD: Immunologic characterization of human malignant lymphomas. Cancer 1974, 34:1488–1503
3. Lennert K, Mohri N, Stein H, Kaiserling E: The histopathology of malignant lymphoma. Br J Haematol 1975, 31:193–203
4. National Cancer Institute sponsored study of classification of non-Hodgkin's lymphoma. Summary and description of a working formulation for clinical usage: The non-Hodgkin's lymphoma pathologic classification project. Cancer 1982, 49:2112–2135
5. Winberg CD, Sheibani K, Burke JS, Wu A, Rappaport H: T-cell-rich lymphoproliferative disorders: Morphologic and immunologic differential diagnosis. Cancer 1988, 62:1539–1555
6. Weinberg DS, Pinkus GS, Ault KA: Cytofluormetric detection of B cell clonal excess: A new approach to the diagnosis of B cell lymphoma. Blood 1984, 63:1080–1087
7. Braylan RC, Fowlkes BJ, Jaffe ES, Sanders SK, Berard CW, Herman CJ: Cell volumes and DNA distributions of normal and neoplastic human lymphoid cells. Cancer 1978, 41: 201–209
8. Williams ME, Innes DJ, Borowitz MJ, Lovell MA, Swerdlow SH, Hurtubise PE, Brynes RK, Chan WC, Byrne GE, Whitcomb CC, Thomas CY: Immunoglobulin and T cell receptor gene rearrangements in human lymphoma and leukemia. Blood 1987, 69:79–86

9. Picker LJ, Weiss LM, Medeiros LJ, Wood GS, Warnke RA: Immunophenotypic criteria for the diagnosis of non-Hodgkin's lymphoma. Am J Pathol 1987, 128:181–201
10. Foon KA, Todd RF: Immunologic classification of leukemia and lymphoma. Blood 1986, 68:1–31
11. Nowell PC: Phytohemagglutinin: An initiator of mitosis in cultures of normal human leukocytes. Cancer Res 1960, 20: 462–466
12. Mann RB, Jaffe ES, Berard CW: Malignant lymphomas—A conceptual understanding of morphologic diversity: A review. Am J Pathol 1979, 94:105–175
13. Tindle BH: Malignant lymphomas. Am J Pathol 1984, 116: 119–174
14. Weinberg DS, Ault KA, Gurley M, Pinkus GS: The human lymph node germinal center cell: Characterization and isolation by using two-color flow cytometry. J Immunol 1986, 137: 1486–1494
15. Braylan RC, Benson NA, Nourse M, Kruth HS: Correlated analysis of cellular DNA, membrane antigens and light scatter of human lymphoid cells. Cytometry 1982, 337–343
16. Kurtin PJ, Pinkus GS: Leukocyte common antigen—A diagnostic discriminant between hematopoietic and nonhematopoietic neoplasms in paraffin sections using monoclonal antibodies: Correlation with immunologic studies and ultrastructural localization. Hum Pathol 1985, 16:353–365
17. Bacus JW, Grace LJ: Optical microscope system for standardized cell measurements and analyses. Applied Optics 1987, 26:3280–3293
18. Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984, 133:1710–1715
19. Glick AD, Leech JH, Waldron JA, Flexner JM, Horn RG, Collins RD: Malignant lymphomas of follicular center cell origin in man. II. Ultrastructural and cytochemical studies. J Natl Cancer Inst 1975, 54:23–36
20. Nieuwenhuis P, Opstelten D: Functional anatomy of germinal centers. Am J Anat 1984, 170:421–435
21. Stein H, Gerdes J, Mason DY: The normal and malignant germinal centre. Clin Haematol 1982, 11:531–559
22. Lennert K, Kohri N: Histopathology and diagnosis of non-Hodgkin's lymphoma, Malignant Lymphomas Other than Hodgkin's Disease. Edited by K Lenert. New York, Springer-Verlag 1978, pp 111–469
23. York JC, Glick AD, Cousar JB, Collins RD: Changes in the appearance of hematopoietic and lymphoid neoplasms: Clinical, pathologic, and biologic implications. Hum Pathol 1984, 15:11–38
24. Cullen MH, Lister TA, Brearley RL, Shand WS, Stansfeld AG: Histological transformation of non-Hodgkin's lymphoma: A prospective study. Cancer 1979, 44:645–651
25. Taylor CR: Classification of lymphoma: 'New thinking' on old thoughts. Arch Pathol Lab Med 1978, 102:549–554
26. Seligman M, Preud'homme J-L, Brouet J-C: Human lymphoproliferative diseases as models of lymphocytic differentiation, Human Lymphocyte Differentiation: It's Application to Cancer. INSERM Symposium 8. Amsterdam, North-Holland Publishing Co. 1978, pp 133–140
27. Anderson DC, Bates MP, Slaughenhoupt BL, Pinkus GS, Schlossman SF, Nadler LM: Expression of human B cell-associated antigens on leukemias and lymphomas: A model of human B cell differentiation. Blood 1984, 63:1424–1433
28. Mason DY: A new look at lymphoma immunohistology. Am J Pathol 1987, 128:1–4
29. Weiss LM, Picker LJ, Copenhaver CM, Warnke RA, Sklar J: Large-cell hematolymphoid neoplasms of uncertain lineage. Hum Pathol 1988, 19:967–973
30. DeFranco AL, Raveche ES, Asofsky R, Paul WE: Frequency of B lymphocytes responsive to anti-immunoglobulin. J Exp Med 1982, 155:1523–1536
31. Muraguchi A, Kehrl JH, Butler JL, Fauci AS: Sequential requirements for cell cycle progression of resting human B cells after activation by anti-Ig. J Immunol 1984, 132:176–180
32. Thompson CB, Scher I, Schaefer ME, Lindsten T, Finkelman FD, Mond JJ: Size-dependent B lymphocyte subpopulations: Relationship of cell volume to surface phenotype, cell cycle, proliferative response, and requirements for antibody production to TNP-Ficoll and TNP-BA. J Immunol 1984, 133: 2333–2342
33. Murray AW: A cycle is a cycle is a cycle. Nature 1987, 327: 14–15
34. Diamond LW, Braylan RC: Flow analysis of DNA content and cell size in non-Hodgkin's lymphoma. Cancer Res 1980, 40: 703–712
35. Shackney SE, Levine AM, Fisher RI, Nichols P, Jaffe E, Schuette WH, Simon R, Smith CA, Occhipinti SJ, Parker JW, Cossman J, Young RC, Lukes RJ: The biology of tumor growth in the non-Hodgkin's lymphomas: A dual parameter flow cytometry study of 220 cases. J Clin Invest 1984, 73: 1201–1214
36. Shackney SE, Skramstad K: A dynamic interpretation of multiparameter studies on the lymphomas. Am J Clin Pathol 1979, 72:756–764
37. Christensson B, Tribukait B, Linder I-L, Ullman B, Biberfeld P: Cell proliferation and DNA content in non-Hodgkin's lymphoma: Flow cytometry in relation to lymphoma classification. Cancer 1986, 58:1295–1304
38. Diamond LW, Nathwani BN, Rappaport H: Flow cytometry in the diagnosis and classification of malignant lymphoma and leukemia. Cancer 1982, 50:1122–1135
39. Braylan RC, Benson NA, Nourse VA: Cellular DNA of human neoplastic B-cells measured by flow cytometry. Cancer Res 1984, 44:5010–5016
40. Kruth WS, Braylan RC, Benson NA, Nourse VA: Simultaneous analysis of DNA and cell surface immunoglobulin in human B-cell lymphomas by flow cytometry. Cancer Res 1981, 41:4895–4899
41. Piwnicka M, Darzynkiewicz Z, Melamed MR: RNA and DNA content of isolated cell nuclei measured by multiparameter flow cytometry. Cytometry 1983, 3:269–275
42. Roti Roti JL, Higashikubo R, Blair OC, Uygur N: Cell-cycle position and nuclear protein content. Cytometry 1982, 3:91–96
43. Gerdes J, Schwab U, Lemke H, Stein H: Production of a mouse monoclonal antibody reactive with a human nuclear

antigen associated with cell proliferation. Int J Cancer 1983, 31:13–20
44. Yen A, Fried J, Kitahara T, Strife A, Clarkson BD: The kinetic significance of cell size. II. Size distributions of resting and proliferating cells during interphase. Exp Cell Res 1975, 95: 303–310
45. Dardick I, Setterfield G, Hall R, Bladon T, Little J, Kaplan G: Nuclear alterations during lymphocyte transformation: Relationship to the heterogeneous morphologic presentations of non-Hodgkin's lymphomas. Am J Pathol 1981, 103:10–20
46. Setterfield G, Hall R, Bladon T, Little J, Kaplan JG: Changes in structure and composition of lymphocyte nuclei during mitogenic stimulation. J Ultrastruct Mol Struct Res 1983, 82: 264–282
47. Braun N, Papadopoulos T, Müller-Hermelink HK: Cell cycle dependent distribution of the proliferation-associated Ki-67 antigen in human embryonic lung cells. Virchows Arch [B] 1988, 56:25–33
48. Dardick I, Sinnott NM, Hall R, Bajenki-Carr TA, Setterfield G: Nuclear morphology and morphometry of B-lymphocyte transformation: Implications for follicular center cell lymphomas. Am J Pathol 1983, 111:35–49

Acknowledgment

The author wishes to thank Ms. Michelle Toder for technical assistance.