*American Journal of Pathology, Vol. 134, No. 2, February 1989*


# Cell Proliferation in Non-Hodgkin's Lymphomas

## *Digital Image Analysis of Ki-67 Antibody Staining*

**Beth R. Schwartz, Geraldine Pinkus, Sarah Bacus, Michelle Toder, and David S. Weinberg**

*From the Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, and Cell Analysis Systems, Inc., Lombard, Illinois*

***Ki-67 is a monoclonal antibody to a nuclear antigen present in cycling human cells but not in resting cells. The authors have performed immunoperoxidase on non-Hodgkin's lymphomas using Ki-67 antibody in order to correlate proliferation rates with tumor grade and type, and compare Ki-67 staining with S-phase content as determined by flow cytometry. Ki-67 staining of 109 sections was quantitated using a digital image analysis system (CAS 100). There was a significant difference among mean overall Ki-67 staining values in Working Formulation low (13.7%), intermediate (42.6%), and high grade tumors (57.9%, P < 0.00001). The level of significance improved when a revised grading system was formulated based on proliferative activity, with the inclusion of diffuse large cell lymphomas in the high grade category. Within nodular and a few diffuse lymphomas, there were well-defined proliferation centers in which Ki-67 staining showed no correlation with grade. Flow cytometric DNA determination was performed on 74 specimens, and there was a positive correlation between Ki-67 positivity and S phase content (r = 0.66). It is concluded that Ki-67 staining of tissue sections is an alternative to flow cytometric quantitation of cell cycle activity in lymphomas, and provides the advantage of revealing histologic patterns of proliferation. By including G1 phase cells, Ki-67 staining allows a more complete determination of total cell cycle activity in lymphomas. (Am J Pathol 1989, 134: 327–336)***

Non-Hodgkin's lymphomas have been classified into subtypes based on histologic criteria. In most classification systems, there is a correlation between histology and clinical behavior.[1] Histology alone is not always predictive of clinical behavior, however, and other biologic parameters such as cytogenetics,[2] tumor ploidy,[3–5] and cell cycle activity[5–7] may prove to be prognostically important. In addition, more objective criteria are needed to type and classify the various non-Hodgkin's lymphomas.

Tumor cell proliferation in lymphomas has been previously assessed by tritiated thymidine uptake, mitotic index, and flow cytometry. Most studies have shown a correlation between proliferation rate and either histologic grade[4–17] or survival[3,16,18–20] in non-Hodgkin's lymphoma. However, these studies use only parts of the cell cycle to measure proliferative activity but do not address the sum of actively cycling cells, and, in particular, do not consider G1 phase content.

Ki-67 is a mouse monoclonal antibody that recognizes a proliferation-related nuclear antigen expressed during G1, S, and G2/M phases of the cell cycle but not in resting (G0 phase) cells.[21,22] By including cell cycle phase G1, Ki-67 expression gives more information than can be determined by standard flow cytometry, tritiated thymidine uptake, or mitosis counting. Studies performed on tissue sections provide the additional advantage of revealing the topographic distribution of cycling cells. Only recently has Ki-67 antibody been used to demonstrate a relationship between cell proliferation in lymphomas and prognosis.[23–25]

We have taken advantage of new quantitative image analysis techniques to examine cell proliferation in non-Hodgkin's lymphomas stained with Ki-67 antibody. This study shows the value of rapid, objective quantitation of cell proliferation in classifying these tumors, and our results are compared with other studies of Ki-67 staining in lymphomas that either count[26] or estimate[27] the number of positively staining cells. Our aims were to correlate Ki-

Supported in part by National Institutes of Health Grant AI23360.

Accepted for publication September 20, 1988.

Presented in part at the 77th Annual Meeting of the International Academy of Pathology, United States-Canadian Division, March, 1988.

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

**Table 1.** *Cases Included in Study and Overall Ki-67 Staining According to Diagnosis*

| Diagnosis | Code | Grade* | Number of cases† | Overall Ki-67 staining‡ | Range |
|---|---|---|---|---|---|
| Small lymphocytic lymphoma | A | Low | 15 | 8.46 ± 1.47 | 22.42 |
| Follicular small cleaved cell lymphoma | B | Low | 16 | 13.36 ± 1.86 | 26.76 |
| Follicular mixed, small cleaved and large cell lymphoma | C | Low | 6 | 29.36 ± 5.81 | 42.47 |
| Follicular large cell lymphoma | D | Int§ | 4 | 38.91 ± 9.83 | 47.63 |
| Diffuse small cleaved cell lymphoma | E | Int | 15 | 23.70 ± 5.77 | 81.31 |
| Diffuse mixed, small and large cell lymphoma | F | Int | 8 | 29.66 ± 6.10 | 49.93 |
| Diffuse large cell lymphoma | G | Int | 18‖ | 68.20 ± 4.36 | 61.71 |
| Large cell immunoblastic sarcoma | H | High | 12¶ | 59.91 ± 5.85 | 73.96 |
| Lymphoblastic lymphoma | I | High | 1** | 47.35 ± 0.00 | — |
| Small noncleaved cell lymphoma | J | High | 3 | 53.60 ± 5.36 | 18.51 |
| Intermediate lymphocytic lymphoma | K | NA†† | 2 | 14.15 ± 6.24 | 12.48 |
| Reactive lymph node with follicular hyperplasia | L | NA | 9 | 12.24 ± 1.97 | 21.77 |

* Grade according to the Working Formulation for non-Hodgkin's lymphomas.
† All cases are phenotypically B cell tumors, except as indicated.
‡ Mean ± standard error.
§ Intermediate grade.
‖ Phenotype: 17B, 1T.
¶ Phenotype: 8B; 4T.
** Phenotype: 1T.
†† Not applicable.

67 determined proliferative activity with tumor grade and subtype according to the Working Formulation, to compare overall Ki-67 staining of non-Hodgkin's lymphomas with cell cycle activity as determined by standard flow cytometry, and to evaluate the topographic distribution of Ki-67 staining with regard to histologic subtype and grade.

## Materials and Methods

### Specimens

One hundred specimens from lymph nodes and spleens diagnosed as non-Hodgkin's lymphomas between 11/10/83 and 11/17/87 were obtained retrospectively from the Department of Pathology at the Brigham and Women's Hospital (Table 1). Cases that had been stored longer than 4 years showed diminished staining, and could not be used. Because storage conditions did not vary, loss of antigenicity was probably related to the age of the specimen. All cases have been immunophenotyped by immunoperoxidase studies at the time of original diagnosis (Rappaport classification), and were re-classified according to the Working Formulation.[1] Ninety-three of the 100 specimens were B cell lymphomas; the remaining seven cases were T cell neoplasms. Tissue used for immunoperoxidase studies had been uniformly snap frozen in dry ice and isopentane, and stored at −80 C. Nine lymph nodes with reactive follicular hyperplasia, frozen and stored in the same manner, were also studied.

### Immunocytochemistry

Cryostat sections were reacted with Ki-67 antibody (DAKO-PC, Dako Corporation, Santa Barbara, CA), using standard immunoperoxidase techniques.[28] Sections were cut at 2-3 $\mu$. At this thickness, only a single layer of nuclei was present and there was not significant nuclear overlap. The sections were fixed for 6 minutes in 1% paraformaldehyde at 20 C. Peroxidase conjugated goat anti-mouse immunoglobulin and swine anti-goat immunoglobulin were used as secondary staining reagents. Controls consisted of sections incubated with buffer instead of the primary antibody. The slides were developed using 3′-3′-diaminobenzidine tetrahydrochloride (Aldrich Chemical Company, Milwaukee, WI) as chromogen, and sections were counterstained with 2% methyl green. In each case, an adjacent section was cut for hematoxylin and eosin (H&E) staining to confirm that representative lesional tissue was present.

### Digital Image Quantitation 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.[29] Analog video signals are digitalized and stored as 256 × 256 pix-

els containing 8 bits of intensity values, sufficient for 256 levels of gray determination. Digitalized light intensity values are converted by an input look-up table to optical density values, based on previous standardization and calibration of the instrument. Thus, image memory contains values for optical density for each pixel. Nuclear Ki-67 staining was measured using software supplied with the instrument. Microscopic fields were selected for measurement using criteria described below. First, using red (650/10 nm bandpass) filter, an image of the total cell nuclei in the microscopic field was obtained. Then, using a green (540/10 nm bandpass) filter, the same field was again captured, revealing nuclei stained brown by the immunoperoxidase reaction (as recognized by their increased optical density compared with unstained cells). The computer automatically calculates the percent of the nuclear field stained by the immunoperoxidase reaction by comparing the images obtained by the red and green filters. All images were obtained using a ×40 objective (numerical aperture 0.66) and a ×10 eyepiece, yielding a final magnification of ×400.

Fields were selected for measurement by two methods. First, all cases were analyzed by random selection of fields, using computer-determined random pairs of coordinates encompassing the tissue section as measured by stage sensors of the CAS 100 System. Second, in cases showing obvious nodular distribution of Ki-67 staining, the nodular areas containing the most abundant staining were selected for measurement. Thus, the first measurement provides assessment of the "overall Ki-67 staining" by a random determination, whereas the second method provides quantitation reflecting the maximum Ki-67 staining within tissues having nodular staining patterns. Field measurements were accumulated and averaged on each slide until additional measurements did not change the average by more than 5%, usually requiring five to ten measurements. In all cases no fewer than four fields were measured.

### *Flow Cytometry*

Sixty-five of the 100 non-Hodgkin's lymphomas had flow cytometric cell cycle analysis performed on fresh tissue at the time of surgery, using previously described methods.[30] Briefly, detergent-isolated nuclei were stained with propidium iodide, and DNA histograms were generated on a FACS analyzer (Becton Dickinson). G0/G1, S, and G2/M phases were quantitated from the DNA histograms, and immunophenotyping was also performed by flow cytometry, confirming the presence of tumor in the tissue studied. Cell cycle analysis data was also available on the nine reactive lymph nodes with follicular hyperplasia.

### *Statistical Analysis*

Statistical analysis was performed using Statgraphics (Plus*Ware, STSC Inc., Rockville, MD), including linear regression analysis and analysis of variance.

## *Results*

### *Ki-67 Antibody Staining*

Immunoperoxidase staining of cryostat sections with Ki-67 antibody revealed distinct nuclear staining of positive cells. Cytoplasmic staining was no greater than the background seen in the immunoperoxidase control. Tissues from cases of non-Hodgkin's lymphoma demonstrated variable reactivity with Ki-67 antibody, ranging from cases with only scattered positive nuclei to cases with predominantly positive nuclei (Figure 1A,B). Six of the diffuse lymphomas and all but three of the follicular lymphomas had distinct nodular zones of increased proliferation, as indicated by focally increased numbers of Ki-67 positive nuclei (Figure 1C). Of 15 small lymphocytic lymphomas, three had distinct pseudo-proliferation centers and two had foci suggestive of increased proliferation. In benign reactive lymph nodes, most staining was restricted to the germinal centers, as has been reported (Figure 1D).[18]

Quantitative image analysis using the CAS 100 system was employed to measure the amount of nuclear staining by Ki-67 antibody, as described in the Materials and Methods section. First, nuclear staining of the entire tissue section was quantitated. Figure 2 demonstrates the overall Ki-67 staining of each specimen, grouped according to histologic type by the Working Formulation. The mean and standard error of staining are shown for each type of lymphoma, as well as for intermediate lymphocytic lymphomas and reactive lymph nodes with follicular hyperplasia (Table 1). Although a trend of increasing Ki-67 positivity is noted among the Working Formulation subtypes, the range of values tends to be large in intermediate and high grade tumors (Table 1) and there is considerable overlap among diagnostic subtypes. In general, low grade (indolent) types of lymphoma show the least Ki-67 staining, and high grade (aggressive) types the greatest. When the histologic subtypes are grouped according to low, intermediate, and high grades as defined by the Working Formulation, there is a significant difference in overall Ki-67 staining among groups (Figure 3, Table 2). The mean values of overall Ki-67 staining for reactive lymph nodes and intermediate lymphocytic lymphomas are similar to those of the low grade lymphomas.

Twenty-six cases of low and intermediate-grade lymphomas exhibited obvious nodular areas of increased Ki-

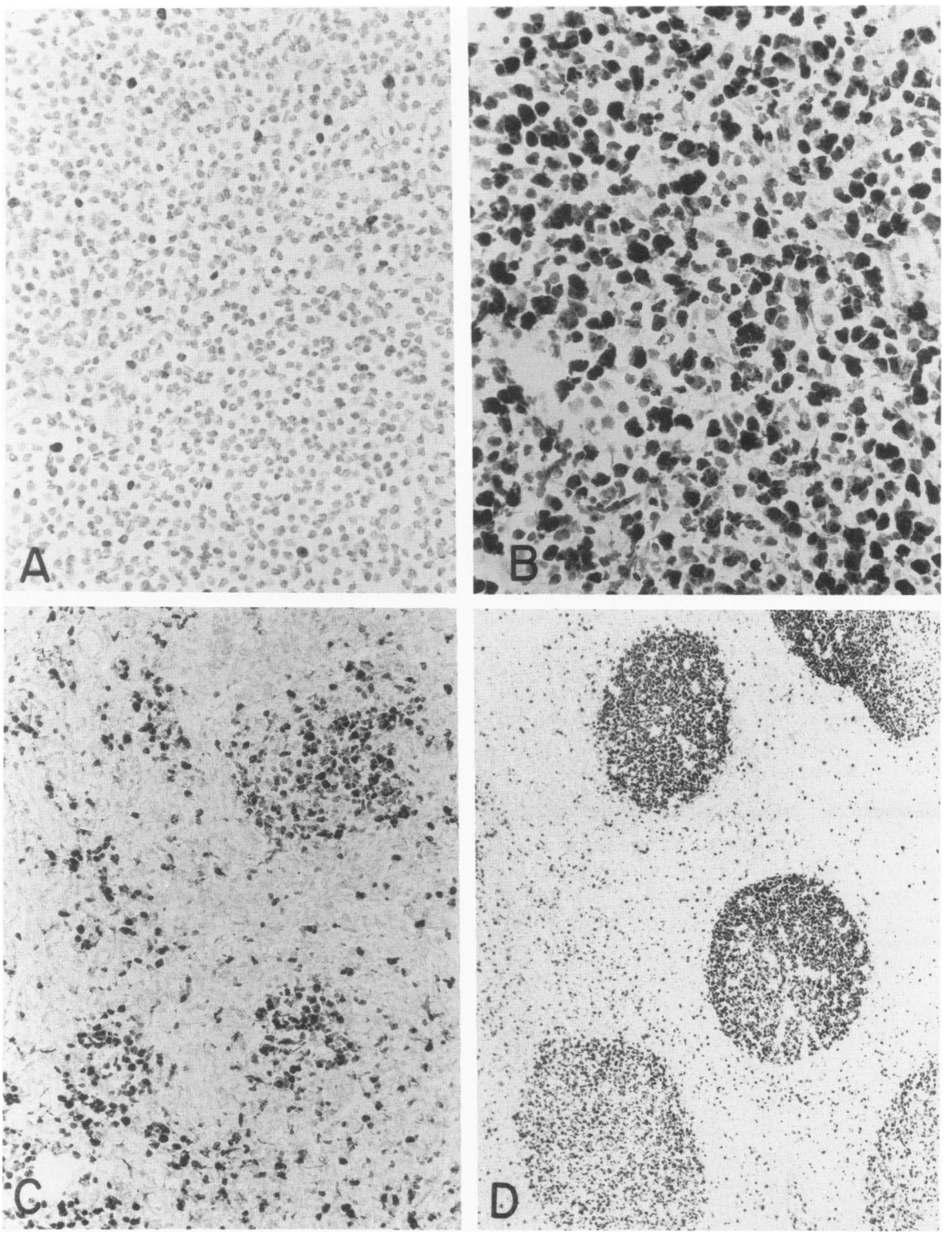


**Figure 1.** *Nuclear staining by Ki-67 antibody (immunoperoxidase method). All tissues were counterstained with methyl green, revealing nuclei only.* **A:** *Small lymphocytic lymphoma (×400).* **B:** *Diffuse large-cell lymphoma (×400).* **C:** *Follicular mixed, small cleaved and large-cell lymphoma with nodular regions of Ki-67 staining (×200).* **D:** *Reactive lymph node with Ki-67 staining primarily in germinal centers (×60).*

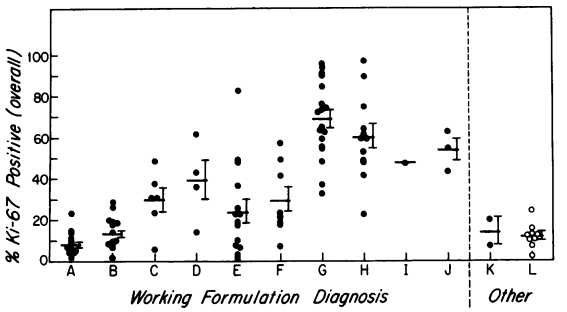


Figure 2. *Percent overall Ki-67 staining of non-Hodgkin's lymphomas subtyped according to Working Formulation diagnoses. (See Table 1 for diagnosis key.) Each point represents an individual case. Mean and standard error bars are indicated for each diagnostic category. The percent staining is calculated by measuring the proportion of total nuclear area stained by the antibody.*

67 staining. The CAS 100 System was used to quantitate the Ki-67 staining in these regions, with the resulting values representing the most highly proliferative areas within these lymphomas. There is no apparent pattern of differences among histologic types when the mean nodular staining for all evaluable diagnostic categories is examined, except for the low value shown for follicular small, cleaved cell lymphomas (B) (Table 3). The mean staining of follicles in reactive lymph nodes is similar to that seen in nodular areas of most lymphomas. The mean Ki-67 staining of the nodular regions of low (41.8%) and intermediate (47.3%) grade lymphomas and reactive lymph nodes (46.9%) did not show significant differences ($P = 0.5$, low *vs.* intermediate grades and low grade *vs.* reactive; $P = 0.9$, intermediate grade *vs.* reactive).

To compare our data with the findings of previous studies,[16,17,30] we also quantitated the highest proliferative

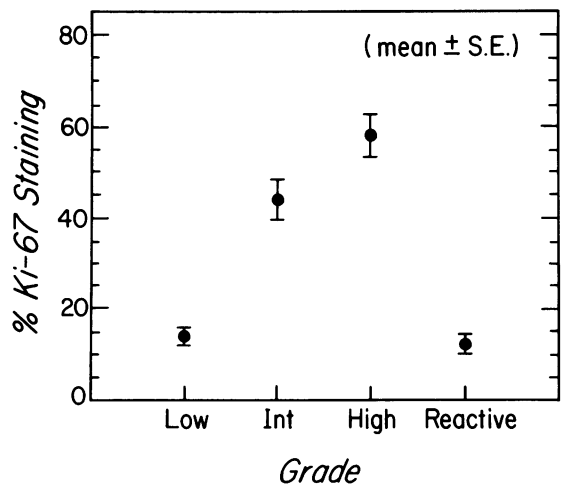


Figure 3. *Percent overall Ki-67 staining according to Working Formulation grade. Points indicate group means, with standard error bars. Reactive lymph nodes are included for comparison.*

Table 2. *Overall Ki-67 Staining According to Histologic Grade*

| Grade | Number of cases | Overall Ki-67* | Significant difference† |
|---|---|---|---|
| Low | 37 | 13.69 ± 10.56 | ‡ |
| Intermediate | 45 | 42.55 ± 28.19 | § |
| High | 16 | 57.94 ± 18.09 | |

* Mean ± standard deviation.
† Significant difference among all three grades is $P < 0.00001$.
‡ Significantly different at $P < 0.00001$.
§ Significantly different at $P = 0.05$.

rates in all cases. These measurements consisted of overall staining in most diffuse lymphomas and nodular staining in all tumors with focally increased staining patterns. Considering the highest proliferative measurement in each case, only low-grade lymphomas exhibited significantly different staining when compared with other histologic grades of lymphoma and reactive lymph nodes (Figure 4).

## Correlation Between Ki-67 Staining and Flow Cytometric DNA Analysis

Quantitative DNA measurements by flow cytometry were performed in 74 cases. In each case, the proportion of cells in G0/G1, S, and G2/M phases of the cell cycle was determined. Because the nuclei measured by flow cytometry represent a random measurement of cells within the tissue, we believed that these values might most directly correspond to the random measurements performed on tissue sections stained with Ki-67 antibody. As shown in Figure 5, although there was a general correlation between overall Ki-67 staining and S-phase content ($r = 0.66$) or S + G2/M phase content ($r = 0.62$), several cases show markedly divergent values. Thus, in some cases, S-phase content is not predictive of Ki-67 staining, which represents the total cell cycle activity within the tissue. When only nodular areas of Ki-67 staining were considered, there was a poor correlation between Ki-67 staining and S-phase content ($r = 0.22$, data not shown).

Table 3. *Measurement of Nodular Areas of Ki-67 Staining*

| Diagnosis* | Number of cases | Nodular Ki-67† |
|---|---|---|
| B | 12 | 34.65 ± 4.54 |
| C | 5 | 63.45 ± 7.86 |
| D | 3 | 47.05 ± 11.91 |
| E | 5 | 54.62 ± 12.15 |
| G | 1 | 50.55 ± 0.00 |
| L | 9 | 46.85 ± 2.27 |

* See Table 1.
† Mean ± standard error.

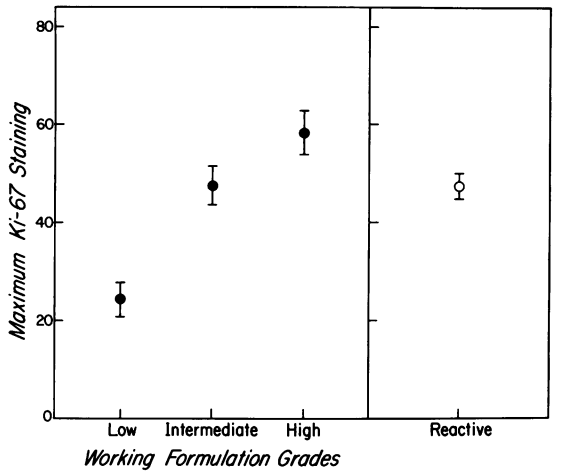


**Figure 4.** *Maximum Ki-67 staining (mean ± standard error) for low, intermediate, and high-grade lymphomas and reactive lymph nodes. The highest proliferation rate (either overall or nodular) of each case was used for calculations. Only the low-grade lymphomas showed significantly different Ki-67 staining compared with other groups when the maximal level of Ki-67 staining was considered (P > 0.0039, low grade* vs. *reactive; P = 0.0001, low grade* vs. *intermediate grade).*

## Discussion

Ki-67 antibody provides a useful alternative to other methods of measuring cell cycle activity. This antibody distinguishes all actively proliferating cells (G1, S, and G2M phases) from resting (G0 phase) cells. The nuclear staining by Ki-67 is easily adapted to the image quantitating capabilities of the CAS 100 system. Objective quantitation provides distinct advantages over other methods used to measure proliferation rates. Autoradiography is technically cumbersome, requiring the use of radioactive materials and incubation periods lasting several days. Flow cytometry, although less time-consuming, destroys the morphology of the tumor and is subject to sampling error. Studies examining mitotic counts have provided inconsistent results, probably due to differences in techniques and interobserver variability.[16,31] Previous quantitative studies of Ki-67 staining have employed either tedious manual counting or estimation,[23,24] and therefore are subject to many of the same technical difficulties already described.

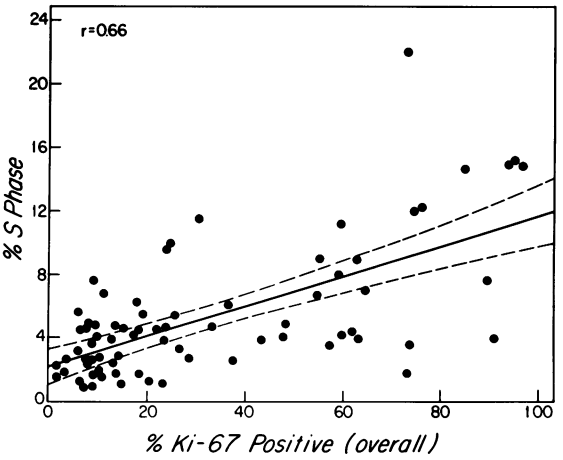


**Figure 5.** *Correlation between overall Ki-67 staining and S phase content. Seventy-four cases were analyzed for both S-phase content by flow cytometry and Ki-67 staining on tissue sections. Dashed lines indicate 95% confidence intervals.*

It should be noted that the quantity of Ki-67 staining measured using the imaging system is a percent of total nuclear area, which may not be precisely the same as percent positive nuclei. Although the entire nucleus is stained in Ki-67 positive nuclei, there is variability of nuclear size in lymphomas, and differences in the proportions of large and small nuclei may influence the values of Ki-67 staining obtained. Our main goal, however, was to correlate the Ki-67 staining value with tumor classification as a system of rapid and objective measurement. The precise correlation between percent positive nuclear area and percent positive nuclei was not determined in this study, but may be of technical interest.

The ability to examine either random or selected fields with the aid of the CAS 100 system provided additional information concerning patterns of proliferation within the tissues. When comparing overall Ki-67 staining to nodular areas of increased staining, we favor overall staining as the most significant indicator of proliferative activity within each tumor. When nodular areas of Ki-67 staining were compared, no significant difference among the tumor grades and germinal centers of reactive lymph nodes was found. This indicates that microenvironments favorable to proliferation within lymph nodes are difficult to distinguish among the broader pathologic groups examined, although follicular small cleaved cell lymphomas have a distinctly lower proliferative rate compared with other specific diagnostic types (Table 3). When we quantitated only the most highly proliferating areas in lymphomas (using overall staining in most diffuse lymphomas and nodular staining in all tumors with focally increased staining patterns), only low-grade lymphomas exhibited a significantly different amount of staining when compared with the other histologic grades and reactive lymph nodes (Figure 4). This finding differs from those of previous papers. Studies that measure mitotic rates in non-Hodgkin's lymphomas have typically analyzed the areas of highest proliferation.[16,17] Not only does this selection ignore lower proliferation zones within the tumors, but mitoses can be technically difficult to measure,[31] and reflect only one component of the cell cycle. Bauer et al found no relationship between mitotic counts by light microscopy and S-phase activity by flow cytometry.[32] Weiss et al also mea-

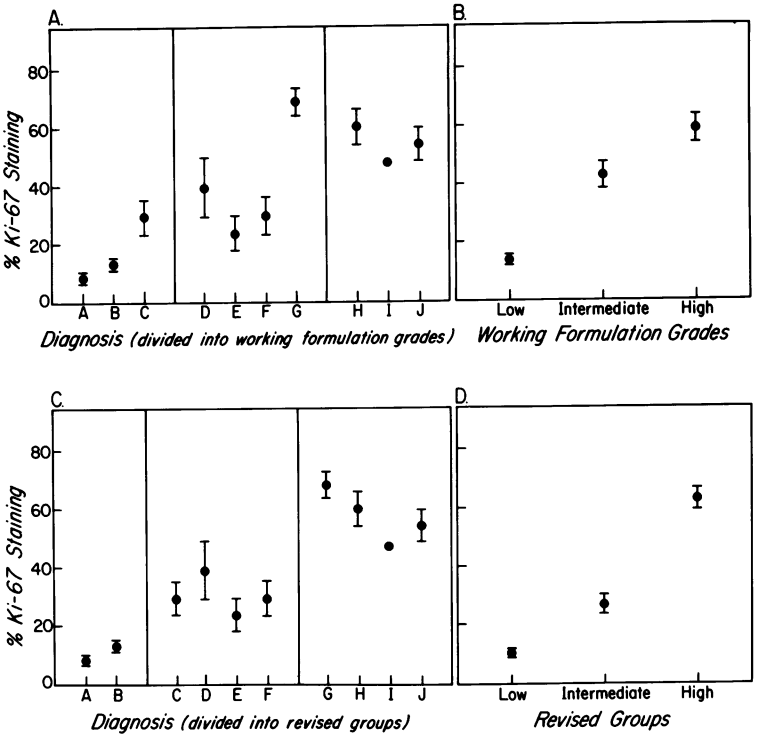


Figure 6. *Overall Ki-67 staining according to Working Formulation grades and regrouped according to revised grades.* **A:** *Percent overall Ki-67 staining (mean ± standard error) for each diagnostic category of the Working Formulation (see Table 1 for diagnosis key). Vertical lines indicate division of diagnoses into Working Formulation low, intermediate, and high grades.* **B:** *Percent overall Ki-67 staining according to Working Formulation grades. Statistical data is presented in Table 3.* **C:** *Percent overall Ki-67 staining for each diagnostic category of the Working Formulation, as in A. Vertical lines indicate division of diagnoses into revised groups.* **D:** *Percent overall Ki-67 staining according to revised groups. There are greater differences between these groups than between Working Formulation grades (P < 0.00001, low vs. intermediate and intermediate vs. high grades).*

sured the areas of greatest proliferation in non-Hodgkin's lymphomas by Ki-67 staining and found good correlation with the Working Formulation grades, although their figures were based on subjective estimates.[27] Our results demonstrate that only overall Ki-67 proliferation rates of the various non-Hodgkin's lymphomas correlate with the Working Formulation grades with a high level of overall significance ($P < 0.00001$), although the difference between intermediate and high-grade tumors is less highly significant ($P < 0.05$, Table 2). The histologic types seem to cluster more naturally when regrouped as shown in Figure 6, and the differences among the revised low, intermediate, and high-grade groups are of greater significance than the differences among the Working Formulation grades.

The revised group with the lowest proliferation rate includes small lymphocytic lymphoma and follicular small cleaved cell lymphoma. Very low Ki-67 staining in small lymphocytic lymphoma with respect to the remaining non-Hodgkin's lymphomas has also been found previously.[26,27] If it were included in the Working Formulation, intermediate lymphocytic lymphoma would also fit into this revised low grade group, which corroborates recent comparisons of this lesion with low-grade neoplasms.[33] Reactive lymph nodes also have overall staining values similar to this revised group. Parallel findings of low S-phase values in reactive nodes are cited in flow cytometric studies,[6,7,10,13] whereas studies reporting mitotic rates[16] or Ki-67 staining index[27,34] examine germinal centers only, and find proliferation rates of reactive lymph nodes to be higher than those of follicular center cell lymphomas.

The second or intermediate grade revised group includes follicular mixed small cleaved and large-cell lymphoma and all of the intermediate lymphomas of the Working Formulation excluding diffuse large cell lymphoma. Of interest is the unexpectedly low proliferative rate of diffuse small cleaved cell lymphoma (23.70%). This fact, together with the much higher Ki-67 staining seen in follicular predominantly large-cell lymphoma (38.91%), supports the finding that small lymphoid cells are predominantly in the resting (G0) phase of the cell cycle.[7-10,16,35]

The revised high-grade group includes diffuse large cell lymphoma, immunoblastic sarcoma, lymphoblastic lymphoma, and small noncleaved cell lymphoma. Of interest here is the grouping of diffuse large-cell lymphoma with the higher grade lymphomas. Similarly, Christensson[6] found significant differences between the S-phases of diffuse large cell lymphoma and two other intermediate grade lymphomas by flow cytometry. On a practical level, diffuse large-cell lymphomas have similar clinical behavior to the Working Formulation high-grade lymphomas, and show a similar response to chemotherapeutic agents as these tumors.

Because only three cases of small noncleaved cell lymphomas could be obtained for examination, there is

insufficient data to compare with other studies that claim that this class of tumor has the highest Ki-67 expression.[26,27,36] Only one case of lymphoblastic lymphoma was obtained for staining, which showed a lower than expected Ki-67 staining of 47.35%. Other studies have found S-phase activity and Ki-67 expression to be more variable than expected in lymphoblastic lymphomas, given their high mitotic activity and grade.

Overall Ki-67 staining shows some correlation with S and S + G2/M phase content as determined by flow cytometry ($r = 0.66, 0.62$, respectively), although a marked disparity was noted in occasional cases. Sampling error during the preparation of suspensions may account for differences in some cases. Loss of cells is another explanation, although unlikely because the nuclei were prepared from intact tissues. In addition, G1 phase content may influence the correlation, because the length of this phase is known to be the most variable part of the cell cycle.[37] To examine G1 phase duration among cell populations, the ratio of Ki-67 staining to S + G2/M may be calculated.[38] Because Ki-67 staining is equivalent to G1 + S + G2/M phase content, this ratio reflects G1 phase content. The values determined for the low, intermediate and high-grade tumors of the Working Formulation are 2.15, 3.86, and 3.87, respectively. For the revised low, intermediate, and high grades discussed in this study, the ratios are 1.78, 3.76, and 4.32, respectively. Thus, the proportion of G1 phase content is lowest in the low-grade tumors and approximately equal in the intermediate and high-grade tumors. The number of cells cycling is therefore most likely to be underestimated by flow cytometry in intermediate and high-grade lesions. Also of interest is the low Ki-67:S + G2/M ratio of reactive lymph nodes (0.93). Although Ki-67 is biologically a more inclusive marker of cell cycle activity than S-phase alone because all proliferating phases of the cell cycle are included, additional studies would be needed to determine whether Ki-67 staining provides additional prognostic information.

The preceding conclusions have been based on the mean Ki-67 positivity for lymphoma subtypes. It must not be overlooked that within each tumor category there is considerable variability (Table 1). Wide ranges of proliferative activity are likewise reported in studies using tritiated thymidine uptake,[18] flow cytometry,[4,6,11,12] mitotic indices,[15,16] and Ki-67.[26,27] The smallest range of values occurs in low-grade lymphomas (revised grouping), reactive lymph nodes, and small noncleaved cell lymphomas. Although these findings must be considered in the context of the limited number of cases examined, other reports also show relatively small variations in proliferation assessments for lower grade tumors[4,12,16,20,26,27] and small noncleaved cell lymphomas.[26,27] In this study, no attempt was made to distinguish neoplastic from non-neoplastic cells. Therefore, some variability may be due to differences in the proportion of non-neoplastic cells present among the cases. Although dual color techniques are available for flow cytometric analysis of specific cell subsets,[39] similar techniques could not yet be applied to tissue sections for quantitative imaging.

The overlapping ranges of Ki-67 positivity found in most lymphoma subtypes suggests biologic heterogeneity that might have prognostic significance for individual cases. For example, it is interesting that of the small lymphocytic lymphomas studied, the highest Ki-67 value (23.7%) occurred in a case of small lymphocytic lymphoma undergoing histologic transformation. Likewise, the only disproportionately high staining small lymphocytic lymphoma in Weiss's study[27] is also an unusual case, manifesting T cell phenotype and more aggressive behavior than expected. Other studies, using flow cytometry[15,32] and mitotic indices,[40–43] have each examined a single subtype of non-Hodgkin's lymphoma and found that within these diagnostic categories, proliferative activity correlates with prognosis. Thus, it is possible that the accurate measurement of proliferation is equally or more indicative of tumor behavior than morphologic diagnosis.[19] A recent study by Grogan et al examined diffuse large-cell lymphomas and found Ki-67 staining to be an independent predictor of survival by multivariate analysis.[24] Work by Gerdes et al also indicates a role for Ki-67 staining in the prediction of clinical outcome in large cell lymphomas.[25] In addition, their study examined 171 cases of lymphomas of all subtypes and found great differences in survival between cases having less than 30% and greater than 70% Ki-67 staining. Hall et al have recently demonstrated that Ki-67 index correlates strongly with survival and relapse rates in lymphomas divided into low and high-grade groups according to the Kiel classification.[43] It would be useful to perform a similar study with respect to the Working Formulation. Finally, the clinical significance of the Ki-67 antibody could be expanded to monitor lymphoma proliferative rates before and after chemotherapy, rendering pertinent information about tumor vulnerability to cell-cycle dependent agents.

## References

1. The Non-Hodgkin's Lymphoma Pathologic Classification Project: National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas: Summary and description of a working formulation for clinical usage. Cancer 1982, 49:2112–2135
2. Yunis JJ, Frizzera G, Oken MM, McKenna J, Theologides A, Arneson M: Multiple recurrent genomic defects in follicular

lymphoma: A possible model for cancer. N Engl J Med 1987, 316:79–84

3. Braylan RC, Diamond LW, Powell ML, Harty-Golder B: Percentage of cells in the S phase of the cell cycle in human lymphoma determined by flow cytometry: Correlation with labeling index and patient survival. Cytometry 1980, 1:171–174
4. Macartney JC, Camplejohn RS, Alder J, Stone MG, Powell G: Prognostic importance of DNA flow cytometry in non-Hodgkin's lymphomas. J Clin Pathol 1986, 39:542–546
5. Diamond LW, Nathwani BN, Rappaport H: Flow cytometry in the diagnosis and classification of malignant lymphoma and leukemia. Cancer 1982, 50:1122–1135
6. Christensson B, Tribukait B, Linder I, 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
7. 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
8. Meyer JS, Higa E: S-phase fractions of cells in lymph nodes and malignant lymphomas. Arch Pathol Lab Med 1979, 103: 93–97
9. Kvaloy S, Godal T, Marton PF, Steen H, Brenhovd IO, Abrahamsen AF: Spontaneous 3H-thymidine uptake in histologic subgroups of human B cell lymphomas. Scand J Haematol 1981, 26:221–234
10. Srigley J, Barlogie B, Butler JJ, Osborne B, Blick M, Johnston D, Kantarjian H, Reuben J, Batsakis J, Freireich EJ: Heterogeneity of non-Hodgkin's lymphomas probed by nucleic acid cytometry. Blood 1985, 65:1090–1096
11. Juneja SK, Cooper IA, Hodgson GS, Wolf MM, Ding JC, Ironside PN, Thomas RJS, Parkins JD: DNA ploidy patterns and cytokinetics of non-Hodgkin's lymphoma. J Clin Pathol 1986, 39:987–992
12. Scarffe JH, Crowther D: Pre-treatment proliferation activity of non-Hodgkin's lymphoma cells. Eur J Cancer 1981, 17:99–108
13. 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
14. Porwit-Ksiazek A, Christensson B, Lindmalm C, Mellstedt H, Tribukait B, Biberfeld G, Biberfeld P: Characterization of malignant and non-neoplastic cell phenotypes in highly malignant non-Hodgkin's lymphomas. Int J Cancer 1983, 32:667–674
15. Lang W, Kienzle S, Diehl V: Proliferation kinetics of malignant non-Hodgkin's lymphomas related to histopathology of lymph node biopsies. Virchows Arch [A] 1980, 389:397–407
16. Donhuijsen K: Mitoses in non-Hodgkin's lymphomas: Frequency and prognostic relevance. Path Res Pract 1987, 182:352–357
17. Ellison DJ, Nathwani BN, Metter GE, Turner RR, Burke JS, Miller TP, Mann RB, Barcos M, Kjeldsberg CR, Winberg CD, Whitcomb CC: Mitotic counts in follicular lymphomas. Hum Pathol 1987, 18:502–505
18. Costa A, Bonadonna G, Villa E, Valagussa P, Silvestrini R: Labeling index as a prognostic marker in non-Hodgkin's lymphomas. J Natl Cancer Inst 1981, 66:1–5
19. Roos G, Dige U, Lenner P, Lindh J, Johansson H: Prognostic significance of DNA-analysis by flow cytometry in non-Hodgkin's lymphoma. Hemat Oncol 1985, 3:233–242
20. Akerman M, Brandt L, Johnson A, Olsson H: Mitotic activity in non-Hodgkin's lymphoma: Relation to the Kiel classification and to prognosis. Br J Cancer 1987, 55:219–223
21. 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
22. Gerdes J, Lemke H, Baisch H, Wacker 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
23. Hall PA, Richards MA, Gregory WM, d'Ardenne AJ, Lister TA, Stansfeld AF: The prognostic value of Ki-67 immunostaining in non-Hodgkin's lymphoma. J Pathol 1988, 154: 223–235
24. Grogan TM, Lippman SM, Spier CM, Slymen DJ, Rybski JA, Rangel CS, Richter LC, Miller TP: Independent prognostic significance of nuclear proliferation antigen in diffuse large cell lymphomas as determined by the monoclonal antibody Ki-67. Blood 1988, 71:1157–1160
25. Gerdes J, Stein H, Pileri S, Rivano MT, Gobbi M, Ralfkiaer E, Nielson KM, Pallesen G, Bartels H, Palestro G, Delsol G: Prognostic relevance of tumour-cell growth fraction in malignant non-Hodgkin's lymphoma (letter). Lancet 1987, 2:448–449
26. Gerdes J, Dallenbach, Lennert, Lemke H, Stein H: Growth fractions in malignant non-Hodgkin's lymphomas (NHL) as determined in situ with the monoclonal antibody Ki-67. Hematol Oncol 1984, 2:365–371
27. Weiss LM, Strickler JG, Medeiros LJ, Gerdes J, Stein H, and Warnke RA: Proliferation rates of non-Hodgkin's lymphomas as assessed by Ki-67 antibody. Hum Pathol 1987, 18:1155–1159
28. Pinkus GS, Said JW: Characterization of non-Hodgkin's lymphomas using multiple cell markers: Immunologic, morphologic, and cytochemical studies of 72 cases. Am J Pathol 1979, 94:349–380
29. Bacus JW, Grace LJ: Optical microscope system for standardized cell measurements and analyses. Appl Opt 1987, 26:3280–3293
30. Lage JM, Driscol SG, Yavner DL, Olivier AP, Mark SD, Weinberg DW: Hydatidiform moles: Application of flow cytometry in diagnosis. Am J Clin Pathol 1988, 89:596–600
31. Donhuijsen K: Mitosis counts: Reproducibility and significance in grading of malignancy. Hum Pathol 1986, 17:1122–1125

32. Bauer KD, Merkel DE, Winter JN, Hauck WW, Marder RJ, Wallemark CB, Williams TJ, Variakojis D: Prognostic implications of ploidy and proliferative activity in diffuse large cell lymphomas. Cancer Res 1986, 46:3173–3178
33. Weisenburger DD, Linder J, Daley DT, Armitage JO: Intermediate lymphocytic lymphoma: An immunohistologic study with comparison to other lymphocytic lymphomas. Hum Pathol 1987, 18:781–790
34. Nathwani BN, Winberg CD, Diamond LW, Bearman RM, Kim H: Morphologic criteria for the differentiation of follicular lymphoma from florid reactive follicular hyperplasia: A study of 80 cases. Cancer 1981, 48:1794–1806
35. Weinberg DS: The relationship between cell size and cell cycle activity in non-Hodgkin's lymphoma (abstr). Lab Invest 1987, 56:85A
36. Payne CM, Grogan TM, Cromey DW, Bjore CG, Kerrigan DP: An ultrastructural morphometric and immunophenotypic evaluation of Burkitt's and Burkitt's-like lymphomas. Lab Invest 1987, 57:200–218
37. Mitchison JM: The Biology of the Cell Cycle. London, Cambridge University Press, 1971, pp 60–61
38. Schrape S, Jones DB, Wright DH: A comparison of three methods for the determination of the growth fraction in non-Hodgkin's lymphoma. Br J Cancer 1987, 55:283–286
39. Braylan RC, Benson NA, Nourse V, Kruth HS: Correlated analysis of cellular DNA, membrane antigens and light scatter of human lymphoid cells. Cytometry 1982, 2:337–343
40. Swerdlow SH, Habeshaw JA, Murray LJ, Dhaliwal HS, Lister TA, Stansfeld AG: Centrocytic lymphoma—a distinct clinicopathologic and immunologic entity: A multiparameter study of 18 cases at diagnosis and relapse. Am J Pathol 1983, 113:181–197
41. Weisenburger DD, Nathwani BN, Diamond LW, Winberg CD, Rappaport H: Malignant lymphoma, intermediate lymphocytic type: A clinicopathologic study of 42 cases. Cancer 1981, 48:1415–1425
42. Evans H, Butler JJ, Youness EL: Malignant lymphoma, small lymphocytic type. Cancer 1978, 41:1440–1455
43. Nathwani BN, Diamond LW, Winberg CD, Kim H, Bearman RM, Glick JH, Jones SE, Gams RA, Nissen NI, Rappaport H: Lymphoblastic lymphomas: A clinicopathologic study of 95 patients. Cancer 1981, 48:2347–2357

## *Acknowledgment*

The authors thank Diana Wauhkonen and Jeffrey Norton for their technical assistance.