Cell Cycle Kinetics in Malignant Lymphoma Studied With In Vivo Iododeoxyuridine Administration, Nuclear Ki-67 Staining, and Flow Cytometry

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MALIGNANT lymphomas constitute a heterogeneous group of lymphoproliferative disorders with respect to presentation, clinical course, response to treatment, and prognosis. Histopathologic classification and clinical staging are considered suboptimal to predict the biologic and clinical behavior of these tumors. Therefore, additional parameters are urgently needed.

The role of proliferative activity has received special attention. Different techniques have been used to study cell kinetics in lymphomas: measurement of the percentage of S-phase cells by DNA flow cytometry (FCM), assessment of the labeling index (LI) by in vitro incorporation of tritiated thymidine and autoradiography, and staining with the Ki-67 monoclonal antibody (MoAb), which reacts with a proliferation-associated nuclear antigen. The clinical impact of cell kinetics with respect to classification and prognosis of lymphomas remains questionable. This might be due to the limitations of the techniques available in a clinical setting. Measurement of the S-phase fraction is difficult to assess in aneuploid tumors. Overestimation of the proliferative activity may occur due to arrested S-phase cells. Assessment of the thymidine LI requires ex vivo undisturbed growth of the tumor cells, and is time-consuming. However, the major limitation of these techniques is that they provide only a partial and static kinetic profile, because only the distribution of cells in certain cell cycle phases at one particular moment can be determined.

Measurement of dynamic kinetic parameters, representing the duration of distinct cell cycle phases, has now become feasible by the introduction of in vivo administration of bromodeoxyuridine (BrdUrd) or iododeoxyuridine (IdUrd) and FCM. BrdUrd or IdUrd are nonradioactive pyrimidine analogues that are incorporated into the DNA during the S-phase. Simultaneous flow cytometric detection of IdUrd/BrdUrd incorporation and DNA content is possible using specific MoAbs directed against these pyrimidine analogues combined with appropriate cytochemical protocols. Using in vivo IdUrd/BrdUrd administration, both static and dynamic growth characteristics, such as the LI, DNA synthesis time (T_s), potential doubling time (T_p), and cell production rate (CPR), can be calculated with data obtained from a single tumor sample. The dose of IdUrd administered to adults for in vivo cell kinetic analysis in this study (200 mg) is far below the concentration that may induce cytotoxic effects. IdUrd/BrdUrd kinetic studies have been performed in various malignancies. Until now, none of these studies has been directed to lymphomas.

We present the results of in vivo IdUrd kinetics in 29 patients with malignant lymphoma and four patients with reactive hyperplasia. We show the feasibility of the technique and relate the kinetic data (LI, T_s, T_p, and CPR) to the histologic classification. In addition, we show how the kinetic information can be extended to data on cell cycle time (T_c) and duration of the G_1-phase (T_{G_1}) by determining the Ki-67-derived growth fraction.

MATERIALS AND METHODS

Patients. Forty-three patients clinically suspected of primary or relapsed malignant lymphoma were eligible for this study and received IdUrd before diagnostic biopsy. None of the patients had received any cytostatic treatment or radiotherapy within 8 weeks before the cell kinetic analysis. Permission to administer IdUrd was obtained by the local Ethical Committees and from the National Cancer Institute (NCI; Bethesda, MD), with the NCI supplying the drug. Written informed consent was obtained from all patients. In six cases, no tissue or insufficient tissue was available to separate...
material for research purposes. In one case, the tumor was localized in the gastric mucosa, and it was not possible to prepare an adequate cell suspension of the tissue. Three tumor biopsies were diagnosed as carcinomas. These 10 cases were excluded from this study, leaving 33 cases for analysis.

In vivo IdUrd labeling. The NCI supplied us with vials of 200 mg freeze-dried IdUrd. The vials were stored at 5°C until use. Before infusion, the IdUrd was dissolved in 30 mL isotonic saline. All patients received 200 mg IdUrd as a 15-minute intravenous infusion. The end of the infusion was defined as time zero (T0).

Tissue sampling and tissue preparation for FCM. Between 3.8 and 7.5 hours (median, 5.5 hours) after in vivo IdUrd labeling fresh tissue samples were obtained from surgically removed lymph nodes (n = 31) or spleen (n = 1). In one case, cells were aspirated from bone marrow (BM) completely infiltrated by lymphoma. The biopsies were immediately submerged in an ice-cold solution of glucose phosphate buffer to stop cell growth. The BM aspirate was collected in ice-cold buffered citrate dextrose formula A (pH 7.0). One part of the biopsy material was processed for histopathology, another part was snap-frozen in liquid nitrogen for immunohistochemistry, and a third part was used for IdUrd/DNA FCM, Ki-67/DNA FCM, and CD3/DNA FCM. The cells from the latter were suspended by mincing and washing in glucose phosphate buffer. Low-density nucleated BM and spleen cells were collected by density centrifugation. All cell suspensions were divided into two portions. The cells from one portion, to be used for IdUrd/DNA FCM, were fixed in cold 75% ethanol, stored at −20°C, and protected from light before analysis. The cells from the other portion, to be used for Ki-67/DNA and CD3/DNA staining, were cryopreserved using a temperature-controlled freezing program.

Histopathology and immunohistochemistry. For routine histologic examination, the biopsy material was fixed in neutral buffered formalin and embedded in paraplast. Sections of 4 μm were stained with hematoxylin and eosin (H&E), Giemsa, methyl green pyronin, periodic acid Schiff, and Laguesse reticulin stain. The non-Hodgkin’s lymphomas (NHL) were typed according to the Kiel classification, with determination of the histologic grade of malignancy according to the criteria of the Working Formulation. For T-cell NHL, we used the updated Kiel classification. In all cases of NHL, the histologic diagnoses and the origin of the tumor cells were established or confirmed by immunohistochemical staining of cryostat sections and paraffin sections with commercially available MoAbs using standard techniques and criteria. Cases of Hodgkin’s disease (HD) were typed according to the criteria of the Rye classification. Immunohistochemical staining with antibodies against CD15 and CD30 resulted in a pattern consistent with HD in all cases.

IdUrd/DNA, Ki-67/DNA, and CD3/DNA staining. Bivariate staining for IdUrd incorporation and DNA content was performed using the simultaneous proteolytic enzyme digestion and acid denaturation technique. The anti-IdUrd HN-IU MoAb, developed at our department, and a fluorescein isothiocyanate (FITC)-conjugated IgG goat antimouse second-step antibody (GAM-FITC; American Qualex International, La Mirada, CA) were used for detection of IdUrd. The HN-IU MoAb shows cross-reactivity with BrdUrd, but not with fluoro-deoxyuridine. It also allows for a good separation of labeled and nonlabeled cells. Propidium iodide was used for DNA staining. IdUrd/DNA staining of the lymphoma cell suspensions was always performed in duplicate. A sample incubated with only the second-step antibody served as the control sample for nonspecific Ki-67 staining.

Bivariate CD3/DNA staining was performed on aliquots of 1 × 10⁶ thawed cells for the determination of the number of reactive T cells in B-cell NHL and their percentage of S-phase cells. A previously described staining protocol was used, producing high-resolution DNA histograms while preserving cell-surface immunofluorescence. Cells labeled with only GAM-FITC served as the negative control.

FCM. The IdUrd/DNA stained samples of the first 20 patients were analyzed on an Ortho 50H Cytolfluorograf (Ortho Instruments, Westwood, CA), with filter settings as described previously. Thereafter, a Coulter Epics Elite (Coulter, Hialeah, FL) was used, equipped with a 40 mW Argon ion laser running at 15 mW, on which the remaining IdUrd/DNA samples, as well as all the Ki-67/DNA- and CD3/DNA-stained samples, were analyzed. Control measurements showed no differences in the results of the IdUrd analyses between the two flow cytometers. A high-pass filter of 610 nm for red fluorescence (propidium iodide), a band-pass filter 525/30 for green fluorescence (FITC), and a dichroic mirror of 550 nm were used. A minimum of 20,000 cells was analyzed in duplicate. In cases with a low LI, this was extended up to 200,000 cells. The fluorescence signals were recorded on a linear (DNA and IdUrd) or logarithmic (Ki-67 and CD3) scale in list mode. Cell debris and cell doublers were eliminated based on their DNA content and the ratio area/peak of the red fluorescence signal.

Calculation of kinetic parameters. The duration of the S-phase (T1) was determined from the bivariate IdUrd/DNA analysis according to the model of Begg et al. This model calculates the T1 from the increase in mean DNA content of the IdUrd labeled nondivided cells since the start of the labeling, assuming that the mean DNA of the labeled cells at time zero is 3n DNA.

The LI was defined as the percentage of IdUrd-labeled cells. A correction was made for the cells that had divided since the start of the IdUrd labeling (actually, the nonlabeled G0 + M cells plus the IdUrd labeled cells that have divided and entered the G1- and early S-phase). The fractions of these cells were halved in both the denominator and the numerator:

\[ LI(%) = \frac{\text{IdUrd}^{+}_{\text{Nondivided}} + (\text{IdUrd}^{+}_{\text{Divided}} \times 0.5)}{100 - (\text{IdUrd}^{+}_{\text{Divided}} \times 0.5) - (G2 + M\text{-Phase} \times 0.5) \times 100} \]

The Tpot represents the tumor doubling time, assuming that no cell loss occurs. The Tpot and the CPR are both indirectly calculated from the Tc and LI:

\[ T_{\text{pot}} (d) = T_{c} \times \frac{100}{LI} \times 24 \]

\[ \text{CPR (cells/100 cells/d)} = \frac{LI}{T_{c}} \times 24 \]

The growth fraction (GF; measured as a percentage) was estimated from the fraction of Ki-67–positive cells. The percentage of positively labeled cells in the control sample was subtracted to correct for nonspecific staining (usually <2%).

The Tc was calculated from the Tc, the LI, and the Ki-67 GF:

\[ T_{c}(h) = \frac{GF}{LI} \times T_{c} \]

Finally, the duration of the G1-phase (Tg1) was calculated from the Tc and the G1-phase fraction of the cycling population (Ki-67–positive cells, Fg1:Cycling):

\[ T_{g1}(h) = T_{c} \times F_{g1:Cycling} \]
In cases of DNA aneuploidy with an admixture of diploid cells, the cell kinetic analysis was performed of the aneuploid population by creating an appropriate window for selecting these cells.

**Analysis of percentage of S-phase cells in the overall population and in CD3+ cells.** DNA histograms of the overall population were derived from the IdUrd/DNA analyses. Calculation of S-phase DNA in cases with unimodal DNA distributions was performed according to the method of Baisch et al, model 41. No appropriate model is available for calculating percentage of S-phase cells in cases with bimodal distributions. However, an estimate of the percentage of S-phase cells in those cases was made by selecting the cells with hyperdiploid and tetraploid DNA content. In B-cell NHL, the percentage of S-phase cells of the reactive T cells was calculated from the DNA histograms of the CD3+ cells, derived from the bivariate CD3/DNA analysis.

**Statistics.** Correlations between cell kinetic parameters were assessed with Spearman’s rank correlation analysis. The Kruskal-Wallis test, a nonparametric test for independent samples, was used to test differences in the values of the kinetic parameters between the three malignancy grades in NHL. Statistical analyses were performed using the software package of SAS Institute Inc (Cary, NC).

**RESULTS**

**Patient characteristics and histopathologic diagnosis.** Table 1 shows some relevant clinical and histologic data. Five histologic categories were defined: reactive hyperplasia (n = 4), HD (n = 7), low-grade NHL (LG-NHL; n = 7), intermediate-grade NHL (IG-NHL; n = 10), and high-grade NHL (HG-NHL; n = 5). Only one NHL of T-cell origin was found; all other NHL were of the B-cell phenotype.

**Cell kinetic data.** The IdUrd LI could be measured in 31 of the 33 patients, and the T, Tpot, and CPR in 30 of the 33 cases. In one case, the DNA analysis was impeded, and in another case, the kinetic analysis was hampered due to a large overlap in DNA content of proliferating aneuploid and diploid cells. In a patient with reactive hyperplasia (RH), the very low LI (0.1%) prohibited the calculation of the T, Tpot, and CPR, although 200,000 cells could be analyzed.

As an example, Fig 1 shows results of DNA/IdUrd analysis in three patients with an HG-NHL. Even in a linear presentation, the clear separation between labeled and unlabeled cells is evident in all three samples. A high LI is seen in Fig 1A and B (24.4% and 34.4%, respectively). However, the transition time through the S-phase is completely different. The labeled cells in Fig 1A have moved relatively fast through the S-phase and after 6.00 hours many have already undergone mitosis and entered the G1-phase. In contrast, the majority of the labeled cells in Fig 1B are still in the S-phase after 5.47 hours. This results in a shorter T, in patient A in comparison with patient B (14.6 and 31.5 hours, respectively). Figure 1C shows an example of an HG-NHL with a low LI (3.6%).

The median values and ranges of the cell kinetic data of each subgroup and the results of the statistical analysis are summarized in Table 2. IdUrd/DNA analysis in NHL showed a low median LI in the LG-NHL (1.0%), a higher median LI in the IG-NHL (9.1%), and the highest value in the HG-NHL (18.4%) (P < .01). However, there was considerable overlap in LI values between these three subgroups. Expectedly, the median percentage of S-phase cells displayed a similar pattern with the lowest values in the LG-NHL and the highest in the HG-NHL (P < .01). In contrast, a significant relationship between malignancy grade and the duration of the S-phase (T,) could not be shown. There was a wide range in the T, values in the NHL (8.4 to 50.2 hours) and the overlap between the three histologically defined subgroups was considerable. A very long median Tpot was found in the LG-NHL (57.2 days) as compared with IG-NHL (6.3 days). The shortest value was seen in HG-NHL (4.6 days) (P = .02). The reciprocal value, CPR, showed an opposite relationship with a short median CPR in LG-NHL and a higher median CPR in IG-NHL and HG-NHL. (P = .02).

The number of HD was too small to study the relationship between IdUrd kinetics and histologic subtyping within this group. The median values of the percentage of S-phase cells and the LI were close to those observed in the LG-NHL. The T, was relatively short in this group with a median value of 10.6 hours. This explains the somewhat shorter median Tpot and higher CPR, as compared with the LG-NHL.

The small number of patients with reactive hyperplasia prohibited a kinetic characterization of this subgroup. Nevertheless, it can be concluded that this group appears to be rather heterogeneous with respect to percentage of S-phase cells, LI, T, Tpot, and CPR.

**Ki-67-derived GF.** Ki-67 discriminates between cycling (G1, S, and G2 + M-phase cells) and resting cells (G0-phase cells). Figure 2 shows examples of bivariate measurements of Ki-67/DNA. It demonstrates that the Ki-67 MoAb reacts with all S-phase cells and the majority of G2 + M-phase cells, except for some residual cell clumps. In the G0/G1-phase the Ki-67 staining discriminates between G1 cells (positively labeled) and G0 cells (negatively labeled).
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Ki-67/DNA data.

The horizontal lines indicate the level used for discrimination between IdUrd-labeled and non-IdUrd-labeled cells. The vertical lines are set to discriminate between IdUrd-labeled cells that had not yet divided since the start of the labeling and those that had already entered G2 and early S-phase. After 5.75 hours, many cells have already undergone mitosis and reentered the S-phase.

Combining the data derived from the IdUrd/DNA and Ki-67/DNA analysis, two additional dynamic kinetic parameters could be calculated: the duration of the cell cycle ($T_c$) and of the $G_1$-phase ($T_{G_1}$) (Table 2). Calculation of $T_c$ and $T_{G_1}$ derived from IdUrd/DNA and Ki-67/DNA data. Combining the data derived from the IdUrd/DNA and Ki-67/DNA analysis, two additional dynamic kinetic parameters could be calculated: the duration of the cell cycle ($T_c$) and of the $G_1$-phase ($T_{G_1}$) (Table 2).

Both IdUrd/DNA as well as Ki-67/DNA kinetic data were available in 29 of 33 patients. There was a wide range in $T_c$ and $T_{G_1}$, but even within each histologic subgroup, the $T_c$ and $T_{G_1}$ showed great variations. In NHL, the longest median durations of the $T_c$ and $T_{G_1}$ were noted in LG-NHL (107 and 80 hours, respectively) and the shortest in HG-NHL (57 and 36 hours, respectively), while the median values in IG-NHL were in between (69 and 47 hours, respectively). However, these differences did not reach statistical significance. In HD, the median $T_c$ (62 hours) and $T_{G_1}$ (37 hours) were close to the values observed in the IG-NHL and HG-NHL. The $T_c$ and $T_{G_1}$ in reactive hyperplasia were successfully assessed in only three of the four patients.

Relationship between distinct kinetic parameters. Statistical analysis showed a significant correlation with a strong $r$ coefficient value between the IdUrd LI and each of the two other static kinetic parameters: the percentage of S-phase cells ($P < .001$, $r = .97$) and the Ki-67 GF ($P < .001$, $r = .91$). However, the ratio of the median Ki-67 GF and the median IdUrd LI decreased with increasing malignancy grade (1:6 in LG-NHL, 1:3 in IG-NHL, and 1:2 in HG-NHL).

Table 2. Summary of Cell Kinetic Data in Malignant Lymphomas

<table>
<thead>
<tr>
<th>Lymphoma Type</th>
<th>S-DNA (%)</th>
<th>LI (%)</th>
<th>GF (%)</th>
<th>$T_c$ (h)</th>
<th>$T_{G_1}$ (h)</th>
<th>$T_{KI}$ (d)</th>
<th>$T_{KI}$ (d)</th>
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<tr>
<td>LG-NHL</td>
<td>1.2</td>
<td>1.0</td>
<td>6</td>
<td>12.8</td>
<td>57.2</td>
<td>2.1</td>
<td>107</td>
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<td></td>
<td>(0.4-4.2)</td>
<td>(3.36)</td>
<td>(6.4-16.5)</td>
<td>(12.5-116.7)</td>
<td>(0.8-8.1)</td>
<td>(22-122)</td>
<td>(16-93)</td>
</tr>
<tr>
<td>IG-NHL</td>
<td>10.2</td>
<td>9.1</td>
<td>26</td>
<td>13.4</td>
<td>63</td>
<td>16.4</td>
<td>69</td>
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<tr>
<td></td>
<td>(1.9-36.2)</td>
<td>(1.4-28.6)</td>
<td>(11-85)</td>
<td>(3.4-96.1)</td>
<td>(1.1-29.6)</td>
<td>(20-270)</td>
<td>(13-215)</td>
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<tr>
<td>HG-NHL</td>
<td>25.2</td>
<td>18.4</td>
<td>34</td>
<td>19.1</td>
<td>4.6</td>
<td>21.9</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>(3.6-37.1)</td>
<td>(3.7-33.5)</td>
<td>(15-93)</td>
<td>(2.6-21.5)</td>
<td>(4.7-38.7)</td>
<td>(21-93)</td>
<td>(11-59)</td>
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<tr>
<td>within NHL</td>
<td>$P = .0006$</td>
<td>$P = .007$</td>
<td>$P = .02$</td>
<td>$P = .22$</td>
<td>$P = .02$</td>
<td>$P = .39$</td>
<td>$P = .32$</td>
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<td>HD</td>
<td>1.5</td>
<td>1.5</td>
<td>7</td>
<td>10.6</td>
<td>29.2</td>
<td>3.5</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>(0.4-4.5)</td>
<td>(0.4-3.4)</td>
<td>(4.37)</td>
<td>(9.6-16.4)</td>
<td>(20.1-140.6)</td>
<td>(0.7-8.1)</td>
<td>(37-180)</td>
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<td>Reactive hyperplasia</td>
<td>2.2</td>
<td>2.0</td>
<td>5</td>
<td>9.4</td>
<td>11.2</td>
<td>8.9</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>(0.6-4.8)</td>
<td>(0.1-3.7)</td>
<td>(1-10)</td>
<td>(7-9-16.7)</td>
<td>(8.9-152.4)</td>
<td>(0.7-11.4)</td>
<td>(21-102)</td>
</tr>
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</table>

Median values are given with ranges in parentheses.

Abbreviation: S-DNA, percentage of S phase cells.

Figure 2A represents a lymphoma with a high GF (95%) and few $G_0$ cells, Fig 2B shows an intermediate GF (19%), and Fig 2C shows a low GF (5%) with many $G_0$ cells. These lymphomas were classified as HG-NHL, IG-NHL, and LG-NHL, respectively. The Ki-67 GF could be adequately measured in 30 of 33 cases. The results are summarized in Table 2. In two cases, the same reasons that hampered the determination of the LI precluded the Ki-67/DNA analysis. In one patient, substantial nonspecific Ki-67 staining was encountered. Within the NHL, a low median GF was found in the LG-NHL (6%), and a high median GF in the IG-NHL (26%) and HG-NHL (34%) ($P = .02$). In HD, a relatively low median GF was observed (7%). Cases with reactive hyperplasia showed both low as well as intermediate values.

Table 2. Summary of Cell Kinetic Data in Malignant Lymphomas
NHL. In addition, a strong correlation was observed between the IdUrd LI and the Tpos (P < .001, r = -.96) and CPR (P < .001, r = .96). There was no significant correlation between the LI and the dynamic kinetic parameters Tc (P = .10), Tg (P = .05), and TGI (P = .06), with poor r coefficient values (Tc, r = .31; Tg, r = -.37; and TGI, r = -.35).

Reactive T cells and their S-phase DNA content in B-cell NHL. IdUrd FCM provides no information on the proliferative activity of distinct subpopulations, eg, nonmalignant reactive cells in B-cell NHL. Therefore, we assessed to what extent the results might have been jeopardized due to cell heterogeneity. The percentage of reactive T cells (CD3+) and their S-phase DNA content were determined retrospectively in 17 cryopreserved NHL. The median number of CD3+ cells was 14% (range, 1% to 70%). In only 4 of 17 NHL, the fraction of CD3+ cells exceeded 30%. The proliferative activity (percentage of S-phase cells) of the CD3+ cells was low (median value, 3.2%; range, 0.1% to 20.7%) compared with the overall cell population (median value, 6.5%; range, 0.6% to 39.4%). This suggests that the low proliferating CD3+ cell population will barely affect the results of analysis of the dynamic parameters (Tc, Tg, and TGI) in the cycling population.

The representativity of the examined cell suspensions was studied by comparing the number of T cells in frozen sections with those found in cytopsin preparations from the cryopreserved cell suspensions, using Pearson’s correlation analysis. This was retrospectively performed in 6 NHL (1 LG-NHL; 3 IG-NHL; and 2 HG-NHL). The numbers of T cells in the histologic sections and in the cytopsin preparations were significantly correlated (P < .01, r = .92). Therefore, a selective loss of tumor cells compared with reactive T cells during the preparation of the cell suspensions is unlikely, as this would result in a relative increase of the reactive cell population.

DISCUSSION

The present study shows the feasibility of obtaining a detailed profile of in vivo growth kinetics in patients with malignant lymphoma using in vivo IdUrd labeling and subsequent bivariate FCM. Data are obtainable from only one single tumor biopsy that has to be performed for diagnostic purposes. No side effects of the IdUrd administration were observed. In our series, we found a wide range in the values of static kinetic parameters, eg, percentage of S-phase cells, IdUrd LI, and percentage of Ki-67–positive cells. In the 22 patients with NHL, there was a statistically significant increase in the median values of these parameters with increasing histologic malignancy grade of the tumor. Most strikingly, the dynamic kinetic parameters, eg, duration of the different cell cycle phases (Tc, Tg, and TGI) did only weakly or not at all correlate with the static kinetic parameters and the histologic malignancy grade. This suggests that the dynamic parameters may represent a new class of independent variables.

Our cell kinetic data confirm the heterogeneity of NHL. Although the classification in three histologic malignancy grades affords a clinically useful guidance with regard to selection of treatment and prognosis, the predictive power is far from perfect. Additional parameters are therefore urgently needed for the identification of distinct subgroups of NHL. Conflicting results have been reported in the literature on the clinical relevance of measurement of the percentage of S-phase cells or the percentage of Ki-67–positive cells. It might not be too unexpected because our data show, in accordance with others, that these kinetic parameters and the histologic classification in three malignancy grades are strongly interrelated: a low percentage of proliferating cells in LG-NHL and significantly higher numbers in the IG-NHL and HG-NHL. Within distinct histologic subgroups the clinical relevance of these parameters is not clearly evident. In LG-NHL, the poor prognostic influence of a high proliferation activity is well documented, while in HG-NHL the relationship with survival, especially on long-term survival, remains equivocal. LG-NHL is characterized by a protracted and, apart from stage I disease, virtually incurable course of disease. This may be related, at least in part, to the relatively low fraction of proliferating cells, making these tumors less susceptible...
to current treatment regimens. Indeed, we found a low median percentage of S-phase cells of 1.2, a low IdUrd LI of 1.0, and a small Ki-67-derived GF of only 6% in the group of LG-NHL. These findings were reflected in a significantly longer median potential tumor doubling time of 57 days, as opposed to 6.3 and 4.6 days in the IG-NHL and HG-NHL, respectively. Unexpectedly, the low proliferative activity of LG-NHL was not accompanied by a prolonged duration of the S- and the G1-phase. Neither did the HG-NHL show a short duration of the S- and the G1-phase, despite their high proliferative activity. So far, we cannot draw definite conclusions from these data because of the relatively low number of patients studied and the wide range in calculated values. However, the remarkable divergence in the duration of the transit times through the different cell cycle phases, which appeared to be independent of the histologic malignancy grade, may reflect distinct characteristics of the respective lymphomas. These results may prove to be useful in understanding biology and clinical behavior.

Several factors may limit the performance of adequate in vivo analysis of cell cycle kinetics in malignant lymphoma. First, IdUrd has to be administered several hours before the surgical procedure, necessitating close cooperation between the investigator and the surgical team. Another prerequisite for this study is the feasibility of obtaining a full kinetic profile. In our study, this was achieved in 29 of 33 cases. Another problem might be the intratumoral heterogeneity of cell populations, raising the question of representativity of the examined tumor specimen. Data reported by Cowan et al3 in lymphomas and of Begg et al41 in solid tumors indicate that the possible bias introduced by supposed intratumor heterogeneity is marginal. A drawback of studying percentage of S-phase cells with conventional methods is the number of arrested S-phase cells that, although measured as S-phase cells, do not actively contribute to proliferation.19 Our data show considerable concordance in the percentage of S-phase cells and the in vivo IdUrd LI. Because arrested S-phase cells do not incorporate IdUrd, these results indicate that arrested S-phase cells occur only infrequently in these NHL.

A major problem in the interpretation of our results might be that the in vivo dynamic data represent characteristics of the overall cell population rather than those of the tumor cells per se. Admixture of nonmalignant cells is well known in NHL and may account for up to 60% of the overall population.2 This feature is largely restricted to the LG-NHL and IG-NHL and is less relevant in HG-NHL.3 In accordance with other reports,2,45 the median proportion of "reactive" T cells in our B-cell NHL was 16% and exceeded 30% in only 4 of 22 cases. More importantly, the percentage of S-phase cells, analyzed separately in the "reactive" T-cell population, accounted for only a median value of less than 50% of the percentage of S-phase cells in the tumor cell population. Therefore, the results of the analysis of the dynamic parameters, eg, the duration of the different cell cycle phases in the overall cell population, will barely be affected because these parameters are calculated exclusively in the population of cycling cells.

In HD, heterogeneity is more complex. In fact, the exact nature of the malignant cell population has not yet been unraveled. Apart from the neoplastic cells, infiltrating B and T cells may contribute considerably to tumor growth. Thus, the results of in vivo dynamic studies in HD may be expected to approximate those obtained in reactive lymph nodes. For comparison, we included data from four patients with a histologic pattern of reactive hyperplasia. Nevertheless, also in HD, proliferative characteristics of the overall cell population analyzed with DNA FCM have been reported to correlate with histologic subtyping11 and survival.6 In our small group of seven patients with HD, the percentage of proliferating cells was low, consistent with other reports.4,6,46 The IdUrd LI of 1% was comparable with the results obtained in LG-NHL. As in the NHL, a wide range in dynamic cell kinetic parameters was observed in HD. The median duration of the G1-phase and the total cell cycle time approximated that observed in the IG-NHL and HG-NHL rather than that of the LG-NHL. This finding might fit in the clinical response to treatment when we assume that kinetic parameters indeed have a prognostic impact on the response to currently available treatment regimens. Obviously, further studies should be directed towards obtaining kinetic data on distinct, immunophenotypically defined, subpopulations of cells, especially in HD.

The kinetic data derived from the IdUrd/DNA analysis were calculated using the single sample model of Begg et al.20 Information on the tumor GF and the fraction of cells in G1-phase is a prerequisite for the calculation of T1 and TG, the GF of the tumor is difficult to assess. Prolonged, continuous labeling with tritiated thymidine combined with multiple tissue sampling may be considered as the standard approach. However, with regard to in vivo measurements in humans, this method is not suitable.47 An estimation of the GF can also be obtained by measurement of IdUrd or BrdUrd incorporation at a single time point after continuous in vivo labeling, especially after sequential administration of IdUrd/BrdUrd.48 Although this method of analysis is more feasible than the tritiated thymidine approach, it is still unsuitable for routine use in vivo. Therefore, we have selected a more indirect but clearly more practical method, eg, staining with the Ki-67 MoAb. Ki-67 reacts with a nuclear antigen expressed in the G2M, S-, and G2 + M-phases, but not in the G0-phase.53 Comparison with GF determination with in vivo continuous thymidine labeling in xenografts showed that Ki-67 provides a reliable estimate of the tumor GF.49,50

Apart from the alleged clinical significance of detailed in vivo cell kinetic profiles in malignant lymphoma, it is tempting to speculate that this method will give new insights in the mechanisms of tumor cell proliferation and progression. Because proliferative activity is, at least in part, regulated by an orderly expression of genes, simultaneous analysis of dynamic cell kinetics in vivo and of altered (onco)-gene expression may show relevant new information.51 Novel staining protocols for simultaneous measurement of IdUrd incorporation, DNA content, and oncogene expression in both the overall cell population as well as immunophenotypically defined subpopulations are likely to
increase the significance of the results and are presently under study.

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REFERENCES

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PP Brons, JM Raemaekers, MJ Bogman, PE van Erp, JB Boezeman, AH Pennings, HM Wessels and C Haanen