DNA Synthesis and Ploidy in Non-Hodgkin’s Lymphomas Demonstrate Intrapatient Variation Depending on Circadian Stage of Cell Sampling

Rune Smaaland, Knut Lote, Robert B. Sothern, and Ole D. Laerum

The Gude Institute, Department of Pathology, Haukeland Hospital, University of Bergen, N-5021 Bergen, Norway; Department of Oncology, Haukeland Hospital, University of Bergen, N-5021 Bergen, Norway; and The Rhythmometry Laboratories, Department of Medicine, University of Minnesota, Minneapolis, Minnesota 55455

ABSTRACT

Significant circadian cell cycle variations with a maximal number of cells in S-phase during the night have been found in a series of 24 patients (18 men and 6 women) with histologically established non-Hodgkin’s lymphomas. Pathological lymph nodes of a total of 26 patients were punctured and aspirated by fine needle technique every 4 h during a single 24-h time span. Twenty-four patients (92.3%) had Stage III or IV disease. Twelve men and 6 women had intermediate grade, and 4 patients (15.4%) had high grade lymphomas according to the Working Formulation. The samples were analyzed by flow cytometry, and DNA synthesis (S-phase) and ploidy were determined according to circadian stage. The individual mean 24-h S-phase varied from 2.2 ± 1.2% (mean ± SD) to 24.0 ± 3.3%. Within the group of patients with low grade lymphomas, a wide range in mean S-phase from 2.4 ± 1.2% to 9.2 ± 2.8% was observed.

The percentage variation within each patient between the lowest and highest S-phase as compared to the lowest value (range of change) during the 24-h time span varied from 21 to 353%, with a mean range of change of 128 ± 19%. When each individual S-phase series was converted to percent of mean and combined for analysis by one-way analysis of variance to test for time-effect across 2 12-h time spans (8 p.m.-8 a.m. versus 8 a.m.-8 p.m.), S-phase variation according to circadian stage was found to be statistically significant (P < 0.004), with higher values found in the 8 p.m.-8 a.m. time span. By single cosinor analysis, S-phase yielded a near significant P value of 0.069 for the least-squares fit of a 24-h cosine to all data as percent of mean, with the acrophase found to be near midnight (0.05 h). For those patients with low and intermediate grade lymphomas and with mean S-phase values <10.0%, we found that mean S-phase was higher during winter (5.8 ± 0.4%) than during spring (3.8 ± 0.3%) or during fall (3.6 ± 0.3%) (P < 0.001, analysis of variance). Twenty-one of the 26 patients (80.8%) had an aneuploid, hypodiploid, or near diploid DNA content, in the same tumor. Such a procedure using repeated 24-h in 26 patients with non-Hodgkin’s lymphoma. Thus, it was established that normal tissues in both animals and humans undergo prominent circadian variations in proliferative behaviour (25-37), malignant tissue has been considered as growing without any systematic time-dependent pattern, with a few exceptions (23, 38-41).

INTRODUCTION

NHL comprises a heterogeneous group of cancers with varied patterns of disease and differing clinical outcomes. The indices most commonly used in the study of NHL, i.e., histological classification and staging, give an estimate of the average prognosis, but do not accurately predict clinical outcome in individual cases. This is reflected within each of the major subgroups of this disease, i.e., low grade, intermediate grade, and high grade lymphomas, in which a great variation in clinical pattern is observed (1, 2). For this reason, there is a need for reproducible quantitative methods of tumor description to confer additional prognostic information to guide the clinician in the selection of the most appropriate therapeutic approach. In addition, an improved definition of prognostic factors for predicting response and ultimately survival and cure rates will be of obvious importance for the evaluation of more specific and improved treatment programs (3).

An objective estimate of proliferative activity in both normal and malignant tissue is today easily obtainable and feasible through the technique of flow cytometry. This method, together with a continuous improvement in preparation and staining techniques, makes it possible to measure a multitude of parameters on thousands of single cells in a short time, not only immediately after sampling, but also at a later stage after adequate fixation of cells.

In several studies, it has been demonstrated that proliferative activity as determined by flow-cytometric analyses may represent a further step in classifying NHL and predicting clinical outcome. Higher proliferative activity has been associated with the more aggressive categories of NHL and a traditionally poorer final outcome (4-15), although modern chemotherapy may have a more profound effect on the clinical course of highly proliferative large cell lymphomas. The prognostic significance of S-phase found in most studies is partly due to the association between this parameter and histological grade. Several authors have noticed a correlation between labeling index and prognosis, as well (16-20). Moreover, in some studies the prognostic power of S-phase has been found superior to histological examination (5, 10, 11). In addition, it has been shown by multivariate regression analyses that the fraction of cells in S-phase can give independent and better prognostic information than usual clinicopathological variables (6, 7, 21).

It is generally acknowledged that antineoplastic drugs exert a greater effect on proliferating cells (22-24), and protocols aimed at affecting neoplastic cells have usually been based on the notion that these cells tend to proliferate at random during the 24-hour period. However, this may not necessarily be the case. Although it is well established that normal tissues in both animals and humans undergo prominent circadian variations in proliferative behaviour (25-37), malignant tissue has been considered as growing without any systematic time-dependent pattern, with a few exceptions (23, 38-41).

Due to the ease with which flow-cytometric examination of cells can be performed, this method is especially suitable for estimating proliferative activity of tissues in a context of sequential sampling. However, because of the obvious practical inconvenience for both the patient and the investigator in obtaining repeated samples for cell kinetic measurements in human tumors in vivo throughout the 24-h time span, it has generally been difficult to study possible circadian stage-dependent variations in tumor cell proliferative activity.

Enlarged pathological lymph nodes in patients with non-Hodgkin’s lymphomas are easily palpable and accessible. We have conducted an around-the-clock study in which lymphoma cells were sampled by fine-needle aspirations from pathological lymph nodes every 4 h for 24 h in 26 patients with non-Hodgkin’s lymphoma. Thus, it was possible to serially obtain repeated measurements of the proportion of malignant lymphoma cells in DNA synthesis, as well as to determine their DNA content, in the same tumor. Such a procedure using re-
peated sampling is also highly relevant with regard to the demonstrated heterogeneity in proliferative activity within each subgroup of NHL (5, 16, 21, 42-45). The finding of comparable flow cytometry results in lymph node biopsies and aspirate material (6) suggests that serial fine-needle aspirations do indeed provide representative material for such temporal studies.

We here show for the first time that non-Hodgkin's lymphomas undergo circadian variations in cell cycle distribution, although it seems to be at variance with normal proliferative behavior. Thus, we challenge the common view that cell proliferation in a malignant tumor is constant during the 24-h time span.

MATERIALS AND METHODS

Patient and Tumor Characteristics. Twenty-six patients (20 men and 6 women) with histologically established non-Hodgkin's lymphomas and palpable lymph nodes in either the cervical, axillary, or groin regions, were included in the study. One patient (3.8%) had Stage I disease, one patient (3.8%) had Stage II disease, 8 patients (30.8%) had Stage III disease, and 16 patients (61.6%) had Stage IV disease, i.e., 92.4% had Stage III or IV disease. Clinical staging was performed according to the Ann Arbor classification system (46).

Median age of the patients was 57 years (range, 23–91 years).

In all cases, the histology was reviewed by one of the authors (O. D. L.) and classified according to the Working Formulation (47) as well as the Kiel system (48). Twelve patients (46.1%) had low grade, 10 patients (38.5%) had intermediate grade, and 3 patients (15.4%) had high grade lymphomas according to the Working Formulation. When using the Kiel system, 22 patients (84.6%) had low grade lymphomas and 4 patients (15.4%) had high grade lymphomas.

Study Protocol. The same s.c. pathological lymph node in each patient was punctured every 4 h during a single 24-h time span, resulting in seven planned sampling times per subject. From the total of 182 samples collected around-the-clock, 161 (88.5%) gave a reliable histogram. Determination of cell cycle distribution was possible in 25 of the 26 patients. In one patient, only 2 time points could be analyzed for cell cycle distribution. Thus, 24 patients were included in the circadian analyses of the different cell cycle phases. Ploidy was determined in all 26 patients. The sampling started at 4 different times of the day, i.e., at 8 a.m., 12 p.m., 4 p.m., or 8 p.m., to reduce a possible stress-related effect on the proliferative activity when analyzing the pooled data. The patients had all followed a regular diurnal schedule in the weeks before admission to the hospital. During the study period, they followed usual hospital routines, with meals at 9 a.m., 1 p.m., and 5 p.m., and lights out between 10 p.m. and 7 a.m. Each patient was awakened briefly for the 12 a.m. and 4 a.m. sampling.

These studies were performed over a 3-year span between October 14, 1985 and October 7, 1988. The study protocol was approved and performed in accordance with the guidelines of the regional medical ethics committee, and all patients gave their informed consent.

Procedure for Lymph Node Aspiration and Sample Handling. All aspirations were done after histological diagnosis had been established and before any treatment was started. A fine needle (0.6-mm outer diameter) on a 10-ml syringe connected to a syringe holder was used to obtain lymphoma cells. The needle was moved around in the lymph node while aspirating with the syringe to obtain a representative sample, and each sampling procedure was performed within 5 min or less. The aspirated cells were immediately fixed in cold 96% ethanol and stored at +4°C until they were assayed. Patient compliance was generally very satisfactory, and no complications occurred during study. Only a single uncomplicated intranasal bleeding in one patient was observed after the study was ended.

Cells and Preparation. At the time of analysis, the fixed cells were spun down at 2000 rpm for 5 min. The pellet was washed twice in 0.9% saline, shaken on a whirlmixer, and thereafter incubated at 37°C for 20 min with 3 ml crude pepticin (Rikshospitalet Apotek, Oslo, Norway), dissolved in 0.2% HCl, for removal of cytoplasm. The isolated nuclei were then washed once in 0.9% saline, and 3 drops of RNase (1 mg/ml in distilled water; Sigma Chemical Co., St. Louis, MO) were added to the pellet. For specific staining of DNA, a solution of ethidium bromide (7.14 mg/ml) plus mithramycin (7.14 mg/ml) in Tris buffer at pH 7.5, together with 3.03 g/liter MgCl2, was added (49).

Flow Cytometry. A Phycy ICP 22 mercury-arc flow cytometer (Ortho Diagnostic Instruments, Westwood, MA), with appropriate excitation and emission filters and attached to a multichannel analyzer (Tectronix 2102, 512 channels), was used for DNA measurements. Each day, before measurements, the instrument was adjusted by use of human peripheral blood lymphocytes that were pooled by pipetting off the buffy coat of EDTA-anticoagulated blood. These lymphocytes were also used as a diploid standard by adding them to the tumor sample as an internal control.

A median of 18,000 nuclei (range, 5,600 to 83,300 nuclei) were analyzed for each sample. The CV, defined as the SD in percent of the mean DNA value of the diploid G0/G1-peak, was 4.9% (mean) and 4.7% (median) for the diploid tumors. The CV of the aneuploid tumors was 6.8% (mean), whereas the CV of the near-diploid tumors ranged from 5.5 to 13.4% (mean, 9.0%) (see "Discussion").

Definitions of DNA Synthesis Phase (S-Phase). Cell cycle distribution was estimated according to a modification of the rectangular method of Baish et al. (50), as described previously (51). A very small percentage of the histograms had debris that could potentially affect the size of the S-phase, and debris therefore was not considered to interfere with the estimation of the S-phase. In the instances in which the S-phases of the diploid and aneuploid population were not separable, the overall proliferative activity of the mixed population was determined. This procedure was considered justifiable since the same tumor was compared with itself at different circadian stages.

Definition of DNA Index. The DNA index of the diploid tumor cells was defined as 1.0 ± 0.1. Tumors were defined as diploid by the presence of a single symmetrical G0/G1 peak in the histogram. A single broad asymmetrical G0/G1 peak was observed in some of the tumors, a phenomenon most likely due to a minor degree of aneuploidy. According to other investigators, we, therefore, considered it appropriate to define these tumors as "near diploid" (52, 53). Tumors with more than one discrete G0/G1 peak were considered aneuploid. The degree of aneuploidy was expressed as a DI, which was defined as the ratio of the channel position of the G0/G1 aneuploid peak to the channel position of the G0/G1 peak of the diploid peak. The number of doublets was estimated by fluorescence microscopy, showing that doublets were negligible (<5%).

Cytological Controls. Samples of the fixed lymphoma cells measured by flow cytometry were smeared on glass slides, dried, and stained by routine Papanicolaou procedure for morphological control of the malignant cells.

Statistical Analysis. Data were analyzed by Student's t test (2-tailed; paired t tests used for paired analysis of groups) and one-way ANOVA, using data both in original units and normalized to percent of the mean. In addition, the individual data for the DNA synthesis were analyzed for circadian rhythm by the fitting of a 24-h cosine by the method of least-squares (cosinor analysis) (54). The rhythm characteristics estimated by this method include the mesor (rhythm-adjusted mean), the amplitude (half the difference between the minimum and maximum of the fitted cosine function), and the acrophase (time of peak value in fitted cosine function). A P value for rejection of the zero-amplitude assumption is determined for each data series, indicating whether or not the cosine model accounts for a significantly greater proportion of the variability in the time series when compared to the total variability around a flat line (the mean). Although the cosinor method involving only a single fitted period may not accurately represent the true characteristics of the actual time-dependent variations if asymmetries exist in a time-series (55), the procedure is nevertheless useful for objectively assessing and quantifying periodicities selected a priori (23). Twelve- and 1-year cosines were also fit to the combined data. A χ² test was used to compare the distribution of S-phase acrophases in NHL cells and normal bone marrow cells within 2 12-h time spans.

RESULTS

S-Phase Variation between Patients. Patient characteristics, histological subcategory, grade, and stage in relation to flow-cytometric data of individual tumors are given in Table 1. The individual mean 24-h S-phase varied from 2.15 ± 1.17% (SD) to 23.99 ± 3.34%. Within the group of 12 patients with low grade lymphomas according to the Working Formulation, a wide range in mean S-phase from 2.4 ± 0.7% to 9.2 ± 2.8% was observed, i.e., nearly a 4-fold variation, with a median of 3.7%. In the 10 patients with intermediate grade lymphomas, the mean S-phase varied from 2.2 ± 1.2% to 24.0 ± 3.3%, i.e., an 11-fold variation, with a median of 5.0%.
CIRCADIAN VARIATIONS IN NON-HODGKIN'S LYMPHOMAS

Table 1 Patient characteristics in relation to DNA ploidy and circadian variation in percentage of cells in DNA synthesis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>No. of data</th>
<th>Histology</th>
<th>Grade</th>
<th>Ploidy</th>
<th>Stage</th>
<th>DNA synthesis</th>
<th>Range</th>
<th>ROC (%)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. K.</td>
<td>47</td>
<td>M</td>
<td>7</td>
<td>Ly/CLL</td>
<td>Low/low</td>
<td>IVA</td>
<td>D</td>
<td>1</td>
<td>1.6-3.7</td>
<td>131</td>
<td>2.44 ± 0.73</td>
</tr>
<tr>
<td>L. O.</td>
<td>23</td>
<td>M</td>
<td>5</td>
<td>CC/CCB, folic.</td>
<td>Low/low</td>
<td>IIIA</td>
<td>4</td>
<td>2</td>
<td>2.1-4.7</td>
<td>124</td>
<td>2.80 ± 1.09</td>
</tr>
<tr>
<td>M. M.</td>
<td>69</td>
<td>M</td>
<td>7</td>
<td>CC/CCB, folic.</td>
<td>Low/low</td>
<td>IIIA</td>
<td>7</td>
<td>1</td>
<td>1.8-4.6</td>
<td>156</td>
<td>2.83 ± 1.10</td>
</tr>
<tr>
<td>B. K. A.</td>
<td>40</td>
<td>F</td>
<td>7</td>
<td>CC/CCB, folic.</td>
<td>Low/low</td>
<td>IIIA</td>
<td>4</td>
<td>1</td>
<td>1.9-5.9</td>
<td>105</td>
<td>2.84 ± 0.62</td>
</tr>
<tr>
<td>K. A. J.</td>
<td>25</td>
<td>M</td>
<td>7</td>
<td>CC/CCB, folic.</td>
<td>Low/low</td>
<td>IIIA</td>
<td>7</td>
<td>1</td>
<td>1.7-7.7</td>
<td>353</td>
<td>2.90 ± 2.17</td>
</tr>
<tr>
<td>O. V.</td>
<td>56</td>
<td>M</td>
<td>6</td>
<td>CC/CCB, folic.</td>
<td>Low/low</td>
<td>IIIA</td>
<td>5</td>
<td>1</td>
<td>2.4-4.3</td>
<td>79</td>
<td>3.35 ± 0.75</td>
</tr>
<tr>
<td>S. B. S.</td>
<td>36</td>
<td>M</td>
<td>7</td>
<td>CC/CCB, folic.</td>
<td>Low/low</td>
<td>IIIA</td>
<td>1</td>
<td>6</td>
<td>2.8-5.6</td>
<td>100</td>
<td>3.97 ± 1.00</td>
</tr>
<tr>
<td>S. A. V.</td>
<td>46</td>
<td>M</td>
<td>6</td>
<td>CC/CCB, folic.</td>
<td>Low/low</td>
<td>IIIA</td>
<td>3</td>
<td>3</td>
<td>3.3-4.5</td>
<td>36</td>
<td>4.15 ± 0.44</td>
</tr>
<tr>
<td>G. H.</td>
<td>49</td>
<td>M</td>
<td>5</td>
<td>CC/CCB, folic.</td>
<td>Low/low</td>
<td>IIIA</td>
<td>5</td>
<td>1</td>
<td>3.6-4.7</td>
<td>31</td>
<td>4.18 ± 0.50</td>
</tr>
<tr>
<td>O. S.</td>
<td>39</td>
<td>M</td>
<td>6</td>
<td>CC/CCB, folic.</td>
<td>Low/low</td>
<td>IIIA</td>
<td>1</td>
<td>5</td>
<td>2.8-5.8</td>
<td>107</td>
<td>4.48 ± 1.25</td>
</tr>
<tr>
<td>L. W.</td>
<td>58</td>
<td>F</td>
<td>7</td>
<td>CC/CCB, folic.</td>
<td>Low/low</td>
<td>IIIA</td>
<td>5</td>
<td>2</td>
<td>2.6-8.5</td>
<td>227</td>
<td>4.73 ± 2.01</td>
</tr>
<tr>
<td>B. A. B.</td>
<td>35</td>
<td>M</td>
<td>7</td>
<td>CC/CCB, folic.</td>
<td>Low/low</td>
<td>IIIA</td>
<td>7</td>
<td></td>
<td>6.0-14.5</td>
<td>142</td>
<td>9.17 ± 2.77</td>
</tr>
<tr>
<td>B. D.</td>
<td>80</td>
<td>M</td>
<td>6</td>
<td>Lymphoplasmacytoid</td>
<td>Low/IM</td>
<td>IA</td>
<td>2</td>
<td>4</td>
<td>1.1-4.4</td>
<td>300</td>
<td>2.15 ± 1.17</td>
</tr>
<tr>
<td>A. T.</td>
<td>48</td>
<td>M</td>
<td>7</td>
<td>CC/CCB, diffuse</td>
<td>Low/IM</td>
<td>IVA</td>
<td>3</td>
<td>4</td>
<td>1.9-4.0</td>
<td>111</td>
<td>2.80 ± 0.78</td>
</tr>
<tr>
<td>H. N. H.</td>
<td>72</td>
<td>M</td>
<td>7</td>
<td>CC, diffuse</td>
<td>Low/IM</td>
<td>IVA</td>
<td>6</td>
<td>1</td>
<td>1.9-6.9</td>
<td>263</td>
<td>4.03 ± 1.83</td>
</tr>
<tr>
<td>A. M. B.</td>
<td>37</td>
<td>M</td>
<td>6</td>
<td>CC/CCB, diffuse</td>
<td>Low/IM</td>
<td>IIIA</td>
<td>2</td>
<td>1</td>
<td>2.1-8.4</td>
<td>300</td>
<td>4.90 ± 2.32</td>
</tr>
<tr>
<td>A. B.</td>
<td>52</td>
<td>M</td>
<td>5</td>
<td>Lymphoplasmacytoid</td>
<td>Low/IM</td>
<td>IVA</td>
<td>5</td>
<td></td>
<td>3.2-7.1</td>
<td>122</td>
<td>5.06 ± 1.63</td>
</tr>
<tr>
<td>A. B. R.</td>
<td>40</td>
<td>M</td>
<td>4</td>
<td>CC/CCB, diffuse /folic.</td>
<td>Low/IM</td>
<td>IVB</td>
<td>2</td>
<td>2</td>
<td>3.2-8.5</td>
<td>166</td>
<td>5.10 ± 2.36</td>
</tr>
<tr>
<td>A. S.</td>
<td>78</td>
<td>F</td>
<td>7</td>
<td>CC/CCB, diffuse</td>
<td>Low/IM</td>
<td>IVB</td>
<td>6</td>
<td>1</td>
<td>4.1-7.8</td>
<td>90</td>
<td>5.76 ± 1.27</td>
</tr>
<tr>
<td>O. L.</td>
<td>71</td>
<td>M</td>
<td>6</td>
<td>CC/CCB, diffuse /folic.</td>
<td>Low/IM</td>
<td>IIIA</td>
<td>6</td>
<td></td>
<td>5.5-8.9</td>
<td>51</td>
<td>7.48 ± 1.09</td>
</tr>
<tr>
<td>S. S.</td>
<td>91</td>
<td>M</td>
<td>7</td>
<td>CC/CCB, diffuse</td>
<td>Low/IM</td>
<td>IVA</td>
<td>7</td>
<td></td>
<td>20-28.8</td>
<td>44</td>
<td>23.99 ± 3.34</td>
</tr>
<tr>
<td>T. S.</td>
<td>83</td>
<td>M</td>
<td>7</td>
<td>CC, diffuse</td>
<td>Low/IM</td>
<td>IVA</td>
<td>7</td>
<td></td>
<td>7</td>
<td>NM**</td>
<td></td>
</tr>
<tr>
<td>T. S. O.</td>
<td>67</td>
<td>M</td>
<td>6</td>
<td>CB, diffuse</td>
<td>High/high</td>
<td>IIIB</td>
<td>3</td>
<td>3</td>
<td>7.7-12.1</td>
<td>57</td>
<td>9.70 ± 1.87</td>
</tr>
<tr>
<td>H. M.</td>
<td>60</td>
<td>M</td>
<td>2</td>
<td>CB, diffuse</td>
<td>High/high</td>
<td>IVB</td>
<td>2</td>
<td></td>
<td>9.7-17.7</td>
<td>21</td>
<td>10.70 ± 1.41</td>
</tr>
<tr>
<td>A. O. B.</td>
<td>74</td>
<td>F</td>
<td>7</td>
<td>CB, diffuse</td>
<td>High/high</td>
<td>IIIB</td>
<td>7</td>
<td></td>
<td>14.8-21.8</td>
<td>47</td>
<td>18.51 ± 2.36</td>
</tr>
<tr>
<td>H. A. L.</td>
<td>71</td>
<td>M</td>
<td>7</td>
<td>CB, diffuse</td>
<td>High/high</td>
<td>IVB</td>
<td>2</td>
<td>5</td>
<td>16.3-24.2</td>
<td>47</td>
<td>18.90 ± 2.59</td>
</tr>
</tbody>
</table>

**WF, Working Formulation; NM, not measurable; IM, intermediate; D, diploid; ND, near diploid; A, aneuploid; HD, hypodiploid; Ly, chronic lymphocytic leukemia; CCL, lymphocytic lymphoma of chronic lymphocytic leukemia type; CC, centrocytic lymphoma; CB, centroblastic lymphoma; folic., follicular.

* More than one aneuploid population seen in some of the samples.

The mean of the lowest S-phase values for those patients with low grade malignant lymphomas was 2.7 ± 0.4% (range, 1.6–6.0%), whereas the mean of the highest S-phase values in this group was 6.0 ± 0.9% (range, 3.7–14.5%). Correspondingly, for the intermediate grade malignant lymphomas, the mean of the individual lowest S-phase values was 5.1 ± 1.8% (range, 1.1–20.0%) and the mean of the individual highest S-phase values was 9.7 ± 2.2% (range, 4.0–28.8%). Thus, the lowest and highest mean S-phase values for the intermediate grade malignant lymphomas were 89% and 62% higher than the corresponding mean values for the low grade malignant lymphomas.

Circadian Variation in S-Phase. The percentage variation in S-phase within each patient between the lowest and highest S-phase as compared to the lowest value (range of change = ROC) during the 24-h time span varied from 21 to 353%, with a mean ROC of 128 ± 19% (median, 107%) (Table 1). Two examples of individual DNA histograms according to circadian stage are shown in Fig. 1. Although the value of the first sample and the last sample taken 24 h later might differ, no statistically significant difference in mean S-phase was found between the first sample (S-phase = 7.6 ± 1.7%) and last sample (S-phase = 7.4 ± 1.6%) for the pooled data of the 17 patients from whom samples were obtained 24 h apart (P = 0.75).

The circadian waveform and phasing (time of day of lowest and highest values) in S-phase varied somewhat between the individual patients and start times, as illustrated in 6 series shown in Fig. 2. However, as indicated by the distribution of the acrophases of the

---

**Fig. 1.** Histograms of DNA cell cycle phase distribution demonstrating difference in S-phase according to circadian stage in 2 different patients. 2C and 4C, G1/G0 and G2/M of the diploid population, respectively.
best-fitting 24-h cosine obtained from each series, the majority of the peaks in S-phase were found to be late in the evening or during the night (Fig. 3). In 18 of 24 patients (75%), location of the acrophase representing higher S-phase was found during evening and night as compared to daytime (8 p.m.-4 a.m. versus 8 a.m.-4 p.m.). This observation was validated when each individual S-phase series was converted to percent of mean and combined for analysis by one-way ANOVA to test for time-effect across 2 12-h time spans (8 p.m.-8 a.m. versus 8 a.m.-8 p.m.). S-phase variation according to circadian stage was found to be statistically significant ($P < 0.004$), with higher values found in the 8 p.m.-8 a.m. timespan (Table 2). A one-way ANOVA across the 6 individual test-times resulted in borderline statistical significance ($P = 0.059$).

When using ANOVA across the 2 12-h timespans for subgroups of patients, a significant time-effect for DNA synthesis was found for low and intermediate grade lymphomas (according to the Working Formulation classification system); i.e., with mean S-phase values <10.0% in the present study, the highest S-phase was found during night ($P = 0.008$). For lymphomas classified as low grade according to the Kiel classification and intermediate grade according to the Working Formulation, the circadian variation was even more pronounced (ANOVA, $P = 0.004$; cosinor analysis, $P = 0.039$). For men and women combined, as well as for men >67 years, a significant circadian variation in S-phase was found both by ANOVA and cosinor analysis (Table 2).

Furthermore, by analyzing the data according to tumor stage, a circadian variation in S-phase was found in lymphomas ≤ Stage III B but not in lymphomas ≥ Stage IVA (Table 2).

By single cosinor analysis of all data combined, S-phase yielded a marginally significant $P$ value of 0.069 for the least-squares fit of a 24-h cosine to all data as percent of mean, with an amplitude of 8.5% and the acrophase found to be near midnight (0.05 h) (Fig. 4A). The circadian pattern of S-phase in NHL thus appears to be out of phase with that reported previously by us (36) in the bone marrow of healthy men (Fig. 4B). When comparing the distribution of acrophases around-the-clock for DNA synthesis in lymphomas in the present study with acrophases for DNA synthesis in human bone marrow cells in our previous study, an apparent difference in the timing of S-phase in the 2 populations can be seen (Fig. 5). It is clear that most S-phase acrophases of NHL are away from the 95% limits computed for the bone marrow S-phases. When comparing the number of acrophases for each group within the 2 12-h time spans, a significant difference in temporal distribution is found by a $\chi^2$ test. Thus, the S-phase acrophase is found between 8 p.m. and 8 a.m. in 15 lymphomas and 3 bone marrow series, whereas there are 9 lymphomas and 16 bone marrow series with S-phase acrophase between 8 a.m. and 8 p.m.
Bone marrow (n = 16 healthy men; 19 24-h periods).

Ploidy According to Circadian Time of Sampling. Twenty-one of the 26 patients (80.8%) had an aneuploid, hypodiploid, or near diploid population in one or several of the repeated samples (Table 1). Examples of individual DNA histograms with varying ploidy according to circadian stage are shown in Fig. 6. Six of the aneuploid populations were seen in only one sample of the 5-7 individual samples from a patient. For the individual tumors with an aneuploid population in several of the histograms, the DI was essentially the same, i.e., the mean variation in DI within the same tumor varied from 1.7 to 5.5%, with a mean variation of 4.0 ± 0.3% (SE).

Seven of the 12 patients (58%) with low grade lymphomas had an aneuploid population in one or more of the samples, and aneuploid populations were found in each of the subgroups of low grade, intermediate grade, and high grade lymphomas.

### Table 2 Statistical evaluation of circadian stage-dependent variation in S-phase in non-Hodgkin’s lymphomas

<table>
<thead>
<tr>
<th>Category</th>
<th>Sex</th>
<th>No. of Patients</th>
<th>Org Units</th>
<th>% of Mean</th>
<th>8 p.m.–8 a.m.</th>
<th>8 a.m.–8 p.m.</th>
<th>Characteristics of 24-h cosine fit (using all data as % of mean):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>P Mesor ± SE Amp ± SE (95% limits)</td>
</tr>
<tr>
<td>All subjects</td>
<td>M + F</td>
<td>24</td>
<td>152</td>
<td>0.61</td>
<td>4.35 ± 0.94</td>
<td>8.40 ± 0.77</td>
<td>0.004 107.9 ± 4.5 84 93.6 ± 2.7 0.069 100.6 ± 2.5 8.5 ± 3.7 0.005</td>
</tr>
<tr>
<td>All subjects</td>
<td>M</td>
<td>18</td>
<td>111</td>
<td>0.81</td>
<td>0.37 ± 0.90</td>
<td>9.00 ± 3.07</td>
<td>0.003 109.5 ± 5.5 61 92.2 ± 2.8 0.069 100.7 ± 3.0 10.1 ± 4.3 23.43</td>
</tr>
<tr>
<td>All subjects</td>
<td>F</td>
<td>6</td>
<td>41</td>
<td>0.01</td>
<td>0.99 ± 0.43</td>
<td>0.51 ± 0.18</td>
<td>0.718 103.5 ± 7.6 23 97.3 ± 6.2 0.770 100.3 ± 4.9 5.3 ± 7.8 0.012</td>
</tr>
<tr>
<td>By age group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23–58 yrs</td>
<td>M + F</td>
<td>15</td>
<td>92</td>
<td>0.03</td>
<td>0.85 ± 0.79</td>
<td>0.37 ± 0.35</td>
<td>0.735 103.4 ± 6.3 51 97.2 ± 3.7 0.795 100.2 ± 3.5 3.5 ± 5.2 0.005</td>
</tr>
<tr>
<td>23–58 yrs</td>
<td>M</td>
<td>12</td>
<td>72</td>
<td>0.13</td>
<td>0.72 ± 1.83</td>
<td>0.18 ± 0.30</td>
<td>0.363 105.6 ± 7.2 39 95.3 ± 3.7 0.674 100.3 ± 3.9 5.1 ± 5.7 23.33</td>
</tr>
<tr>
<td>23–58 yrs</td>
<td>F</td>
<td>3</td>
<td>20</td>
<td>0.14</td>
<td>0.79 ± 0.33</td>
<td>0.53 ± 0.73</td>
<td>0.865 94.5 ± 13.4 12 103.7 ± 10.5 0.886 99.4 ± 8.6 5.6 ± 11.4 0.0714</td>
</tr>
<tr>
<td>67–91 yrs</td>
<td>M + F</td>
<td>6</td>
<td>90</td>
<td>0.76</td>
<td>0.38 ± 1.80</td>
<td>&lt;0.001</td>
<td>0.007 114.8 ± 5.8 33 87.9 ± 3.3 0.007 101.3 ± 3.4 15.9 ± 4.8 23.45 (21:38, 02:00)</td>
</tr>
<tr>
<td>67–91 yrs</td>
<td>M</td>
<td>6</td>
<td>39</td>
<td>1.45</td>
<td>0.23 ± 14.12</td>
<td>&lt;0.001</td>
<td>0.016 117.2 ± 8.0 22 86.7 ± 4.0 0.016 101.8 ± 4.4 19.3 ± 6.3 23.49 (21:28, 02:12)</td>
</tr>
<tr>
<td>67–91 yrs</td>
<td>F</td>
<td>3</td>
<td>21</td>
<td>0.02</td>
<td>0.80 ± 4.20</td>
<td>0.052</td>
<td>0.418 110.7 ± 8.5 11 90.3 ± 6.2 0.418 100.7 ± 5.6 10.3 ± 7.6 23.31</td>
</tr>
<tr>
<td>By grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LL, LI</td>
<td>M + F</td>
<td>20</td>
<td>125</td>
<td>0.65</td>
<td>0.42 ± 7.28</td>
<td>0.008</td>
<td>0.56 108.8 ± 5.4 69 92.8 ± 3.2 0.112 100.7 ± 3.0 9.2 ± 4.4 23.59</td>
</tr>
<tr>
<td>LL</td>
<td>M + F</td>
<td>12</td>
<td>77</td>
<td>0.02</td>
<td>0.89 ± 1.88</td>
<td>0.28 ± 0.01</td>
<td>0.35 104.5 ± 6.9 42 96.3 ± 3.9 0.374 100.4 ± 3.8 7.6 ± 5.4 0.0312</td>
</tr>
<tr>
<td>LI</td>
<td>M + F</td>
<td>8</td>
<td>48</td>
<td>2.04</td>
<td>4.13 ± 9.42</td>
<td>0.004</td>
<td>0.21 116.1 ± 8.4 27 87.5 ± 5.3 0.039 102.2 ± 4.9 17.6 ± 6.7 21.49 (18.44, 00:52)</td>
</tr>
<tr>
<td>HH</td>
<td>M + F</td>
<td>4</td>
<td>27</td>
<td>2.15</td>
<td>0.15 ± 1.72</td>
<td>0.20 ± 0.01</td>
<td>0.36 103.8 ± 4.3 15 97.0 ± 3.3 0.396 100.4 ± 2.7 5.3 ± 5.7 0.0103</td>
</tr>
<tr>
<td>By stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-21HB</td>
<td>M + F</td>
<td>10</td>
<td>62</td>
<td>0.09</td>
<td>0.76 ± 10.57</td>
<td>0.002</td>
<td>0.27 116.0 ± 9.0 35 98.7 ± 3.5 0.046 101.4 ± 4.6 17.0 ± 6.7 0.014 (22:20, 03:48)</td>
</tr>
<tr>
<td>21-31VA</td>
<td>M + F</td>
<td>14</td>
<td>90</td>
<td>0.51</td>
<td>0.47 ± 0.72</td>
<td>0.397</td>
<td>0.41 102.6 ± 4.4 49 97.8 ± 3.7 0.560 100.3 ± 2.9 4.3 ± 4.0 21:11</td>
</tr>
</tbody>
</table>

---

Fig. 4. Circadian variation in DNA synthesis in non-Hodgkin’s lymphoid tumors versus healthy human bone marrow. A. non-Hodgkin’s lymphoid tumors (n = 18 men, 6 women). B. bone marrow (n = 16 healthy men; 19 24-h periods).

---

\( \chi^2 = 9.5; P = 0.002 \). This difference is even more pronounced if the 12-h spans begin at 6 or 7 a.m. (Fig. 5).

**Circannual Variation in S-Phase.** When using the data for those patients with low and intermediate grade lymphomas and with mean S-phase values <10.0%, we found that mean S-phase was higher in the winter (5.75 ± 0.40%, n = 8 patients), than in the spring (3.84 ± 0.30%, n = 8 patients) or the fall (3.61 ± 0.31%, n = 5 patients) (note: no data were obtained in the summer). Thus, an effect of season was detected by ANOVA (F test = 11.6, P < 0.001) and the least-squares fit of a 1-year cosine (P = 0.044). The mesor was computed was indicated by the acrophase was February 8, with 95% confidence limits from January 1 to March 19.
most patients with indolent lymphoma will ultimately die from their disease (2). The generally accepted consensus of postponing treatment until symptom progression is due to the fact that cytotoxic chemotherapy has been considered incapable of curing these low grade malignant diseases with a generally low proliferative activity. Flow-cytometric analysis may thus possibly improve the handling of certain lymphoma subgroups, exemplified within the group of low grade follicular lymphomas, in which properly timed S-phase determinations may lead to an earlier detection of an increase in proliferative activity in the tumor and aid in the decision as to when to initiate therapy in primarily asymptomatic patients.

The present study has demonstrated a large intraindividual variation from lowest to highest S-phase (ROC, 21 to 353%) during the 24-h time span (mean ROC, 128 ± 19%). Overall, a statistically significant circadian stage-dependent variation was found when comparing 2 halves of the day, with highest DNA synthetic activity during evening and night hours. For certain subgroups of patients, this variation was also statistically significant both by ANOVA and/or cosinor analysis. An interesting observation is that a significant circadian rhythm was found for tumor Stages I-IIIB, but not for the most advanced tumor stages, i.e., Stages IVA and IVB.

It could be argued that these demonstrated circadian variations might be due to intratumor heterogeneity in proliferative activity, or possibly sample variations in the ratio of neoplastic to reactive cells, e.g., T lymphocytes. However, it has earlier been demonstrated that the intratumor variation in proliferative index estimated from multiple analyses revealed a variation within ±5% (52), which refutes the possibility that the large variation seen in the present study can be explained solely by tumor cell heterogeneity or by admixture of reactive cells. The near-identical value of the mean S-phase 24 h apart in the present study supports this argument. Another point to be made in this context is that all samples were harvested from the same lymph node in each individual. When harvesting about 20,000 cells each time, a relatively equal distribution of lymphoma cells should be expected. An alternative explanation is therefore that a circadian variation in tumor cell proliferative activity may exist, which is supported by the finding of Klevecz et al. (23, 59), who demonstrated a higher DNA synthesis at a certain time of day for ovarian carcinoma. Values for S or S + G2/M were found to vary from 0.5 to 15% through the 24-h span, i.e., representing a 30-fold difference in the fraction of ovarian cancer cells replicating DNA. A 2- to 4-fold difference over the 24 h was still observed in DNA synthesis after pooling the data across patients for combined analysis (23).

Due to differences between patients, it is necessary to have a minimum of observations in several patients. Thus, in an earlier study we were unable to find a consistent and significant variation during the 24-h time span, except that several patients had highest S-phase during night (60). A significant circadian variation was eventually established when additional patients were included.

The location of the acrophase at midnight for lymphoma cells in S-phase is somewhat in agreement with the timing of ovarian carcinoma S-phase cells reported by Klevecz and Braly (59), who showed an acrophase at midnight and a secondary peak from 11 a.m. to 3 p.m. In the ovarian cancer study, some tumors demonstrated 12-h rhythms of S-phase with peaks at both noon and midnight. When our lymphoma data were analyzed by the least-squares fit of a 12-h cosine, we found 2 patients displaying a significant rhythm at this frequency, but could not detect a significant rhythm for the group as a whole, whether this period was fitted alone (P = 0.505) or concomitantly with a 24-h component (P = 0.578). The partial discrepancy between our study and the study of Klevecz et al. (23, 59) may possibly be explained by the different tissue origin of these tumor cells, from lymphoid and epithelial tissues, respectively. In human normal tissues, rhythms of proliferation 12 h apart have, to our knowledge, not been reported.
The mean S-phase values at the start and end of the study for the pooled data (the 17 patients measured 24 h apart) were almost identical: 7.6 versus 7.4%, i.e., a relative difference of 2.7%, which contrasts with a difference of 82.1% between the mean lowest and mean highest S-phase values during the 24-h sampling span. This finding implies that a tissue response in lymph nodes due to repeated sampling cannot explain the variation observed in proliferative activity during the 24-h sampling procedure. The existence of a circadian variation in proliferative activity with large circadian stage-dependent differences in S-phase is thus more likely.
Such repeated sampling during a 24-h time span may help in the selection of the optimal circadian time of administering cytotoxic drugs for an individual. That this may be a relevant approach is suggested by the findings of Sothern et al. (61), who have shown in a murine plasmacytoma model that tumor remission rate is significantly dependent on the circadian stage of cytotoxic therapy administration. Thus, one could potentially increase the response and survival in patients with NHL, which is a curable malignant disease.

In addition, by obtaining tissue samples for determination of S-phase several times during a 24-h period, a better indication of the proliferative status may be achieved. The finding of a high mean 24-h S-phase and/or a great variation in S-phase during the 24-h time span could possibly identify patients with low grade NHL at risk of transforming to high grade histology. The time of highest DNA synthesis in lymph nodes bearing non-Hodgkin’s lymphomas coincides with the time of lowest DNA synthesis (36) (Fig. 4), as well as lowest number of CFU-GM (37) in healthy human bone marrow. Recently, a circadian rhythm in DNA synthesis in human rectal mucosa has been reported in healthy men (62). In that study, highest proliferative activity, as reflected by in vitro \[^{3}H\]thymidine uptake, was found near the time of awakening at 7 a.m. Together, these findings on differences in the circadian timing of proliferation in healthy and tumorous cells suggest the possibility of achieving the specification of a therapeutic window in time during which an increase in dosage and cytotoxic effect, together with a decrease in undesired toxicity, might occur. The results imply that a chronotherapeutic administration of the whole or main dosage of an S-phase-specific drug (or drug with its main effect on S-phase) during the time of highest DNA synthesis in lymphoma cells and lowest DNA synthesis in healthy tissues, such as the human bone marrow and the gastrointestinal mucosa, i.e., during late evening and night, could result in a maximized therapeutic index.

Nevertheless, as tumor cell proliferation is likely to be more autonomous, depending on differentiation grade, heterogeneity of the tumor, and tumor burden, the timing of cancer therapy should still primarily be adjusted to the temporal pattern of proliferation in normal tissues, e.g., the bone marrow and the gastrointestinal tract, which are the major dose-limiting tissues today relative to cytotoxic therapy. This approach is supported by the fact that circadian variation in normal cell proliferation is coupled to a broad spectrum of circadian rhythmicity in the body (63), which may remain stable even as treatment continues (23). In this context, it is important to be aware of the possibility that treating patients at an optimal circadian time is today feasible and cost-effective through programmable drug delivery systems that allow for a temporally adjusted administration of cytotoxic drugs, mainly in an outpatient setting (64–66).

An incidence of aneuploidy varying from 29 to 80% has been reported in non-Hodgkin’s lymphomas (6, 13, 44, 67–69), and aneuploid tumors have been found to be more common among “high grade” NHL than among “low grade” (21, 42–45, 56). No correlation between ploidy and survival has been generally observed. The association between ploidy status and remission induction and survival has generally not been agreed upon, and their relationship remains controversial in NHL (5, 9, 21, 45), with most studies reporting an absence of a relationship between ploidy and survival (5, 9, 15). However, when interpreting ploidy data and using them as a predictive parameter, it is important to be aware of the intratumor ploidy heterogeneity (70–78). Consequently, an unequivocal determination of the ploidy status of a tumor can only be achieved by several samplings of the same tumor.

In the present study, there was a large intratumor DNA stem cell heterogeneity with regard to ploidy, in addition to the variation in S-phase, more than could be expected as compared to earlier studies (52, 77). Intraindividual differences in ploidy status in one or more samples of the same tumor was observed in 61.5% of the tumors (Table I). However, the distribution of DI observed in the interval from 1.10 to 1.30 and from 1.85 to 2.00 corresponds to what others have found (53). Thus, the demonstration of intratumor heterogeneity is in accordance with the study of Cowan et al. (52), demonstrating ploidy discrepancy within the same tumor in NHL in 16% of all the tumors, the lower percentage of differing ploidy in this study most likely being explained by fewer repeated sampling procedures performed at but a few unspecified circadian stages during the daytime only. Heterogeneity in ploidy has been demonstrated in other types of tumors as well, such as breast cancer (75, 77), leukemia (72), renal cancer (70), colorectal cancer (71, 74), and lung cancer (76). The observation of a frequent intratumor ploidy heterogeneity is also consistent with reported findings that despite similar histological appearance at relapse, ploidy status differed in several of the cases (52, 53). This change in ploidy may be explained by sequential genetic alterations and the predominance of a specific malignant clone with tumor progression (78).

When presenting ploidy data, it, therefore, seems important to be aware of the degree of intratumor ploidy variation (52), as heterogeneity in ploidy may explain the lack of prognostic significance of this parameter in many tumors. It is also important to recognize that larger values of CV may mask minor degrees of aneuploidy, especially if the aneuploid population is only a small percent of the diploid population.
CIRCADIAN VARIATIONS IN NON-HODGKIN'S LYMPHOMAS

In conclusion, a combination of already established prognostic factors together with an optimally timed estimation of DNA synthesis activity as well as DNA content, i.e., ploidy, might increase the predictive accuracy relative to response in individual patients, as well as guiding the selection of dose and a properly timed chemotherapeutic schedule of drug delivery.

ACKNOWLEDGMENTS

We are indebted to our patients for making this study possible.

REFERENCES

CIRCADIAN VARIATIONS IN NON-HODGKIN'S LYMPHOMAS


DNA Synthesis and Ploidy in Non-Hodgkin's Lymphomas Demonstrate Intrapatient Variation Depending on Circadian Stage of Cell Sampling

Rune Smaaland, Knut Lote, Robert B. Sothern, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/53/13/3129

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.