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Ploidy and Proliferative Activity Measurement by Flow Cytometry in Non-Hodgkin's Lymphomas. Do Speculative Aspects Prevail Over Clinical Ones?

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Abstract—*Paraffin-embedded lymph node biopsies from 107 patients with newly diagnosed non-Hodgkin's lymphomas were examined for cell DNA content and proliferative activity (as percentage of S-phase cells) by means of flow cytometry. Patients were diagnosed between 1975 and 1985 and were homogeneously treated according to the grade of histologic malignancy. Cytofluorimetric data were studied with regard to their correlation with histology (classified and reviewed according to both Kiel and Working Formulation criteria), clinical stage, presence of constitutional symptoms, presence of bulky disease, sex, age, and the following laboratory data measured at diagnosis: erythrocyte sedimentation rate, hemoglobin, serum lactic dehydrogenase and serum albumin concentration. Aneuploidy was more frequent in the high grade malignant subtypes and in the miscellaneous group but showed no correlations with the other clinical parameters studied. Proliferative activity demonstrated a wide variation of data but a trend was evident toward higher proliferative values in the more severe histologic subtypes. The survival discrimination allowed by high- and low-grade malignant histology is exactly reproduced when highly and slowly proliferating lymphomas are considered ($>$ or $\leq$ 12% of S-phase cells).*

These results, analyzed with those in the literature, suggest that measurements of ploidy and proliferative activity add little independent information to what is already provided by current histologic classifications, mainly as far as clinical evaluation and prognosis are concerned. Cytokinetic-aided therapeutic choices can be usefully proposed in a restricted number of cases. Improvement of the available lymphoma classifications through a better integration of ploidy and cytokinetic data with immunologic, genetic and histologic findings is still an object to be pursued in cytometric studies.

INTRODUCTION

THE non-Hodgkin's lymphomas (NHL) are characterized by a very wide spectrum of clinical manifestations and prognostic behavior. Classification of these lymphomas according to strictly morphological criteria is now considered inadequate, since an important amount of additional information can be obtained by supplementing morphology with immunologic [1], cytokinetic [2, 3], cytogenetic [4] or even metabolic parameters [5].

During the last 10 years, cell kinetic studies have been arousing a great deal of interest: the first observations [2, 6–8] showed stimulating correlations of cytokinetic findings with both the morphologic data and the overall clinical course of the NHL. Thus, a new trend of research was opened which tried to define better the relation between the proliferative activity of these tumors and their histological aggressiveness [9, 13–30]. A considerably clearer insight was reached into the cytological basis supporting the observed differences in clinical malignancy. Nevertheless, the heterogeneity of the methods employed, of the populations studied and especially of the results obtained, did not allow organization of the remarkable amount of experimental data into a consistent, articulated and reliable classificatory structure.

For these reasons, we determined to study ploidy and proliferative activity in our untreated NHL patients, observed over a decade, by means of

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cytofluorimetric (CFM) analysis of archival lymph node tissue [10–12] biopsied for diagnostic purposes.

The main advantages of such a retrospective analysis, which is now technically feasible, are represented by homogeneous experimental conditions (in both CFM determination and histologic re-evaluation of lymph nodes) and prolonged clinical follow-up of patients. The complete knowledge of the clinical course and fate of each case allows a more reliable study of the clinical and prognostic correlations of ploidy and proliferative activity data.

MATERIALS AND METHODS

Study population

We studied 107 untreated patients with NHL diagnosed between January 1975 and December 1985: 50 males and 57 females, whose mean age was 54.8 years (range: 9–82). They were staged according to guidelines given at the Ann Arbor Conference [31]. All had bipedal lymphangiography, bone marrow core biopsy and either ultrasound exploration or computed tomography of the abdomen. Six patients had stage I disease, 10 stage II, 33 stage III and 58 stage IV disease.

Histologic subtypes were routinely diagnosed according to the Kiel Classification [32] and were retrospectively re-evaluated according to both the Kiel criteria again and the International Working Formulation (WF) scheme [33].

Treatment was primarily related to histologic type: the CVP [34] regimen, or its variant COP [35], was used for patients with low-grade malignant subtypes (according to Kiel), while CHOP(Bleo) [36] chemotherapy was administered to patients with high-grade malignant subtypes.

Preparation of lymph node specimens

All pathological specimens analyzed in this study were obtained at biopsy during the patient's routine diagnostic evaluation and underwent standard formalin fixing and paraffin embedding procedures. Sections, 4 and 30 μm thick, were cut serially from each tissue block. The 4-μm thick paraffin section was processed for routine slides. This section was used for an additional histologic control and to assess the percentage of tumor cells.

DNA flow cytometry

Two to four 30-μm sections from each paraffin block were dewaxed in xylene (twice) for 15 min, rehydrated through a series of alcohols (100%, 95%, 70% and 50%: 10 min for each concentration) and then washed twice with distilled water. The samples were agitated several times during rehydration. The sections were then treated for 30 min at 37°C in a 5 mg/ml solution of pepsin (Sigma) in 0.9% NaCl, adjusted to pH 1.5 with 2 N HCl. The tubes were placed in a waterbath with intermittent vortex mixing. The suspensions thus obtained were passed through a 25-μm gauge needle and then filtered through a 35-μm pore nylon filter. Cell counts were made so as to have more than 10^4 cells/ml per sample. The suspension was centrifuged and the pellet stained with propidium iodide (PI, Calbiochem, Behring Corp., San Diego, CA) at a concentration of 50 μm/ml in H_2O; 0.05% Nonidet P40 (Calbiochem) and RNase (Type 1A, from bovine pancreas, Sigma) were included in the staining solution. A 30-min staining time at room temperature proved to provide the best histogram resolution. Immediately before measurements the cells were sonicated for a few seconds. An aliquot of cells, obtained by processing paraffin-embedded normal lymph nodes in the same manner, was used as a diploid standard in each set of measurements. The reliability of a diploid standard in paraffin-embedded tissues is still an open problem [22, 37]. The closest approximation would be to use blocks containing non-malignant tissue obtained from the same patient, or at least from homologous organs from healthy subjects [38] or even from select regions containing only normal cells from the block being studied using one of the methods described by Oud *et al.* [39] or van Driel-Kulker *et al.* [40]. Admixture with tumor sample would augment the normal diploid G peak, thus making its identification easier if duplicate samples are run.

A Partec PAS II flow cytometer was used and data were recorded as frequency histograms. To construct each DNA histogram 10,000–15,000 cells were analyzed. The measuring conditions were the following: HBO 100 W/2 (Osram) excitation source with KG1 (2 mm) and BG 38 (4 mm) filters; 546 ± 12 nm interference filter, TK 590 dichromatic mirror and K 610 barrier filter to select the emitted red fluorescence.

Ploidy evaluation

Cell suspensions were analyzed either alone or after they were mixed with an aliquot of cells from normal lymph nodes. At first, the normal cells and the tumor population under examination were measured separately. The normal cells were employed to calibrate the instrumentation and also served as diploid standard. The tumor population was then measured under the same experimental conditions with and without the addition of cells from normal lymph nodes (used as internal diploid standard). Aneuploidy was expressed as a propidium iodide DNA index (DNA I = modal channel of the $G_{0/1}$ peak of the studied population/modal channel of the $G_{0/1}$ peak of the normal cells, used as diploid standard).

Theoretically, the DNA I of a diploid tumour population is 1.00. Minor deviations (of the order of 10% or less [41]) from this value were found for the $G_{0/1}$ peak of a number of tumors having either unimodal or bimodal DNA distribution. In these cases, measurements were repeated by mixing the tumor cells with the normal diploid standard [42]. Under these conditions, diploidy was assessed when mixed normal and tumor cells gave a single $G_{0/1}$ peak, with a coefficient of variation (C.V.) identical to those determined for normal or tumor cells measured alone. Near-diploidy was assessed when mixed normal and tumor cells still gave a single $G_{0/1}$ peak, with a C.V. clearly greater than those determined for normal or tumor cells. Aneuploidy was assessed when mixed normal and tumor cells gave two separate peaks. In this study the coefficient of variation of the $G_{0/1}$ peak had a median value of 3.8 (range: 2.2–5.5).

Proliferative activity

Cell cycle analysis was carried out in cases having unimodal DNA distribution. The percentage distribution of cells in the $G_{0/1}$, S and G_2 phases of the cell cycle was obtained using Fried's mathematical model (which fits data with a Gaussian distribution [43]) adapted for a DPS 8 Honeywell computer [44]. This method allows the analysis of histograms with unimodal DNA distribution, i.e. with $G_{0/1}$ and G_2 peaks and S-phase cells between them. Cases with bimodal DNA distribution, i.e. with more than one cell population, cannot be evaluated with any of the available methods used for analysis of DNA histograms.

Statistics

The chi-squared test for contingency tables, Student's *t* test for comparison of means and the life table method for survival, along with the log-rank test for differences in survival were the statistical methods used [45].

RESULTS

Eighty-one out of the 107 patients had normal diploid DNA content, whereas 26 (24.3%) were aneuploid and showed bimodal DNA content: seven tetraploid, 19 hyperdiploid.

The frequency of diploid and aneuploid cases in relation to the characteristics of the patients at diagnosis is reported in Table 1. It is evident that aneuploid cases were more frequent in males, in patients aged more than 55 years, in advanced stages (III or IV), in patients without constitutional symptoms (A category), in those with high-grade

Table 1. Aneuploidy and proliferative activity in relation to the histologic classification and the main characteristics of the patients

Parameters	Total No.	Aneuploid No.	Aneuploid %	Aneuploid χ^2	S-phase cell % No.	S-phase cell % M ± S.D.	S-phase cell % *t*
Histology (Kiel)							
Low-grade types	58	11	18.9	0.162	49	12 ± 6.2	0.045
High-grade types	49	15	30.6		39	16 ± 7.1	
Histology (WF)							
Low-grade types	29	7	24.1	n.s.	24	11 ± 5.9	0.017
Intermediate-grade types	34	5	14.7		30	13 ± 6.1	
High-grade types	38	11	28.9		30	16 ± 7.0	
Miscellaneous group	6	3	50.0		4	20 ± 1.0	
Stage I	6	1	16.6	n.s.	4	11 ± 5.9	n.s.
II	10	2	20.0		7	15 ± 7.2	
III	33	10	30.3		27	13 ± 6.6	
IV	58	13	22.4		50	15 ± 7.2	
Constitutional symptoms							
Absent (A)	64	17	26.5	n.s.	53	14 ± 6.9	n.s.
Present (B)	43	9	20.9		35	14 ± 7.1	
Bulky disease							
Absent	95	22	23.1	n.s.	78	14 ± 7.1	n.s.
Present	12	4	33.3		10	9 ± 4.6	
Males	50	14	28.0	n.s	41	15 ± 7.4	n.s.
Females	57	12	21.1		47	13 ± 6.5	
Aged <55 years	51	9	17.6	0.126	42	13 ± 6.9	n.s.
Aged ≥55 years	56	17	30.3		46	15 ± 6.9	

malignant subtypes according to both the Kiel and the WF classifications and, finally, in patients with bulky disease. None of these differences was statistically significant after the chi-squared test.

No correlations at all were found between aneuploidy and laboratory parameters such as erythrocyte sedimentation rate (ESR), serum lactic dehydrogenase (LDH), hemoglobin and serum albumin concentrations.

A total of 88 histograms was considered suitable for a reliable estimate of the S phase; the median percentage of S-phase cells was 14.2 ± 6.9.

Table 1 also reports means ± standard deviations of S-phase cell percent in relation to the same clinical parameters considered for aneuploidy: no significant difference was found between proliferative activity and sex, age and presence of bulky disease, while the difference in relation to clinical stage or constitutional symptoms (i.e. A or B category) was not statistically significant. A good correlation was evident with malignancy grading according to both the Kiel and WF classifications: the trend is towards higher proliferative activity in the more malignant subtypes, and the differences pointed out between low- and high-grade malignant subtypes according to the Kiel classification, or between low-grade malignant ones and all other subtypes according to WF are statistically significant.

No correlations were found between percentages of S-phase cells and ESR, LDH, hemoglobin and serum albumin.

Investigation of the prognostic significance of CFM findings yielded statistically insignificant data. Figure 1 illustrates that no differences in survival were found between patients with and those without aneuploid tumors.

Figure 2 shows rather different survivals when patients are subdivided according to whether their percentage of S-phase cells was ≤ or >12. This cut-off level was found to be relatively the most

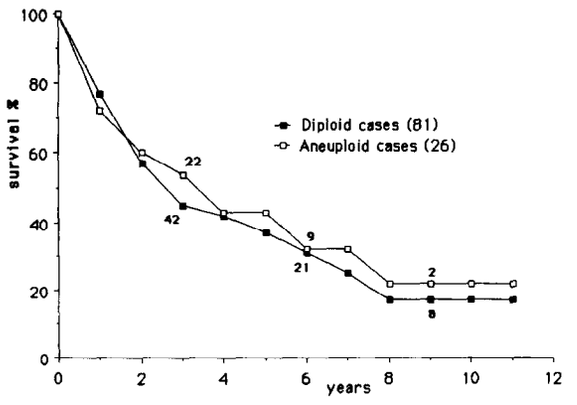

Fig. 1. Survival of 81 NHL patients with euploid DNA content and of 26 others with aneuploid DNA content

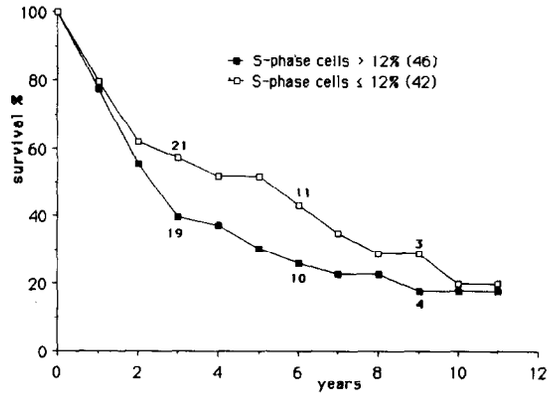

Fig. 2. Survival of 88 NHL patients according to whether percentage of S-phase cells was > or ≤ 12

discriminant after a large number of different percentage values had been systematically tested. The figure suggests a better prognosis for patients with lower than those with higher proliferative activity. This is remarkable for a period of 6–7 years after diagnosis. After this interval the difference in survival decreases quickly, though the number of patients who are present at risk in both curves becomes low for reliable comparisons. As a matter of fact, the survival curves have only a few cases at risk after the eighth year but only the curve of patients with high proliferative activity shows a trend to plateaux after this interval.

The majority of long-survivors (4/6 at 10 years) had high proliferative activity and 3/4 of them belong to the group with high-grade malignancy according to the WF, as is demonstrated by Fig. 3, which illustrates separately the survival of patients with different proliferative activity in distinct histologic subgroups.

DISCUSSION

We can summarize the principal findings of our results in the very poor correlation of aneuploidy with prognosis and the main clinical and laboratory parameters of NHL at presentation; the correlation with histology was without statistical relevance. On the contrary, proliferative activity showed a fairly good correlation with histology and, in a less obvious way, with prognosis.

From a methodological point of view our investigation seemed to be sufficiently comparable with the studies available in the literature as far as number of cases studied, of clinical parameters investigated and length of follow-up were concerned. The flow CFM technique applied to paraffin-embedded biopsy material has already received several confirmations [12, 13]. In particular, the CFM measurements of DNA content were not influenced by a focally variable amount of non-neoplastic cells within the paraffin-embedded specimens since, in this work, the total neoplastic

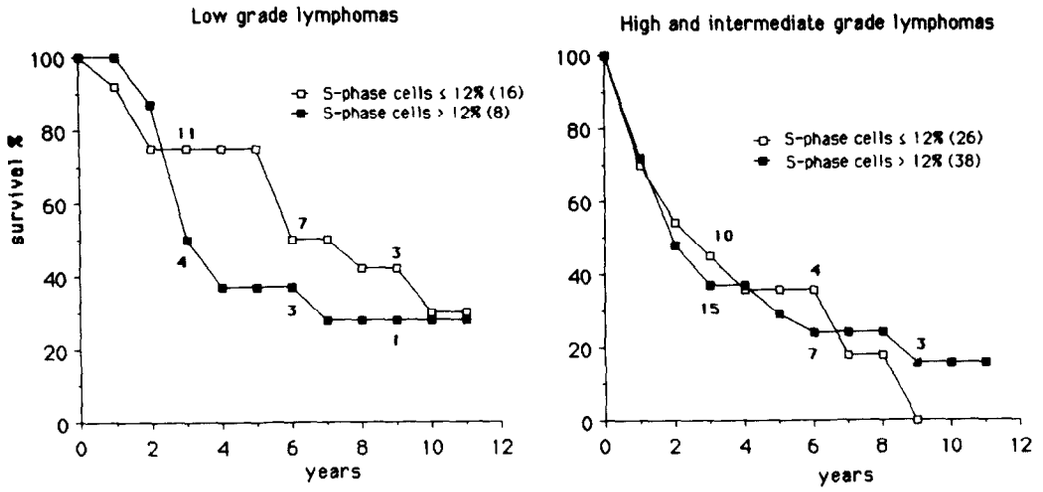

Fig. 3. Survival of patients with low and high proliferative activity (> or ≤ 12% S-phase cell) evaluated separately in the groups with low-grade and intermediate- or high-grade malignant histologic subtypes

Table 2. Incidence of aneuploidy and mean values of proliferative activity reported by the most important recent studies. Number of cases examined, clinical parameters correlated and best cut-off levels used for the discrimination of the population are reported

Authors	No.	Aneupl %	Related parameters	No.	S-phase % (mean)	Cut-off level	Related parameters
Silvestrini *et al.* [8] 1977 (FC + LI)	34	11.7	—	37	6.2	n.g.	Histology
Silvestrini *et al* [23] 1980 (LI)	—	—	—	66	n.g.	4	Histologic pattern Survival at 3 years
Barlogie *et al.* [21] 1980 (FC)	23	56.7	Histology	—	—	—	—
Shackney *et al.* [15] 1980 (FC)	35	31.4	Histology S-phase %	35	6.4	n.g.	Histology
Costa *et al.* [2] 1981 (LI)	—	—	—	81	4.1	4	Histologic pattern Survival at 4 years
Costa *et al.* [3] 1981 (FC)	74	61	Histology	21	6	n.g.	Histology
Diamond *et al.* [20] 1982 (FC)	43	65	Histology	36	9.2	5	Histology
Braylan *et al.* [14] 1984 (FC)	52	78.8	Histology S-phase %	52	8.2	5	Histology Aneuploidy
Roos *et al.* [17] 1985 (FC)	61	23	—	48	3.5	4	Histology Survival at 3 years
Camplejohn *et al.* [12] 1985 (*FC*)	9	11.1	Histology	9	10.4	n.g.	—
Bauer *et al.* [22] 1986 (*FC*)	50	62	—	50	14	20	Survival at 4 years
Christensson *et al.* [24] 1986 (FC)	154	29.2	Histology	154	8.5	n.g.	Histology Cell dimension
Juneja *et al.* [25] 1986 (FC)	115	33	Histology S-phase %	115	5.7	n.g.	Histology Aneuploidy
Macartney *et al.* [9] 1986 (*FC*)	81	34.6	Histology	81	n.g.	8	Histology Conversion to high-grade type
Morgan *et al.* [18] 1986 (*FC*)	178	26	Histology	178	19	20	Histology
Akerman *et al.* [26] 1987 (LI + MI)	—	—	—	101	1.9	2	Histology
Young *et al.* [13] 1987 (*FC*)	111	44	Histology Survival	56	10	10	Survival at 6 years
Veneroni *et al.* [27] 1988 (LI)	—	—	—	35	4.4	4	Histology Ki-67 positivity
Wooldridge *et al.* [28] 1988 (*FC*)	52	56	Survival	52	n.g.	20	Survival at 3 years
McLaughlin *et al.* [29] 1988 (FC)	140	48.6	Survival	140	13.3	10 ÷ 18	Survival at 5 years
Lehtinen *et al.* [30] 1989 (FC)	182	27.5	—	169	8.3	—	Survival at 2–6 years (in low grade types only)
O'Brien *et al.* [46] 1989 (FC)	77	32.5	—	52	n.g.	22	Survival at 4 years (poorly)

Patients studied by Bauer *et al.* and McLaughlin *et al.* were only of the diffuse large cell type (according to Rappaport's classification [47]). In those reported by McLaughlin *et al.* and O'Brien *et al.* cells in the ($S + G_2M$) phase were computed.
FC = flow cytometry; *FC* = FC on paraffin-embedded material; LI = labelling index; MI = mitotic index; n.g. = not given.

involvement of the analyzed specimen was sytematically controlled through examination of thin hematoxylin–eosin stained slides, contiguous to the thicker ones submitted to cytometry. Some authors [14] found somewhat higher proportions of aneuploid cases, mainly due to the use of a selective cell separation technique. However, this did not lead to different results when clinical correlations of the altered DNA content were investigated.

Also, it is possible that flow cytometry on archival material does not identify hypodiploid cases accurately. Nevertheless, the percentage of hypodiploid lymphomas is so low (1–2%) [14–16] that it can be considered negligible [13]. It is well known that such a condition is rare in comparison with other neoplasias [4, 19].

After considering all these methodologic implications, however, it is likely that the great variability in the results available in the literature cannot be fully explained by heterogeneity of experimental techniques. Table 2 summarizes the results of the main studies performed on the DNA content and proliferative activity of NHL in recent years. Even if we analyze only investigations performed with the CFM technique, the percentage of cases with aneuploid DNA content ranges from 11 [12] to nearly 80 [14]. Thus, the aneuploid proportion found in this study (24.3%) corresponds to a middle value within the limits reported by other authors [17, 18].

The ploidy appeared related with histology in many cases but was generally limited to comparisons between low- and high-grade malignant subtypes, and such a relation was statistically significant for only two groups of authors (Diamond *et al.* [20] and Morgan *et al.* [18]). On the other hand, several studies [13, 21, 46] noted no relationship at all between ploidy and histology; in a few others [14, 15, 25] aneuploidy was shown to be mildly related to high proliferative activity and, in two series [13, 30], to a lower probability of surviving at 6 and 5 years, respectively, and in one study to a better 3-year survival [29]. In O'Brien *et al.*'s recent work [46], the relationship between ploidy and survival is somewhat controversial, but there is a trend for aneuploid cases to have a worse 4-year survival.

Similarly, nearly all authors found a good correlation between proliferative activity and histology but, again, according to low- or high-grade malignant subgroups, at the most with an intermediate grade also being included. However, since the promising results of Silvestrini *et al.* [23] and Costa *et al.* [2, 3], who first tried to give a cytokinetic interpretation of the histologic grade of malignancy (nodular vs. diffuse involvement), proliferative activity has shown a widely accepted, significant, but only mild degree of correlation with histology, when a binary variation of S-phase cell percentage around a given discriminant value is evaluated in relation to a binary level of histologic aggressiveness.

Furthermore, the cut-off levels of S-phase cell percentage that in all these studies best discriminated patients with different prognosis varied widely from 2% (Akerman [26]) to 20% (Bauer *et al.*[22], Morgan *et al.* [18] and Wooldridge *et al.* [28]) or even 22% (O'Brien *et al.* [46]). In several studies [2, 3, 8, 13, 17, 22, 19, 30], a statistically significant correlation was found between proliferative activity and overall survival. Generally high proliferative activity is related to a poorer prognosis except in the experience of McLaughlin *et al.* [29]. However, only three reports demonstrated that this prognostic importance was independent of the simultaneous effect exerted by histology on survival: in Bauer *et al.*'s [22] and McLaughlin *et al.*'s [29] works only patients with diffuse large cell lymphomas (according to the Rappaport classification [47]) were studied, while Young *et al.* [13] found that the prognostic value of cytokinetic data persisted after correction for histology when multivariately analyzed (Cox regression). It is remarkable, nontheless, that in all these studies the survival times considered for prognostic evaluation were no longer than 3–6 years. Morgan *et al.* [18], who analyzed survival over a period of 15 years, and our present work, which considered survival times up to 11 years, found no significant difference in survival during the overall interval of the study. Both these latter studies recorded only a temporary prognostic advantage, limited to the first 4–6 years, for patients with lower proliferative activity. Beyond this survival time the difference between high and low proliferative activity curves decreases, or these curves even tend to reverse their course. Such behavior closely resembles that presented by the survival curves of high- and low-grade malignant histologic subtypes. Indeed the relationship between histology and proliferative activity could suggest that the decreasing difference seen in survival over time between high- and low-grade malignant types, as well as between low and high proliferating lymphomas, represents likely the same paradoxical phenomenon explained from different points of view: histology on the one hand, and proliferative activity on the other. As observed by McLaughlin *et al.* [29], the greater sensitivity to antitumor drugs of more actively proliferating tumors is the most likely reason for which a group of cured patients can be found among those affected with high-grade malignant lymphomas. A similar group cannot be observed among patients with low-grade histologic types, whose tumors can have an indolent course, a good response to therapy when they become symptomatic, but a high frequency of relapse and, in conclusion, cannot be eradicated.

We agree with Juneja *et al.*'s opinion [25] that the intrinsic cytologic heterogeneity of lymphomas, among different histologic subtypes and also within the same subtype, contributes to the wide dispersion of the experimental data regarding cell ploidy and kinetics and to the large overlap of their distributions [9, 13–15, 20, 24–26, 28–30, 48]. The possible diagnostic contribution of the CFM assay to the discrimination of cases difficult to classify with conventional techniques is limited by such a dispersion of kinetic data.

A possible source of inaccuracy in this field is given by the microscopical and variable mixture of neoplastic and non-neoplastic cells that very often is presented by the lymphomatous tissue. However, even cell sorting by means of monoclonal antibodies against tumor-associated antigens, as performed by Braylan *et al.* [14], which gives more specific information on tumoral cell DNA and kinetics, on the whole does little to improve discrimination among different types of lymphomas. These authors recognize that still represents a confusion area that low- and high-grade malignant histologic types are equally included within the range between 5 and 10% of S-phase cells; nor is this cytokinetic-aided dichotomous prognostic allocation 100% correct outside these limits.

The assessment of the growth fraction by means of the monoclonal antibody Ki-67, which recognizes a nuclear antigen expressed during all phases of the cell cycle, may offer further possibilities in defining slowly and rapidly proliferating lymphomas. However, its use is still recent and wide investigation is needed. First reports [27] would suggest a good correlation with the [^{3}H]thymidine labelling index.

Thus the analysis of our data, compared with those from the literature, suggests that for the present histologic diagnosis and clinical evaluation in routine practice (for confirmation of diagnosis and for evaluation of prognosis) will not be substantially improved by CFM measurements with respect to the information already furnished by histology itself according to the available classifications. On therapeutic grounds, treatment policy should be better tailored to single-patient characteristics through the evaluation of proliferative activity. Successful use of aggressive chemotherapy in selected subsets of patients with low grade lymphomas [49, 50] is the object of increasing interest. There is no doubt that the therapeutic choice in these cases might be further improved by taking cytokinetic data into account. Moreover, CFM measurements still represent necessary tools, together with cytogenetic, cytoimmunologic and clinical investigation for further validation or refinement of new, more reliable histologic classificatory criteria or for possible identification of unusual new clinical entities.

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