

Contribution of ^3H -thymidine labelling index and flow cytometric S-phase in predicting survival of patients with non-Hodgkin's lymphoma

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Summary The ^3H -thymidine labelling index (^3H -dT LI) of cell suspensions from fresh material and the flow cytometric S-phase (FCM-S) of nuclei recovered from paraffin blocks were determined on the same pathologic lymph node specimen for 190 non-Hodgkin's lymphomas (NHLs). FCM-S was defined by a planimetric method and by an optimization procedure. Poor correlation coefficients were observed among the three cell kinetic variables. All three cell kinetic variables were significant indicators of 8-year survival and median survival time. The life-regression procedure evidenced a significant relative contribution of ^3H -dT LI and FCM-S, thus suggesting a different biologic meaning of the two cell kinetic variables. This finding was further supported by evidence that simultaneous use of ^3H -dT LI and FCM-S can identify groups of patients with different survival better than when either modality is used alone. Multivariate analysis indicated that the risk groups as defined by cell kinetic variables are predictors of survival even in the presence of established factors such as histology and stage.

The prognostic relevance of cell kinetics in malignant non-Hodgkin's lymphomas (NHLs) has been the subject of study for several decades. Cell kinetic studies have consistently shown that ^3H -thymidine labelling index (^3H -dT LI) is an indicator of long-term clinical outcome (Brandt *et al.*, 1981; Costa *et al.*, 1981; Kvaloj *et al.*, 1985), independently of prognostic factors such as histopathology and stage (Silvestrini *et al.*, 1989).

More recently, we showed that the use of bromodeoxyuridine (BUdR) on NHL specimens gives labelling index values superimposable to those obtained with ^3H -thymidine (Silvestrini *et al.*, 1988). However, both approaches are based on the incorporation of DNA metabolic precursors and therefore imply the availability of fresh and viable tumour material. Such a requirement cannot always be satisfied when, as often happens, patients are referred with pathologic material already fixed and another biopsy is not ethically justified or easily obtainable. Moreover, determination of ^3H -dT LI or BUdR LI is limited to prospective collection of fresh biopsies and precludes retrospective analysis of paraffin-embedded samples from series of patients with a long follow-up.

Several studies have shown that the flow cytometric S-phase cell fraction (FCM-S) is a discriminant of clinical outcome in NHL patients (Braylan *et al.*, 1980; Scarffe & Crowther, 1981; Roos *et al.*, 1985; Christensson *et al.*, 1986; Lenner *et al.*, 1987; Griffin *et al.*, 1988; Grierson *et al.*, 1990; Rehn *et al.*, 1990; Joensuu *et al.*, 1990). Flow cytometric analysis is an automatic and quick method to measure the S-phase cell fraction, and an advantage is that stocked paraffin blocks can be used for retrospective analysis.

The present study compared the results obtained when the S-phase cell fraction was measured by autoradiography and by flow cytometry with two mathematical models, i.e., a planimetric method and an optimisation procedure. Moreover, through univariate and multivariate analyses we defined the prognostic power for survival of cell kinetics and clinical and pathologic variables determined in parallel on the same patients.

Materials and methods

Case series

Biopsies were obtained from 190 patients with NHLs at the National Cancer Institute of Milan between 1974 and 1989. The ^3H -dT LI was determined on fresh tissue specimens, and flow cytometric S-phase was determined on the same biopsy sample from paraffin-embedded blocks. All patients were pathologically staged and classified according to the Ann Arbor Staging System (Carbone *et al.*, 1971). Treatment was decided according to different prospective protocols (Monfardini *et al.*, 1980; Monfardini *et al.*, 1984; Bajetta *et al.*, 1988; Tondini *et al.*, 1991) or to the best standard therapy.

Histologic specimens were retrospectively reviewed and classified according to the Working Formulation for clinical usage according with The Non-Hodgkin's Lymphoma Pathologic Classification Project (1982). The new National Cancer Institute clinical distinction (NCI system) between indolent lymphoma (small lymphocytic; follicular, small cleaved cells; follicular, mixed; diffuse, small cleaved cells) and aggressive lymphoma (follicular, large cell; diffuse, mixed; diffuse, large cell; diffuse, immunoblastic) was applied for prognostic analysis (De Vita *et al.*, 1989; Urban *et al.*, 1990).

^3H -dT LI

^3H -dT LI was determined on cell suspensions obtained by mechanical disaggregation of lymph node material as previously described (Costa *et al.*, 1981). Briefly, 6×10^6 cells were incubated with ^3H -thymidine ($4 \mu\text{Ci}$; specific activity, 25 Ci mmol^{-1} ; Radiochemical Centre, Amersham, UK) for 1 h at 37°C . Autoradiography was performed according to the stripping film (AR10 Kodak) technique. The ^3H -dT LI was determined on the entire cell population by scoring 5,000–10,000 cells from different cytopspins for each tumour.

FCM-S

Flow cytometric analysis was performed on cells recovered from paraffin-embedded specimens and fixed in formalin (132 cases) or Zenker's solution (58 cases). Tumor sample adequacy was assessed at the microscope on hematoxylin-eosin-stained sections adjacent to those used for flow cytometric analysis and was verified by a pathologist (R.G.).

The technique of Hedley *et al.* (1983) with minor modifications was used. The nuclei suspension was stained

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with a solution containing propidium iodide (Calbiochem, Boehringer, Corp., San Diego, CA) at the concentration of $50 \mu\text{m ml}^{-1}$ in H_2O , 0.05% Nonidet P40 (Calbiochem) and RNase (Type IA, from bovine pancreas, Sigma).

DNA analysis was performed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA), and a minimum of 30,000 events was acquired on a single-parameter 256-channel integrated fluorescence histogram. Since the use of paraffin-embedded specimens does not allow the use of an internal standard to determine the position of a diploid $G_{0/1}$ population of cells (Schute *et al.*, 1985), an aneuploid cell population was considered to be present only if two distinct $G_{0/1}$ peaks were evident. In such DNA profiles, the DNA index of the aneuploid population was calculated by dividing the mean fluorescence channel of the $G_{0/1}$ peak with the higher intensity by the mean fluorescence channel of the $G_{0/1}$ peak with the lower intensity. A lower median CV was observed for formalin-fixed (4.9%) than for Zenker-fixed specimens (8.9%). Only DNA profiles with a $G_{0/1}$ peak CV less than 8% were considered evaluable, and this criterion was fulfilled in 155 of the original 190 cases.

FCM-S was computed by two mathematical models: a planimetric method (RFIT, Becton Dickinson) and an optimisation procedure (Lampariello & Del Bino, 1988). According to the first model the S phase was calculated from a rectangle, where the height of the rectangle is the average height of mid S phase. For aneuploid tumors, the S-phase fraction of the aneuploid population alone was calculated [i.e. (number of aneuploid S-phase cells/total number of aneuploid cells) \times 100]. The second model represents an optimisation procedure to process DNA histograms containing up to two cell populations and includes an exponential function to subtract background debris. An objective measure of goodness of fit is obtained by computing the chi-square error index. In our experience, chi-square values lower than 3 corresponded to nearly exact fits, as judged by visual inspection.

Statistical analysis

Spearman's correlation coefficient (Hajek & Sidak, 1967) was used to compare S-phase percentages obtained by the two models and to investigate the relationship between FCM-S and $^3\text{H-dT LI}$. The differences in cell kinetics in the various clinico-pathologic subsets were analysed by means of the Wilcoxon rank-sum test.

The role of each of the prognostic variables (univariate analysis) and their joint effect (multivariate analysis) were evaluated by resorting to a Weibull regression model. In this model each regression coefficient (β) is recognisable as the log of the hazard ratio and it is constant with time. For each variable, the 'unadjusted' hazard ratio and its 95% confidence interval were estimated using the putative 'best prognosis' as reference category. To investigate the joint prognostic relevance of the kinetic variables ($^3\text{H-dT LI}$, planimetric and optimisation FCM-S), the 'adjusted' hazard ratios were estimated by resorting to a multiple regression model containing the three variables. The parsimonious regression model was obtained by means of a backward elimination procedure.

Results

FCM-S was computed by the planimetric method and the optimisation procedure on 149 of 155 cases with evaluable DNA histograms. Similar median values (3.7% and 4.0%), ranges and exponential distributions were observed for $^3\text{H-dT LI}$ and FCM-S determined by the optimisation procedure, which implies subtraction of debris contamination. A higher median FCM-S value (12%), as expected, and a log-normal distribution were observed by using the planimetric method without background subtraction.

Statistically significant correlation coefficients between the $^3\text{H-dT LI}$ values and the FCM-S values obtained by the two

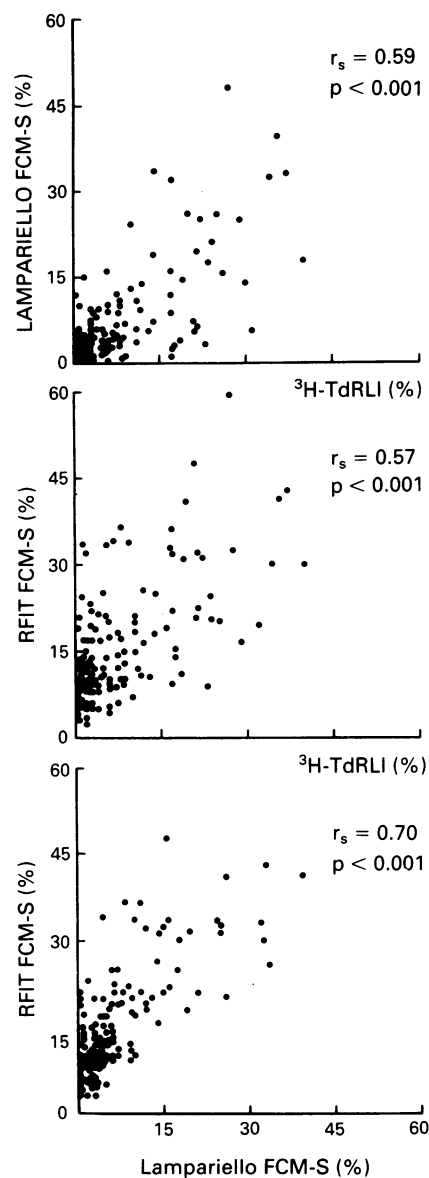


Figure 1 Relationship between cell kinetic variables. a, $^3\text{H-dT LI}$ vs planimetric FCM-S; b, $^3\text{H-dT LI}$ vs optimisation FCM-S; c, planimetric vs optimisation FCM-S. r_s = Spearman's correlation coefficient.

quantification approaches were observed. However, the strength of the association appeared to be rather poor (Figure 1a,b). A significant correlation with a somewhat higher correlation coefficient ($r_s = 0.70$) was also observed between the FCM-S data pairs obtained with or without background subtraction (Figure 1c). Cell kinetics, expressed in the three different ways, was consistently related to histology as defined by the NCI system and unrelated to stage or patient's age. Conversely, FCM S-phase fraction, but not $^3\text{H-dT LI}$, was related to DNA ploidy (Table I).

Survival analysis was performed on the 138 patients with complete kinetic evaluation and follow-up information. The median value of each cell kinetic variable was used as the cutoff between slowly and rapidly proliferating NHLs. Univariate analysis showed that all three variables were correlated to 8-year survival but with a different discriminant power. In fact, survival curves were more diversified for the two kinetic subgroups as defined by $^3\text{H-dT LI}$ than for those defined by FCM-S (Figure 2). Patients with high $^3\text{H-dT LI}$ tumours had about a four-fold shorter median survival than those with low $^3\text{H-dT LI}$ tumours (Table II). Conversely, the difference was only about two-fold when the FCM-S determination was considered. These findings were also reflected

Table I Relationship between cell kinetic variables and biologic, clinico-pathologic features

	³ H-dT LI (%)		Optimisation procedure	FCM-S (median, %)		P value
	Median	P value		P value	Planimetric method	
<i>Working Formulation^a</i>						
Indolent lymphoma	2.0	<0.001	3.0	<0.001	10.0	<0.001
Aggressive lymphoma	9.6		10.3		20.3	
<i>Stage</i>						
I-II	4.5	ns	3.8	ns	12.1	ns
III-IV	3.5		4.5		12.2	
<i>Patient's age</i>						
≤ 60 yrs	3.6	ns	4.4	ns	11.1	ns
> 60 yrs	5.1		4.3		13.0	
<i>DNA ploidy</i>						
Diploid	4.6	ns	3.8	0.05	10.8	<0.001
Aneuploid	3.5		5.8		20.0	

^aAccording to NCI system.**Table II** Prognostic relevance for survival of cell kinetic variables

	Median survival (mo)	Hazard ratio	95% Confidence interval	P ^b value
<i>³H-dT LI</i>				
≤ 4% ^a	80	2.38	1.67-3.39	0.001
> 4%	17			
<i>FCM-S</i>				
<i>Planimetric method</i>				
≤ 12% ^a	62	1.96	1.21-3.16	0.006
> 12%	25			
<i>Optimisation procedure</i>				
≤ 4% ^a	59	1.62	0.98-2.68	0.05
> 4%	26			

^aReference category. ^bBased on Wald statistics.

by the different hazard ratios observed when considering the three different variables (Table II). Relative influence on survival of the three cell kinetic variables was assessed by a multiple regression analysis (Table III). Using a backward method for regression, the value of FCM-S according to the optimisation procedure was removed from the model, leaving ³H-dT LI and planimetric FCM-S in the final step. These two cell kinetic variables retained independent prognostic values even though they were characterised by a different hazard ratio and effect on long-term survival (Table III). Finally, we evaluated whether information provided by these two cell kinetic variables could identify groups of patients with different survival probabilities better than either modality

alone. Plots of the survival curve obtained by the regression model identified three groups of patients with different survival probabilities. Survival curves estimated by the regression model for each group showed that patients with two unfavourable cell kinetic variables (high ³H-dT LI, high FCM-S) did very poorly, with a median survival of only 12 months (Figure 3). In contrast, the median survival of patients with favourable variables (low ³H-dT LI, low FCM-S) was 80 months, and the presence of only one risk factor identified a group with an intermediate median survival of 59 months. Multiple regression analysis of cell kinetic variables, indolent and aggressive lymphomas as defined by the NCI system, and Ann Arbor stage (Table IV) showed that in the present series of patients cell proliferation index retained its prognostic relevance.

Discussion

The proliferation indices, as defined by flow cytometric and autoradiographic methods, have shown their relevance as prognostic indicators in NHLs (Braylan *et al.*, 1980; Brandt *et al.*, 1981; Costa *et al.*, 1981; Scarffe & Crowther 1981; Kvaloj *et al.*, 1985; Roos *et al.*, 1985; Christensson *et al.*, 1986; Lenner *et al.*, 1987; Griffin *et al.*, 1988; Silvestrini *et al.*, 1989; Grierson *et al.*, 1990; Rehn *et al.*, 1990; Joensuu *et al.*, 1990) as well as in other human malignancies (Volm *et al.*, 1985; Volm *et al.*, 1988; Costa *et al.*, 1990; McGuire *et al.*, 1990; O'Reilly *et al.*, 1990; Silvestrini *et al.*, 1990). However, cell kinetic variables have been usually studied independently on different series of patients, thus preventing a clear comparison between the different methods. The present study allowed a direct comparison, on the same biopsy sample, of

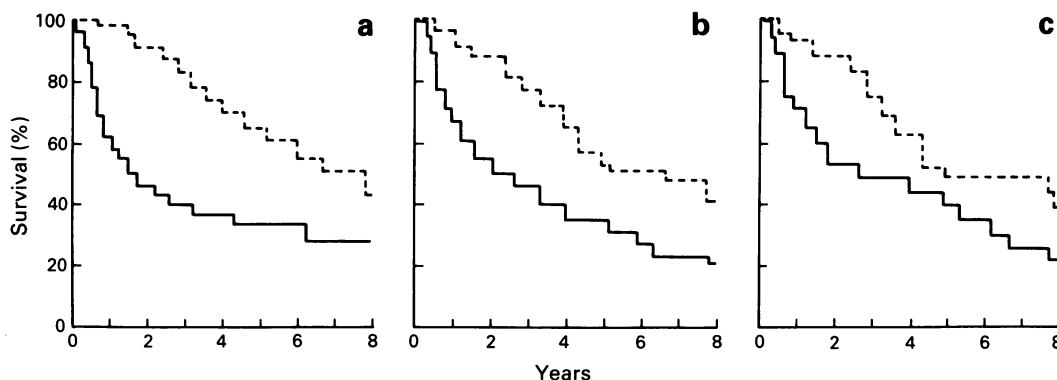
**Figure 2** Eight-year survival as a function of cell kinetic variables. a, ³H-dT LI; b, planimetric FCM-S; c, optimisation FCM-S. (---) slowly proliferating NHLs; (—) rapidly proliferating NHLs.

Table III Multiple regression analysis of cell kinetic variables

Model	Chi square ^a	P value	Hazard ratio	95% confidence interval
<i>Complete</i>				
³ H-dT LI (>4% vs ≤4%)	5.50	0.02	1.83	1.11–3.04
<i>FCM-S</i>				
Planimetric method (>12% vs ≤12%)	3.26	0.07	1.65	0.96–2.85
Optimisation procedure (>4% vs ≤4%)	0.23	n.s.	1.14	0.66–1.97
<i>Final</i>				
³ H-dT LI	8.04	0.005	1.96	1.23–3.13
Planimetric FCM-S	3.96	0.046	1.63	1.01–2.63

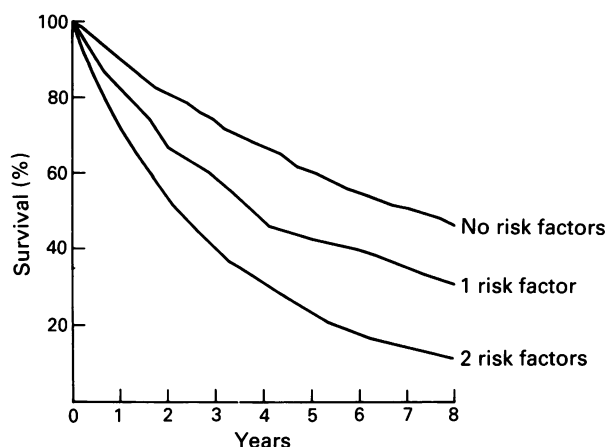
^aBased on Wald statistics.

Figure 3 Eight-year survival as a function of combined ³H-dT LI and FCM-S. Low proliferation index (43 patients): ³H-dT LI ≤4% and FCM-S ≤12%; intermediate proliferation index (46 patients): ³H-dT LI ≤4% and FCM-S >12%, or ³H-dT LI >4% and FCM-S ≤12%; high proliferation index (50 patients): ³H-dT LI >4% and FCM-S >12%.

the interrelationship and of the relative contribution as prognostic indicators of ³H-dT LI and of FCM-S.

The study showed the high feasibility, which was favoured by the high frequency of diploid NHLs (about 70%), of FCM-S determination for paraffin-embedded specimens. Moreover, in agreement with the results of several studies (Griffin *et al.*, 1988; Grierson *et al.*, 1990; Joensuu *et al.*, 1990), the clinical relevance of ploidy was not found (data not shown) and the relevance of FCM-S as a prognostic indicator for survival was confirmed. The results also showed a greater reliability of a planimetric method than of a more sophisticated optimisation procedure with background subtraction to quantify the S-phase cell fraction. We interpret these findings as evidence that exponential debris subtraction could be of greater biologic relevance in other tumor systems than in NHLs. Although debris and contaminating non-neoplastic populations both interfere with FCM-S quantifications, the relative artifactual contribution of each alteration of a true neoplastic proliferative cell fraction is not known. The effect of both variables most likely varies considerably from case to case, depending on a number of factors intrinsic to the individual tumour and technical in nature.

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Table IV Multiple regression analysis for survival

	Chi square ^a	P value
³ H-dT LI, FCM-S	7.12	<0.05
Stage	2.31	ns
Working Formulation ^b	1.04	ns

^aBased on Wald statistics. ^bAccording to NCI system.

Furthermore, the present study suggests that the different accuracy of flow cytometric or autoradiographic methods in predicting clinical outcome is probably due to a different biologic meaning of the two cell kinetic variables. This assumption is supported by their difference in identifying proliferating cells. In fact, ³H-dT as a precursor of thymine is therefore a specific precursor of DNA and it has a functional activity, i.e., it measures the fraction of cycling S-phase cells. As regards FCM-S, such a determination does not discriminate between actually DNA synthesising cells and those with an S-phase equivalent DNA content but quiescent. Moreover, the relation observed between DNA ploidy and the S-phase cell fraction defined on the basis of nuclear DNA content and the lack of a relation with the S-phase cell fraction defined by DNA precursor incorporation could indicate that the two cell kinetic variables are partially complementary. In fact, in combination they accurately identify subgroups at different risk.

Multiple regression analysis including cell kinetic variables, histology (as defined by the NCI system or by the three subsets of the Working Formulation), and stage indicated the relevance of the association of ³H-dT LI and FCM-S in predicting survival. However, owing to the heterogeneity of our population of NHL patients and the different therapeutic approaches used during the study period, we do not feel that the present study can define the correlation between kinetic variables and complete remission. Therefore, the precise role of kinetic variables in defining survival should be interpreted with caution. These findings should be confirmed by prospective studies on adequate series of patients homogeneous for tumour histology and treatment. Such an analysis should necessarily include other biologic factors of established prognostic relevance, such as LDH and β_2 -microglobulin, number of extranodal sites and presence of bulky disease.

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