Eur J Cancer Clin Oncol, Vol. 23, No. 10, pp. 1497–1504, 1987.
Printed in Great Britain

0277-5379/87$3.00+0.00

The Prognostic Significance of Proliferative Activity in Poor Histology Non-Hodgkin's Lymphoma: a Flow Cytometry Study Using Archival Material

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Abstract—*The DNA content and proliferative activity of paraffin-embedded lymph node tissue from* 111 *patients with poor histology non-Hodgkin's lymphoma were measured by flow cytometry. These patients had been entered into a prospective randomized trial which, to date, has shown no survival difference by treatment arm. Forty-four* (40%) *samples showed evidence of aneuploidy with three samples having more than one aneuploid population. The aneuploid populations had a bimodal distribution with one group having a DNA index between* 1.1–1.3 *and the other* 1.8–2.2. *The incidence of aneuploidy did not correlate with age, stage or survival. In* 56 *diploid samples the S phase values below* 10% *had a significantly better survival than those with S phase values above* 10% ($P < 0.011$). *For patients with diffuse large cell histology the corresponding discriminatory S phase value was* 19% ($P < 0.009$).

INTRODUCTION

PATIENTS with non-Hodgkin's lymphoma (NHL) still present a therapeutic challenge, for despite the advances of modern combination chemotherapy a significant proportion of patients will die of their disease. Perhaps no other neoplastic disease has such a wide spectrum of clinical and biological behaviour. The reasons for this heterogeneity remain unclear, but for practical purposes it has become conventional to classify patients with lymphomas into subgroups, usually based on histopathological criteria.

Traditionally speaking, NHL has thus been divided into the so called 'good' and the 'bad' prognostic categories [1, 2], however it is clear that such descriptive terms are by no means absolute, and at times can be misleading. Current survival statistics tend to show that patients with 'good histology' lymphomas have a slow but inexorable progression of their disease that is not greatly influenced by treatment [3]. In contrast patients with 'bad histology' disease have a rapid demise if untreated but a significant proportion, perhaps even 50%, may have a prolonged survival if complete remission is attained by therapy [4].

Attempts to further categorize patients into prognostic categories have met with some success. Immunological [5], cytogenetic [6], kinetic [7] and metabolic [8] approaches have all attempted to provide further prognostic information.

Recently Srigley *et al.* [9] analysed cellular DNA, RNA and double stranded RNA content of lymph nodes and extranodal tissue from 177 patients with NHL and found a correlation between histological grade, aneuploidy, proliferative activity and RNA content. Their report stated that in intermediate and high grade lymphomas, aneuploidy was an unfavourable prognostic feature for attainment of complete remission in previously treated patients but only prior treatment status and high proliferative activity were identified as independent factors affecting survival.

In a previous paper [10] we have shown that reliable ploidy data can be obtained from archival paraffin-embedded tissue when compared to fresh material and a recent report has confirmed this finding in NHL [11]. In this study we have analysed the cellular DNA content of archival lymph node

Accepted 28 April 1987.
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The authors wish to acknowledge support from the National Health and Medical Research Council of Australia, The New South Wales State Cancer Council and members of the Australasian Non-Hodgkin's Lymphoma Study Group (Chairman, Dr I. Cooper, Peter MacCallum Hospital, Melbourne).

material from 111 patients with poor histology NHL who were entered into a randomized prospective trial, and show that proliferative activity determined by the percentage of S phase cells is a significant prognostic indicator for long-term survival, whereas DNA aneuploidy is only weakly correlated with outcome.

MATERIALS AND METHODS

Study population

In 1979, the Australasian Non-Hodgkin's Lymphoma Study Group (ANHLSG) initiated a prospective randomized trial comparing the efficacy of AVTCP vs. ATCP vs. AVCP (A = adriamycin, V = vincristine, T = teniposide, C = cyclophosphamide P = prednisolone) in adults with poor histology non-Hodgkin's lymphoma. The results of this trial showed no significant difference in survival between the three arms [12].

Histological assessment was according to a modified Rappaport classification and only patients with diffuse large cell lymphoma (DLC), diffuse mixed cell lymphoma (DMC), diffuse poorly differentiated lymphocytic lymphoma (DPDL), nodular large cell lymphoma (NLC) and nodular mixed cell lymphoma (NMC) were eligible for study. Transitional variants were also recognized, e.g. mixed cell nodular becoming diffuse (MCT), large cell nodular becoming diffuse (LCT) and lymphocytic poorly differentiated nodular becoming diffuse (LPT). These histological subtypes were included in this study on the basis of the results of a previous ANHLSG randomized trial [13] which identified these subtypes as having a poor prognosis.

From this population group it was possible to obtain the paraffin blocks from the original lymph node biopsy together with survival data in 111 patients. The ploidy status and proliferative activity of the biopsy material and survival of these 111 patients form the basis of this report.

Measurement of cellular DNA content

The method used has been described in detail elsewhere [10] and has been shown to provide reliable results from paraffin blocks up to 10 years old [14]. Briefly, 30 μm sections were dewaxed, rehydrated and a nuclear suspension then produced by digestion with an acidic pepsin solution. The nuclei were then stained with the DNA specific fluorochrome 4',6-diaminido-2-phenyl indole-dihydrochloride (DAPI, Boehringer Mannheim, F.R.G.), and cellular DNA content measured using an ICP22 flow cytometer (Ortho Instruments, Westwood, MA), the results being expressed as frequency distribution histograms.

All histograms had a peak corresponding to the DNA content of G_0/G_1 diploid cells, but in some histograms a second peak was observed, indicating a clonal abnormality of DNA content—'DNA aneuploidy'. The degree of DNA aneuploidy was expressed as DNA index, which is the ratio of aneuploid DNA to diploid DNA content. Because the method used here does not allow the use of an internal standard to identify with certainty the diploid peak, we adopted an accepted convention which assumed that in DNA aneuploid histograms the peak with the lower DNA content was diploid [9]. A small minority of tumours may have been hypodiploid, but erroneously assigned a DNA index of greater than 1.0.

In addition to DNA index, the percentage of cells in S phase was estimated planimetrically using a computer program [15].

Statistical analysis

Data were analysed using standard non-parametric procedures. Survival analysis was performed with BMDP statistical software [16]. Kaplan–Meier survival distributions were obtained with P1L which also tests the equality of survival curves using the Breslow test statistic. Cox model proportional hazards regression analysis for the identification of independent prognostically significant covariates was performed with P2L. The only valid endpoint for the purposes of this study was death attributable to lymphoma, all other patients being censored at the date of last follow up or the date of death from other causes.

RESULTS

1. *Cytometry*

The clinical data on the 111 patients together with cytometry results are detailed in Table 1. The mean coefficient of variation of the diploid G_1 peak, which is a measure of resolving power, was 4.9 ± 1.0 S.D.

(a) *Ploidy status.* Of the 111 patients, 44 (40%) showed DNA aneuploidy, with clustering around a near diploid and tetraploid mode (see Fig. 1). In order to exclude the possibility that some of these tetraploid peaks were artefactual, due to cell doublets or a prominent diploid G_2/mitosis peak, peaks were only considered to be near tetraploid G_1 if the number of cells exceeded 20% of the total and there was no evidence of a triploid peak equivalent to DNA index = 1.5. Three patients (3%) had multiple aneuploid populations. Of 57 patients with diffuse large cell lymphoma (the largest single histological group) 22 (39%) had aneuploid populations. There was no correlation between the presence of aneuploidy and stage of disease, age, sex or histology.

Table 1.

	Ploidy			S phase (diploid tumours only)	
	Diploid	Aneuploid	Multiploid	< 10%	> 10%
Sex					
Male	38	28	2	14	17
Female	29	13	1	15	10
Age					
Under 50	16	13	—		
50+	51	28	3		
Stage					
I	5	6	—	2	3
II	6	4	1	3	3
III	11	5	—	6	3
IV	45	26	2	18	18
Histology					
DLC	35	21	1	8	23
DPDL	5	5	1	3	—
DMC	3	0	—	2	1
MCT	9	3	—	6	1
LPT	4	2	1	3	1
NMC	8	3	—	5	1
LCT	2	5	—	1	—
NLC	1	2	—	1	—
Response					
C.R.	30	21	1	14	9
P.R.	23	12	1	10	11
I.D.	5	6	1	2	2
N.A.	9	2	—	3	5
Survival					
Alive	28	13	1		
Dead	36	26	2		
Lost to follow up	3	2	—		

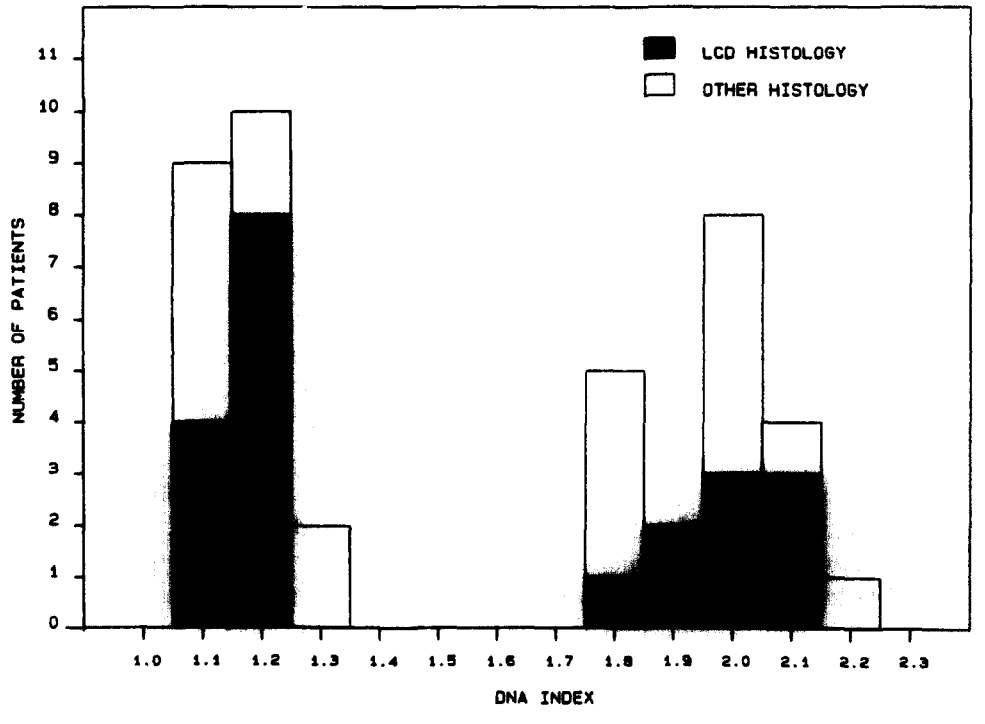

Fig. 1. *Frequency distribution of DNA index in* 44 *aneuploid lymph node specimens.*

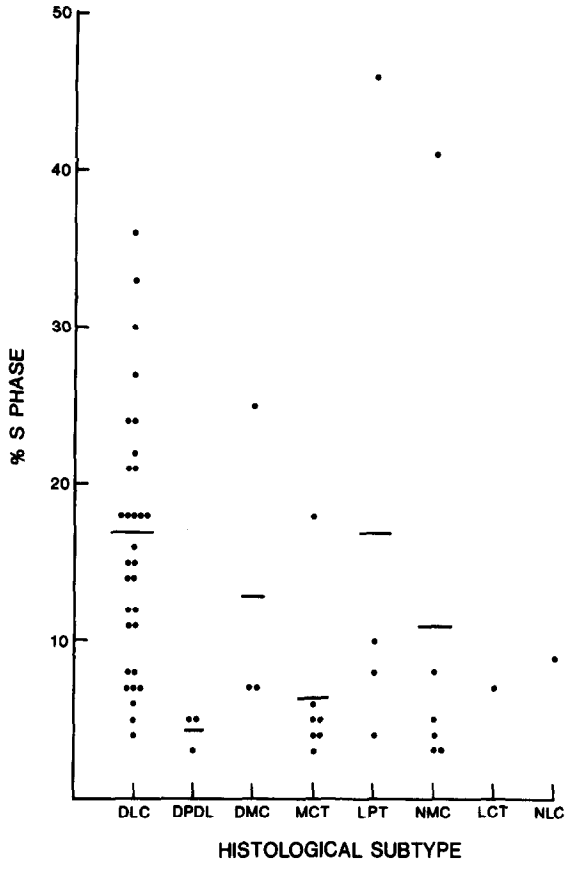

Fig. 2. S phase value from 56 diploid lymph node specimens according to histological subtype. DLC = diffuse large cell lymphoma; DPDL = diffuse poorly differentiated lymphocytic lymphoma; DMC = diffuse mixed cell lymphoma; MCT = mixed cell nodular becoming diffuse; NM = nodular mixed cell lymphoma; LCT = large cell nodular becoming diffuse; NLC = nodular large cell lymphoma. Bars indicate mean values.

(b) *S phase estimation.* A total of 64 histograms were considered to yield a reliable S phase estimate, a proportion not dissimilar to that reported from other series [9, 17]. Common problems which precluded valid estimation of % S phase in the remaining 47 patients were poor resolution due to fixation in Bouin's fluid (seven histograms) and technical problems with a new computer system which in 16 instances resulted in grossly unreliable S phase estimates particularly for DNA aneuploid tumours. With DNA aneuploid tumours additional problems were the presence of multiple aneuploid stemlines (3), excessive cellular debris (8) and overlap with a dominant diploid population (9). Of the 64 evaluable histograms 56 were diploid and only eight aneuploid. The S phase activity of the 56 diploid tumours gave a range of 3–46% with a mean of 10%. When analysed according to histology the samples from patients with diffuse large cell lymphoma had the highest mean (17%) with a range of 11–36% (see Fig. 2).

2. *Cytometry and survival*

(a) *Ploidy and survival.* Patients were allocated to one of three different treatment arms, but the survival curves based on treatment were not significantly different in our series or indeed in the entire clinical trial [12]. Although there was a trend for patients with diploid tumours to show a superior survival (median survival being 59 months compared to 25 months for DNA aneuploid tumours), this was not statistically significant ($P = 0.23$).

Figure 3 shows the survival curves for diploid and aneuploid tumours within the whole group of unfavourable histology NHL, and Fig. 4 shows the corresponding curves for patients with diffuse large cell lymphoma, with median survivals of 25 months for both diploid and aneuploid samples ($P = 0.89$).

The survival curves for patients with DNA index 1.1–1.3 and 1.8–2.2 were also not significantly different (data not shown).

(b) *S phase estimation and survival.* Although aneuploidy was not of significant prognostic value, the estimation of S phase was highly significant with respect to survival. For diploid patients with a calculable S phase, those patients with high proliferation activity, i.e. above the mean of 10%, had a significantly worse survival than those less than or equal to 10% ($P < 0.011$). This is shown in Fig. 5. Inclusion of the S phase estimates from the eight aneuploid histograms gave very similar results ($P < 0.011$). Figure 6 indicates the same analysis in patients with diffuse large cell histology and indicates a similar result. In this histological subtype with a high proliferative activity, patients with an S phase value above 19% had a significantly worse survival than those less than or equal to 19% ($P < 0.009$).

To demonstrate the independent prognostic significance of proliferative activity the data were analysed using Cox Model proportional hazards regression analysis. In the 56 patients with diploid histograms in whom complete data were available, *viz* age, histology, stage, therapy, S phase (greater or less than 10%), ploidy and survival, only S phase ($P = 0.012$) was highly significant as a prognostic factor.

DISCUSSION

In this study we have confirmed the feasibility of performing flow cytometric analysis on archival pathological material and correlating this with a randomized prospective trial in patients with poor histology NHL. The ploidy status and proliferative characteristics derived from this study are in general agreement with previous studies in this area which have employed either flow cytometry [9, 18, 19] or labelling studies using tritiated thymidine uptake

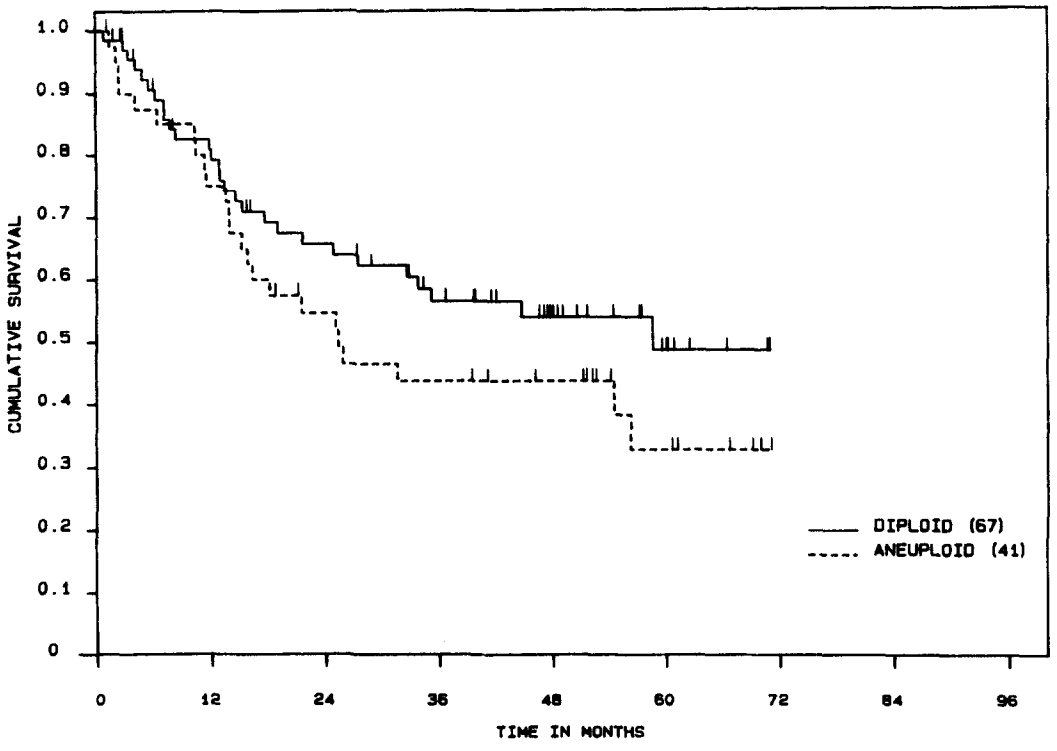

Fig. 3. Survival of patients with poor histology NHL according to ploidy.

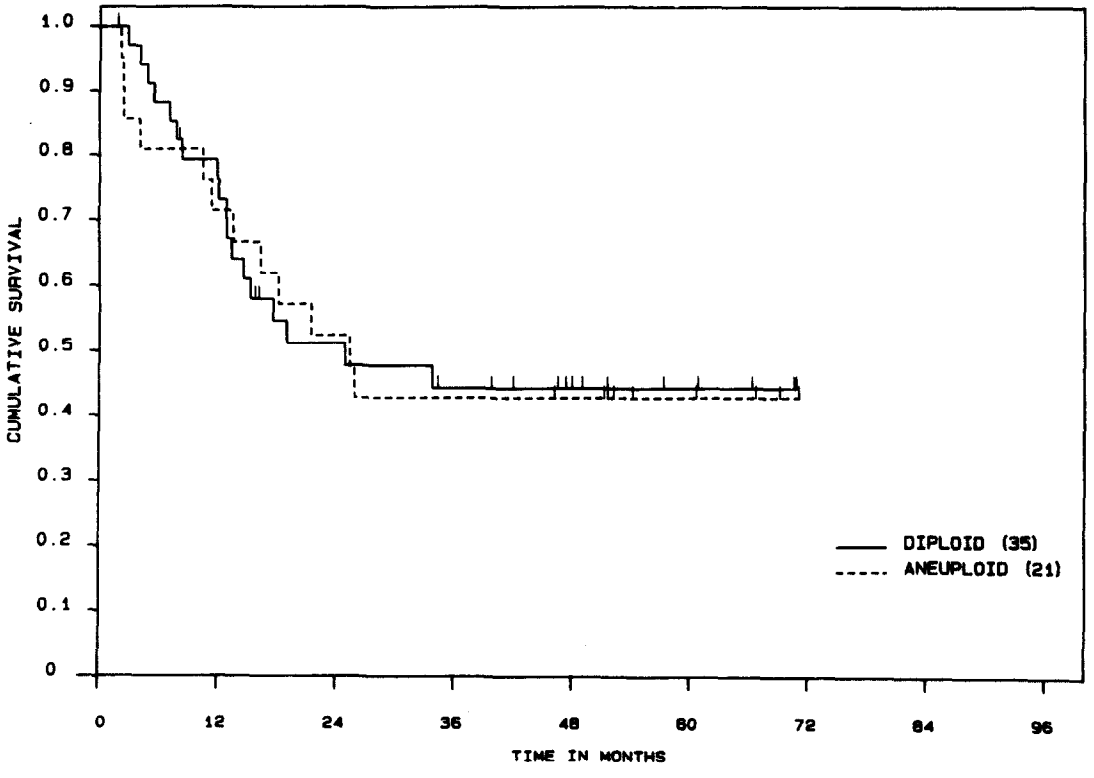

Fig. 4. Survival of patients with diffuse large cell lymphoma according to ploidy.

[7, 17] by fresh tissue. Indeed a recent report by Bauer *et al.* [20], using archival material, has documented very similar results in 50 patients with diffuse large cell lymphoma.

It is important to realise, however, that there remain technical problems in the use of paraffin-embedded material in flow cytometric determination of DNA index and S phase. The resolution obtained here (mean c.v. of G_1 peak = 4.9%) is less than would be expected using fresh unfixed tissue in our hands, and may well have led to near diploid clones being overlooked. A small proportion of human tumours are hypodiploid, but because an internal biological marker cannot reliably define

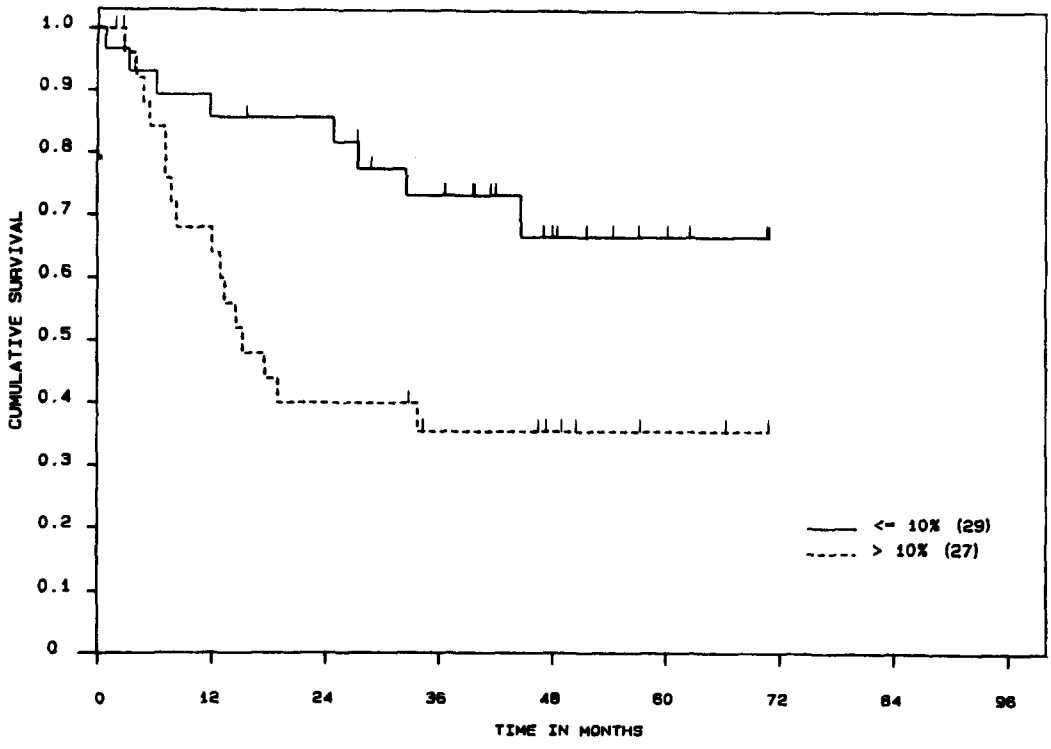

Fig. 5. Survival of patients with poor histology NHL, according to S phase.

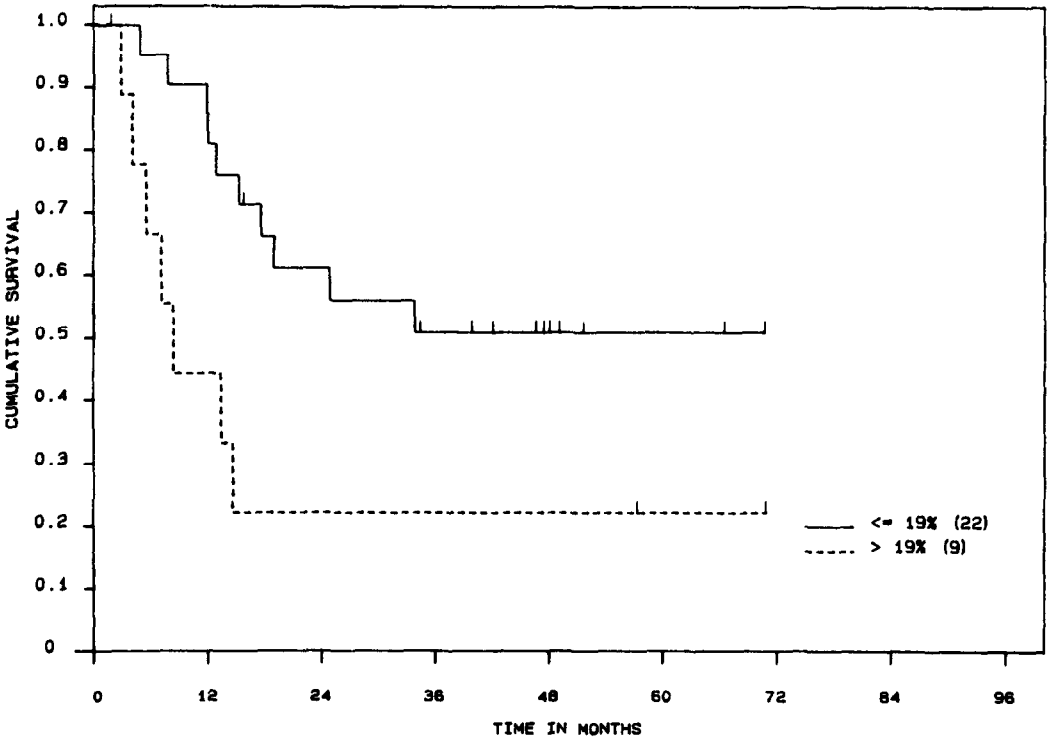

Fig. 6. Survival of patients with diffuse large cell lymphoma according to S phase.

the diploid G_1 peak, hypodiploid tumours cannot be identified using this technique. Using fresh material, Srigley *et al.* [9] found only four hypodiploid tumours in 185 samples from patients with NHL, and thus it is unlikely that this inability to detect hypodiploid tumours using the current technique would significantly influence the results.

Estimation of % S phase using DNA flow cytometry is most reliable for homogeneous populations of cells such as are obtained from tissue culture. Human tumour samples invariably contain a proportion of normal host cells, which are a major potential source of error due to overlap of their DNA histogram with that of the malignant cell population. For DNA aneuploid tumours the relative proportions of normal and malignant cells can

be approximated by comparing the numbers of cells in the two G_1 peaks, but the method used in the present study is incapable of discriminating between the two when the tumour is diploid. Therefore, although we examined the influence on prognosis of S phase % obtained for DNA diploid tumours in the present study, it is uncertain whether this is a true guide to tumour cell proliferation or an index of the proportion of nonmalignant cells present in the sample.

Multivariate analysis for survival identified only the proportion of S phase cells ($P = 0.012$) as an independent factor affecting survival, whereas aneuploidy did not significantly affect survival. It is also noteworthy that the stage of disease did not correlate with survival in our study, but in such a heterogeneous group of patients this is perhaps not too surprising.

In general, flow cytometry produces a rapid and reliable estimate of DNA index, which has been shown to correlate with modal chromosome number. It should be noted, however, that no information is obtained as to which particular chromosomes are abnormal, while the resolution is such that gains or losses of one or two intact chromosomes could be overlooked and traslocations or deletions are effectively undetectable. Thus, considerable chromosomal abnormalities can be harboured in DNA diploid tumour cells.

In this particular study, reliable S phase values were obtained in only 58% of samples. Despite this, in patients with poor-histology NHL, S phase is a more significant prognostic variable than age, sex, stage or histological subtype, and we suggest that the use of this technique in combination with other biological markers may be of benefit in the study of lymphoma and other neoplasms, and may point the way to consider the early introduction of more aggressive therapy in patients with high S phase values.

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