The biology of tumor growth in the non-Hodgkin's lymphomas. A dual parameter flow cytometry study of 220 cases.

S E Shackney, … , S J Occhipinti, J W Parker

J Clin Invest. 1984;73(4):1201-1214. https://doi.org/10.1172/JCI111306.

Research Article

Dual parameter flow cytometry studies (cell DNA content and electronic cell volume) were performed in 220 cases of non-Hodgkin's lymphoma. All cases were characterized as B or T cell malignancies, based on immunologic surface marker characteristics. Aneuploidy by flow cytometry was more common among the B cell lymphomas than among the T cell lymphomas, and was most common among the large B cell lymphomas and B cell lymphomas of intermediate size. Ploidy index distributions showed a prominent hyperdiploid peak, as well as tumor cell populations with near-tetraploid DNA contents. In serial studies, a decrease in ploidy index was observed in association with clinical and histologic transformation in one case. The highest S fractions were observed among the large and intermediate B cell lymphomas and among the aggressive T cell lymphomas. In clinical samples consisting of mixtures of diploid and aneuploid populations, the data on the aneuploid components could often be separated from other components of the mixture in multiparameter studies on the basis of the larger electronic cell volumes of the aneuploid cells. In each case, the aneuploid large cell component almost invariably had a higher S fraction than the residual component(s) of the mixture. Overall, the data are consistent with a model of clonal selection and clonal evolution in the lymphomas in which early cytogenetic abnormalities that involve little […]

The Biology of Tumor Growth in the Non-Hodgkin's Lymphomas

A Dual Parameter Flow Cytometry Study of 220 Cases

Stanley E. Shackney, Alexandra M. Levine, Richard I. Fisher, Peter Nichols, Elaine Jaffe, William H. Schuette, Richard Simon, Charles A. Smith, Sandra J. Occhipinti, John W. Parker, Jeffrey Cossman, Robert C. Young, and Robert J. Lukes

Cell Kinetics Section, Clinical Pharmacology Branch, Medicine Branch, and Biometric Research Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205; Adult Hematologic Neoplasia Service and Department of Pathology, University of Southern California Medical Center, Los Angeles, California 90033; Laboratory of Pathology, Division of Cancer Biology and Diagnosis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205; Applied Clinical Engineering Section, Biomedical Engineering and Instrumentation Branch, National Institutes of Health, Bethesda, Maryland 20205; Division of Medical Oncology, Allegheny General Hospital, Pittsburgh, Pennsylvania 15212

Abstract. Dual parameter flow cytometry studies (cell DNA content and electronic cell volume) were performed in 220 cases of non-Hodgkin's lymphoma. All cases were characterized as B or T cell malignancies, based on immunologic surface marker characteristics. Aneuploidy by flow cytometry was more common among the B cell lymphomas than among the T cell lymphomas, and was most common among the large B cell lymphomas and B cell lymphomas of intermediate size. Ploidy index distributions showed a prominent hyperdiploid peak, as well as tumor cell populations with near-tetraploid DNA contents. In serial studies, a decrease in ploidy index was observed in association with clinical and histologic transformation in one case. The highest S fractions were observed among the large and intermediate B cell lymphomas and among the aggressive T cell lymphomas. In clinical samples consisting of mixtures of diploid and aneuploid populations, the data on the aneuploid components could often be separated from other components of the mixture in multiparameter studies on the basis of the larger electronic cell volumes of the aneuploid cells. In each case, the aneuploid large cell component almost invariably had a higher S fraction than the residual component(s) of the mixture. Overall, the data are consistent with a model of clonal selection and clonal evolution in the lymphomas in which early cytogenetic abnormalities that involve little or no change in total cell DNA content are followed by cell tetraploidization that is associated with cytogenetic instability and chromosome loss over the course of time.

Address reprint requests to Dr. Shackney, Division of Medical Oncology, Allegheny General Hospital.

Received for publication 31 May 1983 and in revised form 22 December 1983.

J. Clin. Invest.

0021-9738/84/04/1201/14 $1.00
Volume 73, April 1984, 1201–1214

Introduction

In recent years it has come to be appreciated that there are important interrelationships among histopathologic features, immunologic properties, and cytogenetic abnormalities in the non-Hodgkin's lymphomas, and that all of these factors bear on the clinical behavior of these malignancies.

Large-cell lymphomas tend to be aggressive clinically (1–4) and they often respond well to therapy, with relatively high proportions of durable complete remissions (3–7). Although small-cell lymphomas are also responsive to treatment, durable complete remissions are not the rule (5, 8). In one recent study it was shown that, among the large-cell lymphomas, the majority of B cell lymphomas respond favorably to treatment, whereas the majority of large T cell lymphomas are refractory (9).

Cytogenetic abnormalities are common in the non-Hodgkin's lymphomas (10–27) and specific cytogenetic abnormalities are often found in association with specific histopathologic subtypes of lymphoma. Small-cell lymphomas often undergo histologic transformation to large-cell lymphomas during the course of disease (28–30). In serial studies in individual patients, it has been shown that histologic transformation is often accompanied by the appearance of new cytogenetic abnormalities (19, 20, 25–27) and/or changes in cell DNA content (19, 27).

The technique of flow cytometry can provide rapid, quantitative measurements of cell DNA content, cell volume, and immunologic surface markers on a cell-by-cell basis. Therefore, this technique could be especially useful in elucidating the biology of tumor growth in the non-Hodgkin's lymphomas, and its relation to cell morphology, immunologic features, and cell DNA content abnormalities in this group of diseases.

A number of flow cytometry studies in the non-Hodgkin's lymphomas have been published in which DNA histograms were obtained alone, or in combination with other measurements (31–39). However, in most of these studies, the number of patients studied was relatively small, and substantial differences in methods among different laboratories have precluded meaningful comparisons or the pooling of data from different studies for analysis.

In this paper we report the results of dual parameter flow cytometry studies in 220 patients with non-Hodgkin's lymphoma. Paired measurements of DNA content and electronic cell volume were obtained on each cell in every patient. Immunologic surface marker characteristics were determined separately in each case. Our studies demonstrate biological differences between the B and T cell lymphomas, and among different subtypes within each group. Our studies provide new information regarding clonal selection and clonal evolution in the non-Hodgkin's lymphomas, and suggest possible mechanisms for the generation of genetic diversity in individual tumors.

Methods

Clinical samples

Clinical samples were obtained from patients with non-Hodgkin's lymphoma seen at the National Cancer Institute in Bethesda, MD, and at the University of Southern California Medical Center in Los Angeles, CA, and surrounding hospitals, from 1977 through 1982. Portions of each specimen were prepared for conventional histopathological examination, for determination of immunologic cell surface markers, and for flow cytometry, respectively. Clinical specimens consisted largely of lymph nodes obtained by excisional biopsy, and also included spleen, soft tissue masses, pleural fluid, involved bone marrow, and leukemic peripheral blood. Basic specimen collection and processing techniques were essentially similar at both institutions and are described elsewhere in greater detail (40, 41).

Histopathological diagnoses were made in accordance with both the Rappaport classification (42) and the Lukes-Collins classification (43) by each of the pathologists at their respective institutions (Dr. Jaffe and Dr. Cossman at the National Cancer Institute and Dr. Lukes and Dr. Nichols at the University of Southern California). Immunologic methods for distinguishing B and T cell lymphomas are described elsewhere in detail (44–48).

Table I. Criteria for Aneuploidy

1. Multiple G_1 peaks
2. Single G_1 peak normal peak position, increased CV*
3. Single G_1 peak, abnormal peak position, normal CV

* CV, coefficient of variation.

Flow cytometry studies

Monodispersed tumor cell suspensions were fixed in 70% cold ethanol for flow cytometry, and stored at 4°C. Both tumor samples and normal lymphocyte control samples were incubated in fixative at 37°C for 2 h before staining to eliminate potential staining artifacts (49, 50).

Flow cytometry studies were carried out on mithramycin-stained cells (mithramycin, 100 μg/ml and $MgCl_2$, 15 mM), using a Los Alamos Flow Cytometer, and data were analyzed using software developed at Los Alamos Scientific Laboratory, Los Alamos, NM (51).

Normal human lymphocytes were used as an external biological diploid reference for each clinical sample. Before each clinical sample run, amplifier gain settings were adjusted so that the mean peak channel of normal human diploid lymphocytes fell in channel 60 on a 256-channel scale (D_1). Then, the clinical sample was analyzed without adjustment of amplifier gain settings. After the clinical sample run, the lymphocyte reference cells were reanalyzed, without adjustment of amplifier gain settings (D_2). If the positions of the G_1 peaks of D_1 and D_2 did not agree within one channel (<2%), the sample run was rejected.

Paired cell-by-cell measurements of cell DNA content and electronic cell volume were carried out on all samples in separate runs, as described previously (34).

Detection and quantitation of aneuploidy by flow cytometry. Three potential criteria for aneuploidy by flow cytometry are noted in Table I. In our studies, virtually all tumor samples were found to contain at least one peak whose position corresponded with that of the external diploid reference standard (see Results). Thus, we did not consider a shift in the position of a single G_1 peak with a normal coefficient of variation (criterion 3, Table I) to be a valid criterion for aneuploidy by flow cytometry.

The coefficient of variation (CV)[1] of the G_1 peak of normal lymphocytes ranged between 2 and 4% in all of our studies, as did those of most diploid clinical samples. Clinical samples with single diploid G_1 peaks with coefficients of variation exceeding 4% were rerun to confirm that the CV was high relative to that of normal lymphocytes. When the CV exceeded 4.5% in multiple runs, criterion 2 for aneuploidy (Table I) was considered to have been met.

The presence of at least two G_1 peaks in a clinical sample (criterion 1, Table I) was considered to be indisputable evidence of aneuploidy by flow cytometry.

The degree of aneuploidy was expressed quantitatively by the ploidy index, where ploidy index = channel number of aneuploid G_1 peak/mean or modal channel number of diploid G_1 peak.

In most samples with a single G_1 peak with a high CV, the presence of a second peak could be detected by mathematical analysis (52), and

1. *Abbreviations used in this paper:* CLL, chronic lymphocytic leukemia; CV, coefficient of variation; FCC, follicular center cell.

the ploidy index could be determined. In cases where the second peak could not be separated mathematically, the ploidy index was assigned a nominal value of 1.02.

Determination of the fraction of cells in S phase. The fraction of cells in S was determined by the method of Ritch et al. (53). In diploid-aneuploid mixtures that could be resolved either graphically or by dual parameter analysis, S fractions were calculated separately for each subpopulation. When a mixture could not be resolved, the S fraction was calculated for the combined population.

Analysis of dual parameter data. Comparisons of electronic cell volumes among different lymphomas were performed on data that were gated on G_1 cells in each tumor in order to eliminate the confounding effects of large cells in S and G_2 phases, as described previously (34).

In mixed samples containing diploid and aneuploid cell populations with overlapping DNA histograms, dual parameter studies were helpful in separating the data on the two populations on the basis of electronic cell volume. The method for separating the diploid and aneuploid components of a cell mixture is illustrated in Figs. 1 and 2. Fig. 1 *A* shows a projection of the bivariate frequency distribution of electronic cell volume and cell DNA content in an experimental tumor cell population. The relationship between cell volume and cell DNA content is not readily appreciated from this view. The boundaries of parallel horizontal slices taken through this surface (Fig. 1 *B*) form a contour map when viewed from above (Fig. 1 *C*). The relationship between cell size and DNA content is readily appreciated from the contour map. Fig. 2 A_1 shows a contour map of the bivariate frequency distribution of cell volume and cell DNA content in a patient with a large B cell lymphoma. The contour map reveals the presence of two aneuploid populations in addition to a diploid population. The data can be separated using computer techniques (51), producing separate files for the large aneuploid population (Fig. 2 C_1) and the residual diploid-aneuploid mixture (Fig. 2 B_1). The data in each file can then be analyzed separately (Fig. 2 B_2, B_3, C_2, and C_3). Additional examples of diploid/aneuploid data separation are given elsewhere (34, 56).

Data presentation and analysis

In the present study, cells were divided into B and T cell lymphomas. The B cell lymphomas were each further subdivided by morphologic cell size characteristics (Table II). The T cell cases were divided into aggressive and indolent subgroups (Table II). Corresponding histopathologic diagnostic categories by the Lukes-Collins system and the Rappaport system and their relation to the International Working Formulation (57) are given in Table II. The aggressive T cell cases consisted predominantly of convoluted lymphocytic (lymphoblastic) lymphoma (21 of 30 cases), and also included six cases of T cell immunoblastic sarcoma and three cases of mycosis fungoides/Sezary syndrome.

For comparing proportions, the Fisher exact test or contingency χ^2 test was used. For comparing quantitative measurements (e.g., S fraction) among groups of samples, the Kruskal-Wallis nonparametric analysis of variance was used (54). Pairwise comparisons between two histologic groups were performed only if the overall heterogeneity test among all histologic groups was statistically significant at the 5%-level. All statistical significance levels are of the two-sided type.

Results

Electronic cell volume distributions of G_1 cells in the lymphomas

Differences in mean cell volume among different tumors could be due either to intrinsic cell size differences that are unrelated to cell position in the cell cycle, or to differing proportions of larger S and G_2 cells in different tumors. Intrinsic differences in cell size among the lymphomas can be assessed separately from cell cycle-dependent differences in cell size by restricting the comparison of distributions of mean electronic cell volume to G_1 cells only among different samples, as described previously (34). The results are summarized in Table III. The G_1 cells of the large B cell lymphomas and B cell lymphomas of intermediate cell size were each larger than those of all the small B cell lymphomas. The largest mean G_1 cell volumes in the series were observed among the large B cell lymphomas. Large G_1 cell volumes were also observed among the aggressive T cell lymphomas, but the difference between this group and the indolent T cell lymphomas was not statistically significant.

Detection of aneuploidy by flow cytometry

All clinical samples contained at least one G_1 peak whose position corresponded with that of the diploid lymphocyte reference standard. In Table IV the ploidy indices of nonneoplastic lymph nodes with reactive hyperplasia are compared with the ploidy indices of all lymphomas containing a single G_1 peak with CV $< 4.5\%$, and all other lymphomas with either single G_1 peaks and broad CVs or multiple G_1 peaks that were at least partially separable. In each and every case of lymphoma there was a G_1 peak that corresponded to the normal diploid reference. Among

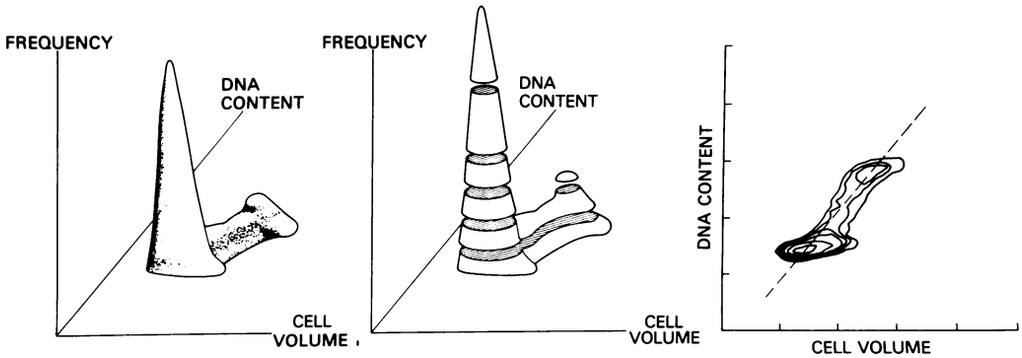

Figure 1. (*Left*) Schematic representation of the bivariate frequency distribution of cell DNA content and electronic cell volume in murine sarcoma 180 cells. (*Middle, right*) Generation of a contour map from the bivariate distribution. For discussion, see text.

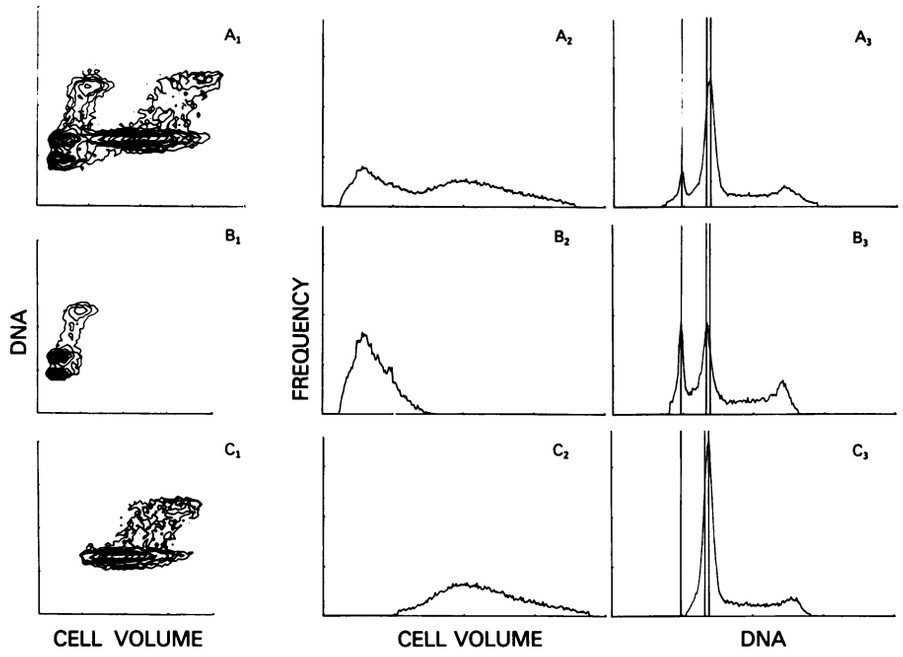

Figure 2. Illustration of the results of computer-aided separation of data in diploid-aneuploid mixtures in a patient with a large B cell lymphoma. (A_1) Contour map of the bivariate frequency distribution of cell DNA content and electronic cell volume in the original diploid-aneuploid mixture. Contour map shows a diploid population and two aneuploid populations. (A_2) Electronic cell volume distribution shows the presence of two populations that differ in size from each another. (A_3) DNA histogram for the original diploid-aneuploid mixture shows a small diploid component and a broad, fused peak containing two aneuploid populations. (B_1) Population containing diploid component and aneuploid component with lower ploidy index separated on the basis of size. (B_2) Electronic cell volume distribution of population with diploid component and aneuploid component with lower ploidy index. (B_3) DNA histogram of population with diploid component and aneuploid component with lower ploidy index. (C_1) Population with aneuploid component with higher ploidy index separated from residual diploid-aneuploid mixture on the basis of size. (C_2) Electronic cell volume distribution of large cell aneuploid component. (C_3) DNA histogram of large cell aneuploid component.

the lymphomas with a single G_1 peak and a normal CV, the position of the G_1 peak fell within two channels of the external diploid reference in 95% of cases. Among the lymphomas with G_1 peaks with broad CVs or multiple peaks, the position of the G_1 peak closest to the origin fell within three channels of the external diploid reference in 95% of cases. The slightly greater variability in G_1 peak position in the latter group of cases is due to distortions produced by the proximity of additional aneuploid G_1 peaks. The variances in ploidy indices of normal cells and the diploid peak in tumor samples were compared by Levene's test and the Ansari-Bradley test for equality of variance. Both tests gave $P > 0.30$, indicating that there is little evidence for believing that the variances are unequal.

Thus, the data do not support the use of a shift in the position of a single G_1 peak with normal CV as a criterion for aneuploidy by flow cytometry (criterion 3, Table I).

The relative stability of diploid G_1 peak position in our lymphoma samples can be attributed both to the inherently low intersample staining variability that is associated with mithramycin (49) and to the elimination of the artifact of time-dependent hyperchromatism by the incubation of samples at 37°C before analysis (50).

The frequencies of aneuploidy by flow cytometry using criteria 1 and 2 (Table I) are given in Table V. Aneuploidy by criterion 2 was much less common than by criterion 1 (13 of 70 aneuploid cases by criterion 2 vs. 57 of 70 aneuploid cases by criterion 1). The greatest number of aneuploid cases by criterion 2 was observed among the B cell lymphomas of intermediate size. Overall, the B cell lymphomas were more frequently aneuploid than the T cell lymphomas ($P < 0.05$). The large B cell lymphomas and B cell lymphomas of intermediate cell size were both more frequently aneuploid than the small B cell lymphomas ($P < 0.05$). Overall, the frequency of aneuploidy among the T cell lymphomas was relatively low (8 of 46 cases, or 17%), and the difference in the frequencies of aneuploidy between the two T cell subgroups was not statistically significant. Three cases of mycosis fungoides/Sezary syndrome were included among the aggressive T cell lymphomas. Of these, one exhibited aneuploidy by flow cytometry.

The frequencies of aneuploidy among cases studied at diagnosis or before treatment and those studied at the time of relapse were 29% (43 of 150) and 39% (24 of 62), respectively. The difference was not statistically significant.

Samples were obtained from multiple lymphoma-bearing tissue sites in 16 patients either on the same day (14 of 16) or within a 2-wk period (2 of 16). Tissue sites included lymph node, spleen, bone marrow, and peripheral blood, grouped in various combinations. In 11 cases, mostly small B cell lymphomas (7 of 11) and small T cell lymphomas (4 of 11), only diploid cells were present at all tissue sites. In five cases, there were differences between tissue sites with respect to presence or absence of aneuploidy, the number of aneuploid populations, and/or the degree of aneuploidy. These cases are summarized in Table VI. All of these were B-cell lymphomas, and all exhibited diffuse histology. Aneuploidy was detectable in only one of two sites in three patients, and different aneuploid populations were

Table II.

Biological grouping	Lukes-Collins classification	Modified Rappaport classification	Working formulation
B cell lymphomas			
Large B cell lymphomas	Immunoblastic sarcoma (B cell) Large, noncleaved cell lymphoma, FCC*	Diffuse histiocytic lymphoma	High grade, large cell immunoblastic lymphoma Intermediate grade, large cell lymphoma
B cell lymphomas of intermediate cell size	Small, noncleaved cell lymphoma, FCC	Diffuse undifferentiated lymphoma	High grade, small, noncleaved cell lymphoma
Small B cell lymphomas	Large, cleaved cell lymphoma, B cell type, FCC Small lymphocytic B-cell lymphoma	Nodular and diffuse mixed lymphoma Intermediate lymphocytic lymphoma Nodular and diffuse, lymphocytic poorly differentiated lymphoma	Intermediate and low grade, follicular and diffuse mixed cell lymphoma Intermediate and low grade, follicular & diffuse large cleaved cell lymphoma Intermediate and low grade, follicular and diffuse small cleaved cell lymphoma
Small B cell lymphomas with maturation/ differentiation	B cell type, small lymphocytic B cell lymphoma	Well-differentiated lymphocytic lymphoma, CLL‡	Low grade, small lymphocytic lymphoma consistent with CLL
	B cell type, plasmacytoid lymphocytic lymphoma	Well-differentiated lymphoma with plasmacytoid features	Low grade, plasmacytoid small lymphocytic lymphoma
T cell lymphomas			
Aggressive T cell lymphoma	Immunoblastic sarcoma (T cell)	Diffuse histiocytic lymphoma Diffuse mixed lymphoma	High grade, lymphoblastic lymphoma Intermediate grade, diffuse mixed lymphoma
	T cell type, convoluted lymphocytic lymphoma	Lymphoblastic lymphoma diffuse, poorly differentiated lymphoma	High grade, lymphoblastic lymphoma Intermediate grade, diffuse mixed cell
	T cell type, Sezary/mycosis fungoides (cerebriform)	Mycosis fungoides/Sezary syndrome	High grade, mycosis fungoides
Indolent T cell lymphoma	T cell type, small lymphocytic lymphoma	Lymphocytic, well-differentiated lymphoma, CLL	Low grade, small lymphocytic lymphoma

* FCC, follicular center cell.
‡ CLL, chronic lymphocytic leukemia.

observed at different sites in two patients. In two patients, two different aneuploid populations were present at the same site. In patient B.G., the lymph node and peripheral blood each exhibited two aneuploid populations, only one of which was common to both sites. The data suggest that clonal selection and clonal evolution in the B cell lymphomas may proceed independently at different tissue sites, despite hematogenous dissemination. This raises the possibility that the sampling of only one of several potential sites of tumor involvement in a given patient may bias the histopathologic diagnosis and classification of lymphoma in that patient. It may also introduce an element of uncertainty in the interpretation of sequential sampling studies in individual patients.

Sequential flow cytometry studies were performed in six

Table III. Mean G_1 Cell Electronic Cell Volume

	Mean G_1 cell volume (channel No.) ±SE	Cases
		n
B cell lymphomas		
Large B cell lymphomas	64±6	25
B cell lymphomas of intermediate size	54±4	20
Small B cell lymphomas	43±1	85
Small B cell lymphomas with maturation/ differentiation	46±2	44
T cell lymphomas		
Aggressive cell lymphomas	48±3	30
Indolent T cell lymphomas	47±2	16

patients, over a period ranging from 2 to 19 mo. In four patients with T cell lymphoma-leukemias and in one patient with plasmacytoid B cell chronic lymphocytic leukemia (CLL), only diploid cells were present in the peripheral blood throughout the entire observation period. In one case (patient E.R., Table VI), a repeat lymph node biopsy was obtained 18 mo after the first, at a time when her disease showed clinical progression. A histopathologic diagnosis of nodular histiocytic lymphoma (Rappaport) or large noncleaved lymphoma (Lukes-Collins) was made at that time. Flow cytometry studies of cells obtained from this lymph node revealed a new aneuploid population with a ploidy index of 1.03.

Multiple aneuploid populations were present in the same tissue in eight cases (Table VII). Six of the eight cases were B cell tumors with diffuse histologies; of these, five of the six were large B cell lymphomas or B cell lymphomas of intermediate cell size. In all the B cell cases, the ploidy indices of both aneuploid components were in the hyperdiploid range; none were in the near-tetraploid range. Among the B cell cases, the ploidy indices of the two aneuploid components in a given tissue were similar; in all cases the two values differed by <10%.

Both T cell tumors with multiple aneuploid populations at the same site were classified as clinically aggressive. Unlike the B cell tumors, the T cell tumors exhibited a spread in ploidy index between the two aneuploid populations in each sample that ranged from near-diploid to hypotetraploid.

The overall distribution of ploidy indices of all aneuploid cell populations that were observed in the B and T cell lymphomas is shown in Fig. 3. Most populations were hyperdiploid, but some were near-tetraploid. A larger proportion of the aneuploid T cell populations (3 of 10, or 33%) had near-tetraploid indices than did the aneuploid B cell populations (5 of 68, or 7%), but the difference was not statistically significant.

The ploidy index distributions for aneuploid B cell populations studied at diagnosis and aneuploid B cell populations studied at relapse are shown in Fig. 4. All of the near-tetraploid B cell populations were observed at the time of diagnosis (Fig. 4 *A*); all of the aneuploid B cell populations studied at the time of relapse were hyperdiploid (Fig. 4 *B*). The overall number of near-tetraploid B cell populations was small, however, and the differences between the ploidy index distributions at diagnosis and relapse were not found to be statistically significant.

Fraction of cells in S phase

The distributions of S fractions among the lymphomas are shown in Fig. 5. The B cell lymphomas of intermediate cell size (small, noncleaved lymphoma, Lukes-Collins; undifferentiated lymphoma, Rappaport) exhibited the broadest range of values, including one tumor sample with an S fraction of 0.5. The large and intermediate B cell lymphomas, in turn, had higher S fractions than the small B cell lymphomas or the indolent T cell lymphomas ($P < 0.05$), but the S fractions of the large and intermediate B cell lymphomas were comparable to those of the aggressive T cell lymphomas. The S fractions of the aggressive T cell lymphomas were significantly higher than those of the indolent T cell lymphomas ($P = 0.05$).

In 24 samples with aneuploidy, S fractions could be calculated separately for the aneuploid and diploid component in each sample, either because there was minimal overlap in the S regions of the two (e.g., with near tetraploidy), or because the aneuploid cells were distinctly larger than the diploid cells in the sample in dual parameter studies (see Methods).

In nearly all cases, the S fraction of the aneuploid component was higher than that of the diploid component (Fig. 6), suggesting that the aneuploid cells had a proliferative advantage over the diploid cells in the mixture. In 7 of these 23 cases (●, Fig. 6), there were two aneuploid components in the mixture, of which only one was separable on the basis of electronic cell volume. In these seven cases, the "diploid" component actually consisted of a residual mixture of diploid and aneuploid cells. One such case is shown in Fig. 2. In these seven cases, the S phase fractions of the residual diploid mixture were higher than those observed in cases with pure diploid components, exceeding 0.1 in every instance (Fig. 6). In contrast, in 14 of 17 cases with a pure diploid component, the S fractions of these diploid components were <0.1.

Table IV. Variation of Diploid G_1 Peak Position in Normal Cells and in the Non-Hodgkin's Lymphomas

	Reactive lymph nodes	Lymphomas with a single G_1 peak	Other lymphomas
Number of cases	11	105	54
Mean ploidy index	0.997	0.999	1.005
Median ploidy index	0.995	0.999	1.002
Standard deviation	0.011	0.015	0.025
Standard error	0.003	0.001	0.003

Table V. Aneuploidy by Flow Cytometry in the Non-Hodgkin's Lymphomas

	Aneuploidy criterion 1		Aneuploidy criterion 2		Aneuploidy criteria 1 and 2	
	(n)	%	*(n)*	%	*(n)*	%
B cell Lymphomas					(62/174)	36
Large B cell lymphomas	(15/25)	60	(3/25)	12	(18/25)	72
B cell lymphomas of intermediate cell size	(9/20)	45	(4/20)	20	(13/20)	65
Small B cell lymphomas	(23/85)	27	(2/85)	2	(25/85)	29
Small B cell lymphomas with maturation/ differentiation	(5/44)	11	(1/44)	2	(6/44)	14
T cell Lymphomas					(8/46)	17
Aggressive T cell lymphomas	(4/30)	13	(3/30)	10	(7/30)	23
Indolent T cell lymphomas	(1/16)	7	(0/16)	0	(1/16)	7

It seems likely that in each of the seven separable cases with a residual diploid-aneuploid mixture, the S fraction of the true diploid component was lower than that observed in the residual mixture, and probably <0.1. By the same token, it is likely that the S fraction of the aneuploid component in the residual diploid-aneuploid mixture was higher than that observed in the residual mixture, and may have approached or exceeded the S fraction of the separable aneuploid component. Indeed, in one case the observed S fraction of the residual diploid-aneuploid mixture exceeded that of the separable aneuploid component (Fig. 6).

In Fig. 5, the S fractions of diploid samples, of samples with nonseparable diploid-aneuploid mixtures, and of the aneuploid components of separable diploid-aneuploid mixtures were combined. The data are presented separately for each category in Table VIII.

The large B cell lymphomas had the largest number of cases with separable aneuploidy, and within this biological grouping the cases with separable aneuploidy were more abundant than the nonseparable aneuploid cases. Among the large B cell lymphomas, the separable aneuploid cases had the highest S fractions, the nonseparable aneuploid large B cell lymphomas had intermediate S fractions, and the diploid cases had S fractions that were in the same range as cells from lymph nodes with reactive hyperplasia.

In general, the highest S fractions within a biological grouping were found in the separable aneuploid category, except for the

Table VI. Discordant Ploidy Indices in Different Tissue Sites in the Same Patient

Case no.	Patient	Biological grouping	Histopathological diagnosis, Lukes-Collins/Rappaport	Sample sources	Ploidy indices
1	L.S.	Large B cell lymphoma	Immunoblastic sarcoma, B cell/diffuse histiocytic lymphoma	Lymph node Spleen	1.02 Diploid
2	A.H.	B cell lymphoma of intermediate cell size	Small, noncleaved FCC B cell lymphoma/diffuse histiocytic lymphoma	Lymph node Spleen	1.02 1.23
3	B.G.	B cell lymphoma of intermediate cell size	Small, noncleaved FCC B cell lymphoma/diffuse poorly differentiated lymphoma	Lymph node Peripheral blood	1.07, 1.10 1.10, 1.19
4	A.S.	Small B cell lymphoma	Small, cleaved FCC B cell lymphoma/diffuse intermediate lymphoma	Lymph node Lymph node	1.03 Diploid
5	E.R.	Small B cell lymphoma with maturation/ differentiation	Plasmacytoid CLL/diffuse well-differentiated lymphoma with plasmacytoid features	Spleen Lymph node	1.17, 1.20 Diploid

Table VII. Multiple Aneuploid Populations at the Same Tissue Site

Case no.	Patient	Diagnosis Lukes-Collins/Rappaport	Tissue site	Ploidy indices
1	W.W.	Immunoblastic B cell sarcoma/diffuse histiocytic lymphoma	Spleen	1.37, 1.42
2	A.B.	Large noncleaved FCC B cell lymphoma/diffuse histiocytic lymphoma	Lymph node	1.06, 1.17
3	A.G.	Large noncleaved FCC B cell lymphoma/diffuse histiocytic lymphoma	Soft tissue	1.06, 1.10
4	A.E.*	Small noncleaved FCC B cell lymphoma/diffuse undifferentiated lymphoma	Lymph node	1.21, 1.21
5	B.G.	Small noncleaved FCC B cell lymphoma/diffuse poorly differentiated lymphoma	Lymph node Peripheral blood	1.07, 1.10 1.10, 1.19
6	E.R.	Plasmacytoid lymphocytic B cell lymphoma/diffuse well differentiated lymphocytic lymphoma with plasmacytoid features	Spleen	1.17, 1.20
7	A.A.	T cell type mycosis fungoides	Lymph node	1.02, 1.65
8	T.D.	T cell type, convoluted lymphocytic lymphoma/lymphoblastic lymphoma	Pleural fluid	1.02, 1.7

* Sample contained two aneuploid populations that differed from each other with respect to electronic cell volume, but were indistinguishable with regard to ploidy index.

B cell lymphomas of intermediate cell size, where all three categories exhibited comparably high S fractions (Table VIII). In general, within a given biological grouping the nonseparable aneuploid cases had S fractions that were smaller than those of the separable aneuploid cases, and were greater than the S fractions of the diploid cases, unless the S fractions of the diploid cases were also high.

It is likely that the intermediate S fractions of the nonseparable aneuploid cases (Table VIII) reflect the admixture in each sample of a diploid population with a low S fraction and a second population with a high S fraction.

Correlations among S fraction, electronic G_1 cell volume, and aneuploidy in individual tumors

The relations among S fraction, electronic cell volume of G_1 cells, and aneuploidy in individual tumors is shown in Fig. 7.

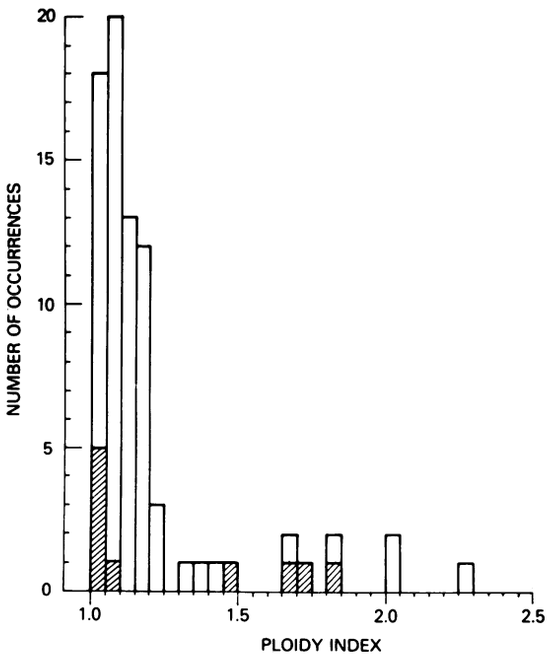

Figure 3. The distribution of ploidy indices among aneuploid cases in the non-Hodgkin's lymphomas. Shaded regions represent aneuploid T cell cases. Unshaded regions represent B cell cases.

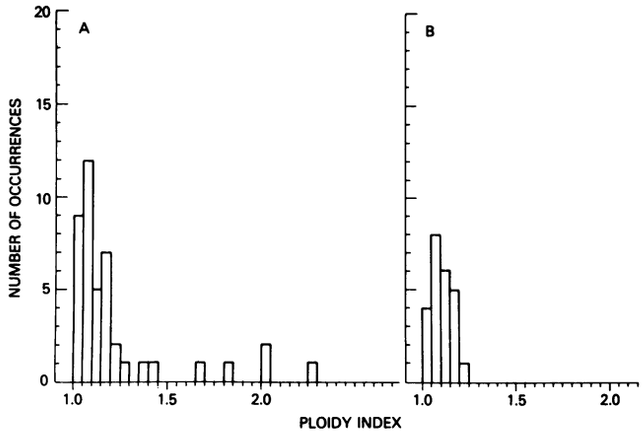

Figure 4. The distribution of ploidy indices among aneuploid B cell non-Hodgkin's lymphomas (*A*) before treatment and (*B*) at the time of relapse.

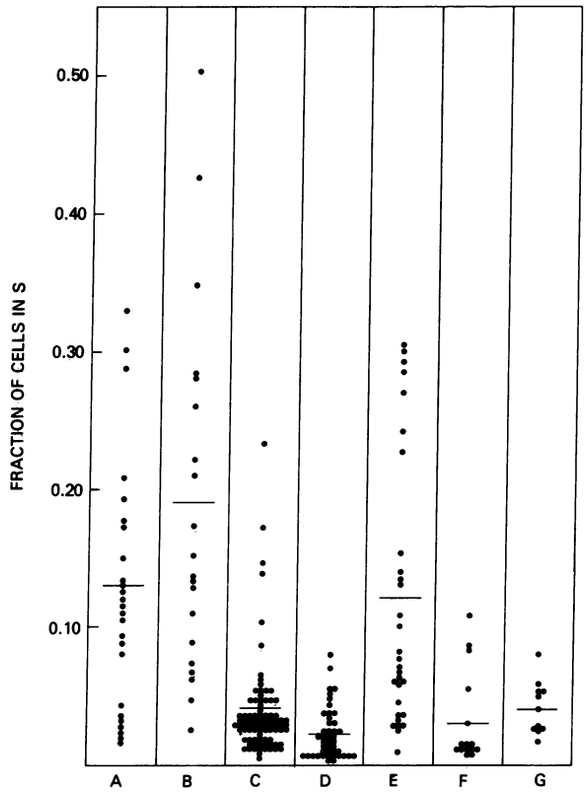

Figure 5. The distributions of S fractions among the non-Hodgkin's lymphomas by biological subgroup. (*A*) Large B cell lymphomas. (*B*) Intermediate B cell lymphomas. (*C*) Small B cell lymphomas. (*D*) Small B cell lymphomas with maturation/differentiation. (*E*) Aggressive T cell lymphomas. (*F*) Indolent T cell lymphomas. (*G*) Reactive lymph nodes.

There was a significant correlation between S fraction and the electronic cell volume of G_1 cells among the large B cell lymphomas (Fig. 7 A_1) (Pearson correlation coefficient 0.497, $P = 0.014$). The overwhelming majority of cases with high S fractions, large G_1 cells, or both were aneuploid (●, Fig. 7 A_1). Conversely, the majority of cases that were classified histopathologically as large B cell lymphomas, but had neither large G_1 cell volumes nor high S fractions, were diploid by flow cytometry.

In contrast, the cases classified as B cell lymphomas of intermediate cell size (Fig. 7 A_2) showed no significant correlation between S fraction and electronic cell volume of G_1 cells (Pearson correlation coefficient 0.038, $P = 0.88$). As in the large B cell lymphomas, the majority of aneuploid tumors had high S fractions, large mean G_1 volumes, or both. The vast majority of cells classified as small B cell lymphomas (Fig. 7 A_3) had both low S fractions and small G_1 cell volumes. Among the tumors with both high S fractions and large G_1 cell volumes, three of five, or 60%, were aneuploid. Among the mature/differentiated small B cell lymphomas (Fig. 7 A_4), nearly all had low S fractions, and, of course, there was no demonstrable correlation between S fraction and G_1 cell volume.

The aggressive T cell lymphomas (Fig. 7 B_1) exhibited both a relatively broad range of S fractions and a relatively broad range of G_1 cell volumes in comparison with the indolent T cell lymphomas (Fig. 7 B_2), but in neither group was there a clear correlation between S fraction and G_1 cell volume. Aneuploidy was relatively infrequent among the T cell lymphomas but, when present, it was likely to be observed in tumors with high S fractions, large mean G_1 cell volumes, or both.

Overall, large G_1 cell volume and/or high S fraction were associated with tumors that generally follow an aggressive clinical course (upper row, Fig. 7). Despite different frequencies of aneuploidy between the B and T cell lymphomas and among subgroups within each immunologic class, within each tumor subgroup it was the aneuploid tumors that were likely to exhibit the higher S fractions, the larger G_1 cell volumes, or both.

Discussion

Evaluation of the data

In the present study there were statistically significant differences in the frequencies of aneuploidy by flow cytometry between the B and T cell lymphomas, and among subgroups within the B cell lymphomas. We have also found statistically significant differences in G_1 cell volumes and in the fractions of cells in S phase among subgroups within the B and T cell lymphomas.

The large B cell lymphomas (immunoblastic sarcomas of B cell type and large noncleaved follicular center cell [FCC] B cell lymphomas, Lukes-Collins; diffuse histiocytic lymphomas, Rappaport) form a distinct group of cases with a high frequency of aneuploidy by flow cytometry (Table V), large G_1 cell volumes (Table III), high S fractions (Table VIII), and a statistically significant correlation between G_1 cell volume and S fraction in

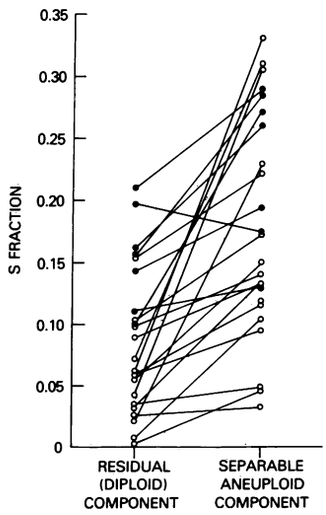

Figure 6. A paired comparison of S fractions in separable aneuploid components and their respective residual components. ○, cases containing a diploid component and a single separable aneuploid component; ●, cases containing a diploid component and two aneuploid components, only one of which was separable on the basis of electronic cell volume.

Table VIII. S Fractions in the Lymphomas in Relation to Presence/Absence and Separability of Aneuploid Populations

Biological grouping	Diploid		Mixed		Separable aneuploid		Total	
	Cases	Mean SF*±SE	Cases	Mean SF±SE	Cases	Mean SF±SE	Cases	Mean SF±SE
	n		*n*		*n*		*n*	
B cell lymphomas								
Large B cell lymphomas	7	0.06±0.02	7	0.10±0.03	11	0.18±0.03	25	0.13±0.02
B cell lymphomas of intermediate cell size	7	0.18±0.07	9	0.2±0.04	4	0.18±0.05	20	0.19±0.03
Small B cell lymphomas	60	0.03±0.003	21	0.04±0.009	4	0.11±0.05	85	0.04±0.004
Small B cell lymphomas with maturation/ differentiation	38	0.02±0.003	5	0.03±0.01	1	0.05	44	0.02±0.003
T cell lymphomas								
Aggressive T cell lymphomas	23	0.09±0.02	3	0.10±0.02	4	0.25±0.04	30	0.12±0.02
Indolent T cell lymphomas	15	0.02±0.007	1	0.11	0	—	16	0.03±0.008
Reactive hyperplasia	11	0.04±0.005	—	—	—	—	11	0.04±0.005

* SF, S fraction.

individual cases (Fig. 7). The B cell lymphomas of intermediate cell size (small noncleaved B cell lymphomas by Lukes-Collins; undifferentiated by Rappaport) are characterized by a high frequency of aneuploidy (Table V), intermediate G_1 cell volumes (Table III), and very high S fractions, but no significant correlation between G_1 cell volume and S fraction in individual cases. The small B cell lymphomas (Table II) are characterized by frequencies of aneuploidy that are almost half of those of the large and intermediate B cell lymphomas (Table V), small G_1 cell volumes (Table III), and low S fractions (Table VIII).

Among the T cell lymphomas, the overall frequency of aneuploidy by flow cytometry was relatively low in comparison with the B cell lymphomas. The frequency of aneuploidy by flow cytometry in the aggressive T cell lymphomas was less than one-third those of the large B cell lymphomas and the B cell lymphomas of intermediate cell size (Table V), and were

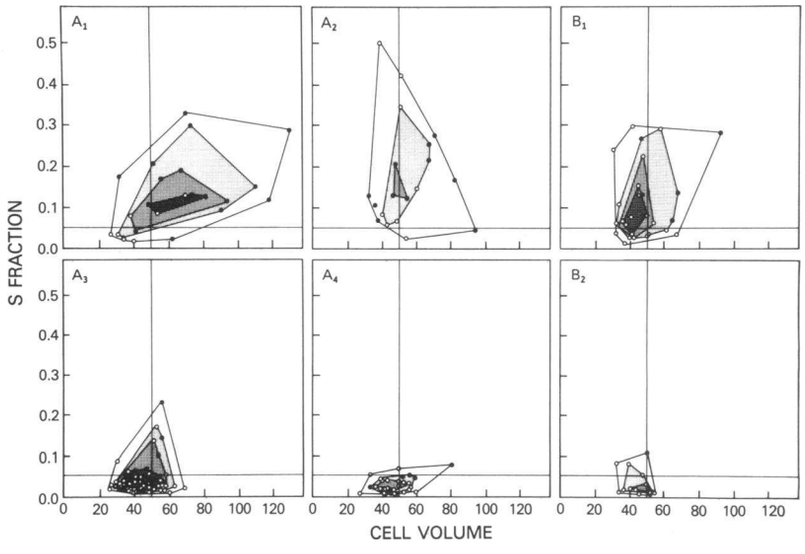

Figure 7. The relations among S fraction (*ordinate*), electronic cell volume (*abscissa*), and the presence of aneuploidy (●) in individual cases of lymphoma. (A_1) Large B cell lymphomas. (A_2) B cell lymphomas of intermediate cell size. (A_3) Small B cell lymphomas. (A_4) Mature/differentiated B cell lymphomas. (B_1) Aggressive T cell lymphomas. (B_2) Indolent T cell lymphomas. Concentric polygons and shading are intended to highlight the relations between extreme values for S fraction, cell volume, or both, and more representative values of both. In each panel the largest convex polygon is generated by connecting the points with the most extreme values. Each successive concentric polygon is formed in the same way using the remaining unconnected points. The ellipsoidal shape of these polygons and their orientation along the diagonal in panel A_1 provide visual confirmation for the correlation between S fraction and electronic cell volume in the large B cell lymphomas.

comparable to those seen among the small B cell lymphomas. It is of some interest that 70% of 46 cases with mycosis fungoides and Sezary syndrome have been reported to be aneuploid by flow cytometry by Bunn et al. (19). Patients with cutaneous T cell lymphomas may represent a special subgroup with a high frequency of aneuploidy by flow cytometry. The aggressive T cell lymphomas were indistinguishable from the indolent T cell lymphomas with regard to G_1 cell volumes (Table III), but the S fractions of the former were significantly higher than those of the latter (Table VIII).

Thus, our flow cytometry measurements support the placement of B and T cell lymphomas in separate categories and support the further subdivision of the B and T cell lymphomas into distinct biological subgroups. The biological implications of these flow cytometry measurements are considered below in greater detail.

Many of the statistically significant differences in cell properties among the lymphoma subgroups that have emerged in the present study were not clearly demonstrated in previous flow cytometry studies of the non-Hodgkin's lymphomas (31–39). This is attributable in part to the much larger number of samples examined in the present study, and in part to substantial differences in methods among the various studies.

Several methodological considerations deserve special comment:

Staining artifacts and the detection of aneuploidy. We have identified a variety of staining artifacts that could be mistaken for aneuploidy in clinical samples. These include a temperature- and fixation time-dependent hyperchromatic shift in the position of the DNA histogram, and the presence of spurious hyperdiploid peaks in cell samples from normal individuals (50). Both of these artifacts can be eliminated by incubating both the clinical sample and the normal diploid reference cells in 70% ethanol at 37°C for 2 h before mithramycin staining and flow cytometry analysis (50). All clinical samples included in the present study were treated in this fashion, and in virtually every case the DNA histogram included a G_1 peak whose position corresponded with that of an external diploid reference standard. Aneuploidy could be identified by the presence of distinct additional G_1 peaks in the hyperdiploid or near-tetraploid region (criterion 1, Table I) or by an increase in the CV of a normally positioned G_1 peak (criterion 2, Table I). Our findings do not support the validity of criterion 3 (Table I) for the detection of aneuploidy in clinical samples (see Table IV and associated discussion). In that virtually all samples contain at least some cells with diploid DNA content, it would seem proper to treat a single, abnormally positioned G_1 peak with a normal CV as a staining artifact until proven otherwise, regardless of the DNA stain that is used.

In many of the published flow cytometry studies of the lymphomas, propidium iodide was used as the DNA fluorochrome rather than mithramycin (19, 32, 35–39), and nucleated chicken erythrocytes were used as an internal ploidy reference standard for each clinical sample (35–38). Reported CVs for relative position of the G_1 peak of propidium iodide-stained normal human diploid cells have ranged from a low of 2% (38) to a high of 6% (37). By comparison, in the present study the CV of G_1 peak position of mithramycin-stained normal human diploid cells was only 1% (Table IV). The greater apparent staining variability observed with propidium iodide may account for many of the published cases reported as aneuploid by criterion 3 of Table I.

Table IX summarizes currently available published data on aneuploidy by flow cytometry in relation to criteria for its detection in the non-Hodgkin's lymphomas. Cases reported as aneuploid by criterion 3 of Table I represented 24–76% of reported aneuploidy by flow cytometry. When these cases are excluded, approximately 33% of all reported cases of non-Hodgkin's lymphoma studied by flow cytometry are aneuploid by this technique.

Grouping of cases. In studies in which cases are grouped solely by the Rappaport classification and/or by a modification of the International Working Formulation, no explicit distinction

Table IX. Aneuploidy in Relation to Criteria for Detection

Reference	Stain	Variation in normal G_1 peak position CV	Aneuploidy by criteria			
			1	2	3	1 and 2
				n		
Diamond and Braylan (35)	Propidium iodide	0.03	7/30 (23)	12/30 (40)	6/30 (20)	19/30 (63)
Costa et al. (37)	Propidium iodide	0.06	11/74 (15)	—	34/74 (46)	11/74 (15)
Diamond et al. (38)	Propidium iodide	0.02	6/43 (14)	14/43 (33)	10/43 (23)	20/43 (47)
Present study	Mithramycin	0.01	59/220 (27)	11/220 (5)	—	70/220 (32)

Values in parentheses are percentages.

is drawn between B and T cell lymphomas, and the lumping of these two immunologic types of lymphoma might obscure their distinctive biological properties. This adverse effect might be expected when one lumps lymphoblastic lymphomas (T cell lymphomas with relatively low frequencies of aneuploidy) together with B cell lymphomas of large and intermediate cell size (with high frequencies of aneuploidy) under the category of high-grade lymphomas in the International Working Formulation. Similarly, the grouping of immunoblastic T cell lymphomas (with relatively low frequencies of aneuploidy and small cell size) together with the large B cell lymphomas (with high frequencies of aneuploidy and large G_1 cell size) under the category of diffuse histiocytic lymphoma in the Rappaport classification might obscure biological differences between the large B cell lymphomas and the small B cell lymphomas (Table III and Table V).

S fraction calculations. In the present study, S fraction calculations were performed on all cases, including those with aneuploidy by criterion 1 of Table I. When the aneuploid component could be separated on the basis of cell size in dual parameter studies, and the S fractions of the two components could be calculated and reported separately, the S fraction of the separable aneuploid component was reported (Table VIII). In the remaining aneuploid cases where the aneuploid component could not be separated, the S fraction was calculated and reported for the mixture (Table VIII). In published flow cytometry studies in the lymphomas where S fractions were not calculated in samples that contained aneuploidy by criterion I of Table I (35, 37, 38), the high S fractions of the large B cell lymphomas may have been underrepresented.

Biological implications of flow cytometry measurements in the non-Hodgkin's lymphomas

Longitudinal studies have shown that a large proportion of indolent small cell non-Hodgkin's lymphomas eventually undergo clinical and histopathologic transformation to more aggressive large cell lymphomas (28–30). Given the statistically significant differences in the frequency of aneuploidy between the small and large B cell lymphomas demonstrated in the present study, it would seem reasonable to suppose that clinical and histopathologic transformation might also be accompanied by changes in ploidy, in keeping with the concept of clonal evolution as proposed by Nowell (58). In our flow cytometry studies, cytogenetic instability can be inferred from the finding of multiple aneuploid peaks in the same sample in eight patients (Table VII), discordant ploidy indices at different tissue sites in the same patient in five cases (Table VI), and changes in ploidy over time during the course of serial sampling in one patient. Multiple aneuploid stemlines and serial changes in ploidy by flow cytometry have also been noted by others in human lymphoid malignancies (19, 27, 59–61).

In the lymphomas, ploidy abnormalities that are detected by flow cytometry involve an increase in cell DNA content above the diploid level (Fig. 3). Thus, one would expect that ploidy abnormalities that accompany clinical and histopathologic transformation from indolent disease to aggressive disease would involve an increase in stem-line DNA content. Thus, the finding in one of our patients of a lower ploidy index at the time of transformation than at the time of initial study might seem surprising. One possible mechanism that could account for the loss of cytogenetic material during the course of transformation to more aggressive disease is that of spontaneous cell tetraploidization, with cytogenetic instability and progressive chromosome loss. The sequence of spontaneous tetraploidization with subsequent loss of chromosomal material is well documented in experimental tumor systems (62). The approximately twofold range of ploidy indices seen in the lymphomas (Fig. 3) and the presence of near-tetraploid stem lines in the B cell lymphomas at the time of diagnosis but not at the time of relapse (Fig. 4) are consistent with such a process. Tetraploidization has been demonstrated directly in serial studies of human lymphoid malignancies (27, 59, 60). A direct assessment of the degree of cytogenetic stability or instability of such tetraploid stem lines must await the collection of more extensive serial flow cytometry data in man.

Overall, the data presented in this paper support the tumor growth model shown in Fig. 8. Non-Hodgkin's lymphomas with favorable histologies (the small lymphocytic B cell lymphomas and indolent T cell lymphomas) are shown as clonal proliferations that undergo growth retardation during late subclinical or early clinical stages of growth (curve *A*, Fig. 8). The clonal nature of these tumors can be deduced from immunologic surface marker patterns and from the characteristic chromosomal abnormalities found in cytogenetic studies. Such tumors exhibit low fractions of cells in S (Fig. 5 and Table VIII), reflecting their indolent growth behavior at the time of biopsy. Among the B cell lymphomas, there is a general correlation between G_1 cell size and proliferative rate, not only among tumors, but among cell subpopulations with the same G_1 DNA content in the same tumor (34). Thus, the relatively small mean G_1 cell

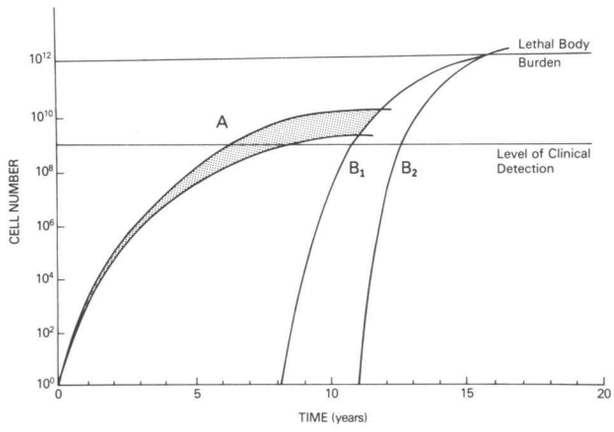

Figure 8. Schematic representation of clonal evolution in the non-Hodgkin's lymphomas. For discussion, see text.

size among the indolent B cell lymphomas (Table III) also reflects the preponderance of slowly proliferating cells in these tumors.

Curves B_1 and B_2 of Fig. 8 represent the emergence over time of increasingly more aggressive clones of tumor cells that are capable of proliferating rapidly and overgrowing preexisting cell populations. About 70% of the intermediate and large B cell lymphomas and 25% of the small B cell lymphomas contain such aneuploid clones, compared with 23% of the aggressive T cell lymphomas and 7% of the indolent T cell lymphomas (Table V). When the DNA measurements of such clones could be separated on the basis of large cell size, the high S fractions of these aneuploid populations were obvious (column 3, Table VIII and Fig. 7); their contribution to the high S fractions of mixed samples could also be appreciated readily (column 2, Table VIII).

While the data presented in this paper are entirely consistent with the model shown in Fig. 8, critical validation of the model must await the collection of a much more extensive set of serial studies.

References

1. Jones, S. E., Z. Fuks, M. Bull, M. E. Kadin, R. F. Dorfman, H. S. Kaplan, S. A. Rosenberg, and H. Kim. 1973. Non-Hodgkin's lymphomas. IV. Clinicopathologic correlation in 405 cases. *Cancer.* 31:806–823.

2. Schein, P. S., B. A. Chabner, G. P. Canellos, R. C. Young, C. W. Berard, and V. T. DeVita. 1974. Potential for prolonged disease-free survival following combination chemotherapy of non-Hodgkin's lymphoma. *Blood.* 43:187–189.

3. Fisher, R. I., V. T. DeVita, B. L. Johnson, R. Simon, and R. C. Young. 1977. Prognostic factors for advanced diffuse histiocytic lymphoma following treatment with combination chemotherapy. *Am. J. Med.* 63:177–182.

4. Fisher, R. I., V. T. DeVita, S. M. Hubbard, D. L. Longo, R. Wesley, B. A. Chabner, and R. C. Young. 1983. Diffuse aggressive lymphomas. Increased survival after alternating flexible sequences of ProMACE and MOPP chemotherapy. *Ann. Intern. Med.* 98:304–309.

5. Anderson, T., R. A. Bender, R. I. Fisher, V. T. DeVita, B. A. Chabner, C. W. Berard, L. Norton, and R. C. Young. 1977. Combination chemotherapy in non-Hodgkin's lymphoma. Results of long-term follow-up. *Cancer Treat. Rep.* 61:1057–1066.

6. Cadman, E., L. Farber, D. Berd, and J. Bertino. 1977. Combination therapy for diffuse histiocytic lymphoma that includes antimetabolites. *Cancer Treat. Rep.* 61:1109–1116.

7. DeVita, V. T., G. P. Canellos, B. Chabner, P. Schein, S. P. Hubbard, and R. C. Young. 1975. Advanced diffuse histiocytic lymphoma, a potentially curable disease. *Lancet.* I:248–250.

8. Portlock, C. S., and S. A. Rosenberg. 1979. No initial therapy for Stage III and IV non-Hodgkin's lymphomas of favorable histologic types. *Ann. Intern. Med.* 90:10–13.

9. Levine, A. M., M. Goldstein, P. R. Meyer, S. J. Forman, J. W. Parker, A. P. Hill, B. Nathwani, and R. J. Lukes. 1982. Lukes-Collins classification predicts for response and survival after BACOP therapy (bleomycin, doxorubicin, cyclophosphamide, vincristine, prednisone) in diffuse histiocytic lymphoma (DHL). *Blood.* 60(Suppl. 1):161*a*. (Abstr.)

10. Coutinho, V., C. Bottura, and R. P. Falcao. 1971. Cytogenetic studies in malignant lymphomas. A study of 28 cases. *Br. J. Cancer.* 25:789–801.

11. Reeves, B. R. 1973. Cytogenetics of malignant lymphomas. *Hum. Genet.* 20:231–250.

12. Kaiser-McCaw, B., A. L. Epstein, K. M. Overton, H. S. Kaplan, and F. Hecht. 1977. The cytogenetics of human lymphomas. Chromosome 14 in Burkitt's, diffuse histiocytic and related neoplasms. *Chromosomes Today.* 6:383–390.

13. Mark, J., C. Ekedahl, and A. Hagman. 1977. Origin of the translocated segment of the $14q^+$ marker in non-Burkitt lymphomas. *Hum. Genet.* 36:277–282.

14. Fleischman, E. W., and E. L. Prirogina. 1977. Karyotype peculiarities of malignant lymphomas. *Hum. Genet.* 35:269–279.

15. Fukuhara, S., J. D. Rowley, D. Variakojis, and D. L. Sweet. 1978. Banding studies on chromosomes in diffuse "histiocytic" lymphomas. Correlations of $14q^+$ marker chromosome with cytology. *Blood.* 52:989–1002.

16. Finan, J., R. Daniele, D. Rowlands, Jr., and P. Nowell. 1978. Cytogenetics of chronic T cell leukemia, including two patients with a $14q^+$ translocation. *Virchows Arch. B Cell Pathol.* 29:121–127.

17. Fukuhara, S., and J. D. Rowley. 1978. Chromosome 14 translocations in non-Burkitt lymphomas. *Int. J. Cancer.* 22:14–21.

18. Fukuhara, S., J. D. Rowley, D. Variakojis, and H. M. Golomb. 1979. Chromosome abnormalities in poorly differentiated lymphomas. *Cancer Res.* 39:3119–3128.

19. Bunn, P. A., J. Whang-Peng, D. N. Carney, M. L. Schlam, T. Knutsen, and A. F. Gazdar. 1980. DNA content analysis by flow cytometry and cytogenetic analysis in mycosis fungoides and Sezary syndrome. *J. Clin. Invest.* 65:1440–1448.

20. van Vloten, W. A., E. A. Pet, and J. P. M. Geraedts. 1980. Chromosome studies in mycosis fungoides. *Br. J. Dermatol.* 102:507–513.

21. Rowley, J. D., and S. Fukuhara. 1980. Chromosome studies in non-Hodgkin's lymphomas. *Semin. Oncol.* 7:255–266.

22. Sandberg, A. A. 1981. Chromosome changes in the lymphomas. *Hum. Pathol.* 12:531–540.

23. Kaneko, Y., R. Abe, K. Sampi, and M. Sakurai. 1982. An analysis of chromosome findings in non-Hodgkin's lymphomas. *Cancer Genet. Cytogenet.* 5:107–121.

24. Yunis, J. J., M. M. Oken, H. E. Kaplan, K. M. Ensrod, R. R. Howe, and A. Theologides. 1982. Distinctive chromosomal abnormalities in histologic subtypes of non-Hodgkin's lymphoma. *N. Engl. J. Med.* 307:1231–1236.

25. Kaneko, Y., Y. Veshima, D. Variakojis, and J. D. Rowley. 1982. Correlation of karyotype with morphology and prognosis in patients with non-Hodgkin's lymphoma. *Blood.* 60 (Suppl. 1):146*a*. (Abstr.)

26. Han, T., H. Ozer, N. Sadamori, G. Gomez, E. S. Henderson, J. Minowada, M. L. Blood, and A. A. Sandberg. 1982. Cytogenetic abnormalities in chronic lymphocytic leukemia (CLL). A clinical correlation. *Blood.* 60 (Suppl. 1):127*a*. (Abstr.)

27. Meijer, C. J. L. M., E. M. van der Loo, W. A. van Vloten, E. A. van der Velde, E. Scheffer, and C. J. Cornelisse. 1980. Early diagnosis of mycosis fungoides and Sezary syndrome by morphometric analysis of lymphoid cells in the skin. *Cancer.* 45:2864–2871.

28. Cullen, M. H., T. A. Lister, R. L. Brearley, W. S. Shand, and A. G. Stansfeld. 1979. Histological transformation of non-Hodgkin's lymphoma. A prospective study. *Cancer.* 44:645–651.

29. Risdall, R., R. T. Hoppe, and R. Warnke. 1979. Non-Hodgkin's lymphoma. A study of the evolution of the disease based upon 92 autopsied cases. *Cancer.* 44:529–542.

30. Acker, B., R. T. Hoppe, T. V. Colby, R. S. Cox, H. S. Kaplan, and S. A. Rosenberg. 1983. Histologic conversion in the non-Hodgkin's lymphomas. *J. Clin. Oncol.* 1:11–16.

31. Barlogie, B., W. Hittelman, G. Spitzer, J. M. Trujillo, J. S. Hart, L. Smallwood, and B. Drewinko. 1977. Correlation of DNA distribution abnormalities with cytogenetic findings in human adult leukemia and lymphoma. *Cancer Res.* 37:4400–4407.

32. Braylan, R. C., B. J. Fowlkes, E. S. Jaffe, S. K. Sanders, C. W. Berard, and C. J. Herman. 1978. Cell volumes and DNA distributions of normal and neoplastic human lymphoid cells. *Cancer.* 41:201–209.

33. Barlogie, B., J. Latreille, E. J. Freireich, C. T. Fu, D. Mellard, M. Meistrich, and M. Andreeff. 1980. Characterization of hematologic malignancies by flow cytometry. *Blood Cell.* 6:719–744.

34. Shackney, S. E., K. S. Skramstad, R. E. Cunningham, D. J. Dugas, T. L. Lincoln, and R. J. Lukes. 1980. Dual parameter flow cytometry studies in human lymphomas. *J. Clin. Invest.* 66:1281–1294.

35. Diamond, L. W., and R. C. Braylan. 1980. Flow analysis of DNA content and cell size in non-Hodgkin's lymphoma. *Cancer Res.* 40:703–712.

36. Kruth, H. S., R. C. Braylan, N. A. Benson, and V. A. Nourse. 1981. Simultaneous analysis of DNA and cell surface immunoglobulin in human B-cell lymphomas by flow cytometry. *Cancer Res.* 41:4895–4899.

37. Costa, A., G. Mazzini, G. Del Bino, and R. Silvestrini. 1981. DNA content and kinetic characteristics of non-Hodgkin's lymphoma. Determined by flow cytometry and autoradiography. *Cytometry.* 2:185–188.

38. Diamond, L. W., B. N. Nathwani, and H. Rappaport. 1982. Flow cytometry in the diagnosis and classification of malignant lymphoma and leukemia. *Cancer.* 50:1122–1135.

39. Braylan, R. C., N. A. Benson, and V. A. Nourse. 1982. Flow cytometry: A new approach toward characterizing lymphomas. *In* B and T Cell Tumors. E. S. Vitetta and C. F. Fox, editors. Academic Press, Inc., New York.

40. Lukes, R. J., C. R. Taylor, J. W. Parker, T. L. Lincoln, P. K. Pattengale, and B. H. Tindle. 1978. A morphologic and immunologic surface marker study of 299 cases of non-Hodgkin's lymphomas and related leukemias. *Am. J. Pathol.* 90:461–486.

41. Jaffe, E. S., E. M. Shevach, M. M. Frank, C. W. Berard, and I. Green. 1974. Nodular lymphomas. Evidence for origin from follicular B lymphocytes. *N. Engl. J. Med.* 290:813–819.

42. Rappaport, H. 1966. Tumors of the Hematopoietic System. Atlas of Tumor Pathology. Sect. 3, Fasc. 8. Armed Forces Institute of Pathology, Washington, DC.

43. Lukes, R. J., and R. D. Collins. 1974. Immunologic characterization of human malignant lymphomas. *Cancer.* 34:1488–1503.

44. Lukes, R. J., J. W. Parker, C. R. Taylor, B. H. Tindle, A. D. Cramer, and T. L. Lincoln. 1979. Immunologic approach to non-Hodgkin's lymphomas and selected leukemias. An analysis of the results of multiparameter studies of 425 cases. *Semin. Hematol.* 15:322–351.

45. Jaffe, E. S., J. A. Strauchen, and C. W. Berard. 1982. Predictability of immunologic phenotype by morphologic criteria in diffuse aggressive non-Hodgkin's lymphomas. *Am. J. Clin. Pathol.* 77:46–49.

46. Lukes, R. J., C. R. Taylor, and J. W. Parker. 1983. Immunologic surface marker studies in the histopathological diagnosis of non-Hodgkin's lymphomas based on multiparameter studies of 790 cases. *In* Advances in Malignant Lymphoma: Etiology, Immunology, Pathology, Treatment. Vol. 3. S. A. Rosenberg and H. S. Kaplan, editors. Academic Press, Inc., New York. In press.

47. Cossman, J., and E. F. Jaffe. 1981. Distribution of complement receptor subtypes in non-Hodgkin's lymphomas of B-cell origin. *Blood.* 58:20–26.

48. Hoffman, F. M., J. W. Parker, R. J. Lukes, P. R. Meyer, and C. R. Taylor. 1983. Immunological techniques. Their practical applications in lymphoma diagnosis. *In* Proceedings of the 15th International Leucocyte Conference. J. W. Parker, and R. J. O'Brien, editors. John Wiley & Sons, Inc., New York. In press.

49. Shackney, S. E., B. W. Erickson, and K. S. Skramstad. 1979. The T-lymphocyte as a diploid reference standard for flow cytometry. *Cancer Res.* 39:4418–4422.

50. Cunningham, R. E., K. S. Skramstad, A. E. Newburger, and S. E. Shackney. 1982. Artifacts associated with mithramycin fluorescence in the clinical detection and quantitation of aneuploidy by flow cytometry. *J. Histochem. Cytochem.* 30:317–322.

51. Salzman, G. C., R. D. Hiebert, and J. M. Crowell. 1978. Data acquisition and display for a high-speed cell sorter. *Comput. Biomed. Res.* 11:77–88.

52. Schuette, W. H., S. E. Shackney, M. A. McCollum, and C. A. Smith. 1983. High resolution method for the analysis of DNA histograms that is suitable for the detection of multiple aneuploid G_1 peaks in clinical samples. *Cytometry.* 3:376–386.

53. Ritch, P. S., S. E. Shackney, W. H. Schuette, and C. A. Smith. 1983. A practical graphical method for estimating the fraction of cells in S in DNA histograms from clinical tumor cells containing aneuploid cell populations. *Cytometry.* 4:66–74.

54. Lehmann, E. L. 1975. Nonparametrics. Holden Day, Inc., San Francisco.

55. Jett, J. H. 1978. Mathematical analysis of DNA histograms from asynchronous and synchronous cell populations. *In* Proceedings of the 3rd International Symposium on Pulse Cytophotometry. D. Lutz, editor. European Press, Ghent, Belgium. 93–102.

56. Shackney, S. E., and K. S. Skramstad. 1979. A dynamic interpretation of multiparameter studies in the lymphomas. *Am. J. Clin. Pathol.* 72:756–764.

57. Rosenberg, S. A. 1982. National Cancer Institute-sponsored study of classifications of non-Hodgkin's lymphomas (for the non-Hodgkin's pathologic classification project). *Cancer.* 49:2112–2135.

58. Nowell, P. C. 1976. Clonal evolution of tumor cell populations. *Science (Wash. DC).* 194:23–28.

59. Clifford, P., J. Gripenberg, E. Klein, E. M. Fenyo, and G. Manolov. 1968. Treatment of Burkitt's lymphoma. *Lancet.* II:517–518.

60. Bunn, P. A., Jr., S. Krasnow, R. W. Makuch, M. L. Schlam, and G. P. Schecter. 1982. Flow cytometric analysis of DNA content of bone marrow cells in patients with plasma cell myeloma. Clinical implications. *Blood.* 59:528–535.

61. Barlogie, B., W. Gohde, D. A. Johnston, L. Smallwood, J. Schumann, B. Drewinko, and E. J. Freireich. 1978. Determination of ploidy and proliferative characteristics of human solid tumors by pulse cytophotometry. *Cancer Res.* 38:3333–3339.

62. Isaacs, J. T., N. Wake, D. S. Coffey, A. A. Sandberg. 1982. Genetic instability coupled to clonal selection as a mechanism for tumor progression in the Dunning R-3327 rat prostatic adenocarcinoma system. *Cancer Res.* 42:2353–2361.