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Perspectives in Cancer Research

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Abstract

The prognosis of patients with cancer is largely determined by the specific histological diagnosis, tumor mass stage, and host performance status. The management of neoplastic disease with the currently available treatment armamentarium may be further advanced if individual patients' risk factors could be better defined. Some of the determinants of tumor response seem to be expressed at the cellular level in terms of degree of tumor cell differentiation, growth kinetics, and hormone receptor expression, which are not readily appreciated by descriptive morphology. Quantitative cytology in the form of flow cytometry has greatly advanced the objective elucidation of tumor cell heterogeneity by using probes that discriminate tumor and normal cells and assess differentiative as well as proliferative tumor cell properties. Abnormal nuclear DNA content is a conclusive marker of malignancy and is found with increasing frequency in leukemia (23% among 793 patients), in lymphoma (53% among 360 patients), and in myeloma (76% among 177 patients), as well as in solid tumors (75% among 3611 patients), for an overall incidence of 67% in 4941 patients. The degree of DNA content abnormality varies according to disease type, with a predominant excess of 10 to 20% in the hematological cancers, whereas ploidy levels in solid-tissue neoplasms span the entire range from almost haploid to hyperoctaploid abnormalities, with near-triploid mean and median DNA content values. The proportion of cells in the S phase of the cell cycle increases with increasing DNA excess in a number of different solid tumors and in acute leukemia. This cytokinetic parameter permits discrimination of low- and high-grade malignant lymphomas. Several reports demonstrate increasing morphological immaturity to be associated with increasing DNA content abnormality and increasing S percentage, all of which adversely affect prognosis. Among phenotypic tumor cell markers, surface membrane antigens have been extensively studied in lymphoid and myeloid neoplasms by the use of hybridoma-generated monoclonal antibodies, which have recently also found *in vitro* and *in vivo* therapeutic application. Cellular RNA content is useful for the objective discrimination of acute leukemias and of multiple myeloma. Newer applications of flow cytometry concern studies in the areas of cytoenzymology and cellular pharmacology. Current research is dedicated to the identification of neoplastic marker probes for DNA-diploid disease (*e.g.*, nucleolar antigen) and additional phenotypic (*e.g.*, hormone receptors) and cytokinetic (*e.g.*, cycle traverse rate, growth fraction) parameters.

From a patient management perspective, a role for flow cytometry is emerging as a tool for diagnosis of cancer (abnormal DNA content), specific histopathological diagnosis (RNA for hematological cancers; surface markers for lymphoid and myeloid neoplasms), prognosis (adverse impact of aneuploidy and high S percentage), and treatment (cytokinetically oriented, monoclonal antibodies, drug pharmacology). The pace of past progress justifies the hope that cytometry may soon provide "fingerprint-type" information of an individual patient's tumor which, if proven prognostically relevant, may provide the basis for treatment selection in the future.

Presently, our understanding of neoplastic disease is mainly descriptive. Thus, we still know very little about the factor(s) initiating neoplastic growth, nor can we diagnose the incipient stages of subclinical disease with certainty. Even once macroscopic cancer is established, histopathological description is semiquantitative, not allowing a definitive discrimination, cell by cell, of the malignant *versus* the normal state within the usually heterogeneous cell population constituting a tumor mass lesion. It is this cellular heterogeneity that, at least in part, may account for the diverse clinical course of comparably staged and treated patients. Recognizing the inadequacy of descriptive morphology for the assessment of tumor cell heterogeneity, several groups have developed automated instruments that are capable of objective and quantitative cell analysis (81, 96, 175, 183). FCM[3] allows high-speed analysis and sorting (1000 cells/sec) according to morphological, molecular, biophysical, and functional cellular features, which can be correlated with each other in multiparameter computer-assisted instruments (51). Essential to all FCM investigation is cell monodispersion, quantitative cell staining, and high-resolution analysis (50). A major requirement for FCM to probe effectively the heterogeneity of human cancer is the unequivocal discrimination of tumor from normal cells, so that the phenotypic diversity of tumor cells and the interaction with neighboring normal cells can be investigated.

This report will address the current status of FCM as a tool to unravel the heterogeneity of human neoplastic disease. This will be accomplished by reviewing cellular parameters suitable for cytometric investigation and pertinent to biological features of potential prognostic importance such as cytogenetics (12), differentiation (2, 144), and proliferation (13, 20, 35, 52, 57, 67, 87, 115, 131). We will provide the reader with a critical evaluation of established and promising new neoplastic, phenotypic, and cytokinetic FCM markers and point to their potential in the context of cancer biology, diagnosis, prognosis, and therapy.

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[3] The abbreviations used are: FCM, flow cytometry; MSKCC, Memorial Sloan Kettering Cancer Center; ALL, acute lymphoblastic leukemia; MDAH, M. D. Anderson Hospital and Tumor Institute; AML, acute myeloid leukemia; FITC, fluorescein isothiocyanate; ER, estrogen receptor; DAPI, 4′-6-diamidino-2 phenylindole.

Neoplastic Markers

DNA. The observation of a clonal chromosomal abnormality probably represents the strongest single evidence for the neoplastic nature of a cell population (130). Such chromosomal abnormalities were found by cytophotometric studies often to be associated with an abnormal DNA content (137, 138). DNA content abnormalities have since been confirmed in extensive microscopic investigations on solid tumors by Atkin and Kay (7). With the advent of DNA-specific fluorescent dyes (20, 82, 101) and the development of FCM, the measurement of DNA content has emerged as a powerful and practical tool to examine the presence of ploidy abnormality in human tumors (13, 14). Unlike mitotic karyotyping, ploidy analysis by DNA FCM is independent of cell proliferative activity, and a successful assay is limited only by cell preparative and staining procedures (50). These problems have been worked out in recent years, so that for most tumors measurements of DNA content can now be performed with high resolution and great reproducibility (13, 176, 177, 178).

FCM studies of normal and reactive tissues and of benign tumors all have revealed a normal diploid DNA content (12). Aneuploidy was noted in some premalignant conditions such as preleukemia (14, 135) and angioimmunoblastic lymphadenopathy (13) and in benign monoclonal gammopathy (18, 105, 107).

There is now a large body of information available on ploidy abnormalities in various human cancers, using a variety of DNA-specific staining methods and different flow instruments (Table 1). We have introduced the term DNA index as a measure of the degree of ploidy abnormality by FCM, denoting the ratio of DNA fluorescence of abnormal to normal G_1-G_0 cells (13, 14). This term is now generally accepted for the quantitative description of DNA-derived ploidy. The choice of reference cells varies among investigators in the field and includes diploid peripheral blood granulocytes and lymphocytes or chicken erythrocytes with lower DNA content. The observation of potential sex (177, 178)- and age (162)-related DNA differences in blood mononuclear cell standards and evidence of variation in stainability as a function of cell type, preparative procedure, and DNA dye leads us to propose, for studying minor ploidy abnormalities, the use of 2 independent standards with different DNA content and possibly of 2 different DNA-staining methods such as ethidium bromide-mithramycin (20) and DAPI (82). While there is considerable variation in the reported incidence of DNA-defined aneuploidy, most studies concur in the observation of diploid DNA content in Hodgkin's disease, chronic lymphocytic leukemia, and benign phase of chronic myelogeneous leukemia; a 15 to 30% aneuploidy rate in acute leukemia and in indolent non-Hodgkin's lymphoma; a 50 to 80% aneuploidy rate in aggressive lymphoma and in multiple myeloma; and a 60 to 100% aneuploidy rate in solid tumors (Table 1). It remains to be seen whether additional refinement leading to improved resolution in FCM measurements will further enhance the aneuploidy yield, particularly in leukemia and in lymphoma. Most published studies, however, do not provide information on the coefficient of variation as an important indicator of the quality of DNA FCM measurements or on the degree of aneuploidy. Data from some investigators, reporting on several different diagnoses, would indicate, however, consistently high aneuploidy rates for all solid-tissue neoplasms in the 80 to 100% range, suggesting that a lower incidence in other studies most probably originates from technically inferior DNA measurements (Table 1).

In order to determine whether there are disease-related ploidy differences, Chart 1 depicts cumulative frequency distributions of DNA index values in lymphoma (Chart 1*A*), in leukemia and myeloma (Chart 1*B*), and in solid tumors (Chart 1*C*). The frequently diploid nature of leukemia and low-grade lymphoma can be readily recognized. High-grade lymphoma and myeloma generally display low-degree hyperdiploid abnormalities. In contrast, ploidy levels in solid tumors evenly span the entire range from hypodiploid to hyperoctaploid values. DNA index frequency distributions are quite similar for different solid tumors, except for a prevalence of triploid abnormalities in germ cell tumors.[4] Chart 1*C* also illustrates a remarkable similarity between results obtained in 2 different laboratories (Munster, Germany, and Houston, Texas) on almost 1500 cases. Surprisingly, the data on human tumors also closely resemble observations on spontaneous canine tumors previously reported by Johnson *et al.* (Chart 1*C*) (94). This interspecies similarity also extends to a low frequency of multimodal DNA stem lines in both human and canine tumors in the 7 to 10% range (12, 15). Finally, there is evidence that increasing DNA index is associated with a rise in the S-phase compartment size both within (93) and across (15) human tumor diagnostic groups, as well as in dog tumors (94) (Table 2). Evaluation of DNA content over time and by disease site thus far has revealed a uniform ploidy pattern in most cases analyzed (15, 140, 141).

Thus, DNA content abnormality, due to its low degree of dispersion, exquisite association with neoplastic lesions, and stable expression, should be useful for tumor diagnosis in general and for detection of rare neoplastic cells in early or residual disease stages in particular. The availability of an unequivocal tumor marker is also relevant to study, by multiparameter analysis, the phenotypic heterogeneity of tumor lesions and the tumor-host cell interaction.

Perhaps the most conclusive investigation into the potential of DNA FCM as a diagnostic tool was conducted at MSKCC in the area of bladder cancer. Examining bladder irrigation specimens from 392 individuals including 100 patients without bladder cancer, Klein *et al.* (98) noted only a 2% false-positive and a 7% false-negative rate (when papillomas were excluded). The demonstration in 18 patients of positive FCM tumor criteria antedating the cystoscopically visible tumor by up to 12 months underscores the clinical usefulness of FCM in disease monitoring.

Clinical programs using high-dose chemotherapy with autologous bone marrow rescue are concerned with the problem of marrow contamination by tumor cells, particularly in cancers with a high propensity of marrow metastasis such as oat cell lung cancer and malignant lymphoma. Even though aneuploidy is found in only 20% of acute leukemias, its presence can be utilized to determine residual disease in morphological remission. Thus, remission duration may be related to the degree of residual disease following remission induction, and the continued cytoreductive effect of maintenance therapy can be ascertained.

In light of current research addressing the ability of chemotherapeutic agents (151) and of biological response-modifying drugs to induce terminal tumor cell differentiation (32), the continued presence of an abnormal DNA stem line would lend direct support for such a mechanism as opposed to eradication of tumor stem cells through cytotoxic effects. The presence of an abnormal DNA stem line in some premalignant conditions should

[4] J. Schumann, unpublished observations.

Table 1
Ploidy abnormalities in various human neoplasms

Diagnosis	No. of patients	% aneuploid	Comments	Ref.
Leukemia				
Childhood acute	152	24	Higher aneuploidy rate in CALLA[a]-positive ALL	Look *et al.* (115)
	119 (ALL)	29		
	33 (ANLL)	6		
	107 (ALL)	40		Suarez *et al.* (165)
Adult acute	72	16	Higher aneuploidy rate in ALL *vs.* AML	Andreeff *et al.* (2)
	264	15	Higher aneuploidy rate in ALL *vs.* AML	Barlogie *et al.* (14, 19)
	194 (AML)	13		
	70 (ALL)	26		
	198	26		Barlogie[b]
	145 (AML)	25		
	53 (ALL)	30		
	793	23		
Lymphoma	19	42		Hagemeister *et al.* (83)
	38	29	Higher aneuploidy rate in FCC + high grade lymphomas	Shackney *et al.* (157)
	44	45	High S% in high-grade lymphoma	Diamond *et al.* (61)
	30	50	High S% in high-grade lymphoma	Diamond *et al.* (60)
	43	65	Higher aneuploidy rate and higher S% in high-grade lymphomas	Diamond *et al.* (62)
	74	61	Higher aneuploidy rate in high-grade lymphomas	Costa *et al.* (49)
	66	45	Higher aneuploidy rate and higher S% in high-grade lymphomas (see Chart 1*A*)	Barlogie[b]
	46	70	Poor prognosis in mycosis fungoides with aneuploidy and high S%	Bunn *et al.* (38)
	360	53		
Myeloma	145	77		Barlogie *et al.* (18)
	32	72	Higher aneuploidy rate with high tumor burden	Bunn *et al.* (36)
	177	76		
Breast cancer	92	92	Higher aneuploidy rate in poorly differentiated and ER-negative tumors	Olzewski *et al.* (132)
	80	85	Low S% in ER-positive tumors	Raber *et al.* (140)
	70	44	Low S% in ER-positive tumors	Kute *et al.* (104)
	79	78	Better prognosis in patients with diploid tumors	Thornthwaite *et al.* (168)
	64	90	Higher S% and higher aneuploidy rate in poorly differentiated and ER-negative tumors	Moran *et al.* (124)
	385	79		
Lung cancer	30	80		Raber *et al.* (139)
	61	91		Bunn *et al.* (37)
	30	79		Vindelov *et al.* (179)
	200	80	Higher aneuploidy rate in non-oat *vs.* oat cell carcinoma. Increasing S% with increasing DNA index	Johnson *et al.* (93)
	32	100	Higher aneuploidy rate in non-oat *vs.* oat cell carcinoma	Olzewski *et al.* (131)
	353	85		
Colon	20	55		Rognum *et al.* (149)
	62	74		Peterson *et al.* (136)
	33	39	Longer survival in patients with diploid and near-diploid tumors	Wolley *et al.* (184)
	20	70	Higher aneuploidy rate in poorly differentiated tumors	Linden *et al.* (113)
	135	62		
Prostate cancer	18	78	Higher aneuploidy rate in poorly differentiated tumors	Bichel *et al.* (26)
	4	100		Goerttler *et al.* (80)
	54	81		Tribukait *et al.* (172)
	40	55	Lower response rate to hormone treatment in aneuploid tumors	Zetterberg *et al.* (186)
	31	36		Rotors *et al.* (150)
	147	62		
Bladder cancer	41	42		Collste *et al.* (46)
	35	87		Collste *et al.* (48)
	123	94		Klein *et al.* (98)
	52	92		Klein *et al.* (97)
	96	86	Higher aneuploidy rate in poorly differentiated tumors	Tribukait *et al.* (171)
	21	85	Higher S% in aneuploid tumors	Tribukait *et al.* (173)
	83	84	Comparable results in biopsies and washings	Tribukait *et al.* (170)
	28	50		Freni *et al.* (76)
	32	72		Levi *et al.* (110)
	511	83		
Testicular cancer	74	93		Schumann[b]
Ovarian cancer	31	45		Krug and Ebeling (103)
Cervical cancer	51	94		Jacobsen *et al.* (90)
	40	98		Linden *et al.* (112)
Skin squamous cell cancer	220	82		Schumann[b]

Table 1—*Continued*

Diagnosis	No. of patients	% aneuploid	Comments	Ref.
Melanoma	38	79	Higher S% in aneuploid tumors; poor prognosis in tumors with high S%	Hansson *et al.* (85)
	605	76		Schumann *et al.* (155)[b]
	643	76		
Sarcomas	41	98		Schumann[b]
Brain tumors	18	78		Hoshino *et al.* (89)
	11	90	Higher rate of relapse in aneuploid tumors	Frederikson *et al.* (74)
	6	83	Medulloblastoma	Frederikson *et al.* (75)
	56	11		Mork and Laerum (126)
	81	48		
Subtotal (solid tumors)	2692	79		
Miscellaneous solid tumors	125	40		Dixon and Carter (64)
	242	69		Frankfurt *et al.* (73)
	482	75		Barlogie *et al.* (12, 15)[b]
	969	80	Only 70 not previously listed	Schumann[b]
All solid tumors	3611	75		
Grand total	4941	67	Includes hematologic neoplasms	

[a] CALLA, common acute lymphocytic leukemia antigen; ANLL, acute nonlymphocytic leukemia; FCC, follicular center cell.
[b] Unpublished observations.

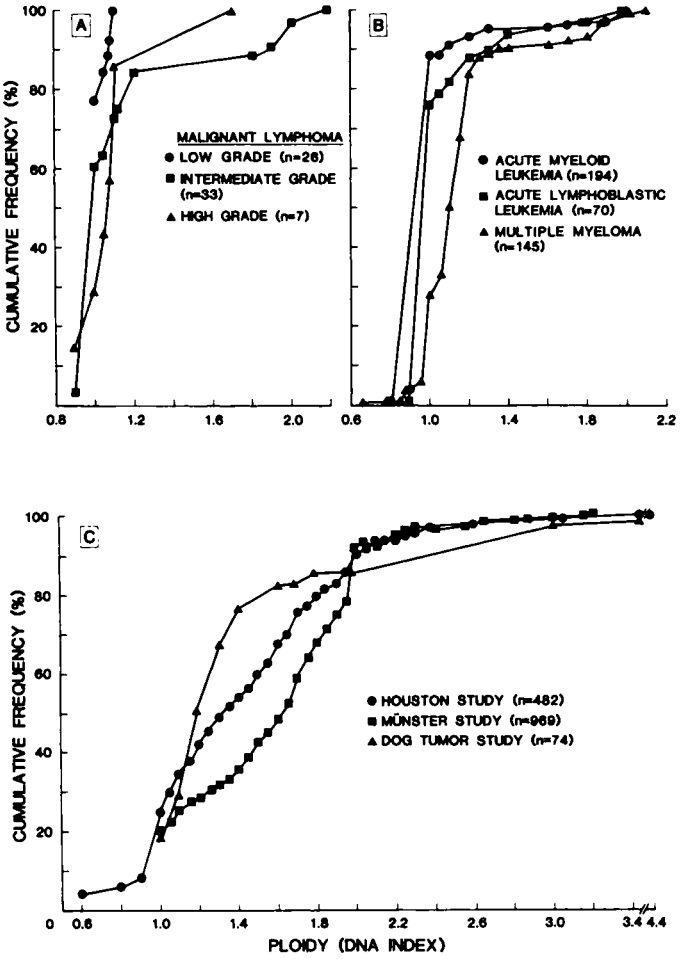

Chart 1. Cumulative frequency distribution of ploidy levels (DNA index; see text) in non-Hodgkin's lymphoma (*A*), in acute leukemia and myeloma (*B*), and in solid tumors (*C*). Note the prevalence of diploid and low-degree hyperdiploid DNA index values in lymphoma, leukemia, and myeloma, contrasting with an even distribution of DNA index values across the entire spectrum from hypodiploidy to hyperoctaploidy in solid tumors. There is a remarkable similarity in DNA index frequency profiles in solid-tissue neoplasms among 2 large human studies performed in Houston and Munster as well as between human and canine tumors (93).

facilitate research into host- or tumor-related features associated with a stable disease course *versus* rapid disease progression (13, 14, 18, 135).

Not infrequently, the question arises as to the primary or metastatic nature of a tumor lesion. Observation of an identical DNA index in a preceding contralateral breast lesion, for example, strongly suggests that the new lesion has the same origin and hence is metastatic.

Several authors have addressed the question of prognostic implications of ploidy (see references in Table 1). In solid-tissue neoplasms, survival appears to be adversely affected by increasing DNA index, as evident in bladder (173), prostate (26, 172), and colon (184) cancer. Unfortunately, some of these studies do not provide details on other prognostically important features such as tumor stage and malignancy grade. In our own study on a large variety of different solid tumors, in which we analyzed DNA index along with diagnosis as an independent variable, hypertriploid abnormality heralded a shorter survival (15). In light of the aforementioned relationship between DNA index and cytokinetic characteristics, the question arises whether the unfavorable prognosis of high-DNA-index disease is mediated by unfavorable cytokinetic features. Available data on both DNA index and cell kinetics are too sparse to answer this important question at the present time. In myeloma, the favorable prognosis of low-labeling-index disease was apparent only in patients with hypodiploid and markedly hyperdiploid abnormalities (108). In mycosis fungoides and Sézary syndrome, Bunn *et al.* (38) noted adverse effects of both aneuploidy and high S percentage on disease-free survival. The prognostic implications of DNA index measurements in acute leukemia are not yet settled. While Andreeff *et al.* (1, 6) report an adverse impact on survival of aneuploid and in particular hypodiploid abnormalities compared to diploid disease, Look *et al.*[5] note a favorable effect of DNA-derived hyperdiploidy in childhood ALL. The MDAH experience in 194 patients with AML indicates a superior response rate (65 *versus* 49%; $p = 0.03$) and longer survival in DNA-diploid disease (17 *versus* 8 months; $p = 0.01$). In 70 adults with ALL, a trend

[5] T. A. Look, unpublished observations.

for higher remission induction (84 *versus* 66%) and longer survival (22 *versus* 12 months) was noted for DNA-aneuploid disease ($p = 0.1$). Thus, it is possible that similar abnormalities in DNA content may have different prognostic implications in different diseases.

The relationship between DNA content and chromosomal abnormalities has been studied to only a limited extent mostly in the leukemias (14, 115). With high-resolution DNA FCM (coefficient of variation, ≤2%), a numerical chromosomal aberration involving more than one chromosome should routinely be detectable. Table 3 summarizes cytogenetic and DNA FCM findings in 284 recently treated adult patients with acute leukemia, 198 of whom were seen at MDAH (80% had AML) and 86 of whom were cared for at MSKCC (all had ALL). As in earlier observations, abnormal DNA stem lines were not confined to cytogenetic categories with numerical aberrations (14, 19) but were observed in 23% of patients with a normal diploid karyotype, in 18% of pseudodiploid abnormalities, and in 42% of cases with insufficient metaphases, with an overall incidence of 30% (27% at MDAH and 35% at MSKCC), comparing to 48% for cytogenetic abnormalities among the 241 patients with evaluable karyotypes. The quantitative relationship between degree of DNA content abnormality (expressed by the DNA index) and numerical chromosomal aberration [expressed by the karyotype index (modal number of chromosomes divided by the normal diploid value of 46)] was examined in 128 patients from 3 cancer centers (St. Jude, MSKCC, MDAH) (Chart 2). A linear correlation between DNA and karyotype index was noted in 85 patients. There were 43 additional cases expressing a distinctly abnormal DNA index in the presence of a normal diploid karyotype (38 patients) or near-diploid karyotype (5 patients with a karyotype index within 2% of a normal value). We have previously explained the discordance between normal diploid karyotype and abnormal DNA content by insufficient growth of leukemic cells compared to residual normal hemopoietic cells, which could be confirmed in a few cases by FCM-concordant chromosomal abnormalities in prematurely condensed G_1 chromosomes (14). However, differences in DNA stainability cannot entirely be ruled out, particularly for the pseudodiploid cases with apparently aberrant DNA fluorescence. It is our recommendation at this moment to use, in the case of DNA index-karyotype index discordance, a second DNA-specific fluorochrome such as DAPI (82). We also expect further clarification of this dilemma from studies of chromatin condensation (3, 54, 88) and histone composition.

Another interesting feature in the comparison between DNA content and karyotype analysis concerns the discrepancy between the high resolution of DNA content distributions in comparison to a generally greater dispersion in chromosome number. This paradox has been related to differences in packaging of chromosomes (100) and to preferential cell loss in mitosis (164), resulting in more uniform karyotypes in interphase cells. The latter hypothesis can be tested using the premature chromosome condensation technique (92). Advances in cytochemistry and instrumentation have also made possible the quantitative analysis of DNA per chromosome in mitotic preparations (79, 133). Clinical application of flow karyotyping is severely limited, however, by the poor mitotic yield in most human tumors and particularly in solid tumors. The possibility of premature chromosome condensation in interphase cells may be exploitable in the future for flow karyotyping of the more abundant G_1 chromosomes (92).

While not allowing the quantitation of DNA in each individual chromosome, the recent demonstration of serum from patients with the CREST syndrome of scleroderma to react with the centromeric region of chromosomes has opened the possibility to relate, in interphase cells, the degree of DNA excess to the number of chromosomes involved (33, 125).

Other Probes Offering the Potential of Objectively Distin-

Table 2

Ploidy and proliferative activity

Significant increase in S% with increasing DNA index, $p < 0.01$.

	Human tumors									
	Lung cancer		Breast cancer		All solid tumors[a]		Childhood CALLA+[b] ALL[c]		Dog tumors[d]	
DNA index	No. of patients	Mean S%	No. of patients	Mean S%	No. of patients	Mean S%	No. of patients	Mean S%	No. of dogs	Mean S%
≤1.0	70	10.5	40	11.1	131	10.3	75	5.5	14	8.4
1.01–1.5	80	15.5	59	19.2	181	16.6	39	8.4	43	11.2
>1.5	54	20.9	56	18.6	160	19.6			18	17.7

[a] Refs. 12 and 15, in part.
[b] CALLA+, common acute lymphocytic leukemia antigen positive.
[c] Ref. 114.
[d] Ref. 92.

Table 3

Cytogenetics by FCM and mitotic karyotype

	No. of patients			Patients with FCM abnormality					
				No.			%		
Karyotype	MDAH	MSKCC	Total	MDAH	MSKCC	Total	MDAH	MSKCC	Total
Normal	88	27	115	22	5	27	25	19	23
Insufficient metaphases	26	17	43	10	8	18	38	47	42
Pseudodiploid	39	16	45	7	1	8	18	6	18
Aneuploid	45	26	71	15	16	31	33	62	44
Total	198	86	284	54	30	84	27	35	30

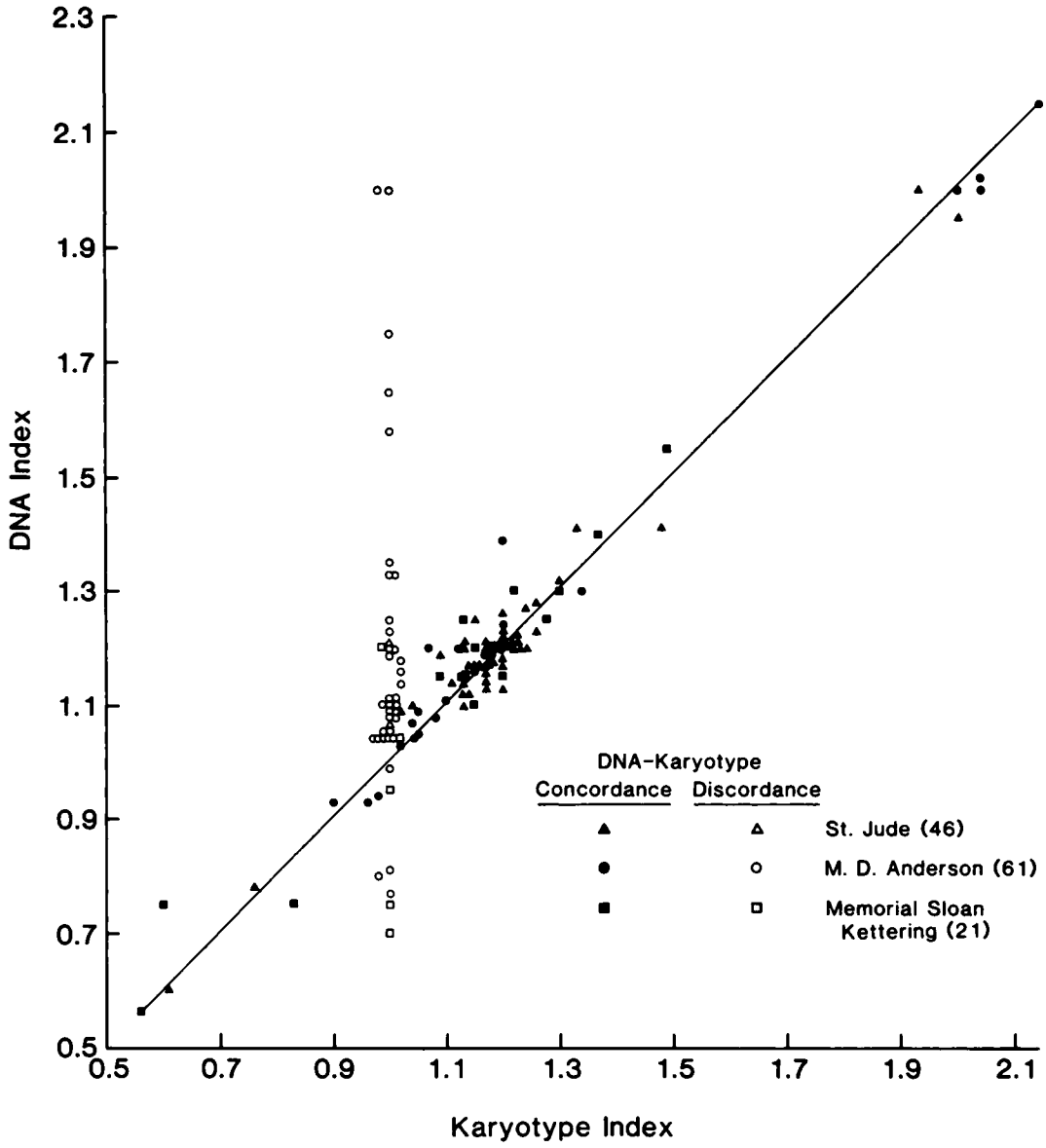

Chart 2. Relationship between DNA content and karyotype in 128 patients with acute leukemia, expressing an abnormal DNA stemline. The data are expressed as DNA index (ratio of modal G_1 DNA content of tumor cells *versus* diploid reference standard) and karyotype index (ratio of modal chromosome number and 46). There is a linear correlation for 85 patients between DNA and karyotype index values (●, ▲, ■). A DNA-karyotype discordance with striking abnormalities in DNA content in the presence of a normal diploid or near-diploid karyotype was observed in 43 patients (○, △, □). These 2 principal subsets of patients with abnormal DNA index were seen in separate studies from 3 different institutions (St. Jude, Thomas A. Look; MSKCC, Michael Andreeff; and MDAH, Bart Barlogie).

guishing Cancer Cells from Normal Cells. The occurrence of tumor cells with a near-diploid DNA content requires alternative neoplastic markers. Demonstration of a monoclonal expansion of cells with defined surface antigen in lymphoproliferative disorders is highly suggestive of neoplasia but can also be associated with profound immunoregulatory abnormalities.

Pathologists have known for over 100 years that the nucleoli are enlarged in actively growing cells and that they are prominent in cancer cells (154). Several investigators have conclusively demonstrated that nucleolar RNA synthesis, and specifically rRNA synthesis, is low in resting cells and increases in cells stimulated to proliferate (23, 39, 111). Biochemical and molecular-biological studies have led to a renewed interest in the role of the nucleolus in the control of cell proliferation and in neoplastic transformation, long suspected by morphological investigations (22). Busch *et al.* (40, 58) have thus recently demonstrated an almost universal expression in human tumors of a nucleolar antigen, which can be detected by means of indirect immunofluorescence with antibody raised against HeLa cell nucleoli. The tumor specificity of the nucleolar antigen has meanwhile been proven in many hundreds of cases of human neoplasia (59, 88, 159) and should therefore be of particular interest as a probe to discriminate DNA-diploid tumors by FCM. Preliminary studies in our laboratory indeed indicate the general feasibility of such measurements (Fig. 1) (19). In this example of correlated analysis of nucleolar antigen (indirect immunofluorescence with FITC-conjugated goat anti-rabbit IgG) and DNA (propidium iodide), hyperdiploid tumor cells express a higher nucleolar antigen den-

sity than do normal diploid marrow cells. In contrast to the well-defined abnormalities in DNA content, however, nucleolar antigen-related fluorescence is considerably more dispersed, thus accounting for overlap between normal and malignant cells. The discrepancy between microscopic results demonstrating nucleolar antigen expression exclusively in neoplastic lesions and quantitative FCM data, possibly lacking such specificity, may reflect differences in spatial distribution of nucleolar antigen density. Advances in FCM technology, particularly time-of-flight correlated fluorescence measurements (95), may help to examine spatial fluorescence distribution and thus improve the discrimination beween normal and malignant cells.

The above observations also stimulated our interest in further investigation of double-stranded RNA content by means of FCM analysis, using propidium iodide staining after DNA digestion (72). We have confirmed Frankfurt's earlier notion that double-stranded RNA content measured in this fashion is a function of cellular proliferation and is high in malignant compared to normal cells. Specifically, tumor cells localize propidium iodide fluorescence predominantly in the nucleoli, and our studies thus far have demonstrated markedly higher levels of double-stranded RNA in tumor cells compared to normal cells (Table 4).

Phenotypic Markers

Total RNA. Studies of RNA content can be conducted readily using either the metachromatic fluorescent dye acridine orange (169) or pyronine Y (158). The Sloan-Kettering group has published its large experience with acridine orange FCM (46, 55, 56, 57, 132), and Andreeff *et al.* (2) have introduced this method to distinguish AML from ALL. In cell separation studies of human marrow, we have found RNA content to be related to both cell type and proliferative activity, as previously reported by Darzynkiewicz for stimulated lymphocytes (17, 57, 109). Thus, a progressive increase in RNA content was noted in granulocytes-lymphocytes, erythroid precursors, myeloid precursors, and plasma cells, in this sequence.

On the basis of extensive observations in acute leukemia, Andreeff *et al.* (5) have proposed the distinction of leukemias according to their RNA phenotype and DNA genotype into mono- or biphenotypic, mono- or bigenotypic groups, a classification that is supported by the observation of terminal transferase activity in AML (121). In our own experience of several hundred cases of adult leukemia, RNA content analysis by acridine orange FCM did permit the distinction of myeloblastic from lymphoblastic leukemia in the majority of patients (19).

RNA mesurements have also been found to be useful for the distinction of low- and high-grade non-Hodgkin's lymphomas, both in the MSKCC and in the MDAH experience (4, 28). Of particular value, however, was acridine orange FCM for the distinction of myeloma plasma cells from the remaining normal marrow cells (17, 108). The additional information on RNA content allowed the quantitation also of DNA-diploid plasma cells (18). Indeed, we have recently demonstrated that the quantitation of marrow tumor involvement by acridine orange FCM in myeloma provides a direct assessment of tumor burden and, along with the level of RNA content, prognostic information for both remission induction and survival (9). Thus, high-RNA-content myeloma with low marrow plasmacytosis, *i.e.*, low tumor burden, identified a good risk group of patients.

Table 4

Double-stranded RNA excess in human neoplasms

Values are in relationship to normal donor lymphocytes, using propidium iodide after DNA digestion (see Ref. 72).

Diagnosis	No. of patients	Double-stranded RNA excess		% of patients with double-stranded RNA excess
		Mean	Range	
Leukemia	29	44	7–91	100
Multiple myeloma	16	37	3–83	100
Lymphoma	10	36	10–86	100
Solid tumors	6	36	8–94	100

Cytoplasmic Immunoglobulin. For multiple myeloma, the determination of cytoplasmic immunoglobulin as a characteristic feature of plasma cells can be performed readily by means of direct or indirect immunofluorescence (185). This approach is particularly useful for the classification of nonsecretory myeloma. In contrast to measurements of RNA content, cytoplasmic immunoglobulin determination holds the potential for better separation of DNA content-derived cell cycle distribution of tumor and normal marrow cells, due to the restriction of cytoplasmic immunoglobulin to plasma cells [and to pre-B-cells (see "Cell Surface Markers")].

Phenotypic Markers Related to Cell Size. A number of cellular properties are related to cell size, such as total protein and RNA content as well as forward-angle light scatter. In the case of myeloma, however, RNA content is predominantly an expression of the specialization of plasma cells for protein synthesis. The most direct measurement of cell size is afforded by electronic Coulter volume analysis which, until recently, has not been available in most commercially available flow cytometers (77). Studies by Shackney *et al.* (157), however, have demonstrated the usefulness of 2-parameter analysis of DNA content and Coulter volume for the distinction of low- and high-grade lymphomas. Lymphomas composed of cells with larger Coulter volumes were characterized by higher proliferative activity and corresponded to the higher-grade histological subtypes (156).

Forward-angle light scatter analysis is generally used as an approximative measurement of cell size (152) and has been quite useful, along with right-angle light scatter measurement for texture analysis, to distinguish peripheral blood cells and several major marrow lineages. In multiparameter studies using, in addition, lectin-binding properties, Nicola *et al.* (128) have demonstrated enrichment of hemopoietic stem cells by these parameters.

Cell Surface Markers. The rapid expansion of cell surface marker research and its application to normal and malignant hemopoiesis and lymphopoiesis have recently been reviewed (71, 114). While various T- and B-cell neoplasms have been successfully characterized according to their presumed normal cell origin along the differentiation pathway, similar studies of myeloid malignancies are still in their infancy (44). Among the lymphoid neoplasms, B-, pre-B-, and various T-cell lymphomas and leukemias have been identified (84, 116, 181). Mycosis fungoides and Sézary syndrome, more recently jointly classified as cutaneous T-cell lymphomas, generally express the helper T-cell phenotype (86), while T-cell ALL appears more heterogeneous in nature (25, 144). Bernard *et al.* (25) have noted differences between T-cell lymphoblastic lymphoma and T-cell ALL, in that the former disease generally expresses antigens more

common to early and common thymocytes, whereas in T-cell ALL at least one-third of patients have late thymocyte characteristics. Cytoplasmic IgM in ALL defines a separate group of children with pre-B-cell leukemia whose prognosis is comparable to that of patients with "null cell" or common ALL antigen-positive leukemia (180). FMC-7 has recently been found to distinguish prolymphocytic and chronic lymphocytic leukemia (42). Nodular lymphomas in general have high-density monoclonal surface membrane immunoglobulin and Ia, BA1, and B1 antigens (71). They commonly express the common ALL antigen, while most diffuse lymphomas do not (145). Similar to B-cell chronic lymphocytic leukemia, there is evidence for reactivity of nodular lymphomas with pan-T monoclonal antibodies (153). Fifty to 60% of large-cell lymphomas also show B-cell phenotypic characteristics, while approximately 15% have T-cell markers and 15 to 25% do not react with either T- or B-cell markers ("null cell"). Bloomfield *et al.* (29) have recently demonstrated that both B- and T-cell-type lymphomas recognized on morphological grounds, but not expressing immunological surface markers, have a poor prognosis compared to those with marker expression.

An interesting new application of surface membrane immunoglobulin analysis has been introduced by Ault *et al.* (8) demonstrating monoclonal light chain excess in peripheral blood lymphocytes from patients with malignant lymphoma. The exact nature of this phenomenon is unclear, but similar findings in our laboratory in myeloma coupled with the observation of circulating DNA abnormal cells (18) raise the interesting possibility that such monoclonal excess may be expressed on circulating tumor cells. In large-cell lymphomas, the disappearance of light chain excess in complete remission was associated with longer disease-free survival, whereas persistence of the abnormality was typical for nodular lymphomas (160).

Another new dimension of FCM analysis of surface markers has come with the therapeutic use of monoclonal antibodies for the treatment of lymphomas and leukemias (122, 123, 146, 148). Monitoring of antibody titer and of antibody binding to target cells can be accomplished and may provide invaluable therapeutic guidance. A related application pertains to monoclonal antibody treatment *in vitro* to remove residual leukemic cells from remission marrow before its use for autologous rescue after high-dose chemoradiotherapy (127, 147, 182). FCM analysis can be used to verify the efficacy of such purging procedures.

As is the case for other phenotypic markers, the biological and clinical relevance of cell surface marker studies is greatly enhanced by multiparameter FCM analysis and its ability to relate several different phenotypic characteristics to each other and to a tumor cell marker. This is perhaps the best approach of unraveling the full heterogeneity of a tumor cell phenotype and its relationship to the normal differentiation pathway, which has become so important in the understanding of the malignant lymphomas.

Hormone Receptors. A number of human tumors have been known to express hormone receptors, thus providing valuable guides for therapeutic intervention. Breast cancer is the most thoroughly studied human tumor for which the biological role of hormone receptor expression has been clearly established. Thus, compared to patients with ER-negative primary breast cancer, those with ER-positive tumors have a longer disease-free survival after radical mastectomy (99). Similarly, palliation of metastatic breast cancer with endocrine therapy can be accomplished in approximately 70% of ER-positive and in only <5% of the ER-negative tumors (120). The determination of ER quantity is derived from cytosol measurements, which provide information on average ER values representative of tumor tissue containing varying proportions of normal ER-negative cells and possibly tumor ER-negative subpopulations as well (91). With the availability of abnormal DNA content in approximately 90% of patients with breast cancer (140), biparametric FCM analysis of DNA and hormone receptor content should afford quantitative ER information per tumor cell. ER quantitation by fluorescence can be performed by different methods (for review, see Ref. 43). Studies at MDAH with FITC-conjugated estradiol (kindly provided by G. Barrows, Louisville, Ky.) (21) revealed the general feasibility of such measurements (Fig. 2) (143). We have been able to demonstrate that FITC-conjugated estradiol conforms to the requirements of a receptor ligand (43, 45) and appears to be a promising tool to study receptor heterogeneity, in relationship to different tumor cell genotypes, to tumor cell proliferation, and to the acute or chronic influence of cytotoxic or hormonal therapy.

Other Markers. There are a number of additional phenotypic parameters of potential biological and clinical interest that are amenable to FCM investigation, including carcinoembryonic antigen in colon and other tumors (27), α_1-fetoprotein in hepatocellular and germ cell tumors, and a variety of hormones accounting for paraneoplastic clinical syndromes associated with a number of human tumors, predominantly the "apudomas" (134). The field of cytoenzymology holds great promise for the understanding of tumor and normal cell heterogeneity (for review, see Ref. 65). Coupled with the measurement of time, Swartzendruber *et al.* (167) have recently demonstrated the feasibility of FCM analysis of enzyme kinetics.

Cytokinetic Markers

Extensive experimental studies *in vitro* and *in vivo* have demonstrated that the major cytokinetic determinants for tumor cell kill include growth fraction, cell cycle distribution, and cycle traverse rate (for review, see Refs. 11 and 68). While cell cycle distribution can be readily measured by FCM analysis of DNA content, functional studies of tritiated thymidine uptake are required to assess the proportion of slowly cycling or noncycling cells.

Growth Fraction. Recently, Darzynkiewicz *et al.* (54) have demonstrated the feasibility of cytochemical growth fraction analysis, using acridine orange to measure single- *versus* double-stranded DNA as a result of partial chromatin denaturation by acid or heat treatment *in situ*. Chromatin susceptibility to *in situ* denaturation was found to be greater in noncycling (and mitotic) than in cycling cells and accounts for a higher proportion of acridine orange-related red-green fluorescence in G_0 than in G_1. Such studies were initially performed on human lymphocytes under various culture conditions. We and others have successfully adapted this technique to routine laboratory use and demonstrated its feasibility to measure reproducibly the proliferative state of various human tumor cell lines in culture (142). Application of this method to the study of clinical specimens has been met with mixed success. Differential chromatin denaturation patterns were noted in 17 of 21 cases of various leukemias, either on fresh preparations (9 samples) or only after short-term culture (8 samples) (Chart 3) (19). Similar studies in multiple

myeloma and solid tissue neoplasms have not yet proved satisfactory, in that a separation into discrete subpopulations with acridine orange staining characteristics typical for cycling and quiescent lymphocytes was infrequently observed. Further physicochemical and biochemical data are needed pertaining to the role of chromatin and histone structural and chemical changes during the cell cycle, in order to explain inconsistencies observed in various laboratories applying the acridine orange DNA denaturation technique to the study of tumor growth kinetics.

Andreeff *et al.* (3) have recently reported that the differential chromatin denaturation method can also be used to distinguish more sensitive ALL from relatively more resistant myeloid disease.

Cycle Time. Quantitative determination of cytodynamics has become possible through the use of DNA precursors that modify fluorescence emission of DNA-specific fluorochromes. Thus, 5-bromodeoxyuridine incorporation quenches acridine orange-related green fluorescence and modifies fluorescence emission from other DNA stains (30, 53, 166). We have utilized this approach to measure the *in vitro* cycle traverse rate of marrow cells from patients with morphologically normal marrow and various types of leukemia (19). While not providing information on the *in vivo* unperturbed cycle times, such FCM measurements of fluorescence quenching confirm earlier studies utilizing radioisotope technology, in that in general normal marrow cells are characterized by shorter generation times than are marrow cells from patients with acute and chronic myeloid leukemia (163). Compared to observations on normal marrow, studies in a patient with hyperdiploid AML revealed a markedly reduced cycle traverse rate of residual diploid normal cells (19), a finding which is consistent with the recent evidence of suppression of normal hemopoietic cell proliferation by leukemic inhibitory activity released by myeloid leukemic cells (34).

Cell Cycle Stage Distribution. Autoradiographic studies have demonstrated that the *in vitro* tritiated thymidine pulse-labeling index provides a useful estimate of overall proliferative activity and has, at least in some authors' experience, also prognostic implications particularly in leukemia (for review, see Ref. 67). With the advent of DNA FCM as an expedient means for rapid cell cycle analysis (52), many investigators have pursued this application with the following objectives: (*a*) characterization of cytokinetic properties of various hematological and solid tissue cancers; (*b*) assessment of *in vivo* and *in vitro* cell cycle effects of various antitumor agents; and (*c*) more rational design of combination chemotherapy regimens. Having actively participated in all 3 areas ourselves, we conclude that DNA FCM has contributed greatly to the understanding of perturbation of cycle progression by antitumor agents and its relationship to lethal cellular effects (for review, see Ref. 10). On the other hand, cytokinetically directed antitumor therapy has met with little success, mostly due to oversimplification of cell synchronization and recruitment concepts, requiring more extensive experimental approaches including cytodynamic and clonogenicity measurements (for review, see Ref. 11). Prognostic studies, finally, have

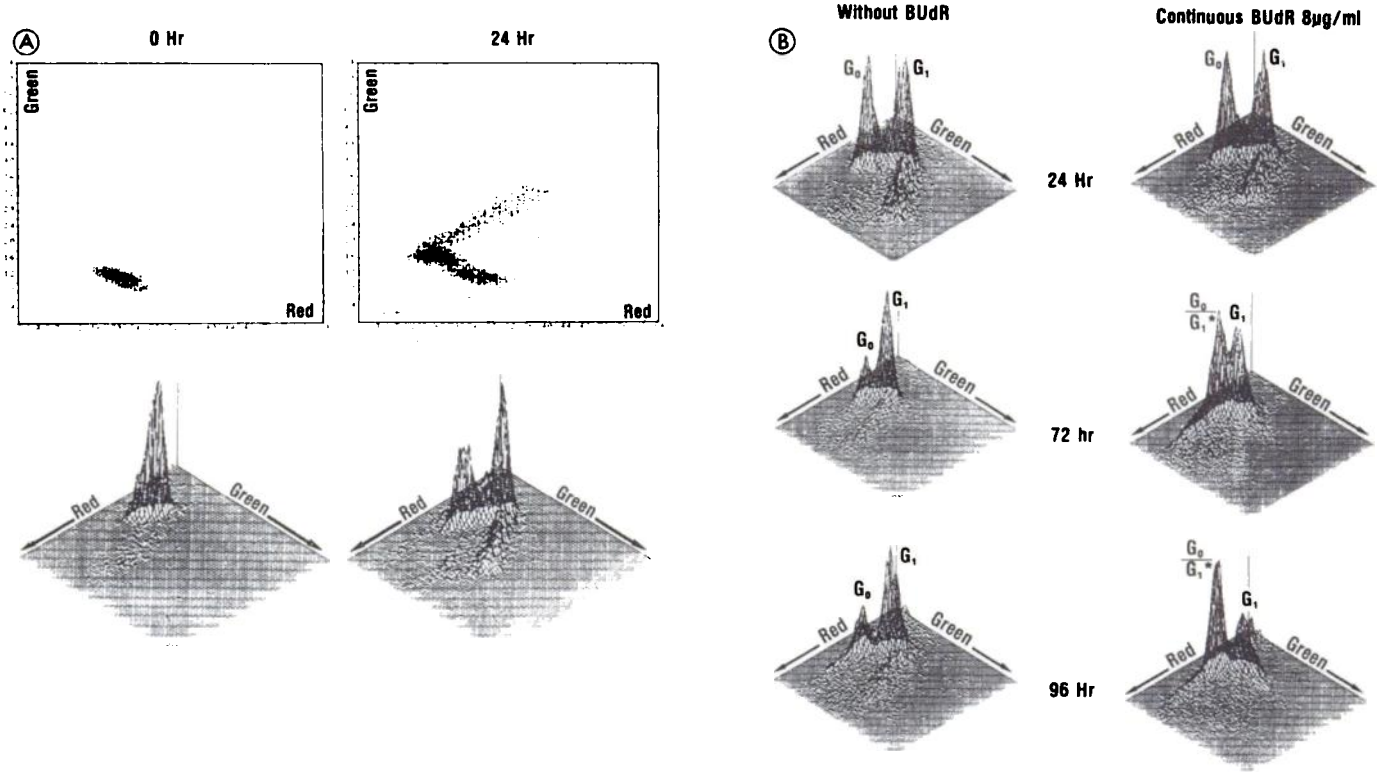

Chart 3. Acridine orange FCM of RNase-treated AML bone marrow cells following acid denaturation for growth fraction determination. *A*, effect of *in vitro* culture on red-green fluorescence distribution pattern. While the direct sample reveals a single-cell population, 24 hr culture in Roswell Park Memorial Institute Medium 1640 results in emergence of G_1 cells with higher green and lower red fluorescence intensity compared to the original sample. Also note the population of S-phase cells originating from the high-green-, low-red-fluorescence G_1 population. *B*, further evidence for $G_{1/0}$ discrimination by the acridine orange staining technique. In the continuous presence of 5-bromodeoxyuridine (*BUdR*), a marked redistribution of peak populations can be observed, resulting from quenched green fluorescence of cycling G_1 cells (G_1^*) which overlap with the G_0 compartment. This experiment provides direct evidence for the cytokinetic difference between the 2 populations with acridine orange staining characteristics typical for G_0 and G_1 lymphocytes (56).

been fraught with problems of sampling (marrow aspiration with variable admixture of peripheral blood) (66, 87), quality of measurement, and clinical trial design (comparability of patients and treatment) (11). Pertaining to the latter problem, it appears imperative to analyze cell cycle distribution data in light of the entire host of other already established prognostic factors.

A few important observations, however, have emerged from DNA FCM cytokinetic studies. In the area of malignant lymphoma, Braylan *et al.* (31), Diamond *et al.* (60, 62), and our own group (28, 83) have compiled evidence that measurement of the S-phase compartment size provides objective discrimination of low-, intermediate-, and high-grade lymphomas (Chart 4). There is not as yet sufficient experience available to assess whether pretreatment cycle stage distribution affects remission induction and duration in patients undergoing comparable therapy.

The prognostic implications of pretreatment kinetics are probably most controversial in acute leukemia (see Refs. 67 and 87). Using marrow biopsy material (66), we have recently found that indeed pretreatment S-phase compartment size affects remission induction, but only when analyzed in the context of patient age: whereas response rates were comparable for patients below and above age 50 in case of (S + G_2-M) ≤15% (65%), values >15% implied >90% likelihood of remission in young and only a 40% response rate in old individuals (16). In addition, early (S + G_2-M)-phase increment predicted prompt remission after one course of therapy. Low values of (S + G_2-M) percentage prior to treatment and high values in complete remission both favored prolonged remission duration in myeloblastic leukemia (16). Hiddemann *et al.* (87) have introduced a method to determine treatment-induced cytoreduction by determining the cell density per unit volume of pure marrow. The latter was computed by correcting for admixed peripheral blood in marrow aspirates, using differences in cell cycle distribution between marrow biopsy and aspirate and peripheral blood as well as differences in RBC hematocrits of marrow and blood from the same patient.

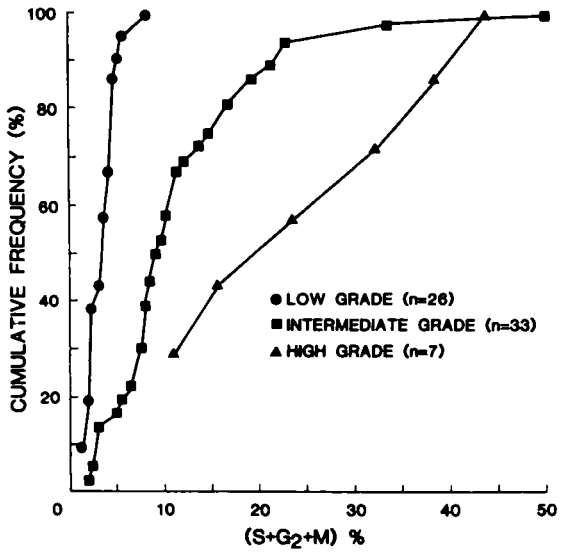

Chart 4. Cumulative frequency distribution of proliferative activity (S + G_2-M percentage) in non-Hodgkin's lymphoma. Low-grade histologies (diffuse well-differentiated lymphocytic, nodular poorly differentiated lymphocytic, nodular mixed lymphoma) (62) generally display values of (S + G_2-M) percentage ≤5 and can readily be distinguished from intermediate (diffuse poorly differentiated lymphocytic, nodular large cell, diffuse mixed, and diffuse large cell lymphoma) and high-grade (immunoblastic, undifferentiated, lymphoblastic lymphoma) lymphomas.

Cytokinetic studies in human solid tumors are currently under way in many institutions (see Ref. 106), and exciting results with regard to the diagnostic and prognostic implications of such measurements should be forthcoming soon.

Cellular Pharmacology and Biochemistry

Several investigators have reported on FCM analysis of fluorescent antitumor agents, particularly anthracyclines (102, 129, 161). Recent work by van den Engh *et al.* (174) suggests that FCM analysis can recognize differential uptake of various anthracyclines into different cell types. When coupled with a measurement of time (117), studies of cellular uptake rates and retention times should be feasible in the near future, thus possibly offering the potential of an *in vitro* drug sensitivity test. Of equal clinical relevance is the development of an assay to determine abrogation of reproductive capacity, *i.e.*, of cell kill, by means of FCM. We have preliminary evidence that DNA-reactive drugs such as *cis*-platinum may alter the sensitivity of chromatin to denaturation (63), which can be detected by FCM using acridine orange as a chromatin probe (19, 41). There was a linear correlation between the degree of *cis*-platinum-induced DNA cross-linking and increasing degree of resistance to chromatin denaturation, both of which in turn correlated with clonogenic survival. A similar correlation with reduction in clonogenic survival by various antimetabolites was observed by Bernal *et al.* using the mitochondrial probe Rhodamine 123 (24a).

Perspective

From the preceding discussion, it is apparent that FCM is a powerful tool in the transition from descriptive to quantitative cytology. Having reviewed the cellular markers suitable for FCM investigation of neoplasia, differentiation and proliferation, the following section presents a synopsis of the application of FCM to the major clinical problems of (*a*) diagnosis of neoplasia, (*b*) specific tissue diagnosis, (*c*) assessment of prognosis, and (*d*) selection or direction of treatment (Table 5).

Diagnosis of Malignancy. The almost universal presence of abnormal DNA stem lines in solid tissue cancers makes DNA FCM a powerful diagnostic tool in difficult areas of cytology, *e.g.*, bladder irrigates, effusions, sputum, and bronchial washings. Occasionally, disseminated neoplasia will be associated with marrow necrosis and pancytopenia, and the demonstration of an abnormal DNA stem line in the marrow and/or blood may clarify a diagnostic puzzle. Similarly, morphological assessment of residual disease after primary cytotoxic therapy is difficult and may be supplemented by DNA FCM. Persistence of aneuploid cells would constitute the need for further adjuvant therapy. Detection of a DNA marker in recurrent leukemia will be important in the understanding of the failure mechanisms involved in allogeneic bone marrow transplantation biology.

Alternative neoplastic markers such as double-stranded RNA (72) and nucleolar antigen (40, 58, 59) for the diagnosis of DNA-diploid disease are still undergoing investigation. Cell populations with high RNA index are highly suggestive of neoplasia, particularly of myeloma, although this feature can be expressed in reactive plasmacytosis as well (18). A low S-phase proportion in the marrow of an otherwise normal individual should raise the suspicion of pre- or oligoleukemia (17, 19).

Table 5
Perspectives

Problem	FCM parameter	Comment
Cancer diagnosis	DNA index	Abnormal in about 80% of solid tumors, 80% of myeloma, 50% of lymphoma, and 20% of leukemia
	Nucleolar antigen	To be examined as cancer marker for DNA-diploid disease
	Double-stranded RNA	Same; seems to identify residual leukemia in remission
Differential diagnosis	DNA index	Near-diploid in hematological cancers Higher values commonly in solid tumors Indolent *vs.* aggressive lymphoma Recurrence of same *vs.* second neoplasm
	RNA index	Distinguishes 70–80% of ALL *vs.* AML Identifies near 100% of myeloma
	S phase	Lower in active leukemia than in remission marrow Separates, with DNA index, indolent and aggressive lymphoma
	Clg[a]	Monoclonality identifies myeloma and pre-B-cell disease (IgM)
	Smlg	Monoclonality identifies B-lymphoma
	Monoclonal light chain excess	Suspicious of lymphoma
	T antigens	Identify T-cell lymphomas and leukemias
	TdT, ER, CEA, etc.	Under investigation
Prognosis	DNA index	High values; unfavorable for survival; exception (?), ALL Hypodiploid leukemia, myeloma with poor prognosis
	S phase	High values; favorable for response in ALL and AML less than 50 yr, unfavorable for response duration and survival
	RNA index	High values; favorable for response in myeloma, under investigation in leukemia
	Monoclonal light chain excess	Unfavorable during remission of aggressive lymphomas
Treatment	DNA index	Persistence in leukemia remission marrow: more treatment? Persistence in bladder irrigates or effusions; more treatment Selection of patients without abnormalities for autologous marrow support
	S phase	May become major criterion for treatment choice in lymphoma and other solid tumors
	RNA index	Low values in myeloma; investigational therapy
	Surface markers	Selection of monoclonal antibodies for treatment

[a] Clg, cytoplasmic immunoglobulin; Smlg, surface membrane immunoglobulin; TdT, terminal deoxynucleotidyl transferase; CEA, carcinoembryonic antigen.

Specific Diagnosis. A specific histological diagnosis can be made with some degree of certainty only in the lymphoproliferative disorders expressing a monoclonality of surface or cytoplasmic properties, such as immunoglobulin and B- or T-cell markers. High RNA index in marrow cells is typical for myeloma (18); intermediate values are seen in myeloid leukemia (2) and in cases of marrow involvement by aggressive lymphoma (4, 28). Relatively uniform and low RNA index values are commonly observed in ALL and chronic lymphocytic leukemia (2). High S-phase values in lymphomatous lymph nodes are consistent with a diagnosis of aggressive lymphoma, particularly when accompanied by an abnormal DNA stem line (28, 62). The presence of a high-degree-DNA-content abnormality in a bone marrow infiltrated with immature and cytologically unclassifiable cells makes a nonhematological cancer with marrow metastasis more likely (12, 19). Research for specific monoclonal antibodies recognizing melanoma and other tumors is currently under way and may in the near future enable FCM to provide a specific tissue diagnosis.

Because long-term disease control is no longer exceptional, late relapses of acute leukemia and solid tumors raise the question of second primary cancer *versus* reactivation of the original neoplasm. A different DNA index in the new lesion would be consistent with a second primary cancer, possibly induced by a different carcinogenic mechanism such as chemo- or radiotherapy.

Prognosis. Although extensively studied, the prognostic implications of various FCM-derived cellular parameters are only slowly emerging. This is a consequence of the heterogeneity of human tumors and rapidly changing treatment strategies. By and large, however, high S-phase and high DNA index values, seemingly even interrelated (15, 93, 115), have emerged as adverse prognostic factors for survival in lung (37, 93, 131), breast (132, 140), bladder (171), prostate (26, 172), and colon (184) cancer. Aneuploidy and high S-phase values are also likely to be important predictors of short survival in lymphoma (28, 31). Controversy exists with regard to the prognostic value for remission induction of pretreatment cytokinetic characteristics in acute leukemia. There may be age- and karyotype-related differences in the prognostic impact of high S-phase disease (16). For remission duration, low pretreatment S-phase seems to emerge as a favorable factor in leukemia (16), myeloma (108), and lymphoma (31). In myeloma, high RNA index and low marrow tumor infiltrate both are favorable features for both remission induction and survival (9). Thus, analysis of DNA and RNA content by one single fluorescent dye, acridine orange, provides considerable insight into the cellular heterogeneity of several human neoplasms and as such seems to account for some of the observed heterogeneity in their clinical behavior.

At a time when cancer therapy regularly produces long-term disease control in some tumors including Hodgkin's disease, lymphomas, leukemias, germ cell tumors, and breast cancer, more attention is focused on host tissue toxicity. In this regard, the studies by Evenson *et al.* (69, 70) probing the chromatin function of sperm cells as a potential quantitative tool to assess male fertility deserve wider exploration.

Treatment Design. If the aforementioned prognostic factors are confirmed, they can be used for treatment stratification. Serial studies during a patient's disease course of DNA index, cytokinetics, etc., may be helpful in delineating residual disease and thus determining the need for further therapy. Many pilot studies have been conducted to gauge chemotherapy according to drug-induced perturbation effects on cell cycle progression (for review, see Ref. 11). Although they have not demonstrated a superiority over empirically chosen schedules, we see a role for such investigations, when DNA FCM for assessment of cell cycle distribution can be routinely coupled with measurements of cycle traverse rate (53) and growth fraction (54). These studies should be helpful for optimization of scheduling of drugs such as high-dose 1-β-D-arabinofuranosylcytosine and methotrexate. Likewise, strategies of normal host tissue protection by agents preferentially blocking normal cells can be investigated (11).

Cellular pharmacology studies by FCM analysis of fluorescent drugs such as anthracyclines may gain importance as *in vitro* drug sensitivity assays, if a correlation between *in vitro* cellular drug uptake and *in vivo* chemotherapy sensitivity can be demonstrated.

The development of a FCM assay for hormone receptor anal-

ysis should be of great help in determining the feasibility of pharmacological manipulation of hormone receptor expression and/or of cell cycle kinetics in an effort to maximize the antitumor effect of subsequent hormonal or chemotherapy.

FCM monitoring of tumor cell contamination of bone marrow to be used for autografting should become a routine part of transplantation research. FCM analysis of surface membrane characteristics of tumor cells will be an indispensable tool in programs using monoclonal antibodies for *in vitro* or *in vivo* therapy (146–148). Similarly, evaluation of blood lymphocyte subsets by FCM will be necessary to monitor the efficacy of biological response-modifying agents aimed at restoring immunodeficiency or imbalance.

For any of the above applications of FCM to become routine clinical practice, standardization of cell processing, staining, and FCM analysis is mandatory. Efforts in this direction have been initiated at the recent Society of Analytical Cytology meeting in Elmau, Germany, in 1982. In addition, close interaction between pathologists and clinical oncologists is vital to establish firmly the role of FCM as a prognostic tool aiding in treatment selection. This requires prospective studies of FCM parameters along with established prognostic factors in a defined patient population undergoing comparable therapy. Such investigations are currently under way at major cancer centers. When complete, the results should form the basis for determining whether or not research-oriented clinical laboratories can justifiably take on FCM as a routine cytological tool.

In summary, an objective and quantitative approach to the elucidation of an individual patient's cancer has become possible since the introduction of automated cytology permitting quantitative measurements of tumor genotypic and phenotypic properties, in analogy to a "SMA profile" in laboratory chemistry. Together with advances in humoral tumor marker research facilitating the quantitative assessment of tumor burden, FCM appears to be an expedient means to determine relevant tumor and host cell features underlying the heterogeneity of human cancer in terms of different metastatic spreading patterns and differences in therapeutic response, thus hopefully identifying patients with a high prospect for cure with available treatment and pointing the direction for treatment research to control notoriously unresponsive disease. Investigation of premalignant disorders and recognition of cellular features associated with the transition to overt malignancy may provide clues for cancer prevention. At a time of exciting progress in the areas of tumor virology (78) and molecular genetics (24, 118, 119), it is hoped that by FCM distinctive phenotypic tumor features can be identified that relate to more fundamental abnormalities in gene expression present in cancer *versus* normal cells.

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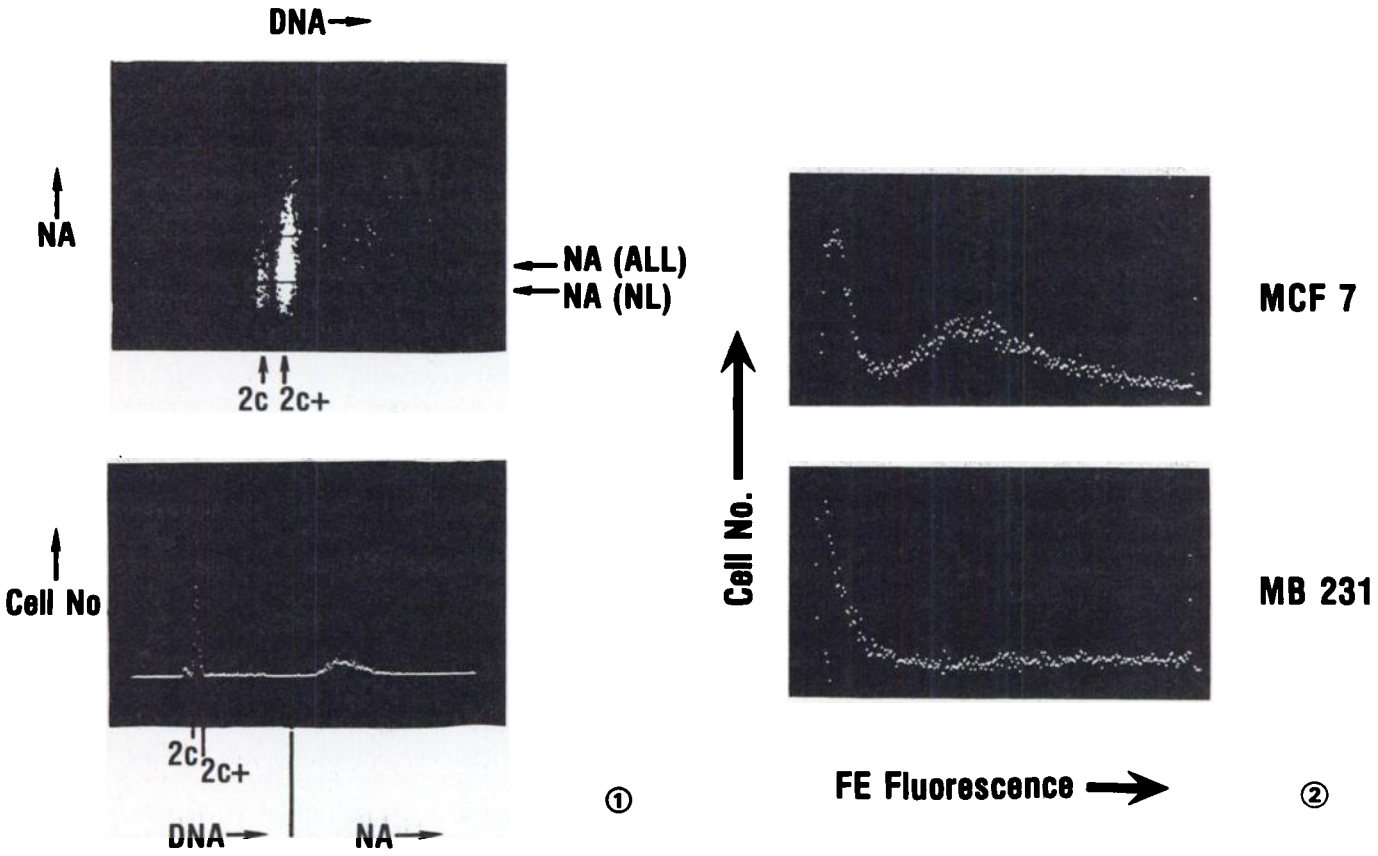

Fig. 1. Correlated FCM analysis (FACS II) of DNA (propidium iodide) and nucleolar antigen (*NA*) (indirect immunofluorescence with FITC) in a case of hyperdiploid ALL. *Top*, scattergram; *bottom*, 2-dimensional histograms. Note the markedly higher nucleolar antigen expression in hyperdiploid tumor (*2C+*) compared to diploid normal (*NL*) hemopoietic cells (*2C*).

Fig. 2. FCM assessment of ER density in human breast cancer cell lines, utilizing FITC-conjugated estradiol (*E-FITC*) at a concentration of 10^{-10} M which is in the range of the K_d value (10^{-9} M) for the type I receptor. Note the striking difference between cytosol-positive MCF-7 and negative MB-231 cell lines.