Flow Cytometry in Clinical Cancer Research

Barthel Barlogie, Martin N. Raber, Johannes Schumann, Tod S. Johnson, Benjamin Drewinko, Douglas E. Swartzendruber, Wolfgang Gohde, Michael Andreeff, and Emil J. Freireich

The University of Texas System Cancer Center M. D. Anderson Hospital and Tumor Institute [B. B., M. N. R., T. S. J., B. D., E. J. F.], Houston, Texas 77030; University of Munster Medical School, Munster, Germany [J. S., W. G.]; University of Colorado, Colorado Springs, Colorado 80907 [D. E. S.]; and Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [M. A.]

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 hydridoma-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 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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2 To whom requests for reprints should be addressed.
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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 cytogenetic 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 G1-G0 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, Chart 1 depicts cumulative frequency distributions of DNA index values in lymphoma (Chart 1A), in leukemia and myeloma (Chart 1B), and in solid tumors (Chart 1C). 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 hypo- 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 1C 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 1C) (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

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4 J. Schumann, unpublished observations.
Table 1

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<td>Vindelov et al. (179)</td>
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<td>Tribukait et al. (172)</td>
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<td>Lower response rate to hormone treatment in aneuploid tumors</td>
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<td>Klein et al. (98)</td>
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<td>Klein et al. (97)</td>
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<td>Comparable results in biopsies and washings</td>
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<td>Linden et al. (112)</td>
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<td>Skin squamous cell cancer</td>
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<td>82</td>
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<td>Schumann et al. (96)</td>
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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 hypodiploid 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

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Table 1—Continued

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<th>Comments</th>
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<td>Sarcomas</td>
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<td>Schumann et al. (155)</td>
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<td>Higher rate of relapse in aneuploid tumors</td>
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<td>Only 70 not previously listed</td>
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<td>80</td>
<td></td>
<td>Schumann et al. (155)</td>
</tr>
<tr>
<td>All solid tumors</td>
<td>3611</td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grand total</td>
<td>4941</td>
<td>67</td>
<td>Includes hematologic neoplasms</td>
<td></td>
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* CALLA, common acute lymphocytic leukemia antigen; ANLL, acute nonlymphocytic leukemia; FCC, follicular center cell.
* Unpublished observations.

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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 hyperoctoploidy 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 (83).
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, $\pm 2\%$), 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 G0 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 G0 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-
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 between 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 Andreff 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-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 measurements 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.

**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- and 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 (96), 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 charac-
teristics. 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 la, BA1, and B1 antigens (71).
They commonly express the common ALL antigen, while most
diffuse lymphomas do not (145). Similar to B-cell chronic lymph-
phocytic 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 character-
istics, 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 expres-
sion.

An interesting new application of surface membrane immuno-
globulin analysis has been introduced by Ault et al. (8) demon-
strating monoclonal light chain excess in peripheral blood lym-
phocytes 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 therapeu-
tic guidance. A related application pertains to monoclonal anti-
body treatment in vivo 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 en-
hanced 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 availa-
bility 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 infor-
mation 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 dem-
onstrate that FITC-conjugated estradiol conforms to the require-
ments 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 an-
tigen in colon and other tumors (27), α-fetoprotein in hepatocel-
lar and germ cell tumors, and a variety of hormones account-
ing for paraneoplastic clinical syndromes associated with a num-
ber of human tumors, predominantly the “apudomas” (134). The
field of cytoenzymology holds great promise for the under-
standing 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 demon-
strated 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 re-
quired 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 anal-
ysis, 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 G3 than in G1.
Such studies were initially performed on human lymphocytes
under various culture conditions. We and others have success-
fully adapted this technique to routine laboratory use and dem-
onstrated its feasibility to measure reproducibly the proliferative
state of various human tumor cell lines in culture (142). Applica-
tion 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 inef
curately observed. Further phys
ticochemical 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 dena
turation 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 dis
ease.

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-relat
ed 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 radio-

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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 cyto
dynamic 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 G1 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 G1 population. B, further evidence for G1-G2 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 G1 cells (G1) which overlap with the G2 compartment. This experiment provides direct evidence for the cytokinetic difference between the 2 populations with acridine orange staining characteristics typical for G0 and G1 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 + G2-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 + G2-M)-phase increment predicted prompt remission after one course of therapy. Low values of (S + G2-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.

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 antime tabolites 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).


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Table 5

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<th>Problem</th>
<th>FCM parameter</th>
<th>Comment</th>
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| Cancer diagnosis | DNA index     | Abnormal in about 80% of solid tumors, 80% of myeloma, 50% of lymphoma, and 20% of leukemia (2) 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 node biopsies are consistent with a diagnosis of aggressive lymphoma, particularly when accompanied by an abnormal DNA stem line (28, 62). The presence of 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 of remission induction of pretreatment cytogenetic 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, and 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-
y 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 immuno-nodificiency 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.

Acknowledgments

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References

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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 $10^{-8}$ M which is in the range of the $K_d$ value ($10^{-8}$ M) for the type I receptor. Note the striking difference between cytosol-positive MCF-7 and negative MB-231 cell lines.
Flow Cytometry in Clinical Cancer Research

Barthel Barlogie, Martin N. Raber, Johannes Schumann, et al.


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