Flow Cytometric Analysis of DNA Content of Bone Marrow Cells in Patients With Plasma Cell Myeloma: Clinical Implications

By Paul A. Bunn, Jr., Steven Krasnow, Robert W. Makuch, Mark L. Schlam, and Geraldine P. Schechter

DNA content analysis by flow cytometry was performed in 32 patients with plasma cell myeloma and 3 patients with Waldenstrom's macroglobulinemia to determine the biologic and potential clinical usefulness of this technique. Hyperdiploid tumor DNA content was found in 23 myeloma patients (72%) during the course of illness, including 16/28 at presentation, but in none of 3 patients with Waldenstrom's macroglobulinemia. There was no significant association of aneuploidy in myeloma patients with age, sex, race, or M-protein class. Myeloma patients with aneuploid tumor cells were more likely to have advanced stage \( (p = 0.032) \) than patients with diploid plasma cells, and all patients with renal failure had aneuploid tumors. Pretreatment factors significantly associated with survival included stage \( (p = 0.01) \), serum creatinine \( (p = 0.003) \), and tumor DNA content \( (p = 0.005) \). Multivariate analysis using the Cox life table regression procedure indicated that the significant relation of tumor DNA content with survival remained after adjusting for stage \( (p < 0.005) \). Myeloma patients with diploid tumors at diagnosis frequently had aneuploid plasma cells at the time of relapse, indicating a possible relationship of chromosomal alterations in the tumor to clinical drug resistance. We conclude that aneuploid tumor cells at the time of diagnosis of myeloma are of independent prognostic significance, and the development of aneuploidy is a frequent occurrence at clinical relapse, suggesting the changes in DNA content are of biologic and clinical significance.

FLOW CYTOMETRY (FCM) can rapidly analyze multiple cellular parameters including DNA content independently of cell proliferative state. This technique has recently been employed in the study of a variety of lymphoproliferative malignancies. In the acute leukemias and non-Hodgkin's lymphomas, 10%--20% of tumors have abnormal DNA content, while a larger percentage have abnormal RNA content. There is mounting evidence in the lymphoproliferative malignancies that aneuploidy is associated with unfavorable histology and a more aggressive clinical course. FCM can also analyze the fraction of cells in various stages of the cell cycle \( (G_0, S, G_2 + M) \), and it has been suggested that the fraction of cells in S-phase correlates well with the expected clinical aggressiveness of lymphoproliferative malignancies. The proliferative rate of myeloma has been measured previously only by autoradiographic techniques. There is evidence that high pretreatment labeling indices predict a poor prognosis in myeloma patients.

Cytogenetic studies in multiple myeloma have been limited by the low proliferative fraction in most patients, particularly at diagnosis. The sensitivity of FCM in documenting DNA content in myeloma cells was first shown by Latreille et al., who demonstrated aneuploidy in the bone marrow sample of 67% of all the myeloma patients studied and 76% of patients with “active” disease. Serial studies were performed, however, in a minority of cases. In this report, we describe the results of FCM studies of tumor DNA content in bone marrow samples of 32 patients with multiple myeloma and 3 patients with Waldenstrom's macroglobulinemia. These investigations include serial studies in nearly all patients, and the results are correlated with the patients' clinical course.

MATERIALS AND METHODS

Patients

The diagnosis of a plasma cell malignancy was established by the criteria of the Chronic Leukemia and Myeloma Task Force. The median age of the patients was 60 yr, with a range of 31--76 yr. There were 23 males and 12 females; 28 patients were white and 7 were black. FCM analyses of cellular DNA distribution in 95 bone marrow aspirations from these 35 patients were performed. Thirty-one patients (28 with myeloma) were studied at diagnosis prior to treatment and 4 patients were studied first at relapse (Table 1). Studies after initial drug therapy were repeated in 23 of the 28 myeloma patients studied at diagnosis and 10 of these 28 patients were studied following relapse of disease. Serial studies were also performed on 3 of the 4 patients first studied at relapse. Patients studied after drug therapy had not been treated within 3 wk of FCM analysis. The clinical staging classification of Durie and Salmon was used to define the extent of disease. Criteria of response ( \( \geq 50\% \) decrease in M-protein levels) and progression after therapy were those of the Chronic Leukemia-Myeloma Task Force. All symptomatic patients were initially treated with an intermitted schedule of melphalan and prednisone. The majority of patients were treated on a protocol that included an alternating four-drug regimen (vin-cristine, cyclophosphamide, adriamycin, prednisone) with the melphalan and prednisone at 15-wk intervals. A plateau phase was defined as stable M-protein levels (\( \leq 23\% \) increase or decrease) for at least 3 mo.
Statistical Considerations

Remission duration and survival were calculated from the first day of treatment, or from the date of diagnosis for untreated patients, using the product limit method of Kaplan and Meier. Differences in remission duration and survival between groups were compared using the log rank test. The Cox life-table regression method was used to evaluate the effect of tumor DNA content and stage of disease on survival in a multivariate manner. All significance tests were based on a chi-square analysis, and all p-values in this report are two-sided.

Sample Collection

Bone marrow needle aspirates from all patients and peripheral blood specimens from two patients were collected in preservative-free heparin and subjected to Ficoll-Hypaque density separation. The mononuclear cells from the interface layer were collected and washed with calcium-free and magnesium-free phosphate-buffered saline (PBS, Gibco, Grand Island, N.Y.).

Flow Cytometry

For immediate DNA analysis, cells were stained with 5 mg/dL propidium iodide (Sigma, St. Louis, Mo.) in 0.1% sodium citrate by the method of Krishan. When DNA analysis was to be delayed, the cells were fixed in 50% ethanol and stored at 0°C–4°C. Immediately prior to analysis, fixed cells were stained with 5 mg/dL propidium iodide after RNase treatment by the method of Crissman. The DNA content of 50,000–100,000 cells was measured in a Coulter TPS-I cell sorter (Coulter Electronics, Hialeah, Fla.) with an exit orifice diameter of 75 μ. Peripheral blood mononuclear cells obtained from normal donors were used as diploid standard cells. The electronics of the instrument were adjusted so that the modal channel of the diploid standard cells was 40 (128 total channels). Subsequently, the sample was analyzed with the same instrument settings. Whenever two discrete peaks were present a mixture of the diploid standard lymphocytes and sample cells were stained and analyzed together to determine the DNA content of aneuploid cells relative to the diploid standard (Fig. 1). In these instances, the DNA index, defined as the ratio of the modal channel number of the aneuploid peak to the modal channel of the diploid peak, was calculated.

The coefficient of variation (CV) for the G1 peak was determined for each sample from the formula CV = HM × 100/V × 2.35, where HM is the width of the G1 peak at half-maximum and V is the modal channel number of the G1 peak. Over the course of this study, the CV varied from 2.0 to 5.6 (average 4.0) for the diploid standard and 2.1–6 for the bone marrow samples. Cell cycle stage distribution analysis of the DNA distribution was conducted by a planimetric integration method. The aneuploid fraction of cells was derived by dividing the number of aneuploid G1 cells by the number of aneuploid plus diploid G1 cells.

Table 1. DNA Content Abnormalities in Myeloma Patients by Time of Study

<table>
<thead>
<tr>
<th>Time of Study</th>
<th>No. of Patients</th>
<th>No. Aneuploid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis*</td>
<td>28</td>
<td>16 (57)</td>
</tr>
<tr>
<td>Remission</td>
<td>23</td>
<td>9 (39)</td>
</tr>
<tr>
<td>Relapse (progression)</td>
<td>14</td>
<td>11 (79)</td>
</tr>
<tr>
<td>Total†</td>
<td>32</td>
<td>23 (72)</td>
</tr>
</tbody>
</table>

*Prior to treatment. All 3 patients with Waldenstrom's macroglobulinemia had diploid tumor cells.
†Many patients were studied sequentially.

RESULTS

Frequency of Aneuploid Tumor Cells

Hyperdiploid DNA content (aneuploidy), indicated by two distinct G1 populations, was detected in 23 of 32 (72%) myeloma patients and 0/3 Waldenstrom's patients over the course of this study (Fig. 1, Table 1). Aneuploidy was detected in all phases of illness; at diagnosis, during chemotherapy-induced remission, and most frequently, in patients with progressive disease. At the time of diagnosis, 16 of 28 myeloma patients (57%) had hyperdiploid tumors, and at the time of relapse or progressive disease, 11 of 14 (79%) were hyperdiploid. Nine of 23 (39%) patients who had...
In 13 patients with aneuploid tumors who had sequential studies, response to therapy could be monitored by FCM. Overall, at diagnosis, an average of 40% of bone marrow cells were aneuploid (36% in responders and 51% in nonresponders to chemotherapy). On entering a plateau phase, the average fell to 8% (range 0%-16%), with 8/9 responders showing substantial decreases in the aneuploid fraction. In the single responding patient without a substantial reduction in the aneuploid fraction, there were 12% aneu-

Abnormalities in DNA content were always hyperdiploid. Hypodiploid DNA contents were not observed. The median DNA index was 1.25, a DNA index in excess of 1.35 was found in only 5 patients (Fig. 2). In 2 patients with plasma cell leukemia, the bone marrow and peripheral blood myeloma cells had the same abnormal DNA index. Another patient developed leptomeningitis with cerebrospinal fluid plasma cells having the same abnormal DNA index.

Correlation of FCM With Clinical Course

In 13 patients with aneuploid tumors who had sequential studies, response to therapy could be monitored by FCM. Overall, at diagnosis, an average of 40% of bone marrow cells were aneuploid (36% in responders and 51% in nonresponders to chemotherapy). On entering a plateau phase, the average fell to 8% (range 0%-16%), with 8/9 responders showing substantial decreases in the aneuploid fraction. In the single responding patient without a substantial reduction in the aneuploid fraction, there were 12% aneu-
ploid cells at diagnosis and during the plateau, and the aneuploid fraction doubled at the time of relapse. In 3 patients, aneuploid cells were no longer detectable following chemotherapy-induced remission. One of these patients subsequently relapsed with a hyperdiploid population identical in DNA content to the original tumor (Fig. 3). The 4 nonresponders had little or no change in the fraction of aneuploid cells, except for one patient who had a 25% reduction in M-protein level and a decrease in the aneuploid fraction from 28% to 12%.

Sequential studies were also obtained in 3 of the 4 myeloma patients first studied at relapse. Following chemotherapy, 1 of these 3 had a 25% reduction in M-protein accompanied by a decrease in the aneuploid fraction (58% → 26%), one had no change in M-protein level or aneuploid fraction (22% → 20%), and one had a progressive increase in M-protein and aneuploid fraction (27% → 45%).

Single parameter DNA content analysis was not useful in following response to chemotherapy for patients with diploid tumors until the patients relapsed. Four of six relapses in these patients were associated with the new development of aneuploidy (Fig. 4). The increased fraction of patients with relapse or progressive disease who had aneuploidy is due in part to the development or detection of these new aneuploid clones.

Association of Aneuploidy With Other Prognostic Factors

In 28 myeloma patients with pretreatment DNA analysis, the presence of hyperdiploidy was associated with renal failure and advanced disease stage but not with other patient characteristics (Table 2). Using the Durie-Salmon staging classification we found that only 1 of 8 patients with low or intermediate stages (I and II) had aneuploidy, whereas 15/20 with advanced stage (III) disease had aneuploidy (p < 0.01). All 5 patients presenting with renal failure (stage B, creatinine ≥ 2.0 mg/dl) had aneuploid G1 populations. There were no significant differences with respect to age, sex, or race for those with or without aneuploid G1 populations at diagnosis. All heavy and light chain types were represented in both the aneuploid and diploid patients. Although aneuploidy was more frequent in patients with IgA heavy chains, this association was not statistically significant.

The results of DNA content analysis by FCM appeared to have prognostic importance in regard to length of remission and survival (Fig. 5). Patients with hyperdiploid myeloma cells at diagnosis had significantly shorter survival than those with only diploid or near diploid cells (p = 0.005). Excluding the 2 patients with "indolent myeloma," who were not treated, the responding patients with aneuploid tumors had significantly shorter chemotherapy-induced remission durations (p = 0.004), which may account for their shorter survival. When the survival analysis is restricted to patients with stage IIA or IIIA disease, survival times for aneuploid patients were also shorter than for patients with diploid tumors (p = 0.052).

Table 2. Associations Between FCM Results and Clinical Characteristics at Diagnosis

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>DNA Content</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diploid (n = 12)</td>
<td>Aneuploid (n = 16)</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>59 (32–73)</td>
<td>58 (29–76)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>7/5</td>
<td>10/6</td>
</tr>
<tr>
<td>Race (W/B)</td>
<td>10/2</td>
<td>12/4</td>
</tr>
<tr>
<td>Stage</td>
<td>I and II</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>A‡</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0</td>
</tr>
</tbody>
</table>

*All p-values derived from Pearson chi-square analysis adjusted for continuity where appropriate, except age for which the Wilcoxon rank sum test was used.
†NS, not significant at p = 0.40 level.
‡A, serum creatinine < 2.0 mg/dl; B, serum creatinine ≥ 2.0 mg/dl.
Other pretreatment factors that were significantly related to survival were stage \((p = 0.01)\) and creatinine levels \((p = 0.003)\). Stage III patients did worse than stage I and II patients combined; the same held true for patients with elevated \((\geq 2.0 \text{ mg/dl})\) creatinine levels. The M-protein levels, serum calcium and hemoglobin were not significantly related to survival \((p > 0.1)\).

Using Cox’s multivariate life table regression analysis, we found tumor DNA content remained significantly associated with survival after accounting for stage of disease \((p < 0.005)\). The association between the stage of disease and survival did not retain its significance once tumor DNA content was included \((p = 0.49)\), indicating that tumor DNA content was a useful prognostic factor of survival for this study population, which contributed significant information above that provided by stage of disease.

Objective response following chemotherapy with melphalan and prednisone was higher in myeloma patients with diploid cells \((10/11)\) than with hyperdiploid cells \((10/15)\), however, this difference is not significant.

Our treatment protocols call for discontinuation of chemotherapy after 14–20 mo for patients whose disease has reached a stable plateau phase. Another measure of the prognostic importance of aneuploidy is to examine the duration of these unmaintained remissions. When all patients are included in this analysis, patients with aneuploid tumors had significantly shorter duration of unmaintained remission \((p = 0.004)\). Chemotherapy could only be discontinued in 6/12 at risk with aneuploid tumors, whereas all patients with diploid tumors had a period without chemotherapy. When only those patients who had any period of unmaintained remission were included for analysis, the same conclusion holds.

The fraction of cells with abnormal DNA content was compared to the fraction of plasma cells in the same marrow sample independently counted by light microscopic examination of Wright-Giemsa stained smears of the marrow aspirate. The results are shown in Fig. 6 and show a relatively close correlation \((r = 0.648)\). Inspection of this figure, however, reveals that while the fraction of aneuploid cells never exceeded the fraction of plasma cells by more than 10%, there were many instances where the percentage of plasma cells far exceeded that of aneuploid cells. This strongly suggests that malignant plasma cells of two DNA classes (near-diploid and hyperdiploid) are present in some patients.

**DISCUSSION**

We have shown that the majority of patients \((23/32)\) with multiple myeloma develop hyperdiploidy during the course of their illness. The fraction of patients
with hyperdiploid tumors varies with time. At diagnosis, 57% had hyperdiploid tumor cell populations. The frequency decreased during remission due to clearing of marrow plasma cells and increased at relapse due to development of new aneuploid clones. The overall frequency of aneuploidy, the frequency of aneuploidy in remission and at relapse are similar to the report of Latreille et al.9 The slightly higher frequency of aneuploidy at diagnosis in their report appears to be due in part to the increased sensitivity of their instrumentation where more narrow coefficients of variation were routine, or to differences in the prognostic features of the patient groups.

In this report we have demonstrated that the DNA histograms provide clinically useful information. Myeloma patients with tumor cells having diploid or near diploid DNA content at the time of diagnosis have a more indolent clinical course than patients with hyperdiploid DNA contents as evidenced by significantly longer remission duration, significantly longer survival, and higher response rates (not significant). Myeloma patients with hyperdiploid cells were also significantly more likely to have advanced stage, and all 5 patients with a serum creatinine ≥ 2.0 mg/dl had hyperdiploid myeloma cells. While stage and creatinine levels were also significantly associated with survival time, multivariate analysis indicated that the significant relation of tumor DNA content with survival length remained after adjusting for disease stage.

DNA content analysis was useful in following response to treatment, particularly in patients with aneuploid tumors, where the fraction of aneuploid cells always fell with remission and increased with progressive disease. In patients with diploid myeloma cells, the development of progressive disease and resistance to chemotherapy was associated with the development of new aneuploid clones in 4 of 6 instances.

Durai et al. have recently reported that patients with a low labeling index have superior survival compared to patients with a high labeling index when considering all patients or patients with high cell mass. Unfortunately, single parameter DNA analysis did not permit determination of the S fraction in patients with diploid tumors or in the majority of patients with aneuploid DNA content with near diploid values (DNA index < 1.3). The future use of dual parameter FCM studies employing cytoplasmic immunofluorescence and nuclear DNA measurements may allow for independent quantitation of the proliferative fraction of the normal diploid cells and the diploid and/or aneuploid myeloma population.20 Such studies may also prove useful for distinguishing the effects of chemotherapy on the normal and malignant marrow populations.

Because of the low proliferative fraction, standard cytogenetic techniques are frequently unsuccessful in the majority of patients with myeloma at diagnosis.23 The chromosomal abnormalities reported in the literature include pseudodiploid changes with 46 chromosomes containing structural abnormalities in 11% of cases, hypodiploid and hyperdiploid chromosome numbers in 26% and 44% of cases, respectively, and both hypodiploid and hyperdiploid cells were reported in 19%. DNA content analysis from this study and others8 shows that aneuploidy is present in the majority of patients from the time the diagnosis is established and that hypodiploidy is uncommon. The discrepancies may be explained in part by the presence of large chromosomes and the inability to obtain metaphases from the malignant plasma cells.

There is considerable evidence to suggest clonal heterogeneity within the malignant plasma cell dyscrasias. We have demonstrated that a new hyperdiploid population developed at relapse in at least 4 patients. The finding of a disproportionately small fraction of aneuploid cells when compared to the number of plasma cells in several other patients suggested the presence of multiple stem lines in these patients as well. Latreille et al. found 4/123 patients with multiple myeloma had multiple hyperdiploid stem lines.9 Clonal heterogeneity as defined by FCM has been found in other tumors as well, including small cell lung cancer.
and non-Hodgkin's lymphomas. It is likely that the frequency of heterogeneous DNA stem lines is underestimated by present techniques, since clones representing as much as 30% of a population can be missed if the difference in DNA content from normal is small. Additionally, clones smaller than <1% of the population cannot be detected even with widely separated G1 peaks. The emergence of drug-resistant clones, as seen in several of our patients, may be a general phenomenon and may explain the inability of chemotherapy to "cure" tumors that initially appear responsive.

The results of this study must be put into the context of our current treatment policies for multiple myeloma and other lymphoreticular malignancies. Systemic chemotherapeutic and/or radiotherapeutic approaches are not curative in myeloma nor in patients with advanced favorable histology non-Hodgkin's lymphomas. It has been suggested that these therapies can be withheld in at least some patients with non-Hodgkin's lymphomas until severe symptoms intervene.

Therapeutic studies in multiple myeloma suggest that different approaches might be preferred for patients with "good" risk versus "poor" risk characteristics. For protocol study purposes, DNA content analysis by FCM may be another useful parameter for defining patient risk categories. FCM may also prove to be useful in determining patients most likely to benefit from unmaintained remissions. Alexanian and coworkers have shown that patients with low stage and/or with significant remissions (>90% reduction in M-protein levels) have the longest periods of unmaintained remission. Our results show that unmaintained remissions are also longer in patients with diploid tumors than in patients with aneuploid tumors.

ACKNOWLEDGMENT

The authors are indebted to Dr. John D. Minna for helpful suggestions and to Judy Arriaga for typing the manuscript.

REFERENCES


Flow cytometric analysis of DNA content of bone marrow cells in patients with plasma cell myeloma: clinical implications

PA Jr Bunn, SKrasnow, RW Makuch, ML Schlam and GP Schechter