TUMOR CYTOGENETICS and cellular proliferative activity encompass important intrinsic features of neoplastic cells. We have previously reported that the analysis of cellular DNA content by flow cytometry readily provides quantitative information on cell ploidy as well as on cell cycle distribution of both normal and malignant cells. Abnormalities of cellular DNA content (hereafter referred to as “ploidy abnormalities”) were found with increasing frequency in leukemia, lymphoma, multiple myeloma, and in solid tumors in this order. We stressed the independence of flow cytometric ploidy measurements from proliferative activity, since cells with a G0 DNA content served as the target cell population for such determinations. In this article, we evaluate cellular DNA and RNA content and tritiated thymidine labeling index in patients with monoclonal gammopathies in order to define the biologic significance of intrinsic tumor cell properties such as ploidy and proliferative activity and to relate these findings to clinical and other laboratory parameters of recognized prognostic importance.

MATERIALS AND METHODS

One-hundred and forty-three patients with monoclonal gammopathy consenting to bone marrow aspiration for DNA flow cytometry were studied between June 1978 and June 1980. The study population included 127 patients with multiple myeloma, of whom 47 patients were studied at diagnosis, 32 at relapse, 36 during primary treatment failure, and 12 at the time of remission. We employed diagnostic, staging, and treatment criteria previously reported by Alexanian et al. Treatment consisted of melphalan-prednisone alone or in combination with cyclophosphamide, Adriamycin, vincristine, and nitrosoureas, according to various Southwest Oncology Group protocols. Seven patients with Waldenström’s macroglobulinemia were treated with chlorambucil and prednisone. Nine patients fulfilled the criteria of benign monoclonal gammopathy.

While DNA flow cytometric examination was performed on all patients, the following additional laboratory studies were largely limited to patients with multiple myeloma. Thus, combined DNA/RNA analysis was available in 18 patients. Autoradiographic determination of the plasma cell tritiated thymidine labeling index was performed in 50 patients at diagnosis and in 5 patients with benign monoclonal gammopathy.

Two to three milliliters of heparinized bone marrow were collected from the posterior iliac crest and subjected to Hypaque-Ficoll gradient separation (density, 1.078 g/ml) as previously described, washed once in 0.9% NaCl, and counted in an electronic particle counter. A cytocentrifuge preparation stained with May-Grünwald-Giemsa was used for differential counts. For ploidy analysis by DNA flow cytometry, ethanol-fixed cells were stained with ethidium bromide and mithramycin and measured in an ICP 11 flow cytometer (Ortho Instruments, Boston, Mass.) as previously described. The degree of ploidy abnormality was expressed as DNA index, representing the ratio of modal channel numbers of tumor cells and normal granulocytes. Bivariate analysis of DNA and RNA contents was accomplished on acridine orange-stained cells with an ICP 22 flow cytometer (Ortho Instruments, Boston, Mass.) as previously reported. For quantitation of RNA content, a RNA index was derived that represents the ratio of modal channel numbers of target G1,0 cells and unstained human lymphocytes.

For plasma cell labeling index determination, another aliquot of the Hypaque-Ficoll buffy coat cell suspension was incubated for 1 hr at 37°C in RPMI 1640 growth medium (Grand Island Biological Co., Grand Island, N.Y.) containing tritiated thymidine at a final concentration of 5 μCi/ml (specific activity, 6.7Ci/mM), washed, and cytocentrifuged. Autoradiographic processing involved dipping in Kodak NTB 2 emulsion (diluted 1:3 in distilled water), exposing for 24 hr, developing by standard techniques, and staining with May-Grünwald Giesma. Between 200 and 400 plasma cells were

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scored, and the percentage of cells containing more than 5 silver grains overlying the nucleus determined the labeling index.

Statistical analysis involved the use of a generalized Wilcoxon test, and survival curves were constructed according to the method of Kaplan and Meier.

RESULTS

Ploidy Analysis by DNA Flow Cytometry

Figure 1 illustrates a representative DNA distribution histogram of bone marrow cells from a patient with multiple myeloma. There are 2 closely adjacent cell subpopulations with different DNA content, corresponding to the G₁₀₀ cells of normal and malignant cells. The incidence of ploidy abnormality in 143 patients with monoclonal gammopathy was 66% (Table I). Aneuploidy was noted in 4 of 9 patients with benign monoclonal gammopathy and in 80 of the 115 patients with active multiple myeloma. In contrast, all 12 patients with multiple myeloma in remission and the 7 individuals with Waldenström's macroglobulinemia had a diploid DNA content. Ninety-six percent of the 115 patients with active multiple myeloma had unimodal tumor cell populations; 4 individuals had 2, and 1 patient had 3 distinct subpopulations with abnormal DNA content. DNA index values for the 110 patients with unimodal tumor cell populations ranged from 0.9 to 1.95, with a mean value of 1.15 (Fig. 2). The majority of patients (83%) had DNA index values clustering between 1.0 and 1.2. The ploidy level of the 4 patients with aneuploid benign monoclonal gammopathy ranged from a DNA index value of 1.05 to 1.37 and thus matched the general distribution pattern observed in the multiple myeloma population.

For the myeloma population, there was an excellent correlation between the percentage of cells with abnormal DNA content and the fraction of morphologically identifiable plasma cells (Fig. 3, r=0.84). There were, however, 3 individuals with an exceedingly higher percentage of cells with abnormal DNA content, documented during repeat observations in 2 individuals, the exclusion of whom improved the correlation coefficient significantly to r=0.96. We also noted a close correlation between the fraction of morphologically identifiable plasma cells (range 3%-20%; mean 9%) and the proportion of aneuploid G₁₀₀ cells in the 4 patients with aneuploid benign monoclonal gammopathy (4%-20%; mean 8%).

![Fig. 1. Representative DNA distribution of bone marrow cells from a patient with multiple myeloma. Note the hyperdiploid tumor G₁₀₀ population with a DNA index of 1.2. Normal human granulocytes were used to identify the diploid nature of G₀ cells peaking in channel 30.](image-url)
Serial observations in 36 patients with active multiple myeloma did not disclose a change in DNA index values (Table 2). Patient follow-up is still too short to comment on the stability of DNA index values during the development of drug resistance. There was, however, no significant difference in the DNA index distribution between the groups of patients studied at diagnosis and at relapse (Table 3). We likewise cannot yet comment on the incidence of diploid conversion with the attainment of complete remission in previously aneuploid myeloma.

**DNA/RNA Flow Cytometry**

Biparametric analysis of DNA and RNA content was effected on bone marrow specimens from 18 patients with multiple myeloma and from 1 patient with reactive plasmacytosis during chemotherapy for acute myeloid leukemia. Bone marrow plasmacytosis exceeded 15% in all and 30% in 12 patients. Figure 4 illustrates both scattergram and 3-dimensional displays of the DNA/RNA frequency distribution in a case of Bence-Jones multiple myeloma at diagnosis. The hyperdiploid tumor $G_{1/0}$ population is characterized by a markedly higher RNA content (RNA index = 6) compared to normal diploid bone marrow cells (average RNA index = 2.5). In this example, there is complete separation of tumor and residual normal hemopoietic cells, so that individual cell cycle distributions can be readily appreciated. RNA index values of plasma cells were readily determined in 13 of 18 patients with ploidy abnormalities (DNA index $\neq 1$) and ranged from 5.2 to 18 with a mean value of 10.8, thus considerably exceeding modal RNA content values of residual normal bone marrow (1.8–4.0, mean 2.5) (Fig. 5A). Discrete high RNA index subpopulations were also recognized in the 5 patients with diploid myeloma (all of whom had $>20\%$ bone marrow plasmacytosis) (Fig. 5B). The RNA index in this group ranged from 3.8 to 16.0, with a mean value of 10.0. We also had the opportunity to perform DNA/RNA flow cytometry on a bone marrow sample from a patient with acute myeloid leukemia with reactive plasmacytosis (Fig. 6). There was a well defined subpopulation of diploid cells with a high modal RNA index of 8 and thus clearly outside the range noted for morphologically normal marrow and for normal cell subpopulations in myeloma (see Fig. 5). Thus, bone marrow DNA/RNA flow cytometry identified plasma cell populations in all 18 cases with multiple myeloma and in the 1 case with reactive plasmacytosis.

**Cytokinetic Studies**

While DNA content measurements provide both ploidy and cell cycle distribution data, cytokinetic information on individual cell subpopulations is complicated even for those cases with a DNA content marker because of overlap of cell cycle compartments of normal and abnormal cells. Therefore, only autoradiographic data were included in this analysis. Such data were available in 50 patients with multiple myeloma at diagnosis and in 5 patients with benign monoclonal gammopathy. The plasma cell labeling index for patients with multiple myeloma ranged from 0%–36%, with a mean of 2.1%, whereas it varied from 0%–1.7%, with a mean of 0.5%, in the benign monoclonal gammopathy group.

**Relationship of Ploidy and Plasma Cell Labeling Index to Clinical and Laboratory Disease Parameters in Multiple Myeloma**

Examination of relationships among laboratory parameters under investigation in this report revealed...
a trend for higher plasma cell labeling index in the higher DNA index group (DI > 1.15; \( p = 0.18 \)). With regard to the relationship of study parameters to other laboratory and clinical parameters of established prognostic significance, a plasma cell labeling index >1% was associated with significantly higher albumin-corrected serum calcium concentrations (\( p = 0.01 \)).

We failed to find any other correlation between autoradiographic or DNA flow cytometric data and clinical or laboratory parameters of multiple myeloma.

**Prognostic Significance of Ploidy and Plasma Cell Labeling Index in Multiple Myeloma**

The magnitudes of pretreatment plasma cell labeling index and DNA index did not affect rate and duration of remission. Survival, however, was significantly longer in patients with labeling index values \( \leq 1\% \) compared to the remainder of patients with labeling index values >1% (Fig. 7; median of 62 versus 28 mo, \( p = 0.009 \)). Of the 50 patients with pretreatment labeling index determination, concurrent DNA index values were available in 23 individuals. Because of the previously noted lack of change in individual DNA index values within patients (Table 2) and in DNA index distribution between groups studied at diagnosis and at relapse (Table 3), DNA index values of 21 additional patients with active disease were included to permit a combined analysis of the prognostic impact of both labeling and DNA index (Fig. 8). Except for the subgroup of patients with DNA index values of 1.01–1.15, labeling index values >1% significantly decreased survival in the remaining DNA index groups (\( p = 0.019 \)).

According to classical kinetics, the tumor growth fraction decreases with increasing tumor burden. We have therefore examined the relationship between plasma cell labeling index as a potential representative of overall tumor proliferative activity and tumor mass computed from laboratory and radiologic data.7,17

**Table 2.**

<table>
<thead>
<tr>
<th>No. of Patients with Multiple Observations</th>
<th>No. of Observations per Patient</th>
<th>Time Interval in Months between Observations</th>
<th>No. of Patients with Change in DNA Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>Mean</td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>36</td>
<td>2–14</td>
<td>3</td>
<td>1–15</td>
</tr>
</tbody>
</table>

**Table 3. DNA Index Distribution at Diagnosis and Relapse**

<table>
<thead>
<tr>
<th>Time of Study</th>
<th>No. of Patients</th>
<th>Percent Distribution of Patients With DNA Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \leq 1 )</td>
<td>1.01–1.15</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>47</td>
<td>26</td>
</tr>
<tr>
<td>Relapse</td>
<td>32</td>
<td>25</td>
</tr>
</tbody>
</table>

\( p = 0.63 \)
While there was no relationship between tumor burden and plasma cell labeling index (and DNA index), there was an independent impact of both labeling index and tumor burden on survival duration (Fig. 9). Finally, multivariate regression analysis revealed that survival duration was determined by tumor mass (high versus intermediate and low) \( (p = 0.022) \), plasma cell labeling index (\( \leq 1\% \) versus \( >1\% \)) \( (p = 0.045) \), and by DNA index (1.01–1.15 versus \( \leq 1.0 \) and \( >1.15 \)) \( (p = 0.086) \) in this order of significance. The following hazard function was derived:

Fig. 4. DNA/RNA frequency distribution of bone marrow cells from patient with kappa light chain multiple myeloma. The hyperdiploid tumor G\(_{1,0}\) population has a markedly higher RNA content (RNA index = 6) compared to normal diploid bone marrow cells (RNA index = 2).

Fig. 5. RNA index of plasma and normal marrow cells according to DNA index. In both DNA index groups (A: aneuploid, B: diploid), the plasma cell RNA index considerably exceeds that of the remaining normal cells.

Fig. 6. DNA/RNA distribution of marrow sample from a patient with reactive plasmacytosis in the course of remission induction for acute myelogenous leukemia. Note the cell population with a high modal RNA index of 8, which falls in the range noted for diploid and aneuploid myeloma (see Fig. 5).
100'

75.

50

CS

0

'C.

'5

25

No. of Patients

Total

Dead

32

5

18

11

50

16

25

Alive

48

Months

72

dcx.

48

Fig. 7. Survival duration in 50 patients with multiple myeloma according to pretreatment plasma cell labeling index.

\[ \ln \left( \frac{\lambda(t,p)}{\lambda_0(t)} \right) = 1.73 \times (\text{tumor mass} - 1.41) \]
\[ - 1.25 \times (\text{labeling index} - 1.57) \]
\[ + 1.09 \times (\text{DNA index} - 2.61) \]

where \( \frac{\lambda(t,p)}{\lambda_0(t)} \) ratio expresses the instantaneous risk of death; where high tumor mass equals 1 and intermediate plus low tumor mass equals 2; where labeling index \( \leq 1\% \) equals 1 and labeling index >1\% equals 2; where DNA index (1.01–1.15) equals 2 and other values equal 1.

DISCUSSION

In a large patient population, we have confirmed our previous results indicating that cellular DNA content abnormalities are a frequent feature of the active phase of multiple myeloma. The excellent correlation between the proportion of morphologically identifiable plasma cells and the fraction of \( G_{10} \) cells with abnormal DNA content in 110 samples indicates that the DNA content difference relates directly to the plasma tumor cells in question. The discrepancy in 3 patients displaying a significantly higher proportion of cells with abnormal DNA content remains unresolved. We have previously hypothesized that we might measure morphologically nonidentifiable plasma cell precursors or that other normal-appearing bone marrow cells may also be involved in the malignant disease process. Compared to our investigations on solid tissue malignancies, multiple myeloma (and benign monoclonal gammopathies) were characterized by a low-degree hyperdiploid abnormality in a fashion similar to that previously reported for adult acute leukemia and non-Hodgkin's lymphoma. The stability of DNA content abnormality in a limited number of patients studied serially and the lack of shift in DNA index distribution in a comparison of patients studied at diagnosis and at relapse strongly suggest that cellular DNA content is a stable marker of
malignancy, not reflecting the development of drug resistance. This observation has been previously reported in solid tissue malignancies as well as in acute leukemia.2,4

The absence of DNA content abnormalities in Waldenström's macroglobulinemia is noteworthy and is similar to our findings in chronic lymphocytic leukemia, where we noted exclusively diploid DNA contents among 28 patients studied (Barlogie, unpublished results).

Of particular interest is the observation of ploidy abnormality in 4 of 9 patients with benign monoclonal gammopathy. In an extensive review of 241 patients with monoclonal gammopathy of undetermined significance, Kyle noted a definite progression into overt myeloma, macroglobulinemia, or amyloidosis in only 11%.5 Our patient population also included 8 patients fitting the category of indolent or smouldering myeloma,17,18 and of these, 75% had an aneuploid DNA index. In view of the tumor cell marker-like properties of abnormal DNA index in hematologic and solid tissue neoplasms,4,12 the observation of aneuploidy in benign monoclonal gammopathy and in indolent myeloma may help to identify those patients definitely carrying the potential for disease progression into overt multiple myeloma. The observation of aneuploidy in "benign" plasma cell dyscrasias is reminiscent of data previously published in patients with preleukemia and smouldering leukemia.2,12

While measurements of cellular DNA content provide cell cycle distribution analysis, information on plasma cell proliferative activity cannot be derived in cases of diploid myeloma because of sample heterogeneity, nor in case of the majority of aneuploid myelomas because of overlapping cell cycle distributions originating from normal and tumor cells. Biparametric investigation of DNA and RNA contents suggests that the additional separation of plasma cells
<table>
<thead>
<tr>
<th>No. of Patients</th>
<th>Tumor Mass</th>
<th>Labeling Index (LI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>High</td>
<td>( \leq 1% )</td>
</tr>
<tr>
<td></td>
<td>Intermediate + Low</td>
<td>( \leq 1% )</td>
</tr>
<tr>
<td>19</td>
<td>High</td>
<td>( \leq 1% )</td>
</tr>
<tr>
<td></td>
<td>Intermediate + Low</td>
<td>( \leq 1% )</td>
</tr>
<tr>
<td>7</td>
<td>High</td>
<td>( &gt; 1% )</td>
</tr>
<tr>
<td></td>
<td>Intermediate + Low</td>
<td>( &gt; 1% )</td>
</tr>
<tr>
<td>11</td>
<td>Intermediate</td>
<td>( &gt; 1% )</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>( &gt; 1% )</td>
</tr>
</tbody>
</table>

Fig. 9. Survival in multiple myeloma according to pretreatment labeling index and tumor mass. Note the independent impact of plasma cell labeling index on survival duration, regardless of tumor burden.

on the basis of a significantly increased RNA content compared to the remainder of marrow cells\(^{13}\) may permit individual cell cycle analysis of plasma cells and residual marrow cell populations. It remains to be seen whether the magnitude of plasma cell RNA content relates to disease activity and to prognosis. The finding of a similarly elevated RNA content in a case of reactive plasmacytosis indicates that RNA content per se cannot be used as a marker of multiple myeloma. This is also true for the generally increased RNA content in acute myeloid leukemia, since cell separation experiments revealed a similarly increased content in normal myeloblasts.\(^{12}\)

A limited number of patients with multiple myeloma had pretreatment tritiated thymidine labeling index studies performed. Plasma cell labeling index did not correlate with myeloma tumor burden and did not predict for the incidence and duration of remission. In a representative group of patients with multiple myeloma, we did, however, find a significant impact of pretreatment labeling index on survival, regardless of pretreatment tumor burden. These data largely confirm a recent report of Durie et al.\(^{19}\) There was, however, a subgroup of patients with DNA index values of 1.01–1.15 whose survival did not appear to be affected by the magnitude of the labeling index. The biologic importance of a significant correlation between plasma cell labeling index and degree of
hypercalcemia in the absence of a correlation to tumor mass is not understood. It may reflect a higher incidence of patients with hypercalcemia in the poor prognosis group with high labeling index. The available information in 5 patients with benign monoclonal gammopathy revealing lower labeling indices is in keeping with their long benign course without evidence of disease progression, as has been previously noted by Durie et al.19

Through technical advances in the area of multiparameter flow cytometry, it has been possible to identify myeloma plasma cells on the basis of both DNA and RNA content.14 As has been illustrated in this article, combined DNA and RNA analysis may permit a separate description of cellular kinetics of normal and myeloma tumor cells by automated cytology, thus avoiding more tedious and subjective autoradiographic technology. Another direction of plasma cell identification employs the use of fluorochrome-tagged heavy and light chain antibodies.20 Like RNA content, such measurements per se will not permit distinction of malignant from benign plasma cells. Thus, we are currently evaluating the feasibility of nucleolar antigen determination by flow cytometry, so that an independent marker of malignancy may be available for the minority of patients with diploid multiple myeloma.21

In conclusion, the results of our present study indicate that differences in myeloma tumor burden at diagnosis are not reflected in differences at the genetic level or in the degree of tumor cell proliferation. Yet, both proliferative activity and DNA index appear to have independent prognostic implications.

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