Human Myeloma In Vitro Colony Growth: Interrelationships Between Drug Sensitivity, Cell Kinetics, and Patient Survival Duration

By Brian G. M. Durie, Laurie A. Young, and Sydney E. Salmon

Ninety-seven patients with multiple myeloma evaluated serially had both a tritiated thymidine labeling index of bone marrow plasma cells (LI%) and in vitro myeloma stem cell culture performed. Thirty-three patients with myeloma colony growth had in vitro drug sensitivity testing carried out. Eighteen had a thymidine suicide assessment. The LI% and the likelihood of in vitro myeloma colony growth were highly correlated: the higher the LI%, the more likely was colony or cluster growth ($p < 0.001$). The tritiated thymidine suicide of myeloma stem cells was usually very high. There was excellent correlation between in vitro and in vivo drug sensitivity. Both pretreatment drug resistance and selective sensitivity (e.g., interferon, bisantrene, methotrexate, vincristine) at the time of relapse were accurately detected and correlated well with survival duration ($p = 0.01$ Wilcoxon). Although LI% and in vitro sensitivity were clearly independent variables, a high LI% ($>3\%$) plus in vitro resistance were associated with a subsequent survival duration of $<6$ mo. The studies allowed dissection of the complex interrelationship between cell kinetics and drug sensitivity.

A system has been developed that permits the growth of human myeloma cells in soft agar. With this direct in vitro colony assay it has proved possible to measure the drug sensitivity of clonogenic myeloma cells to anticancer drugs. The further application of this technique has stimulated the more detailed assessment of the kinetics of myeloma colony growth both in terms of the likelihood of myeloma colony growth and the relationship to drug sensitivity and patient survival.

This article describes the results of measurements of the tritiated thymidine labeling of bone marrow myeloma cells, the tritiated thymidine suicide index of myeloma colonies and clusters, in vitro drug sensitivity of cells that give rise to clonogenic myeloma cells, and correlations with response to treatment and survival duration.

MATERIALS AND METHODS

A total of 106 patients with plasma cell myeloma as defined by the Chronic Leukemia-Myeloma Task Force of the National Cancer Institute were studied. All patients had baseline staging and follow-up as previously reported. Bone marrow aspiration was performed at least 6 wk following pulse chemotherapy and from sites not currently or previously irradiated. Bone marrow samples were divided into aliquots for comparative studies of the tritiated thymidine labeling index, soft agar culture (including thymidine suicide) and drug sensitivity studies. Ninety-seven patients evaluated serially had both a tritiated thymidine labeling index of the plated cells and agar culture performed. Eighteen had a thymidine suicide assessment, and 33 had in vitro drug sensitivity testing. Only patients who had clinical trials that were evaluable for response, as well as sufficient in vitro data, are included for drug sensitivity analysis. Drug sensitivity results on some of the patients included in this series have been reported in part previously. Nine patients had in vitro drug sensitivity testing subsequent to the serial evaluation of 97 consecutive patients with respect to LI% and in vitro growth. Previously untreated patients with multiple myeloma received bifunctional oral alkylating agents singly or in combination. Our standard protocol for patients in relapse from oral alkylating agents was with carmustine (BCNU) 30 mg/sq m combined with Adriamycin 30 mg/sq m. The Southwest Oncology Group criteria of response were used: a complete response required a 75% reduction in tumor mass as well as definite improvement in other clinical features. A partial response required a 50%-74% tumor mass reduction plus clinical improvement.

Collection of Cells

After informed consent was obtained, bone marrow cells were aspirated into a syringe containing preservative-free heparin. A single cell suspension was then prepared as previously described.

Tritiated Thymidine-Labeling Index Studies

Labeling index studies were performed on aliquots of the single cell suspensions prepared for soft agar culture. Heparinized bone marrow cell suspensions ($0.5 \times 10^6$-$1.0 \times 10^6$ cells/ml of cell suspension) were incubated for 1 hr at 37°C with high specific activity (40-60 Ci/m mole) tritiated thymidine (dose, 5.0 $\mu$Ci/ml of cell suspension). Cytocentrifuge smears were made on gelatin-coated slides and fixed with methanol. The tritiated thymidine-labeling index was then determined using our previously published high-speed scintillation autoradiography method with control comparison to the standard method. The amount of 2,5-diphenyloxazole (PPO) used in the scintillator solution was 5 g/500 ml of dioxane.

Soft Agar Culture

The culture system has previously been described in detail. In brief, cells to be tested were suspended in 0.3% agar in enriched CMRL 1066 medium, supplemented with 15% horse serum to yield a final concentration in the range of $2-5 \times 10^3$ cells/ml. Freshly prepared 2-mercaptoethanol was added at a concentration of $5 \times 10^{-5} M$ immediately before triplicate plating of the cells. One milliliter of the mixture was pipetted into a 35-mm plastic Petri dish containing conditioned medium in a 1-ml agar feeder layer. Because the plating efficiency in myeloma was $0.01\%-0.2\%$, a plating

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Blood, Vol. 61, No. 5 (May), 1983: pp. 929-934
concentration of 5 × 10^3 cells was normally used. Cultures were incubated at 37°C in 5% carbon dioxide in humidified air. Colonies (collections of more than 30 cells) appeared in 10–21 days, and plates were counted for drug effects after 14–21 days, with use of an inverted phase microscope.

Direct staining of the plates for peroxidase was used to identify occasional contaminating granulocyte-macrophage and eosinophil colonies present in the bone marrow culture. At least 30 tumor colonies/plate were required in the control plates to assure an adequate range for measurement of drug effect. Drug effects were scored only for reduction in the number of colonies.

Control studies for development of the drug assay were carried out with the 8226 human myeloma cell line.10 In experiments with melphalan, a 1-hr exposure was optimal, with increasing lethality at increasing doses. Repeated drug assays with this line initiated on different days yielded similar survival curves.

Examination of Cells in Colonies

Individual colonies were aspirated with a fine capillary pipette and suspended in a drop of heat-inactivated fetal calf serum and dried on slides. Myeloma colonies were stained with the Wright-Giemsa method, 0.5% orcein in 60% acetic acid, and methyl green pyronin, and for peroxidase, nonspecific esterase, plasma-cell acid phosphatase activity, and cytoplasmic immunoglobulin.2

Determination of Percentage of Cells in DNA Synthesis by 3H-TdR Suicide

The 3H-TdR suicide method was used to measure the proportion of myeloma colony-forming cells in the DNA synthetic (S) phase of the cell cycle.11 Samples of 2 × 10^4 cells suspended in HBSS and 10% heat-inactivated FCS were added to 1.0 ml of HBSS containing 40 μCi of 3H-TdR (23 Ci/m mole, Amersham Searle, Arlington Heights, IL). Control samples were added to HBSS. Cell suspensions were incubated for 60 min at 37°C and washed twice with 20 ml of cold HBSS containing 100 μg/ml of unlabeled thymidine and 10% FCS. Each suicide and control suspension was cultured in 4 replicate plates at a concentration of 5 × 10^4 cells/plate.

In Vitro Exposure of Myeloma Cells to Drugs

The drug assay procedure has been reported previously.7 Stock solutions of intravenous formulations of melphalan, carmustine, adriamycin, vinblastine, methotrexate, vincristine, and other agents were prepared in sterile buffered saline or water and stored at −70°C in aliquots sufficient for individual assays. Subsequent dilutions were made in saline for cell incubation. Myeloma cells were suspended at 4 × 10^5 cells/ml in the presence of the appropriate drug dilution or control medium. Each drug was tested at a minimum of 3 dose levels, including low concentrations that we calculated (from pharmacokinetic data) to be achievable pharmacologically in vivo. Final concentration ranges (in μg/ml) were 0.038–0.37 for melphalan, 0.08–2.72 for carmustine, 0.006–0.370 for adriamycin, 0.001–0.90 for vincristine, 0.05–5.66 for vinblastine, 0.01–0.1 for cisplatinum, 0.000–0.1 for vindesine, 0.04–4.16 for methotrexate, 0.1–1.0 for bisantrene, 0.01–0.1 for mitoxantrone, and 50–500 U for leukocyte interferon. The bifunctional alkylating agent, cyclophosphamide, is inactive in vitro, and therefore, melphalan was used as the index bifunctional alkylating agent in the in vitro studies and was assumed to have similar effects to other mustard-like alkylating agents, including cyclophosphamide. Cells were incubated with drugs for 1 hr at 37°C in Hank's balanced salt solution. The cells were then centrifuged at 150 g for 10 min, washed twice in the balanced salt solution, and prepared for culture.

Data Analysis

The laboratory technologist entered and stored all assay data on a Wang 2200 C laboratory computer disk file. Cloning efficiencies were calculated from the total number of cells plated and not corrected for the proportion of nontumor cells in the sample. The standard error of the mean for individual data points averaged 5% of the mean.

A quantitative “sensitivity index” of myeloma stem cells to drugs was determined graphically from linear survival by drug concentration curves. The area under the curves was calculated for each patient to a defined upper concentration. The upper concentration was specified as 0.1 μg/ml (1 hr) for melphalan and vincristine and 0.2 μg/ml (1 hr) for the remaining drugs except for leukocyte interferon for which 50 U/ml (continuous exposure) was used. For each drug, areas under the survival curve were ordered, limits of sensitivity selected based on a “training set” of observations of in vitro/in vivo data,7 and patients classified as sensitive if their corresponding area was less than or equal to the selected limit and resistant if above it. The sensitivity limits were 5.0 survival-drug-concentration area units for melphalan and 10 units for the other drugs. Statistical association of the in vitro and in vivo results was obtained with the Fisher exact test.12 The method of Kaplan and Meier was used to calculate actuarial survival curves, and tests of the differences were based on the Gehan modification of the generalized Wilcoxon test.13,14 Comparison in the significance of survival differences using the Wilcoxon and log-rank methods was carried out as previously reported.8

RESULTS

Ninety-seven patients had both tritiated thymidine labeling indices of bone marrow plasma cells (LI%) and soft agar culture performed in serial fashion. Sixty-four percent of the patients had myeloma cell growth in agar culture: 28% formed myeloma colonies (>30 cells) and 36% produced clusters (10–30 cells). Thirty-six percent had no significant colony or cluster growth. Figure 1 shows the LI% results for each group. As can be seen, there was a statistically significant difference in labeling indices (p < 0.001): the higher the LI%, the greater the likelihood of cluster or colony growth.

Eighteen patients in addition to having labeling indices had suicide indices performed on the myeloma colony-forming cells. Figure 2 shows the relationship between LI% and suicide index of myeloma colony-forming cells. Although a majority of patients had high suicide indices (>50%), there was not a linear relationship between the LI% and the suicide index. A minority of patients (4%) had both low labeling indices and negligible or no suicide.

Tables 1 and 2 summarize the details of the stage and type of myeloma, LI%, subsequent chemotherapy, plus response and survival. Patients have been divided into two groups: those studied before any treatment was administered (Table 1) and those studied at the time of relapse (Table 2). All patients had both a tritiated thymidine labeling of the plated myeloma cells and in vitro drug sensitivity tested on their
The relationship between the myeloma colony growth and the tritiated thymidine labeling index (LI%) of the bone marrow plasma cells is shown diagramatically. Myeloma colony growth is divided into no growth, clusters (10 to <30 cells), and colonies (≥30 cells). Each ● indicates a single determination of the LI% performed on an aliquot of the same sample used for myeloma stem cell culture. As indicated, the differences between the three groups are highly significant (p ≤ 0.001).

Clonogenic myeloma cells. Patients in each group are listed in order of their overall survival.

In evaluating the LI% and in vitro sensitivity, it was clear that there was no simple overall relationship. However, the previously untreated patients who were sensitive in vitro to melphalan (Table 1, category A) had the lowest LI%; mean value 1.7% compared to mean values of 4.3% (Table 1B), 3.5% (Table 2A), and 4.8% (Table 2B) for the other subgroups in Tables 1 and 2 (p = 0.02). Conversely, patients with an LI% of >3% who were also resistant in vitro to melphalan represented a very high risk subgroup with a median survival of 2.5 mo. Of interest, the relapse patients resistant in vitro (Table 2, category B) were predominantly kappa (κ) M-component subtype (9/10, 90%).

In the previously untreated patients (Table 1), the significance of in vitro sensitivity to melphalan, the standard alkylating agent in myeloma, was evaluated. Since most patients received combination induction therapy, melphalan sensitivity was a marker of overall sensitivity. The correlation between the in vitro findings and response to therapy and survival duration was...
Table 2. Relapse Patients (17): Interrelationships Between Stage, Type, LI%, In Vitro Sensitivity, Response to Therapy, and Subsequent Survival Duration

<table>
<thead>
<tr>
<th>Stage</th>
<th>Type</th>
<th>LI%</th>
<th>In Vitro Sensitivity</th>
<th>Regression</th>
<th>Survival Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitive or Intermediate</td>
<td>Resistant</td>
<td></td>
</tr>
<tr>
<td>A. Sensitive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III A</td>
<td>IgG x</td>
<td>5%</td>
<td>A*, B*, V*</td>
<td>M</td>
<td>80%</td>
</tr>
<tr>
<td>III A</td>
<td>IgA x</td>
<td>3%</td>
<td>IL*</td>
<td>M, Mitox, Velb, Cis p</td>
<td>90%</td>
</tr>
<tr>
<td>III A</td>
<td>IgG x</td>
<td>2%</td>
<td>A*</td>
<td>M, B</td>
<td>20%</td>
</tr>
<tr>
<td>III A</td>
<td>IgA x</td>
<td>2%</td>
<td>Bis*</td>
<td>M, Mitox, Velb, Cis p</td>
<td>46%</td>
</tr>
<tr>
<td>III A</td>
<td>IgG x</td>
<td>4%</td>
<td>Cis p*</td>
<td>M, A, B, Velb</td>
<td>20%</td>
</tr>
<tr>
<td>III A</td>
<td>IgA x</td>
<td>2.4%</td>
<td>MTX†, Velb‡, V</td>
<td>M</td>
<td>50%</td>
</tr>
<tr>
<td>III A</td>
<td>IgG x</td>
<td>6%</td>
<td>M*, A*, B*, V*</td>
<td>—</td>
<td>30%</td>
</tr>
<tr>
<td>B. Resistant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III A</td>
<td>IgG x</td>
<td>2%</td>
<td>—</td>
<td>M*, A*, B*, P*</td>
<td>20%</td>
</tr>
<tr>
<td>III A</td>
<td>IgG x</td>
<td>0.6%</td>
<td>—</td>
<td>M*, A*, B*, V*</td>
<td>10%</td>
</tr>
<tr>
<td>III A</td>
<td>IgG x</td>
<td>8%</td>
<td>—</td>
<td>A*, B*, V*</td>
<td>6</td>
</tr>
<tr>
<td>III A</td>
<td>IgG x</td>
<td>3%</td>
<td>—</td>
<td>M*, A*, V*</td>
<td>5</td>
</tr>
<tr>
<td>III A</td>
<td>IgA x</td>
<td>7%</td>
<td>IL</td>
<td>A*, B*</td>
<td>4</td>
</tr>
<tr>
<td>III A</td>
<td>IgG x</td>
<td>11%</td>
<td>A*</td>
<td>B*</td>
<td>3</td>
</tr>
<tr>
<td>III A</td>
<td>IgG x</td>
<td>3.4%</td>
<td>MTX, Vind, B</td>
<td>M*, A*</td>
<td>2</td>
</tr>
<tr>
<td>III A</td>
<td>IgG x</td>
<td>4%</td>
<td>—</td>
<td>M*, A, B</td>
<td>2</td>
</tr>
<tr>
<td>III A</td>
<td>IgG x</td>
<td>2%</td>
<td>—</td>
<td>A*, B*</td>
<td>2</td>
</tr>
<tr>
<td>III A</td>
<td>IgG x</td>
<td>7%</td>
<td>—</td>
<td>M*, A*, B*</td>
<td>1</td>
</tr>
</tbody>
</table>

*Drugs used in treatment immediately following in vitro study. In one patient († ‡), methotrexate was used first and produced response (50% regression) and velban used subsequently.

†As for Table 1, see Materials and Methods.

excellent. All but one patient sensitive in vitro to melphalan had >50% tumor regression. However, in patients resistant in vitro to melphalan, there were some discrepant results. Two patients had >50% tumor regression and survival of >4 yr. One patient had a very brief 90% regression followed by rapid relapse and death at 5 mo. The overall correlations with survival are shown in Fig. 3. The differences in survival duration between patients sensitive and resistant in vitro were highly significant by both the Wilcoxon and log-rank methods of analysis (p < 0.01 for both).

In the relapse patients it was possible to evaluate the correlation between in vitro sensitivity or resistance and clinical response to specific drugs (Table 2). Again, the correlation between in vitro sensitivity and response was quite good, in that all patients sensitive in vitro had at least some tumor regression with the corresponding drug(s). Two patients who had intermediate sensitivity in vitro to adriamycin and cisplatinum had only 20% regression with these agents, but had clear clinical benefit and prolongation of survival. One patient who had retained in vitro sensitivity to melphalan, adriamycin, BCNU, and vincristine had an overall regression of only 30% and survival of 8 mo. However, each time this patient received therapy, substantial cell kill occurred, but therapy was always delayed by over 6 wk because of thrombocytopenia with platelet antibodies, CNS bleeding, and other problems. This patient is listed as sensitive in the overall correlations (Table 3). For patients resistant in vitro, the correlations were also reasonably good. One patient clearly resistant to all agents tested had a 20% regression and prolonged survival (33 mo). Otherwise,
response and survival were very poor in this group. The overall correlation with survival is shown in Fig. 4. Although the total survival for all patients was not good, there was an early survival advantage for those patients sensitive in vitro to a specific agent (or agents) reflected by the Wilcoxon p value = 0.01.

The overall sensitivity and resistance patterns are shown in Table 3. With strict adherence to the criteria for in vitro and in vivo response, there were several patients giving discordant results (SR or RS) as discussed above. One patient sensitive in vitro to bisantrene had a 46% regression (Table 2, category A), but is listed as SR. Nonetheless, the 73% sensitivity and 83% resistance predictions allowed a clear association between in vitro and in vivo findings (p < 0.01 by Fisher exact test).

**DISCUSSION**

The results presented above allow a number of conclusions to be drawn. Firstly, the kinetics of the plated myeloma cells as reflected by the tritiated thymidine labeling index (LI%) clearly influences the likelihood of growth in soft agar. The subgroup of patients with growth sufficient for in vitro drug sensitivity testing have higher LI% values: 91% > 1%; 52% > 3%. The tritiated thymidine suicide index of myeloma colony-forming cells was also usually extremely high (Fig. 2). These findings are similar to those in acute leukemia. Unfortunately, because of difficulties with reproducible serial myeloma colony growth in recent months, possibly related to problems with the BALB/c mice used for preparation of the myeloma conditioned medium, these studies have not been further extended.

Nonetheless, it is possible to draw additional conclusions from the results presented concerning the significance of in vitro sensitivity and resistance patterns. The LI% does not in itself predict for in vitro sensitivity or resistance. However, in the previously untreated patients (Table 1), in vitro sensitivity plus a predominantly low LI% (all <3%) is

**Table 3. Overall In Vitro/In Vivo Correlations**

<table>
<thead>
<tr>
<th>SS</th>
<th>In Vivo</th>
<th>Rs</th>
<th>In Vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive Both In Vitro and In Vivo</td>
<td>Sensitive In Vivo, but Resistant In Vivo</td>
<td>Resistant In Vivo, but Sensitive In Vivo</td>
<td>Resistant Both In Vitro and In Vivo</td>
</tr>
<tr>
<td>(True Positive)</td>
<td>(False Positive)</td>
<td>(False Negative)</td>
<td>(True Negative)</td>
</tr>
<tr>
<td>Number of patients</td>
<td>11</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Percent</td>
<td>73%</td>
<td>27%</td>
<td>17%</td>
</tr>
</tbody>
</table>

With the Fisher exact test, the association between in vitro and in vivo results was significant (p < 0.01).

*In vitro sensitive includes intermediate also. In vivo response defined as >50% tumor regression. Only clinical trials immediately following the in vitro test are included. Early deaths (<2 courses of treatment) are excluded.
associated with excellent survival. Conversely, in the relapse group (Table 2), in vitro resistance plus a high LI% of >3% is a very poor risk combination, with all such patients surviving <6 mo. Thus, patient survival is influenced both by intrinsic drug sensitivity and by cell kinetics. Of interest, the thymidine incorporation per myeloma cell (as indicated by grain count on autoradiography) reflects both parameters and has allowed more direct prediction of sensitivity and resistance.18

The correlations between in vitro sensitivity or resistance and patient response and survival duration largely confirm prior reports from our group and others.3,19-22 There was excellent correlation between in vitro results and percent regression and survival duration, as shown in Table 3 and Figs. 3 and 4. In the previously untreated patients, in vitro sensitivity to melphalan, the standard alkylating agent in myeloma, corresponded with drug responsiveness and a good prognosis. The discrepant results in a few patients may represent a combination of technical factors plus the effects of myeloma cell kinetics or other parameters not evaluated. In the relapse patients it was possible to identify specific sensitivity to several agents, including leukocyte interferon, methotrexate, and bisantrene. This sensitivity, associated with short-term survival benefit, occurred in the face of clinical and in vitro resistance to other therapy.

Thus, as has been concluded in other studies, the use of the clonogenic assay system provides promise for the evaluation of in vitro drug sensitivity in multiple myeloma. Identification of both general and specific drug sensitivity or resistance is possible. Clearly, further studies are necessary to improve myeloma cloning efficiency and to clarify and extend the above observations.

ACKNOWLEDGMENT

We gratefully acknowledge the help of Anne Hamburger, Ph.D., Barbara Soehnlen, and Linda Vaught in carrying out these studies. The statistical assistance of Thomas Moon, Ph.D., was greatly appreciated, as well as the secretarial assistance of Margaret Sieras.

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