Expansion of the Growth Fraction in Multiple Myeloma With Alkylating Agents

By Sydney E. Salmon

Patients with IgG multiple myeloma underwent serial studies of tumor cell kinetics including (1) estimation of the total body myeloma cell number (TBMC), (2) measurement of the myeloma cell thymidine labeling index (LI), and (3) calculation of the total number of myeloma cells undergoing DNA synthesis. Intermittent courses of chemotherapy with cycle-non-specific agents such as melphalan resulted in a marked increase in the LI of myeloma cells in patients who had a 75% reduction in TBMC. The long "plateau" phase of partial remission of myeloma in these patients was associated with a continued high LI; this suggests that the plateau resulted from a balance between the cytoreductive effects of chemotherapy and expansion of the growth fraction (GF) of the tumor. Preliminary attempts to capitalize therapeutically on this expansion of the GF in several patients included administration of the cycle-active agents vincristine and cytosine arabinoside. Vincristine appeared to induce a further reduction in tumor in several patients, although cytosine arabinoside appeared to be ineffective despite clear evidence of its inhibition of DNA synthesis in myeloma cells in vivo. Further clinical studies of the effects of cycle-active drugs on myeloma appear to be warranted; however, successful exploitation of the dynamic change in myeloma cell kinetics with chemotherapy will require the use of cycle-active agents with marked selective toxicity for myeloma cells.

Thus far, the development of effective chemotherapy for disseminated human tumors has been a difficult challenge. Although many single agents have marked oncolytic activity within the range of acceptable toxicity to the host, they usually induce only partial regression of the tumor despite continued treatment. In experimental animal tumor systems, this form of drug resistance can be understood in terms of cell cycle kinetic principles. In man, such apparent resistance is typically observed with single alkylating agent therapy for multiple myeloma. Multiple myeloma has been of signal importance because it has many characteristics of a "model tumor." Specifically, myeloma cells secrete a characteristic marker (the M-component, a monoclonal immunoglobulin), measurements of which can be used to calculate the total body burden of tumor cells. Additionally, the tumor cells can be readily sampled serially from the bone marrow for immunologic and cytokinetic studies. Our previous studies defined the clinical range of myeloma as associated with a body burden of from approximately $2 \times 10^{11}$ to approximately $5 \times 10^{12}$ myeloma cells and indicated that there was a proportionality between the number of tumor cells present, symptoms of disease, and patient survival time. We analyzed the kinetics of the growth and regression of myeloma and found that...
the tumor grows in accord with Gompertzian kinetics, often displaying an intriguing "plateau" phenomenon after initial regression with a fixed schedule of intermittent or continuous therapy with an alkylating agent. Inasmuch as we had determined that both tumor growth and tumor regression can be described with Gompertzian kinetics, we reasoned that both retardation of growth and drug-induced regression might be attributable to dynamic shifts in the growth fraction (GF) of the tumor.

In order to test this latter hypothesis, we performed serial studies on a group of patients with IgG myeloma. In addition to calculating the total body tumor cell number and assessing tumor kinetics, we measured the in vitro tritiated thymidine labeling index (LI) of myeloma cells. (The LI is a useful indicator of the percentage of cells going through scheduled DNA synthesis and is generally accepted as a direct correlate of the GF.) In a few cases, preliminary pharmacologic "manipulations" were carried out in vivo with cycle-active agents to determine whether increased susceptibility to cycle-active agents could be induced with an expansion of the GF of the tumor. Our observations provide evidence that alkylating agent therapy can expand the tumor's GF. Expansion of this proliferative compartment may at least partially explain the important "plateau" phenomenon of partial remission in patients with myeloma. In addition, the studies provide support for the use of cycle-nonspecific drugs to attack the overwhelming mass of nonproliferating cells usually present at the time of diagnosis, as well as a strong rationale for empirical trials of cycle-active drugs in the "plateau" phase of myeloma in an attempt to further reduce the residual myeloma cell mass.

MATERIALS AND METHODS

Patient Studies

Previously untreated patients with newly diagnosed multiple myeloma were selected for study. The clinical and immunologic criteria used for the diagnosis and staging of disease in these patients have been described in detail.

Bone marrow aspirates were collected in heparin for in vitro LI studies; short-term tissue cultures were also prepared for studies of cellular synthesis of myeloma protein. Newly synthesized M-component was measured with the "sandwich" radioimmunoassay technique. Cellulose acetate electrophoretic and densitometric studies of serum proteins were performed at least monthly. The concentration of M-component in the serum was determined by careful analysis of the electrophoretic patterns. Plasma volume was measured every 3 mo with the 125I-labeled albumin technique. The total body synthetic rate for the M-component was calculated from its concentration in the serum, the plasma volume, and the body weight as described previously.

Initial chemotherapy for all of the patients studied was with 4-day courses of melphalan and prednisone, administered once every 4-6 wk. Bone marrow aspirates for in vitro LI studies were obtained prior to the initial course of chemotherapy and immediately prior to subsequent "pulse" courses of alkylating agent-prednisone therapy. Although marrow aspirates were occasionally obtained for in vitro studies at other times in the treatment cycle, this was not done routinely.

Storage of patient records, computation of metabolic turnover data for M-component synthetic rates and total body tumor cell number, and curve-fitting and tumor kinetic studies were carried out with a General Electric 260 digital computer via the GE Mark III time-sharing service (General Electric Co., ISB Div., Schenectady, N.Y.). The theoretical background, mathematical equations, and nature of the programs used have been detailed previously.

Particularly relevant to the present study is an additional computer program based on this theoretical background which was written to calculate the theoretical proliferative fraction at
any given tumor size. The equation for calculation of the theoretical proliferative fraction ($p_i$) is

$$p_i = A_i \cdot t_g$$

where $A_i$ is the slope of the line tangent to the growth curve at time $t_i$, and $t_g$, the theoretical doubling (generation) time, is assumed to be constant. Development of this concept is detailed in reference 2.

Remission of myeloma was defined as a reduction in the total body tumor cell number of at least 50%, and significant regression was defined as an approximate 1 log reduction.

Calculation of the Total Body M-Component Synthetic Rate and Total Body Myeloma Cell Number (TBMC)

The total body synthetic rate for the M-component was calculated from the fractional catabolic rate ($f$) for the specific immunoglobulin, its concentration in the serum, plasma volume, and body weight. In IgG myeloma cases, the equation of Waldmann and Strober for the calculation of $f$ was used as described in our previous kinetic analysis. TBMC was derived from the calculated total M-component synthetic rate and the measured cellular M-component synthetic rate per myeloma cell in vitro, as described previously.

Myeloma Cell LI Studies

A 1-3 ml specimen of bone marrow was aspirated aseptically into a syringe containing heparin. One-quarter volume of 3% dextran was added to the sample in order to sediment the red cells. The supernatant plasma, which contained the marrow cells, was transferred to a sterile centrifuge tube and diluted to 45 ml with Hanks' Balanced Salt solution with 10% fetal calf serum (HBSS-FCS) and centrifuged for 10 min at 600 g at room temperature. The cell button was then resuspended in HBSS-FCS, and the centrifugation and cell washing procedure was repeated two times. A 2-ml aliquot of the washed cell suspension was placed in a sterile plastic tissue culture tube and warmed to 37°C in a waterbath. One microCurie of tritiated thymidine (specific activity, 18 Ci/m mole) was added to the cell suspension. After 1 hr of incubation, the suspension was washed free of the unincorporated tritiated thymidine by two additional centrifugations and washes with HBSS-FCS. The cell preparations were free of clumping, and, at the completion of the incubation, viability was from 98% to 100%. Slides for radioautography and microscopic examination were prepared with the Shandon cytocentrifuge. After methanol fixation, radioautographs were prepared for exposure by dipping in Kodak NTB-3 emulsion and then exposed for 7 days at 2°C. Standard developing techniques were used, and the slides were stained with acid Giemsa stain. Radioautographs prepared in this fashion were of exceedingly high quality with a background median grain count of less than 35 grains per 100 cells.

All marrow cells which were morphologically in the lymphoid-plasma cell series were defined as myeloma cells. Special care was taken in staining and microscopic examination to assure that plasma cells could be distinguished morphologically from early red cell precursors. Cells containing at least five grains over the nucleus were considered labeled. One thousand cells were counted in order to determine the myeloma cell LI, which was expressed as a percentage. To ensure the accuracy of the counting procedure, slides on patients with less than 8% myeloma cells in the bone marrow were not analyzed. The median grain count of labeled myeloma cells measured with this LI technique was in the class of >50 grains per cell nucleus. Statistical considerations regarding threshold grain counts and relation to background were as described by Clarkson et al.

A simple extrapolation from the LI and tumor cell number data was the calculation of the total number of labeled myeloma cells in the body (the size of the compartment of DNA-synthesizing tumor cells), TBLMC. TBLMC equals TBMC x LI.

RESULTS AND DISCUSSION

Proliferative Kinetics in a Previously Untreated Patient with Myeloma

Patient R.D., a 43-yr-old man, was discovered to have an M-component when electrophoresis was performed as part of a health screening program. He
was entirely asymptomatic, and his physical examination, skeletal x-rays, and
peripheral blood laboratory values were normal. Immunoelectrophoresis of the
serum identified the M-component as IgG kappa. Electrophoresis of a urine
concentrate showed no evidence of Bence Jones proteinuria. A bone marrow
aspirate contained 20% plasma cells, some of which were binucleate. The pa-
tient's physician considered him to have a benign monoclonal gammopathy, but
referred him for evaluation of immunoglobulin synthesis and cell kinetics.
The patient was studied four times over a 136-day period with serial bone mar-
row studies of immunoglobulin synthesis and plasma volume, hematologic, and
electrophoretic measurements. Within that time there was slightly more than a
doubling of the TBMC and a halving of the LI. Thus, despite the increase in
total tumor cell mass, the total number of myeloma cells synthesizing DNA
(TBLMC) remained essentially unchanged at approximately $1 \times 10^{11}$ cells. A
diagnosis of multiple myeloma was considered established, and chemotherapy
was begun. Studies of the patient's kinetic measurements are listed in Table 1.

It is clear from these measurements that only a small fraction of myeloma
cells were undergoing DNA synthesis during the phase of tumor growth with
$>10^{11}$ myeloma cells and a tumor doubling time of 6 mo. Back calculation from
this patient's growth curve would suggest an initial doubling time (generation
time) of about 3 days. Confirmation of this estimate would require precise mea-
surement of the myeloma cell generation time with labeled thymidine tech-
niques, although this estimate is in general accord with the few data that have
been published.9,10 Additional measurements of the myeloma cell generation
time in vivo are clearly needed to develop firm knowledge of the range of Tc in
myeloma.

**Effects of Intermittent Chemotherapy on the LI**

Eight patients with good responses to alkylating agent–prednisone therapy
(at least 75% tumor regression) underwent serial LI studies initiated at the time
of diagnosis. Labeling was most frequently observed in cells which would be
classified as either plasmablasts or “small” myeloma cells (<18μ in diameter),
but it was occasionally observed in larger myeloma cells. Binucleate or multi-
nucleate plasma cells took up label only infrequently, but when they did, all
nuclei were tagged. Characteristic LI patterns were observed: Prior to inter-
mittent alkylating agent chemotherapy, the LI was low, ranging from 2% to 7%
in the group studied. In contrast, in association with significant tumor regress-
ion, the LI rose progressively with a series of courses of chemotherapy until a

<table>
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<th>Day</th>
<th>IgG M-component (g/100 ml)</th>
<th>Total No. of Myeloma Cells ($x 10^{11}$)</th>
<th>Labeling Index (%)</th>
<th>Total No. of Myeloma Cells Synthesizing DNA ($x 10^{11}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.4</td>
<td>1.7</td>
<td>6.0</td>
<td>0.10</td>
</tr>
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<td>47</td>
<td>4.6</td>
<td>2.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>107</td>
<td>5.0</td>
<td>2.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>136</td>
<td>6.2</td>
<td>3.6</td>
<td>3.1</td>
<td>0.11</td>
</tr>
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maximal value was obtained (in the range of 15%-40%). Inasmuch as each LI was measured 4–6 wk after the previous course of chemotherapy, the LI results would appear to reflect prereplicative DNA synthesis rather than DNA repair (which has a time course of minutes to hours after injury). These LI observations are summarized in Fig. 1. In most (but not all) instances, the highest value was obtained at a time of maximum tumor regression and persisted during the “plateau” phase of myeloma, at which time the tumor remained constant in size despite repetition of the cycles of chemotherapy. These data were taken as evidence of expansion of the tumor’s proliferative compartment as a result of alkylating agent chemotherapy.

Serial Observations on Chemotherapy With Cycle-nonspecific and Cycle-specific Agents

Seven patients with IgG myeloma underwent serial TBMC and LI studies. Response to chemotherapy in the patients chosen for this analysis varied, with some patients having excellent response and others failing to respond. Two of the patients who had excellent responses also received trials of cycle-active drugs in the “plateau” phase of remission.

Figure 2 shows the serial data on three previously untreated patients with relatively difficult cases who received pulse courses of melphalan-prednisone therapy. Patient H.S. showed clear evidence of tumor cell kill with each course of treatment but had rapid rebound in tumor cell number after each course in association with a brisk increase in the LI. In patient B.D., there was virtually no difference in sensitivity to alkylating agents between his myeloma cells and normal hematopoietic precursors, which were unusually sensitive to melphalan. Only two courses of therapy could be administered over a 150-day period because of limiting bone marrow hypoplasia. Patient R.K. represents an unusual phenomenon: Only minimal regression in myeloma cell mass occurred with full doses of melphalan; however, the LI rose markedly during this period. Therefore, it would appear that in this latter patient, the proliferative compartment had expanded enough to promptly counterbalance the cytoxic effects of chemotherapy. An additional patient (data not shown) who did not have tumor regression failed to have a significant increase in LI with treatment. In this patient, drug resistance did not appear to have a cytokinetic basis.
Patient E.M. (Figs. 3 and 4), a 75-yr-old man, had severe anemia and osteoporosis when first examined. He had a presenting TBMC of $2.9 \times 10^{12}$ tumor cells and an LI of 3.6%. Intermittent courses of melphalan and prednisone resulted in a 72% reduction in TBMC within 100 days. By that time, the patient had achieved a "plateau" stage of remission, with a stable, residual myeloma cell mass of $9 \times 10^{10}$ myeloma cells. With further pulse courses of therapy, the "plateau" cell mass persisted at this level for an additional 250 days. By this time, the LI had increased to 22%, and the TBLMC number had more than doubled (from $8.7 \times 10^{10}$ to $2.0 \times 10^{11}$ cells), despite a 72% reduction in TBMC. In an attempt to perturb the tumor with a cycle-active agent, 1-mg injections of vincristine were added to the cycles of melphalan-prednisone therapy. The tumor then regressed an additional 39% from the first plateau level and appeared to plateau again at $5.5 \times 10^{11}$ myeloma cells (i.e., a total tumor regres-
sion of 81% from the TBMC at presentation). The total effective cell kill was \(2.35 \times 10^{12}\) myeloma cells. Six weeks after the third vincristine injection, the LI had increased to 42% and the TBLMC compartment had a further increase to \(2.3 \times 10^{11}\) cells. Several days after the LI of 42% had been obtained, the patient was treated with cytosine arabinoside administered as a continuous intravenous infusion at a dose of 100 mg/sq m/day for 5 days. Twelve hours after termination of the infusion, a repeat LI was less than 0.1%. This latter result indicates that cytosine arabinoside entered proliferating myeloma cells and inhibited their DNA synthesis. Although a transient regression in tumor was observed, rebound to a level of \(5 \times 10^{11}\) cells occurred within several weeks. This result suggests that either the cytosine arabinoside inhibited the DNA synthesis without being lethal or that additional cells not undergoing DNA synthesis at the time of the infusion were able to repopulate the tumor very rapidly. Despite this rebound phenomenon, the plateau level could be maintained with melphalan–prednisone–vincristine therapy, and the patient has remained asymptomatic. The total period of followup in this case as of July 1, 1974 is 1406 days.

Patient L.W. (Fig. 5), a 58-yr-old woman, had marked bone pain and anemia and a TBMC of \(8.5 \times 10^{11}\) tumor cells when first referred for treatment. A prompt regression of 88% occurred with melphalan–prednisone combination therapy, and the patient then received daily maintenance treatment with melphalan (administered orally). A “plateau” phase in excess of 800 days then persisted with a concomitant increase in the LI of residual myeloma cells. Injections of vincristine were administered intermittently. Repeated 2-mg injections caused a prompt fall in the residual myeloma cell mass; however, sporadic injections of 0.5 mg had much less effect. No other cycle-active drugs have been tried in this patient, although periodic injections of vincristine have been used to reinforce the effects of alkylating agents. The total followup in this patient as of July 1, 1974 is 2302 days from the time of presentation, with the patient remaining in an excellent remission.
Fig. 5. Effects of addition of vincristine in the "plateau" phase of myeloma in patient LW. Arrows indicate time at which doses were administered. Dose levels (in milligrams) are indicated.

GENERAL COMMENTS

The clinical explorations described in this paper provide some insights relative to success and failure in the treatment of disseminated neoplasms in man. Many of these features had already been ascertained in experimental tumor systems in animals; our studies confirm the occurrence of the same kinetic events in myeloma in man. Very recent observations on human myeloma cells by Alberts and Golde provide additional evidence that the measured LI of myeloma cells reflects prereplicative DNA synthesis, rather than DNA repair. The in vitro effects of cytosine arabinoside and hydroxyurea that they observed on myeloma cell DNA labeling are compatible with effects on scheduled DNA synthesis, as opposed to unscheduled DNA repair.

The serial measurements of the LI, TBMC, and TBLMC in Patient R.D. (Table 1) provide evidence that the approach to "plateau" phase during the growth of a monoclonal is associated with a reduction of the fraction of myeloma cells undergoing DNA synthesis. This decrease in the LI probably reflects a reduction in the growth fraction and a reciprocal expansion of the G₀ compartment, with perhaps some increase in spontaneous cell death. The measured LIs in this patient's case were significantly larger than would be anticipated from calculation of the theoretical proliferative fraction. One distinct possibility to explain this is that the difference between the "theoretical LI" and the measured LI is a function of the tumor's spontaneous death rate. In a sense, such comparisons can be used to approximate the size of the spontaneous cell death compartment.

As detailed in our previous kinetic analysis, myeloma regression during treatment with a fixed dosage schedule of a cycle-nonspecific alkylating agent follows a "reverse Gompertzian curve." The present studies show that the LI (and undoubtedly the GF) rise progressively during tumor regression. Since the time of our original report on the change in the myeloma cell LI with alkylating agent therapy, confirmatory observations have been made by
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Drewinko et al. 17 as well as by Alberts and Golde. 15 Similar observations have recently been made in acute leukemia. 18 These studies all indicate that a dynamic increase in the size of the OF of tumor cells follows the induction of remission with chemotherapy. Although the precise mechanism responsible for this expansion in the OF after chemotherapy remains obscure, it may well result from a feedback regulatory system. Our detailed analysis of the growth and regression of myeloma suggested that a simple feedback inhibitor produced by myeloma cells (e.g., a chalone 3) could account for the retardation of growth of myeloma prior to therapy, whereas release from the inhibitory effect (after antitumor therapy) could result in the triggering of myeloma cells into cycle. The biochemical nature of such an inhibitor or chalone must still be established, although one potential candidate, interferon, has already been demonstrated to be produced by human myeloma cells in vitro. 19 It is of interest that the measured LIs always exceeded what would have been anticipated on comparison with the plot of the theoretical proliferative fraction as shown in Fig. 6. The theoretical proliferative fraction is a projection of the minimum size that the OF would have to be to account for the tumor's kinetic behavior at any given tumor size. The percent of cells in the OF will always be greater than those enumerated with the LI, as that is an index only of S-phase cells and not of all cells in cycle. During the course of alkylating agent chemotherapy, the cytotoxic effects of treatment clearly increase the rate of cell death over that which would occur spontaneously. The result of these dynamic alterations in the size of the growth and death fractions of the tumor during treatment is often the development of a "plateau" of partial remission, in which these two opposing kinetic forces are in a delicate balance.

In view of the results of our preliminary attempts to increase the forces favoring cell death by adding cycle-active drugs once the LI has increased, further explorations with cycle-active agents appear warranted. Although worthy of trial, this approach with cycle-active agents will not necessarily "break the logjam" of partial regression of myeloma. Normal hematopoietic elements and myeloma cells may display similar sensitivity to cycle-active agents, with a resultant narrow therapeutic index. Indeed, studies by Ogawa...
and Bergsagel\textsuperscript{20} which have shown a marked increase in the sensitivity of mouse myeloma cells (as compared to hematopoietic cells) to melphalan have shown no differential sensitivity to the cycle-active drug 5-fluorouracil. Viewed in this perspective, optimal chemotherapy requires not only the availability of suitable markers of tumor burden and exploitation of kinetic alterations such as those which we have observed, but also the availability of new drugs with a marked selective toxicity for myeloma cells.

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