Drug Resistance in Multiple Myeloma Associated With High In Vitro Incorporation of $^3$H-Thymidine

By Victor Hofmann, Sidney E. Salmon, and Brian G. M. Durie

In multiple myeloma, tumor cell mass and labeling index correlate with subsequent survival duration, but do not predict for response to treatment. In the present study we have autoradiographically measured the incorporation of $^3$H-thymidine as determined by the number of grains over the myeloma nuclei in bone marrow aspiration samples. In 33/37 patients with <50% tumor regression or progressive disease, the pretreatment grain count was $>20$/myeloma nucleus. Conversely, values of <20 were found in 27/29 patients who had >50% cell mass reduction. Survival duration was significantly better ($p < 0.001$) in patients with grain counts $<20$. Sixty percent of the patients with both a low labeling index (<3%) and grain count (<20) were alive at 48 mo, whereas 15/17 patients with a high labeling index and grain count had a median survival of <6 mo. In a subset of 22 patients, there was a significant correlation between in vitro resistance to melphalan, adriamycin, and vincristine as tested in the myeloma stem cell colony assay system and a grain count of >20. We can only speculate as to the reasons for the increased $^3$H-thymidine uptake by myeloma cells resistant to treatment, however, it could be associated with accumulation of excess DNA and/or increased unscheduled DNA synthesis following injury from alkylating agents.

PROGNOSIS in multiple myeloma has been demonstrated to be related to measured tumor cell mass. For routine prognostic classification, a clinical staging system based on prior cell mass determinations has allowed separation into stages I, II, and III (low, intermediate, and high cell mass). An additional subclassification for serum creatinine [group A (<2 mg/100 ml) or B (≥2 mg/ml)] helps in identifying patients with good or poor prognosis. More recently, the labeling index (LI%) from pulse incubation with $^3$H-thymidine (TdR) has been shown to be an independent prognosticator for survival duration. A low LI% has also been found in patients with stable remission, as opposed to patients with unstable or progressive disease. However, neither tumor cell burden, nor cell kinetic parameters predict for response to treatment. The development of a myeloma stem cell assay has allowed the study of differential sensitivity to specific antineoplastic agents. The method, although a powerful tool, is time consuming and only possible in specialized laboratories. Myeloma colony growth occurs more frequently with a LI% > 3%. However, the LI% does not relate to subsequent in vitro drug sensitivity. While reviewing the relationship between myeloma cell labeling and in vitro sensitivity or resistance we were impressed by the frequent heavy labeling of cells in patients with subsequent in vivo resistance. It was therefore decided to systematically analyze the quantitative $^3$H-TdR uptake, as reflected by the grain count over the cell nucleus, with respect to sensitivity or resistance. This article outlines the relationship between grain count and subsequent response to treatment. In addition, the grain count was related to patient clinical and laboratory features and actual percentage tumor regression with therapy plus subsequent survival duration.

MATERIALS AND METHODS

Patients

Seventy-three patients with plasma cell myeloma evaluated at the University of Arizona between 1976 and 1979 were reviewed in this study. All were carefully staged by measuring the tumor cell mass. Early deaths were included. Multiple (plasma cell) myeloma was defined as by the Chronic Leukemia-Myeloma Task Force of the National Cancer Institute. Of the 73 patients, 47 had IgG myeloma, 14 IgA myeloma, and 12 pure Bence-Jones myeloma. Fifty-nine patients were stage III, 9 were stage II, and 5 were stage I. Of the 73 patients, 66 had complete data sufficient for analysis of both response (percent tumor regression) and overall survival; 7 had serial grain counts and disease activity evaluation. Therapy consisted of courses of melphalan or cyclophosphamide plus prednisone. In the newer SWOG protocols, combination therapy with BCNU, adriamycin, or vincristine were used in addition. Myeloma cell mass changes were calculated using a computerized system. Response was expressed in terms of changes of total body myeloma cell mass.

Myeloma Cell Labeling Index (LI%) Measurement

Samples for the LI% were obtained from either untreated patients, patients in relapse off treatment, or at least 3–4 wk after the last treatment course. One to three milliliters from the bone marrow were aspirated aseptically into a syringe containing heparin...
as described in detail in our previous papers. Critical steps were as follows: 5 μCi of ²H-TdR (specific activity 40-60 Ci/mmmole) were added to the cell suspension. After exactly 1 hr of incubation at 37°C in a waterbath, the suspension was washed free of the unincorporated ²H-TdR. Such cell preparations were free of clumping, and after incubation, the viability was > 98%. Slides for autoradiography and microscopic examination were prepared with a cytocentrifuge. After methanol fixation, autoradiographs were prepared for exposure in Kodak NTB-3 emulsion and further processed using our previously published method of high speed scintillation autoradiography (HSARG). Slides used in this study were incubated for 24 hr. Standard developing techniques were used, and the slides were stained with acrid Giemsa stain. The background median grain count was less than 35/100 cells. All marrow cells that were morphologically in the lymphoid-plasma cell series were defined as myeloma cells. With the described staining technique and careful microscopic examination plasma cells could be distinguished from the early red cell precursors. Plasma cells containing >5 grains over the nucleus were considered labeled. One-thousand cells were counted to determine the L1%, which was then expressed as a percentage. Statistical considerations, regarding threshold grain counts and relation to background were as described by Clarkson, Ohkita, Ota, and Fried.

**Grain Count Measurement and Nuclear Sizing**

The number of grains over each labeled cell (>5 grains/nucleus) was counted. Grain counts over 50 were arbitrarily given a value of 50. In addition, the size of the major axis of the labeled nucleus was measured. Although 1000 cells were counted, in cases with low L1% and number of labeled cells, a minimum of 5 cells was required for evaluation of the grain count and nuclear sizing as part of this study.

**Myeloma Stem Cell Culture Assay and In Vitro Drug Sensitivity Testing**

For the 22 patients included in this study, the myeloma stem cell assay and drug sensitivity tests were performed as described previously. The 4 drugs—melphalan, BCNU, Adriamycin, and vincristine—were tested in at least 3 concentrations, covering the clinically achievable range. Drug survival curves were drawn for each drug. From the area under the survival curve, an in vitro sensitivity index was graphically derived and calculated for each patient to a defined upper boundary. This system of analysis expresses in one value the area under the survival curve, reflecting the degree of cell kill; a low sensitivity index indicates a high cell kill and good anticipated clinical response.

**Statistical Methods**

Actuarial survival curves were calculated using the method of Kaplan and Meier. Differences were tested using a generalized Wilcoxon test or Breslow test. A nonlinear regression procedure was used to determine the combination of patient characteristics related to survival duration and response to treatment. A logistic regression procedure was used to determine which patient characteristics were important in predicting 50% or greater tumor regression. Duplicate autoradiographs were counted and compared using the methods of Livingston, Ambus, George, Freireich, and Hart. Two-fold differences were concluded to be statistically significant (p < 0.05).

**RESULTS**

Figure 1 shows the correlation between pretreatment grain count and subsequent maximum tumor cell mass reduction with a particular treatment schedule. The 33/37 patients who did not respond to treatment (<50% tumor regression or progressive disease) had grains counts >20. Conversely, 27/29 patients who had >50% tumor cell mass reduction had values <20. In patients who did not respond to treatment, there was a wide range of L1 values: the L1 was >3% in 16 patients categorized by grain count. Survival is significantly better versus (p = 0.007); (A) 10–19 versus (B) >30 (p = 0.003).
and <3% in 21. A low LI was predominant (23/29) in the responding group sensitive to treatment.

For both previously untreated and relapse patients survival duration was also related to the grain count. As shown in Fig. 2, there was a highly significant difference in survival \( p < 0.001 \) for categories of patients with more than or less than 20 grains per myeloma cell nucleus. The major difference between the previously untreated and relapse patients was the frequency of patients with grain counts of 20 and associated drug resistance. Seven of 20 previously untreated patients (35%) compared with 30/46 relapse patients (65%) had grain counts >20, poor response to therapy, and poor survival. Figure 3 demonstrates, as previously published,\(^4\) that the LI was also a prognosticator for survival duration in this group of 66 patients. However, no relationship was demonstrable between the LI and tumor cell mass reduction. By combining a low LI with a grain count <20, a group of patients with a particularly good prognosis emerged (Fig. 4): 60% of the patients in this subset of 25 patients were alive at 48 mo. Conversely, 15/17 patients with both high LI and grain count had a median survival of less than 6 mo.

Figure 5 demonstrates the change in grain counts in
7 patients who were initially sensitive to treatment and had a grain count <20. These patients subsequently developed resistance to treatment after 5–30 mo accompanied by a marked increase in the grain count. Looking simply at the LI no consistent pattern was evident.

To further analyze whether resistance was specific for one antineoplastic agent or nonspecific (being present for several classes of drug), grain counts were correlated with the in vitro sensitivity index of the four most commonly used drugs in multiple myeloma. Melphalan (Fig. 6), adriamycin (Fig. 7), and vincristine (Fig. 7), but not BCNU (Fig. 6) showed a significant correlation between grain count and sensitivity index. The combined in vitro sensitivity index (Fig. 8), which was obtained by adding the sensitivity index of the four drugs tested separately in vitro, but simultaneously in vivo, again showed a significant correlation ($p < 0.05$). In most cases, sensitivity or resistance could be confirmed in vivo. In those cases where the drugs were only tested in vitro, comparable sensitivity or resistance was demonstrated in vivo with other cytostatic agents.

Since the size of the nucleus was considered to be a marker for the degree of differentiation, and hence, possibly related to prognosis, we measured the size of the major axis of the labeled myeloma nuclei. The size varied between 7 and $19.9\mu$ (median $11.6\mu$). Using Cox’s nonlinear regression procedure, nuclear size, grain count, labeling index, and additional patient characteristics related to survival duration were evaluated as predictors of subsequent survival duration. Grain count proved to be the most important independent predictor, although nuclear size, labeling index, and prior therapy added additional predictive capacity.

**DISCUSSION**

This study reveals that high $^3$H-TdR incorporation, as expressed by the grain count of bone marrow myeloma cells, is correlated with both in vivo and in vitro resistance to treatment with the commonly used drugs in multiple myeloma. The significant correlation between high grain count ($\geq 20$ grains per nucleus) and resistance to treatment was surprising in view of the fact that the proportion of labeled cells (expressed as LI%), although a prognosticator for survival dura-
tion did not appear to predict the percent tumor cell mass reduction with treatment. The correlation was strengthened by the serial studies indicating transition from low to high grain counts with acquisition of drug resistance.

In this study the immunoglobulin type and treatment status (initial induction or relapse) were additional independent factors delineated by multivariate analysis as predictive of response to treatment. This is in line with the known better survival duration of IgG myeloma in comparison to IgA myeloma or pure Bence-Jones myeloma. Relapse patients had much poorer survival than previously untreated patients and were more likely to have developed both in vitro and in vivo drug resistance.

From the current study we can only speculate as to the possible mechanisms underlying the association of high levels of \(^3\)H-TdR incorporation and both in vivo and in vitro resistance to the four drugs evaluated. Conventional concepts would rather suggest increased sensitivity (particularly to cell-cycle-specific drugs) for cells with a large S-phase compartment. However, patients with a high LI and as noted in this study, high grain counts have an extremely poor prognosis, possibly due to expansion of the population of resistant myeloma stem cells with, in addition, a large growth fraction. Nonetheless, it must be kept in mind that \(^3\)H-TdR incorporation into DNA is not necessarily associated with ongoing cell proliferation. For instance, in a recent study in cultures of epidermal keratinocytes, Davison, Liu, and Karasek failed to demonstrate a correlation between cell proliferation and \(^3\)H-TdR uptake by DNA. There are in fact several possible reasons for incorporation of \(^3\)H-TdR, including DNA synthesis concomitant with cell replication, ongoing sister chromatid exchange and presence of, and incorporation into, excess DNA accumulated in drug-resistant myeloma cells. DNA content abnormalities as measured by flow cytometry have in fact recently been demonstrated to be a frequent finding in multiple myeloma, especially with advanced or resistant disease. The prominent feature was hyperdiploidy, and it is not unreasonable to speculate that some of the excess DNA may be responsible for both increased \(^3\)H-TdR uptake and resistance. Unscheduled DNA synthesis and repair has recently been shown to be strikingly increased in myeloma cells injured by alkylating agents. Other mechanisms reflecting chromatid instability such as sister chromatid exchange could also be responsible for other pathways of \(^3\)H-TdR uptake. We are currently investigating the various possible mechanisms for high in vitro incorporation of \(^3\)H-thymidine.

The described resistance both in vivo and in vitro against different classes of cytostatic agents seems to indicate that the mechanism is nonspecific, in contrast to resistance to, for example, methotrexate, which has been shown to be associated with gene amplification and greatly increased dihydrofolate reductase synthesis. From the present results, it appears that resistance to one agent may indicate overall resistance against several drugs, suggesting that there may be some common resistance mechanisms at a cellular or molecular level. However, both from in vivo studies and the in vitro myeloma stem cell assay, it appears that potential active single agents or combinations can still be found in a percentage of cases.

By combining the sensitivity index with the grain count, it may be possible to improve the reliability of prediction of response to treatment. This may be particularly true for the group of patients with intermediary in vitro sensitivity indices who may be responders or nonresponders. However, in the current study, the numbers were insufficient to substantiate the exact additional value of the grain count.

Whatever the true mechanism responsible for the denoted phenomenon, there is no doubt that a marker of resistant disease has been observed and deserves further investigation.

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