

The Growth Fraction of Human Myeloma Cells

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Greater reductions of tumor load in patients with multiple myeloma may result from therapeutic strategies that are based on a better knowledge of growth kinetics. We have previously shown that the labeling index of myeloma cells remains unchanged when tumor mass is reduced and that the cells of relapsing patients have different biologic properties than the cells present before melphalan-prednisone therapy. This study investigated the growth fraction (GF) of myeloma cells at various disease stages using continuous i.v. infusions of tritiated thymidine. We studied 17 patients on 22 occasions (4 untreated, 2 unresponsive, 6 in remission, and 10 in relapse). All untreated and unresponsive patients and 5 of 6 patients in remission had a GF of less than 4%. GF was defined in these studies as the maximum percentage of labeled plasma cells exposed

continuously to tritiated thymidine. Relapsing patients, with the most rapid tumor doubling times, had GF ranging from 14% to 83%. The plasma cell transit time through the proliferative compartment for all of the relapsing patients ranged from 6.6 to 11.9 days and the calculated intrinsic cell loss ranged from 50% to 86%. These findings support our model for the growth kinetics of multiple myeloma that assumes that the entire tumor mass issues from a small proportion of proliferating cells and that the growth kinetics of myeloma cells in relapsing patients differ from those in untreated and unresponsive patients. Therapeutic trials with cycle-active agents need further investigation in selected relapsing patients who are likely to have a high growth fraction.

ONE APPROACH towards improving the treatment of multiple myeloma (MM) consists of designing therapeutic strategies based on a growth kinetics rationale.¹⁻⁶ Tumors are composed of two kinetically-distinct cell classes, proliferating and nonproliferating (quiescent) cells whose relative proportions change as tumor growth progresses. Because proliferating cells are more sensitive to most chemotherapeutic agents than quiescent cells, changes in their respective proportions will influence the design of treatment regimens, especially those that include cell cycle dependent drugs.⁶

The evolution of untreated MM is associated with a continuously decreasing proportion of proliferating cells so that the fraction of nonproliferating plasma cells is largest at the time of diagnosis.⁷⁻¹³ After successful therapy with melphalan and prednisone, the usual clinical course consists of an initial decrease in tumor load followed by a short duration of stabilized tumor mass, and then a final relapse with an increasing population of myeloma cells that are usually resistant to most antitumor drugs.¹ We have previously proposed that all untreated MM patients possess two neoplastic populations: a sensitive and a resistant fraction.^{9,10,12} With continuing therapy, the proportion of sensitive cells decreases and the tumor mass consists primarily of resistant cells. If the growth kinetics (i.e., proportion of proliferating cells) of sensitive and resistant cells differ, treatment regimens for relapsing patients must be modified in accordance with the properties of the dominant cell population.

The present study was designed to evaluate the growth fraction (GF) and other growth kinetics parameters in patients with MM using prolonged intravenous infusions of tritiated thymidine (³H-TdR). Results in 17 patients indicated that a markedly elevated GF occurred only during relapse

suggesting that further trials with cycle active agents should be conducted only at this phase of disease.

MATERIALS AND METHODS

Twenty-two studies were conducted in 17 patients with MM. Sixty-five percent were male and the median age was 54. All but 2 patients had an IgG myeloma protein peak to facilitate calculation of tumor mass doubling times as previously described.^{10,12} Three patients were studied more than once. One patient (A.H.) was evaluated once during remission and again during relapse; O.R. was evaluated during two successive relapses, the first after melphalan-prednisone and the second after a response to adriamycin. W.S. was evaluated before initiating treatment, during remission and at two different stages of relapse. In another patient (F.V.), studies were conducted on bone marrow and pleural fluid plasma cells simultaneously.

All patients received detailed information concerning the investigational nature of the procedure and were required to sign an informed consent approved by the Human Surveillance Experimentation Committee of the Institution. Patients were given a single intravenous injection of 10 mCi of ³H-TdR (sp. act. 40-50 Ci/mmole) followed by a continuous daily i.v. infusion of 10 mCi of ³H-TdR/day in 5% dextrose (1000 ml/day) dispensed by an IVAC 500 infusion pump (IVAC Corp., San Diego, Calif.) for 8-10 days. Bone marrow aspirates were obtained 1 hr after the initial injection and at about 2-day intervals thereafter. Marrow aspirates were placed in a centrifuge tube containing 5 ml of McCoy's 5A medium with 660 U of heparin/ml and mixed by repeated pipette aspira-

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tions. Smears were prepared by centrifuging cells directly onto slides by means of a cytocentrifuge and processed for autoradiography by the liquid emulsion technique with the use of Ilford K5 emulsion (Polysciences, Inc., Warrington, Penn.). After an exposure interval of 4–6 mo, the smears were developed, fixed, and dyed with May-Grunwald stain. Differential counts were made and 200–1000 plasma cells were examined. Cells were considered labeled when they exhibited at least 5 grains overlying the nucleus. Increments in labeling index were plotted as a function of time elapsed from the initial dose of ^3H -TdR.

In five relapsing patients with large GF, approximate estimates of cell cycle transit times were calculated from the continuous labeling curve using a heuristic growth kinetics model based on the following assumptions. (1) The entire myeloma cell population is comprised essentially of two distinct compartments, proliferating and nonproliferating cells. (2) The flux from the nonproliferating to the proliferating compartment is negligible over the duration of the experimental interval. (3) Following mitosis, half of the daughter cells in the proliferative compartment enter in a maturation phase, while the remaining daughter cells re-enter the G_1 phase, leading to subsequent division. (4) Cells in the maturation phase are lost at the rate at which they enter, so that the proliferative compartment during the experimental interval is in a steady state. (5) Variability of the durations of the phases of the proliferative cell cycle is considered to be negligible.

The assumed theoretical scheme of proliferation is illustrated in Fig. 1. Under these circumstances, it is a simple matter¹⁴ to express mathematically the theoretical consequences of a continuous labeling regimen (See Appendix). The schematic form of the resulting labeling index curve is shown in Fig. 2. The following features of the curve should be noted: (A) The "plateau" or asymptotic value of the labeling index, LI_∞ is an upper boundary of the GF which, strictly speaking counts only cells in G_1 , S and G_2 phases. In what follows we interpret the LI_∞ as the GF, even though we recognize the approximate nature of the identification. (B) The initial labeling index, $LI(0)$ is given as $LI_\infty T_s/T$ where T_s is the duration of S phase and T is the transit time through the proliferative compartment:

$$T = T_{G_1} + T_s + T_{G_2} + T_M$$

(C) The transit time T may be estimated from the slope of the rapidly rising portion of the LI curve, which has the theoretical value $2 LI_\infty/T$. Knowing T , we can determine T_s from the initial labeling index, $LI(0)$.

In addition to the above indicated parameters, the growth rate of a tumor is influenced by the rate of cell attrition from the malignant population.¹⁵ Classical methods to measure this rate of attrition are based on models that assume a homogeneous population and could, therefore, not be strictly applied to our myeloma cell population. Yet, in the interest of providing an approximate estimate of such values for MM tumors, we decided to treat the malignant plasma cells as a homogeneous population within the constraints of this evaluation.

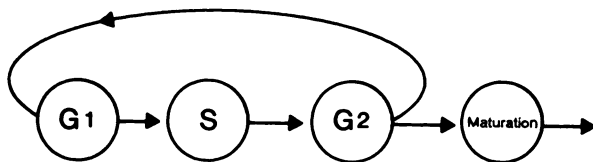


Fig. 1. Diagram demonstrating the assumed phases of the proliferative compartment (G_1 ; S; G_2 and a maturation phase). The mitotic phase is assumed to be part of G_2 . The mean durations of these phases are: T_{G_1} ; T_s ; T_{G_2} ; and T_M , respectively.

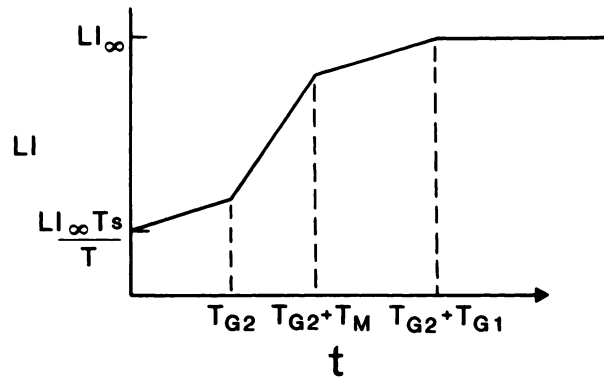


Fig. 2. Schematic representation of the labeling index (LI) as a function of the time (t), based on equation 8 (See Appendix) with $T_M < T_1$. The slope on the first and third segments of the curves is LI_∞/T_1 , while for the second segment, it is $2 LI_\infty/T$.

Based on generation time values calculated for the discrete experimental interval, further cytodynamics were estimated under the assumption of an exponential tangent to a Gompertzian function. The potential doubling time (T_{DT}) was estimated by $T_{DT} = T_c/GF$; and the observed tumor doubling time (T_{DO}) was computed from the curve depicting sequential increments in M-protein production rate.^{9,10} Cell loss was estimated as described by Steel,¹⁵ cell loss factor = $1 - T_{DP}/T_{DO}$ and considered independent of treatment since the doubling times were calculated from curves fitted to the changing M-protein production rate during relapse.

RESULTS

Two types of continuous labeling curves were obtained in patients receiving intravenous infusions of ^3H -TdR (Fig. 3). One untreated patient shows the very low proportion of labeled cells (1%) attained after 8 days of infusion in contrast to the very high level (47%) observed for a patient in relapse. The labeling index curves for patients in relapse were interpreted in accordance with the theoretical model described above.

Variations in the GF of the myeloma tumor mass as

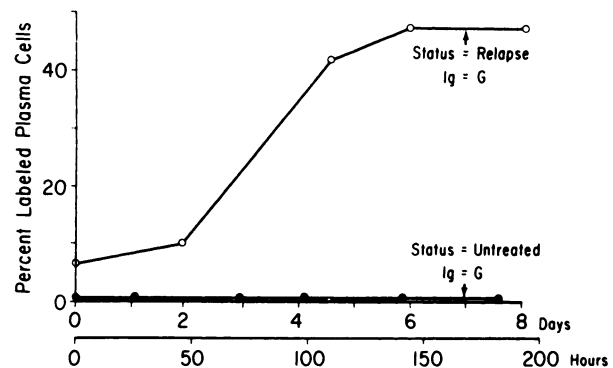


Fig. 3. Increments in labeling index during prolonged infusions of tritiated thymidine. The figure contrasts the level of 47% labeled cells for a relapsing patient to the nearly constant value in an untreated patient.

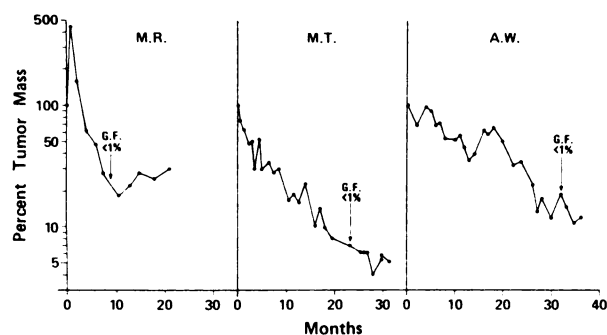


Fig. 4. Growth fraction for patients in remission. Changes in myeloma protein production rate, as an index of tumor mass are correlated with durations of chemotherapy.

a function of stage of disease are shown in the next series of representative graphs. The GF was low ($<1\%$) for untreated patients and during the initial period of tumor mass reductions (Fig. 4). When rapid tumor relapse developed, GF values were very high (Fig. 5). During the last phase of relapse when the recurrent tumor mass exceeded the pretreatment level and growth rate slowed down, the GF was very low (Fig. 6). Figure 7 shows results for patients in whom at least two evaluations of GF were performed. The upper panel presents results for a patient evaluated during remission (GF = 8%) and then at relapse (GF = 18%); the mid-panel shows a GF of 20% during relapse that declined to 1% during a second remission. The lower panel summarizes the data of 4 separate GF evaluations. The GF was less than 1% before treatment and remained low (4%) during remission. As relapse began, the GF was 5.6% and increased to 14% as a higher tumor burden developed.

Table 1 summarizes results from 22 studies

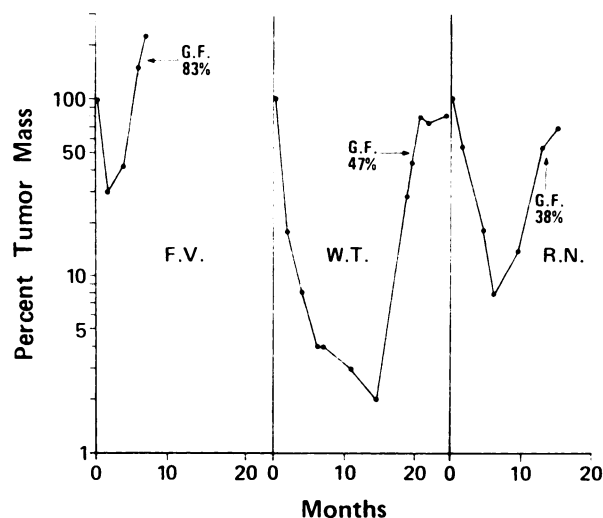


Fig. 5. Growth fraction for patients in rapid relapse.

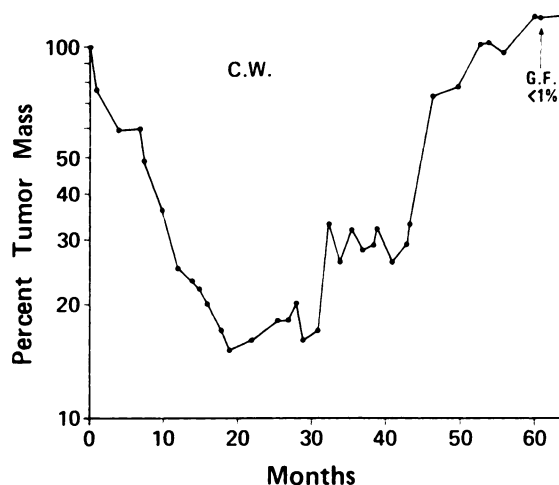


Fig. 6. Growth fraction determined for a relapsing patient entering a period of slow tumor growth.

obtained in 17 patients. Although a low GF always corresponded to a very low initial LI, the reverse was not always true and a high GF may be present despite the low 1-hr LI.

The correlation of GF values with stage of disease demonstrated that in all 4 untreated patients, in both unresponsive patients and in 5 of 6 patients in remission the GF was less than 4% (usually less than 1%). In 7 or 10 patients in relapse, the GF exceeded 14%. All 7 patients were in the phase of rapid relapse. Only during very early and late phases of relapse was a low GF found.

Table 2 presents values for growth kinetics parameters calculated for 4 patients with a GF exceeding 20%. Data obtained for 3 other relapsing patients were

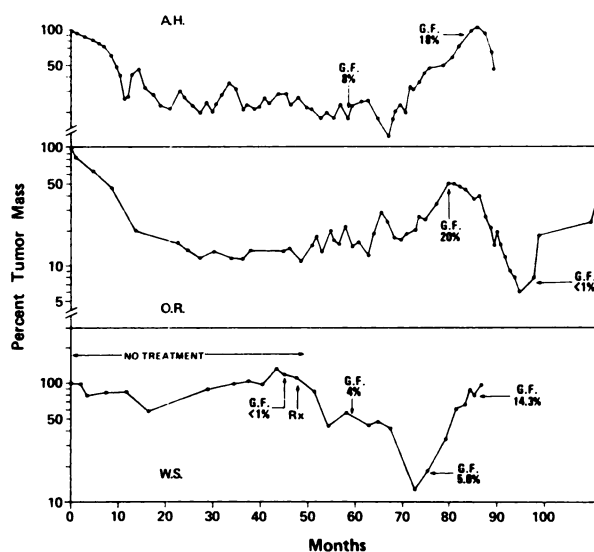


Fig. 7. Individual patients with multiple growth fraction determinations.

Table 1. Growth Fractions of Patients With Multiple Myeloma Determined After Intravenous Infusions of Tritiated Thymidine

Disease Stage	Patient	Labeling Index at 1 Hr	Growth Fraction (%)
Untreated	W.S.	0.2	<1
	E.M.	0.2	<1
	C.R.	0.2	<1
	H.S.	2.4	3.7
Unresponsive	D.C.	0.4	<1
	R.J.	0.6	2.6
Remission	A.W.	0.2	<1
	O.G.	0.2	<1
	M.R.	0.2	<1
	M.T.	0.2	<1
	W.S.	0.2	4
Early or Late Relapse	A.H.	0.8	8
	W.S.	1.6	5.6
	O.R.	0.2	1
	C.W.	0.2	1
Rapid relapse	O.R.	5.7	20
	W.T.	6.6	47
	R.N.	13.8	38
	F.V.	23.4	83
	A.H.	0.4	18
	C.M.	4.4	21
	W.S.	2.4	14.3

inadequate for these calculations. In one patient (F.V.), simultaneous studies were conducted for the myeloma cells present in the bone marrow and in the pleural effusion induced by a rib lesion. The transit time through the proliferation compartment of bone marrow myeloma cells ranged between 6.6 and 11.9 days and the calculated S phase time ranged from 1.2 to 3.3 days. The potential doubling time for the tumor mass of each of these patients ranged from 0.3 to 1.1 mo, values markedly shorter than the actual doubling times (1–8 mo) measured from changes in myeloma protein production rates. These differences are attributed to the marked degree of cell loss experienced by the tumor mass computed at higher than 50% for all patients.

Growth kinetics parameters of the myeloma cells in

the pleural fluid of patient F.V. differed markedly from those defined for the bone marrow. Thus, the transit time (7.2 days) and S phase duration (0.8 days) were only 61% and 24% of the values defined for the bone marrow cells. The presence of numerous mitotic figures in the pleural fluid permitted an approximation of the length of G₂ phase (11 hr) at the level of 50% labeled mitoses. After 22 hr, all mitoses were labeled.

DISCUSSION

To evaluate the percentage of proliferating plasma cells, patients with MM in different stages of disease received a prolonged intravenous infusion of ³H-TdR. If all marrow plasma cells participated in the proliferative process, the percentage of labeled cells would approach 100% asymptotically.¹⁵ In practice, the LI attains a "plateau" phase within the constraints of the experimental period of observation, and at a level that is usually significantly less than 100%. We believe that this feature reflects a fundamental inhomogeneity in the constituency of the plasma cells, and has led to our advocacy^{9,10,12} of the concept that plasma cells in MM patients, in the first approximation, should be considered to behave as two distinct neoplastic populations, namely, proliferative (drug sensitive) and nonproliferative (drug resistant). A mathematical realization of this concept is presented herein and forms the basis of a quantitative kinetic evaluation of MM patients. While this model represents a very simplistic version of a biphasic proliferating-nonproliferating cell population, it is doubtful whether a more elaborate version is either deserved by the data, or could alter treatment strategy in any significant degree.

In our studies, the nonproliferating fraction determined in this manner contains true quiescent cells, some proliferating cells with very long intermitotic times, and some proliferating cells that have entered the maturation phase. Although this approach underestimates the size of the proliferative compartment, it provides valid information for potential clinical applications; thus, if a cell does not traverse the cycle during an 8–10 day interval (as shown by ³H-TdR uptake), it should be considered out of cycle for purposes of sensitivity to antitumor agents delivered either as a bolus or in courses of less than 8 days. Conversely, the GF calculated from the "plateau" of labeled cells will be an overestimate because labeled proliferating cells that enter the maturation compartment are no longer destined to proliferate and indistinguishable from cells in the proliferating compartment. Should quiescent cells reenter the proliferating compartment during the experimental interval, or if mature cells fail to disintegrate or leave the marrow,

Table 2. Growth Kinetics of Myeloma Cells in Relapsing Patients

	O.R.	W.T.	R.N.	F.V.	
				Bone Marrow	Pleural Fluid
Growth fraction, LI _∞ (%)	20	48	38	83	95
LI (%) at 1 hr	5.7	6.6	13.8	23.4	10.5
Length of S phase*	1.9	1.2	2.9	3.3	0.8
Transit time*	6.6	8.9	7.9	11.9	7.2
Potential doubling time*	1.1	0.6	0.7	0.5	0.3
Observed doubling time†	8	1.2	2	1.3	1.3
Cell loss (%)	86	50	65	62	77

*Days.

†Months.

achievement of a plateau of labeled cells will be delayed.

While more elaborate models have been constructed to calculate kinetic parameters from LI curves following continuously labeling,^{15,16} the sampling frequency required by such analyses precludes their routine application in clinical procedures. Furthermore, these models are based on the assumption that the cell population under investigation consists entirely of proliferating cells, which we have seen is not supported by the data.

In previous studies using the halving time of the median grain count of pulse labeled myeloma cells of relapsing patients, the generation time was longer than the study period of 4 days.¹² We concluded that the generation time of resistant myeloma cells was probably much longer than the 2–4 days defined for sensitive plasma cells of untreated patients by Killman et al.,¹⁶ Pileri et al.¹⁸ and Riccardi et al.¹⁹ This conclusion was confirmed by the present studies where the calculated transit time ranged from 6.6 to 11.9 days in 4 relapsing patients. Patient F.V. had different growth kinetics values for the myeloma cells growing in the bone marrow and the pleural fluid. The LI of the pleural fluid myeloma cells was less than half of the bone marrow cells, probably because a large fraction of these cells (included in the denominator of LI equations) consisted of shed nonviable elements. In contrast, the generation time and length of S phase were much shorter for the pleural fluid cells. These results were similar to those previously observed for the pleural effusion (79 hr) and bone marrow cells (> 4 days) of another patient with multiple myeloma in whom results were defined from the halving time of the median grain count.¹² Thus, secondary lesions appear to have shorter generation times than bone marrow plasma cells, perhaps because of reduced S phase transit time. This rapid proliferation rate could account for the increased sensitivity and more rapid reduction of tumor cells in secondary lesions than in the primary sites.

The potential tumor doubling time calculated for relapsing patients was about 1 mo, a value considerably shorter than the actual doubling times determined from increments in myeloma protein synthesis. These differences are attributed to the marked degrees of cell loss inherent in each tumor. Our data on relapsing

patients support the evidence presented by Pileri and Tarocco for untreated patients with MM where a marked cell loss was considered responsible for the slow growth rate of the tumor mass.¹⁷ We do not know whether this cell loss issues from the proliferating or the quiescent pool; we also ignore whether it is a random process conditioned by as yet unexplained host-tumor cell immunologic relations or the consequence of normal maturation and senescence usually associated with cell renewal systems such as hemopoietic tissues. What is most apparent is that the growth rate characteristics of the myeloma tumor mass are affected mostly by the GF and by the rate of inherent cell loss, and only partially by the generation time of the proliferating plasma cells.

The magnitude of the LI did not correlate with the size of the GF. Although a low GF was always associated with a low LI, an initially low LI could correspond to large proportions of proliferating plasma cells suggesting that the size of the proliferating compartment varies greatly from patient to patient. These results were similar to those reported by Pileri et al.²⁰ and by Riccardi et al.¹⁹ using a different method for determining the GF. These findings reemphasize the inappropriateness of the LI for comparing differences in growth kinetics parameters among individual patients.^{5,12,21}

Our results also demonstrated that the GF of malignant plasma cells increases substantially only during the rapid period of relapse and it is only during this stage that the use of cell cycle sensitive drugs or treatment regimens based on a growth kinetics rationale (i.e., synchronization) are appropriate. Furthermore, these results support our contention^{10,12} that the entire tumor mass of untreated and remission patients is maintained by a very small fraction of proliferating cells as also proposed by others.^{5,7} Hence, declining tumor loads may not be detected for long periods after treatment with effective agents that sterilize only the proliferating cells. To increase tumor cell kill beyond that presently obtained with conventional chemotherapy, agents must be developed that will kill nonproliferating plasma cells or regimens must be designed that will recruit quiescent plasma cells into the proliferating pool. These approaches might be enhanced by techniques for evaluating antitumor drugs on primary²² or permanent²³ cultures of myeloma cells.

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APPENDIX

Assume that cells are traversing the phases of the proliferating compartment in a steady state manner (see Fig. 1). The mean transit times through these phases are designated T_{G_1} ; T_S ; T_{G_2} ; and T_M , respectively. With n equal to the steady state mitotic rate, the population of the phases of the proliferative compartment are: $N_{G_1} = n T_{G_1}$; $N_S = n T_S$; $N_{G_2} = n T_{G_2}$; and $N_M = n T_M$. The total population N of the proliferative compartment is $N = n T$, where T is the total transit time.

$$T = T_{G_1} + T_S + T_{G_2} + T_M \quad (1)$$

At time $T = 0$, continuous labeling of all cells in S commences until all cells in the proliferative compartment are labeled. Designating labeled cells by an asterisk, the labeled population is given as:

$$N_{G_1}^*(t) = n \begin{cases} 0 & , & 0 < t \leq T_{G_2} \\ t - T_{G_2} & , & T_{G_2} < t \leq T_{G_1} + T_{G_2} \\ T_{G_1} & , & T_{G_1} + T_{G_2} < t \end{cases} \quad (2)$$

$$N_S^*(t) = n T_S \quad , \quad 0 < t \quad (3)$$

$$N_{G_2}^*(t) = n \begin{cases} t & , & 0 < t \leq T_{G_2} \\ T_{G_2} & , & T_{G_2} < t \end{cases} \quad (4)$$

$$N_M^*(t) = n \begin{cases} 0 & , & 0 < t \leq T_{G_2} \\ t - T_2 & , & T_{G_2} < t \leq T_{G_2} + T_M \\ T_M & , & T_{G_2} + T_M < t \end{cases} \quad (5)$$

$$\text{The labeling index, LI (t) is defined as } LI(t) = \frac{N^*(t)}{N + N_0} \quad (6)$$

Where N^* is the total number of labeled cell ($N_{G_1}^* + N_S^* + N_{G_2}^* + N_M^*$); N is the total population of the proliferative compartment; and N_0 is the total population of the nonproliferative compartment. Note that asymptotically, all of the cells in the proliferative compartment will be labeled so that $N^* \rightarrow N$ and $LI \rightarrow 1$, which is essentially the growth fraction of the population. Hence the ratio N_0/N can be expressed as

$$N_0/N = \frac{1 - LI \infty}{LI \infty} \quad (7)$$

By substituting equations (1) through (5), and (7) into (6), and assuming that $T_M < T_1$, we obtain:

$$LI(t) = \frac{LI \infty}{T} \begin{cases} T_S + t & , & 0 < t \leq T_{G_2} \\ T_S - T_{G_2} + 2t & , & T_{G_2} < t \leq T_{G_2} + T_M \\ T_M + T_S + t & , & T_{G_2} < t \leq T_{G_2} + T_{G_1} \\ T & , & T_{G_2} + T_M < t \end{cases} \quad (8)$$

The above equation is illustrated schematically in Fig. 2. Two features of the above equation should be noted. One is that T_M and T_{G_1} must be interchanged if $T_1 < T_M$. The other, is that if the concept of a maturation phase of the proliferative compartment is to be discarded, so that $T_M = 0$ in equation (8), then $LI(t)$ becomes a ramp function represented by a single straight line of slope $LI \infty / T$ in the interval $0 < t < T_{G_1} + T_{G_2}$. The latter possibility is an alternate interpretation of experimental curves such as that shown in Fig. 3, with "mending" of the curves due to cell-to-cell variability of phase duration.