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# A Comparison of Analytical Methods for the Generation Time of Cultured Human Myeloma Cell Line, KMM-1

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**ABSTRACT.** The generation time (GT) of a human myeloma cell line (KMM-1) producing λ-type Bence-Jones protein[1] was analyzed by the following methods; calculation from the growth curve, autoradiographic analyses of continuous $^{3}$H-thymidine labeling and of a fraction of labeled mitoses, the sister chromatid exchange method, time-lapse cinemicrography, and flow cytometric measurement. The GTs obtained by each method differed considerably. The GT calculated from the cell growth curve was 29.3 hr, while those analyzed by other methods were as follows: 16.6 hr by a fraction of labeled mitoses, 22.9 hr by sister chromatid exchange, and 26.4 hr by time-lapse cinemicrography, and 18 to 20 hr by flow cytometric analysis. The mean GT of the cultured myeloma cells, when averaged from these results, was 22.8 hr. The reasons for differences in the GTs obtained by these different analytical methods were suggested in Discussion.

**Key words : cell growth kinetics — human myeloma — cell synchronization**

Since the pioneering work of Howard and Pelc,[2] several methods have been developed to determine the proliferation characteristics of normal and malignant cells. These include the calculation of population doubling time (PDT) from growth curves,[3] autoradiographic analyses (ARG) of continuous $^{3}$H-thymidine labeling (CL)[4,5] and of a fraction of labeled mitoses (FLM),[4,6] sister chromatid exchange (SCE) method after exposure to bromodeoxyuridine (BudR),[7-10] time-lapse cinemicrography (CMG),[11] and flow cytometric (FCM) measurement.[12,13]

In spite of the development of these methods, it is still relatively difficult to obtain accurate information on the cell growth kinetics of certain proliferative tissues in an adult mammalian bodies,[14] because tissues such as those from the epidermis, testis, small intestine or bone marrow show a steady loss of cells through differentiation or balanced replacement from their stem cell compartment.

Human myeloma cells originate from plasma cells which are terminally differentiated B-lymphocytes. Therefore, learning what types of growth kinetics myeloma cells show should be interesting. For this reason, using the above-mentioned several methods, we analyzed the growth kinetics of a relatively differentiated human myeloma cell line (KMM-1) which continuously produces λ-type Bence-Jones proteins and is not tumorigenic in nude mice. In addition, we discussed the causes of the differences in the GT obtained by each method.

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## MATERIALS AND METHODS

### Cell culture

A human myeloma cell line, KMM-1, was established in the Department of Pathology of Kawasaki Medical School[1] from a subcutaneous tumor of a patient with BJP (λ-chain) type multiple myeloma. This cell line produces λ-chains in culture and does not grow in soft agar, and does not form tumors in nude mice.[15] KMM-1 was grown in RPMI-1640 with 10% fetal bovine serum and 100 μg/ml of kanamycin at 37°C in a humidified atmosphere with 5% $CO_2$.

### Growth curve and PDT

$10^5$ cells were seeded in 35 mm culture dishes with 2 ml of culture medium per dish. On days 1 and 4 the cell number was counted by means of a hemocytometer after staining with 1% crystal violet in 0.1 M citric acid. PDT was calculated from the growth curve by adopting the following formula:

$$GT = t/n, \quad b = a \times 2^n$$

GT: generation time, t: culture time, n: frequencies of cell division, a: cell number at initial time, b: cell number after t hr culture.

### Continuous labeling methods (CL)[4,5]

The exponentially growing cells were labeled with 0.01 μCi/ml of $^3$H-TdR (824 GBq/mmol, Amersham, England) from the initiation of culturing, and 24 hr later the same dose of $^3$H-TdR was added to the cultures. The labeled cells were harvested for smear preparation for ARG at 24 and 48 hr after culturing.

### FLM method

We used the method described by Steel, G.G.[6] Briefly, cells were pulse-labeled by 2 μCi/ml of $^3$H-TdR for 30 min. Thereafter, smears for ARG were prepared every 2 hr. In order to clearly define mitotic figures the cells were treated with a hypotonic solution of 0.075 M KCl for 20 to 30 min before the preparation of smears.

### ARG

After washing by 10% cold trichloroacetic acid solution and fixation by methanol, smears were filmed with konica emulsion and exposed for 2 wk to CL and 1 wk to FLM at 4°C. Then ARG were developed in Konidol X at 20°C for 5 min.

### SCE method

BudR was added to cultures at a final concentration of 1 μg/ml, and samples were harvested every 12 hr. Air-dried chromosome preparations were made by the conventional technique, stained with Hoechst 33258 (0.5 μg/ml) for 12 min, mounted in citrate-phosphate buffer (pH 7.0), exposed to black light on a hot plate at 50°C for 60 min, and stained in 5% Giemsa. Metaphases were classified as either first, second, or third divisions using criteria previously described.[7]

CMG analysis

To prevent the escape of cells from the optic field of a microscope the cells were grown in fibrin clots, which were made of 0.5% fibrinogen (Calbiohem-Boehringer, Mannheim, Germany) with 1 unit of thrombin (Sigma). Pictures were taken at 1 fr/ 2 min for 6 days.

FCM analysis

FCM analysis was performed according to previously described methods[12,13] using a Becton Dickinson FACstar. Briefly cells were exposed to 2.5 $\mu$g/ml of BudR for 30 min, harvested every 2 hr, and fixed in 70% cold ethanol. After incubation in 4 N HCl at 20°C for 20 min, cells were incubated in 1 ml PBS containing 0.5% Tween 20 (Sigma Chemical Co.) and 10 $\mu$l of a FITC-labeled monoclonal anti-BudR antibody (Becton-Dickinson Co.) at 20°C for 20 min. After DNA staining in 5 $\mu$g/ml of propidium iodine (PI, Sigma) clumps of cells were removed by passing the specimens through 50-$\mu$m nylon mesh and single cells were subjected to FCM analysis.

Cell synchronization

Cell synchronization was performed by the following schedule using a DNA polymerase $\alpha$ inhibitor, aphidicolin[16,17] (APC, Wako Pure Chem. Ltd., Osaka). On Day 0, $10^6$ cells were seeded in 60 mm dish containing 5 ml culture medium with 3 ml of an ammonia absorbent, silica-alminium ZCP-50.[18] After 4 days, at which time the culture reached a plateau phase, the cells were collected by centrifugation and reseeded in the fresh culture medium supplemented with 20% conditioned medium (CM) and 0.5 $\mu$g/ml of APC. The conditioned medium was harvested from the exponentially growing cultures of the same cells. After 24 hr, APC was removed by centrifugation, and cells were reseeded into the culture medium containing 20% CM. Then the FCM analysis described above was done every 2 hr.

## RESULTS

Based on the growth curve of KMM-1 cells shown in Fig. 1, PDT was calculated to be 29.3 hr by the formula described in Materials and Methods. The percentage of labeled mitoses is shown in Fig. 2. The generation time was inferred to be 16.6 hr from the duration period from point A (the time when FLM was reached to one half of the first peak) to C (the time at one half of the second FLM peak). The generation time analyzed by the SCE method was presumed to be 22.4 hr from the duration period between the first and third division (Fig. 3). As shown in Fig. 4, 18 cells were fully observed from their initial division to the next division and the mean generation time of these 18 cells was 26.4 hr. The results of FCM analysis are shown in Fig. 5. The horizontal axis indicates the time after removal of pulse-labeled BudR and the vertical axis shows the percentage of 2c, 3c, and 4c cells among the total cells positively stained with anti-BudR antibody. The generation time was indicated as approximately 16 hr from the time from the first to second peak of 3c cells.

Fig. 1.

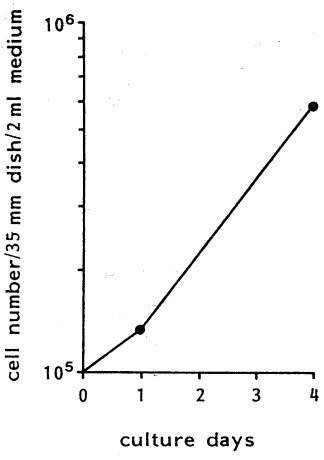


Fig. 2.

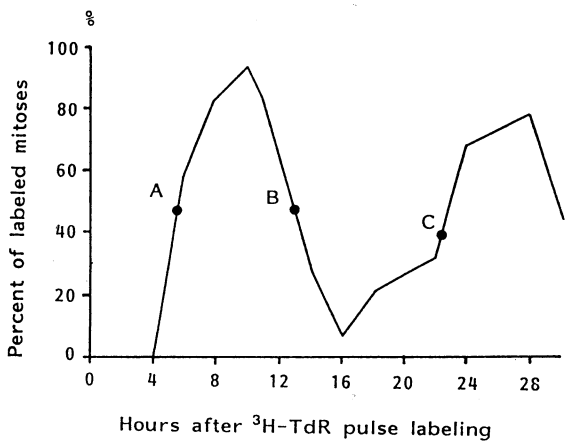


Fig. 1. Cell growth curve of KMM-1 cells

Fig. 2. Cell cycle analysis of KMM-1 cells by a fraction of labeled mitoses. A: time at one half of the first ascending curve, B: time at one half of the first descending curve, C: time at one half of the second ascending curve

Fig. 3.

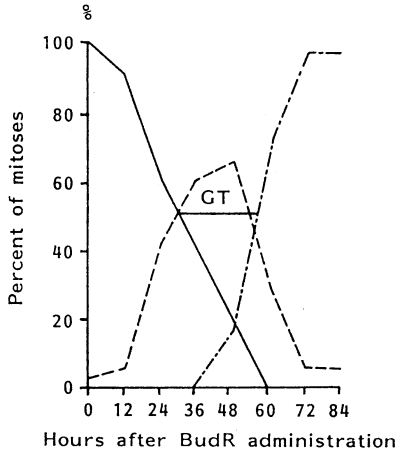


Fig. 4.

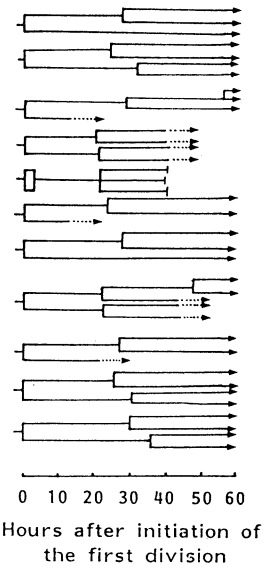


Fig. 3. Cell cycle of KMM-1 analyzed by SCE. Distribution of first, second, and third division mitoses. The generation time (GT) was calculated from the duration period between when the first and the third division mitoses.
——— : first division, - - - - : second division, — — — — : third division.

Fig. 4. Family trees of KMM-1 cells which were observed from their initial division to the next division by time-lapse cinemicrography.
⟶ : alive, —⊣ : dead, ·····→ : indistinguishable for increasing of cell number

Fig. 5.

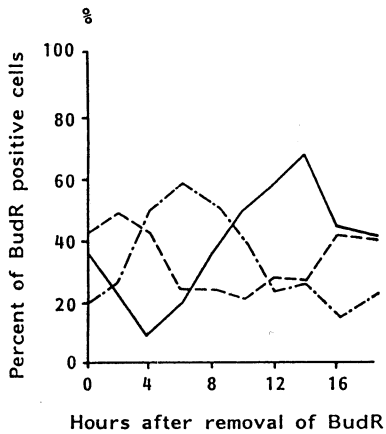


Fig. 6.

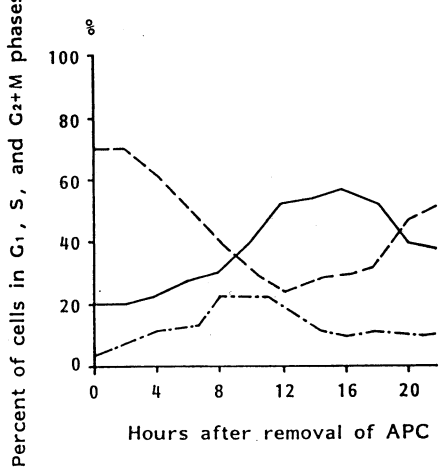


Fig. 5. Cell cycle of KMM-1 cells analyzed by FCM.
——— : 2c, ---- : 3c, —·—·— : 4c

Fig. 6. FCM cell cycle analysis of KMM-1 cells synchronized by treatment with aphidicolin.
——— : $G_1$-phase, ---- : S-phase, —·—·— : $G_2$+M-phase

TABLE 1. Comparison of GT of KMM-1 analyzed by various methods

| Method | Generation time (hr) |
|---|---|
| Population doubling time | 29.3 |
| Fraction of labeled mitoses | 16.6 |
| Sister chromatid exchange | 22.9 |
| Time-lapse cinemicrography | 26.4 |
| Flow cytometry | 18~20 |
| | 22.8 (Mean) |

The results of cell synchronization are shown in Fig. 6. After 4 days of culturing, KMM-1 cells were supposed to be in the plateau phase. Then they were transferred into the fresh medium supplemented with 20% CM with APC for 24 hr. At this stage the cells were assumed to be in the $G_1$/S boundary. Immediately after removal of APC approximately 70%, 20%, and 10% of the cells were in the early S-, $G_1$-, and $G_2$+M-phases, respectively (Fig. 6). The second early S-phase was observed about 20 hr after APC removal.

The generation times obtained by these methods are summarized in Table 1. The GT calculated from the cell growth curve was longer than those obtained by other methods. The GT from the growth curve was based on an assumption that whole cells were in the proliferative fraction. However, it was demonstrated by the other methods that a certain number of cells were not proliferative but rather in the $G_0$ or degenerative state. For example, the percentage of non-labeled cells, when examined by CL, was 28.2% at 24 hr and 21.1% at 48 hr. Therefore, the other methods used could detect only the proliferative fraction. This may explain why GT from the cell growth curve was longer than those analyzed by the other methods.

## DISCUSSION

The difference in the generation time of KMM-1 cells obtained by the several methods used in the present study may be due to differences in the cell population with each method could measure. The results of PDT were obtained from a heterogeneous cell population of proliferative and non-proliferative cells, but the other methods could measure only the proliferative fraction of the cells. Unless the cells were cultured under unsuitable conditions, the results achieved by the FLM or FCM method, in particular, may indicate the real generation time of proliferative KMM-1 cells. It was confirmed that the short time labeling of $^{3}$H-TdR or BudR did not disturb the cell growth kinetics for measurement of the generation time only for one cell cycle.

The generation times obtained by the remaining two methods, CMG and SCE, were also calculated from only the proliferative cell population. Using CMG we could actually observe the cell division and pursue the generation time under a microscope. On the other hand, we could detect only mitotic figures of cells by the SCE method, so these cells were assumed to be progressing through the cell cycle. However, the generation times obtained by these two methods were longer than those measured by FLM and FCM. This difference may be due to the use of different procedures. For CMG, the KMM-1 cells presumably had to be cultured under unfavorable conditions; i.e., in fibrin clots which kept the cells in the optic field of the microscope. Therefore, the cell growth was delayed a little longer than it was for cells cultured under the usual suspension culture conditions employed for the other analytical methods. With the SCE method, the cytotoxicities of BudR may have prolonged the generation time, because we had to continuously treat the cells with BudR for at least 72 hr to observe the third division metaphases, based on the assumption that the generation time of the cells was 24 hr. This continuous treatment of the cells with BudR may have disturbed the progression of the cell cycle. In fact, approximately 15% growth inhibition was observed when KMM-1 cells were cultured with 1 $\mu$g/ml of BudR for 96 hr, the concentration of which was used to analyze the cell cycle (Data is not shown).

CL or FCM analysis of the synchronized cells showed that approximately 20 to 30% of the KMM-1 cells were in the non-cycling fraction. As mentioned before, the characteristics of the KMM-1 cells were a little more differentiated; e.g., the inability to grow in soft agar and to form tumors as compared with other malignant B cell lymphomas. In addition, KMM-1 cells produce a relatively large amount of immunoglobulin. Although the myeloma cells in the present study were malignant, about one fifth of them were losing their proliferative capacity in culture. Therefore, it would seem of interest to learn whether or not the non-cycling cells in the cell population are at a certain point of differentiation of plasma cells which do not divide any more. To elucidate the cellular mechanisms by which growing cells leave the proliferative cell cycle may help us understand biological characteristics of malignant myelomas and be beneficial in future therapies.

It is valuable to study the growth patterns of various B-cell lines derived from different stages of differentiation; e.g., Burkitt lymphoma (Raji,[19] EB 3[20]), EBNA positive lymphoblastoid cell lines (KMS-9,[15] KMS-10[15]), less differentiated and more malignant plasma cell line (KMS-5[15]), and other myeloma cell lines

(U266,[21] ARH-77[22]), because knowledge of the growth kinetics of these cell lines should provide us with significant information about the relationship of differentiation and the malignant characteristics of B-cells as well as therapeutic clues to the treatment of B-cell lymphomas.

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