The Thymidine-$^{14}$C and -$^{3}$H Double-labeling Technic in the Study of the Cell Cycle of L1210 Leukemia Ascites Tumor in Vivo

Robert C. Young, Vincent T. DeVita, and Seymour Perry

Medicine Branch, National Cancer Institute, NIH Bethesda, Maryland 20014

SUMMARY

A study was performed to evaluate the double-labeling technic with thymidine-$^{14}$C and -$^{3}$H in the determination of the duration of DNA synthesis, duration of mitosis, and generation time of L1210 leukemia ascites tumor. The results were comparable with data obtained from labeled mitoses curves. The average duration of DNA synthesis was 9.0 hours, and the average duration of mitosis was 0.20 hours.

Prolongation of generation time on the seventh day of tumor growth (18.1 hours) compared with the sixth day (13.3 hours) was noted. This difference is highly significant ($P < 0.001$). Evidence was presented to suggest that the prolongation was related to a lengthened $G_{1}$.

INTRODUCTION

The double-labeling technic was first described by Pilgrim and Maurer (11) and by Wimber and Quastler (15) independently.

Consecutive injections of thymidine -$^{3}$H and -$^{14}$C are used, and the method depends upon recognition of cells leaving DNA synthesis in the interval between the two injections. The number of cells labeled with tritium alone is proportional to the time between injections, and the total cells labeled with $^{14}$C is proportional to the duration of DNA synthesis.

Certain assumptions concerning the cell population are required in order for the double-label technic to be a valid tool (11, 15, 16). The population of cells must be asynchronous, and a steady state must exist; cell loss must occur after mitosis and prior to DNA synthesis, and variation in generation time must be small. On autoradiographs, the cells must be well separated.

Several correction factors have been proposed for exponentially growing populations (4, 5, 9), and some controversy exists over their general applicability (2). Nevertheless, they can be used to adapt the conventional equations to exponentially growing populations, such as the ascites form of L1210.

L1210 murine leukemia is one of the tumors used in the primary drug screen by the Cancer Chemotherapy National Service Center of the National Cancer Institute and, as such, is the source of a considerable amount of data on the antitumor effect of various chemotherapeutic agents. Little information, however, has been available about the effects of these agents on the cell cycle of the tumor, and controversy exists as to whether alterations of generation times actually occur in response to drug therapy (6, 7, 13). This study was undertaken to compare the double-label technic in L1210 leukemia to data on the phases of cell cycle which had been determined previously by standard but more tedious methods. It was felt that, once the validity of the double-label technic had been established on this tumor, it would be possible to use the method to focus on the effects of various chemotherapeutic agents on the $S$ phase. The results indicate that the method is easily performed and yields comparable and reproducible data in L1210.

MATERIALS AND METHODS

CDF$_1$ mice (average weight 20 grams) were used throughout the experiment. L1210 ascitic cells for inoculation were obtained from CDF$_1$ donor mice on the 7th day of tumor growth. Two to 3 × $10^6$ cells were injected intraperitoneally into each of twenty mice used in the study. Ten mice were arbitrarily selected for study on the 6th day (Group I) and the 7th day (Group II) of tumor growth.

Twenty $\mu$g thymidine-$^{3}$H (specific activity 1.9 c/mmole, Schwarz BioResearch, Orangeburg, N. Y.), diluted to a total volume of 0.2 ml with sterile saline, were injected intraperitoneally into each of eight mice on each day. One hour later 5 $\mu$g thymidine-$^{14}$C (53.6 mc/mmole, Schwarz BioResearch, Orangeburg, N. Y.), diluted to a total volume of 0.5 ml with sterile saline, were injected into the same mice in a similar manner. In each set of experiments one control animal was injected with thymidine-$^{14}$C and one with thymidine-$^{3}$H alone.

One hour after the second injection, the mice were killed by cervical traction. Ascitic fluid was withdrawn into 12-ml centrifuge tubes containing 0.5 ml 1% sodium citrate and kept in an ice bath. The abdomens were washed with 1% sodium citrate solution, and all fluid obtained was added to the original centrifuge tubes. The supernatant was discarded and the cell button gently resuspended with a methyl alcohol: glacial acetic acid solution (4:1). Centrifugation was repeated, and the cell button was resuspended as before. The suspensions were allowed to stand for 10 minutes; 3 drops were placed on alco-
hol-cleaned slides, allowed to spread, and blown forcefully to spread cells evenly. The slides were allowed to dry at room temperature for at least 12 hours and then AR10 stripping film (Kodak Limited, London, England) was applied. The slides were stored in light-tight plastic boxes at 4°C for 10–14 days and developed with Kodak 19D developer. After fixation and drying, the slides were stained with Giemsa stain for 27 minutes.

Mitotic and labeling indices were determined by counting 1000–2000 cells. Cells labeled with thymidine-3H alone and 14C-labeled cells were determined by analyzing 1000–2000 labeled cells in each of the 20 animals.

Recognition of cells labeled with 3H alone and 14C (with or without additional 3H) is accomplished by utilizing the difference between the energies of the β-particles of the two radioisotopes. The mean energy of the 3H β-particle is 5.5 KeV and the mean distance traveled is 1 μ. Therefore, cells labeled with thymidine-3H demonstrate grains sharply localized above the nucleus in one plane. The β-particle from 14C has a mean energy of 49 KeV and the mean distance traveled is 50 μ. Therefore, grains from 14C appear in many planes above and outside the nucleus and usually beyond the cell itself.

The accuracy of the distinction between 3H- and 14C-labeled cells was established initially in the following manner. Two control animals were injected with 20 μ of thymidine-3H alone, and 2 were injected with 5 μ of 14C. Two thousand labeled cells from each animal were counted. Cells with >20 grains over the nucleus and <5 grains over the cytoplasm were counted as tritium labeled. The significance of extranuclear label increased with increasing distance from the nucleus. Cells with >20 grains over the nucleus and >10 grains over the cytoplasm and beyond were considered 14C-labeled. Rare cells did not satisfy one of the above criteria and were excluded from the counts. Using these criteria, in singly-labeled control slides, 1% of the 3H-labeled cells and 0.3% of the 14C-labeled cells were incorrectly identified. If these two sources for error occur simultaneously during counting, their effect is in the opposite direction, i.e., errors in 14C counting increase the value for H and errors in 3H counting decrease the value for H. This has the effect of partially cancelling the error that occurs from incorrect identification. If an error of 0.7% is applied to the absolute values of H determined in the experiment in Table 1, and the t s is calculated, the range is within the standard deviation of the method.

RESULTS

In the steady state system described by Wimber and Quastler, if t s > t a, and the interval between injections is equal to or shorter than G 2 + 89 M, the S phase may be calculated using the formula

\[
\frac{H}{C} = \frac{t_a}{t_s} \quad \text{or} \quad t_s = \frac{C}{H} \times t_a
\]

where t s = duration of DNA synthesis (S phase); t a = the time interval between injections; H = number of cells labeled with thymidine-3H alone; and C = all cells labeled with 14C (with or without additional 3H).

In an exponentially growing population such as L1210 ascites tumor, a correction (K) is necessary for a more accurate estimate of the duration of DNA synthesis. We have used the following formula (5):

\[
K = \ln 2 \times 2 \left(1 - \frac{t}{t_c}\right)
\]

where t c = mitotic cycle time and t = the average age of cells in the phase (expressed in terms of t c). t/t c reduces to 0.125 in the L1210 system where 75% of the cell cycle time is spent in S phase. The formula therefore reduces to

\[
K = \ln 2 \times 2^{\frac{1}{3}} = 0.825
\]

where t s (actual) = K \times t s (observed)

Calculations of t c and t m are then based upon the corrected value for t s.

The duration of mitosis can be calculated using the formula supplied by Wimber and Quastler (15)

\[
\frac{C + H}{t_s + t_a} = \frac{M}{t_m}
\]

Table 1

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>Labeling index (%)</th>
<th>Mitotic index (%)</th>
<th>H/C</th>
<th>t s (hours)</th>
<th>t m (hours)</th>
<th>t c (hours)</th>
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<td>0.098</td>
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<td>14.1</td>
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</table>

Mean 65.1 2.1 Mean + S.D. 8.7 ± 0.9 0.20 ± 0.03 13.3 ± 0.8

In vivo mitotic cycle on 6th day of tumor growth. H/C, Cells labeled with 3H alone / cells labeled with 14C (with or without additional 3H). t s = Duration of DNA synthesis; t m = duration of mitosis; t c = cell cycle time.
where $M$ is the number of mitoses seen and $t_m =$ the duration of mitosis.

**Group I: Cell Cycle Data on the 6th Day of Tumor Growth**

The average L1210 DNA synthesis period in these 10 mice was 8.7 ± 0.9 hours. Individual calculated L1210 cell cycle times appear in Table 1. The average cell cycle time was 13.3 ± 0.8.

Average duration of mitosis was 0.20 ± 0.03 hours. Individual results can be seen in Table 1.

**Group II**

In this group of 10 mice, cell cycle data were obtained on the 7th day of tumor growth. At that time the animals were double labeled as above. Results are shown in Table 2. The average DNA synthetic period = 9.3 ± 0.7 hours and the average cell cycle time was 18.1 ± 1.7 hours with an average duration of mitosis of 0.19 ± 0.05 hours.

The differences between the $S$ phase for the 6th and 7th day of tumor growth are insignificant ($P > 0.1$), as are the differences between values for the duration of mitosis ($P > 0.4$). The prolongation of the cell cycle time between the 6th and 7th days of tumor growth is highly significant ($P \leq 0.001$).

**DISCUSSION**

The duration of DNA synthesis and the cell cycle time, as determined by the double-labeling technic yields results in substantial agreement with the findings of others using more conventional technics. Mitotic cycle time in L1210 6 days after inoculation was determined by Skipper et al. (12) to be 14.8 hours compared to 13.2 hours by the present technic. The $S$ phase of 9.0 hours compared well with the 8.9 hours reported by Yankee et al. (17) using labeled mitoses curves. A doubling time of 14.5 hours, reported previously from this laboratory on October 3, 2017. © 1969 American Association for Cancer Research.

The prolongation of the cell cycle time to be shorter than the observed doubling time by a factor representing either cell death, migration of cells from the abdomen, or both.

The generation time of L1210 on the 7th day of tumor growth was found to be prolonged to 18.1 hours. This is in agreement with results found previously using labeled mitoses curves (21.0 hours) (17). Cell cycle prolongation occurs in the absence of a significant change in duration of either DNA synthesis or mitosis. Variation must, therefore, occur in either $G_1$ or $G_2$. Information obtained previously in this laboratory utilizing labeled mitoses curves indicates that $G_2 + \frac{1}{2} M$ is not prolonged (17). This suggests that the prolongation in generation time is due to a lengthened $G_1$. A lengthening of the cell cycle at the later stages of growth brought about by prolongation of $G_1$ has been described (3, 10) in other tumor systems although it is unclear as to whether this is the only cause. Lala (8), using Ehrlich ascites tumor, found the prolongation of the cycle to be shared proportionately by $S$ and $G_2 + M$, findings which differed from Baserga's using the same tumor system (1). Prolongation of cell cycle time is only one of the factors affecting tumor growth at its later stages. Progressive decline in growth fractions (8) and increasing cell loss (14) also contribute their effects.

The double-labeling technic has several major advantages for the investigation of tumor systems to which the method is applicable. It requires only one sampling of tissue after the 2 injections, and information about $S$ phase and generation time of an individual tumor can be obtained from the single sample. The technic eliminates the need for counting labeled mitoses on serial samples, which may be tedious and is inconclusive if few mitoses are seen in any one sample. The technic is rapid and the time needed for the completion of the experiment is short enough that very few labeled cells will pass through mitosis. $S$ phase and generation time calculations can be made from data obtained in individual animals rather than depending upon curves constructed from multiple animal experiments. It also eliminates the need for multiple aspirations of ascites fluid, a procedure which may accelerate the growth rate (8).

The double-label technic is useful for focusing study on the duration of DNA synthesis and can be used to test the effects of physical and chemical alterations in the environment on cell kinetics (15). In L1210 leukemia, the use of this technic may

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</table>

| Mean      | 51.3               | 1.8               | Mean ± S.D. | 9.3 ± 0.7 | 0.19 ± 0.05 | 18.1 ± 1.7 |

*In vivo* L1210 mitotic cycle on 7th day of tumor growth. $H/C$: Cells labeled with $^3$H alone / cells labeled with $^{14}$C (with or without additional $^3$H). $t_s =$ Duration of DNA synthesis; $t_m =$ duration of mitosis, $t_c =$ cell cycle time.
provide a simple method of investigating the effect of antineoplastic agents on the cell cycle of this model tumor system.

REFERENCES

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