Kinetic Study on Cell Proliferation of Ehrlich Ascites Tumor Cells

Jun Tsuchiya
College of Medical Care and Technology
Gunma University

SUMMARY

Cytokinetic analysis was performed on the Ehrlich ascites tumor cells of hypotetraploid subline at the 4th and 7th day after the intraperitoneal inoculation of $1 \times 10^6$ cells, and the mean length of $G_1$, $G_2$ and S period was estimated by autoradiographic method using simultaneous administration of Thymidine-$6^{-}^{3}H$ and Colcemid. The results obtained are as follows: mean length of $G_1$ period is 3 and 4.5 hours, and S period is about 8.5 hours and 9.0 hours, respectively. The mean duration of $G_2$ period is about 1.5 hours for both ages of tumor cells. The dormant cell population is 16 and 25%, respectively. Assuming that the mean length of mitosis is one hour, generation time ($i.e.$, sum of $S + G_2 + M + G_1$) is 14 hours and 16 hours, respectively.

From these results, it is concluded that the slowing of growth rate of Ehrlich ascites tumor cells is brought about by an increase in the dormant cell population and lengthening of the $G_1$ period.
INTRODUCTION

It has been noted in recent studies on ascites tumors that the initial exponential proliferation of growth is always followed by a progressive deceleration, (5, 8, 9, 14) and finally the growth curve reaches a plateau. The reasons for these shifts in growth are far from clear. To elucidate the mechanism underlying the changes in growth rate, a cytokinetic analysis at several different stages in tumor growth are necessary.

In this report, the results obtained from a cytokinetic analysis of Ehrlich ascites tumor cells at the 4th and the 7th day after intraperitoneal inoculation will be presented.

MATERIALS AND METHODS

The Ehrlich ascites tumor was a hypotetraploid subline which has been propagated in this laboratory by weekly transfers of approximately $1 \times 10^6$ cells to healthy female mice of ddN strain weighing 25-30 grams. Ascites tumor cells used in the present study were obtained fresh from population in the peritoneal cavity 4 or 7 days after inoculation of $1 \times 10^6$ cells. The mean length of the $G_1$, $G_2$ and S period was estimated directly by the method described below. The details of the method were reported elsewhere (13).

Measurement of the growth of tumor cell population

Fifteen mice received a single intraperitoneal inoculation of $1 \times 10^6$ cells of Ehrlich ascites tumor. At daily intervals after inoculation, the total number of Ehrlich ascites tumor cells and eosin-stained cells in the peritoneal cavity of each
5 mice was determined by the method of Patt et al. (16) with slight modification.

Estimation of the mean length of G1 period and percentage of dormant cells

Five μc of Thymidine-6-³H (TdR-³H) with specific activity of 5 c/mM was injected intraperitoneally into each of ten mice bearing Ehrlich ascites tumor on 4th or 7th day of growth. One-tenth mg of Colcemid (deacetyl-N-methyl-colchicine) per 100 gm of body weight was simultaneously administered intravenously. Thereafter, the same quantity of TdR-³H was administered intraperitoneally at hourly intervals and Colcemid was injected every half an hour for 2 hours after the first injection of TdR-³H and thereafter at hourly intervals for 4 to 5 additional hours. Smears of the ascites fluid were made on glass slides previously coated with a thin layer of egg-albumin. Smears thus obtained were fixed in methanol and autoradiograms were prepared by the dipping method using Sakura NR-M2 emulsion, which were then exposed for 2 weeks at 4°C and developed with D-19-b for five minutes at 18°C. After development they were stained with Giemsa solution. Cells having more than 4 grains on their nuclei were regarded as labeled cells (12) since the background was less than 1 grain per single cell area. From 1,000 to 2,000 of the tumor cells were examined. Irrespective of cells in mitotic phase or in interphase, changes in the percentage of unlabeled cells during each time course of the experiment were obtained. If most of the tumor cells except for those in dormant phase go through four phases of the cell cycle at a constant rate, the percentage should decrease linearly to a value equivalent to the percentage of cells in pro-metaphase of mitosis, G2 phase and dormant phase at the time of pulse-labeling, and then it should maintain the
same level. The time spent for reaching this fixed value from the first injection of TdR$^-{}^3$H should be the mean length of ana-telophase of mitosis plus $G_1$ phase. In essence, this can be regarded as the length of $G_1$ phase, since ana-telophase is of such short duration.

Dormant cells should be detected as unlabeled cells in interphase at the time when the percentage of unlabeled cells both in interphase and mitotic phase reaches a plateau.

Estimation of the mean length of $G_2$ and S period

Ten $\mu$c of TdR$^-{}^3$H was injected intraperitoneally into each of 10 mice bearing either 4-day (5 mice) or 7-day-old tumor (5 mice) and one-tenth mg of Colcemid per 100 gm of body weight was simultaneously injected intravenously. An equal amount of Colcemid was administered intravenously every 4 hours, while no further injection with TdR$^-{}^3$H was performed. Smears of ascites fluid were made every half hour for 2 hours, thereafter every hour for 7 hours after the injection of TdR$^-{}^3$H. The ratio of the labeled to unlabeled cells among 1,000-2,000 of the tumor cells in interphase was determined on the autoradiograms prepared as described above.

The ratio should decrease slightly for a time equivalent to the length of ana-telophase of mitosis, and then increase linearly to a certain peak value. Thereafter, the ratio decreases linearly to zero as time progresses. The time required to reach the peak value should be the mean length of $G_2$ phase, while the time from the peak to zero should be the mean length of S phase.

RESULTS
Characteristics of the growth curve

Changes in the total number of Ehrlich ascites tumor cells in the peritoneal cavity of mice at different times following an inoculation of $1 \times 10^6$ cells are shown in Chart 1.

A decline in growth rate which was fitted by an exponential initially became apparent between 3rd and 4th day after inoculation, and this continued until the terminal stage.

The total number of large eosin-stained cells slowly increased as time elapsed.

Mean length of G₁ period and percentage of dormant cells

The results obtained are shown in Charts 2 and 3. The mean percentage of unlabeled cells among 1,000-2,0000 tumor cells half an hour after the first injection of TdR-$^3$H was 39.5% and 48.7% for the tumor cells obtained in their 4th and 7th day of growth respectively. Thereafter, it decreased linearly and diminished to 22.8% by the 3rd hour in the former and 29.4% by the 5th hour in the latter. No further change was evident up to 6 or 7 hours after beginning the experiment. From the results obtained, the mean length of G₁ period was estimated as 3 and 4.5 hours respectively. Some of the unlabeled cells were still in interphase when the percentage of unlabeled cells reached a plateau, and no change was observed in the number of these cells during further observation. From these results, 16% of the tumor cells were in the dormant stage on the 4th day after the intraperitoneal inoculation of $1 \times 10^6$ tumor cells (Chart 2). In the tumor cells obtained in their 7th day of growth, 25% of the cells were in the dormant stage (Chart 3).
Estimation of the mean length of $G_2$ and S period

As shown in Chart 4 and 5, the ratio of labeled to unlabeled cells in interphase averaged 2.04 for the 4-day-old and 1.51 for the 7-day-old tumor cells by half an hour after the administration of TdR-$^3$H, and increased to 2.92 in the former and 1.98 in the latter by an hour and half. Thereafter, it decreased linearly and diminished to 1.41 and 1.01 respectively by 6th hour after the TdR-$^3$H injection. Hence, 10.0 and 10.5 hours would be required, respectively, from the injection of TdR-$^3$H to the time when this decreasing line crossed the axis of abscissa. From these results, the mean length of $G_2$ period is equal in both cases and estimated to be about 1.5 hours. While the duration of S period is about 8.5 and 9.0 hours respectively. The results are summarized in Table 1.

DISCUSSION

The growth characteristics of ascites tumors consist of an initial phase of exponential growth followed by a second phase of retardation in growth rate with time. This has been explained by either an increase in dormant cell population, a prolongation in duration of the cell cycle, an increase in the rate of cell loss or their combination. Several papers devoted to the kinetics of cell proliferation in Ehrlich ascites tumor have already been presented (1, 2, 3, 4, 11). Lala and Patt (10) pointed out recently a tendency for the progressive prolongation of the cell cycle during tumor growth, in addition to a progressive decline in the growth fraction. They suggested that the prolongation resulted from a lengthening of $S$ and $G_2$ time and there was no detectable $G_1$ period in Ehrlich ascites tumor cells of hyperdiploid line at any stage of their growth.
The absence of $G_1$ phase in Ehrlich ascites tumor cells was pointed out by Hornsey and Howard (6) previously. Baserga and Lisco (2) have also reported the absence of $G_1$ phase in Ehrlich ascites tumor cells growing in male mice, although they have noted that Ehrlich ascites tumor cells growing in female mice have 5 hours of $G_1$ phase.

In the present study, we corroborated not only the presence of $G_1$ period in Ehrlich ascites tumor cell but also the fact that the mean length of this period was evidently longer in the tumor cells obtained in their 7th day of growth than those of 4th day as summarized in Table I. A mean length of $G_1$ phase of 3.5 or 4.0 hours coincided well with the results reported by Edwards et al. (4) and Kim and Evans (7). The latter used female mice for the recipient of Ehrlich ascites tumor. However, the presence of $G_1$ phase is not characteristic feature of the Ehrlich ascites tumor cells growing in female mice, since Lennartz and Maurer (11) have reported the results of the presence of $G_1$ phase in the Ehrlich ascites tumor cells growing in male mice.

In the present study, no significant difference was detected between tumor cells obtained at two different stages of their growth in the mean length of $G_2$ and $S$ period, although the latter had a tendency to increase with time after inoculation of the tumor cells. Quite similar results have been reported by Baserga (1, 2) using either the mitosis chase method or double labeling method. Lennartz and Maurer (11) have also pointed out that there was no marked difference in the length of DNA synthesis phase in Ehrlich ascites tumor cells at different stages of tumor growth. Thus, our observations differ from those reported by Lala and Patt (10) who found a gradual prolongation of $S$ and $G_2 + M$ phase.

As to the mean length of $G_2$ phase, the results presented here are the shortest among several papers devoted to the cell-
kinetics of Ehrlich ascites tumor. The exact reasons why there are so many different results as to the mean duration of each phase of the mitotic cell cycle are still unknown. However, the differences may be due, in part at least, to both technical reasons, the difference in Ehrlich ascites tumor subline subjected and the size of inoculum. It has been pointed out by Lala and Patt (10) that the exponential growth can be maintained by repeated paracentesis. In the present study, however, the amount of ascites aspirated by repeated paracentesis was so small that it might not accelerate the cell growth. As to the reliability of the method employed in the present study has already been discussed in detail in the previous report (13).

The results presented here correspond well to the frequent generalization that the duration of $S$, $G_2$ or $M$ period, particularly that of $S$, is relatively constant for mammalian cells of both normal and malignant (3, 17, 20) and that a variation of the cell cycle is brought about by that in $G_1$ time (3, 15, 18, 19). In the method employed, a dormant cell is defined as a cell in interphase which does not take up TdR$^3$H significantly during the whole course of observation. Analysis of the results obtained revealed that the percentage of dormant cells was evidently higher in tumor cells of 7-day-old than that of 4-day-old. This is one of the factors contributing to the progressive deceleration in the growth rate of Ehrlich ascites tumor.

Frindel et al. (5) reported that increasing cell death contributes to the slowing of tumor growth in a solid tumors. In Ehrlich ascites tumor, Lala and Patt (10) revealed that the rate of cell loss did not differ significantly at various stages of the growth of Ehrlich ascites tumor. Cell death is one of the important parameters and should be estimated in cytokinetic studies. However, it may not have an important role in the retardation of Ehrlich ascites tumor growth since the
percentage is small.
(A part of these results has been presented at the 26th annual meeting of the Japanese Cancer Association in 1967)
REFERENCES


Chart 1. Change in the total number of Ehrlich ascites tumor cells and eosin-stained cells in the peritoneal cavity following an inoculation of $1 \times 10^6$ cells. Each point represents the average of cell counts in five animals and vertical lines indicate ±1 standard error of the mean.
Chart 2. Percentage of unlabeled cells in Ehrlich ascites tumor at various hours after the first injection of Tdr-3H and Colcemid.

(1) Results obtained on 4-day-old tumor cells. Each point represents the average of measurement in five animals and vertical lines indicate ±1 standard error of the mean. Dotted line represents proportion of unlabeled cells in interphase (dormant cells).
Chart 3. Percentage of unlabeled cells in Ehrlich ascites tumor at various hours after the first injection of TdR-^{3}H and Colcemid. (2) Results obtained on 7-day-old tumor cells. Each point represents the average of measurement in five animals and vertical line indicate ±1 standard error of the mean. Dotted line represents proportion of unlabeled cells in interphase (dormant cells).
Chart 4. Ratio of labeled to unlabeled cells of Ehrlich ascites tumor in interphase at various hours after the injection of TdR-3H.

(1) Results obtained on 4-day-old tumor cells. Each point represents the average of measurement in five animals and vertical lines indicate ±1 standard error of the mean.
Chart 5. Ratio of labeled to unlabeled cells of Ehrlich ascites tumor in interphase at various hours after the injection of TdR-3H.

(2) Results obtained on 7-day-old tumor cells. Each point represents the average of measurement in five animals and vertical lines indicate ±1 standard error of the mean.
<table>
<thead>
<tr>
<th>Growth Parameters</th>
<th>Age of Tumor</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 days</td>
<td>7 days</td>
</tr>
<tr>
<td></td>
<td>3.0 hr</td>
<td>4.5 hr</td>
</tr>
<tr>
<td>G&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>8.5 hr</td>
<td>9.0 hr</td>
</tr>
<tr>
<td>G&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.5 hr</td>
<td>1.5 hr</td>
</tr>
<tr>
<td>M</td>
<td>(1.0)hr</td>
<td>(1.0)hr</td>
</tr>
<tr>
<td>G</td>
<td>14.0 hr</td>
<td>16.0 hr</td>
</tr>
<tr>
<td>Ns/N</td>
<td>0.61 ± 0.03*</td>
<td>0.51 ± 0.02*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% of Dormant Cells</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
</tr>
</tbody>
</table>

Table 1. Growth parameters at different stages of tumor growth after inoculation of 1 x 10<sup>6</sup> cells of Ehrlich ascites tumor.

Ns: Number of cells in DNA synthesis.
N: Total number in or out of a mitotic cycle.
G: Generation time.
G<sub>1</sub>: Mean duration of G<sub>1</sub> period.
S: Mean duration of S period.
G<sub>2</sub>: Mean duration of G<sub>2</sub> period.
M: Mean duration of mitosis.
*: Mean ±1 standard error.