Autoradiographic and Cytophotometric Analyses of the Resting Stages of the L1210 Ascites Tumor

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SUMMARY

In the murine L1210 ascites tumor the resting stages were investigated on Days 5, 6, and 8 after transplantation of $10^5$ cells.

The experimental work consisted of continuous labeling with thymidine-3H combined with cytophotometric determination of the single-cell DNA content of unlabeled tumor cells. The present experimental data and earlier experiments with percentage of labeled mitoses and continuous labeling were analyzed by a computer method based on 2 alternative mathematical models for the cell kinetics in the tumor.

In the 5-day tumor, no resting cells were found, while 4.2 and 44.8% were noncycling cells in the 6- and 8-day tumors, respectively. The new finding was the demonstration of a small but significant fraction of noncycling cells with G2 DNA content in the 6- and 8-day tumors (0.6 and 0.9%, respectively). Furthermore, the investigations confirmed the previous finding of noncycling cells with G1 DNA content in the 6- and 8-day tumors (3.6 and 43.9%, respectively). The study provided evidence in support of the assumption of cell loss in the L1210 tumor as an age-specific elimination of resting cells with G1 DNA content.

INTRODUCTION

In a previous cytokinetic study of the L1210 ascites tumor at different stages of growth, the experimental data (PLM curve, CL curve, and growth curve) were analyzed by a computer method based on 3 alternative mathematical models for the cell kinetics (11). The analysis showed a decrease in the growth fraction and an increase in the cell loss with increasing tumor mass, while the mean cell cycle time increased at first but was again shortened in the most advanced stage. It was demonstrated that the increasing fraction of resting cells consisted of cells with G1 DNA content, and the analysis rendered it likely that the mode of cell loss was an age-specific elimination of these resting cells. However, the available experimental data did not allow a more accurate qualitative description of the noncycling cells.

MATERIALS AND METHODS

Experimental Work. The strain of leukemia L1210 used was obtained in 1969 from Southern Research Institute, Birmingham, Ala., and has since been maintained in DBA/2 mice by weekly i.p. inoculations of $10^5$ cells.

First-generation hybrids of female random-bred Swiss mice and inbred DBA/2 male mice were used in the experiments. All the animals were males weighing from 18 to 22 g.

An inoculum of $10^5$ tumor cells in a volume of 0.2 ml was given to each animal (8). The mean survival time after inoculation of $10^5$ tumor cells had previously been estimated to be 7.9 days (8). If, in this study, the mean survival time of a control group (20 mice) differed from 7.9 days by more than 0.5 day, the experiment was excluded.

The CL experiments combined with cytophotometry were carried out on Days 5, 6, and 8 after transplantation. TdR-3H (specific activity, 5.0 Ci/m mole), obtained from the Radiochemical Centre, Amersham, England, was injected i.p. in the amount of 5 μCi after dilution in 0.9% NaCl solution to 25 μCi/ml every 2 hr over a period of 8 to 16 hr. Aspiration of less than 0.05 ml ascites fluid was performed on 5 to 10 mice 15 min after the TdR-3H injections except on Day 5, when the mice were killed in order to obtain enough tumor cells. The tumor cells were smeared on cleaned, gelatin-coated glass slides and then dried and fixed for 1 hr in a mixture of 85% methanol, 10% formalin, and 5% acetic acid.
For the cytophotometric determination of DNA, hydrolysis with 5 N hydrochloric acid at 25 °C for 35 min was performed to ensure maximal dye binding. The slides were then incubated for 1 hr in Schiff’s reagent (pH 2.5), prepared with p-rosaniline free of acridine (Chroma, Stuttgart, West Germany), rinsed 3 times for 10 min in SO2 water, and dehydrated. The slides were thereafter prepared for autoradiography as earlier described (8).

The fraction of labeled cells was determined in 1000 tumor cells/mouse. Single-cell DNA content was determined by means of a scanning microscope photometer 05 (Cytoscan, Carl Zeiss, Inc., Oberkochen, West Germany).

In each tumor, about 100 consecutive unlabelled cells (<3 grains) were measured at maximal absorption (560 nm) using immersion oil with a refractive index of 1.515 (Carl Zeiss, Inc.). Single poles of anaphases and telophases were used to determine the pre-DNA-synthetic DNA values (G1, 2n) and late prophases and metaphases for the post-DNA-synthetic DNA values (G2, 4n). Lymphocytes and segmented granulocytes were used as an internal standard; the coefficient of variation was less than 3%. The determination of single-cell DNA content in unlabeled tumor cells allows separation between cells with G1 and G2 DNA content, but does not allow distinction between resting and cycling cells.

The MI was obtained by counting 3000 tumor cells on smears stained with May-Grünwald-Giemsa (late prophase to early telophase included).

Student’s t test was used in the statistical evaluation.

The Computer Method. The experimental data were analyzed on the basis of 2 new mathematical models, Model IV and Model V (Chart 1), developed from those previously described (11) by the addition of a Q2 compartment and the introduction of an alternative mode of cell loss.

Q1 and Q2 are defined as resting compartments with an influx of cells with G1 and G2 DNA content, respectively. The cells remain in these compartments for an infinitely long time except in Model IV, in which the Q1 cells have a limited transit time due to cell loss. The cell loss is age specific in Model IV, in the sense that cells of a certain age are lost from Q1; while in Model V, newly generated cells are lost immediately after mitosis.

The transit times in the different compartments are stochastic quantities that are expressed by their PDF’s, T(G1), a(G1), T(G2), a(G2), T(Q1), a(Q1), T(C), and a(C) are the mean value and standard deviation of the transit times in G1, S, G2, Q1, and the proliferative pool, respectively. The transit time (T(M)) in the mitosis (M) is assumed to be the same for all cells. T(D) is the doubling time.

The basic assumptions behind the models are exponential growth and transit times distributed according to PDF’s, which do not change during the time of the study and are independent of each other. The PDF’s are simulated with normal distributions. The experimental data were analyzed on an IBM 370/165 computer, and the programs were written in FORTRAN IV.

The ratio between the percentages of unlabeled cells with G2 DNA content (G2 + Q2) at 16 (8 hr for the 5-day tumor) and 0 hr, respectively, after the start of continuous labeling was calculated from the cytophotometric data (Table 1). Based on this ratio, the MI, and cell cycle parameters estimated in the earlier PLM experiment (11), the computer was used to obtain the best fit between a theoretical CL curve and the corresponding experimental data. The initial point on the theoretical CL curve was chosen to be identical with the experimental labeling index, and the amount of cell loss was altered until the best fit was obtained. The experimental CL data have been published earlier (11).

With the best fit, all desired kinetic parameters were estimated, and the fractions of cells in G1 + Q1 and G2 + Q2 were separated in their respective compartments.

The last step in the analysis was the calculation of the percentages of unlabeled cells with G1 and G2 DNA content, respectively, as a function of time after the start of CL. The calculations constitute a direct control of the models when the theoretically determined decrease in the percentage of unlabeled cells is compared with the corresponding experimental data. The calculated T_D is also a control when compared with the experimental T_D.

RESULTS

Table 1 shows the number of tumors and the number of unlabeled tumor cells measured in the 5-, 6-, and 8-day tumors at 0, 8, and 16 hr after the initiation of CL. In the same table is also recorded the mean percentage distribution of labeled cells in the tumors, unlabeled cells in G1 + Q1 and G2 + Q2, respectively, and MI.

In the 5-day tumor immediately after the start of CL, 30% of the cells had G1 DNA content and 4% had G2 DNA content. However, after CL for 8 hr, only 3% (p < 0.01) of the cells were unlabeled with G1 DNA content, and only 0.05% (p < 0.1) was unlabeled with G2 DNA content.

After the initiation of CL, the 6-day tumor revealed 34%
unlabeled cells with G1 DNA content and 3% unlabeled cells with G2 DNA content. Of all the cells, 4.4% remained unlabeled with G1 DNA content (p < 0.001) after 16 hr of CL. Simultaneously, 0.3% (p < 0.001) was unlabeled cells with G2 DNA content.

In the 8-day tumor immediately after the start of CL, 45% of the cells had G1 DNA content and 6% had G2 DNA content. After CL for 16 hr, 10.5% (p < 0.001) were unlabeled cells with G1 DNA content, while only 0.7% (p < 0.025) was unlabeled with G2 DNA content.

Chart 2 shows the experimental CL data (11) and the corresponding theoretical curves from the L1210 tumor on Days 5, 6, and 8 after transplantation. For the 5-day tumor, the 2 models gave identical fits. The best fit (—..—) obtained with Model V gave for the 6-day tumor a somewhat shorter T_D of 25 hr. Chart 2 also shows for the 6- and 8-day tumors a theoretical CL curve (- - - -) obtained with Model V, but calculated with the same parameter values as estimated with Model IV.

Chart 3 shows the experimental determined decrease in the percentages of unlabeled cells with G1 DNA content and G2 DNA content, respectively, plotted as a function of time after the start of CL. Chart 3 also shows the control curves calculated by the computer. The control curves for the unlabeled cells with G1 DNA content are identical for the 5-day tumor, show minor differences for the 6-day tumor, and reveal distinct differences for the 8-day tumor. The models gave identical control curves for the unlabeled cells with G2 DNA content in the 5- and 6-day tumors, while for the 8-day tumor minor differences were present.

The correspondence between experimental data and theoretical curves (Charts 2 and 3) does not favor any of the models in the 5- and 6-day tumors. However, for the 8-day tumor, it is obvious that the best result was obtained with Model IV. Because it is most unlikely that a shift in the mode of cell loss should occur from Day 6 to Day 8, the overall best fit was obtained with Model IV.

All parameter values corresponding to the theoretical curves obtained with Model IV are recorded in Table 2. The table also shows doubling times and cell cycle parameters as estimated in a previous study (11).

In the 5-day tumor, nearly all cells were proliferating. In the 6-day tumor, 3.6% and 0.6% were noncycling cells with G1 and G2 DNA content, respectively. However, in the 8-day tumor, 44% were noncycling cells with G1 DNA content and 0.9% were noncycling cells with G2 DNA content.

DISCUSSION

The new finding in this study is the demonstration of a small but significant fraction of Q2 cells in the L1210 ascites tumor, with evidence that this fraction increases with tumor age. Furthermore, the investigations confirm the earlier finding of an increasing fraction of Q1 cells with increasing tumor mass and lend support to the assumption of cell loss as an age-specific elimination of resting cells with G1 DNA content (11).

The main argument for the presence of Q2 cells is the good agreement between the experimentally determined decrease in the percentage of unlabeled cells with G2 DNA content and the calculated control curves. After CL for a period of approximately $T_{G1} + T_{M} + 2o_{Q2}$, both the experimental and the theoretical curves decreased very slowly as the unlabeled cells with G2 DNA content consisted only of Q2 cells.

The question arises whether Q2 cells can be long-living G2 cells. From the parameter values in Table 2 it is possible to calculate the expected fractions of unlabeled cells with G2 DNA content at 8 and 16 hr after the start of CL, assuming that Q2 cells are not present (N. R. Hartmann and P. Dombernowsky, unpublished data). The calculated fractions are a factor of 36 to 146 less than the corresponding experimental quantities in Table 1. An exception is the 5-day tumor, where the experimental value. Taking into account that all experimental values differ.
Chart 2. Experimental CL data from the L1210 ascites tumor and the theoretical curves obtained from the models. ●, experimental data; ——, Model IV; ——, Model V; ——, Model V using the same parameter values as estimated with Model IV. Error bars, S.E.

Chart 3. The percentage distribution of unlabeled cells with G1 DNA content and G2 DNA content in the L1210 ascites tumor as a function of time after the start of CL. The chart shows the experimental data and the theoretical curves obtained from the models. ●, experimental percentage of unlabeled cells with G1 DNA content; ○, experimental percentage of unlabeled cells with G2 DNA content; ——, Model IV; ——, Model V; ——, Model V using the same parameter values as estimated with Model IV. Error bars, S.E.
fractions of unlabeled cells recorded in Table 1 are significantly higher than zero ($p < 0.001–0.1$), the calculations clearly support the existence of a small fraction of $Q_2$ cells in the 6- and 8-day tumors, while the existence of $Q_2$ cells in the 5-day tumor is more doubtful. These considerations are independent of any assumption as to the transit time in $Q_2$, which means that even a limited transit time in $Q_2$ would not stress the actual demonstration of $Q_2$ cells in the L1210 ascites tumor.

We would be in serious error to maintain the existence of $Q_2$ cells if the unlabeled cells with $G_2$ DNA content measured in the CL experiments were simply cells that had passed through the S phase between 2 TdR-$^3$H injections. From the models and the parameter values in Table 2 it is possible to calculate the percentage of the total population that is able to complete DNA synthesis in a time interval of 2 hr, which is equal to the period between 2 TdR-$^3$H injections. These fractions are 0.0005 and 0.003% of the total population in the 6- and 8-day tumors, respectively. Compared with the estimated fractions of $Q_2$ cells in the same tumors ($0.6$ and $0.9$), the calculations do not indicate any serious error.

The calculations above are not strongly dependent on normally distributed transit times for 3 reasons: (a) calculations based on skew PDF’s with parameter values recorded in Table 2 would allow an even smaller fraction of unlabeled cells to pass through the S phase between 2 TdR-$^3$H injections, since skew PDF’s favor cells with long transit times; (b) experience shows that, when used in PLM analysis, skew distributions give mean values that are identical to, if not slightly larger than, those obtained with normal distributions (Ref. 25; N. R. Hartmann, C. W. Gilbert, B. Jansson, P. D. M. MacDonald, G. G. Steel, and A. J. Valleron, Cell Tissue Kinet., in press); (c) the estimated cell cycle parameters $T_{G_1}$, $T_S$, $T_{G_2}$, and $T_M$ in Table 2 fit well to the theorem that, independent of choice of PDF’s, the PLM curve approximates a level equal to $T_S: T_C$ as the time increases ($25, 29$). These theoretical levels are 0.68 and 0.82 for the 6- and 8-day tumors, respectively, corresponding very well to the experimental PLM curves ($11$). The theorem means that the ratio $T_S: (T_{G_1} + T_S + T_{G_2} + T_M)$ in the 8-day tumor is approximately 0.82, and as the $T_S$, $T_{G_2}$, and $T_M$ values in Table 2 are within reasonable limits the $T_{G_1}$ value must be as short as $0.4$ hr independent of the choice of PDF. A skew distribution with a $T_{G_1}$ value 10 times higher, for example, would give a $T_S: T_C$ value of about 0.6 to 0.7, which is inconsistent with the experimental PLM curve.

Noncycling cells with $G_2$ DNA content or cells with extremely long $T_{G_1}$ have been demonstrated in animal tissues and tumors and in human acute leukemia ($3–5, 7, 9, 10, 13, 14, 17, 18, 20–22, 24$). One can only speculate as to the biological significance of the $Q_2$ cells. It has, however, been reported that, during compensatory growth of the epidermis, kidney, duodenum, and ascites tumors, some of these $Q_2$ cells are released into mitosis ($3, 10, 14, 17, 18$). DeCosse and Gelfant ($6, 7$) observed in the ELD ascites tumor that molecular weight below 10,000 daltons is capable of blocking the cells in $G_2$ ($2$); and (c) the normal accumulation of $Q_2$ cells with increasing tumor mass is preserved after whole-body irradiation ($P$. Bichel and P. Dombernowsky, unpublished observation).

The present study confirms the previous findings of no $Q_1$ cells in the 5-day tumor, a small fraction in the 6-day tumor, and a large fraction of $Q_1$ cells in the 8-day tumor ($11$). The results from the 8-day tumor are also obvious without computer analysis, inasmuch as 26.7% of all cells remained unlabeled and had $G_1$ content after 8 hr of CL, although the

### Table 2

Model IV growth parameters of the L1210 ascites tumor on Days 5, 6, and 8 after inoculation of $10^4$ cells

<table>
<thead>
<tr>
<th></th>
<th>5-day tumor</th>
<th>6-day tumor</th>
<th>8-day tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell population (millions)</td>
<td>7.5</td>
<td>210</td>
<td>490</td>
</tr>
<tr>
<td>$T_{G_1}$ (hr)</td>
<td>10.0</td>
<td>25.0</td>
<td>75.0</td>
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<tr>
<td>$T_{G_2}$ (hr)</td>
<td>12.5</td>
<td>23.0</td>
<td>88.6</td>
</tr>
<tr>
<td>$T_S$, $Q_1$ (hr)</td>
<td>3.1, 3.4</td>
<td>5.4, 2.6</td>
<td>0.4, 0.01</td>
</tr>
<tr>
<td>$T_{G_1}$, $Q_2$ (hr)</td>
<td>8.4, 2.9</td>
<td>14.8, 3.5</td>
<td>12.5, 3.4</td>
</tr>
<tr>
<td>$T_Q$, $Q_3$ (hr)</td>
<td>0.8, 0.4</td>
<td>0.9, 0.8</td>
<td>1.8, 0.9</td>
</tr>
<tr>
<td>$T_M$ (hr)</td>
<td>0.6</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>$T_{Q_1}$, $Q_2$ (hr)</td>
<td>22.8, 11.4</td>
<td>15.9, 6.5</td>
<td></td>
</tr>
<tr>
<td>$T_C$, $Q_3$ (hr)</td>
<td>12.9, 4.4</td>
<td>21.8, 4.4</td>
<td>15.3, 3.5</td>
</tr>
<tr>
<td>$G_1$ cells (%)</td>
<td>30.7</td>
<td>29.8</td>
<td>1.5</td>
</tr>
<tr>
<td>$G_2$ cells (%)</td>
<td>60.9</td>
<td>61.0</td>
<td>45.6</td>
</tr>
<tr>
<td>$G_3$ cells (%)</td>
<td>4.7</td>
<td>2.7</td>
<td>6.0</td>
</tr>
<tr>
<td>MI (%)</td>
<td>3.6</td>
<td>2.3</td>
<td>2.1</td>
</tr>
<tr>
<td>Growth fraction (%)</td>
<td>99.9</td>
<td>95.8</td>
<td>55.2</td>
</tr>
<tr>
<td>Cell production (%/hr)</td>
<td>5.6</td>
<td>3.1</td>
<td>3.4</td>
</tr>
<tr>
<td>Cell loss (%/hr)</td>
<td>0.0</td>
<td>0.13</td>
<td>2.6</td>
</tr>
</tbody>
</table>

* $a$ Calculated from the growth curve.
* $b$ Calculated from the mathematical models.
mean transit time in $G_2 + M + G_1$ was less than 3 hr and the standard deviation was less than 1 hr. As the fraction of $G_1$ cells is roughly proportional to $T_{G_1}$, a considerably longer $T_{G_1}$ value in the 8-day tumor would not eliminate the need for a $Q_1$ compartment. If $T_{G_1}$ were increased, for example, to 4 hr (a value absolutely inconsistent with the experimental PLM data), the fraction in $G_1$ would increase to approximately 15% of the population, which is only about one-third of the estimated fraction in $Q_1$.

The existence of $Q_1$ and $Q_2$ L1210 cells is in agreement with studies of the NTCT 2472 ascites tumor by Frindel et al. (13) and with studies of the JB-1 tumor by Bichel and Dombernowsky (3) and Dombernowsky et al. (9, 10). In studies of the Ehrlich ascites tumor at different stages of growth, only $Q_1$ cells were found (16, 19).

It is uncertain whether $Q_1$ and $Q_2$ cells are triggered into the cell cycle during the time of tumor growth, a phenomenon observed in an autogenous rat sarcoma (22). A major recycling would be detected by this method, while a minor recycling would not.

The overall best fit to the experimental data in Charts 2 and 3 was obtained with Model IV, which has an age-specific cell loss from $Q_1$. Cell loss immediately after mitosis in Model V has the effect that the CL curve (- - -) increases more slowly than a CL curve (—) calculated from Model IV.

With Model IV the theoretical curve for the decrease in the percentage of unlabeled cells with $G_1$ DNA content decreases more rapidly than a curve calculated with Model V. The difference is clearly demonstrated by the solid and broken lines for $G_1$ DNA content in Chart 3. However, the theoretical curve for the decrease in percentage of unlabeled cells with $G_2$ DNA content is not influenced by the mode of cell loss, because no loss is supposed to occur from the $Q_2$ compartment (Chart 3).

As early as 1969, Steel et al. (27) proposed alternative modes of cell loss, and in a subsequent study Steel and Hanes (28) showed that a shift in the localization of the cell loss had the same effect on the theoretical CL curve as demonstrated in the present study. It was also demonstrated in their work that random cell loss from both proliferating and resting cells gives a CL curve lying between the theoretical curve based on an age-specific elimination of $Q_1$ cells and the curve based on cell loss immediately after mitosis. A theoretical CL curve lying between the solid line (Model IV) and the broken line (Model V) in Chart 2 would definitely give an inferior fit to the experimental data compared with Model IV. This indicates that at least the major cell loss in the L1210 ascites tumor is an age-specific elimination of resting cells with $G_1$ DNA content ($Q_1$).

Our hypothesis for the mode of cell loss is in agreement with observations in the Ehrlich ascites tumor, in which an age-specific elimination of noncycling cells with postmitotic DNA content and a small amount of mitotic death have been found to occur (15).

Most authors dealing with mathematical models for systems with nonproliferating cells take into account only resting cells with $G_1$ DNA content, with the usual procedure being the analysis of experimental PLM and CL data by a computer method (23, 26–28). Some authors have also included resting cells with $G_2$ DNA content in their models (12, 22, 24), using as their experimental tool a CL curve combined, for example, with a PLM curve (12, 24) or a double labeling technique and a grain count experiment (22). However, none of the aforementioned investigators has subjected a PLM curve and a CL curve combined with cytophotometric data to computer analysis, a method that has proved very useful in the estimation of the fraction of resting cells with $G_1$ and $G_2$ DNA content.

A condition for using the previous PLM and CL experiments is that no alteration in the cell kinetics has taken place since the 1st investigation (11). The good agreement between the CL data in both studies indicates no change.

It is not necessary to reanalyze the previous PLM data, because the development of the models by addition of a $Q_2$ compartment and the introduction of an alternative mode of cell loss has no influence on the PLM analysis. However, the presence of the $Q_2$ compartment leads to a CL curve that approaches unity more slowly than does a CL curve calculated without this compartment, so reanalysis of the previous CL data was necessary.

An error in the experimental work is the grain limit of 3 or more grains used in the evaluation of the autoradiographs. This grain limit is low and only slightly above the mean background (average 1 to 2 grains). Consequently, the experimental error is an overestimation of the percentage of labeled cells and an underestimation of the percentage of noncycling cells.

A possible error in the interpretation of the cytophotometry could be caused by the presence of a clone of tumor cells with abnormal ploidy. This possibility was excluded, however, since only tetraploid cell was detected after cytophotometric determination of DNA in more than 5000 cells measured in this study.

It can be concluded from this investigation that both a small fraction of $Q_2$ cells and a larger fraction of $Q_1$ cells are present in the L1210 ascites tumor. This demonstration and quantitation of resting cells with $G_2$ and $G_1$ DNA content, respectively, has been possible by the combination of CL and cytophotometry.

This method may be of therapeutic interest, since it has been previously shown by Dombernowsky et al. (10) that resting cells are rapidly triggered into the cell cycle after reduction of the tumor mass.

ACKNOWLEDGMENTS

The excellent technical assistance of J. Andersen, N. Arnkjaer, and I. Kobbernagel is gratefully acknowledged.

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Cancer Res 1974;34:3296-3302.

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