Relationship between Growth and Radiosensitivity in the P388 Murine Leukemia

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SUMMARY

Growth of the P388 lymphocytic leukemia as an ascites tumor in DBA/2 mice is characterized by lengthening of the mean cell-cycle time from 8.5 hr on Day 1 to 22.0 hr on Day 5. This increase reflects increases in S phase from 6.2 to 14.8 hr, in $G_1$ from 0.2 to 4.2 hr, and in $G_2$ from 1.6 to 3.0 hr; the greatest change thus occurs in $G_1$. The proportion of cells in S decreases from 70% on Day 2 to <10% on Day 8, the last day of growth. This decrease is reversed 16 hr after transplantation of 10⁶ cells to fresh hosts. Since the mitotic index does not change during the 16-hr lag period, the cells that surge into S between the 16th and 48th hr after transplantation probably originate in $G_0$. The characteristics of the P388 tumor make it a useful model for investigating the relationships between cell kinetic parameters and sensitivity to X-radiation or chemotherapeutic drugs. We find that P388 cells collected from late-plateau-phase tumors and irradiated in air in vitro are more radioresistant than are cells harvested from exponentially growing tumors.

INTRODUCTION

The P388 murine lymphocytic leukemia has been used in numerous investigations of cytocidal agents, including ionizing radiations (3–6, 12, 18) and chemicals (1). It is particularly advantageous for these studies because it grows as an ascites and because survival can be assayed accurately by classical in vivo titration techniques (3). Despite its widespread use, however, the P388 has not yet received a complete cytokinetic analysis. Since the therapeutic efficacy of "cell-cycle-specific" cytocidal agents varies with the relative proportion of cells in resistant and sensitive phases of the cell cycle, we analyzed the growth kinetics of this tumor and correlated changes in cytokinetic parameters during growth with the in vitro radiosensitivity of cells harvested from exponential and late-plateau-phase tumors.

MATERIALS AND METHODS

Tumor. A P388 tumor, obtained from Dr. J. Brennan (University of Pennsylvania), was passaged weekly by i.p. inoculation of 10⁶ cells into male DBA/2 mice (The Jackson Laboratories, Bar Harbor, Maine) weighing approximately 20 g. Whenever possible, blood-free tumors were selected for passage, but despite this precaution tumors became increasingly bloody by the 6th day of growth. Assay of the inoculum required a TD₅₀ confirmed a figure of 2 to 3 cells in DBA/2 mice (3). However, despite similar TD₅₀'s this tumor is more virulent than the P388 that we obtained some years ago from Dr. J. Belli and used in earlier experiments (12). An inoculum of 10⁶ cells from the tumor used in the present experiments kills >90% of the hosts on the 7th postinoculation day and all of the mice by the 8th day, whereas Belli and Andrews (3) report that their P388 did not kill the hosts until the 12th day.

Assay of Tumor Growth and Labeling with ³H-Labeled Thymidine. Growth curves were constructed by washing the peritoneal cavities of tumor-bearing mice repeatedly with up to 50 ml of heparinized 0.9% NaCl solution. These wash fluids were diluted in volumetric flasks and cell concentrations were determined with a Model B Coulter counter. Coulter counts were checked against hemocytometer counts and differential counts on stained smears. Viability was tested by the standard nigrosin exclusion test (14).

P388 cells were labeled by i.p. injection of ³H-labeled thymidine (Schwarz/Mann, Orangeburg, N. Y.) at 0.1 to 1 µCi/g; specific activity ranged from 3.7 to 17 Ci/m mole, depending on the experiment. (The lowest specific activity and dose were used for labeled mitosis and repeated-labeling experiments.) Twenty min after injection of ³H-labeled thymidine, cells were collected and washed, resuspended in a few drops of cell-free ascites fluid, and smeared on clean slides. These were then dried, fixed in absolute methanol, and processed for autoradiography as described elsewhere (11).

X-irradiation. Cells were harvested from donors of appropriate age, washed once, resuspended in Tris-buffered 0.9% NaCl solution (pH 7.0), and irradiated at room temperature in a Petri dish. X-irradiation was performed with a G.E. Maxitron unit at 300 kVp and 20 ma with 2-mm copper filtration; the dose rate was 500 rads/min. After irradiation, 10⁴ cells were injected i.p. into fresh mice, which were sacrificed for tumor cell count at appropriate intervals. Cell survival was determined by extrapolating the growth curves back to the ordinate.

Calculation of Cytokinetic Parameters. Cell-cycle times

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The abbreviation used is: TD₅₀, 50% tumor takes.
were determined by the labeled mitosis method (19) using the 50% points on the curve. The growth fraction\(^4\) and cell loss factor were computed by standard formulations (2, 16, 17, 21, 23).

**RESULTS**

**Growth of the P388 Leukemia as an Ascites Tumor in DBA/2 Mice.** Sixteen hr after inoculation of \(10^6\) P388 cells into DBA/2 mice, the number of cells in the peritoneal cavity began to increase (Chart 1A); the population-doubling time was 8.3 hr between Day 2 and Day 3. The growth curve plateaued at \(4 \times 10^3\) cells/mouse and the host usually died on the 7th day postinoculation. The initial lag in the growth curve (Chart 1A) does not reflect the presence of dead cells in the inoculum since nigrosin exclusion tests and TD\(_{50}\) assays indicated that essentially 100% of the inoculated 7-day cells were viable and tumorigenic.

As shown in Chart 1B, the 7-day tumor contained few S cells (approximately 12%). However, when cells from a tumor this age were transplanted into fresh mice there was a lag of at least 16 hr and then a sustained rise in the proportion of S cells. By Day 2, approximately 70% of the cells were in S phase. The level remained high for a day or so and then fell again as the tumor entered plateau phase. The experimental data suggest a 2nd plateauing of the labeling index between Days 4 and 5, but this was ignored in constructing the curve in Chart 1B. The finding of an extended lag period in the labeling index after transplantation is reminiscent of our earlier results with Ehrlich ascites tumors (11).

One possible explanation for the age-related lag period is that the precursors for DNA synthesis are more severely depleted in old tumors than in young ones. We are investigating this possibility and can report that injection of deoxyribonucleotides (9 nmoles each of deoxyadenosine, deoxycytosine, and deoxyguanosine) directly into 7-day P388 tumors has no effect on the rate of thymidine incorporation, at least within the 1st 3 hr, in contrast to a report for Ehrlich ascites tumors (8).

Labeled mitosis curves initiated 1 and 5 days after inoculation of \(10^6\) cells indicate that the cycling cells, which constituted only about one-third of the population on Day 1 (see below), had a mean cycle time of 8.5 hr on Day 1 and 22 hr on Day 5 (Chart 2). This lengthening of the cell cycle during growth is caused by a doubling of S and G\(_2\) and about a 20-fold increase in G\(_1\) from 0.2 to 4.2 hr (Table 1). This pattern is typical of many ascites tumors (9, 10, 13, 24).

From the data in Charts 1 and 2 we computed a growth fraction of 0.36 for the Day 1 tumor (Table 1). The cell-cycle time of the proliferating cells in this population was 8.5 hr and the essential identity of this figure and the population-doubling time on Day 2 (8.3 hr) indicates that the growth fraction rose to unity before the 48th hr after transplantation. The labeling data in Chart 1B suggest that this occurred between Hr 16 and 48. As the population began to enter plateau phase, the growth fraction declined again, falling to 0.78 on Day 5 and to 0.36 on Day 7 (Table 1). This analysis is substantiated by repeated-labeling data.

\(^4\)Growth fraction is defined according to the standard mathematical formulations used to calculate it (17). Since “noncycling” cells in ascites tumors may be delayed in G\(_1\), rather than entering a true G\(_0\) compartment (7, 13), we will use “growth fraction” in the general sense, without intending to imply that the “noncycling” cells necessarily halt completely or enter a true G\(_0\).
J. W. Harris, B. Shon, and J. Meneses

Table 1

<table>
<thead>
<tr>
<th>Day of growth*</th>
<th>Cell No.</th>
<th>Population-doubling time (hr)</th>
<th>$T_c$*</th>
<th>$T_0$</th>
<th>$T_s$</th>
<th>$T_{ci}$</th>
<th>Growth fraction</th>
<th>Cell loss factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$3.4 \times 10^4$</td>
<td>19.2</td>
<td>8.5</td>
<td>0.2</td>
<td>6.2</td>
<td>1.6</td>
<td>0.36</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
<td>$5.7 \times 10^4$</td>
<td>11.3</td>
<td>3.1</td>
<td>1.3</td>
<td>14.8</td>
<td>3.0</td>
<td>0.78</td>
<td>0.35</td>
</tr>
<tr>
<td>5</td>
<td>$280 \times 10^4$</td>
<td>36.0</td>
<td>22.0</td>
<td>4.2</td>
<td>14.8</td>
<td>3.0</td>
<td>0.78</td>
<td>0.35</td>
</tr>
</tbody>
</table>

* Inoculum, $10^6$ cells.

* Examination of the labeled mitosis curve for 5-day cells (Chart 2) shows that the trough in the curve occurs in the region of 40 to 50% labeled cells and that the data do not precisely define the curve after the 15th hr. Therefore, the durations of $T_c$ and $T_{ci}$ in the 5-day tumor must be regarded as only approximate (cf. Ref. 22).

wherein 100% of the cells in a 2-day tumor were labeled within 1 cell-cycle time when $^3$H-labeled thymidine was administered every 2 to 4 hr (unpublished data), whereas 5- and 7-day tumors reached a labeling index of only 78% within 1 cell-cycle time and only 85% even after extended exposure to $^3$H-labeled thymidine (Chart 3).

These and other cytokinetic parameters are summarized in Table 1. The cell loss factor of 35% on Day 5 probably reflects migration of tumor cells out of the peritoneal cavity rather than cell death. This is supported by the observation of Belli and Andrews (3) that there were P388 cells in spleen, lymph nodes, and liver after the 3rd day of growth (3).

**Relationship between Tumor Growth Stage and Radiosensitivity.** Since the labeling index of the P388 falls sharply between Days 3 and 7 (Chart 4) and, since S cells are usually more radioresistant than G1 cells (at least late G1 cells), we investigated the radiation sensitivity of cells harvested from tumors at these 2 extremes of age.

These experiments show that the response of P388 cells to 1000 rads of X-irradiation *in vitro* has a clear relationship to the age of the tumor from which they were harvested (Chart 4). Cells harvested 3 days after inoculation of $10^5$ cells ($^3$H-labeled thymidine index, 70%) were approximately 2.7 times more sensitive than those harvested after 7 days ($^3$H-labeled thymidine index, 30 to 40%) and 7.5 times more sensitive than those harvested 7 days after inoculation of $10^4$ cells ($^3$H-labeled thymidine index, 10%). In contrast to this result at 1000 rads, 3- and 7-day cells apparently do not differ in their response to a lower dose (550 rads). This apparent discrepancy between the responses to high and low doses is not unexpected: 550 rads is on the shoulder of the survival curve for these cells (3), and even large differences in sensitivity that are reflected in slope changes are often indiscernible in this region (cf. Fig. 1 of Ref. 5), whereas 1000 rads is well down on the linear portion of the curve.

**DISCUSSION**

The P388 lymphocytic leukemia growing as an ascites tumor in DBA/2 mice exhibits many of the characteristics that are common to other ascites tumors (e.g., Refs. 9, 13, 16, and 24): the cell cycle elongates during growth (primarily because of lengthening of G0), the growth fraction declines, and the cell loss factor increases (at least in late growth). After transplantation the tumor exhibits a lag period of about 16 hr before cells reenter the cell cycle and synthesize DNA. We previously hypothesized (11) that this lag period reflects the time needed for resynthesis of some macromolecule (e.g., a protein) that has decayed below a critical concentration during prolonged residence in plateau phase. We know little about this process or the nature of these hypothetical molecules, except that the process apparently uses preexisting mRNA and is at least partially oxygen dependent (11).

Shortening of the P388 cell cycle after transplantation precedes the increase in the growth fraction. Indeed, the growth fraction is actually higher in early plateau phase (Day 5) than during the 1st day of growth, even though the cell cycle time of the proliferating cells in the old tumor is 2.5 times greater than in the young one. This analysis indicates that cell-cycle time and the growth fraction change independently of each other in the P388, despite their coordinated changes in some tumors (13).

Perhaps the most significant result of these experiments is...
the observation that cells harvested from late-plateau-phase tumors are more radioresistant than cells from exponentially growing tumors (Chart 4). Since the validity of this conclusion rests on the assumption that extrapolation of the growth curves is a valid measure of cell survival in this tumor (it is not for all tumors (e.g., Ref. 20)), it should be emphasized that the survival percentage obtained for cells in exponential growth (Chart 4) is nearly identical to that estimated by the TD50 technique (3, 4, 12).

Our finding that cells from late-plateau-phase tumors are significantly more resistant to irradiation in air than cells from exponential tumors is consistent with results obtained by Hahn and Bagshaw (10) with Chinese hamster cells in vitro. They observed that cells accumulated in plateau-phase G1 by growth in low-serum medium were more resistant to X-rays than were exponentially growing cultures (10). Chinese hamster cells blocked in an analogous G1 (or G0) position by anoxia are also significantly more radioresistant than normal G1 cells (15). Taken together, these data suggest that cells deprived of oxygen and/or essential nutrients stop cycling at some point after mitosis and before S phase and that in this "quasi-G1" (or G0) state they are more resistant to X-radiation than are normal G1 cells. The implications of this resistance for the radioreponsiveness of tumors containing appreciable numbers of these cells are obvious.

In apparent contrast to our results, earlier studies of the P388 by Belli and Andrews (3) disclosed no difference in the radiosensitivity of cells grown for 1 or 7 days in vivo. This disagreement is only apparent, however; Belli and Andrews' growth curve and the fact that their animals did not die until Day 12 both indicate that their study encompassed only exponential and very-early-stage-plateau-phase cells (3). Our cytokinetic data suggest that only minimal differences in the distribution of cells within the cell cycle would be expected to occur during this interval. In our study, which compares exponential and late-plateau-phase cells, a difference in radiosensitivity is evident (Chart 4). Since these late-plateau-phase cells may be typical of those found within large solid tumors, we are extending our studies. Preliminary results suggest that the "biochemical status" of late-plateau-phase cells, rather than their physical position in the cell cycle, is responsible for their radioresistance.

ACKNOWLEDGMENTS

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J. W. Harris, B. Shon, and J. Meneses


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