Initiation of DNA Synthesis in Ehrlich Ascites Tumor Cells in Their Plateau Phase of Growth

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SUMMARY

The E2 hypotetraploid Ehrlich ascites tumor reached its peak size at $1 \times 10^9$ cells on the 14th day after i.p. inoculation of $0.30 \times 10^8$ cells. During this period the % nonviable Ehrlich ascites tumor cells did not change. Autoradiographic studies during the first 14 days of Ehrlich ascites tumor growth demonstrated no decrease in the % tumor cells incorporating tritiated uridine, proline, phenylalanine, or leucine. The % Ehrlich ascites tumor cells in DNA synthesis (thymidine-$^3$H incorporation), however, decreased with increasing tumor age. Azepitation of most of the tumorous ascitic fluid from a 14-day-old Ehrlich ascites tumor host resulted in recurrent growth of the remaining Ehrlich ascites tumor. Recurrent growth of the Ehrlich ascites tumor was characterized by a significant increase in the thymidine index (% Ehrlich ascites tumor cells incorporating thymidine-$^3$H in 20 min). The peak thymidine index of recurrent growth, 51.8%, occurred between the 14th and 24th (19th) hr after aspiration of most of the 14-day-old tumorous ascitic fluid. During recurrent growth of the Ehrlich ascites tumor a lag period of at least 4 hr occurred between the time of ascites removal and the increase in the thymidine index. Thymidine index determinations made after transplantation of saline-washed 14-day-old Ehrlich ascites tumor cells to new hosts also demonstrated (a) a 4 hr lag period before the thymidine index began to increase and (b) a peak thymidine index, 56.1%, 19 hr after inoculation. The data are discussed in relation to the events in the cell cycle.

INTRODUCTION

Recurrent growth of malignant tissue after apparently incomplete surgical removal is a major clinical problem (4, 5, 22, 36, 37). It is, therefore, important to study recurrent tumor growth in an experimental tumor-host system that permits quantitative measurements of growth. The Ehrlich ascites tumor is such a system (6, 7, 9, 11–13, 20, 25, 26, 29, 30, 31, 41, 43, 53).

It is the purpose of this paper to describe experiments which were specifically designed to investigate growth characteristics of the Ehrlich ascites tumor in the late stage of intraperitoneal growth. Data from these experiments suggested further experiments which describe the initiation of Ehrlich ascites tumor cell DNA synthesis (a) during recurrent growth of the Ehrlich ascites tumor and (b) after transplantation of 14-day-old Ehrlich ascites tumor cells to new hosts. An ancillary portion of this work has been published (18).

MATERIALS AND METHODS

The Ehrlich ascites tumor used was the E2 single cell clone, hypotetraploid line. It was transplanted by weekly i.p. inoculations of $0.30 \times 10^8$ saline-washed cells, the subsequent growth of which will be referred to as standard intraperitoneal growth (SIG). Hosts were CD1 female mice from 1–3 months of age. Animals were given Purina Mouse Chow and tap water containing terramycin (3.6 gm/gal) ad libitum.

Thymidine-$^3$H (1.9 c/mmole), uridine-$^5$H (2.0 c/mmole), L-phenylalanine-$^3$H (2.5 c/mmole), L-leucine-$^3$H (1.8 c/mmole), and L-proline-$^3$H (2.0 c/mmole) were obtained from Schwarz BioResearch, Inc. The time interval between intraperitoneal injection of the tritiated precursor and sacrifice of the animal via decapitation was always 20 min. Tumorous ascitic fluid was smeared on clean glass slides, air dried, and fixed in 10% buffered formalin or methanol. Ehrlich ascites tumor cells labeled with thymidine-$^3$H had synthesized DNA essentially the same in each case, statistical comparisons ($t$ test) were made, where appropriate, between the means of the number of labeled cells. It was assumed that Ehrlich ascites tumor cells labeled with thymidine-$^3$H had synthesized DNA (42) and would have undergone subsequent mitosis (8); however, exceptions to this general rule have been reported (9, 28,
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48). Ehrlich ascites tumor cells labeled with uridine-3H were considered to be cells that had synthesized RNA although this assumption has been questioned, at least in the isolated thymic nuclei system (1).

Total Ehrlich ascites tumor cell counts were made essentially according to the method described by Klein and Revesz (29). Nonviable cells were determined according to the method of Schrek (47).

RESULTS

Growth Curve. This Ehrlich tumor (solid circles in Chart 1) reached its peak size on the 14th day of SIG. As expected for a hypotetraploid cell line, the peak was approximately \(1 \times 10^9\) cells (25, 26) contained in 15–25 ml of bloody ascitic fluid. There was essentially no change in the % nonviable cells (solid triangles in Chart 1) from the 7th to the 14th day of SIG. These data (Chart 1) are similar to data obtained by other investigators (25, 26, 29, 30, 43, 53).

Biosynthetic Processes. Thymidine-3H incorporation studies (Chart 2) demonstrated that as this tumor aged there was a progressive decrease in the thymidine index. Similar observations have been reported (6, 7, 31). The occurrence of a minimal number of 14-day-old Ehrlich ascites tumor cells in DNA synthesis without any further increase (there is actually a small decrease) in the total number of cells may be explained by the observation that some tumor cells attach to peritoneal surfaces and invade abdominal viscera (3, 20).

These observations raised a question as to the biosynthetic state of the Ehrlich ascites tumor cells on the 14th day of SIG. What % of the 14-day-old Ehrlich ascites tumor cells would incorporate uridine-3H or tritiated amino acids? Baserga (6) reported no change in the % of cells incorporating radioactive uridine from the 4th to the 13th day of Ehrlich ascites growth. This finding was confirmed by the results in Chart 2 (uridine-3H curve). Chart 2 further demonstrates that from the 5th to the 14th day of SIG no change occurred in the % of Ehrlich ascites tumor cells incorporating various tritiated amino acids. The degree of incorporation (grain counts) of all tritiated precursors, however, decreased as the tumor reached its peak size (Table 1).

The data presented in Charts 1 and 2 indicated that (a) this Ehrlich tumor, the \(E_2\), had markedly decreased its growth by the 14th day of SIG, (b) the Ehrlich ascites tumor cells, however, remained viable, and (c) the cells continued to incorporate RNA and protein precursors. Apparently the energy
Table 1

<table>
<thead>
<tr>
<th>Precursor</th>
<th>7-day-EAT*</th>
<th>14-day-EAT*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Phenylalanine-³H</td>
<td>147*</td>
<td>17</td>
</tr>
<tr>
<td>L-Leucine-³H</td>
<td>131</td>
<td>25</td>
</tr>
<tr>
<td>L-Proline-³H</td>
<td>26</td>
<td>14</td>
</tr>
<tr>
<td>Uridine-³H</td>
<td>56</td>
<td>32</td>
</tr>
<tr>
<td>Thymidine-³H</td>
<td>150</td>
<td>16</td>
</tr>
</tbody>
</table>

Autoradiographic grain count data on 7- and 14-day-old Ehrlich ascites tumor (EAT) cells.

* Exposed to 25 microcuries (μCi) of tritiated precursor.
* Exposed to 100 microcuries (μCi) of tritiated precursor.
* Mean grain count of 3 different cases based on 30 EAT cells/case. Grain count corrections were unnecessary because of the absence of autoradiographic background.

and precursor requirements for the synthesis of Ehrlich ascites tumor RNA and protein were met by the 14-day-old Ehrlich ascites tumor host.

Recurrent Growth. If the precursors available from the host for Ehrlich ascites tumor cell RNA, protein, and DNA synthesis are not significantly decreased or altered, one might expect any particular Ehrlich ascites tumor host to support, metabolically, more tumor growth than that which is usually observed for a hypotetraploid Ehrlich ascites tumor, i.e., 1 X 10⁹ cells. To investigate this possibility, animals with Ehrlich ascites tumor for 14 days were anesthetized with ether, and, under aseptic conditions, as much tumorous ascitic fluid as possible (inconstant amount) was aspirated using a 20-ml hypodermic syringe and a 19-21 gauge needle. The animals became bloated again 2-3 days after aspiration of most of the 14-day-old Ehrlich ascites tumor cells and associated ascitic fluid. The aspiration procedure was repeated every 2-3 days until the animal died. Dead animals were not subjected to a final aspiration and therefore contained Ehrlich ascites tumor cells that were not counted. The results of this experiment are presented in Table 2. The data indicate that animals which have supported Ehrlich ascites tumor growth for 14 days may produce up to approximately 4 times as much Ehrlich ascites tumor if they suffer frequent removal of tumorous ascitic fluid.

The thymidine-³H indices of recurrent growth of the Ehrlich ascites tumor are presented in Chart 3. The thymidine indices at 2 (26.9%) and 4 (25.6%) hr after aspiration were essentially the same as the thymidine index of a 14-day-old Ehrlich ascites tumor during SIG (24.0%). After the 4th hr of the newly initiated recurrent growth, the thymidine index began to rise and reached its peak between the 14th and 24th (19th) hr of recurrent growth. The thymidine index during the 19th hr of recurrent growth of the Ehrlich ascites tumor was 51.8%, which was not different from the thymidine index of a 7-day-old Ehrlich ascites tumor in SIG (47.2%). A comparison of the thymidine indices between 14-day-old Ehrlich ascites tumor and recurrent Ehrlich ascites tumor at its 19th hr indicated

Table 2

<table>
<thead>
<tr>
<th>Hosts</th>
<th>Number of aspirations</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>A</td>
<td>815.5*</td>
<td>735.0</td>
</tr>
<tr>
<td>B</td>
<td>910.0</td>
<td>575.0</td>
</tr>
<tr>
<td>C</td>
<td>752.5</td>
<td>700.0</td>
</tr>
<tr>
<td>D</td>
<td>750.0</td>
<td>655.0</td>
</tr>
<tr>
<td>E</td>
<td>470.0</td>
<td>345.0</td>
</tr>
<tr>
<td>F</td>
<td>1037.5</td>
<td>510.0</td>
</tr>
<tr>
<td>G</td>
<td>800.0</td>
<td>1162.5</td>
</tr>
<tr>
<td>H</td>
<td>995.0</td>
<td>625.0</td>
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<tr>
<td>I</td>
<td>987.5</td>
<td>1195.0</td>
</tr>
<tr>
<td>J</td>
<td>1197.5</td>
<td>757.5</td>
</tr>
<tr>
<td>K</td>
<td>787.5</td>
<td>1127.5</td>
</tr>
</tbody>
</table>

Quantity of Ehrlich tumor cells produced by hosts following repeated aspiration at 2- to 3-day intervals. All Hosts had the Ehrlich tumor for 14 days at the beginning of the experiment.

* All numbers are X 10⁶.
that there were twice as many Ehrlich ascites tumor cells in DNA synthesis in the recurrent tumor.

Transplantation of 14-day-old Ehrlich Ascites Tumor Cells to New Hosts. After aspiration of most of the 14-day-old Ehrlich ascites tumor, a lag period of at least 4 hr occurred before the % of Ehrlich ascites tumor cells incorporating thymidine-3H began to increase. Will a similar lag period be observed when 14-day-old Ehrlich ascites tumor cells are transplanted to a new host? To answer this question, 70 x 10^6 saline-washed, 14-day-old Ehrlich ascites tumor cells were inoculated i.p. into new hosts. The recipient animals received 25 µc of thymidine-3H at 10 min, 2, 4, 9, 19, 48, and 72 hr after the tumor cell inoculation. The results of this experiment are presented in Chart 4.

A lag period of at least 4 hr occurred before the % of Ehrlich ascites tumor cells incorporating thymidine-3H began to increase. The peak thymidine index (56.1%) occurred at the 19th hr after the inoculation of the saline-washed, 14-day-old Ehrlich ascites tumor cells into new hosts, and the thymidine index did not change appreciably thereafter. These observations do not agree with those reported by Baserga and Gold (12). They observed an immediate increase in the % of labeled Ehrlich ascites tumor cells when 55 x 10^6 Ehrlich ascites tumor cells (20 days old and not saline-washed) with a thymidine index of 40% were immediately transplanted to 3 recipient mice (thymidine indices of 54%, 58%, and 57%). The only apparent difference between the 2 experiments was that Baserga and Gold (12) did not use saline-washed Ehrlich ascites tumor cells. Experiments are in progress using saline-washed and unwashed 14-day-old Ehrlich ascites tumor cells inoculated into new hosts.

The DISCUSSION

The data in this paper demonstrated that, in the 14-day-old Ehrlich ascites tumor, almost all of the cells were viable and synthesizing RNA and protein. Few very of these cells, however, were engaged in DNA synthesis. Removal of most of the 14-day-old Ehrlich ascites tumor or transplantation of 70 x 10^6 14-day-old Ehrlich ascites tumor cells to a new host resulted in an increase in the number of Ehrlich ascites tumor cells in DNA synthesis, after at least a 4-hr lag period.

The time required (after application of the appropriate stimulus) to obtain the maximum number of cells in DNA synthesis has been determined in several different experimental systems: 60 hr after nondividing adult kidney tissue is explanted in vitro (34), 24 hr after subtotal hepatectomy (15, 16), 20 hr after the medium is changed on contact-inhibited 3T3 normal fibroblasts (32), approximately 20 hr after wounding of rabbit lens epithelium (24), and, as reported in this paper, 19 hr after aspiration of most of the 14-day-old Ehrlich ascites tumor cells or inoculation of 70 x 10^6 of these cells into new hosts.

Present knowledge of the cell cycle and its various phases indicates that the control of growth is actually the control of the initiation of DNA synthesis (8). This turns our attention to the pre-DNA synthetic phase (G_1) in the cell cycle. Factors inhibiting the initiation of DNA synthesis block the cell in the G_1 phase (17, 32, 40, 44). It should be noted that there are instances in which control of cell division may operate in the DNA synthetic phase (S phase) or in G_2, the post-DNA synthetic phase (23, 27, 35, 38, 39). These observations indicate a series of temporally associated molecular events in G_1 leading to the initiation of DNA synthesis. RNA and protein synthesis are intimately involved in the initiation of DNA synthesis (9, 10, 14, 21, 32, 33, 34, 50, 51, 52). Baserga et al. (9, 11) recently demonstrated an actinomycin D-sensitive step in the G_1 phase of the Ehrlich ascites tumor cell.

These observations suggest that, in the 14-day-old Ehrlich ascites tumor, a significant proportion of the tumor cells may be blocked in G_1, i.e., a nonproliferating population. The magnitude of the drop in the mean grain count (thymidine-3H) between Days 7 and 14 of SIG (Table 2) suggests a lengthening of the S phase and thus the cell cycle in the smaller proliferating fraction of 14-day-old Ehrlich ascites cells (31). Release from this situation and subsequent initiation of recurrent growth of the Ehrlich ascites tumor may be affected by removing (aspiration of most of the tumorous ascitic fluid or transplantation of saline-washed 14-day-old Ehrlich cells to a new host) the majority of the tumor cells. Thus, this situation may be analogous to normal tissue or organ regeneration (45).
The data presented in this paper demonstrated that recurrent growth of the Ehrlich ascites tumor (E<sub>2</sub>, hypotetraploid, single cell clone) was consistently produced following aspiration of most of the 14-day-old ascitic fluid. However, after daily tapping of Ehrlich ascites tumor, beginning 7-10 or 12-14 days after inoculation of 2.0-2.5 x 10<sup>6</sup> Ehrlich ascites tumor cells, Apffel et al. (2) obtained “recovery with immunity.” They did not obtain recurrent growth of the Ehrlich ascites tumor. Although there were many differences in experimental technic, one of the significant points seems to be the presence or absence of the acellular ascitic fluid. If enough ascitic fluid remained in the animal after paracentesis or acellular ascitic fluid was immediately reinjected into a previously aspirated animal, the host immunologic response was thwarted and the tumor grew and killed the host (2).

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REFERENCES


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