Estimation of Tumor Growth Fraction in Murine Tumors by the Primer-available DNA-dependent DNA Polymerase Assay

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SUMMARY

The tumor growth fraction measured by the percentage labeled mitoses method has been determined in transplantable solid and ascites murine tumors, the latter being measured at different times after transplantation. These values were compared to an in vitro autoradiographic assay that determines the fraction of cells in a given population (primer-available DNA-dependent DNA polymerase index) that have both nuclear DNA-dependent DNA polymerase and DNA capable of acting as primer-template. It appears that almost all cells with a short G1 phase duration (<19 hr) that are within the proliferative cycle are primer-available DNA-dependent DNA polymerase positive. The results of the comparison indicate that the primer-available DNA-dependent DNA polymerase index estimation of growth fraction is very nearly identical to the growth fraction measured by the percentage labeled mitoses method.

INTRODUCTION

When exogenous DNA is added to cell-free systems in the assay for DDDP, the activity of the cytoplasmic enzyme(s) exhibits variation according to the position of the cell in the proliferative cycle (3, 7). Quiescent and noncyling systems show little activity (3, 12). Nuclear DDDP activities, on the other hand, do not appear to change appreciably during the cell cycle (3, 5, 7) and may remain detectable in quiescent cells (3). If sole reliance for DNA synthesis is placed upon endogenous primer-template being present, nuclear DDDP activity does exhibit considerable fluctuation in synchronized mammalian cell populations (5). This activity is also discernibly higher in cells from logarithmically growing cultures than in cells from stationary phase cultures (5). Conversely, nuclear DDDP activity is lost in differentiated cells such as muscle (21) and avian erythrocytes (22).

The fact that test tube assays measure an average DDDP activity in all cells of the studied cohort precludes the use of this method to measure the proportion of cells with DDDP activity or the activity in any specific cell. We have recently described (11) an in vitro method for detecting the presence of DDDP in individual nuclei, utilizing the cell's own endogenous nuclear DNA as primer-template. This in vitro assay is called the PDP assay, and it allows the determination of the fraction of PDP-positive cells in a given population, the PDP index. We now postulate that, in tumors with short G1 duration, the fraction of cells whose nuclei contain DDDP, and DNA in a state that can prime and act as template for DNA synthesis, is a close estimate of that fraction of cells in active cycle (GF). This communication describes the comparison of the PDP estimate of GF with the measurement of GF by the PLM method in murine tumors.

MATERIALS AND METHODS

Two groups of tumors have been utilized for these studies. Group A has had PLM curves performed entirely at this laboratory. Calculations of their cytokinetic time parameters were made by hand by the authors and were computer fitted by the method of Takahashi et al. (20).

Group B tumors consist of several tumors in which PLM curves were done elsewhere, or GF was estimated in a different manner. Although their results are included in this paper to show the data on all the tumors we have studied, the linear least-square regression line of Chart 1 was determined without use of the Group B data.

GF's of Tumors with Computer-fitted PLM Curves

T1699 Mammary Tumor. T1699 mammary tumors were passaged in 6- to 8-week-old male DBA/2J mice by s.c. implantation of approximately 25 mg of tumor tissue and, subsequently, were studied 14 days later when the solid tumor size reached approximately 1 cu cm. The mice and tumor were obtained from The Jackson Laboratory, Bar Harbor, Maine. At frequent intervals after i.p. injection of [3H]Tdr, 3 mice were sacrificed by cervical dislocation and the tumors were excised and fixed in cold Carnoy’s solution. The [3H]Tdr had a specific activity of 1.9 Ci/mmmole, was administered at 1 μCi/g body weight, and was obtained from Schwarz/Mann, Orangeburg, N. Y. After routine histological processing, 4-μm sections were cut and stained by Feulgen method. Autoradiographs were prepared with a 1:1 dilution of NTB2 liquid emulsion (Eastman Kodak, Rochester, N. Y) and exposed for 45 to 90 days.
Two methods of counting the PLM were used. The 1st involved starting at one edge of the tumor and proceeding to the opposite edge until 200 or more consecutive mitoses were observed, and then repeating at right angles to the initial count. Once started, a count across the tumor was completed even if 200 mitoses were observed. The 2nd method involved counting 200 consecutive mitoses only at the periphery of the tumor. Since there was little difference in the shape of the PLM results, data from both methods were combined for final cytokinetic calculations. Thus, a total of at least 600 mitoses were evaluable for each tumor studied.

Both the [3H]TdR LI and the calculated GF’s at the periphery of the tumor were consistently higher than that found centrally. Thus, for the purpose of these calculations, the LI determined by the 1st method was used, as it is more representative of the whole tumor.

The growth curve was obtained by daily caliper measurements of individual tumors and by calculating the approximate prolate spheroid volume by L x W x H/2. The tumor-doubling time was determined from the growth curve by using the tumor volume on the day of analysis and 2 days on either side, and calculating the best straight line by a linear least-squares method.

The cytokinetic time parameters were derived from the PLM curve (Chart 2) drawn by eye fit and the GF was determined by Steel’s formulations (18, 19). Additionally, a computer-fitted curve was determined as described (20). The results of this analysis, and the subsequent PLM curves, are shown in Table 1.

**GF’s of Other Tumors**

The techniques and cytokinetic results of the Group B tumors will not be reported in detail. Only their estimated GF determinations and the PDP indices from this laboratory will be compared (Table 2).

**Sarcoma 180 Solid Tumors.** Cytokinetics values for this tumor were obtained from the literature, as reported by Dr. Linda Simpson-Herren (17). Two 5-day tumor PLM curves were reported, with attendant time parameters. They are sufficiently similar to allow averaging of results for this purpose. It should be noted that the host animals are different; Swiss mice (Dr. Simpson-Herren) versus C3H/HeJ mice (this laboratory).

**C3H/HeJ Spontaneous Mammary Tumors.** Sixteen tumors developing in retired breeders of the C3H/HeJ line (The Jackson Laboratory) were allowed to reach 1 cu cm in volume. At that time, animals were given injections of [3H]-TdR, 2 μCi/g body weight, and were sacrificed 5 days later when desynchronization of the curve was assumed to have occurred. Autoradiographed paraffin sections were analyzed for their GF by the method of Mendelsohn, i.e., labeled cells per total cells divided by labeled mitoses per total mitoses.

**Rat 13762 Mammary Tumor.** This tumor was obtained from Dr. Arthur Bogden, Worcester Research Institute, Worcester, Mass. It is passaged in Fischer 344 rats obtained from A. R. Schmidt Co., Madison, Wis. A PLM curve was performed in our laboratory but has not yet been analyzed by computer. The GF was determined 14 days after transplantation (9).
Table 1

Cytokinetic values of murine tumors by hand (H) and computer (C) fit

<table>
<thead>
<tr>
<th>Tumor</th>
<th>GF</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoma 180</td>
<td>H</td>
<td>C</td>
</tr>
<tr>
<td>T1699 (Day of study, 14; [3H]TdR LI, 0.135; Tn, 54.2)</td>
<td>3.1</td>
<td>5.4</td>
</tr>
<tr>
<td>Ehrlich ascites</td>
<td>H</td>
<td>C</td>
</tr>
<tr>
<td>T1699 (Day of study, 2; [3H]TdR LI, 0.667; Tn, 19.0)</td>
<td>5.8</td>
<td>5.3</td>
</tr>
<tr>
<td>T1699 (Day of study, 4; [3H]TdR LI, 0.496; Tn, 30.5-33.6)</td>
<td>3.7</td>
<td>3.9</td>
</tr>
<tr>
<td>T1699 (Day of study, 6; [3H]TdR LI, 0.135; Tn, 14.0)</td>
<td>0.5</td>
<td>0.5&quot;</td>
</tr>
<tr>
<td>T1699 (Day of study, 9; [3H]TdR LI, 0.454; Tn, 50)</td>
<td>13.1</td>
<td>15.0</td>
</tr>
<tr>
<td>GF</td>
<td>0.30</td>
<td>0.40</td>
</tr>
<tr>
<td>SD</td>
<td>0.13</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Table 2

Comparison of measured tumor GF's with the PDP index

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Tumor age (days)</th>
<th>Measured GF</th>
<th>PDP index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1699 solid</td>
<td>14</td>
<td>0.40</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>Sarcoma 180 ascites</td>
<td>2</td>
<td>1.00</td>
<td>0.88 ± 0.02</td>
</tr>
<tr>
<td>Sarcoma 180 ascites</td>
<td>4</td>
<td>0.98</td>
<td>0.88 ± 0.04</td>
</tr>
<tr>
<td>Ehrlich ascites</td>
<td>2</td>
<td>0.79</td>
<td>0.80 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.80</td>
<td>0.70 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.59</td>
<td>0.61 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.61</td>
<td>0.62 ± 0.02</td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcoma 180 solid</td>
<td>5</td>
<td>0.62</td>
<td>0.51 ± 0.06</td>
</tr>
<tr>
<td>C3H/HeJ spontaneous</td>
<td>1 cu cm (vol)</td>
<td>0.18</td>
<td>0.17 ± 0.08</td>
</tr>
<tr>
<td>Solid</td>
<td>T3762, solid, rat</td>
<td>14</td>
<td>0.75</td>
</tr>
<tr>
<td>Glioma, solid, rat</td>
<td>21</td>
<td>0.35-0.46</td>
<td>0.34 ± 0.02</td>
</tr>
</tbody>
</table>

* Computer-derived GF values.
* Mean ± 2 S.E.

**Rat Glioma.** This tumor was obtained from Dr. Robert Selker, Montefiore Hospital, Pittsburgh, Penna., who maintains it in tissue culture until i.e. implantation in Fischer 344 rats. The tumor was originally obtained from the laboratory of Dr. Charles Wilson, Department of Neurological Surgery, University of California, San Francisco, Calif., and Barker et al. (2) reported the PLM and cytokinetic parameters that we compared with our PDP data.

**PDP Measurements**

Single cell suspensions were obtained from solid tumors, on the days indicated, by mincing the tumor tissue in culture medium. Cells of Sarcoma 180 and Ehrlich ascites tumors were harvested directly from the peritoneum. All cell suspensions were washed in 0.9% NaCl solution or in culture medium, resuspended, smeared onto acid-cleaned slides, and air-dried. Slides were dipped in 0.25% solution of agar at 40 °C and again air dried. This process appears to disrupt the cytoplasm, leaving only nuclei adherent to the slide. The slides were used in a modification of the PDP assay described previously (11). Briefly, 0.5 ml of the incubation mixture was added to chambers made by affixing a glass ring to each slide. The incubation mixture contained the deoxy-5' triphosphates of adenosine, guanosine, and cytidine, 5 mM MgCl2, Ficoll (Sigma Chemical Co., St. Louis, Mo.) and 2 μCi [3H]TdR triphosphate (specific activity, 17 to 19 Ci/m mole; Schwarz/Mann) in 0.02 M Tris (pH 7.4 at 37°C). After 45 min at 37°C, the reaction was terminated on ice, the glass rings were removed, and the cells were fixed for 10 min each in 3 changes of acidic formaldehyde. The subsequent processes of rinsing, dehydration, and autoradiography were performed as described previously. Following autoradiography, the cells were stained with either Giemsa or hematoxylin stain. Labeling indices derived from counts of 500 cells were determined for at least 6 tumors of each type studied.

**RESULTS**

Table 1 shows the cytokinetic values of the Group A animals on which computer-derived data are available. The data in Table 2 clearly indicate the close approximation of the in vitro PDP index to the in vivo determination of GF in these tumor systems. The regression of the PDP index on GF (Chart 1) is a straight line with a slope of 0.925 and an intercept of -0.003 and a correlation coefficient of 0.950. If a regression line were drawn using all the data, including the non-computer-derived and miscellaneous material, the slope would be 0.935 with a Y intercept of -0.017 and a correlation coefficient of 0.974.

**DISCUSSION**

The PDP index measures the fraction of cells that contain nuclear DDPD and have DNA capable of being active as primer-template for DNA synthesis. We have found this fraction of a tumor population is always greater than the fraction of cells labeled in vivo or in vitro with [3H]TdR. All cells in DNA synthesis are PDP positive as measured in hydroxyurea-synchronized Sarcoma 180 cells (14). Approximately one-half of mitoses are PDP positive, with early mitotic figures more so than anaphases and early telophases. The fraction of cells represented by PDP-negative mitotic fig-
ures is sufficiently small not to seriously influence the results. Our evidence, to date, is that out-of-cycle cells (peripheral blood lymphocytes and granulocytes) and dead cells (45° heat killed Sarcoma 180 cells) are not PDP positive (14). Lymphocytes stimulated into cycle by phytohemagglutinin become PDP positive 5 to 11 hr prior to the start of DNA synthesis (15); HeLa cells synchronized by mitotic shaking, with a G1 of 9 hr, are PDP positive. We have found that the PDP index of Sarcoma 180 ascites tumor cells decreases after 4 days postimplantation, although the measured GF remains at unity (16). This discrepancy is explainable by an increase of cells in long G1, some of which are PDP negative. A model of Sarcoma 180 ascites tumor growth suggests that we can estimate all cells in S and G2, 23% in M, and a proportion of cells corresponding to approximately 8 hr of G1. The model is based upon synchronization of Sarcoma 180 ascites cells of various implantation ages, by doses of hydroxyurea, during which [3H]TdR labeling indices reach 100% and PDP indices are also found to be 100%. This information leads us to believe that we can rely on cell systems with a short G1 (<9 hr) to manifest the linear relationship of GF to PDP index. Most solid murine and rat tumors would fit into this category.

The GF estimates themselves may be subject to considerable error, and will reflect the grain thresholds, emulsion exposure duration, and sampling variations of the tumor. The GF of the computer-fitted PLM curves in this paper was determined by comparing the observed and expected labeling index using the method of Mendelsohn and Dethlefsen (8).

At this stage of development, the PDP assay is too crude to evaluate the presence of specific DNA polymerases. We may, in fact, be detecting more than one enzyme, although the evidence seems to indicate that the nuclear enzyme DNA polymerases are the most likely candidates (1). This does not exclude the possibility that we may detect a soluble cytoplasmic enzyme which becomes associated with DNA primer-template during specific phases of the cell cycle (4). The PDP assay also presumes, as one of its variables, that the cell’s nucleus is capable of having priming-template activity. This is probably not identical to transcriptional activity for RNA synthesis on DNA template, although a recent study (10) using an acridine orange probe for template activity shows results similar to ours during the mitotic phase of the cell cycle.

Although the biochemistry of the PDP reaction is not completely understood, the assay may prove to be a valuable tool in designing the initial therapy with anticancer agents, as well as a way of following the results of their application. Although these data refer only to unperturbed systems of transplantable tumors, we have compelling evidence that the PDP index does change in these tumors after chemotherapeutic drugs or radiation (14).

ACKNOWLEDGMENTS

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REFERENCES

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