Cell kinetics of rat 9L brain tumors determined by double labeling with iodo- and bromodeoxyuridine


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Rats with 9L brain tumors received intraperitoneal injections of iodo-deoxyuridine (IUdR) and bromodeoxyuridine (BUdR) to estimate the duration of the deoxyribonucleic acid (DNA) synthesis phase (Ts) and the potential doubling time (Tp) of individual tumors. Different sequences and intervals (2 or 3 hours) of IUdR and BUdR administration were evaluated. After denaturation, tumor sections were reacted with Br-3, a monoclonal antibody that identifies only BUdR, and then were stained immunohistochemically by the avidin-biotin complex method. An antibody that recognizes both IUdR and BUdR, IU-4, was applied to the sections and identified by the alkaline phosphatase-antialkaline phosphatase method. Nuclei labeled only with IUdR stained blue, while those labeled with BUdR or with BUdR and IUdR stained brown. The fraction of cells that either left or entered the S-phase during the time between administration of IUdR and BUdR was measured to calculate Ts and Tp, assuming that the labeled cohort completed the DNA synthesis at a constant rate. The Ts was 8.8 hours (coefficient of variation (cv) = 0.05) and the Tp was 64.2 hours (cv = 0.08). The sequence and interval of administration of IUdR and BUdR had a minimal effect on Ts and Tp. In studies of 9L cells in monolayer culture, the Ts was 9.6 hours (cv = 0.08) and the Tp was 30.6 hours (cv = 0.06). Double labeling with BUdR and IUdR allows the duration of the S-phase and potential doubling time of individual brain tumors to be estimated in situ from a single biopsy specimen.

KEY WORDS: cell kinetics • brain neoplasm • bromodeoxyuridine • iododeoxyuridine • monoclonal antibody • immunohistochemistry

CELL kinetics studies were greatly facilitated by the development of a monoclonal antibody against halogenated pyrimidines. Although two thymidine analogues, iodo-deoxyuridine (IUdR) and bromodeoxyuridine (BUdR), are available, this antibody alone cannot be used for double-labeling studies because it reacts equally with BUdR and IUdR. Recently, more specific monoclonal antibodies against halogenated pyrimidines have been created. The monoclonal antibody Br-3 identifies only BUdR, whereas the IU-4 antibody cross-reacts with both BUdR and IUdR. Theoretically, all cell kinetics studies that previously used radioactive thymidine are now feasible using these agents without the hazard or pollution involved in the use of radioactive thymidine. To take advantage of these two antibodies, we have developed a double-labeling method with BUdR and IUdR. This report describes the use of that method to estimate the duration of the deoxyribonucleic acid (DNA) synthesis phase (Ts) and the potential doubling time (Tp) of rat 9L brain tumors in situ.

Materials and Methods

In Vitro Studies

For the in vitro studies, rat 9L gliosarcoma cells (4 × 10⁴ cells/0.5 ml of medium) growing in monolayer culture in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum were transferred to each chamber of tissue culture/chamber slides. The cells were incubated at 37°C in a 100% humidified atmosphere with 95% air/5% CO₂. To estimate the BUdR labeling index (LI), cells were exposed to 10 μM...
Double labeling for cell kinetics studies

B UdR in 0.5 ml of medium for 30 minutes at 37°C in a 100% humidified atmosphere. For the single-labeling study, the cells were fixed in 4°C 70% ethanol for 30 minutes, denatured with 4 N HCl, and stained with Br-3 by the avidin-biotin complex (ABC) method. For double-labeling studies, the cells were incubated first with 10 μM IUdR for 2 hours and then with 10 μM BUdR for 30 minutes. After fixation in chilled 70% ethanol, the cells were denatured with 4 N HCl.

In Situ Studies

Eighteen male Fisher 344 rats, each weighing about 200 gm, were injected intracerebrally with approximately 4 × 10⁴ 9L gliosarcoma cells in 10 μl of complete medium using a standard stereotactic technique. Near the terminal stage, when motor palsy or pigmentation of the eyelids was evident (approximately 16 to 21 days after implantation), the rats were separated into four groups. Groups 1 and 2 received intraperitoneal injections of IUdR and BUdR (80 mg/kg each) at intervals of 2 and 3 hours, respectively. Groups 3 and 4 received equal doses of the same agents in reverse order at intervals of 2 and 3 hours, respectively. All rats were sacrificed 1 hour after the second injection, and the tumors were excised immediately.

All of the tumors were approximately 6 mm in diameter and weighed more than 200 mg. Each specimen was fixed in chilled 70% ethanol for 12 hours, embedded in paraffin, and cut into sections 5 μm thick. The sections were deparaffinized in xylene and rehydrated in 100% ethanol, 95% ethanol, and phosphate-buffered saline (PBS). The slides were incubated for 15 minutes in 0.3% H₂O₂ to block endogenous peroxidase and for 10 minutes in 4 N HCl to denature DNA.

Immunohistochemistry

After DNA denaturation, the cells in the LabTek slides and tissue sections were rinsed three times for 5 minutes each in PBS and incubated with Br-3 diluted 1:250 in PBS containing 1% bovine serum albumin (BSA) and 0.5% Tween 20 for 1 hour at room temperature in a 100% humidified atmosphere. The slides were rinsed with PBS and covered with a 1:200 dilution of biotinylated antimouse immunoglobulin G. After 30 minutes, they were rinsed in PBS, incubated with a 1:100 dilution of avidin DH and biotinylated horseradish peroxide H complex (ABC)† for 30 minutes, and then reacted for 5 to 10 minutes with 0.5 mg/ml of diaminobenzidine and 0.01% H₂O₂ in 0.05 M Tris-buffered saline (TBS).

To measure the BUdR LI, the slides were counterstained with hematoxylin. For double staining with BUdR and IUdR, the slides were left overnight in 5% acetic acid to inactivate immunoglobulin G and incubated with IU-4 diluted 1:5000 in PBS containing 1% BSA and 0.5% Tween 20 for 1 hour at room temperature in a 100% humidified atmosphere. The slides were rinsed in TBS and covered with rabbit anti-mouse immunoglobulin for 30 minutes, rinsed again in TBS, and reacted with alkaline phosphatase-antialkaline phosphatase complex‡ for 30 minutes at room temperature. The slides were then incubated for 5 to 10 minutes at room temperature in filtered substrate solution containing 5 mg of naphthol AS MX phosphate dissolved in 250 μl of dimethyl sulfoxide, 10 mg of fast blue BB base dissolved in 250 μl of 2 N HCl and 250 μl of 4% sodium nitrite, and 10 mg of levamisole in 40 ml of 0.1 M Tris-HCl buffer (pH 8.5). The BUdR-labeled cells contained brown reaction product, the IUdR-labeled cells stained blue, and double-labeled cells contained brown reaction product against a blue background (Fig. 1).

To calculate the BUdR LI, approximately 3000 cells from viable areas in which the labeled cells were evenly distributed were examined in each tissue section. Approximately 1000 cells from monolayer cultures were examined.

Calculation of Ts and Tp

The duration of the S-phase (Ts) was estimated using the equation Ts = t/dS, where “t” is the time interval between administration of IUdR and BUdR and dS is the fraction of S-phase cells that entered or left the S-phase during time “t.” The fraction of cells that entered or left the S-phase (dS) was estimated as the ratio of cells labeled only with IU-4 (blue) to those labeled only with Br-3 or with both Br-3 and IU-4 (brown or mixed). Approximately 1000 labeled cells in each section or culture slide chamber were analyzed. The potential doubling time (Tp), which is the time required for a tumor to double its size in the absence of cell loss, was calculated as k × 100 × Ts/LI, where k is approximately 0.7 if the expected doubling time is over 50 hours and 0.8 if it is 30 to 50 hours.²⁰

Results

In Vitro Studies

Double-labeling studies showed a dS value (dynamic fraction of the S-phase) of 0.21 (coefficient of variation

Table:

<table>
<thead>
<tr>
<th>Definitions of Abbreviations</th>
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<tbody>
<tr>
<td>BSA = bovine serum albumin</td>
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<tr>
<td>BUdR = bromodeoxyuridine</td>
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<tr>
<td>cv = coefficient of variation</td>
</tr>
<tr>
<td>DNA = deoxyribonucleic acid</td>
</tr>
<tr>
<td>IUdR = iododeoxyuridine</td>
</tr>
<tr>
<td>LI = labeling index</td>
</tr>
<tr>
<td>PBS = phosphate-buffered saline</td>
</tr>
<tr>
<td>TBS = Tris-buffered saline</td>
</tr>
<tr>
<td>Tp = potential doubling time</td>
</tr>
<tr>
<td>Ts = duration of tumor S-phase</td>
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Notes:

† Vectastain ABC kit manufactured by Vector Laboratories, Burlingame, California.

‡ Universal alkaline phosphatase-antialkaline phosphatase complex kit manufactured by DAKO Corp., Santa Barbara, California.
FIG. 1. Photomicrographs showing the results of the double-staining technique in cultured 9L cells (left) and in a rat 9L tumor section (right). Nuclei stained blue are labeled only with iododeoxyuridine. Nuclei stained brown are labeled with bromodeoxyuridine only. Nuclei with brown reaction products against a blue background are double-labeled with both iodo- and bromodeoxyuridine. × 240.

TABLE 1

Cell kinetic parameters of rat 9L brain tumors determined by double labeling with iodo- and bromodeoxyuridine*

<table>
<thead>
<tr>
<th>Group</th>
<th>Mode of Administration</th>
<th>No. of Studies</th>
<th>Sequence</th>
<th>Time Interval (hrs)</th>
<th>Dynamic Fraction of S-Phase</th>
<th>Labeling Index (%)</th>
<th>Duration of S-Phase (hrs)</th>
<th>Potential Doubling Time (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IUdR, BUdR</td>
<td>5</td>
<td>IUdR, BUdR</td>
<td>2</td>
<td>0.242 (0.16)</td>
<td>9.6 (0.27)</td>
<td>8.3 (0.16)</td>
<td>60.6 (0.31)</td>
</tr>
<tr>
<td>2</td>
<td>IUdR, BUdR</td>
<td>6</td>
<td>IUdR, BUdR</td>
<td>3</td>
<td>0.317 (0.18)</td>
<td>9.8 (0.21)</td>
<td>9.5 (0.18)</td>
<td>67.8 (0.28)</td>
</tr>
<tr>
<td>3</td>
<td>BUdR, IUdR</td>
<td>4</td>
<td>BUdR, IUdR</td>
<td>2</td>
<td>0.234 (0.18)</td>
<td>9.6 (0.28)</td>
<td>8.5 (0.18)</td>
<td>62.0 (0.33)</td>
</tr>
<tr>
<td>4</td>
<td>BUdR, IUdR</td>
<td>3</td>
<td>BUdR, IUdR</td>
<td>3</td>
<td>0.332 (0.15)</td>
<td>9.5 (0.20)</td>
<td>9.0 (0.16)</td>
<td>66.3 (0.25)</td>
</tr>
</tbody>
</table>

* Values in parentheses represent the coefficient of variation. IUdR = iododeoxyuridine; BUdR = bromodeoxyuridine.

(cv) = 0.08) and an average LI of 22% (cv = 0.1). The Ts was calculated as 9.6 hours (cv = 0.01), and the Tp was estimated as 34.9 hours, assuming that k is 0.8.

In Situ Studies

Table 1 shows cell kinetic parameters in each group of rats. The Ts was fairly constant, approximately 8.8 hours, despite the different sequences and time intervals in the administration of IUdR and BUdR. There were no statistically significant differences in the Ts between groups. The average LI was approximately 9.6% ± 0.1% (± standard error of the mean). The mean Tp was approximately 64.2 ± 3.0 hours, assuming that k is 0.7.

Discussion

The fraction of S-phase cells, the LI, and the growth fraction of various tumors have been estimated immunohistochemically utilizing monoclonal antibodies that identify IUdR or BUdR incorporated into cellular DNA during DNA synthesis.9,23 Using new, more specific antibodies against these halogenated pyrimidines, Shibui, et al.,19 developed a double-staining technique for analyzing cell cycle progression from a single specimen labeled with IUdR and BUdR at an appropriate interval. We have used this technique to measure the Ts and Tp of the rat 9L brain-tumor model.

The Ts, measured by the various combinations of sequence and interval (2 to 3 hours) of IUdR and BUdR administration in situ, yielded a fairly constant value (8.8 hours). This value was reasonably close to that of 9L cells in vitro (9.6 hours), but was slightly higher than previously found in vitro (8.2 hours),22 perhaps because the cells were almost confluent in our study. Thus, the Ts appears to be fairly constant whether cells are grown in situ or in vitro. The Ts determined in situ was also similar to that of 9L tumors determined autoradiographically (7.6 to 10 hours).1,7,13 Although the fraction cells entering or leaving the S-phase (dS) estimated by administering IUdR first followed by BUdR theoretically should be smaller than that estimated by administering these two agents in the reverse sequence (because of the cell cycle age distribution of exponentially
Double labeling for cell kinetics studies

growing population), this slight difference was not detected in the current study.

The Tp determined in situ in the present study, 64.2 hours, was higher than that measured earlier in this tumor model (42 hours). One reason for this difference is that the tumor LI in the current study was approximately 10%, whereas that of the earlier study was 18%. There are two possible explanations for the lower LI: growth retardation due to biological changes in cells during the long interval between the two studies and a decreased growth fraction and/or a decreased regional blood flow in a tumor at its terminal stage. The calculated Tp (64.2 hours) seemed to contradict an actual doubling time (40 hours) reported previously because, theoretically, the Tp should be smaller than the actual doubling time. One explanation for this contradiction is that the actual doubling time was measured from an extrapolation of the growth curves (3 to 100 mg of tumor weight) 8 to 15 days after tumor implantation. After this period, the growth rate may become much slower. Because all tumors used in our study were excised 16 to 21 days after tumor implantation, the actual doubling time at this stage might be larger than at an earlier stage.

The Ts determined by the double-labeling method was also fairly stable. Because it is calculated independent of heterogeneity in labeling or LI, it is not affected by the heterogeneity of tissue within a tumor. The LI itself does not directly indicate the rate of tumor growth, although it is important in estimating the Tp. Further accumulation of data regarding a relationship between the LI and tumor growth from various kinds of tumors is certainly required for a better understanding of the Ts. On the other hand, the Tp is a direct indicator of tumor growth. Although the Tp is fairly variable because it depends on the LI, which varies from site to site and at different times, the smallest and largest Tp's can be estimated from the labeled areas showing the greatest and least amount of labeling, and the average Tp could be calculated. We believe this double-labeling method provides improved characterization of tumor cell kinetics at any specified stage of tumor growth from a single biopsy specimen.

Determining the proliferative potential of individual tumors is extremely important in predicting the prognosis precisely and in designing treatment modalities for individual patients with brain tumors. In the past several years, BUdR LI has been measured in many types of brain tumors. The BUdR studies appear to predict the proliferative potential of individual tumors more precisely than does histopathological diagnosis and may even correlate with survival better than with histology. The BUdR LI, however, does not predict the actual rate of tumor growth or other important cell kinetic parameters. Double-labeling studies with BUdR and IUdR allow some of these parameters to be determined very quickly from a single biopsy specimen. Such studies may help us to understand further the biological as well as the clinical nature of individual tumors.

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