In Vitro Labeling and Gold Activation Autoradiography for Determination of Labeling Index and DNA Synthesis Times of Solid Tumors

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SUMMARY

In vitro labeling and gold activation autoradiography were used to determine the $[^{3}H]$thymidine ($[^{3}H]$TdR)-labeling indices and DNA synthesis times for C3H/He spontaneous mammary tumors.

Three variations of the $[^{3}H]$TdR, $[^{14}C]$thymidine ($[^{14}C]$TdR) double-labeling method, together with double-emulsion autoradiography, were used to determine the DNA synthesis times ($T_{s}$). Tumors labeled totally in vivo (in vivo-in vivo method) and tumors labeled with $[^{3}H]$TdR in vivo and subsequently labeled with $[^{14}C]$TdR in vitro showed similar $T_{s}$ values. DNA synthesis times for tumors determined totally in vitro by double labeling (in vitro-in vitro method) were significantly longer than those observed in vivo; however, identical samples subjected to Hypaque-Ficoll gradient separation after double labeling showed $T_{s}$'s similar to those found in vivo. Furthermore, the interval between $[^{3}H]$TdR and $[^{14}C]$TdR administration had no effect on $T_{s}$ estimates in vitro. Gold activation autoradiography was used in the present experiments to reduce autoradiographic exposure times. This method, together with in vitro labeling, permits $[^{3}H]$TdR labeling index and $T_{s}$ determinations after 6-hr and 7-day exposures, respectively.

INTRODUCTION

Since many therapeutic modalities currently used in cancer therapy exert their effect during specific phases of the cell cycle, cell kinetic information on tumor cell populations may be important for the future design of treatment protocols. The percentage labeled mitoses method has yielded valuable information on a variety of human solid tumors; however, it is limited in application to situations where multiple biopsies are feasible. In addition, in vivo administration of $[^{3}H]$TdR may not only be a potential hazard in patients with good prognosis but may also preclude follow-up or serial studies throughout treatment. Furthermore, the long autoradiographic exposure times required in these studies render the results of little or no benefit in planning the course of therapy for the patient.

As a result of these limitations, in vitro methodologies have been developed whereby cell kinetic information for individual solid tumors can be obtained. Studies utilizing oxygen-enriched atmospheres (4) or hyperbaric oxygen conditions (5, 8, 10) to facilitate the uptake of $[^{3}H]$TdR into tissue fragments have been relatively successful. Livingston et al. (17) recently reported a method utilizing the Hypaque-Ficoll system described by Boyum (2) and high specific activity $[^{3}H]$TdR to rapidly determine LI's for human melanoma and lung cancer from single-cell suspensions labeled in vitro.

The $[^{3}C]$TdR, $[^{14}C]$TdR double-label technique (36) for the determination of the $T_{s}$ has been adapted for use in human tumors by administering the 2nd label, $[^{14}C]$TdR, in vitro to dis-aggregated cells from tumors labeled with $[^{3}H]$TdR in situ. This in vivo-in vitro method has proven useful in cell kinetic studies of bone marrow cells (15, 36), spleen cells (18, 31), ascites tumors (15), lymphocytes (24, 31), leukemias (12, 36), and melanoma cells in the bone marrow (11). Although the $T_{s}$ values determined by this method for these easily dissociable tumors are in good agreement with values found by in vivo methods, autoradiographic exposure times of up to 6 weeks are often required.

Fabrikant (7, 8) and Fabrikant and Cherry (9), utilizing hyperbaric oxygen to facilitate the uptake of $[^{3}H]$TdR and $[^{14}C]$TdR into tissue fragments in vitro, devised a totally in vitro double-labeling method to measure $T_{s}$'s for human solid tumors and normal tissues (in vitro-in vitro method). Although the results using this method correlate well with other studies, the method is time consuming in that paraffin sections and long autoradiographic exposure times are required. Others, using the in vitro-in vitro method at ambient conditions, have measured $T_{s}$'s for easily dissociable tissues such as tonsillar lymphocytes (32), bone marrow cells (14, 16), and melanoma cells in bone marrow (11). Although in vitro-in vitro $T_{s}$'s for these cells types were in good agreement with results obtained with other methods, equivocal findings have been observed (35) with this method for human breast cancer.

In attempts to reduce autoradiographic exposure times, methods to improve the efficiency of both the autoradiographic procedure and the developing process have been devised. Panayi and Neill (26) and others (1, 8) exposed autoradiograms in liquid scintillation counting fluids, effectively decreasing the exposure times. At the electron microscope level, gold salts have been used to intensify the latent image in sensitive emulsions (29, 30). Rechenmann et al.

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2 Abbreviations used are: TdR, thymidine; LI, labeling index; NARG, normal autoradiography; GARG, gold activation autoradiography.

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(27, 28) reported a similar method to increase the efficiency of developers and better resolve nuclear tracks resulting from $^{14}$C $\beta$ emissions (38).

Prior to our initiation of studies with human tumors, the feasibility of performing LI and $T_{\alpha}$ measurements, totally in vitro, was explored in the spontaneous mammary tumor of the C3H/He retired breeder. It was also felt that a rigorous comparison of in vitro with in vivo measurements should be performed.

In the present studies, the in vitro labeling method of Livingston et al. (17) was used and modified to determine the $[^{3}H]$TdR LI and the $T_{\alpha}$ by $[^{3}H]$TdR, $[^{14}$C]$TdT$ double-labeling and double-emulsion autoradiography. Furthermore, an improved autoradiographic technique, using activation of the nuclear track emulsion, was used in an attempt to reduce exposure times to within a clinically useful range.

**MATERIALS AND METHODS**

**Tumor System.** Tumor-bearing C3H/He (JAX) mice were housed 4 to 6 per cage, fed standard mouse chow (Ralston Purina Company, St. Louis, Mo.) and water ad libitum. Tumors were measured in 3 dimensions twice weekly with calipers to determine the volumetric growth characteristics for each tumor. The mean volume for tumors used in these studies was $1.26 \pm 0.13$ cu cm.

**In Vivo Labeling.** In single-label studies, tumor-bearing animals were labeled for 1 hr with a single i.p. injection of 40 $\mu$Ci of $[^{3}H]$TdR (14 to 17 Ci/m mole; Amersham/Searle, Arlington, Heights, Ill.). In double-label studies, similarly treated animals were labeled for an additional 30 min with 2.5 $\mu$Ci of $[^{14}$C]$TdT$ (54$\mu$Ci/m mole; Amersham/Searle). Immediately following the labeling periods, the animals were sacrificed by cervical dislocation and the tumors were excised and placed on ice.

The tumors were minced with fine scissors in McCoy's medium with 20% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.). Tissue fragments were further disaggregated with a Pasteur pipet and the suspension was filtered through a stainless steel screen. The cells were washed in fresh medium and resuspended in 0.1 M citric acid for 1 min at 4°. Drop preparations (35) were made by resuspending the cells in Clarke's fixative for 5 min at 4°, and then applying 1 on 2 drops of the suspension to acid-cleaved slides. The slides were air dried, washed for 20 min in running tap water, then washed briefly in 3 changes of triple distilled water and air dried in a dust-free area.

**In Vitro Labeling.** Single-cell suspensions for in vitro single- and double labeling were prepared in a manner similar to that described above. Tumors were minced with fine scissors in McCoy's medium with 20% fetal calf serum at room temperature, the suspension was filtered, and the cells were resuspended (3 to 5 x 10$^7$ cells/ml) in fresh media. Three-m1 aliquots were incubated at 37° with 2.5 $\mu$Ci $[^{3}H]$TdR per ml for 60 min. In double-label experiments, the cells were labeled for an additional 30 min with $[^{14}$C]$TdT$ at a final concentration of 0.25 $\mu$Ci/ml. At the end of the labeling period, the reactions were stopped on ice, the suspension was centrifuged, and the cells were resuspended in calcium-free Eagle's minimal essential medium (Grand Island, Biological Co.). This medium was used to minimize the formation of cell aggregates. In some experiments, drop preparations were made immediately after the labeling period; however, in most experiments, the suspensions were applied to Hypaque-Ficol gradients (2) and centrifuged at 4° for 20 min at 400 x g. Cells confined to a narrow band at the gradient-medium interface were carefully pipetted off and diluted in 7 to 8 volumes of fresh media. The viability determined by trypan blue exclusion in postgradient samples was consistently greater than 80%, while only 20% or less of the cells were trypan negative in pre-gradient samples. The cells at the gradient-media interface were primarily large mononuclear cells, while cells at the bottom of the gradient consisted primarily of polymorphonuclear leukocytes, erythrocytes, and trypan-positive mononuclear cells. Only 2% or less of the cells in this fraction were trypan negative. Drop preparations of the cells collected from the gradient interface were prepared as described above.

**In Vivo-in Vitro Double Labeling.** Tumor-bearing animals were given i.p. injections of 40 $\mu$Ci $[^{3}H]$TdR and sacrificed 60 min later. Single cell suspensions were prepared as described above, and cells were incubated for 30 min at 37° with $[^{14}$C]$TdT$ (0.25 $\mu$Ci/ml). The reactions were terminated on ice and the suspensions were applied to Hypaque-Ficol gradients and centrifuged for 20 min at 400 x g at 4°. Drop preparations from cells collected from the interface of the gradient were made as described above.

**Autoradiography.** In all studies, Kodak NTB-2 liquid emulsion was applied by dipping at 45°. The autoradiograms were dried at room temperature for 1 hr and exposed in light-tight boxes at 4° for from 6 hr to 30 days.

NARG (utilizing conventional developing techniques) and GARG were used in these experiments. In the latter, a solution of gold salts (13) was used to activate the latent image produced in the photographic emulsion prior to development. The gold solution was prepared by dissolving 75.6 mg KAuCl$_4$ (Varilacoid Chemical Co., Elizabeth, N. J.) and 100 mg KSCN in 20 ml of triple-distilled water (Fisher Scientific, Pittsburgh, Pa.). This solution was then heated to 5° and 300 mg KBr were added. When the solution had cooled, the volume was brought to 500 ml with triple-distilled water. Since the shelf life of this solution is very short, it was prepared just before use to ensure greatest activity and lowest background.

Autoradiograms processed by the NARG procedure were developed in Kodak D-19 for 10 min at 15°, fixed in acid fixer (10 min), washed in tap water (30 min), and air dried. Autoradiograms processed by the GARG procedure were incubated in 3 changes of triple distilled water and developed at 15° in Kodak amidol developer. The slides were fixed, washed, and air dried as in the NARG method. For both methods the developed autoradiograms were stained with Harris hematoxylin. In single-label studies, the LI was determined by counting at least 1000 cells/tumor.

Double-label samples were subjected to double-emulsion autoradiography since it has been shown to be the most accurate autoradiographic method for distinguishing between cells labeled with only $[^{3}H]$TdR and cells labeled with $[^{14}$C]$TdT$ (12). In these studies, the 1st and 2nd emulsion...
layers were separated by a thin layer of celloidin (5% paraloidin in ether:alcohol, 1:1) applied by dipping. This layer not only protects the staining of the cells during subsequent steps, but also prevents the weaker tritium β particle from penetrating into the 2nd emulsion layer. The 2nd emulsion was then applied, exposed and developed (NARG or GARG) like the first. The $T_\text{s}$, calculated from the equation of Wimber and Quastler (37)

$$T_\text{s} = \frac{[{}^{14}\text{C/}{}^3\text{H}]T_\text{A}}{4}$$

where $[^14\text{C}]$ is the number of cells labeled in both emulsion layers, $[^3\text{H}]$ is the number of cells labeled in only the bottom layer and $T_\text{A}$ is the interval (hours) between administration of the isotopes ($[^3\text{H}]$TdR and $[^14\text{C}]$TdR). This result was determined by examining at least 500 labeled cells for each tumor. The relatively few peripheral blood cells (mostly small lymphocytes) in these preparations were easily recognized morphologically and disregarded.

Background in both single- and double-emulsion autoradiograms was consistently less than 2 grains per cell, regardless of the developing method (NARG or GARG) used, and cells labeled with 10 or more grains per cell were considered positively labeled. In mouse in vitro experiments, however, grains over labeled cells were generally too numerous to count. Double-emulsion autoradiograms of cells labeled with only $[^3\text{H}]$TdR showed essentially background labeling in the 2nd emulsion regardless of the method (NARG or GARG) or exposure time (between 6 and 30 days) used.

RESULTS

Table 1 shows the in vitro $[^3\text{H}]$TdR LI’s for 23 consecutively studied C3H/He spontaneous mammary tumors. Samples from the same labeled cell suspension for each tumor were subjected to both the NARG and GARG procedures. In addition, samples from most tumors were subjected to GARG analysis after both a 6-hr and 1-day exposure period. In this study in vitro, LI’s ranged from 15.8 to 4.2%. In vitro LI’s for most tumors, obtained by GARG procedure, were similar to those obtained by the NARG method. The correlation coefficients for NARG versus GARG (24 hr) and NARG versus GARG (6 hr) were 0.703 and 0.681, respectively, both values representing highly significant correlations ($p < 0.01$). Grains per labeled cell were too numerous to count in all samples except GARG (6 hr), where approximately 30 to 40 grains per labeled cell were noted.

Also shown in Table 1 is the mean in vivo LI for 24 consecutively studied C3H/He spontaneous mammary tumors. Although in vivo and in vitro LI’s were not determined for every tumor, obtained by GARG procedure, were similar to those obtained by the NARG method. The correlation coefficients for NARG versus GARG (24 hr) and NARG versus GARG (6 hr) were 0.703 and 0.681, respectively, both values representing highly significant correlations ($p < 0.01$). Grains per labeled cell were too numerous to count in all samples except GARG (6 hr), where approximately 30 to 40 grains per labeled cell were noted.

The overall results comparing the 3 double-label methods for determination of the $T_\text{s}$ for C3H/He spontaneous mammary tumors are shown in Table 2. A total of 51 spontaneous tumors were studied. In vitro double labeling resulted in a mean $T_\text{s}$ value that was similar to values found by double labeling totally in vivo by the vivo-vitro double-labeling method. Furthermore, the range of $T_\text{s}$ values were quite similar for all 3 groups.

Twelve of the 26 tumors studied totally in vitro and reported in Table 2 were not only autoradiogrammed by the NARG method, but also with the GARG technique. In addition, $T_\text{s}$ values for these tumors were determined from double-labeled cell suspensions prior to Hypaque-Ficoll gradient separation. The results (Table 3) indicate that, although autoradiographic exposure times were reduced to within 1 week, using the GARG method, there was no significant difference between $T_\text{s}$ values for the 2 autoradiographic methods. Samples not subjected to Hypaque-Ficoll gradient separation consistently showed longer $T_\text{s}$ values than those observed for cells following Hypaque-Ficoll separation.
Since cells held in vitro during the labeling period could undergo changes in the DNA synthesis rate, or changes in the rate at which cells enter S phase, 2 experiments were done to test the effect of our culture conditions on DNA synthesis in C3H/He spontaneous mammary tumor cells. In the 1st experiment, duplicate cell suspensions were prepared from each tumor as described in the "Materials and Methods." Both were incubated at 37°, one with [3H]TdR and one without. After 1 hr, the [3H]TdR-labeled cells were harvested, while the duplicate culture was labeled for 30 min with [14C]TdR. At the end of each labeling period, the cells were subjected to Hypaque-Ficoll separation and single- ([3H]TdR cells) or double- ([14C]TdR cells) emulsion autoradiography (GARG). The results (Table 4) show similar LI's for both the [3H]TdR- and [14C]TdR-labeled samples, suggesting that cells segregated by the Hypaque-Ficoll gradient continue to enter S phase after 1 hr in vitro at a rate similar to that seen for cells labeled immediately.

Table 3

In vitro T5's for 12 C3H spontaneous mammary tumors determined from double emulsion autoradiograms

<table>
<thead>
<tr>
<th>Tumor</th>
<th>NARGa</th>
<th>GARGb</th>
<th>Pregradientc</th>
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<tr>
<td>231</td>
<td>10.2</td>
<td>10.5</td>
<td>13.8</td>
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<td>10.7</td>
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</tr>
<tr>
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<td>11.1</td>
<td>10.8</td>
<td>15.1</td>
</tr>
<tr>
<td>254</td>
<td>10.7</td>
<td>10.5</td>
<td>17.6</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td>10.6 ± 0.3</td>
<td>10.8 ± 0.1</td>
<td>14.8 ± 0.4</td>
</tr>
</tbody>
</table>

a Autoradiographic exposure times of 1 day and 21 days for the 1st and 2nd emulsion, respectively.
b Sample not subjected to Hypaque-Ficoll separation or NARG procedure.
c Numbers in parentheses, number of tumors.

DISCUSSION

In the present study, [3H]TdR LI's and T5 values were determined for C3H/He spontaneous tumors in vivo and in vitro. In vitro LI and T5 values for this tumor were similar to those observed in vivo and in good agreement with previously reported values (19-25). However, both in vivo and in vitro LI's were in the low range of values reported by Bresciani (3). This discrepancy may be a result of different sampling methods, since his LI's were reflective of cell populations near the cortical shell of the tumor and might not reflect the total cell population.

In vitro duplication of T5 estimates made by double labeling of tumor cells in situ was, in the present studies, contingent upon the use of the Hypaque-Ficoll gradient separation technique (2). That others (11, 14, 16, 24, 32, 36) have demonstrated representative in vitro-in vitro T5's may be due in part to the nature of the tissues studied. Since bone marrow cells, peripheral blood cells, and lymphoid tissue can be easily disaggregated, suspensions with high viability and few damaged cells can be easily prepared. In the present experiments, where extensive mincing was necessary for adequate disaggregation, only 20 to 30% of the cells were trypan-negative prior to the labeling periods. That the postgradient viabilities were consistently in excess of 80% and the total number of trypan-negative cells recovered from the gradients was usually about one-third of the total trypan-negative cells applied would suggest that, aside from nonviable cells, damaged cells possibly synthesizing DNA at subnormal rates were also removed. Thus in the present studies as in previous vitro-vitro studies, T5 determinations made on samples relatively free of damaged and nonviable cells are the most reliable.

The finding that the [3H]TdR LI's for cells during the 1st 60 min in vitro were similar to that for cells incubated at 37° for 1 hr prior to [14C]TdR would suggest that the cells separated by the Hypaque-Ficoll gradients are progressing from G1 into DNA synthesis at or near normal rates. Furthermore, the calculated DNA synthesis times were similar for all T5's.
between 30 and 90 min, suggesting that in Hypaque-Ficoll segregated cells the rate at which cells complete DNA synthesis is also constant during the labeling period.

Although cell suspensions obtained after Hypaque-Ficoll gradient separation are not totally devoid of nontumor cells (lymphocytes and macrophages), the similarity between the results form in vitro and in vivo labeling studies suggests that the cell population isolated from the Hypaque-Ficoll gradients were reflective of the cell population labeled in vivo. Furthermore, other studies (unpublished data) from this laboratory using the 13762 transplanted rat mammary tumor, a tumor line with a relatively high [3H]Tdr LI (approximately 30%) and a short DNA synthesis time (approximately 6 hr) have also shown good agreement between LI and Td values determined in vivo and in vitro.

GARG proved to be an extremely useful tool to shorten autoradiographic exposure times. Rechenmann and Wittendorp (29) found that gold activation could increase grain density 2- to 3.5-fold, depending on the emulsion type, isotope, developer, and developing times. In the present studies, a similar autoradiographic enhancement was noted, although excessive background (>5 grains/cell) was observed for developing times greater than 20 min.

Although the autoradiographic sensitivity can be substantially increased with gold activation, the resolution is reduced. This is a result of the increased size and close proximity of the developed silver grains. In studies where grain counting or definitive localization of labeled compounds is of primary interest, emulsions diluted 1:1 with distilled water may be used with somewhat better resolution and only slightly less autoradiographic enhancement.

Recently, an in vitro method for the estimation of the growth fraction for solid tumors has been reported (33). This method, together with the time-saving features of GARG and the ease and reproducibility of our in vitro labeling techniques, may render cytokinetic analysis of human tumors a more practical clinical consideration. In addition, these methods may represent a faster and less tedious approach to the study of solid tumor cytokinetics following perturbations with chemotherapeutic agents or ionizing radiations.

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