Cellular Sources of Thymidine Nucleotides: Studies for PET

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The relative utilization of endogenously synthesized thymidine nucleotides and exogenously supplied thymidine analog was compared in a number of mammalian cell lines, tissues, and tumors. To measure the relative utilization, cells were incubated in tissue culture media containing the thymidine analog [^{3}H]-5-bromo-2′-deoxyuridine (BUDR). After extraction of the DNA, the degree of substitution of the thymidine by BUDR was determined by density gradient centrifugation. All the cell lines and tissues tested utilized both exogenous BUDR and endogenous thymidine sources to a similar extent. The relative utilization of the exogenous pathway could be manipulated by varying the exogenous concentration of BUDR. Our results demonstrate that one can predict the relative utilization of these two pathways and can calculate the effective specific activity of the intracellular thymidine nucleotide pool. Such information is needed in interpreting ^{11}C-labeled thymidine uptake as measured by positron emission tomography.

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We are interested in the development of carbon-11-(^{11}C) labeled thymidine as an imaging agent for positron emission tomography (PET), but the interpretation of such images requires a detailed knowledge of the biochemistry and kinetics of thymidine uptake (*1–3*). Some of the sampling and heterogeneity problems that have affected in vitro measurements of cell kinetics and limited their usefulness in predicting tumor response to therapy may be overcome by using ^{11}C-labeled thymidine with PET.

Cells synthesize thymidine nucleotides de novo and utilize them in DNA synthesis. This source of endogenous thymidine nucleotides can compete with labeled exogenous thymidine for incorporation into DNA and, in this way, affect measurements of cell growth. An area of controversy we have studied is the relative contributions of the endogenous and exogenous pathways to cellular thymidine nucleotide pools. One investigator has found that in HeLa S-3 cells the internal pool of thymidine nucleotides was entirely bypassed and external thymidine was used preferentially in DNA synthesis (*4*). Others have found that thymidine nucleotides from exogenous and endogenous sources mix in a single intracellular pool (*5–7*). We sought to overcome some of the limitations of these previous studies by directly and quantitatively measuring the relative activities of these two pathways, and to examine primary tissues and tumors as well as cell lines. We have approached this problem by incubating cells from tumors and normal tissues in the presence of the radioactively labeled thymidine analog, 5-bromo-2′-deoxyuridine (BUDR).

Previous workers have demonstrated that cells readily incorporate both thymidine and BUDR into DNA, but our PET imaging model requires quantitative knowledge of how exogenous thymidine or one of its analogs effects the endogenous pathway (*5,8*). In competition experiments no preference for thymidine over BUDR was found (*5,8*), and this allowed us to use labeled-BUDR in our measurements in place of exogenous thymidine. The average relative concentration of BUDR in DNA can be calculated from the buoyant density of the DNA (*9*) and, in this way, we have calculated the relative utilization of exogenous and endogenous sources of thymidine nucleotides.

Unlike other tracers used for PET that generally measure cellular energetics, thymidine is used in DNA synthesis. We are presently studying the biochemistry, kinetics, and mathematic models of thymidine nucleotide metabolism in order to convert the time-dependent

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tissue radioisotope concentration into quantitative measurements of cellular proliferation. We are beginning by using the four-factor model of thymidine metabolism developed by Cleaver and Quastler (*10*). Using this model, the DNA synthetic rate from PET images could be calculated if one could also measure some important associated parameters. The first parameter is the plasma levels of labeled and unlabeled thymidine that can be measured by HPLC analysis of blood samples obtained after injection of ^{11}C-labeled thymidine. Second, we must know what proportion of the labeled thymidine is incorporated into the cell; this is measured by the regional time-activity curve of ^{11}C contained in tissue. Finally, one must have information about the sources of intracellular thymidine nucleotides. In other words, one must know the specific activity of the intracellular thymidine nucleotide pools. It is this final point that we have addressed in this manuscript and that has not been addressed, to the best of our knowledge, by previous studies.

MATERIALS AND METHODS

Cells, Tissues, and Tumors

Cultures of HeLa M-1 and B-16 F1 were obtained from Drs. J. Lewis (Hutchinson Cancer Center, Seattle, WA) and J. Mayo (NCI, Frederick, MD), respectively. HeLa S-3 cells were obtained from the American Type Culture Collection. SL2 (*11*) and Cf2Th (*12*) were obtained from Dr. F. Yoshimura (University of Washington, Seattle). HeLa S-3, B16-f1, and Cf2Th cells were grown as monolayers in Eagle's Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (IFCS). HeLa M-1 cells were grown in MEM with 5% IFCS. SL2 cells were grown as a suspension culture in Roswell Park Memorial Institute medium 1640 (RPMI 1640) supplemented with 10% IFCS.

A colony of inbred AKR/J mice from commercial breeders* is maintained at our research center. The mice were held until signs of spontaneous tumors developed (i.e., enlarged lymph nodes, ruffled fur, humped back), generally at 6 to 14 mo. Normal murine tissues were obtained from 6-wk-old C57BL/6 mice. Dogs with spontaneous tumors were referred by veterinarians, with the permission of the owner, to our institution (*13*). Lymph node biopsies were obtained under aseptic conditions. Mouse thymus and spleen, AKR murine lymphoma, and dog lymphoma cell suspensions were obtained by mincing the tissues with a scalpel in RPMI 1640 with 10% IFCS, forcing the cells through a stainless steel screen (250 microns) and then aspirating the cells through a 22-gauge needle. Mouse marrow cells were obtained by flushing the femur with 2 ml of RPMI 1640 medium with 10% IFCS. The cell suspensions were then labeled in vitro as described below. In vivo labeling was not possible because of competition from plasma thymidine and difficulty in maintaining a constant plasma level of BUDR.

Labeling Conditions

Cells growing as monolayers, usually in 25 cm^2 flasks, were labeled when subconfluent. Prior to radiolabeling, all cells were washed three times with the indicated media supplemented with dialyzed sera to remove residual unlabeled thymidine. Cell suspensions were washed by spinning at 900 rpm (140 *g*) for 2 min and aspirating the supernatants. Cells were then incubated for 60 min at 37° in the dark in medium containing the indicated concentration of BUDR including 10 μCi of [6-^{3}H]BUDR[2] (27.2 Ci/mmol)† per 2.5 ml of medium. Incorporation of nucleoside was halted by washing the cells with cold phenol/ethanol (2/70%), followed by two washes of 95% ethanol. Cells growing as monolayers were lysed in the flask and then the lysate was transferred to a test tube using a rubber policeman. The cells were lysed, treated with pronase‡ (140 units/ml), and the RNA removed by hydrolysis with KOH according to the procedure of Kuebbing and Werner (*4*). The lysate was then neutralized and dialyzed for 2 days at 4° against two changes of 0.05*M* Tris/0.05*M* EDTA (pH 7.4). Samples were maintained in dark vessels and handled in a dark room to prevent excision of bromouracil induced by uv light.

DNA fully substituted with BUDR was prepared as a standard by growing cells overnight in the presence of medium containing 16 μM unlabeled BUDR, 1 μM amethopterin, 100 μM hypoxanthine, and 10 μM glycine prior to the addition of radiolabeled BUDR, generally for 4 hr. We have demonstrated that the use of amethopterin results in a 95% decrease in the synthesis of endogenous thymidine (data not shown). The endogenous pool should not affect the uptake of BUDR, since this nucleotide pool turns over with a half-life of ~5 min (*6*). Furthermore, growth of cells in amethopterin, whether for 2 or 48 hr, resulted in an almost identical specific activity for the intracellular thymidine nucleotide pool and the culture medium (*6*). A DNA standard of normal density was prepared by incubating cells with [methyl-^{14}C]thymidine† (50.5 mCi/mmol). In most experiments we used standards obtained from HeLa M-1 cells. As expected the density of human HeLa M-1, murine SL2, or dog Cf2Th DNA standards that had been similarly prepared were not significantly different when we analyzed them in parallel gradients (*14*).

Cesium Sulfate Gradients

As an internal marker [methyl-^{14}C]thymidine-labeled DNA was added to a sample of the lysate and 1 ml dialysis buffer. The mixture was denatured by heating for 20 min in boiling water followed by rapid chilling on ice. This sample was then added to a polyallomer tube§ containing 2.5 ml of saturated cesium sulfate and brought to a 5.5 ml final volume with dialysis buffer. A parallel tube was run that had been similarly treated but only contained the standards of uniformly [^{3}H]-BUDR-labeled DNA and [^{14}C]thymidine-labeled DNA. The specimens were then centrifuged in a VTi65 rotor§ for 15.5 hr at 40,000 rpm (140,000 *g*) and 4°C. Fractions of 100 μl were then collected directly from the bottom of the tubes into scintillation vials. After the addition of 1 ml of water and 10 ml of scintillation cocktail,¶ each vial was counted by liquid scintillation spectroscopy. The relative substitution of thymidine by BUDR was calculated as a linear function of the density of the DNA relative to the standards (*9*). Generally, we visually chose the peak fraction in these calculations; however, using a weighted mean average to identify the peak did not alter the overall results (data not shown). The DNA analyzed in the experiments reported here had not been purposely sheared. Preliminary studies demonstrated that

shearing of the DNA by sonication did not shift the position of the peak on the gradient, but did result in broadening of the peak (data not shown).

RESULTS

In the initial experiments, the relative utilization of exogenous BUDR was compared with the utilization of endogenous thymidine nucleotides in a number of different cell lines labeled with [^{3}H]BUDR in 2.4 μM BUDR. This concentration was chosen so that results would be comparable to previously published work (*4*). DNA from the SL2 murine lymphoma cell line was obtained under these labeling conditions, and analyzed by density gradient (Fig. 1). The gradient demonstrated that the DNA was of an intermediate density between the standards; it had utilized both the endogenous thymidine nucleotides and exogenous BUDR. A similar analysis of two strains of HeLa cells, murine B16-F1 cells, and dog Cf2Th fibroblasts all demonstrated that both pathways were utilized (Table 1, Section A). Although the average degree of BUDR utilization was not significantly different between the various cells lines ($p > 0.50$ by F-test), the density distribution was very broad and a pooled estimate of the standard deviation was 12%. Part of this variability (28%) was due to imprecision in the gradient measurements and the rest was attributable to variability between preparations.

The initial studies of exogenous thymidine utilization were done with 2.4 μM BUDR in the tissue culture medium, but in most mammalian species the usual level of plasma thymidine is <1 μM (*15–16*). Therefore, we determined if lower or higher concentrations of exogenous BUDR in the tissue culture media altered the relative utilization. Experiments were done with three different cell lines; human HeLa M-1, mouse SL2 lymphoma, and dog Cf2Th fibroblasts. These cells lines were chosen because they are from three different mammals as well as three different tumor types. Each cell line was labeled in the presence of three different BUDR concentrations (0.14, 2.4, and 23 μM). The gradient of the dog Cf2Th cells was typical (Fig. 2) and demonstrated that the higher the exogenous concentration of BUDR the greater the relative utilization of the exogenous pathway. The three cell lines demonstrated similar rates of utilization of the exogenous pathway at all three concentrations of BUDR (Fig. 3).

Since the above experiments were done with cell lines, it is possible that their metabolism of thymidine may have been altered during in vitro passage. Therefore, we extended our studies to normal murine tissues and lymphomas from mice and dogs (Table 1, Section B). As in the cell lines, all the tissues and tumors used both the exogenous and endogenous pathways when grown in 2.4 μM BUDR.

DISCUSSION

Many investigators have attempted to quantitate the number of cells in each phase of the cell cycle and the cellular proliferation rates (*17*). These measurements are often dependent upon the uptake of labeled thymidine which is exogenously supplied and incorporated into DNA by way of the salvage pathway. Factors other than the growth rate of the cells may affect these measurements of thymidine uptake, especially when obtained in vivo. For example, such measurements may be affected by blood flow, permeability, thymidine reutilization from dying cells, and the dose level of labeled thymidine employed (*3,18–20*). Another problem relates to the relative utilization of the two sources of thymidine nucleotides available to the cell; thymidine

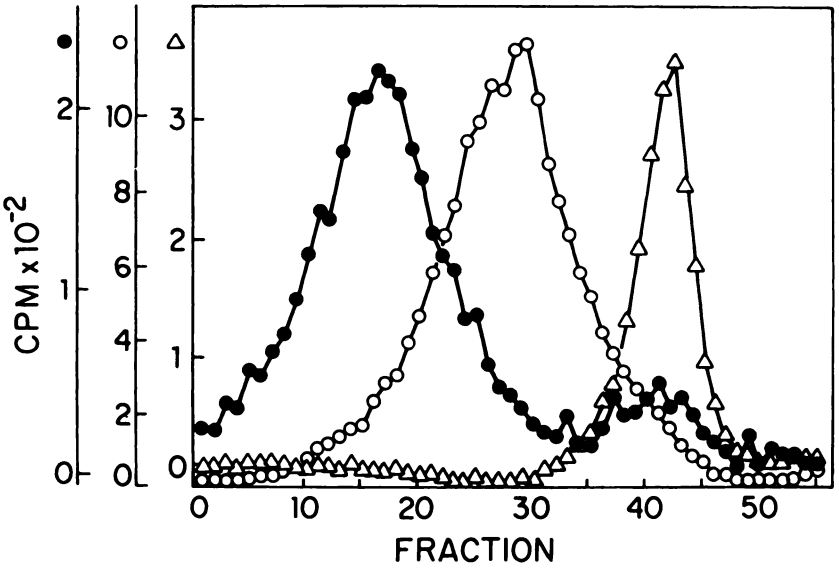

FIGURE 1
Cesium sulfate density gradient of DNA from SL2 cells incubated with 2.4 μM [^{3}H]BUDR (○). The standards include DNA of a normal density from HeLa cells grown in [^{14}C]thymidine (△) and DNA fully substituted with BUDR from cells grown in [^{3}H]BUDR and amethopterin (●).

TABLE 1
Utilization of Exogenous Thymidine Analog*

DNA source	% Exogenous thymidine analog (mean)	Range	N†
Cell lines			
Human			
HELA M-1	45	(52–38)	2
HELA S-3	41	(64–29)	4
Mouse			
B16-F1	41	(42–40)	2
SL2	43	(50–35)	3
Dog			
CF2TH	41	(49–16)‡	5
Tissues			
Mouse			
Thymus	46	(49–41)	4
Marrow	57	(61–51)	3
Spleen	58	—	1
Lymphoma	46	(55–38)	4
Dog			
Lymphoma	41	(48–33)	6

*Cells were incubated for 60 min in 2.4 μM [^{3}H]BUDR. The relative utilization of exogenous BUDR compared with endogenous thymidine was determined by analysis of the extracted DNA on a cesium sulfate gradient.

† N represents the number of different DNA preparations analyzed. The mouse lymphoma data is from four different metastatic lesions in one mouse. The dog lymphoma measurements are from multiple samples taken from areas of lymphomatous nodes in two animals. In some cases a single DNA preparation was analyzed more than once on a cesium gradient.

‡ There was one low value of 16, the other four values ranged from 44 to 49.

nucleotides can be obtained from de novo synthesis within the cell and from thymidine uptake from the extracellular fluid.

We have carried out measurements of the relative utilization of these two pathways in a number of cell lines, tissues, and tumors. We found that all the cells tested utilized both pathways when a thymidine analog was available in the extracellular fluid. This result supports previous studies demonstrating mixing of these two pools within the cytoplasm (*5–7*). These previous studies were limited, however, because they generally did not directly measure incorporation into DNA and only used one or two cell types. On the other hand, our results are inconsistent with the results of Kuebbing and Werner (*4*), whose results we initially sought to reproduce and whose technique we followed. Kuebbing and Werner only tested one cell line (HeLa S-3) at one BUDR concentration (2.4 μM), and found that the thymidine in the external medium blocked use of de novo thymidine. However, we included that cell line obtained from the same source (American Type Culture Collection) and used the same BUDR concentration in our studies. The most likely explanation for the discrepant results, is that the cell line they used may have undergone some phenotypic change during passage in culture and resulted in a marked decrease in the activity in the de novo pathway.

What is of greater importance is the fact that our results demonstrate that one can quantitatively predict the relative utilization of these two pathways independent of the tissue, tumor, or species being studied. With this information one can calculate the effective specific activity of the intracellular thymidine nucleotide pool from a knowledge of the extracellular thymidine concentration and specific activity. Along with a knowledge of the amount of radioactivity incorporated into DNA, this will allow one to calculate an absolute DNA synthetic rate. Our experiments are based on the assumption that BUDR and thymidine are taken up and utilized by the cells in a kinetically similar manner. No difference in the rate of uptake or incorporation into DNA has been found in competition experiments using thymidine and BUDR (*5,8*). Furthermore, experiments have demonstrated that the affinity of human and murine thymidine kinase is similar for BUDR and

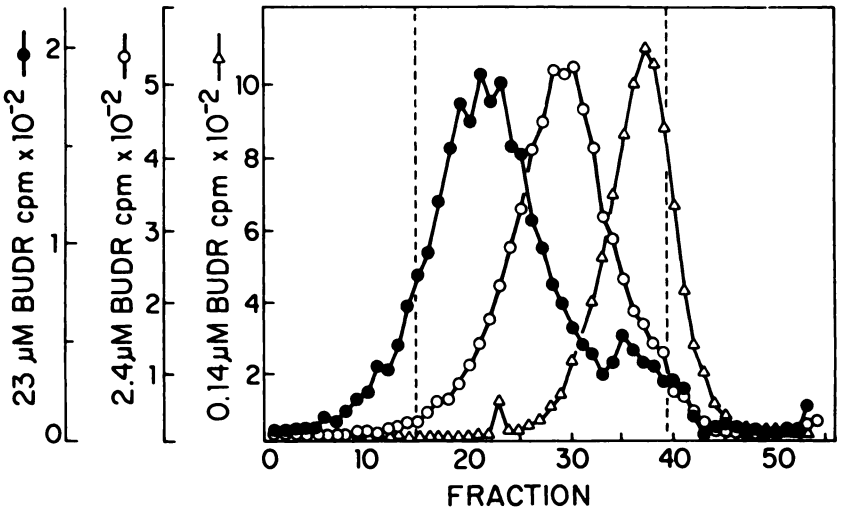

FIGURE 2
Effect of varying concentrations of BUDR on the density of DNA in dog cells (Cf2Th). Cells were incubated for 60 min in the indicated concentration of [^{3}H]BUDR, and the purified DNA analyzed on cesium sulfate gradients. (---) = Positions of the peak activity of the standards.

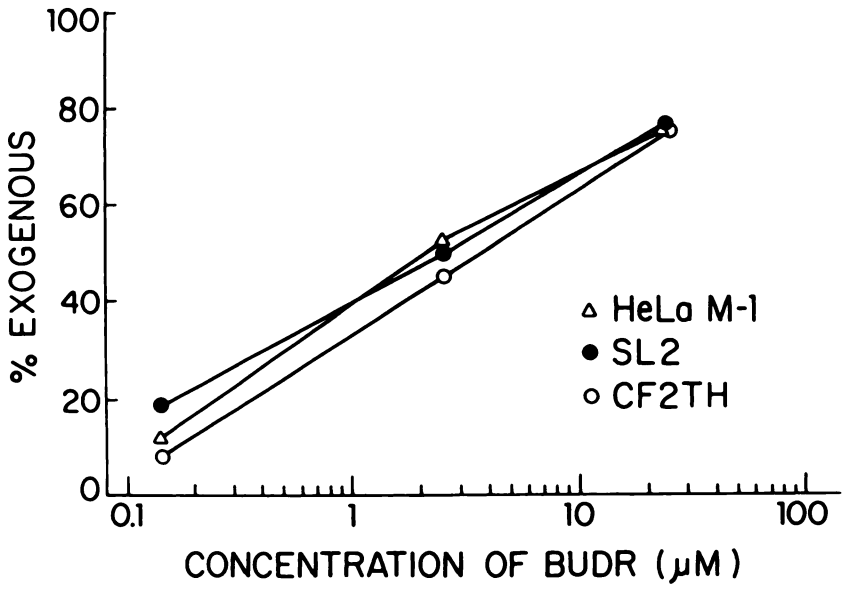

FIGURE 3
Relative utilization of the exogenous thymidine analog (BUDR) at variable concentrations. The indicated cell lines were grown for 60 min in the indicated concentration of [^{3}H]BUDR and the purified DNA analyzed on cesium sulfate gradients. The data for each cell line was obtained using plates of cells labeled in parallel on the same day and by analyzing them on parallel gradients in a single centrifuge run.

thymidine (*21,22*). On the other hand, detailed studies have found a preferential incorporation of thymidine into a minor fraction of the cellular DNA (moderately repetitive sequences), but this should not quantitatively affect our studies (*23,24*). BUDR also appears to be toxic to cells in long exposures (at least 2 days); however, this is unlikely to affect our results that were obtained during 1-hr incubations (*25,26*).

Our initial experiments were done with an external concentration of BUDR of 2.4 μM. For measurement purposes this concentration proved convenient since it resulted in the uptake of 40–50% BUDR. Studies measuring thymidine uptake use varying concentrations of external thymidine, therefore, it was also important to look at the extreme ranges of concentration. We utilized the lowest possible BUDR concentration (based on the specific activity this was 0.14 μM) and a high concentration of BUDR (23 μM) and found that the uptake could be manipulated by such changes.

Our results demonstrated a fair amount of variability in that we had an s.d. of 12% in the calculated determination of the percentage of BUDR used. Much of this variability was due to the broadness of the peaks. Approximately 25 fractions separated the two standards meaning that a one-fraction error in the peak determination equals ~4% error in the measurement of the exogenous thymidine. Nevertheless, our results clearly answered our primary goal in demonstrating that both endogenous and exogenous sources of thymidine are available for DNA synthesis and that mixing occurs rapidly. Furthermore, we demonstrated that by varying the exogenous concentration one could alter the extent of utilization by this pathway. Future experiments will need to address the importance of the exogenous pathway in vivo by varying the concentration of labeled thymidine in the plasma.

The generality of our results is suggested by the fact that we used cell lines from three different species and varying cell types. We also sought, however, to determine if similar results could be obtained from tissues and tumors obtained in vivo, since the passage of cell lines in vitro may alter their metabolism. When we studied fresh tissue and tumor samples labeled in vitro, we found results similar to those obtained in cell lines, further demonstrating the general applicability of our conclusion.

In order to interpret the images obtained using [^{11}C] thymidine and PET one must have a detailed knowledge of the utilization of thymidine. This information is used to construct kinetic models that convert the time-dependent concentration of ^{11}C obtained with the PET into measurements of cellular proliferation. We have previously shown that although thymidine is rapidly cleared from the circulation, its distribution is primarily dependent on metabolism rather than blood flow (*3*). This study demonstrated that the relative utilization of the endogenous pathway can be predicted from the exogenous thymidine concentration. In this way, the effective specific activity of the intracellular thymidine nucleotide pool can be calculated. This simplifies the model of thymidine metabolism of Cleaver and Quastler (*10*), since the endogenous pathway is no longer an independent variable. Furthermore, in rapidly growing tissues the degradation pathway does not appear to be very active (*3,27*, unpublished data). This further reduces the model to three compartments similar to that developed for deoxyglucose metabolism (*28*), where the compartments represent extracellular thymidine, intracellular thymidine nucleotides, and thymidine contained in DNA. Data will be fitted to this model using the blood time-activity curve and the tissue time-activity curve obtained with PET.

NOTES

* Jackson Laboratories, Bar Harbor, ME.
† NEN Research Products, Boston, MA.
‡ Calbiochem-Behring, San Diego, CA.
§ Beckman Instruments, Inc., Palo Alto, CA.
¶ Packard Instrument Company, Downers Grove, IL.

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