Variations in the [³H]Thymidine Labeling of S-Phase Cells in Solid Mouse Tumors

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ABSTRACT

To determine whether all tumor S-phase cells incorporate [³H]thymidine, we labeled the cells in three mouse tumors (MCa-11, colon-26, and colon-51) in vivo for ½ h with [³H]thymidine (10 μCi/g of body weight). Cells from the tumors, as well as control cells from the bone marrows of the tumor-bearing mice, were then placed onto slides and Feulgen stained. The positions of these Feulgen-stained cells were mapped with a computerized scanning stage, and their nuclear DNA content and nuclear areas were determined by absorption cytophotometry. Next, the slides were processed for autoradiography and exposed for 32 or 64 days to obtain plateau labeling. The cells were then relocated, and the areas of the autoradiographic grains over each nucleus were measured. We found that 99% of the S-phase bone marrow cells were labeled. The 5-mm tumors, however, showed a wide range of S-phase labeling, with 94, 89, and 85% of the MCa-11, colon-51, and colon-26 S-phase cells, respectively, being labeled. The same mice bearing both 5- and 20-mm MCa-11 tumors, however, showed 95 and 57% labeling of the S-phase cells in the small and large tumors, respectively. These results show that the [³H]thymidine labeling of S-phase cells varies greatly for experimental mouse tumors of different size and type, and they suggest that labeling index and flow cytometric DNA measurements may not give valid estimates of the actual proportions of cycling S-phase cells in such tumors.

INTRODUCTION

It has generally been thought that, once cells begin DNA replication, they progress without interruption through S phase and incorporate added [³H]thymidine into their newly synthesized DNA. This has led to the use of the LI, as defined as the fraction of labeled cells present upon autoradiography of [³H]thymidine-labeled cells, for estimates of the size of the S-phase compartment in various tumors. The underlying assumption in this assay is that [³H]thymidine is incorporated by all S-phase tumor cells. Early studies in which incorporation of [³H]thymidine in vivo was shown for many different types of nontransformed S-phase cells (1) appeared to support this premise. In addition, Cooper reported on a study of 19 cases of Burkitt's lymphoma in which he combined Feulgen densitometry and autoradiography with [³H]thymidine and showed that almost all S-phase cells were labeled in vitro (2). Several recent observations, however, seem to cast doubt on the assumption that all S-phase tumor cells incorporate [³H]thymidine.

Wickramasinghe et al. studied the bone marrows of patients with vitamin B₁₂ deficiency and sideroblastic anemia and found that 6–24% of the erythroid precursor cells with S-phase DNA content were not labeled in vitro with a 0.5-h pulse of [³H]thymidine (3, 4). Ernst et al. reported that as many as 80% of erythroid precursor cells with S-phase DNA content in the marrow of patients with myeloid leukemia were unlabeled in vitro (5, 6). Plateau labeling curves were not reported in these studies; however, and small amounts of [³H]thymidine incorporation might have been detected if longer autoradiographic exposures had been used (7).

In addition, Sigdestad and Girdna (8) observed a discrepancy between the LI and flow-cytometric DNA distributions for hypoxic cells from a mouse fibrosarcoma grown in vivo. Dreeswinko et al. (9) have recently made a similar observation of low [³H]thymidine labeling in starved monolayer cultures of six colon carcinoma lines containing significant proportions of S-phase cells by flow cytometric measurements. Freyer and Sutherland (10) have measured both the LIs and the flow-cytometric DNA distributions of EMT6 cells grown in vitro as tissue culture spheroids. They found that the inner, presumably hypoxic, cells of the spheroids contained a higher percentage of S-phase cells as determined by flow cytometry than were labeled with [³H]thymidine. This finding, also, suggested that some hypoxic cells in S phase did not incorporate [³H]thymidine. Finally, our own direct measurements of the DNA content and of [³H]thymidine labeling of the same spheroid cells, carried out under plateau labeling conditions, have shown that some S-phase cells remain unlabeled even after 24 h of incubation with [³H]thymidine (11).

These results suggested that a study of the S-phase labeling of tumor cells grown in vivo would be of interest. Therefore, we grew MCa-11, C-26, and C-51 tumor lines in nude mice and then labeled the tumors in vivo with [³H]thymidine. The labeling of nontransformed S-phase cells from bone marrow of tumor-bearing mice was used as a control. We tested several autoradiographic exposure periods for slides of all tumor samples to ensure that the exposures were sufficiently long to yield plateau labeling conditions (7). The DNA content, nuclear areas, and [³H]thymidine incorporation (autoradiographic grains) of the same nuclei were determined by sequential absorption cytophotometry of Feulgen-stained cells before and after autoradiographic processing (12). We found significant variations in the labeling of S-phase nuclei among the different tumors studied.

MATERIALS AND METHODS

Outline of Experiments. MCa-11, C-26, and C-51 solid tumors were grown in nude mice (BALB/c background; Charles River, MA) by s.c.
We labeled the tumors by giving each tumor-bearing mouse an i.v. injection of \( ^{3}H \) thymidine (10 \( \mu \)Ci/g of body weight, 43 Ci/mmol; Amersham Searle, Arlington Heights, IL). One-half h after isotope administration, separate suspensions of tumor cells from the same mice and nontransformed BM cells were prepared and placed onto slides. At least three tumor-bearing mice were studied for each experimental group.

The cells were Feulgen stained, and the positions of approximately 150 cells on each slide were mapped with a computerized scanning stage. During mapping, we took care to select a high proportion of S-phase nuclei by morphological criteria. The DNA content and nuclear areas of the mapped cells were determined by absorption cytophotometry (13). The slides were then processed for autoradiography and exposed for 32 or 64 days. After development of the autoradiographs, the same nuclei were relocated, and the area of the autoradiographic grains over each nucleus was determined by cytophotometry at 625 nm (12). The DNA content and the fraction of nuclear area covered by autoradiographic grains (GAP) for each nucleus were then linked in a computer file for subsequent sorting and data analysis.

**Cell and Slide Preparation.** Cell nuclei were disaggregated by gentle mechanical disruption of the tissues in a nuclear isolation medium consisting of 0.6% Nonident P40, 0.9% phosphate-buffered saline, and 2% PEG (M, 8000; Sigma, St. Louis, MO) (14). Bone marrow cells were flushed directly from femurs into normal phosphate-buffered saline. Chicken erythrocytes were added to each cell suspension as an internal standard for DNA content (13). The cells were placed onto slides with a cytocentrifuge and fixed in cold 1% paraformaldehyde (0.1 \( \times \) phosphate buffered, pH 7.2) for 15 min.

The MCA-11 tumor line, derived from a mouse mammary adenocarcinoma, was a gift from Dr. John Yuhas (15) and has been maintained in our strain of nude mice used.

**Cytocentrifuge.** A Vickers M85 microdensitometer (Vickers Instruments, Malden, MA) was used for measurements of integrated absorbance and areas of Feulgen-stained nuclei at 580–605 nm, with a 0.4-\( \mu \)m scanning spot and a 2% instrument glare level. Integrated absorbance values were automatically corrected for stain darkness by a microcomputer (13). Autoradiographic grain areas over the nuclei were measured at 625 nm, with a 0.10 threshold of absorbance; with these measuring conditions, the underlying Feulgen stain is not detected (12). The slides were uniformly labeled, and no corrections were made for background grains. Since it is possible that some S-phase cells which had not incorporated isotope were counted as labeled in this assay because of nonspecific background grains, the estimates of nonlabeled S-phase cells in the tumors must be regarded as minimal.

**RESULTS**

**Cell Labeling.** Labeling curves for the 5-mm tumors after different autoradiographic exposure periods are given in Chart 1. Exposures for 8 or more days led to plateau labeling at a threshold of 5 nuclear grains for discriminating of labeled from nonlabeled cells. The DNA content of each cell was linked in a computer file to the fraction of the nuclear area of that cell covered by autoradiographic grains (GAP). For example, a cell with 1% of its nuclear area covered by autoradiographic grains would have a GAP value of 0.01. In previous work, we labeled mouse bone marrow cells as in this study and found that a GAP value of 0.10 gave excellent discrimination between S-phase (labeled) and G0-Gi-G2-M (nonlabeled) cells present in autoradiographs exposed for 4 days (12). To make the detection requirements for possible nonlabeled S-phase cells in the solid tumors more stringent, and to utilize the results of the plateau labeling...
curves (Chart 1), we used 32- and 64-day autoradiograph exposures and selected the GAP value of 0.05 as a threshold to distinguish between labeled and nonlabeled cells in this study. In Chart 2, the distribution percentage of GAP values obtained for all nuclei measured is shown. There were two types of nuclear labeling: (a) a homogeneous peak of nonlabeled cells (GAPs < 0.05), and (b); a skewed distribution of labeled nuclei covered by varying amounts of autoradiographic grains. We therefore selected the GAP value of 0.05 as a threshold to distinguish between labeled and nonlabeled cells.

Labeling of Nonmalignant S-Phase Cells. Chart 3 shows the DNA distributions of the unlabeled (A) and labeled (B) bone marrow cells taken from mice bearing the three types of tumors. It can be seen that 99.5% (201 of 202) of the S-phase BM cells (those cells with DNA content between 8 and 12.7 pg) were labeled (Chart 3). Thus, there were no systemic factors in the tumor-bearing mice that prevented [3H]thymidine incorporation into S-phase bone marrow cells.

Labeling of S-Phase Tumor Cells. The DNA distributions of nonlabeled (Chart 4A) and labeled (Chart 4B) cells harvested from mice bearing MCa-11 tumors (5 mm) are plotted. The large proportion of host cells in the MCa-11 tumors is unexplained, but it has been confirmed by flow cytometric DNA measurements. Four separate peaks for nonlabeled cells are apparent: those for (a) diploid host cells in G0-G1 (7 pg of DNA, A1); (b) aneuploid MCa-11 cells in G2-M (11.2 pg of DNA, A2); (c) diploid host cells in G2-M (or possibly a second stemline of tetraploid tumor cells) at 14 pg of DNA (A3); and (d) MCa-11 cells in G2-M (23 pg of DNA, A4). The range of DNA content within which only S-phase MCa-11 cells were found was 15.5–20.5 pg. Of the MCa-11 cells having DNA content in this range, 94% (124 of
132) were labeled (Chart 4). The percentage of unlabeled MCa-11 cells in S phase (6%) differed significantly from that of the almost completely labeled nontransformed S-phase BM cells (P < 0.01, Table 1).

In Chart 5, the DNA distributions of the nonlabeled (A) and labeled (B) cells prepared from the C-51 tumors (5 mm) are plotted. The diploid cells in G0-G1 (A1) are seen at 7 pg. The G2-M peak at 14 pg may be partially merged with the G2-M tumor cell peak (A2), which had an average DNA content of 12.8 pg. The G2-M tumor cell peak (A2) is also apparent at 25 pg. The DNA interval in which only S-phase C-51 cells were present lies between 15.5 and 23.5 pg. Of the S-phase cells within this DNA content range, 89% (140 of 157) were labeled. The 11% of nonlabeled C-51 cells in S phase differed significantly from the almost complete labeling found for the BM (P < 0.001, Table 1).

For the DNA distributions of nonlabeled (Chart 6A) and labeled (Chart 6B) cells present in the C-26 tumors (5 mm), a small diploid (2C) peak of nontransformed host cells is seen at 7 pg of DNA (A1). The diploid host cells in G2 are mixed with the G2-M peak of the C-26 tumors at 14 pg of DNA (A2). For the C-26 cells, the S-phase range was 15.7-26.0 pg of DNA; however, only 85% (174 of 205) of the C-26 tumor cells in S phase were labeled. The proportion of unlabeled C-26 S-phase cells was significantly higher than the proportions of unlabeled S-phase cells found in the MCa-11 tumors and BM cells (P < 0.001, Table 1), but not different from the labeling of the S-phase C-51 cells (Table 1). Fig. 1 shows two adjacent C-26 cells (A, B) with S-phase DNA content, only one of which was labeled (B).

To see if the tumor microenvironment influenced the labeling of S-phase cells, mice bearing both 5- and 20-mm MCa-11 tumors were given injections of [3H]thymidine, followed in 0.5 h by the preparation of tumor cell suspensions (Chart 7). The DNA distributions of the nonlabeled (A) and labeled (B) cells harvested from the 5-mm MCa-11 tumors show that 95% (98 of 103) of the MCa-11 S-phase cells (15.5-20.5 pg of DNA) were labeled, an identical result to that obtained for mice bearing solitary 5-mm tumors (6.1%, 8 of 132) or on mice also bearing large tumors (4.9%, 5 of 103) was identical, these results have been combined for statistical analysis.

### Table 1

<table>
<thead>
<tr>
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<th>Extent of labeling of S-phase bone marrow and tumor cells</th>
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<tbody>
<tr>
<td></td>
<td>% of nonlabeled S-phase cells</td>
</tr>
<tr>
<td>BM</td>
<td>0.5 (1/202)</td>
</tr>
<tr>
<td>MCa-11○ (5 mm)</td>
<td>5.5 (13/235)</td>
</tr>
<tr>
<td>C-51 (5 mm)</td>
<td>10.8 (17/157)</td>
</tr>
<tr>
<td>C-26 (5 mm)</td>
<td>15.1 (31/205)</td>
</tr>
<tr>
<td>MCA-11 (20 mm)</td>
<td>42.9 (51/119)</td>
</tr>
</tbody>
</table>

*, Number of nonlabeled S-phase cells.
○ Since the percentage of labeling of S-phase MCa-11 cells from 5-mm tumors grown as solitary tumors (6.1%, 8 of 132) or on mice also bearing large tumors (4.9%, 5 of 103) was identical, these results have been combined for statistical analysis.
NS, not significant.

### DISCUSSION

The existence of S-phase cells that do not incorporate [3H]-thymidine can be inferred from experiments with cells grown either in vivo as solid tumors (8) or in vitro as starved monolayers (9) or spheroids (10). Such experiments showed that the proportion of S-phase cells measured by flow cytometry was higher than indicated by the LI of the same cells. However, the comparison of flow-cytometric and LI values is somewhat indirect, in that the two assays were performed on two different samples of tumor cells.

McDivitt et al. recently noted a correlation between flow cytometric estimates of the S-phase fraction and the LIs for some, but not all, human breast cancers (20). In this study, blockade of thymidylate synthetase and the choice of the algorithm to calculate the percentage of cells in S phase from the flow cytometric DNA measurements may have helped to contribute to the correspondence of the two assays. Also, measuring the LI on tissue sections may help in the discrimination between neoplastic and nonneoplastic cells. If tumor and host cells cannot be distinguished, the presence of replicating host cells in tumors grown in vivo complicates the interpretation of both flow-cyto-
metric and LI data. Specifically, detection of autoradiographic grains over nontumorous S-phase cells may elevate the LI and create an artificial correspondence with the flow-cytometric estimate of the tumor S-phase fraction, even when nonlabeled S-phase tumor cells are present. Alternatively, if many unlabeled host cells are present, the LI would be lowered. Also, overlap of the host and tumor DNA distributions, as well as the presence of unlabeled host G2 cells (or minor tumor stemlines), may increase the difficulty of determining the true proportion of tumor S-phase cells by flow cytometric DNA measurements alone. The complexity of tumors containing mixtures of replicating tumor and host cells is demonstrated in Chart 4, where host and tumor cells in all phases of the cell cycle are apparently present.

In the present study, we have shown directly that certain S-phase cells of some tumors do not incorporate \[^{3}H\]thymidine in vivo (Charts 4 to 8). The autoradiographic exposures were sufficiently long to result in plateau labeling conditions (Chart 1). The absence of label in varying proportions of the S-phase cells from MCa-11, C-51, and C-26 tumors, compared to the almost complete labeling that we found for S-phase cells from nontransformed bone marrow (Table 1), shows that the S-phase cells from different types of experimental mouse tumors can vary in the extent of incorporation of exogenous thymidine into DNA. The finding of a very small proportion of unlabeled bone marrow (0.5%) S-phase cells may have been due to technical errors in cell preparation or DNA measurements, metabolic derangements in the tumor-bearing mice, or lend some support to observations that small numbers of certain nontransformed rodent S-phase cells may not incorporate \[^{3}H\]thymidine immediately (21, 22). In general, however, previous studies have indicated that most nontransformed cells incorporate \[^{3}H\]thymidine (1). It is therefore possible that the presence and proportions of S-phase cells which cannot be labeled with \[^{3}H\]thymidine in a tumor are a new and quantifiable parameter that may be associated with malignant growth.

The origin of the S-phase tumor cells that do not incorporate
UNLABELED S-PHASE TUMOR CELLS

[3H]thymidine is not clear. In a flow-cytometric study, Darzynkiewicz demonstrated "quiescent" S-phase cells with altered RNA and chromatin in two experimental leukemias and in one case of human chronic myeloid leukemia during blast crisis (23, 24). The relationship of such quiescent leukemic cells to the nonlabeled S-phase cells found in this study is not known. We have found that 99% of the S-phase Ehrlich's ascites tumor cells, grown as ascites, were labeled. The finding, that a very high proportion of unlabeled S-phase cells (43%) are found in large (20 mm) MCa-11 tumors when compared to the proportion found for small (5 mm) MCa-11 tumors (5%; Chart 7; Table 1), also suggests that the solid tumor microenvironment plays an important role in the generation of nonlabeled S-phase cells. We therefore believe that the possible occurrence of such nonlabeled S-phase cells in hematogenous neoplasms, which do not have solid tumor microenvironments, needs further clarification.

Several factors in the solid-tumor microenvironment may cause the failure of S-phase tumor cells to incorporate [3H]thymidine. Sigdestad has shown that a large percentage of S-phase mouse fibrosarcoma cells, which are presumably hypoxic, do not incorporate [3H]thymidine in vivo (8). We have demonstrated nonlabeled S-phase cells in large (500 μm) MCa-11 and V2-79 tissue culture spheroids, which have cells adjacent to a necrotic central core that are not cycling because of hypoxia, nutrient deprivation, and/or toxic factors originating in the region of necrosis (11). Also, Taylor has shown that S-phase arrest can occur in cultured monolayers of cells if the pH of the medium is lowered (25). Gradients in pH which are sufficiently steep to cause S-phase arrest may exist in the microenvironments of some solid tumors (26), presumably attributable to mismatches between the blood supply and the metabolic requirements of the tumor (27, 28).

Perhaps some of the S-phase cells that do not incorporate [3H]thymidine synthesize DNA via enzymatic pathways that do not utilize exogenous thymidine (29, 30). Hamilton and Dobbin reported an increase in the LI for NT adenocarcinoma cells that were incubated with [3H]deoxyuridine compared to the LI after [3H]thymidine labeling (31). Presumably, the NT cells were synthesizing DNA via the thymidylate synthetase pathway (29, 30).
It is not known whether the preferential use of this enzymatic pathway by the NT cells was induced by the tumor microenvironment or was due to a genetic difference between this cell line and other types of tumors and normal tissues. We are presently trying to determine whether the nonlabeled S-phase cells observed in our study were synthesizing DNA by the thymidylate synthetase pathway.

Regardless of the mechanism involved, the existence of S-phase cells that do not incorporate [3H]thymidine increases the difficulty of kinetic analyses of tumors by means of the LI or measurement of the DNA distribution by flow cytometry. Even if the problem of overlap of replicating host and tumor cells can be resolved, the existence of variable proportions of S-phase cells in tumors of different types and sizes that cannot be labeled with [3H]thymidine could complicate these assays in several ways. If such nonlabeled S-phase cells are dying, their presence will lead to overestimates of the functional S-phase compartment by flow cytometry. Even if the nonlabeled S-phase cells are synthesizing DNA by the thymidylate synthetase pathway, or if such cells can be recruited back into cycle as a result of changes in the tumor microenvironment or treatment, then LI determinations based upon [3H]thymidine incorporation will lead to underestimates of the potential S-phase compartment.

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REFERENCES

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