LABELING OF MURINE MASTOCYTOMA CELLS IN VITRO WITH PLASMA TRITIATED THYMIDINE-LABELED ANIMALS

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ABSTRACT

40 min after injecting tritiated thymidine into an animal, 20–30% of the total plasma radioactivity is nonvolatile. This fraction decreases to about 6% 10 hr after the injection and 3% 24 hr after the injection. There appears to be material in this nonvolatile fraction that can label mastocytoma cells in culture. The labeling indices decrease with time after injection in the same way as the nonvolatile fraction. The 40 min plasma sample contains sufficient material to allow accurate assessment of the fraction of cells in S in culture after a 6 wk exposure. The circulating material is not apparently available for incorporation into those cells in cycle in the donor animal. The material appears to be related to the G_0 cell-specific pool that has been described elsewhere. The trichloroacetic acid–soluble or ethanol-soluble nonvolatile activity appears to contain thymine, and some thymidine-phosphorylated compounds.

INTRODUCTION

The injection of 50 μCi of tritiated thymidine (TdR-^{3}H)[1] into an animal is believed to approximate to a single short exposure to the labeled precursor. It is generally assumed that the TdR-^{3}H either is incorporated into the DNA of those cells synthesizing DNA at the time of the injection or is catabolized. Thus it is common practice to terminate the exposure and to take samples of tissue 30–60 min after the initial injection. The major site of catabolism of TdR-^{3}H is believed to be the liver (1–3) and the process results in a rapid release of catabolites, the major one being tritiated water which is eliminated from the body with a half-life of 1.13 days (4). Tritiated water is thus the major component of the large fixative-soluble tritium pool reported to persist for several days in many tissues of the mouse.[2] However, it appears that this tritium pool contains material capable of labeling the nuclei of cells in vivo up to 48 hr after the initial injection of TdR-^{3}H into the mouse (5, 6). Paper chromatography of several extracts from labeled animals showed peaks at rate of flow values consistent with the presence of thymidine, phosphorylated thymidine derivatives, and thymine.[2] The experiments to be reported in this paper investigated the labeling properties of plasma from mice injected with TdR-^{3}H 40 min, 10 hr, and 24 hr earlier. Paper chromatography of the plasma proved to be negative for identification of thymidine, though peaks were observed consistent with

[1] *Abbreviations:* TCA, trichloroacetic acid; TdR-^{3}H, tritiated thymidine.

[2] Potten. C. S., R. F. Hagemann, and J. C. Schaer. 1. 1. Presence of non-DNA radioactivity several days after pulse labeling with tritiated thymidine. Manuscript in preparation.

the presence of thymine, and phosphorylated thymidine derivatives.[2] The labeling properties of the plasma were tested on murine mastocytoma cells in vitro.

MATERIALS AND METHODS

In Vivo Procedures

Male DBA-1 or DBA-2 mice (7–8 wk old) were used. 50 μCi of tritiated (methyl-^{3}H) thymidine (Schwarz Bio Research Co., Orangeburg, N.Y., or The Radiochemical Centre, Amersham, England) were injected intraperitoneally, and blood was removed from the brachial region 40 min, 10 hr, or 24 hr after the injection. The specific activity was 6.0 Ci/mmole and, since the mice weighed about 20 g, approximately 2.5 μCi/g was the injected dose. Heparinized glassware was used. The blood was centrifuged for 15 min at 3000 rpm at 4°C, and the pooled plasma was collected. Samples of the plasma were counted in a liquid scintillation spectrometer.

Liquid Scintillation Procedures

Two scintillation counters were used and the procedures differed slightly according to which instrument was used.

(*a*) 100 μl samples were dissolved in 1 ml of Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.) overnight. 10 ml of scintillation mixture were then added (5.0 g 2,5-diphenyloxazole, 0.2 g 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1000 ml toluene) and the cooled vials were counted at 5°C for 10 min in a Packard Liquid Spectrometer (model 3380). Each vial was quench corrected using the Absolute Activity Analyzer (model 544). The techniques have been described in detail elsewhere (7, 8).

(*b*) 10 μl samples were dissolved in Soluene and treated similarly. Before the scintillation mixture was added, 3 drops of concentrated HCl were added to each vial to quench the chemiluminescence encountered when the vials are not counted at low temperatures. The vials were counted at ambient temperature in a Packard Liquid Spectrometer (model 3375). The external standard ratio method was used to calculate the absolute activity. Under these conditions the counting efficiencies and backgrounds were very similar for the two counting procedures, 25–30% and about 24 cpm, respectively.

In Vitro Procedures

A transplantable murine mastocytoma cell line (P-815-X2) was used. The cultures were kindly provided by Professor R. Schindler; they were grown from frozen cells which had been tested and found to be free of mycoplasma contamination. This cell line was derived (9, 10) from the original P-815 tumor (11) by a selection process and two in vitro consecutive cloning procedures (9, 10). The culture techniques (9) and the medium (12) have been previously described. Except for a small fraction of the total population, these cells do not attach to the surface of the culture vessels (9).

Radioautographic Procedures

4 ml of a cell suspension (2×10^5 cells/ml) were incubated at 37°C for 2 hr with 0.5 ml of plasma (diluted 1:1 with 0.15 M NaCl), 0.5 ml of TdR-^{3}H solution (0.4 μCi/ml), or 0.5 ml of 0.15 M NaCl. Subsequently, the cells were fixed by adding 4.5 ml of fixative (ethanol:water:acetic acid, 5:3:2 v/v), centrifuged, washed in distilled water, and smeared onto slides. Approximately 2×10^4 fixed cells were smeared on a microscope slide which was then Feulgen stained, dried, and dipped in Kodak NTB 2 emulsion. The slides were exposed for 4 or 6 wk, developed, then stained with fast green. In each sample, the percentage of labeled cells was determined by counting 300 cells. The number of grains per cell was also counted. The fraction of the cells showing one or more grains was determined, and the fraction of cells with one or more grains from the sample where 0.5 ml of saline was added to the medium was subtracted to give the labeling index.

Biochemical Techniques

Plasma samples were cooled in an ice bath and mixed with an equal volume of 10% cold trichloroacetic acid (TCA). Subsequently, the TCA precipitate was fractionated according to the procedure of Schmidt and Thannhauser (13) modified by Schneider (14). This permitted the separation of acid-soluble material, lipids, RNA, DNA, and proteins. The radioactivities of the different fractions were measured in a liquid scintillation counter, as described above. The fraction of the samples that was volatile and could be attributed to tritiated water was determined by sealing a known volume of the sample in a Petri dish and then warming the base to evaporate the water which was immediately frozen on the lid by solid carbon dioxide (15). The frozen water was then melted and samples were counted. The residue could be redissolved and samples counted. The separation efficiency of the biochemical techniques has been discussed elsewhere (16, 17).

Chromatographic Analysis of the Nonvolatile Radioactivity in Plasma

Plasma obtained 40 min after TdR-^{3}H injection, 70% ethanol- and 5% cold TCA-soluble fractions of plasma were spotted on No. 1 Whatman-filter paper and separation was achieved with isobutyric acid: water:ammonium hydroxide:ethylenediaminetetra-

acetate, (0.1 M), 100:55.8:4.2:1.6, overnight. The paper was cut in strips which were placed in scintillation vials. A drop of water was added to the vial followed by 1 ml of Soluene. The vials were counted according to liquid scintillation procedure (*b*). The cpm were not corrected for absolute activity. The results were compared to the following standards: TdR-^{3}H, thymine-^{3}H, thymidine-^{3}H mono-, di-, and triphosphate (The Radiochemical Centre).

RESULTS

Table I shows the results of the biochemical fractionation of the pooled plasma from 6 DBA-1 mice bled 40 min, 10 hr, and 24 hr after an injection of 50 μCi of TdR-^{3}H. The total radioactivity in the plasma clearly decreases with time. This decay has been shown to be exponential and has a half-life of 2.02 $\pm$ 0.21 days[2] (15). There is probably a more rapid decay component over the first 24 hr (15). 40 min after an injection, 99.2% of the radioactivity is cold TCA-soluble and 71% is apparently tritiated water. Of the 29% nonvolatile radioactivity, none is apparently in proteins, or DNA, but a very small quantity may be found in the RNA and lipid fractions.

Table II shows some further analyses of the 40 min postinjection plasma. In this case the plasma was pooled from 10 DBA-2 mice. About 90% of the total blood radioactivity is saline soluble, i.e., can be regarded as plasma. About 99% of the plasma radioactivity is soluble in 5% cold TCA. 80% of the total DBA-2 plasma radioactivity was volatile. Only 11.6% of the nonvolatile activity could be measured after evaporation. This is probably due to the difficulty in redissolving the residue in saline after evaporation. Chromatography of the concentrated nonvolatile fraction failed to detect any thymidine as such. However, there were peaks that might represent thymine or phosphorylated thymidine derivatives. At 10 and 24 hr after the TdR-^{3}H injection the level of nonvolatile radioactivity is reduced (Table I). The decay is apparently exponential (Fig. 1). The fraction of the total plasma activity that is TCA soluble does not apparently change, although there is a small increase in the quantity of radioactivity found in several of the fractions. The nonvolatile fraction decays with time after injection but is still clearly detectable 24 hr after the injection. It appears that in all three cases the nonvolatile radioactivity is part of the TCA-soluble activity and therefore is not DNA, RNA, protein, or lipid.

Table III shows the results of the in vitro labeling experiment. The absolute amount of radioactivity to which the cells were exposed (measured in an 0.1 ml portion) varied slightly in the three plasma samples, all three containing slightly more radioactivity than the thymidine controls. Two separate thymidine controls were exposed for 4 wk and produced similar labeling indices; the mean values are shown. These results agree well with preceding labeling experiments with these cells (unpublished data). The saline controls had no cells with three grains or more and only 1 or 2% with two grains. The three plasma samples all clearly show evidence of labeled cells. 4 wk exposure is adequate to demonstrate the presence of labeling material in the plasma. It is clear that the 40 min sample contains sufficient material to label

TABLE I

Plasma Analysis

TCA fractionation, 50 μCi, DBA-1 males, 6 animals/time interval, 100 μl samples counted, Packard 3380. Units: dpm/ml of plasma $\times$ 10^6.

	40 min		10 hr		24 hr	
		% of total		*% of total*		*% of total*
Acid soluble	3.5646	99.3	2.5175	98.3	2.3279	98.8
Lipids	0.0220	0.6	0.02348	0.9	0.01492	0.6
RNA	0.00522	0.1	0.01843	0.7	0.01187	0.5
DNA	0	—	0.000285	—	0	—
Proteins	0	—	0.00272	0.1	0.00221	0.1
Total	3.5919	100.0	2.5624	100.0	2.3569	100.0
^3H_2O	3.268	71.	2.682	94.	2.541	97.
Residue	1.333	29.	0.170	66.	0.084	3.
Total	4.601	100.	2.852	100.	2.624	100.
Whole plasma	4.641	—	2.802	—	2.563	—

those mastocytoma cells in S and give a labeling index almost as high as that of the pure thymidine samples when the exposure of the plasma emulsion was 6 wk. Fig. 1 shows the nonvolatile plasma radioactivity and the labeling indices expressed as percentages plotted against the time after the TdR-^{3}H injection. The labeling indices have been divided by 2.18 to plot them on the same scale. It

TABLE II

Blood Analysis

50 μCi, 40 min, 10 DBA-2 males, 10 μl samples counted, Packard 3375. Units: dpm × 10^6

dpm/ml of blood	3.1616	
dpm/ml of plasma	3.4974	
Plasma, dpm/ml of blood	1.4699	
Total saline soluble activity, dpm/ml of blood	2.8573	(90.4% of the blood)
Total 5% TCA-soluble activity, dpm/ml of plasma	3.4346	(98.2% of the plasma)
^3H_2O, dpm/ml of blood	1.2761	(86.8% of the blood)
^3H_2O, dpm/ml of plasma	2.8045	(80.2% of the plasma)
Nonvolatile component of plasma, dpm/ml of plasma	0.4059	(11.6% of the plasma)
Chromatography		
Thymidine	Negative	
Thymine	Positive	
Phosphorylated thymidine	Positive	

TABLE III

Labeling of Murine Mastocytoma Cells In Vitro with Plasma from TdR-^{3}H-Labeled Animals

50 μCi per animal, DBA-1 ♂, 4–6 wk exposure of NTB2 emulsion

Labeling material	Exposure	Culture medium	Number of cells with 0–6$^+$ gains per nucleus							Cells with one or more grains (L.I.)	Labeled cells − Saline L.I.
			0	1	2	3	4	5	6$^+$		
	wk	*μCi/ml*								%	%
TdR-^{3}H	4	0.0465	117	35	18	13	23	15	113	65.0	56.0
NaCl	4	0	300	23	6	1	0	0	0	9.0	—
	6	0	300	18	2	1	0	0	0	6.2	—
Plasma, 40 min	4	0.1215	170	78	42	26	10	4	3	48.9	39.9
	6	0.1215	124	55	21	25	20	19	90	64.9	58.7
Plasma, 10 hr	4	0.0774	250	52	11	8	1	1	4	23.5	14.5
	6	0.0774	250	63	6	3	2	0	4	23.7	17.5
Plasma, 24 hr	4	0.0698	275	47	9	2	1	0	0	17.6	8.6
	6	0.0698	225	17	8	1	1	0	0	10.7	4.5

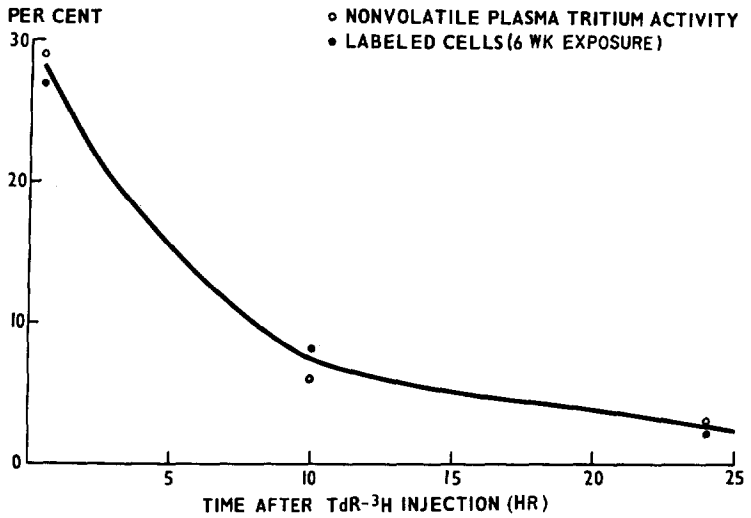

FIGURE 1 Nonvolatile plasma tritium activity expressed as a fraction of the total plasma radioactivity plotted against the time after TdR-^{3}H injection (open circles). The mastocytoma cell labeling index after correction for the saline background values plotted against the time after TdR-^{3}H injection (solid circles, 6 wk exposure). The labeling indices (percentage of labeled cells) were divided by 2.18 to plot them on the same scale.

TABLE IV

Labeling of Jejunum in Plasma Donors

Units: dpm/mg (mean ± SE, 6 animals per value)

Time after injection	Fresh jejunum	Fixed jejunum
40 min	18647 ± 1042	14385 ± 694
10 hr	16823 ± 808	14276 ± 465
24 hr	16366 ± 609	14562 ± 1177

is clear that the labeling efficiency of the plasma changes in the same way as the nonvolatile fraction, suggesting that the material causing the radioautographic labeling is to be found in the nonvolatile fraction of the plasma radioactivity.

Table IV shows how the fresh and fixed dpm/mg values for the jejunum of the plasma donors vary with time after the TdR-^{3}H injection. The fresh tissue values fall slightly over the period of 24 hr but the fixed tissue values remain constant. Thus the plasma material capable of labeling the mastocytoma cells in culture does not apparently label the jejunal crypt cells in the gut of the donor animals.

The paper chromatography of the plasma showed no peaks. The radioactivity remained at the origin, apparently bound in the proteinous material. Both the 70% ethanol-soluble and the 5% TCA-soluble fractions of plasma showed two peaks of radioactivity, one in the region usually occupied by thymine and the other in the region usually occupied by the phosphorylated derivatives of thymidine. The peaks were rather broad, which did not permit an accurate assessment of the nature of the plasma material. There was no detectable radioactivity in the thymidine region of the chromatograms.

DISCUSSION AND CONCLUSIONS

It is clear that the plasma contains some tritium-labeled material in the nonvolatile component that is capable of labeling murine mastocytoma cells in culture. This material decreases in quantity with time after the initial injection of TdR-^{3}H, but is still present in the plasma in sufficient quantities 24 hr after the injection to label cultured cells (giving a labeling index 4–8% above background). The plasma material is not tritiated water since the labeling curves follow so closely the nonvolatile activity decay curve (Fig. 1). It is also apparently soluble in cold TCA and is therefore unlikely to be DNA, RNA, protein, or lipids. Thymidine, some of its catabolites, and its phosphorylated compounds are all probably to be found in the TCA-soluble fraction.

The 40 min plasma clearly contains sufficient

labeling material to allow fairly accurate assessment of the fraction of cells in S and in fact gives values for this fraction very close to those obtained with thymidine when the emulsions are exposed for 6 wk.

It is remarkable that the dpm/mg values for the fixed jejunum do not increase with time after injection, at least for the 10-hr samples. The jejunum is one of the most active tissues proliferatively, and thus it would be expected to utilize any material capable of labeling. This observation has been confirmed elsewhere;[2] the dpm/mg values for fixed jejunum apparently reach a peak value after a 40 min exposure and then decay slowly with time after injection.

It has recently been suggested that part of this radioactive pool in the animal is somehow restricted to G_0 cells (5).[2] Cells in the skin in G_0 are capable of utilizing this pool when stimulated into S up to 48 hr after the initial injection of TdR-^{3}H (5). The skin cells in cycle during this period are apparently incapable of utilizing this same radioactive material. The present data suggest that this G_0-specific pool does in fact circulate in the plasma and is capable of labeling cells in vitro which are not presumably G_0 cells. This further suggests that the specificity of this pool may be due to some differences in permeability to the circulating pool material of the proliferating cells and the G_0 cells. Cells in culture, though not in G_0, may have the same cell permeability to the pool material. The cells in culture are completely surrounded by the precursor material.

It has been suggested that late labeling of cells might be explained by the reutilization of thymidine released initially from labeled cells (18–21). This reutilization does not occur until labeled cells die and thus liberate their labeled material. This is believed to occur in significant quantities only after times often well in excess of 10 hr; for example, it may occur in the jejunum when the labeled crypt cells are lost from the tip of the villus approximately 40 hr after the initial pulse label (22). It is unlikely that the plasma used in the present experiments contained material released from dying labeled cells since it was observed 40 min after the injection and decayed progressively in quantity with time, i.e., it is much more likely to be some residual activity from the injection.

The chromatography demonstrated the presence of material in the plasma after precipitation of the proteins that has a mobility similar to that of thymine and some of the phosphorylated derivatives of thymidine (in particular the mono- and triphosphates). It is possible that either or both of these materials may be responsible for the mastocytoma cell labeling and may be the long-lived pool in question. Since thymine should be freely permeable to all cells, it would appear unlikely that this is the G_0-specific material. These experiments do not distinguish between thymine and the phosphorylated compounds alone and these compounds bound to other molecules. The precipitation may separate these bindings. There is preliminary evidence from other experiments that saline extracts of TdR-^{3}H–labeled tissues contain some radioactivity that is apparently bound to proteins and that this binding is broken during TCA precipitation.[3]

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REFERENCES

1. POTTER, V. R. 1959. Discussion of the reutilization of DNA and DNA breakdown products. *In* Kinetics of Cellular Proliferation. F. Stohlman, editor. Grune & Stratton, Inc., New York. 110.
2. POTTER, V. R. 1960. Nucleic Acid Outlines. Burgess Publishing Co., Minneapolis, Minn.
3. ADELSTEIN, S. J., and C. P. LYMAN. 1968. Pyrimidine nucleoside metabolism in mammalian cells: an *in vitro* comparison of two rodent species. *Exp. Cell Res.* **50**:104.
4. RICHMOND, C. R., W. H. LANGHAM, and T. T. TRUJILLO. 1962. Comparative metabolism of tritiated water by mammals. *J. Cell. Comp. Physiol.* **59**:45.
5. POTTEN, C. S. 1971. Early and late incorporation of tritiated thymidine into skin cells and the presence of a long-lived G_0-specific precursor pool. *J. Cell. Biol.* **51**:855.
6. MOFFAT, G. H., and S. R. PELC. 1966. Delay after plucking of hairs between the appearance of ^{3}H-thymidine in cells and its incorporation into DNA. *Exp. Cell Res.* **42**:460.
7. POTTEN, C. S., B. A. JESSUP, and M. B. CROXSON. 1971. Incorporation of tritiated thymidine into the skin and hair follicles. I. Oscillatory changes through the hair growth cycle. *Cell Tissue Kinet.* **4**:241.

[3] Potten, C. S., and J. C. Schaer. Manuscript in preparation.

8. POTTEN, C. S. 1971. Tritiated thymidine incorporation into hair follicle matrix and epidermal basal cells after stimulation. *J. Invest. Dermatol.* **56**:311.
9. SCHINDLER, R., M. DAY, and G. A. FISCHER. 1959. Culture of neoplastic mast cells and their synthesis of 5-hydroxytryptamine and histamine *in vitro*. *Cancer Res.* **19**:47.
10. GREEN, J. P., and M. DAY. 1960. Heparin, 5-hydroxytryptamine, and histamine in neoplastic mast cells. *Biochem. Pharmacol.* **3**:190.
11. DUNN, T. B., and M. POTTER. 1957. A transplantable mast-cell neoplasm in the mouse. *J. Nat. Cancer Inst.* **18**:587.
12. SCHINDLER, R. 1963. Biochemical studies of the division cycle of mammalian cells: evidence for the premitotic period. *Biochem. Pharmacol.* **12**: 533.
13. SCHMIDT, G., and S. J. THANNHAUSER. 1945. A method for the determination of deoxyribonucleic acid, and phosphoproteins in animal tissues. *J. Biol. Chem.* **161**:83.
14. SCHNEIDER, W. C. 1946. Phosphorus compounds in animal tissue. III. A comparison of methods for the estimation of nucleic acids. *J. Biol. Chem.* **164**:747.
15. RUBINI, J. R., E. P. CRONKITE, V. P. BOND, and T. M. FLIEDNER. 1960. The metabolism and fate of tritiated thymidine in man. *J. Clin. Invest.* **39**:909.
16. SCHAER, J. C., A. GRIEDER, H. J. HEINIGER, and R. SCHINDLER. 1969. Comparison of ^{3}H-cytidine and ^{3}H-5-uridine as precursors of RNA. *Exp. Cell Res.* **56**:449.
17. MUNRO, H. N., and A. FLECK. 1966. The determination of nucleic acids. *In* Methods of Biochemical Analysis. D. Glick, editor. Interscience Pubs., Inc., John Wiley & Sons, Inc., New York. **14**:113–176.
18. BRYANT, B. J. 1965. The delayed uptake of ^{3}H-thymidine by Ehrlich ascites tumor cells. *Exp. Cell Res.* **37**:490.
19. DIDERHOLM, H., K. E. FICHTELIUS, and O. LINDER. 1962. Availability time of ^{3}H-label after administration of ^{3}H-thymidine *in vivo*. *Exp. Cell Res.* **27**:431.
20. RIEKE, W. O. 1962. The *in vivo* reutilization of lymphocytic and sarcoma DNA by cells growing in the peritoneal cavity. *J. Cell Biol.* **13**:205.
21. STEEL, G. G. 1966. Delayed uptake by tumours of tritium from thymidine. *Nature* (*London*). **210**:806.
22. SIGDESTAD, C. P., R. F. HAGEMANN, and S. LESHER. 1970. A new method for measuring intestinal transit time. *Gastroenterology*. **58**:47.