PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 526-529 (1977)

Varying Effects of Deoxynucleosides on the Incorporation of Thymidine into the DNA of Monolayer and Suspension Cultures (39709)[1]

SAMUEL WAXMAN AND CAROL SCHREIBER

Cancer Chemotherapy Foundation Laboratories, Division of Medical Oncology Department of Medicine, Mount Sinai School of Medicine, The City University of New York, Fifth Avenue at 100th Street, New York, New York 10029

Summary. Deoxyuridine will normally suppress the incorporation of subsequently added tritiated thymidine into the DNA thymine of cells grown in suspension culture to less than 10%. Conversely, deoxyuridine appears to stimulate thymidine incorporation, at times approaching thousandfold excess, when the cells are maintained as monolayers. This stimulation is time, temperature, and, to a lesser extent, pH dependent, but is not directly related to the density of the monolayer. Disruption of the monolayer reverts the cells to normal deoxyuridine suppression of thymidine incorporation into DNA. This study extends earlier evidence of variations in *de novo* and salvage DNA synthesis between suspension and monolayer cell cultures. We caution the equating of data obtained with suspension cell systems to solid tumor analysis.

Introduction. De novo DNA thymine synthesis has been monitored in short-term human bone marrow cultures by measuring the incorporation of tritiated thymidine ([^{3}H]TdR) into the acid-insoluble material (1). In normal tissues 0.1 μmole/ml of deoxyuridine (UdR) added prior to the addition of [^{3}H]TdR will effectively suppress the incorporation of [^{3}H]TdR into DNA to less than 10% (2). Lessening of the UdR suppression signifies defective *de novo* DNA synthesis and can be used as a sensitive indicator of abnormal folate metabolism (3). This UdR suppression test has been used to differentiate rapidly between folate and vitamin B_{12} deficiency in megaloblastic marrow (4) and also to evaluate the efficacy of the various folate antagonists such as methotrexate, pyrimethamine, and triamterene (5, 6). These data have since been extended to include drug effects on human solid tumor tissue (7, 8). We now report the unexpected finding of a stimulation of [^{3}H]TdR incorporation by preincubation with UdR or deoxycytidine in intact monolayer cultures. We suggest that the utilization of deoxynucleosides may vary significantly when monolayer rather than suspension cultures are used, and may serve to partially explain resistance to some forms of chemotherapy.

Methods and materials. Cell preparations: L1210 leukemia cells were harvested into Hank's balanced salt solution (HBSS) containing heparin (500 U/ml) from the ascitic aspirates of DBA/J2 mice 7 days postinoculation with 1×10^6 cells ip. The cells were washed three times with HBSS to remove the heparin and ascitic fluid and resuspended in HBSS containing 33% dialyzed fetal calf serum (FCS) to a concentration of $55\text{-}65 \times 10^6$ cells/ml. Cells isolated from human malignant ascitic and pleural effusions were treated as above. Human bone marrow was aspirated from the iliac crest and, after passage through a wire mesh to break up cell clumps and exclude spicules, was treated as described above. The HeLa cells were grown in monolayers on plastic tissue culture plates (LUX) in Minimal essential medium – Earle's salts containing 10% FCS and 4 m*M* glutamine. Human cell lines were prepared by trypsinization of solid tumor specimens obtained at surgery and maintained through at least six passages as monolayer cultures in RPMI 1640 containing 10% FCS and 4 m*M* glutamine. The cell lines derived from the solid tumors have not been carefully evaluated and may not represent malignant cells. Chromosome karyotypes were determined essentially by the method of Broder *et al.* (18). The number of chromosomes counted in the vinblas-

[1] This research was supported by NIH Grants AM 16690-03, CA 14491-02A2, and The Chemotherapy Foundation of New York, Inc.

ISSN 0037-9727

tine-treated cells ranged from 38–48. The cells were determined to be of human origin by the presence of the marker chromosome and numerous metacentrics. Isoenzyme analysis was used to exclude HeLa contaminants. Cell viability was determined by the trypan blue dye exclusion test and all studies performed with human tissues were done according to the guidelines of the Declaration of Helsinki. Informed consent was obtained for the collection of the bone marrow samples.

Incorporation studies. 1–5 × 10^6 L1210 cells, human bone marrow, and cells from malignant pleural and ascitic effusions were preincubated with 0.1 μmol/ml of UdR for 1 hr at room temperature. [^{3}H]TdR (1 μCi; ICN Pharmaceuticals; 18.6 Ci/mmole) was added and the reactions were allowed to proceed at 37° for 3 hr in a metabolic shaker (6). All reactions were performed in triplicate and the total volume was 1.0 ml of HBSS. Cells grown in monolayer were plated into 16 × 75-mm tissue culture plates and used when the cells were just short of being confluent. The plates were washed three times with HBSS and the intact monolayer incubated as described above. At the end of the incubation time the cells were harvested by gentle scraping with a rubber policeman and transferred to test tubes for acid precipitation. In some experiments the cells were removed from the monolayer before the start of the experiment and treated in the same manner as the suspension cultures.

Acid precipitation. The acid-insoluble material was precipitated with cold 10% trichloroacetic acid. Erythrocytes from the bone marrow cultures were removed by shock lysis prior to acid precipitation. The resultant material was dissolved in 1 ml of NCS reagent (Amersham/Searle) and counted in 15 ml of scintillation cocktail (toluene containing 30% ethanol, 0.6% PPO, and 0.3% dimethyl POPOP) in a Beckman LS 250 liquid scintillation system to a counting error of 2% or less.

Results. The presence of UdR significantly inhibits the incorporation of the [^{3}H]TdR in cells from malignant pleural and ascitic effusions, in the bone marrow system, L1210 cells, and established cell lines in suspension (Table I). In contrast to the suspension culture, there is a marked stimulation of [^{3}H]TdR incorporation by UdR in all monolayer cultures which in some instances reaches thousandfold increases (Table II). When the monolayers have been trypsinized and treated as suspension cultures, the [^{3}H]TdR incorporation is significantly suppressed by UdR (Table II). When the identical reactions were run at 4° there was no evidence of UdR stimulation of [^{3}H]TdR. The incorporation of [^{3}H]TdR into monolayer cultures is maximal at 15 min and remains unchanged over 2 hr, while the incorporation of [^{3}H]TdR in the presence of UdR is time dependent with maximal stimulatory effects not yet reached at 2 hr (Table III). The stimulatory effect of UdR on [^{3}H]TdR incorporation occurs in the HeLa cell monolayer whether they are confluent or in a growth phase of prolifera-

TABLE I. UdR SUPPRESSION OF [^{3}H]TdR INCORPORATION INTO THE DNA OF SUSPENSION CELLS.

Cell type	Diagnosis	[^{3}H]TdR incorporated in the presence of 0.1 μmole/ml of UdR (%)[a]
Pleural fluid (N.C.)	Mesothelioma	16.0
Ascitic fluid (G.K.)	Mesothelioma	2.0
Ascitic fluid (B.S.)	Adenocarcinoma, ovary	27.0
Ascitic fluid (H.W.)	Adenocarcinoma, ovary	5.0
Ascitic fluid (T.L.)	Adenocarcinoma, ovary	30.0
Ascitic fluid (D.S.)	Adenocarcinoma, ovary	11.0
Ascitic fluid (S.V.)	Adenocarcinoma, breast	20.0
Bone marrow (average of 30 samples)	Normal	4.5
L1210 (average of 4 studies)		12.1
Human lymphocytes in culture (PGLC 33H)		3.0
Friend leukemia cells (745A)		13.0

[a] Value of 100% is [^{3}H]TdR incorporation in the absence of UdR.

TABLE II. EFFECT OF UdR ON THE [^{3}H]TdR INCORPORATION IN INTACT VS TRYPSINIZED MONOLAYERS.

Cell type	Condition	[^{3}H]TdR incorporated in the presence of 0.1 μmole/ml of UdR (%)
HeLa	a	439.0
	b	8.0
	b	14.0
Ca rectum (A.F.)	a	293.0
	a	379.0
	b	8.0
	b	38.0
Ca lung (T.M.)	a	>8000.0
	b	56.0
	b	39.0
Ca lung (R.R)	a	7266.0
	b	25.0
RD 114	a	360.0
	b	16.0
DBT II	a	445.0
	b	30.0
Normal lung (R.R)	a	169.0
	b	12.0
Ca lung (L.S.)	a	456.0
	b	12.0
Ca breast (Y.Y.)	a	151.0

a, intact monolayers.
b, trypsinized monolayer studied in suspension.

tion. Three-hour incubations in HeLa cell monolayers show that [^{3}H]TdR incorporation is pH dependent. In contrast, the [^{3}H]TdR incorporation in the presence of UdR is less pH dependent in the range of 7.27 to 8.32 (Table IV). [^{3}H]TdR incorporation in HeLa cell monolayers appears to increase with the degree of monolayer disruption while the reverse is true when UdR is present in the media (Table V).

BUdR (0.1 μmole/ml) blocks the stimulatory effect of 0.1 μmol/ml of UdR and, to a lesser extent, blocks [^{3}H]TdR incorporation in the absence of UdR in HeLa cell monolayer cultures. The stimulatory effect of UdR on the [^{3}H]TdR incorporation in the HeLa cell monolayer is still evident when the level of UdR ranges from 0.001 to 1.0 μmole/ml. This same phenomenon was observed when deoxycytidine (0.1 μmole/ml) was used instead of UdR. Uracil (0.1 μmole/ml) showed no inhibition or stimulation of [^{3}H]TdR incorporation while 0.1 μmole/ml of TdR suppressed the incorporation of the [^{3}H]TdR as expected.

Discussion. These studies measure different patterns of deoxynucleoside utilization for *de novo* DNA synthesis in monolayer and suspension cell cultures. Cells normally in suspension such as bone marrow or cell lines in suspension culture demonstrate UdR suppression of TdR incorporation into DNA. In contrast, cells grown in monolayer or those derived from solid tumors, whether loosely or densely populated, are apparently stimulated to incorporate more TdR when in the presence of deoxynucleosides such as UdR and deoxycytidine but not in the presence of uracil. This stimulation is time, temperature, and, to a lesser extent, pH dependent.

The intact HeLa monolayer incorporates less TdR per cell than when it is disrupted or in comparison to cell lines grown in suspension. Moreover, the monolayer cell lines reach an early plateau of TdR incorporation (30 min) as compared to cells in suspension (60–90 min). The reasons for these differences are not clear but suggest differences in the cell membrane transport system for deoxynucleosides. There is a distinct mem-

TABLE III. EFFECT OF TIME ON THE INCORPORATION OF [^{3}H]TdR IN MONOLAYERS (Ca LUNG).

Time (min)	[^{3}H]TdR (CPM)	[^{3}H]TdR in the presence of 0.1 μmole/ml of UdR (cpm)	Incorporated (%)
15	2770	1048	37.0
30	3039	2690	87.0
60	2292	4307	188.0
120	2384	6143	258.0

TABLE IV. EFFECTS OF pH ON THE INCORPORATION OF [^{3}H]TdR INTO THE DNA of HeLa CELL MONOLAYERS.

Start of incubation pH	[^{3}H]TdR (cpm)	[^{3}H]TdR in the presence of 0.1 μmole/ml of UdR (cpm)
7.27	9853	44346
7.25	9806	33655
7.50	12128	48453
7.90	18521	47195
8.32	19792	45743

TABLE V. EFFECT OF CELL DAMAGE ON THE [^{3}H]TdR INCORPORATION INTO THE DNA OF HeLa CELL MONOLAYERS.

Status	[^{3}H]TdR (cpm)	[^{3}H]TdR in the presence of 0.1 μmole/ml of UdR (cpm)	Incorporated (%)
Intact monolayer	1843	13636	740.0
Disrupted monolayer	19435	7880	48.0
Disrupted monolayer removed from plate and assayed in test tube	22673	4337	21.0

brane alteration and structural change in the monolayer cells when adapted to suspension cultures (10) which may be concomitant with alterations in the patterns of cellular metabolism. Myers and Feinendegen (9) have described different patterns for combinations of deoxynucleoside utilization for DNA synthesis depending on whether the cells were grown in suspension or on a glass surface. Moreover, the different patterns found in the monolayer reverted to normal when the monolayer was trypsinized and the cells suspended. We have found similar effects utilizing disrupted HeLa cell monolayers. It is apparent, therefore, that the relative rates of incorporation of TdR and other deoxynucleosides are controled by a labile system and that discrimination in favor of one precursor can only be defined for one particular set of conditions.

There does not appear to be any single rate-limiting step in the incorporation of exogenous nucleosides into DNA. TdR appears to enter the cell by active transport and by diffusion (12–14) and share a carrier-mediated transport with UdR but not deoxycytidine (15). Phosphorylation occurs rapidly after uptake (12, 13, 16) and these derivatives are finally utilized for DNA synthesis. Any of these steps could be crucial under different conditions (14, 17).

The UdR stimulation of [^{3}H]TdR incorporation may be due to a greater dependence on the salvage pathway for DNA synthesis in monolayer cells and in cells derived from solid tumors as compared with cells normally in suspension. Reduction of TdR catabolism by UdR, a known competitive inhibitor of thymidine phosphorylase (11) could cause the monolayer cells (and solid tumor cell) to incorporate greater amounts of TdR in the presence of UdR. BUdR inhibition of the UdR stimulatory effect in monolayer cells supports the concept that the reaction occurs prior to or during the phosphorylation of TdR. These observations reveal the necessity for extreme caution in correlating the data obtained from suspension cell studies to solid tumor analysis. Further studies are under way to determine the biochemical explanation for the difference in utilization of deoxynucleosides for *de novo* and salvage DNA thymine synthesis in monolayer and suspension cultures.

1. Metz, J., Kelley, A., Swett, V., Waxman, S., and Herbert, V., Brit. J. Haematol. **6,** 575 (1968).
2. Killman, S. A., Acta Med. Scand. **175,** 483 (1964).
3. Waxman, S., Metz, J., and Herbert, V., J. Clin. Invest. **48,** 284 (1969).
4. Waxman, S., and Herbert, V., New Eng. J. Med. **280,** 1316 (1969).
5. Waxman, S., Corcino, J., and Herbert, V., Amer. J. Med. **48,** 599 (1970).
6. Bruckner, H., Schreiber, C., and Waxman, S., Cancer Res. **35,** 801 (1975).
7. Waxman, S., Schreiber, C., and Herbert, V., Clin. Res. **18,** 477 (1970).
8. Tisman, G., Herbert, V., and Edlis, H., Cancer Chem. Reports **57,** 11 (1973).
9. Myers, D. K., and Feinendegen, L., J. Cell Physiol. **86,** 621 (1975).
10. Springer, E. L., Hackett, A., and Nelson-Rees, W., Intl. J. Cancer **17,** 407 (1976).
11. Razzell, W. E., and Casshyap, P., J. Biol. Chem. **29,** 1789 (1969).
12. Hare, J. D., Cancer Res. **30,** 684 (1970).
13. Plagemann, P. G. W., and Erbe, J., J. Cell Biol. **55,** 161 (1972).
14. Roller, B. A., Hirai, K., and Defendi, V., J. Cell Physiol. **84,** 333 (1974).
15. Plagemann, P. G. W., and Erbe, J., J. Cell Physiol. **83,** 337 (1974).
16. Feinendegen, L. E., and Bond, V. B., Exp. Cell Res. **27,** 474 (1962).
17. Adams, R. L. P., Exp. Cell Res. **56,** 49 (1969).
18. Broder, S. W., Glade, P. R., and Hirschhorn, K., Blood **35,** 539 (1970).

Received September 22, 1976. P.S.E.B.M. 1977, Vol. 154.