PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 158, 666-670 (1978)

Inhibition of [^{3}H]TdR and [^{3}H]CdR Incorporation in an Established Human Lymphoma Cell Line by Trypsin Treatment[1] (40270)

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Techniques for determining the growth fraction in both perturbed and unperturbed human cancer cells are required for determining kinetic parameters useful in the design of chemotherapeutic protocols which employ cycle-specific agents that act on cells within the proliferating pool. One of the methods most commonly used to assess the growth potential of a cancer cell population is the tritiated thymidine labeling index (LI). However, the LI may not accurately reflect the growth fraction of a cell population since it is very sensitive to changes in cell cycle stage transit times (1). Nelson and Schiffer (2) have developed the primer-template-available, DNA-dependent DNA polymerase (PDP) assay which purportedly is a direct measurement of cells in the proliferative pool and is reflected by the proportion of nuclei that incorporate [^{3}H]TdR. We attempted to use this assay on an established line of human lymphoma cells (T_1 cells) in order to validate this technique, since the growth fraction of T_1 cells was known (3), but failed to observe any incorporation of radiolabeled-DNA precursors. The only difference in the method we followed from the technique reported by Nelson and Shiffer was the harvesting procedure step employed for the cultured cells. We reasoned that the enzyme treatment or media changes undergone by T_1 cells during harvesting may inhibit DNA synthesis and conducted a series of experiments to explore this possibility. Results indicated that enzyme treatment, but not medium change, inhibited incorporation of radiolabeled precursors into intact T_1 cells. We believe that probable causes for this inhibition might be a reversible inactivation of DNA polymerase or of specific deoxyribonucleoside kinases, or an alteration of the cellular membrane induced by the enzymes.

[1] Supported by Grant No. CA 14528 of the NCI, DHEW.

Materials and methods. Cell lines. Cells utilized in this investigation are from: (a) a human immunoglobulin-producing cell line (T_1 cells) derived from a patient with lymphocytic lymphoma (4); (b) a human melanin-producing melanoma cell line (S26-4 cells) derived from a patient with malignant melanoma (5); and c) Hela cells. T_1 cells are maintained as monolayer cultures in Ham's F-10 medium supplemented with 20% fetal calf serum, 1% glutamine, 1% MEM vitamins, 100 units/ml penicillin, and 60 μg/ml tylocin. Biological characteristics, harvesting methods, and kinetic parameters have been previously described (3, 6, 7). Melanoma cells are maintained as monolayer cultures in MEM supplemented with 15% fetal calf serum, 1% tricine buffer, 0.7% MEM vitamins, 0.7% chlortetracycline, and 0.7% glutamine. Biological characteristics and kinetic properties have been previously described (5, 8). HeLa cells are maintained as monolayer cultures in MEM supplemented with 10% fetal calf serum, 1% sodium pyruvate, 1% glutamine, and 1% nonessential amino acid solution.

Both melanoma and HeLa cultures were harvested as monodispersed cell suspensions using 0.25% trypsin in solution A (Grand Island Biological Company, Grand Island, NY) at 37° for 6 min. T_1 cells were harvested as previously described (7). Briefly, 1 ml Viokase (a pancreatic extract from Grand Island Biological Company, Grand Island, NY) was added to the plates for 1 min at room temperature and decanted; 1 ml trypsin (0.25% in solution A, Grand Island Biological Company, Grand Island, NY) was added for 6 min at 37°; and 0.5 ml fresh medium was used to dislodge the cells.

Cell suspensions were counted in an electronic particle counter (Coulter Counter model ZBI, Coulter Electronics, Inc., Hialeah, FL). For autoradiography, cytocentri-

0037-9727/78/1584-0666$01.00/0

fuge preparations of cell suspensions were fixed, stained in 2% aceto-orcein, and processed using a 50% solution of Ilford K5 emulsion (Polysciences, Inc., Warrington, PA). Slides were exposed for 2 weeks at 4° and developed in Kodak D-19 developer (Eastman Kodak, Rochester, NY). Background was estimated from cell-free areas of the slide. Approximately 300 cells per slide were scored as labeled or unlabeled (labeled cells > 8 grains). Labeling index (LI) was expressed as the ratio of labeled cells to total cells counted.

For DNA histogram distribution studies on T_1 cells utilizing pulse cytofluorometry (PCP) techniques, cultures were processed as described before using a Phywe ICP II cytophotometer (9). DNA histograms were evaluated using a modification of the Fried model (10).

Effect of harvesting procedures on the incorporation of radiolabeled DNA precursors. A. Controls. T_1 cells (5×10^5 cells/dish) were seeded into 60 mm petri dishes containing 3 ml of fresh medium and placed in a 37°, 5% CO_2 in air humidified atmosphere for 48 hr to achieve exponential growth. Samples designated as control 1 were pulse labeled for 30 min at 37° with either 1 μCi/ml [^{3}H]TdR (S.A. 6 Ci/m*M*, Schwarz/Mann Radiochemicals, Orangeburg, NY) or with 1 μCi/ml [^{3}H]CdR (S.A. 5 Ci/m*M*, Schwarz/Mann Radiochemicals, Orangeburg, NY). After incubation, cells were washed twice with Hanks' balanced salt solution, harvested, and processed for autoradiography. Cells designated as control 2 were harvested before pulse-labeling, placed in centrifuge tubes, washed, and resuspended in fresh medium with either 1 μCi/ml [^{3}H]TdR or 1 μCi/ml [^{3}H]CdR for 30 min at 37°. After incubation, cell suspensions were washed twice in saline solution; cytocentrifuge preparations were made and processed for autoradiography.

B. Medium change. To observe the effect of medium changes on the LI of T_1 cells, exponentially growing cells were washed twice with fresh medium. Three ml of fresh medium containing either 1 μCi/ml of [^{3}H]TdR or 1 μCi/ml [^{3}H]CdR were added to the plates for 30 min at 37°. Cells were then washed twice with Hanks' balanced salt solution, harvested, and processed for autoradiography. In other experiments, supernatant of 48 hr cultures was collected, cells were harvested, centrifuged, washed in saline solution, and resuspended in their own supernatant medium containing either 1 μCi/ml [^{3}H]TdR or 1 μCi/ml [^{3}H]CdR for 30 min at 37°. After incubation, cell suspensions were centrifuged, washed twice in saline solution, and processed for autoradiography.

C. Enzyme treatment. T_1 cells were harvested before pulse-labeling using only Viokase (3 ml for 6 min at room temperature) and using only trypsin (0.01, 0.1 and 0.25% for 6 min at 37°). Cell suspensions were labeled with either 1 μCi/ml [^{3}H]TdR or 1 μCi/ml [^{3}H]CdR for 30 min at 37°, washed with Hanks' balanced salt solution, and processed for autoradiography. In other experiments, cells were harvested before pulse-labeling using only a solution containing 100 μg/ml of crystalline trypsin that had been purified three times and lacked endonuclease activity (11) (Worthington Biochemical Corp., Freehold, NJ).

D. Recovery. T_1 cells in exponential growth were harvested, washed, and replated in petri dishes. Either [^{3}H]TdR (1 μCi/ml) or [^{3}H]CdR (1 μCi/ml) was added to cultures 30 min before reharvesting at regular intervals for 32 hr. Aliquots were processed for PCP and autoradiography at each time point.

E. Enzyme treatment on other human cell lines. After reaching exponential growth, both melanoma and HeLa cells were pulse-labeled with either [^{3}H]TdR (1 μCi/ml) or [^{3}H]CdR (1 μCi/ml) for 30 min at 37°. Cultures were then washed with medium and processed for autoradiography. Other exponentially growing cultures were harvested using 0.25% trypsin at 37° for 6 min or with Viokase at room temperature for 1 min followed by trypsin at 37° for 6 min. Cells were centrifuged, resuspended in medium containing either [^{3}H]TdR (1 μCi/ml) or [^{3}H]CdR (1 μCi/ml) for 30 min at 37°, washed in saline solution and processed for autoradiography.

F. Reproducibility. All of the above described experiments were conducted at least twice with duplicate control and test points, each with duplicate readings per sample.

Results. The LI was determined for cells exposed to the emulsion for at least 2 weeks. This period is long enough to detect grains even in lightly labeled cells as shown in ex-

periments where sequential pilot slides were developed at 2-day intervals. The LI for both radiolabeled nucleosides increased during the first 6 days and plateaued after day 8.

The LI of T_1 cells pulse-labeled with [^{3}H]TdR before harvesting (control 1) ranged from 20 to 46 (mean 31.5) while the LI of cells labeled with [^{3}H]CdR ranged from 15 to 39 (mean 26.8). Although in most experiments the LI recorded for cells incubated with both radiolabeled nucleosides were similar (within 2% approximation), in some instances there was a considerable difference (~ 6%) for replicate cultures labeled simultaneously with these DNA precursors. In these cases, the LI of cells labeled with [^{3}H]-CdR was always lower than that defined for [^{3}H]TdR labeled cells suggesting that this difference is not the result of random changes in the proportion of S-phase cells in replicate cultures but possibly due to factors involving differences in the intracellular nucleotide pools.

There was no significant effect on radiolabeled DNA precursor incorporation induced by enzyme treatment on both melanoma and HeLa cells (Table I). However, enzyme treatment of T_1 cells had a profound inhibitory effect on the incorporation of both radiolabeled nucleosides. For trypsin concentrations of 0.25% and 0.1%, no labeled nuclei were observed. For cells treated with 0.01% trypsin only 5–6% of the population became labeled. Cells treated with 100 mg/ml of Worthington's trypsin or harvested with Viokase alone had no labeled cells.

The LI for T_1 cells washed with fresh medium and incubated with tritiated nucleosides before harvesting was similar to that of cells pulse-labeled before harvesting without previous medium change (control 1 cells). However, there were no labeled nuclei for cells harvested by enzyme treatment and subsequently resuspended in their own medium before incubation with tritiated nucleosides. These experiments demonstrated that changes in growth medium (with moderate changes in pH and temperature) does not inhibit radiolabeled DNA precursor incorporation.

Increments in LI as a function of time elapsed from harvesting is shown in Fig. 1. Recovery of [^{3}H]TdR and [^{3}H]CdR incorporation was slow and reached pre-enzyme treatment levels only after 12 hr. PCP studies demonstrated that the proportion of enzyme-treated cells with S-phase DNA content was not significantly different from that of control cells throughout the experiment (Fig. 2).

Discussion. Detection of DNA synthesis by autoradiographic procedures depends on dosage levels and specific activity of the radiolabeled precursors, emulsion exposure duration, emulsion background noise, specimen thickness, and emulsion sensitivity. According to Shackney (12), DNA synthesis may not be restricted to a discrete phase of the cell cycle (S-phase) but may occur throughout the entire cycle (except mitosis) with the G_1 and G_2 phases representing periods where DNA synthesis falls below the detection threshold.

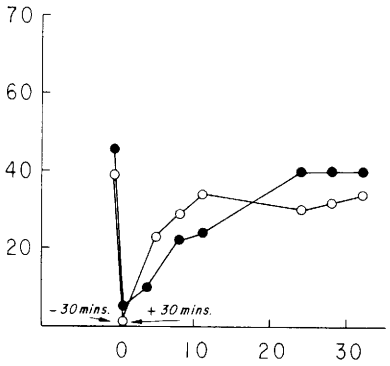

FIG. 1. Abscissa: time after harvesting (hours); ordinate: percent labeled cells. ●—●, [^{3}H]TdR, ○—○, [^{3}H]CdR. Uptake of ^{3}H-thymidine and ^{3}H-deoxycytidine by T_1 cells before (0.5 hr) and after (0.5–32 hr) trypsinization. Cells were pulse labeled with the labeled precursor for 30 minutes prior to washing and fixing.

TABLE I. EFFECT OF ENZYME TREATMENT ON THE LABELING INDEX OF CULTURED MELANOMA AND HELA CELLS.

	Preharvesting		Harvesting with trypsin alone		Harvesting with viokase and trypsin	
Cell line	TdR	CdR	TdR	CdR	TdR	CdR
Melanoma	23.5	20.5	18.5	23	21	23
HeLa	25.5	26	31.5	27	25	27

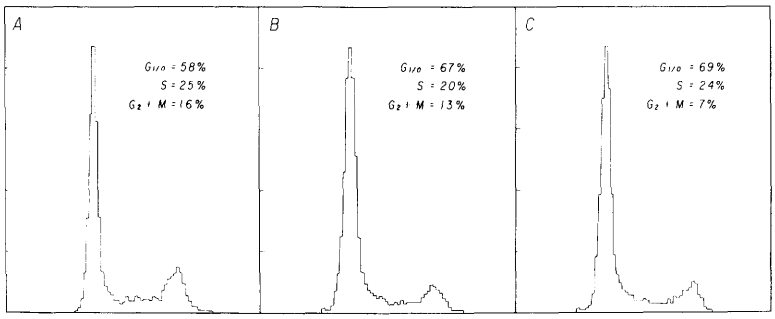

FIG. 2. Abscissa: channel number; ordinate: frequency DNA histograms obtained by PCP analysis of T_1 cells before trypsinization (A) and after trypsin treatment at 0 hr (B) and 4 hr (C).

Thus, selecting a high background threshold for measuring the LI may discriminate against lightly labeled cells when short periods of exposure are employed (13). However, the chosen threshold becomes less critical as the interval of exposure to the emulsion is prolonged and the LI tends to reach a plateau (13). In our study, LI's reached a plateau after 8 days. Hence, experimental autoradiograms were evaluated after two weeks of exposure to prevent the scoring of possible lightly labeled cells as unlabeled.

Considerable interest has been devoted in recent years to the role played by proteases in the regulation of cell proliferation (14, 15). A model was proposed whereby treatment with proteolytic agents would induce cell proliferation and this effect would be counteracted by normal cells capable of synthesizing protease repressors (16). Our results demonstrate that treatment with proteolytic agents does not necessarily lead to cell proliferation and that, in fact, it may result in inhibition of active DNA synthesis.

Our studies showed that both trypsin and a pancreatic extract reversibly inhibited the incorporation of [^{3}H]TdR and [^{3}H]CdR into permanently established tissue cultured human B-lymphoid cells. Treatment with these proteolytic agents did not inhibit or stimulate DNA synthesis in our human melanoma and HeLa cell lines. These results are in contrast to the findings of others for cultured rodent cells (17), especially B-lymphoid elements (18–20), but are similar to the data described by Robinson for Chinese hamster cells (21). Whether the inhibition of radiolabeled precursor incorporation into the DNA of human B-lymphoid cells results from alterations in physicochemical properties of the cell surface and/or from alterations of intrinsic cell processes due to intracellular penetration of the proteolytic agents is unclear. The uptake of thymidine is a mediated process that can be inhibited by a number of structurally diverse compounds that interfere with membrane transport mechanisms (22). Hence, the effect of proteolytic agents on the cell membrane of our T_1 cells could be manifested by decreased transport of exogenous nucleosides. Alternatively, trypsin-induced alteration of physicochemical properties of the cell membrane may interfere indirectly with the regulatory mechanisms of cell division believed to be mediated by the membrane (23), especially in malignant cells (24).

On the other hand, Hodges *et al.* have demonstrated intracellular penetration of trypsin and suggested that this phenomenon may affect intrinsic cellular processes (25). Thus, the observed decreased incorporation of DNA precursors may result from its proteolytic effects on DNA polymerases and/or deoxyribonucleoside kinases. DNA replication in eukaryotic cells requires at least two types of DNA polymerases. Investigators have demonstrated, in several lines of tissue cultured cells, the presence of a low molecular weight DNA polymerase found predominantly in the nucleus and a high molecular weight DNA polymerase found mainly in the cytoplasm (26–28). The latter enzyme is considered the polymeric precursor to the nuclear DNA polymerase [29, 30]. It is also known that incorporation of the exogenous nucleosides into nuclear DNA requires the participation of the salvage pathway through which deoxyribonucleosides are phosphorylated by

the appropriate cytoplasmic kinase. Thus, the inhibitory effect of the proteolytic agents could be the result of enzymatic digestion of the polymerase precursor and/or the cytoplasmic kinases while the observed delay in recovery a reflection of the time required to resynthesize these enzymes.

The fact that following trypsin treatment the incorporation of [^{3}H]TdR into naked nuclei of T_1 cells was also inhibited (PDP assay) indicates that the effects of the proteolytic agents extend further than merely the surface membrane and/or cytoplasm. Thus, it is possible that the enzymes may interfere with nuclear membrane transport mechanisms as well as penetrate the nucleus, preventing either the uptake of the nucleotides or the function of the nuclear DNA polymerase.

Summary. [^{3}H]TdR and [^{3}H]CdR incorporation into intact human lymphoma cells was reversibly inhibited by brief treatment with proteolytic agents (trypsin and Viokase). Neither enzyme affected the incorporation of the tritiated nucleosides by HeLa or human melanoma cells. These results are different from those described for rodent B-lymphoid cells and indicate that protease treatment does not invariably lead to increased DNA synthesis.

The authors express their gratitude to Ms. Judy Johnson for excellent secretarial assistance in preparing this manuscript, to Dr. E. Stubblefield for providing the crystalline trypsin, to Ms. Leslie Smallwood for assisting in pulse cytofluorometry techniques, and to Dr. Pam Roper for helpful discussions during the course of this study.

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Received January 31, 1978. P.S.E.B.M. 1978, Vol. 158.