Bull. Yamaguchi Med. Sch., Vol. 28, Nos. 1-2, 1981

Immunologic Measurement of DNA Synthesis Rate in Individual Mouse L Cells

Tomozo Oku

Department of Pathology, Yamaguchi University School of Medicine, Ube, Yamaguchi 755, Japan.
(Received February 2, 1981)

Abstract. DNA synthesis of mouse L cells *in vitro* was studied by immunofluorescence and immunoperoxidase techniques using anti-BUdR antibody to identify cells which incorporate BUdR. The fraction of fluorescence- or peroxidase-positive cells agreed well with ^{3}H-thymidine labeling index. In synchronized cell populations, DNA synthesis rate as measured by fluorescence intensity increased from immediately after block and after plateau of a 4-hr period returned to the initial level in 8 hr. At the mid S phase when DNA synthesis rapidly increased, fluorescence intensity of individual cells varied significantly.

Key Words: anti-BUdR antibody, double thymidine block, DNA synthesis rate, mouse L cell

Introduction

Autoradiography of ^{3}H-thymidine (TdR)-labeled cells has been used for measuring DNA synthetic activity of individual cells[1-3]. However, grain counting in this method is sometimes difficult. Gratzner et al[4-6] have recently introduced an immunologic technique which uses a specific antibody against an incorporated thymidine analogue, bromodeoxyuridine (BUdR). Their method is advantageous to other fluorescence methods that use quenching of fluorescence[7,8], because the amount of incorporated BUdR can be measured more sensitively by microfluorometry.

The present paper describes the analysis of the DNA synthesis in mouse L cells *in vitro* by the above immunologic technique. BUdR-labeling index paralleled ^{3}H-TdR labeling index in synchronized cell populations.

Materials and Methods

Cell culture and synchronization

A monolayer culture of mouse L5 cells (given by Dr. A. Tsuboi, National Institute of Radiological Science, Chiba, Japan) was used[9]. The culture medium was Ham's F 12 (Nissui Seiyaku Co., Tokyo) supplemented with 10% calf serum and 60 μg/ml Cefamedine (Fujisawa Seiyaku Co., Osaka).

To synchronize cells at various times within the S phase, the cell cycle was blocked for 22 hr with medium containing 2 mM of cold thymidine and after succeeding 10 hr of culture without thymidine, the cells were exposed again to the same concentration of thymidine for another 12 hr.

Pulse-labeling of synchronized cells

At various times after release from block, cells were pulse-labeled for 30 min with either 0.1 μCi/ml ^{3}H-TdR (specific activity 2 Ci/mmol, New England Nuclear) or 10 μM BUdR (Sigma) plus 1 μM fluorodeoxyuridine (Sigma). The cells were then detached by trypsinization, centrifuged and resuspended in cold phosphate buffered saline (PBS) to be used for immunofluorescent and immunoperoxidase staining, autoradiography and scintillation counting.

Antibody preparation

Bromouridine was coupled to bovine serum albumin (BSA) according to the method of Erlanger & Beiser[10] as follows: 100 mg 5-bromouridine (Sigma) was mixed with 0.1 M sodium periodate at room temperature to oxidize the vicinal hydroxyl groups on sugar moiety and the mixture was then added slowly with stirring to 10 ml of an aqueous solution of 280 mg BSA which had been adjusted to pH 9–9.5 with 5% K_2CO_3. Stirring was continued for 45 min during which period the pH was kept at 9–9.5 with K_2CO_3. At the end of this period, 10 ml of a 1.5% $NaBH_4$ solution was added to reduce the complex and left for 18 hr to form a stable conjugate. Five ml of 1 M formic acid was added, followed one hour later by adjustment of the pH to 8.5 using 1M NH_4OH. The solution was dialyzed for 36 hr against cold running water and lyophilized.

The conjugate in saline (4 mg/ml) was emulsified with an equal volume of complete Freund's adjuvant (Difco), and 1 ml of the emulsion was injected into each of 4 rabbits every other week for 8 weeks. Rabbits were bled 1 week after the final injection. Antibody titer in undiluted sera was measured by Ouchterolony double diffusion method[11].

Immunologic staining

The synchronized cells were fixed in methanol acetic acid (3:1), smeared and air dried. In order to denature DNA, the smear was incubated either in 0.025 N NaOH for 2 min at room temperature or 99% formamide at 60°C for 1 hr, washed in 70% ethanol and air dried. The sections were coated with 1:30 diluted serum containing anti-BUdR antibody and after 1 hr incubation at 37°C, they were rinsed three times with PBS for 45 min. Further, the sections were coated with FITC-labeled goat anti-rabbit IgG (Miles-Yeda) or goat-anti-rabbit IgG labeled with horse radish peroxidase (Miles-Yeda) diluted to 1:30 and kept at 37°C for 1 hr.

Immunologically bound peroxidase was stained for 10 min with 0.03% 3,3′-diaminobenzidine and 0.003% H_2O_2 in 100 ml PBS. Fluorescence intensity of FITC-labeled antibody was measured in 56 cells per sample by a digital microfluorometer (Nikon, SPM-RFL system) equipped with a DC-200 mercury light source to estimate the incorporated BUdR. The results were expressed in arbitary units.

Autoradiography

Smears of labeled cells were treated with 5% trichloroacetic acid (TCA) at 37°C for 1 hr, rinsed in water, coated with photographic emulsion Type ET 2F (Fuji Photo. Film Co., Tokyo) and exposed in refrigerator for 7–14 days.

Scintillation counting

Cells were trypsinized and harvested on a Whatman GFC filter, washed 3 times with cold PBS, 3 times with cold 5% TCA and once 100% ethanol. The filter was then dried, and cells were dissolved in Soluen 100 (Packard). Radioactivity was counted in a Packard liquid scintillation specrometer.

Results

Double-diffusion gel techniques revealed a precipitation line between rabbit antiserum and 5-bromouridine-BSA conjugate but a slight cross-reaction with BSA was also noted. This BSA-antibody was absorbed by BSA.

As shown in Fig. 1, BUdR incorporated into nuclei was clearly demonstrated by an indirect immunofluorescence technique in the synchronized cell populations. Nucleolar and cytoplasmic regions were not stained. Furthermore, specificity of this reaction was shown by the decrease of fluorescence to the background level after addition of excess BUdR to the serum.

Labeling index with ^{3}H-TdR shortly after release from thymidine block was approximately 5%. The index increased rapidly to 80% within 2 hr, decreased slowly for 6 hr to reach 60% and then dropped rather abruptly

to 10% in the succeeding 2 hr (Fig. 2). If one assumes that the S phase is the period in which the cell populations acquired more than a half of the maximal LI (82%), the S phase length was estimated to be 8 hr. Immunoperoxidase positivity index (IPI) of synchronized cells pursued a course similar to ^{3}H-TdR labeling index. The IPI was slightly lower than LI in its declining portion. This is probably because diffuse brown discoloration due to peroxidase reaction does not stand out so clearly as silver grains.

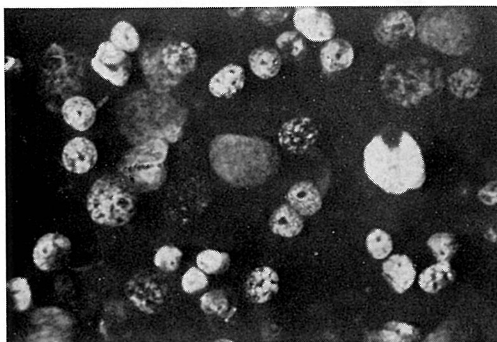

Fig. 1 Synchronized mouse L cells (immunofluorescence picture) Cells were pulse-labeled for 30 min with 10 μM BUdR at 6 hr after release from double thymidine block and the incorporation of BUdR was demonstrated by an indirect immunofluorescence technique utilizing 1:30 diluted anti-BUdR antibody.

The rate of BUdR incorporation at varing times after the release from the block was measured by the immunofluorescence intensity in a digital microfluorometer. Fig. 3 shows frequency histogram of the fluorescence intensity. Despite marked variation in individual cells, the average BUdR incorporation rate could be calculated and taken as the average rate of *de novo* DNA synthesis. The mean DNA synthesis rate estimated by this method increased immediately after release, reached the maxium within 6 hr and then dropped drastically (Fig. 4). Thus, DNA synthesis rate accelerated rapidly by about threefold from early- to mid-

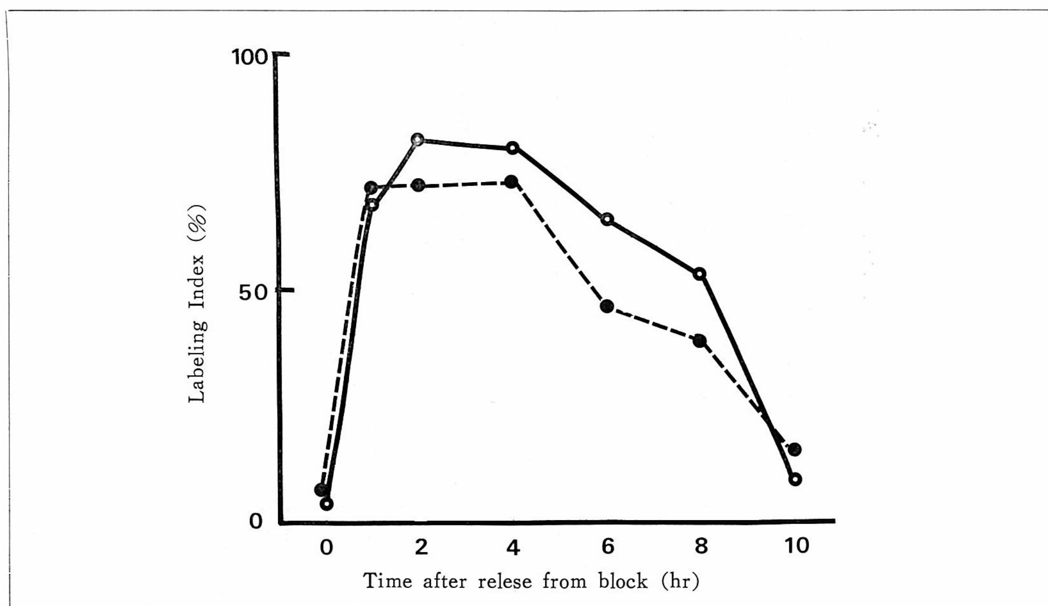

Fig. 2 ^{3}H-TdR labeling indices (LI) (○——○) and immunoperoxidase positivity indices (IPI) (●······●) at various times after release from block.

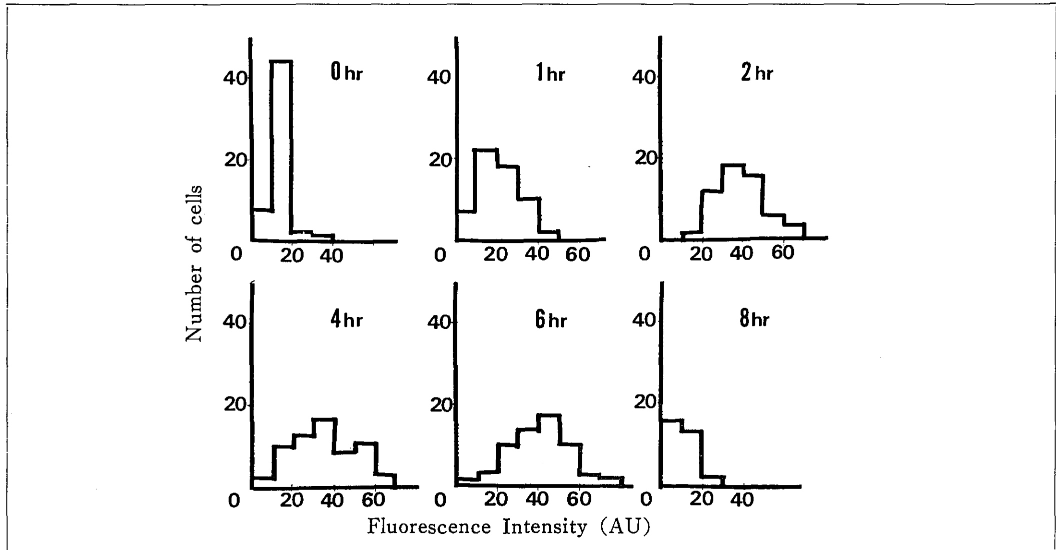

Fig. 3 Fluorescence intensity distributions of nuclei pulse-labeled with BUdR.
The time in each frame refers to the period after release from the double thymidine block.

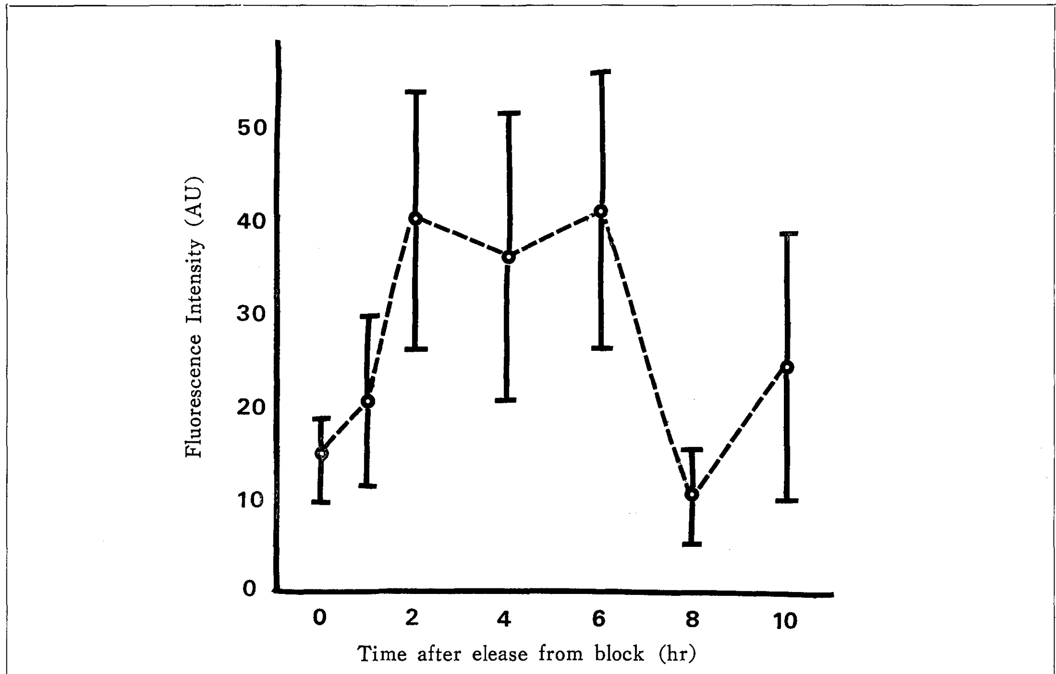

Fig. 4 Changes of the BUdR incorporation rate during the S phase.
Cells were pulse-labeled for 30 min with 10μM BUdR at various times after release from block and the immunofluorescence intensity of the individual nucleus was measured by a digital microfluorometer.

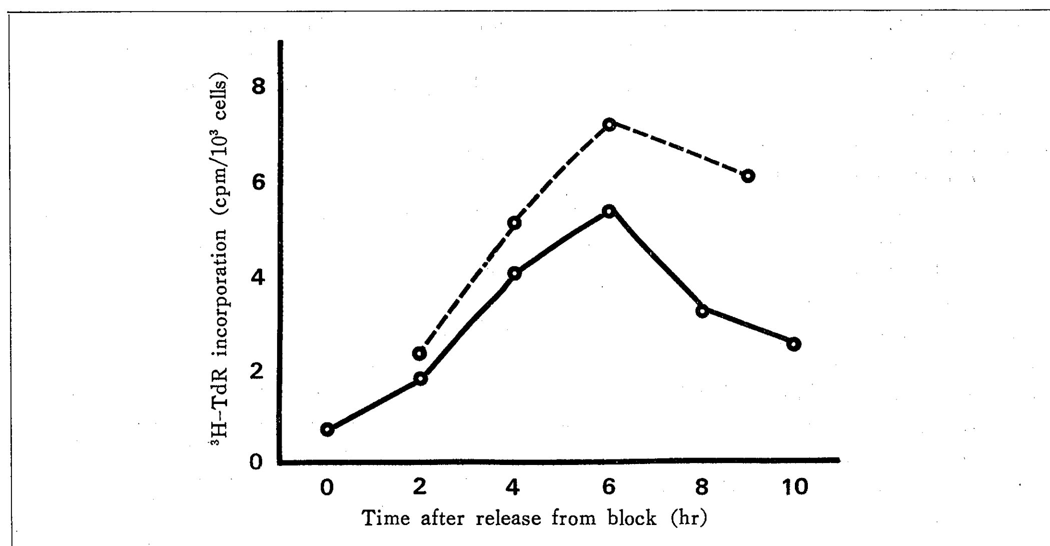

Fig. 5 The amount of ^{3}H-thymidine incorporated per total cell or averaged for the S phase cells at various times during the S phase.

At various times after release from the double thymidine block, cells were pulse-labeled for 30 min with 0.1 μCi/ml ^{3}H-TdR. ^{3}H-TdR incorporation per S phase cell (O······O) was estimated by dividing total amount of ^{3}H-TdR incorporated (O——O) by LI.

S phase and decreased abruptly at late-S phase.

The overall rate of ^{3}H-TdR incorporation into DNA increased progressively from early S phase, showing a peak at 6 hr and decreased thereafter (Fig. 5). The mean ^{3}H-TdR incorporation rate calculated per S phase cell was lower at the early S and higher at the late S phase than that of the mean fluorescence intensity.

Discussion

The most favored technique for the measurement of DNA synthesis rate in the S phase is to use synchronized cell culture and to measure radioactivity by scintillation counter after pulse-labeling the cells at various periods[12-14]. DNA cytophotometry can be combined with autoradiography to locate cells in the cell cycle for the analysis of DNA synthesis in exponentially growing cell populations[1-3].

The method developed by Gratzner et al[4-6] which uses anti-BUdR antibody to identify BUdR incorporated into DNA is obviously advantageous over the grain count method in several respects; (1) fluorescence can be observed immediately after pretreatment of a few minutes whereas autoradiography must await several days at least for exposure, (2) procedures without radioactive isotope allow clinical application, and (3) the positive immunofluorescence of BUdR can be used in a flow cytometric measurement of DNA synthesis rate.

Although it is sometimes difficult to identify the immunofluorescence positive and negative cells, the immunofluorescence (or immunoperoxidase) positivity index and the ^{3}H-TdR labeling index run in parallel with each other after release from double

thymidine block. Therefore, this technique can relace thymidine labeling method. The present author extended the application of this method to the measurement of DNA synthesis rate in individual cells. In accordance with the reports of other investigators [1,14], the mean fluorescence intensity curve showed that DNA synthesis rate increases progressively from early- to mid-S phase until it drops at the late S phase. The same result was obtained irrespective of the synchronization procedures used (unpublished data). However, the higher DNA synthesis rate in the early S phase and the lower rate in the late S phase were observed as compared with the results of TdR incorporation (Fig. 4 and 5).

Possible explanations for such discrepancy in results due to different methods may be as follows: (1) Because of strong alkaline treatment for DNA denaturation, some fraction of the incorporated BUdR might be lost from the nucleus of the late S phase cells. (2) Chromatin condensation due to heterochromatinization in the late S phase may also interfere with the nuclear fluorescence.

A marked variation of fluorescence intensities was observed in the individual cells sampled at the same period of time, especia -lly at the mid S phase. A similar variation has also been reported in experiments with grain count method as well[1-3,15,16]. Most investigators considered that the data scatter reflects variability of thymidine kinase activity[15], varied endogenuous thymidine pool[16] as well as the variability of DNA synthetic activity itself[2]. Disintegration of the fluorescein and consequent fading during observation should not be neglected. It may also be attributed to the incomplete synchronization by thymidine block[12,17,18].

Quantitative evalution of DNA synthesis rate in single cells measured by the immunofluorescence method is potentially more precise than the grain-count method and will be the method of choice for the *in vitro* and *in vivo* cell kinetic analysis, especially by flow cytometric system.

Acknowledgement

The author wishes to thank professor M. Takahashi for valuable discussions and advice on data analysis.

References

1) Dendy, P.P. and Cleavear, T.E.: An investigation of (a) variation in rate of DNA-synthesis during S-phase in mouse L-cells, (b) effect of ultra-violet radiation on rate of DNA-synthesis. *Int. J. Rad. Biol.*, 8 : 301-315, 1965.

2) Dörmer, P., Brinkmann, W., Born, R. and Steel, G.G.: Rate and time of DNA synthesis of individual chinese hamster cells. *Cell Tissue Kinet.*, 8 : 399-412, 1975.

3) Hirt, A. and Wagner, H.P.: Nuclear incorporation of radioactive DNA precursors and progression of cells through S. *Cell Tissue Kinet.*, 8 : 455-466, 1975.

4) Gratzner, H.G., Leif, R.C., Ingram, D.J. and Castro, A.: The use of antibody specific for bromodeoxyuridine for the immunofluorescent determination of DNA replication in single cells and chromosomes. *Exp. Cell Res.*, 95 : 88-94, 1975.

5) Gratzner, H.G., Pollack, A., Ingram, D.J. and Leif, R.C.: Deoxyribonucleic acid replication in single cells and chromosomes by immunologic techniques. *J. Histochem. Cytochem.*, 24 : 34-39, 1976.

6) Gratzner, H.G., Young, I.T. and Sher, S.E.: An immunocytochemical approach to cell kinetics automation. *J. Histochem. Cytochem.*, 27 : 496-499, 1979.

7) Kato, H.: Spontaneous sister chromatid exchanges detected by a BUdR-labelling method. *Nature*, 251 : 70-72, 1974.

8) Latt, S.A.: Microfluorometric detection of deoxyribonucleic acid replication in metaphase chromosomes. *Proc. Natl. Acad. Sci. U.S.A.*, 70 : 3395-3399, 1973.

9) Tsuboi, A., Kurotsu, T. and Terasima, T.: Changes in protein content per cell during growthof mouse L cells. *Exp. Cell Res.*, 103 :

257-261, 1976.

10) Erlanger, B. F. and Beiser, S.M.: Antibodies specific for ribonucleosides and ribonucleotides and their reaction with DNA. *Proc. Natl. Acad. Sci. U.S.A.*, 52 : 68-74, 1964.

11) Carpenter, P.L.: *Immunology* and *Serology*, Saunders, U.S.A., 1975, p. 323-324.

12) Bostock, C.J., Prescott, D.M. and Kirkpatrick, J.B.: An evaluation of the double thymidine block for synchronizing mammalian cells at the G1-S border. *Exp. Cell Res.*, 68 : 163-168, 1971.

13) Kapp, L.N. and Painter, R.B.: Multiple thymidine incorporation peaks in the S phase of synchronous human diploid fibroblasts. *Exp. Cell Res.*, 107 : 429-431, 1977.

14) Schaer, J.C., Ramseier, L. and Schindler, R.: Studies on the division cycle of mammalian cells.: Incorporation of labeled precursors into DNA of synchronously dividing cells in culture *Exp. Cell Res.*, 65 : 17-22, 1971.

15) Brent, T. P.: Periodicity of DNA synthetic enzymes during the HeLa cell cycle. *Cell Tissue Kinet.*, 4 : 297-305, 1971.

16) Stewart, P.A., Quastler, H., Skougaard, M.R., Wimber, D.R., Wolfsberg, M.F., Perrotta, C.A., Ferbel, B. and Carlough, M.: Four-factor model analysis of thymidine incorporation into mouse DNA and the mechanism of radiation effects. *Radiat. Res.*, 24 : 521-537, 1965.

17) Meyn, R.E., Hewitt, R.R. and Humphrey, R. M.: Evaluation of S phase synchronization by analysis of DNA replication in 5-bromodeoxyuridine. *Exp. Cell Res.*, 82 : 137-142, 1973.

18) Studzinski, G.P. and Lambert, W.C.: Thymidine as a synchronizing agent. 1. Nucleic acid and protein formation in synchronous HeLa cultures treated with excess thymidine. *J. Cell. Physiol.*, 73 : 109-118, 1969.