DNA Synthesis and Multinucleation of Mouse Cells Infected with SV40 in the Presence of Cytochalasin B

By TAKASHI KURIMURA AND AKIKO HIRANO

Department of Virology, Tottori University School of Medicine, Yonago 683, Japan

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SUMMARY

DNA synthesis and nuclear division of mouse cells, BALB/3T3, infected with SV40 were studied and were compared with those of SV40-transformed mouse cells, mKS-A TU-7. Both SV40-infected and SV40-transformed cells behaved similarly in the presence of cytochalasin B and differently from normal non-infected cells, BALB/3T3. The chemical inhibited cytokinesis of all the cell groups but the nuclear division was inhibited only in the case of non-infected BALB/3T3. With SV40-infected BALB/3T3, multinucleation occurred with the increase of input m.o.i. by SV40. SV40-infected BALB/3T3 could enter the second S phase after release from double thymidine block in the presence of cytochalasin B, while BALB/3T3 could not enter the second S phase. In bi- or multinucleated cells of SV40-infected BALB/3T3, asynchrony of DNA synthesis among nuclei in a cell was evident, as was the case with mKS-A TU-7.

INTRODUCTION

Introduction of genomes of animal viruses into normal cells, that is into cells which do not contain exogenous virus genomes, evokes many changes in these cells and they behave differently from normal cells under various conditions (Tooze, 1973). Limitation of nuclear division occurs in normal cells in the presence of cytochalasin B, an alkaloid which inhibits cytoplasmic division of cells (Wright & Hayflick, 1972; Hirano & Kurimura, 1974; O'Neill, 1974). Escape from the limitation of nuclear division in the presence of cytochalasin B is not observed in cells transformed by RNA viruses (O'Neill et al. 1975). Consequently, multinucleation or unlimited DNA synthesis of virus-transformed cells in the presence of cytochalasin B can be interpreted as the result of the effect of the virus functions, especially early virus functions, of DNA viruses. To confirm this assumption, several approaches can be made, for example utilization of early conditional lethal mutants of viruses or of abortive infection systems. In this paper the effect of cytochalasin B on nuclear division and on DNA synthesis of mouse cells infected with SV40, an abortive infection system, is reported.

METHODS

Virus. SV40 virus Td205d was kindly supplied by Dr S. Uchida, NIH of Japan, and further propagated in CV-1 cells, an African green monkey kidney cell line, in this laboratory. The infectivity of the virus was determined by plaque formation on CV-1 cells.

Cells and tissue culture medium. BALB/3T3 clone 4 of passage level between 12 and 23, which is re-cloned in this laboratory, was used throughout this experiment. The SV40-
transformed mouse cell line mKS-A TU-7 (Hirano & Kurimura, 1974, 1975) was used at passage level 37. Eagle’s minimum essential medium (Nisui, Tokyo, Japan) supplemented with 10% foetal bovine serum (Microbiological Associates, Walkersville, Md., U.S.A.) was used as the tissue culture medium.

**Estimation of DNA synthesis.** DNA synthesis in cells was estimated by two procedures. (1) Incorporation of $^3$H-thymidine (5 Ci/mmol, 1 to 2 $\mu$Ci/ml) into 5% cold trichloroacetic acid-insoluble fraction within 30 min at 37°C. (2) Autoradiography of the cells, labelled with $^3$H-thymidine (1 $\mu$Ci/ml) for 1 h was performed using Kodak emulsion NTB2.

**Cytochalasin B.** Cytochalasin B was purchased from ICI (Cheshire, England) and dissolved in dimethylsulphoxide (DMSO). The stock solution was diluted with tissue culture medium to a final concentration of 1 $\mu$g/ml of cytochalasin B and 0.4% DMSO. This concentration of cytochalasin B was the minimum which was sufficient to inhibit cytokinesis of the cells used. For the control groups, medium containing 0.4% DMSO was used.

**Synchronization of the cells.** For the synchronization of the cells, the double thymidine block procedure (Puck, 1964; Pages et al. 1973; Kaplan et al. 1975) was employed. Four to 6 h after inoculation of the cells into 30 mm plastic Petri dishes (Toyoshima Seisakujo, Tokyo, Japan), the medium was replaced with medium containing 5 mM and 10 mM-thymidine, respectively for BALB/3T3 and mKS-A TU-7. After incubation for 16 h the medium containing thymidine was sucked off, the cells were rinsed twice with the medium and then medium without thymidine was added for 8 h. After this period, the cells were again incubated in the presence of thymidine for 16 h. The doubling times of the cells were 20 to 21 and 17 to 18 h and the periods of the S phase were 7.5 and 6.4 h, respectively, for BALB/3T3 and mKS-A TU-7; the timing of this procedure is therefore adequate for both cell lines.

**RESULTS**

**Multinucleation of the cells in the presence of cytochalasin B**

BALB/3T3 clone 4 was infected with SV40 at various multiplicities between 3.7 and 183 p.f.u./cell and 24 h afterwards, cytochalasin B (1 $\mu$g/ml) was added to the cells. Three days after the addition of the chemical, the cells were stained and the extent of multinucleation was examined. Fig. 1 shows the distribution of the numbers of nuclei per cell of SV40-infected or mock-infected cells. As is evident from this figure, the tendency to form multinucleate cells increased in parallel with the input m.o.i. At a multiplicity of 183, 72 h after addition of cytochalasin B the mean number of nuclei per cell was 3.6, while that of mock-infected cells was 1.96.

**Autoradiographic studies of DNA synthesis in cells treated with cytochalasin B**

As a result of the inhibition of cytokinesis by cytochalasin B, cells containing multiple nuclei appeared as shown in Fig. 1. De novo DNA synthesis was followed by incorporation of $^3$H-thymidine into DNA and was also determined by autoradiography. Of these cells, binucleate cells were selected for analysis of synchrony of DNA synthesis since they would be the most likely to show synchrony of DNA synthesis between nuclei in one cell. Forty-eight h after addition of cytochalasin B to the tissue culture medium, binucleate BALB/3T3 cells were autoradiographically examined by microscopy and 67 out of 838 cells exhibited de novo DNA synthesis. All of these 67 cells contained similar numbers of silver grains over both nuclei, while binucleate mKS-A TU-7 cells tended to exhibit asynchronous DNA synthesis, that is silver grains over one nucleus and not the other as shown in Fig. 2(a), and 97 out of 235 cells underwent DNA synthesis and 13 of these 97 cells contained appreciable numbers of grains over only one of two nuclei. This tendency towards
SV40-infected cells and cytochalasin B

Fig. 1. Distribution of the number of nuclei per cell in BALB/3T3 infected with SV40 at different m.o.i. BALB/3T3 was infected with SV40 at a m.o.i. of (a) mock, (b) 3.7, (c) 18.3, (d) 37 and (e) 183. Twenty-four h p.i. the culture medium was replaced with fresh medium containing 1 μg/ml of cytochalasin B and 72 h afterwards the cells were fixed and stained. The mean values of the number of nuclei/cell were (a) 1.96, (b) 2.01, (c) 2.25, (d) 2.6 and (e) 3.6. More than 500 cells per group were observed.

Fig. 2. Asynchronous DNA synthesis in multinucleate mKS-A TU-7. Cells were treated with 1 μg/ml of cytochalasin B for 3 days and labelled with 3H-thymidine (1 μCi/ml, 5 Ci/mmol) for 1 h. At the end of the labelling period, cells were fixed, dipped in photographic emulsion, developed and stained. (a) Asynchronous DNA synthesis in a binucleate cell. (b) Asynchronous DNA synthesis in a cell with five nuclei.
Fig. 3. Asynchrony of DNA synthesis in a cell. BALB/3T3 infected with SV40 was treated with 1 \( \mu \)g/ml of cytochalasin B for 2 days and was labelled with 1 \( \mu \)Ci/ml of \(^{3}H\)-thymidine for 1 h. At the end of the labelling period, cells were processed as described in Fig. 3. All the binucleate cells were checked for DNA synthesis. The input m.o.i.'s were: (a) mock, (b) 3-7, (c) 37 and (d) 183. The numbers of the binucleate cells observed were: (a) 453, (b) 547, (c) 337 and (d) 511. □, Both nuclei were unlabelled; □, both nuclei were labelled; □, one of the two nuclei was labelled.

Fig. 4. DNA synthesis of synchronized cells in the presence or absence of cytochalasin B. Cells were synchronized as described in Methods. Time zero corresponds to the time of release from thymidine block. At the times indicated \(^{3}H\)-thymidine (1 \( \mu \)Ci/ml) was added and the cells were incubated at 37 °C for 30 min and then radioactivity in cold 5 % TCA-insoluble fractions was measured. (a) BALB/3T3, (b) mKS-A TU-7, (c) mock-infected BALB/3T3. (d) BALB/3T3 was infected by SV40 at a multiplicity of 180 p.f.u./cell and at 5 h p.i. the double thymidine block procedure was started. ●—●, In the absence of cytochalasin B; ○—○—○, in the presence of cytochalasin B.
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asynchrony of DNA synthesis among nuclei in one cell became prominent in parallel with the increase of the number of nuclei in one cell. Fig. 2(b) shows asynchronous DNA synthesis in a multinucleate cell, where three out of five nuclei exhibited DNA synthesis. Autoradiographical studies on binucleate BALB/3T3 cells infected with SV40 were also performed and the results summarized in Fig. 3. Addition of ³H-thymidine was made at 48 h after addition of cytochalasin B, that is 72 h after infection by SV40 at various input multiplicities. In cells infected with SV40 at high input multiplicities, 4.5% (5/116) and 5.0% (13/259) of DNA-synthesizing binucleate cells exhibited asynchronous DNA synthesis, respectively, for input multiplicities of 37 and 183 p.f.u./cell, while all of 167 DNA synthesizing cells exhibited synchronous DNA synthesis with an input multiplicity of 3.7.

The rate of occurrence of asynchronous DNA synthesis among nuclei in a cell increased in the case of multinucleate cells, that is cells with more than two nuclei, compared with binucleate cells. The ratios of the number of the multinucleate cells exhibiting asynchronous DNA synthesis over total DNA synthesizing multinucleate cells were 0/169 (0 %), 24/214 (11%) and 68/374 (18%), respectively, for the cells infected with SV40 at the input multiplicities of 3.7, 37 and 183 p.f.u./cell.

DNA synthesis in cells, which are synchronized by the double thymidine block procedure, in the presence of cytochalasin B

BALB/3T3, uninfected or infected with SV40, and mKS-A TU-7 were synchronized by the double thymidine block procedure and DNA synthesis in these cells was followed after release from the block in the presence or absence of cytochalasin B. Fig. 4 shows the profile of ³H-thymidine incorporation into 5% cold TCA-insoluble fractions of the cells. As usual, cells entered the first S phase immediately after the release from the block and mKS-A TU-7 or BALB/3T3 infected by SV40 entered the second S phase in the presence of cytochalasin B as non-treated control. But BALB/3T3 could hardly enter the second S phase after traversing the first S phase and this coincides with the fact that BALB/3T3 could form mainly binucleate cells but not multinucleate cells in the presence of cytochalasin B.

DISCUSSION

In this paper we provided data which showed that mouse cells infected with SV40 as well as SV40-transformed cells responded similarly to cytochalasin B while 'normal' mouse cells behaved differently from them. Since we expected that synchrony of DNA synthesis within a cell could be best maintained in binucleated cells, we examined DNA synthesis in those cells by autoradiography. As shown in Fig. 3, asynchrony of DNA synthesis in binucleate cells occurred when the cells were infected by SV40 at high input multiplicity (37 and 183 p.f.u./cell) but not at low input multiplicity (3.7 p.f.u./cell). The cause of this phenomenon and of the multinucleation shown in Fig. 1 might be related to the expression of early function(s) of SV40, considering that similar phenomena could be observed in the case of mKS-A TU-7. In the presence of early function of SV40, mouse cells undergo a normal amount of DNA synthesis with concomitant cessation of cytokinesis after addition of cytochalasin B (Kelly & Sambrook, 1974). This phenomenon is apparently common in the case of cells transformed by DNA viruses (Hirano & Kurimura, 1974), but cells transformed by retroviruses usually halted their DNA synthesis, karyokinesis and cytokinesis after addition of the chemical (O'Neill et al. 1975), although there are some exceptions (Altenburg & Steiner, 1979). The results shown in Fig. 4 indicate that 'the critical stage' within the cell cycle where normal cells are affected by cytochalasin B was not included between G1/S and S phase, since cells were halted at G1/S by the double thymidine block procedure and
since normal BALB/3T3 could traverse the first S phase in the presence of cytochalasin B after release from the block. Early SV40 function(s) might enable the cells to overcome the effect of cytochalasin B and continue DNA synthesis and multinucleation.

The mechanism by which cytochalasin B affects the cells has not been well clarified, but much evidence supports the idea that the chemical affects the cells via its binding to the cell surface (Mayhew et al. 1974; Tannenbaum et al. 1977), although part of the drug bound to intracytoplasmic membrane structures (Tannenbaum et al. 1975). If we postulate that the main target site of the chemical is the cell surface, it is of interest to speculate that regulation of cell DNA synthesis by tumour viruses is performed differently by two groups of viruses, DNA and RNA viruses, and that RNA viruses usually affect cell DNA synthesis via cell membrane while DNA viruses directly affect the nucleus. This idea may be supported by the fact that there existed asynchronous DNA synthesis among multiple nuclei in a cell transformed or infected by SV40, although they shared common cytoplasm and cytoplasmic membrane.

One of the aims of recent studies on tumour viruses is to analyse the early functions of viruses which are responsible for tumourigenesis. The system we employed in this paper, that is DNA synthesis and/or karyokinesis in the presence of cytochalasin B, is undoubtedly one of the useful biological assay systems for early functions of DNA viruses.

Another application of this system is the detection of virus functions in cells persistently infected by some of the DNA viruses. This type of study is now in progress using the cells of human patients.

REFERENCES


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