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REGULATION OF SV40-INDUCED CELL DIVISION AND TUMOR ANTIGEN BY DIBUTYRYL ADENOSINE 3′-5′-MONOPHOSPHATE[1]

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Simian virus 40 (SV40) induces cell division in microcultures of sparsely plated nongrowing mouse BALB/3T3 cells during acute infection at moderate multiplicities of infection (MOI = 10–100). The infected cells are killed when a MOI of 1,000 is used. SV40 tumor (T) antigen is synthesized in the infected cells, but viral DNA, virion antigen, and progeny virions are not synthesized (abortive infection). The addition of exogenous dibutyryl adenosine 3′-5′-monophosphate (dbcAMP) at the time of infection stimulates the SV40-induced cell division at all MOI and inhibits SV40-induced cell death at high MOI. The percentage of T antigen-positive cells, as monitored by immunofluorescence, is also increased by the addition of dbcAMP at the time of infection. This regulation of SV40-induced cell division and T antigen formation by exogenous dbcAMP occurs within the first 6 hr after infection at 37°C and is dependent upon both the MOI and the concentration of added dbcAMP. The addition of dbcAMP to productively infected TC7 monkey cells has little effect on the SV40-induced cell death or T antigen formation.

INTRODUCTION

Growth of mammalian cells in culture can be regulated by both the cellular cyclic nucleotide system (1–3) and infection or transformation with simian virus 40 (SV40) (4, 5). The SV40 A gene product, tumor (T) antigen (6–8) appears to be responsible for the initiation and regulation of cell division in SV40-transformed cells (9–14). The cellular cyclic nucleotide system also regulates cell division in SV40-transformed cells (15, 16).

The mechanisms underlying the interactions that must occur between one or more SV40 proteins and the cellular cyclic nucleotide system are not known. My approach to elucidating these mechanisms has been to investigate the regulatory effects of exogenous dibutyryl 3′-5′ adenosine monophosphate (dbcAMP) on SV40-induced cell division and T antigen formation during abortive infection in BALB/3T3 cells and productive infection in TC7 monkey cells. The data presented in this paper demonstrate a stimulation of SV40-induced cell division and T antigen formation in infected microcultures of sparsely plated, nongrowing BALB/3T3 cells in Tricine-buffered medium when dbcAMP was added early after infection. Little effect was observed in infected TC7 cells.

[1] This is publication no. 4 in a series on SV40-host cell intereactions. Publication no. 3 is Robb J.A., Cold Spring Harbor Symp. Quant. Biol. 39:277–281 (1974).

METHODS

Plastic Microtest plates (Falcon) were used for all experiments. BALB/3T3 (4) mouse cells, clone A31, and TC7 (17) monkey cells were grown in modified Dulbecco-Vogt-Eagle's medium supplemented with 9% calf serum for BALB/3T3 cells (Colorado Serum Co.) or 9% fetal bovine serum for TC7 cells (Flow Labs.) and buffered at pH 7.4 with 60 mM N-Tris glycine (Tricine, Sigma Chemical Co.) and 0.5gm/L $NaHCO_3$ in WEDCO humidified air incubators as previously described (18). Wild-type SV-S (19) and the temperature-sensitive mutant, *tsD*101* (20, 21), viruses were grown in TC7 cells from a beginning MOI of 0.001 at 33°C, and viral lysates were prepared as previously described (22). The TC7 cell lysate used for mock infections was prepared in exactly the same manner as the viral lysates, except for the absence of viral infection. The cells were infected by removing them from 250-ml plastic flasks (Falcon) with 0.05% twice-crystallized trypsin-0.02% EGTA, mixing them in suspension with mock or viral lysates, and plating them into Microtest wells as previously described (23). Mock infections were carried out using the same concentration of TC7 cell lysate as was present in the MOI = 1,000 dilution of viral lysate. The cells were plated at a concentration that gave about 60 attached cells per well bottom at 6 hr after plating. Drugs were added at the time of virus addition or at later times. After 72 hr of incubation at 37°C, the cells were fixed and stained for SV40 T antigen and analyzed by microimmunofluorescence as previously described (23). After the number of T antigen-positive nuclei per well bottom was determined, the cells were stained with Giemsa and the total number of nuclei per well bottom was determined. Ten wells were averaged for each point. The mean value for each point from two independent experiments is given in the figures.

RESULTS

SV40-Induced Cell Death in Abortive BALB/3T3 and Productive TC7 Cells

Cell death did not occur in microcultures of sparsely plated, nongrowing BALB/3T3 cells infected with SV40 at MOIs of 1–100. At a MOI of 1,000, however, the cells were readily killed by 72 hr postinfection (pi) as shown in Fig. 1. Infection with *tsD*101* did not kill the cells, because this mutant is blocked in the uncoating stage and does not activate any known viral-induced processes at restrictive temperature (20, 21, 24). The addition of 0.2–1.0 mM dbcAMP at the time of infection completely prevented the killing (Figs. 1 and 3). The addition of 0.2–1.0 mM butyric acid or AMP had no effect on the killing. The killing of the productive TC7 monkey cells by the virus was not inhibited by the addition of even 5.0 mM of dbcAMP (Fig. 2). The dbcAMP did inhibit cell division in both the mock and virus infected TC7 cells at 72 hr pi (Fig. 2).

SV40-Induced Cell Division and T Antigen Formation in Abortive BALB/3T3 Cells

Infection of confluent, slowly growing BALB/3T3 cells with SV40 results in a stimulation of DNA synthesis and at least one round of cell division (4). Some of the infected cells continue indefinitely to divide and maintain T antigen synthesis. This process is termed stable transformation. Most of the infected cells, however, stop dividing after a few divisions, stop T antigen synthesis, and behave as if they were cured of their infection. This process is termed abortive transformation (25).

Initial experiments using microcultures of both sparsely plated and confluent cells indicated that although cell division was induced in the confluent cultures, the data derived

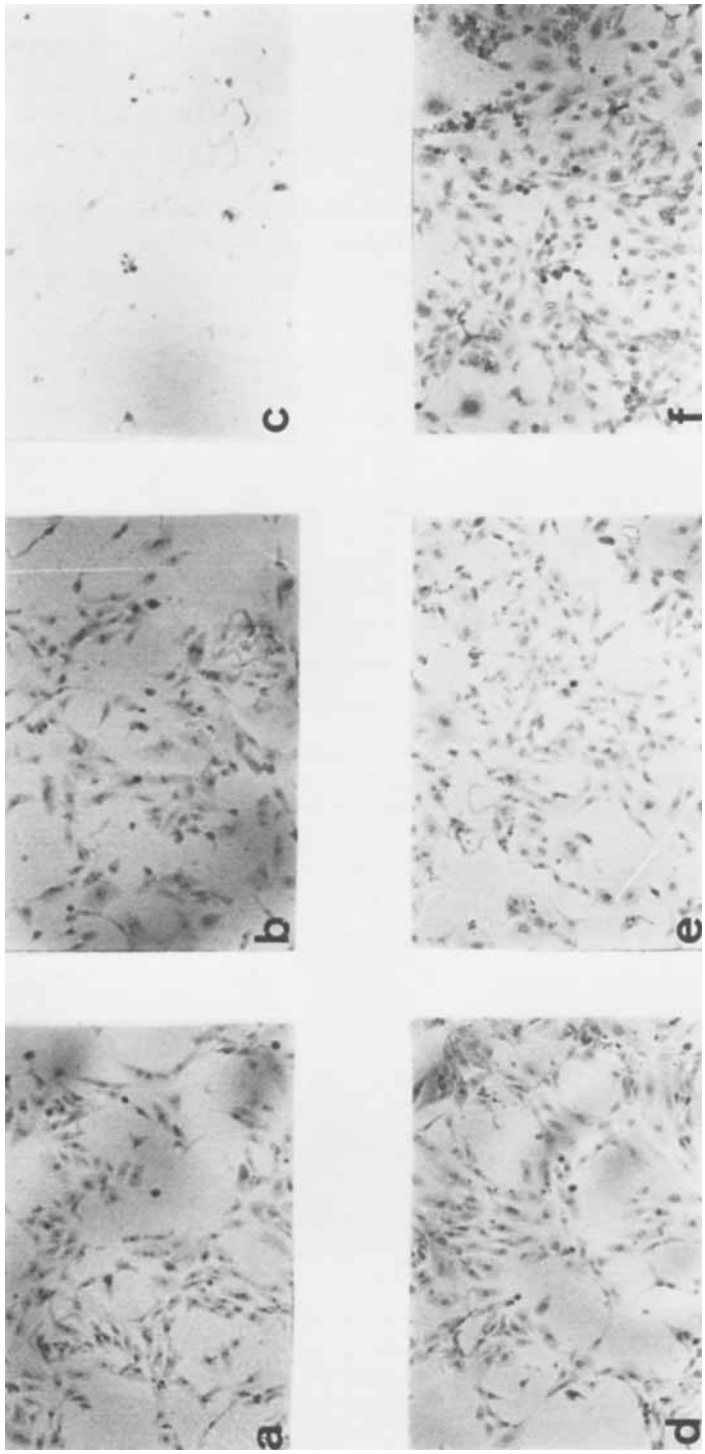

Fig. 1. Prevention of SV40-induced cell killing during abortive infection at high MOI by exogenous dbcAMP. Nongrowing, nonconfluent BALB/3T3 cells were incubated at 37°C for 72 hr pi with: (a, d) mock; (b, e) *tsD*101*; and (c, f) wild-type lysates. The MOI of the virus infections was 1,000. The infected cells in d, e, and f were incubated from the time of infection with 1.0 mM dbcAMP. The cells were stained with Giemsa. Original magnification was 320.

from them was unreliable, due to the very fatiguing process of microscopically counting more than 700 cells per well bottom (in some cases up to 2,000 cells per well bottom). This problem will be overcome through the use of a flow microfluorometer that quantitatively measures the amount of T antigen in each cell at the rate of 100,000 cells per minute. The system using microcultures of sparsely plated, nongrowing cells was chosen because the data were reliable, and the SV40-induced cell division was clearly delineated. Tricine buffer was used to minimize the drop in pH during the 3-day incubation of the 10-μl cultures.

The data in Fig. 3a demonstrate that the stimulation of cell division by SV40 was not very efficient and at high multiplicity the cells were killed. The data in Fig. 3b demonstrate that the addition of 0.2 mM dbcAMP at the time of infection greatly

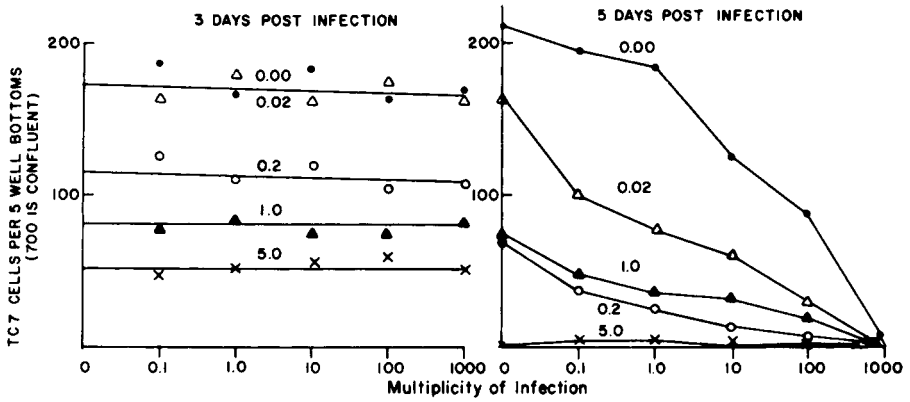

Fig. 2. Effect of dbcAMP on cell division during SV40-productive infection at 37°C in TC7 monkey cells. Growing, nonconfluent TC7 cells were counted at 72 and 120 hr after infection with wild-type SV40. The numbers above the curves indicate the concentration of dbcAMP in mM.

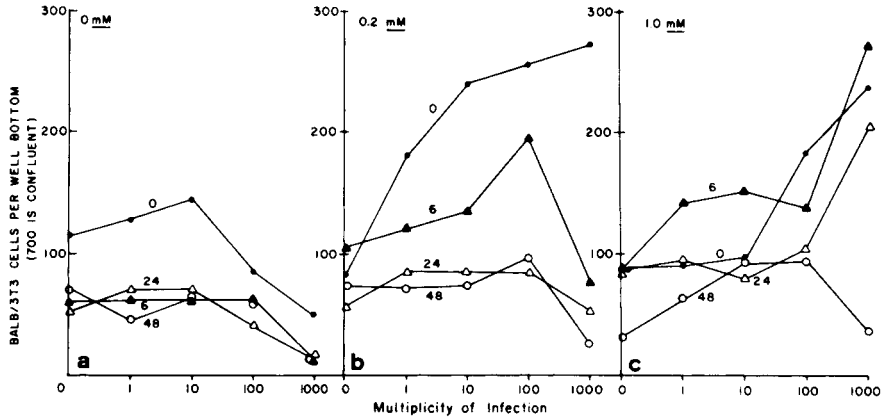

Fig. 3. Effect of dbcAMP on cell division 72 hr after SV40-abortive infection in BALB/3T3 mouse cells at 37°C plotted to emphasize the relationship betwen cell number and MOI. Nongrowing, nonconfluent cells were counted 72 hr after infection with wild-type SV40. The numbers above the curves indicate the time of addition of dbcAMP in hr pi. The drugs were added by putting 2 μl of serum-free medium with or without drug into the 10 μl of medium in the Microtest well with a Hamilton repeating dispenser. The final concentration of drug was 0 (a), 0.2 (b), or 1.0 (c) mM dbcAMP.

stimulated SV40-induced cell division and prevented killing at high multiplicity. Addition of 0.2 mM dbcAMP at 6 hr pi was only partially successful in stimulating cell division and inhibiting cell death, while the additions at 24 and 48 hr pi had no effect. The data in Fig. 3c demonstrate that the addition of 1.0 mM dbcAMP at 0 hr pi was inhibitory to cell division at low MOI, but stimulatory at high MOI. The 6 hr pi addition was stimulatory at all MOI, the 24 hr pi addition was effective only at the highest MOI, and the 48 hr pi addition had no effect. These data indicate that the drug operates early after infection, within 6 hr pi, when a low concentration (0.2 mM) is used, but can still affect the SV40-induced cell division and cell death even 24 hr pi when higher concentrations are used (1.0 mM). The addition of 0.2–1.0 mM butyric acid or AMP any time after infection had no effect.

The data in Fig. 4 are the same as in Fig. 3, but plotted to emphasize the relationship between the time of addition of drug and cell division rather than the relationship between MOI and cell division. The loss of effect of 0.2 mM dbcAMP by 6 hr pi is well demonstrated on this graph as well as the longer period of activity of the 1.0 mM concentration. The addition of 5.0 mM dbcAMP at 0 hr pi was toxic to the mock-infected cells, but not to the virus-infected cells.

The data in Fig. 5 demonstrate the effect of the drug on the appearance of T antigen in cells infected at a MOI of 100 and 1,000. These data are derived from the same wells as are the data in Figs. 3 and 4. At a MOI of 100, the maximal stimulation of T antigen occurred when 1–5 mM dbcAMP was added at 6 hr pi. The addition of 0.2 mM dbcAMP at 6 hr pi produced suboptimal stimulation. Although little effect was observed at a MOI of 1,000 with the 0 and 6 hr pi additions, a stimulation was observed by the 1.0 and 5.0 mM concentrations added at 24 hr pi and by the 5.0 mM concentration added 48 hr pi. These results indicate a complicated relationship between the MOI, dbcAMP concentration, and the time of addition of the drug. The data derived from the addition of 0.2–1.0 mM butyric acid or AMP at 6, 24, and 48 hr pi were similar to the data derived from the drug-free infections.

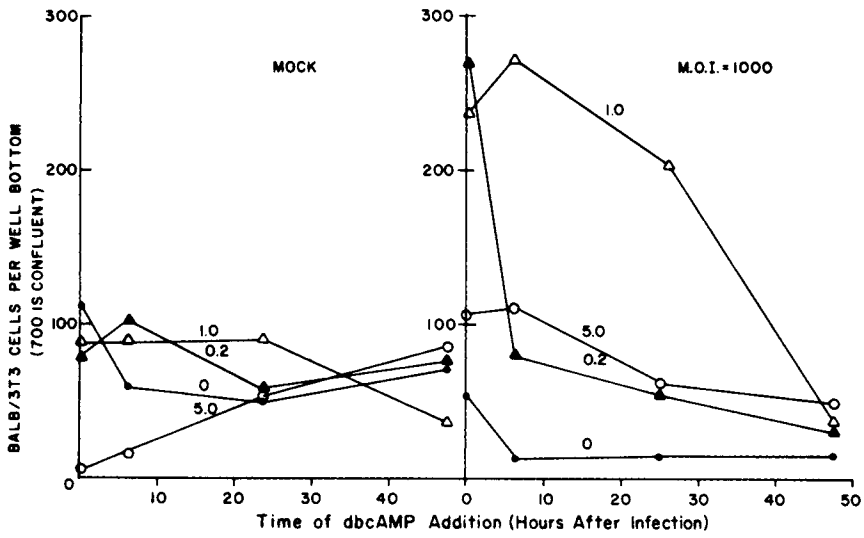

Fig. 4. The same data as in Fig. 3 plotted to emphasize the relationship between cell number and time of addition of dbcAMP. The numbers above the curves indicate the concentration of dbcAMP in mM.

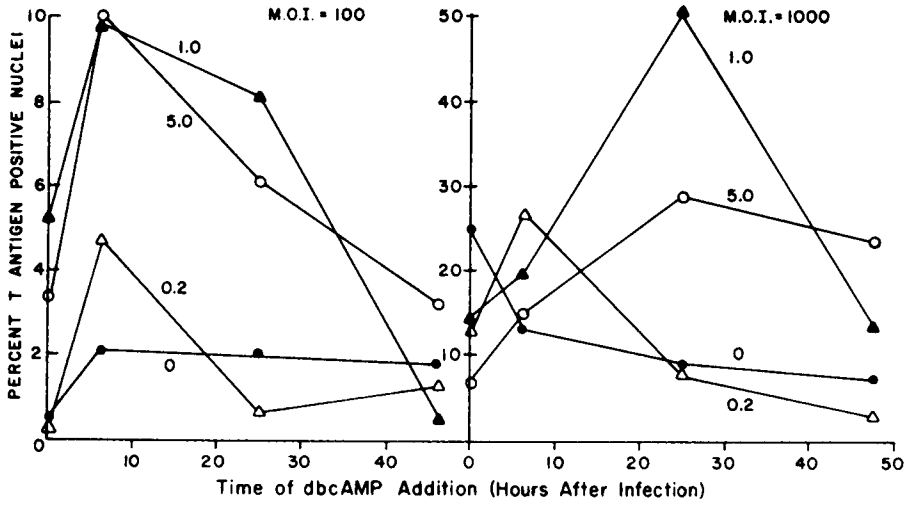

Fig. 5. Effect of dbcAMP on formation of SV40 T antigen 72 hr after SV40-abortive infection in BALB/3T3 cells at 37°C. These data are derived from the same wells as the data in Figs. 3 and 4. The numbers above the curves indicate the concentration of dbcAMP in mM.

DISCUSSION

The presence of exogenous dbcAMP during the early period of SV40 infection in microcultures of sparsely plated, nongrowing BALB/3T3 cells in Tricine-buffered medium produces a strong, but complicated regulatory effect on SV40-induced cell death, cell division, and T antigen formation. The effect of the drug depends upon the drug concentration, the multiplicity of infection, and the time of addition of the drug.

The SV40-induced cell death in BALB/3T3 cells was observed in microcultures of both sparsely plated and confluent cells (data not shown) at a MOI of 1,000. A mouse cell is not killed by SV40 infection using low to moderate MOI, while a monkey cell is almost always killed by SV40 infection independent of the MOI. The SV40-induced BALB/3T3 cell death observed in the cells infected at very high MOI has probably not been reported by other investigators, because very high multiplicity infections have not been used. The mechanism of SV40-induced cell death in the BALB/3T3 cells is not known, but might be due to the integration of large numbers of infecting SV40 genomes into cellular DNA or to extensive endonuclease cleavage of the cellular DNA. Although cAMP has a regulatory role in the integration of bacteriophage DNA into bacterial DNA (26, 27), the mechanism by which dbcAMP inhibits SV40-induced BALB/3T3 cell death, but not TC7 cell death, is not known.

The mechanism underlying the cyclic nucleotide regulation of animal cell division is not known. A working hypothesis has been that a lowered intracellular concentration of cAMP acts as a signal to initiate cell division (15, 16). Compatible with this hypothesis is the finding that the intracellular concentration of cAMP in mouse cells is rapidly lowered after SV40 infection (28). There has also developed a growing concern about the role of cGMP in the regulation of cell division (3). Furthermore, Coffino et al. (29) have recently stated that "cAMP cannot be required for or determine progression through the cell cycle."

In general, exogenous cAMP inhibits cell division including that of SV40 transformed cells. This impression has been derived from bicarbonate-CO_2-buffered macro-

cultures. In my experiments using Tricine buffered microcultures, SV40-induced cell division was separated from normal cell division. Under these conditions, exogenous dbcAMP stimulated SV40-induced cell division, but not normal cell division. Preliminary experiments using the conventional bicarbonate-CO_2 buffering system with microcultures demonstrate an inhibition of SV40-induced cell division by both mbcAMP and mbcGMP (1.0 mM added at the time of infection). The reason for the differences is not known, but the type of buffering system employed appears to influence the cyclic nucleotide regulation of SV40-induced cell division.

The appearance of SV40 T antigen was also regulated by dbcAMP. A working hypothesis to explain the regulation of SV40 T antigen and SV40-induced cell division by the cellular cyclic nucleotide system is as follows. SV40 T antigen binds to SV40 DNA at the origin of transcription (30) and probably regulates its own synthesis (6) by inhibiting the transcription of the early SV40 mRNA used for the translation of T antigen. T antigen regulates cell division by an undefined mechanism. T antigen is phosphorylated (P. Tegtmeyer, personal communication) and therefore meets the definition of a phosphorylated nonhistone DNA binding protein. The binding affinity of T antigen to DNA may depend upon its phosphorylation state in a manner similar to phosphorylated nonhistone mammalian chromosomal DNA binding proteins, as suggested by Stein et al. (31). The phosphorylation state of T antigen could be regulated by the cellular cyclic nucleotide-dependent protein kinases. One or more of the phosphorylated SV40 virion proteins (32) could also have a multifunctional role and regulate the transcriptional activity of the SV40 genome through a similar mechanism.

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