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Cordycepin Inhibits Induction of Murine Leukovirus Production By 5-Iodo-2′-deoxyuridine

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ABSTRACT **Cordycepin (3′-deoxyadenosine), an inhibitor of poly(A) synthesis during the processing of nuclear heterogenous RNA, blocks the production of RNA viruses induced by 5-iodo-2′-deoxyuridine in BALB/3T3 and BALB/K-3T3 cells. This inhibitory activity is not a result of either nonspecific cell killing or general cytotoxicity by cordycepin; rather, it appears to be specific, because cordycepin acts only at a critical time to inhibit virus production. These findings, together with the finding of poly(A) sequences in viral RNAs, suggest that RNA tumor viruses replicate via a transcription of proviral DNA.**

The discovery of RNA-dependent DNA polymerase by Baltimore (1) and by Temin and Mizutani (2) supports the idea that the genetic information of RNA tumor viruses can exist in an infected cell (or transformed cells) in a form of DNA termed a "provirus" (3, 4). To propagate this genetic information (stored in the provirus), transcription of the proviral DNA into RNA is obviously required. A simple model of viral replication is that the RNA product of transcription can serve as a messenger for viral-specific protein synthesis, and that this same source of RNA can also be packed into the viral particles where, on further infection, it will serve as a template for RNA-dependent DNA polymerase. Two pieces of information suggest that the 70S RNA of RNA tumor viruses has properties of some cellular mRNA. One—from the work of Siegert *et al.* (5)—is that AMV RNA can be translated to synthesize virus-specific proteins (group-specific antigens) in a cell-free lysate of *Escherichia coli*. The other—from the work of Lai and Duesberg (6), Gillespie, Marshall, and Gallo (7), and Green and Cartas (8)—is that the RNA of RNA tumor viruses contains a sequence of poly(A). The size of the poly(A) in the C-type RNA tumor viruses is greater than that found in any nontumor RNA viruses (7). This latter finding has triggered the hope of that this property can be used as a new footprint for RNA tumor viruses in cells (7); more importantly, it has cast some light on the mechanism of viral replication.

The poly(A) sequence was suggested to play an important role in the processing of heterogenous RNA and in the maturation of mRNA, both in cellular mRNA (9) and in the mRNA of DNA viruses (10). Darnell *et al.* (9) reported that poly(A) synthesis could be preferentially blocked by cordycepin (3′-deoxyadenosine). This compound has been a useful tool in the study of mRNA of adenoviruses (10). It was of obvious interest to see if cordycepin also inhibited replication of RNA tumor viruses. In this communication, cordycepin was used to study the possible role of the poly(A) sequence in virus production induced by 5-iodo-2′-deoxyuridine IdU(11), both from seemingly uninfected murine fibroblasts and from Murine Sarcoma Virus-transformed nonproducing cells. Our results show that cordycepin inhibits induction of viral production by IdU (11, 12) in BALB/3T3 and BALB/K-3T3 cell lines.

EXPERIMENTAL PROCEDURES

Cell Culture. Clone no. A31 of BALB/3T3 cells (13) (courtesy of Dr. A. Rein, Litton Bionetics, Inc.) and a Kirsten Murine Sarcoma Virus nonproducer, designated BALB/K-3T3(14) (kindly supplied by Dr. S. Aaronson, National Cancer Institute, Bethesda), were used for this study. All cells were grown in Dulbecco Modified Eagle's Medium supplemented with 10% fetal-calf serum. They were incubated at 37° with 10% CO_2.

Induction of Virus Production from Cell Lines. The induction of virus production with 5-iodo-2′-deoxyuridine, reported by Aaronson *et al.* (12), was confirmed. Cells in exponential growth were exposed to 40 μg/ml of IdU (P. L. Biochemicals) for 24 hr. Cordycepin (50–100 μg/ml, depending on the batches of cordycepin, Sigma Chemical Co.; or 20 μg/ml, Merck & Co., Inc., courtesy of Drug Development Branch, National Cancer Institute) when used, was also added to the culture at the time indicated for 24 hr. Medium was changed daily. The harvested media were stored at −20°, and the relative amount of virus particles was measured by determination of RNA-dependent DNA polymerase activity in particles released into the medium.

Measurement of RNA-Dependent DNA Polymerase Activity. Viral RNA-dependent DNA polymerase can efficiently use $(dT)_{12-18} \cdot (rA)_n$ as template-primer, while cellular DNA polymerases use it minimally or not at all (15, 16). This is by no means an absolute criterion (17), but particles released into media that have high activities can be assumed to be virus, since numerous controls of normal cells, as well as nonproducing cells, fail to show significant polymerase activity with $(dT)_{12-18} \cdot (rA)_n$ in particulate fractions of media in this assay (unpublished results). For the assay, 10 ml of harvested culture medium was first centrifuged at low speed (4000 rpm, Sorval SS-34 rotor) or passed through a Swinney filter unit (Millipore, pore size, 0.45 μm) to remove cellular debris. Virus particles were pelleted by centrifugation at 82,000 × *g* for 60 min at 4°. The pellet was resuspended in 0.3 ml of buffer containing 10 mM Tris·HCl (pH 7.9)–20 mM KCl–1 mM EDTA–1 mM dithiothreitol–50% glycerol. To assay RNA-dependent DNA polymerase activity, the 100-μl reaction

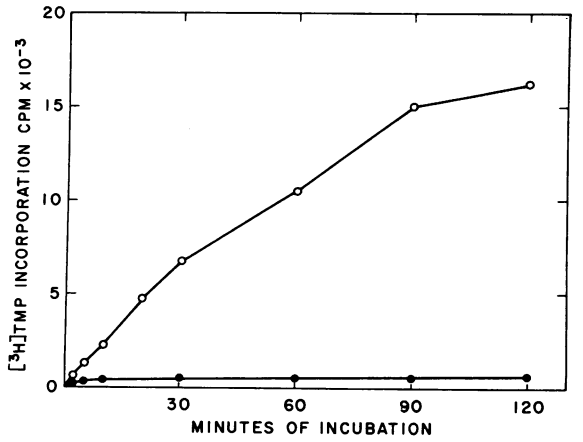

FIG. 1. "RNA-dependent DNA polymerase" activity in IdU-induced leukoviruses from BALB/K-3T3 cells. Rate of synthesis of poly(dT) with $(dT)_{12-18} \cdot (rA)_n$ as template-primer. O——O, activity from IdU-induced culture; ●——●, activity from uninduced culture.

mixture contained 40 mM Tris·HCl (pH 7.9), 60 mM KCl, 1 mM dithiothreitol, 1.5 mM Mn acetate, 0.2 mM EDTA, 0.1% Triton X-100, 20 μg/ml of $(dT)_{12-18} \cdot (rA)_n$ (Collaborative Research) 7.2 nM [^{3}H]TTP (14 Ci/mmol, Schwartz Biochemical) and 20–50 μl of virus solution. DNA was synthesized at 30° for 90 min. The rate of poly(T) synthesis in this reaction is shown in Fig. 1. Without induction, poly(T) synthesis stayed at the background level, while with IdU, synthesis continued to increase for 90 min. The ratio of the polymerase activity with $(dT)_{12-18} \cdot (rA)_n$ as template to that with oligo-$(dT) \cdot (dA)_n$ as template was about 5–10. This ratio is characteristic of most RNA-dependent DNA polymerase (15, 16). The reaction was stopped by chilling and by adding 3 ml of 10% trichloroacetic acid–0.02 M Na pyrophosphate. The acid-precipitable radioactivity was measured.

Determination of Cell Number. Monolayer cells were trypsinized, and the cell number was counted in a hemocytometer. Duplicate plates were always used for cell counts.

RESULTS

Cordycepin Inhibits Induction of Leukovirus Production. If induction of leukovirus production in nonproducing cells requires transcription of proviral DNA and synthesis of a poly(A) sequence in the early period of induction, the presence of cordycepin in this critical period should prevent successful induction. As shown in the Fig. 2 with BALB/K-3T3 cells, RNA-dependent DNA polymerase activity, which measures the relative amount of virus production, reaches a maximum 3–4 days after induction. The time required to reach maximum virus production varied in different experiments, ranging from 2–5 days after induction. The amount of virus production also varied in different experiments. These variations may reflect the stage of the cell cycle of different cell populations during induction. Virus production was completely blocked when 100 μg/ml of cordycepin was given simultaneously with IdU for 24 hr (Fig. 2). The degree of inhibition also varied in different experiments, normally ranging from 80–99% inhibition. Similar results were obtained when BALB/3T3 cells were used. Thymidine and actinomycin D can also block induction of virus production by IdU. Thymidine presumably blocks

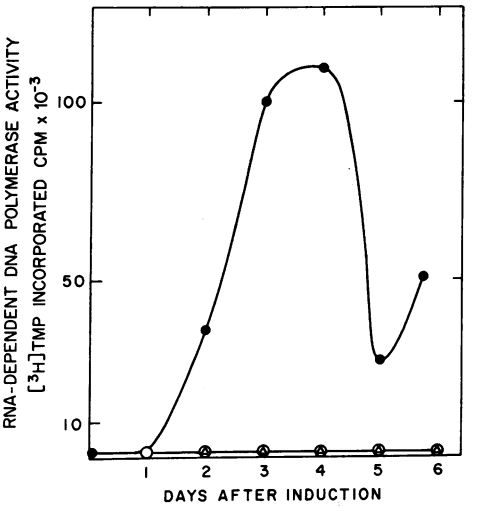

FIG. 2. Rate of induction of virus production by IdU, and its inhibition by cordycepin in BALB/K-3T3 cells. 10^6 cells were plated in a 100-mm petri dish. IdU was added 2 days after plating the cells. Days of induction start with the addition of IdU. ●——●, IdU (5-iodo-2′-deoxyuridine); Δ——Δ, IdU and cordycepin; O——O, Control.

the incorporation of IdU into DNA (11), and actinomycin D caused extensive cell killing (Fig. 3). However, as will be discussed below, the mechanism of action of cordycepin resembles neither of these two cases.

Effect of Cordycepin, IdU, and Actinomycin D on Cell Growth. Since poly(A) synthesis is necessary for normal cell growth (9), inhibition of poly(A) synthesis by cordycepin (9) would be expected to depress cell growth. In fact, this is the case, as shown in Fig. 3. Exposure to cordycepin alone or simultaneously with IdU caused a transient depression of cell growth followed by a higher saturation cell-density. This is in contrast to the effect of IdU alone, which caused a long-term

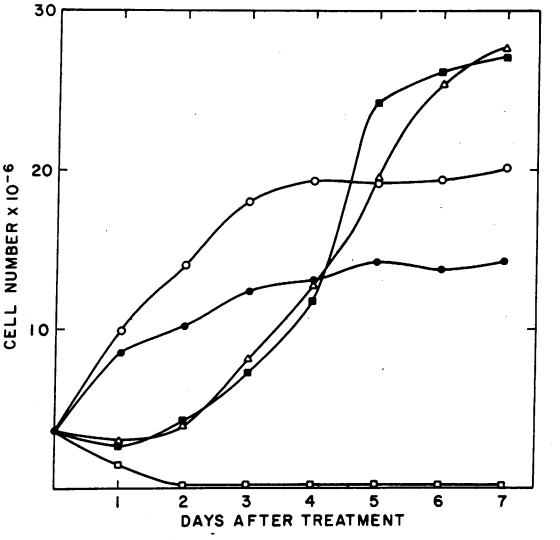

FIG. 3. Cell count of BALB/3T3 cells treated with IdU, cordycepin, and actinomycin D for 24 hr. O——O, control; ●——●, IdU (40 μg/ml); Δ——Δ, IdU and cordycepin (40 μg/ml and 100 μg/ml, respectively); ■——■, cordycepin (100 μg/ml); □——□, actinomycin D (0.04 μg/ml).

TABLE 1. *Prior treatment of cells with cordycepin does not inhibit induction of leukoviruses by IdU*

Experiment	Treatment* 0–24 hr	Treatment* 24–48 hr	Relative virus production† per 10^6 cells [^{3}H]TMP incorporation, cpm
Control	—	None	560
	—	IdU	7500
	—	IdU + Cordycepin	550
Prior treatment	Cordycepin	—	560
	Cordycepin	IdU	7550
	Actinomycin D	—	—‡

* The concentrations of the compounds were as follows: IdU 40 μg/ml; cordycepin, 100 μg/ml; actinomycin D, 0.04 μg/ml.

† The relative virus production was measured by RNA-dependent DNA polymerase activity.

‡ Induction not performed, due to death of cells.

effect of a lower saturation cell-density. Such a toxic effect might be caused by incorporation of IdU into DNA and/or by virus production. At the time when maximal virus production occurred (3–4 days after induction), the cell number in an IdU-induced culture was only about 60% that of an uninduced culture, while the cell number in a cordycepin-treated cultured was comparable to that in the IdU-induced culture. Therefore, the transient depression of cell number by cordycepin cannot account for the dramatic inhibition of induction. The effect on cell growth of cordycepin is quite different from that of actinomycin D. Actinomycin D, at a concentration as low as 0.04 μg/ml, killed almost all of the cells in 2 days (Fig. 3). It is obvious that the inhibition of induction by cordycepin is not a result of a similar nonspecific cell killing.

To further support the argument that the initial depression of cell growth by cordycepin is not the main cause of the inhibition of induction, an experiment was designed in which cells were treated with cordycepin for 24 hr, after which cordycepin was removed and IdU was added for a further 24 hr. If just a depression of cell growth could cause an inhibition of induction, the culture treated with cordycepin should not be inducible; alternatively, if a specific inhibition of cell growth [such as inhibition of poly(A) synthesis] was required for inhibition of induction, this cordycepin-treated culture should be inducible. The results of this experiment, shown in Table 1, indicate that the treated culture was inducible. This means that nonspecific depression of cell growth is not the cause of blockage of induction of virus production by cordycepin at the concentration used (100 μg/ml) for most experiments. The last row of this table shows that prior treatment of cells with a low concentration of actinomycin D resulted in so much cell killing that induction of virus production was no longer possible. Again, this result is quite different from the case of treatment with cordycepin.

Naturally Occurring Nucleosides that Contain Adenine Do Not Inhibit Virus Production. In tissue culture, exposure to a relatively high concentration of naturally occurring nucleosides is toxic to cells. Since cordycepin is an adenine-containing nucleoside, we tested whether this nucleoside-related toxicity was a cause of inhibition of leukovirus induction by cordycepin. The effect of adenosine, 2′-deoxyadenosine, and cyclic AMP, as well as cordycepin, on IdU induction of leukovirus production was studied. Table 2 shows that none of the nucleosides tested, except cordycepin, inhibits the induction of leukovirus production to a significant degree in BALB/3T3 and BALB/K-3T3 cells. The concentration of all compounds was 100 μg/ml (about 0.3 mM), which is cytotoxic. Apparently, cordycepin acts rather specifically to inhibit virus production, and nucleoside-related cytotoxic effect is not a cause of this inhibition.

Cordycepin Acts Only at a Critical Time Interval in the Early Period of Induction. In order to provide more evidence that the inhibition of induction by cordycepin is not due to a toxic effect and, more importantly, to further study the specificity of cordycepin, cordycepin was given at various times during the course of induction and its effect on virus production was measured (Fig. 4). At each time point, cordycepin was given for 24 hr. The maximum inhibition of induction was obtained only when cordycepin was added simultaneously with IdU. This inhibitory effect decreased when cordycepin was given later than 24 hr after induction. This result suggests that cordycepin works only at an early critical period, which varied from 24 to 48 hr, probably depending on the phase of the cell cycle of the cell populations. The lack of inhibition of virus production by later exposure to cordycepin is additional evidence that cytotoxicity is unlikely as the cause of inhibition of induction.

Since both IdU and cordycepin were present in the medium in the first 24 hr of treatment, cordycepin might simply block the incorporation of IdU into DNA, and cordycepin inhibi-

TABLE 2. *Effect of cordycepin, other adenine-containing nucleosides, and cyclic AMP on IdU induction of BALB/3T3 and BALB/K-3T3 cells*

Compound	BALB/3T3 RNA-dependent DNA polymerase* (cpm)	BALB/3T3 % Inhibition† of induction	BALB/K-3T3 RNA-dependent DNA polymerase (cpm)	BALB/K-3T3 % Inhibition of induction
None	8700	0	4000	0
3′-Deoxyadenosine	1020	88	620	85
2′-Deoxyadenosine	6200	29	5268	0
Adenosine	5700	34	4058	0
Dibutyryl-cyclic AMP	6000	31	—	—

* The data for RNA-dependent DNA polymerase activity were taken from the peak activity of each culture, as shown in Fig. 2.

† The percentage of inhibition of induction was calculated as follows:

$$\left[1 - \frac{\text{Enzyme activity from cordycepin-treated induced culture}}{\text{Enzyme activity from untreated induced culture}}\right] \times 100\%$$

tion may have little to do with the effect on transcription of viral RNA. In order to rule out this possibility, an experiment was designed such that the cultures were exposed to IdU for various time intervals (8, 12, 18, and 24 hr). The IdU was then removed, and cordycepin was added for 24 hr. As a control, one set of cultures was exposed for the same time intervals to only IdU and another set was exposed for the same time intervals to both IdU and cordycepin simultaneously. The results (Table 3) show that exposure to IdU for 18 hr is required for maximum induction (second column). Probably this is the time required for most of the cells to go through one cell cycle in this experiment. When cordycepin was given simultaneously with IdU in the same interval (see third column), no inhibition of induction was observed in the 12-hr treatment, but complete inhibition was observed both in the 18- and 24-hr treatments. This finding suggests that cordycepin acts at a time interval between 12 and 18 hr after induction. If cordycepin was given for 24 hr after the removal of IdU at various intervals (see last column), complete inhibition of induction was obtained when cordycepin treatment was started at 8 and 12 hr, but only slight inhibition was noted if treatment was started at 18 and 24 hr. This observation confirms the results shown in the third column of this table, that cordycepin inhibition is most effective between 12 and 18 hr, and that this inhibition can occur after removal of IdU from the medium. This experiment demonstrates clearly that the critical time for cordycepin action is 12–18 hr after induction and that the presence of IdU during this time interval is not required for cordycepin action. As mentioned before, since the status of the cell cycle of the cell population may affect the rate of virus production, the effective time period for cordycepin action may also be affected by the cell status in the cell cycle. Therefore, cordycepin most likely acts specifically to block some important event leading to virus production. This event is likely the transcription of proviral DNA and the maturation of mRNA, in which poly(A) synthesis probably plays an important role.

DISCUSSION

Virus production in murine cell lines can be achieved by induction of nonproducers (BALB/K-3T3) and seemingly uninfected cells (BALB/3T3) with IdU. This induction procedure provides a relatively simple system to study gene expression of proviral DNA. The fact that cordycepin, an inhibitor of poly(A) synthesis, inhibited the induction of virus production supports the idea that virus production requires a successful transcription of the provirus genome. This successful transcription requires the synthesis and processing of heterogenous RNA, a process in which poly(A) sequences play an important role. To prevent this successful transcription, cordycepin could act either directly to block the poly(A) sequence of unprocessed viral RNA, or indirectly to abort transcription of some messengers whose protein products are required for expression of the proviral genome. Our results do not distinguish between these two alternatives. However, the finding of poly(A) sequence in viral 70S RNA suggests that the former possibility is more likely the case. Furthermore, interpretation of our findings based upon this possibility predicts a post-transcriptional addition of poly(A) to unprocessed viral RNA (10). In fact, this prediction has been supported by the recent findings of Reitz, Gillespie, and Gallo (unpublished data) that the poly(A) sequence of viral 70S RNA is not copied in a DNA synthesis reaction performed *in vitro* primed by endogenous viral RNA. This conclusion was based on the lack of the hybridization between poly(A) and the DNA products. If these DNA products do represent the proviruses in cells, the RNA obtained from transcription of the provirus DNA will not contain a poly(A) sequence. For this RNA to obtain a poly(A) sequence, post-transcriptional addition must be required, as in the case of processing of cellular heterogenous RNA. Therefore, our findings, and those of many others, support a model of RNA tumor virus replication via transcription. This model can accommodate both the protovirus hypothesis of Temin (18) and the oncogene hypothesis of Huebner and Todaro (4).

We also find that cordycepin depresses virus production in some virus-producing cells and that it inhibits focus formation by Murine Sarcoma Virus (manuscript in preparation). Therefore, the above-mentioned mechanism of viral replica-

TABLE 3. *IdU induction and cordycepin inhibition*

Period of treatment with IdU or cordycepin (hr)*	Relative virus production†		Period of treatment*		Relative virus production†
	with IdU	with IdU and cordycepin	with IdU‡	with cordycepin‡	
0–8	110.4	—	0–8	8–32	1.7
0–12	220.8	199.8	0–12	12–36	1.6
0–18	297.4	1.5	0–18	18–42	118.2
0–24	285.3	1.0	0–24	24–48	210.4

* Time 0 is the time of addition of IdU.

† As indicated by RNA-dependent DNA polymerase activity (cpm $\times 10^{-3}$). The data are based on activity at the time of maximum virus production. (see Fig. 2).

‡ In these experiments, the cells are first treated with IdU for the indicated periods; the IdU is then removed and cordycepin is added for the subsequent intervals as indicated.

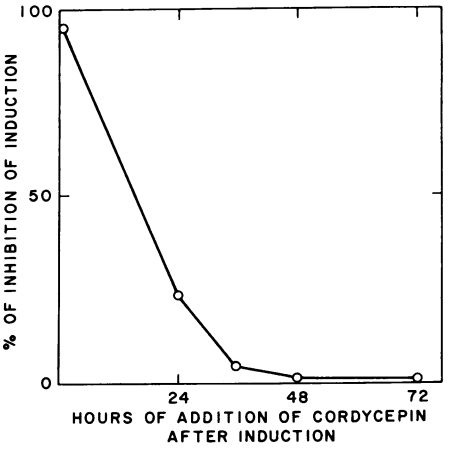

FIG. 4. Effect of time of addition of cordycepin during the course of leukovirus induction of BALB/K-3T3 cells by IdU. The procedure is the same as Fig. 2, except that cordycepin was given to the culture for 24 hr at various times during the course of virus induction. The RNA-dependent DNA polymerase activity from the peak of each induced culture was taken for calculation of the percentage of inhibition of induction (see Table 1).

tion may also be applicable to conditions of viral production other than those induced by IdU.

A transient exposure to cordycepin caused a transient depression in cell growth. This finding is different from the irreversible cell killing caused by low concentrations of actinomycin D. However, neither depression of cell growth nor nonspecific cell killing is the cause of cordycepin inhibition of leukovirus production induced by IdU. This inhibition appears to be relatively specific, because of the lack of inhibition by other adenine-containing nucleosides and, more importantly, because of the requirement of a certain critical time early in induction for effective action of cordycepin; however, the mechanism of action of cordycepin on the inhibition of virus production is uncertain. Most likely it acts to block poly(A) synthesis, but general inhibition of RNA synthesis cannot be ruled out, because cordycepin, although it preferentially blocks poly(A) synthesis of nuclear heterogenous RNA, can also inhibit mitochondrial heterogenous RNA synthesis (19, 20) and ribosomal RNA synthesis (20). Our findings do not bear on the location of the provirus nor the location of its transcription.

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