PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 150, 650–653 (1975)

Cordycepin Inhibition of 3-Methylcholanthrene-Induced Transformation *In Vitro*[1] (39098)

PAUL J. PRICE, WILLIAM A. SUK, ROBERT L. PETERS, CAROLYN E. MARTIN, TERESA M. BELLEW, AND ROBERT J. HUEBNER

Microbiological Associates, and the National Cancer Institute, Bethesda, Maryland 20014

We have described previously a Fischer rat embryo system that can be used to identify carcinogenic chemicals (1). This cell line (F1706) and its clone (H43) are negative for type "C" RNA virus expression (as determined by testing for gs antigen and RNA dependent DNA polymerase) prior to transformation. However, the gs-1 antigen of the endogenous type C RNA rat virus is often expressed in the transformed cultures (2), and virus can be induced by 5-iodo-2′-deoxyuridine (IdU) in both transformed and control cultures (I. Shif, personal communication). The antiviral antibiotic streptonigrin (3, 4) was shown by us to inhibit at nontoxic levels not only the stimulation of endogenous virus by IdU but also the *in vitro* transformation of the cells by the polycyclic hydrocarbons 3-methylcholanthene (3MC), 7,12-dimethylbenzanthracene (DMBA), and benzpyrene (BP), and to render the cells nontumorigenic in newborn rats (5). We report here that a second antiviral antibiotic, cordycepin (3-deoxyadenosine, Sigma Chemical) (6–8), at levels of 5–10 μg/ml, inhibits the induction of endogenous virus by IdU and protects the H43 Fischer rat embryo cells from transformation by 3MC.

Materials and methods. Reduction in plating efficiency relative to a medium control was used to determine the toxicity of cordycepin (Cd). One hundred cells (H43 P_{14}) in 5 ml of complete medium were added to each 60 mm plastic dish (Lux). After a 2-hr incubation at 37°C (to allow cell attachment), the medium was decanted and replaced with a fresh medium now containing Cd. Five days later the cells were fixed and stained (Giemsa), and macroscopic colonies were counted.

In the first experiment, Cd at a dose of 10 μg/ml was incorporated into the medium 24 hr prior to and during treatment of the cells with 0.1 μg/ml of 3MC and at every feed and transfer thereafter. 3MC was only incorporated for 1 wk (one transfer). In the second experiment, half of the cultures were given Cd at a concentration of 5 μg/ml at every feed and transfer while the other half was treated with medium containing Cd only during the 1-wk treatment with 3MC. In this experiment, 3MC was used at final concentrations of 0.1 and 1.0 μg per ml. The growth medium consisted of Eagle's minimal essential medium in Earle's salts (EMEM) suplemented with 5% dialyzed calf serum, 2 m*M* L-glutamine, and 100 μg gentamycin per ml. The 3MC was diluted in acetone to 1000 μg/ml, and further diluted in the medium to the desired concentration. H43 (a clone derived from the F1706 cell line at P_{104}) was treated at subculture 10 in the first experiment and at passage 17 in the second experiment. At each subculture, one set of flasks was set aside to be held for 4 wk without subdivision (holding or "horizontal" series) and the other set subdivided 1:2 weekly to provide two new sets of cultures, one for the holding series and one for subdivision. Transformed cultures were characterized by the appearance of progressively growing foci of cells lacking contact inhibition and orientation. Tumorigenicity was determined by subcutaneous inoculation of 1×10^6 cells into newborn Fischer rats (F344/f Mai).

For induction of endogenous virus, the H43 cells were planted at a concentration of 100,000 cells/ml and 24 hr later (cultures about 80% confluent) treated with 20 μg/ml IdU (in the dark) and either 0, 5, or 10 μg Cd per ml. After an additional 48-hr incuba-

[1] This work was supported by Contracts NO1-CP-43240 and NO1-CP-33248 within The Virus Cancer Program of the National Cancer Institute.

TABLE I. TOXICITY OF CORDYCEPIN (Cd) AS DETERMINED BY REDUCTION IN PLATING EFFICIENCY[a]

Dose (Cd/ml)	Average number of colonies/ three dishes	Relative plating efficiency (%)
Media control	36	100
100 μg	0	0
50 μg	3.3	10
25 μg	9	30
10 μg	29	80
5 μg	34.66	96

[a] Relative plating efficiency is the percentage of cells giving rise to macroscopic colonies, relative to the control, which is arbitrarily set at 100%.

tion, the cultures were washed and the medium replaced with a growth medium still incorporating 0, 5, or 10 μg Cd per ml. Twenty-four hours later virus was assayed by testing the supernatant for the virus-associated RNA-dependent DNA polymerase (reverse transcriptase) (9).

Results. Using reduction in plating efficiency and colony size as the test for toxicity, the maximum dose of Cd producing no toxicity was found to be 5 μg/ml. At a level of 10 μg/ml, the plating efficiency was reduced by about 20%, but the cells appeared healthy and no reduction in the number of cells per colony was evident. Levels of 25 μg/ml or higher were quite toxic (Table I).

Experiment 1. Duplicate cultures of H43 P_{10} treated with 0.1 μg/ml of 3MC in the absence of Cd showed transformed foci four subcultures after treatment. In contrast, cultures treated with acetone alone (1:1000), Cd alone (10 μg/ml), or 3MC (0.1 μg/ml) in the presence of Cd (10 μg/ml) were negative for transformation at the time of animal inoculation nine subcultures after removal of the carcinogen. Cultures treated with 3MC in the absence of Cd produced undifferentiated sarcomas at the site of inoculation in all of 11 Fischer rats by 29 days postinoculation. Cultures treated with 3MC in the presence of Cd produced a tumor in only one of nine rats (day 63). The remaining eight rats were negative for tumors at the termination of the experiment 90 days postinoculation (Table II).

Experiment 2. Cultures of H43 P_{17} treated with either 0.1 or 1.0 μg 3MC per ml showed transformed foci three subcultures after removal of the carcinogen. When Cd (5 μg/ml) was incorporated into the medium 24 hr prior to and during carcinogen treatment and at every medium change after carcinogen treatment, the cultures were protected from transformation. All of the nine rats inoculated with cells treated nine subcultures earlier with 0.1 μg 3MC and all of the 10 rats inoculated with cells treated with 1.0 μg 3MC in the absence of Cd had undifferentiated sarcomas at the site of inoculation 27 and 50 days postinoculation. In contrast, tumor incidence in the rats inoculated with the cultures which had been treated with 0.1 or 1.0 μg 3MC/ml in the presence of Cd was reduced to one in nine and one in six, respectively (Cd incorporated in the medium prior to, during, and after 3MC treatment). When Cd (5 μg/ml) was incorporated in the medium only during 3MC treatment and then removed (at the same time as 3MC), cultures treated with 0.1 μg 3MC/ml showed no transformed foci seven subcultures after treatment. When inoculated into the newborn rats (nine subcultures after treatment), two of six rats had tumors at 60 days. The incorporation of Cd (5 μg/ml) only during the treatment period with 1.0 μg 3MC/ml did not protect the cells from transformation. Duplicate cultures showed transformed foci three subcultures after treatment. However, tumor incidence in animals was reduced to three in 10 (compared to 10 in 10 when the cells had been treated in the absence of Cd) at the termination of the experiment 60 days postinoculation (Table II).

As can be seen in Table III, Cd inhibited the induction of virus (as evidenced by reverse transcriptase activity) by IdU. At a level of 5 μg/ml (the maximum nontoxic dose for Cd), the reverse transcriptase activity was inhibited by about 56%. At a level of 10 μg/ml of Cd, the reverse transcriptase activity was reduced approximately another 10%.

Discussion. Wu *et al.* (10) have previously shown that cordycepin (3-deoxyadenosine), an inhibitor of poly (a) synthesis (11, 12), blocks the induction by 5-iodo-2′-deoxyuridine (IdU) of the type "C" RNA virus in BALB/c-3T3 and BALB/K-3T3 cells. The

TABLE II. CORDYCEPIN (Cd) PROTECTION FROM TRANSFORMATION

Treatment	Transformation (passage first observed)	Tumors[a, b]
Expt. 1		
0.1 μg 3MC/ml	+ (4)	11/11 (29)
10 μg Cd/ml	− (9)	0/11 (90)
0.1 μg 3MC + 10 μg Cd/ml[c]	− (9)	1/9 (90)
Acetone control	− (9)	0/12 (90)
Expt. 2		
0.1 μg 3MC/ml	+ (3)	9/9 (27)
1.0 μg 3MC/ml	+ (3)	10/10 (50)
(Cd included in medium 24 hr prior to, during 3MC, and continuously.)		
5 μg Cd	− (7)	1/9 (60)
5 μg Cd + 0.1γ 3MC	− (7)	1/9 (60)
5 μg Cd + 1.0γ 3MC	− (7)	1/6 (60)
(Cd included in medium 24 hr prior to, during 3MC, then omitted.)		
5 μg Cd (off)	− (7)	0/9 (60)
5 μg Cd + 0.1γ 3MC (off)	− (7)	2/6 (60)
5 μg Cd + 1.0γ 3MC (off)	+ (3)	3/10 (60)

[a] No. animals with tumors/no. inoculated (by no. of days postinoculation).
[b] Newborn Fischer rats inoculated with 1×10^6 cells from Subculture 9.
[c] Cd included in the medium 24 hr prior to, during 3MC treatment, and continuously thereafter.

inhibitory activity was found not to be a result of general toxicity. They found that virus production was completely blocked when 100 μg cordycepin (Cd) per ml was given simultaneously with IdU for 24 hr. Cd has also been shown at a level of 25 μg/ml to cause a 65–75% reduction in the number of transformed foci induced by the Rauscher pseudotype of the Moloney strain of murine sarcoma virus in BALB/c mouse embryo fibroblasts (6); and at a level of 5–10 μg/ml, it causes a twofold reduction in foci formation and a seven to 35-fold decrease in virus production in NRK cells (13). Cd is equally toxic to both normal and transformed cells (14), but markedly reduces the number of cells producing virus (13).

We report here that Cd at dosages of 5 μg/ml (nontoxic) or 10 μg/ml (20% toxicity) inhibits the IdU induction of endogenous virus in our Fischer rat embryo cells and protects the cells from transformation by the polycyclic hydrocarbon 3-methylcholanthrene (3MC). Whenever Cd was incorporated into the medium throughout the experiment, protection against 3MC transformation appeared to be essentially complete. However, if Cd was removed at the

TABLE III. INHIBITION OF 5-IODO-2'-DEOXYURIDINE (IdU) INDUCED "C" TYPE VIRUS EXPRESSION (REVERSE TRANSCRIPTASE) BY CORDYCEPIN (Cd)

Treatment	Cpm of ^{3}(H) TMP incorporated per 1 ml of tissue culture fluids[a]	Percent inhibition of reverse transcriptase activity
Subculture 4[b]		
Cells + IdU	1797	54
Cells + IdU + 5 μg Cd/ml	833	
Subculture 8[c]		
Cells + IdU	7062	59
Cells + IdU + 5 μg Cd/ml	2884	
Subculture 4[b]		
Cells + IdU	1300	74
Cells + IdU + 10 μg Cd/ml	336	
Subculture 8[c]		
Cells + IdU	2455	60
Cells + IdU + 10 μg Cd/ml	976	

[a] Cpm incorporated minus H43 control (no treatment) cpm.
[b] H43 control = 497 cpm.
[c] H43 control = 277 cpm.

same time as the 3MC, oncogenicity was reduced but not eliminated (particularly at the 1.0 μg/ml level of 3MC).

Summary. Cordycepin (3-deoxyadenosine), an inhibitor of poly (a) synthesis, was found to inhibit the induction of the endogenous type "C" RNA virus by 5-iodo-2′-deoxyuridine in a line of Fischer rat embryo cells (H43) grown *in vitro*, and when continuously incorporated into the medium at those same concentrations, it was found to protect the cells from transformation by the chemical carcinogen 3-methylcholanthrene.

The authors are deeply grateful to Drs. Aaron E. Freeman, Mina Lee Vernon, and Janet Hartley for their advice in the preparation of this manuscript, and to Dr. Bernard Sass for the pathological diagnosis of the tumors.

1. Freeman, A. E., Weisburger, E. K., Weisburger, J. H., Wolford, R. G., Maryak, J. M., and Huebner, R. J., *J. Nat. Cancer Inst.* **51,** 799 (1973).
2. Freeman, A. E., Gilden, R. V., Vernon, M. L., Wolford, R. G., Hugunin, P. M., and Huebner, R. J., *Proc. Nat. Acad. Sci. USA* **70,** 2415 (1973).
3. Chirigos, M. A., Pearson, J. W., Spahn, G. J., and Rutman, R., *Bibl. Haemat.* **39,** 1208 (1973).
4. Chirigos, M. A., Pearson, J., Woods, W., and Spahn, G., *in* "Virus Tumorigenesis and Immunogenesis," pp. 335–339. Academic Press, New York (1973).
5. Price, P. J., Suk, W. A., Spahn, G. J., Chirigos, M. A., Lane, J. A., and Huebner, R. J., *Proc. Soc. Exp. Biol. Med.* **145,** 1197 (1974).
6. Lovinger, G. G., Klein, R. A., Gliden, R. V., and Hatanaka, M., *Virology* **55,** 524 (1973).
7. Paran, M., Gallo, R. C., Richardson, L. S., and Wu, A. M., *Proc. Nat. Acad. Sci. USA* **70,** 2391 (1973).
8. Wu, A. M., Reitz, M. S., Paran, M., and Gallo, R. C., *J. Virol.* **14,** 802 (1974).
9. Sarma, P. S., Jain, D. K., Mishra, N. K., Vernon, M. L., Prehn, S. P., and Pomeroy, B. S., *J. Nat. Cancer Inst.*, in press.
10. Wu, A. M., Ting, R. C., Paran, M., and Gallo, R. C., *Proc. Nat. Acad. Sci. USA* **69,** 3820 (1972).
11. Darnell, J. E., Philipson, L., Wall, R., and Adesnik, M., *Science* **176,** 507 (1971).
12. Penman, S., Rosbash, M., and Penman, M., *Proc. Nat. Acad. Sci. USA* **67,** 1878 (1971).
13. Richardson, L. S., Ting, R. C., Gallo, R. C., and Wu, A. M., *Int. J. Cancer* **15,** 451 (1975).
14. Cholon, J. J., and Studzinski, G. P., *Science* **184,** 160 (1974).

Received June 13, 1975, P.S.E.B.M. 1975, Vol. 150.