Potentiation by 2'-Deoxycoformycin of the Inhibitory Effect by 3'-Deoxyadenosine (Cordycepin) on Nuclear RNA Synthesis in L1210 Cells in Vitro

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ABSTRACT

The effect of the adenosine deaminase inhibitor, 2'-deoxycoformycin, on the inhibitory effect of cordycepin on nuclear RNA synthesis was examined in L1210 cells in vitro. The median inhibitory dose for the effect of deoxycoformycin on adenosine deaminase was 4 x 10^-7 M, and 100% inhibition was achieved at 5 x 10^-7 M. Pretreatment of cells for 30 min with 1 x 10^-6 M deoxycoformycin resulted in a reduction of the median inhibitory dose for cordycepin from 2.5 x 10^-4 to 1.8 x 10^-3 M, as assessed by the measurement of [3H]uridine incorporation into total RNA. Measurement of the synthesis of nuclear ribosomal RNA, nonpolyadenylic acid heterogeneous RNA, and polyadenylic acid heterogeneous RNA revealed potentiation by 2'-deoxycoformycin of the inhibitory effect of cordycepin on all species of RNA, as well as on polyadenylic acid synthesis. No differences were noted in the size of nuclear polyadenylic acid obtained from cells treated with cordycepin in either the presence or the absence of the adenosine deaminase inhibitor. These results suggest that the potentiation by 2'-deoxycoformycin of the cytotoxic and antitumor effects of cordycepin on L1210 cells in vivo is related to inhibition of nuclear RNA synthesis.

INTRODUCTION

The nucleoside antibiotic, cordycepin (3'-deoxyadenosine), is a potent inhibitor of nRNA synthesis in both normal (10, 29) and neoplastic (5, 18, 24, 27, 33) tissues. Although its inhibitory activity in tumor cells has been shown to be selective for RNA and poly(A) synthesis (5, 18, 24, 27, 33), this distinction has not been found in normal or regenerating liver (10, 29). One factor that is responsible for these differences is the sharp dose dependence of the inhibitory effect of cordycepin on certain species of nRNA (18). Another contributing factor may be the relative degree of catabolism of cordycepin via adenosine deaminase in various cell types. That deamination of cordycepin and other adenosine analogs plays a significant role in their inactivation has been established by the marked potentiation that inhibitors of adenosine deaminase have on their growth-inhibitory activities (1, 3, 17, 22, 28). Neoplastic cells that have high levels of adenosine deaminase (17, 20) are more sensitive to the cytotoxic action of cordycepin when pretreated with an adenosine deaminase inhibitor (17). In contrast, the action of cordycepin is only marginally increased by the adenosine deaminase inhibitor, dCF, in regenerating liver, a tissue with a 10-fold lower level of adenosine deaminase (11).

Therefore, to test whether the activity of cordycepin is qualitatively or quantitatively affected by the high level of adenosine deaminase in L1210 cells, we measured the synthesis of different types of nRNA in vitro in the presence or absence of the highly specific adenosine deaminase inhibitor, dCF.

MATERIALS AND METHODS

Materials. Cordycepin, formycin A, and dCF were kindly supplied by Dr. Harry B. Wood, Jr., Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, Md. [5,6-3H]uridine (47.9 Ci/mmol), [2,8-3H]adenosine (31 Ci/mmol), and [methyl-3H]thymidine (64.7 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. Actinomycin D was obtained from Sigma Chemical Co., St. Louis, Mo.

Animals. L1210 cells were inoculated into BALB/c x DBA/2 F1 mice at an inoculum of 10^5 cells/0.1 ml Hanks' balanced salt solution. Cells were harvested 6 days after inoculation and freed of RBC by lysis for 5 min at 4°C in 0.14 M NH4Cl:0.017 M Tris-HCl (pH 7.2) (2). Cells were washed once in Dulbecco's medium and were further diluted with the same medium to 2 x 10^7 cells/ml.

Incubations. Incubations of L1210 cells were carried out at 37°C in a shaking water bath at 100 rpm and consisted of 5 ml Dulbecco's medium, 0.25% glucose, either 5 μCi [3H]uridine (50 mCi/mmol) or 5 μCi [3H]thymidine (50 mCi/mmol), cordycepin and/or 1 x 10^-6 M dCF as indicated, and 1 x 10^7 cells. In instances in which RNA was extracted, each assay was increased 5-fold with respect to the number of cells, volume of medium, and amount of isotope.

Isotope Incorporation. Incorporation of [3H]uridine and [3H]thymidine into total RNA and DNA, respectively, was measured by cooling the incubation flasks on ice for 15 min and adding ice-cold trichloroacetic acid to a final concentration of 10% (w/v). Precipitates were then collected on glass fiber filter discs and washed 3 times with 95% ethanol. Discs were dried and counted in 10 ml of Aquasol with a Searle Mark III liquid scintillation system.

RNA Extraction. After incubation cells were centrifuged at 2000 x g for 15 min at 4°C and washed once with...
Dulbecco’s medium. Nuclei were prepared according to the procedure of Daskal et al. (6) with Triton X-100 as the detergent. Nuclear rRNA, non-poly(A) hnRNA, and poly(A) hnRNA were extracted by the sodium dodecyl sulfate:phenol extraction method previously described (10). With this procedure rRNA is extracted with equal parts of 0.1% sodium dodecyl sulfate:0.1 M sodium acetate (pH 5.1):0.005 M EDTA and phenol [phenol:m-cresol:HoO (7:2:1, v/v) containing 0.1% 8-hydroxyquinoline] followed by extraction of hnRNA from the phenol-precipitated interface with an equal volume of 0.1% sodium dodecyl sulfate:0.1 M Tris-HCl (pH 9.0):7 M urea (15). Poly(A) hnRNA was isolated on polyuridylic acid-Sepharose as described (10).

Poly(A) Isolation. Poly(A) hnRNA was digested with 20 
µg of RNase A and 20 units of RNase T1 according to the method of Eiden and Nichols (9). Incubations were similar to those with [3H]uridine except that 250 
µCi of [3H]adenosine (31 Ci/mmol) were used as labeled precursor.

Electrophoresis. Disc gel electrophoresis of either nRNA or poly(A) was carried out by the method of Dudov et al. (8) with 1.75 or 2.5% agarose, respectively, in 6 M urea:25 mM Na2HPO4:0.83 mM citric acid (pH 8.0) with 25 mM Na2HPO4:0.83 mM citric acid (pH 8.0) as the running buffer. Electrophoresis was performed at 4° at 2 ma/gel. Gels were dissolved in 0.3 ml of 60% perchloric acid and mixed with 10 ml of Aquasol. Radioactivity was determined in a Searle Mark III liquid scintillation system.

Adenosine Deaminase Assay. L1210 cells were incubated with dCF for 30 min in Dulbecco’s medium fortified with 0.25% glucose. After incubation cells were washed 3 times with ice-cold Dulbecco’s medium and centrifuged at 2000 × g. Adenosine deaminase was assayed in the supernatant fluid prepared from washed L1210 cells homogenized in 0.1 M potassium phosphate buffer (pH 7.4) and centrifuged at 100,000 × g for 1 hr at 4°. Assays contained, in a volume of 1.0 ml, 0.3 mM formycin A as substrate, 70 mM potassium phosphate buffer (pH 7.4), and approximately 0.5 mg of supernatant protein. Activity was assayed at 37° at 305 nm in a Gilford recording spectrophotometer. Specific activity was calculated with an extinction coefficient of 7.0 mm⁻¹·cm⁻¹ (32). The use of formycin A as substrate served to enhance the sensitivity of the assay since deaminase activity could be measured at a wavelength devoid of interference by proteins and nucleic acids in the 100,000 × g supernatant.

RESULTS

Incubations of L1210 cells in vitro for 30 min with dCF resulted in a dose-dependent inhibition of adenosine deaminase activity assayed in the 100,000 × g supernatant fluid (Chart 1). The ID50 for dCF was 4 × 10⁻⁸ M, but 1 × 10⁻⁶ M dCF was used in all further experiments to ensure complete inhibition of the enzyme. Moreover, although inhibition by dCF occurred within 5 min a 30-min preincubation period was selected to ensure complete inhibition of adenosine deaminase activity. Prior studies have shown that preincubation of adenosine deaminase with dCF is necessary for maximal inhibition (17). In addition, the ID50 for dCF was independent of whether adenosine, cordycepin, or formycin A served as substrate (1). L1210 cells were next incubated with [3H]uridine and [3H]thymidine to obtain an approximate measure of total cellular RNA and DNA synthesis, respectively (Chart 2). Fifty % inhibition of RNA synthesis occurred in the presence of 2.5 × 10⁻⁴ M cordycepin; however, more than a 10-fold decrease in the ID50 of cordycepin was attained by preincubation of L1210 cells for 30 min with dCF (Chart 2A). DNA synthesis was only marginally affected by cordycepin after 2 hr of incubation with the drug but was also potentiated by dCF pretreatment (Chart 2B). In the latter case the ID50 for cordycepin in the presence of dCF was approximately 10-fold higher than that required to inhibit RNA synthesis.

The time course of inhibition of RNA synthesis by cordycepin showed an even level of inhibition throughout 2 hr of labeling. In the presence of dCF, inhibition occurred more

Chart 1. Inhibition of adenosine deaminase activity by dCF. L1210 cells (10⁷ cells/flask) were incubated for 30 min in Dulbecco’s medium fortified with 0.25% glucose. After incubation cells were washed 3 times with ice-cold Dulbecco’s medium and centrifuged at 2000 × g. Adenosine deaminase was assayed in the supernatant fluid prepared from washed L1210 cells homogenized in 0.1 M potassium phosphate buffer (pH 7.4) and centrifuged at 100,000 × g for 1 hr at 4°. Assays contained, in a volume of 1.0 ml, 0.3 mM formycin A as substrate, 70 mM potassium phosphate buffer (pH 7.4), and approximately 0.5 mg of supernatant protein. Activity was assayed at 37° at 305 nm in a Gilford recording spectrophotometer. Specific activity was calculated with an extinction coefficient of 7.0 mm⁻¹·cm⁻¹ (32). The use of formycin A as substrate served to enhance the sensitivity of the assay since deaminase activity could be measured at a wavelength devoid of interference by proteins and nucleic acids in the 100,000 × g supernatant.

Graph 1

Chart 2. Dose-response effects of cordycepin on RNA and DNA synthesis. L1210 cells (10⁷ cells/flask) were incubated for 30 min with dCF, and adenosine deaminase levels were assayed in the 100,000 × g supernatant fluid as described in "Materials and Methods." Each value represents the mean ± S.E. of 4 to 6 assays.

Graph 2
dCF Potentiation of Cordycepin Inhibition of nRNA Synthesis

rapidly and reached a plateau sooner (Chart 3A). DNA synthesis was not inhibited by cordycepin alone until 4 hr after labeling. Yet, in the presence of dCF, inhibition was achieved within 2 hr (Chart 3B).

To test the specificity of action of cordycepin on RNA synthesis, we isolated 3 species of nRNA. Electrophoresis in agarose:urea gels was used to characterize the RNA fractions (Chart 4). Cordycepin treatment predominantly inhibited nuclear rRNA synthesis as shown by the equal suppression of labeling of 28S and 18S rRNA, the main rRNA species detected after a 30-min labeling period (Chart 4A). Non-poly(A) hnRNA was heterodisperse in nature, and in contrast to rat liver hnRNA (11, 15) was not of high molecular weight (Chart 4B). The inhibitory effect of cordycepin on non-poly(A) hnRNA was considerably less than on rRNA and was not restricted to a specific region of the gel. In contrast to non-poly(A) hnRNA, poly(A) hnRNA revealed a modal distribution in size of 11S, and the effect of cordycepin appeared to be restricted to this poly(A) RNA species (Chart 4C).

For further corroboration of the designation of the nRNA fractions, cells were incubated with a low concentration of actinomycin D to inhibit rRNA selectively (30). Agarose:urea gel electrophoresis of the 3 species of nRNA following treatment with actinomycin D indicated that 75% inhibition of rRNA occurred (Chart 5A), whereas non-poly(A) hnRNA and poly(A) hnRNA were inhibited 22 and 20%, respectively (Chart 5, B and C).

rRNA and poly(A) hnRNA were significantly inhibited by 2.5 × 10^{-8} M cordycepin, while non-poly(A) hnRNA was not (Chart 6). More interesting, however, was the marked potentiation of inhibition upon all 3 species of nRNA achieved with 2.5 × 10^{-8} M cordycepin in the presence of dCF. At 2.5 × 10^{-4} M cordycepin, approximately the same effect occurred as with 2.5 × 10^{-8} M cordycepin in the presence of dCF.

The kinetics of inhibition by cordycepin upon the 3
species of rRNA also differed qualitatively (Chart 7). While inhibition of rRNA (Chart 7A) and non-poly(A) hnRNA (Chart 7B) remained relatively constant throughout the 2 hr of labeling, inhibition of poly(A) hnRNA (Chart 7C) appeared to diminish with time. In the absence of dCF, no significant inhibition by cordycepin on non-poly(A) hnRNA occurred by 30 to 60 min of labeling (Chart 7B).

Agarose-urea gel electrophoresis of poly(A) obtained by digestion of poly(A) hnRNA with RNases A and T₁ indicated a size of approximately 4S in control and cordycepin-treated cells (Chart 8). No noticeable reduction in the mean distribution of poly(A) species was found, regardless of whether cells were preincubated with dCF. However, dCF alone was noted to increase the relative incorporation of [³H]adenosine into poly(A), an effect undoubtedly related to its inhibitory effect on adenosine deaminase. The effects of cordycepin and dCF are shown quantitatively in Table 1. The percentage of inhibition of poly(A) synthesis by cordycepin alone or in the presence of dCF indicates that the sensitivity of poly(A) was comparable to that of non-poly(A) hnRNA but less pronounced in comparison to the synthesis of rRNA and poly(A) hnRNA (Chart 6). The degree of potentiation of the effect of cordycepin by dCF was of the same magnitude as that found for total RNA and nRNA.

DISCUSSION

The results of this study indicate that the adenosine deaminase inhibitor, dCF, quantitatively potentiates the inhibitory action of cordycepin on rRNA synthesis while having a significant but lesser secondary effect on DNA synthesis. The degree of potentiation of cordycepin by dCF is of the same order of magnitude as that found for total RNA and nRNA.
postulated to involve chain termination of nascent RNA synthesis (19, 31). This hypothesis was based, in part, on the data of Klenow and Frederiksen (19), who found that there was no detectable incorporation of \([α-32P]3’\)-dATP into RNA with the use of Ehrlich ascites tumor RNA polymerase. Shigeura and Boxer (31) provided evidence that \([^3\text{H}]\)cordycepin has been found to be extensively incorporated into internal and terminal positions of the total RNA of a human tumor cell line (4), as well as into non-poly(A) RNA, poly(A) RNA, and poly(A) of L5178Y cells (25). Moreover, 3’-dATP was not found to cause premature chain termination of poly(A) synthesis catalyzed by liver poly(A) polymerase (26), and 1 \(× 10^{-4}\) M cordycepin produced only a 15% reduction in chain length of poly(A) synthesized in Sarcoma 180 cells (24). This study also has not given any evidence that cordycepin results in chain shortening of either RNA or poly(A).

The first studies of cordycepin documented that this inhibitor appeared to have a lesser effect upon hnRNA versus rRNA and poly(A) (5, 18, 24, 27, 33). This has been confirmed in our study only for low doses of cordycepin (2.5 \(× 10^{-5}\) M) at short incubation times (Charts 4, 6, and 7). Higher concentrations of drug or longer incubations with lower amounts of cordycepin indicated that inhibition of non-poly(A) hnRNA occurred. In addition, dCF appeared to cause primarily a quantitative change in the degree of inhibition of the rRNA fractions. The effect of dCF is undoubtedly related to the increased intracellular concentration of cordycepin or its nucleotide metabolites, or both, via reduction in the formation of 3’-deoxynosine. However, the exact site of action in vivo of this nucleoside inhibitor is unknown. Several studies have clearly shown that all eukaryotic RNA polymerases and poly(A) polymerase are equally affected by 3’-dATP in vitro (7, 16, 23, 25, 26). Recently, competitive inhibition by cordycepin of nuclear protein kinase activities has been demonstrated (12, 14), as well as inhibition of the phosphorylation of nonhistone chromosomal proteins (21). In the latter study the inhibitory concentrations of cordycepin were less or equal to the concentrations of 3’-dATP required to inhibit RNA polymerases. Thus, it is equally probable that the action of cordycepin is mediated, in part, by inhibition of other regulatory processes that might affect transcription. Alternatively, cordycepin has recently been shown to inhibit 2’-O-methylation of nRNA in L1210 cells to a greater extent than nRNA synthesis (13). This phenomenon may account for the sensitivity of RNA to this drug and suggests that inhibition of posttranscriptional processing of nuclear RNA may also be involved in the antitumor activity of cordycepin.

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