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Effect of (−)-Δ^9Tetrahydrocannabinol on Nucleoside and Amino Acid Uptake in Reuber-H-35 Hepatoma Cells

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SUMMARY

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(−)-Δ^9-tetrahydrocannabinol (Δ^9THC) was found to be a noncompetitive inhibitor for the uptake of thymidine into TCA soluble material by Reuber H-35 hepatoma cells. It also reduced uptake rates of adenosine, guanosine and cytidine. A 50% inhibition of thymidine uptake was produced with Δ^9THC concentrations of 70 μM while cytidine uptake was inhibited to the same extent at 30 μM Δ^9THC. The drug had no significant effect on the uptake of uridine, leucine and proline. Intracellular thymidine-containing nucleotide pools, isolated by thin layer chromatography after pulse labeling of the Δ^9THC treated cells with ^{3}H thymidine, were significantly diminished. Concentrations of 70 μM Δ^9THC reduced radioactive dTMP, dTDP and dTTP levels to 50% of those of the control cells. As thymidine kinase activity was not inhibited by Δ^9THC, it is concluded that Δ^9THC inhibits thymidine uptake at some step prior to formation of dTMP.

INTRODUCTION

Δ^9THC[1] has been reported to retard tumor growth in mice bearing the Lewis lung adenocarcinoma (1, 2). Studies using cells in tissue culture showed that this antineoplastic effect might be due to an inhibition of macromolecular synthesis (DNA, RNA and protein) by Δ^9THC (3-6). The mechanism by which Δ^9THC reduces macromolecular synthesis is subject to speculation. In addition different cell lines have been found to respond differently to the drug. In Lewis lung tumor cells Δ^9THC was reported to inhibit only thymidine incorporation (3), while in cultured lymphocytes both uptake and incorporation of thymidine were repressed (5). Stimulated by these findings we were led to study the effect of Δ^9THC on nucleoside and amino acid uptake by hepatoma cells. In this communication we show that Δ^9THC inhibits the uptake of various nucleosides by Reuber H-35 hepatoma cells. Furthermore, our results indicate that Δ^9THC reduces the level of intracellular thymidine nucleotides without affecting thymidine kinase activity.

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[1] The abbreviations used are: Δ^9THC, (−)-Δ^9tetrahydrocannabinol; HBSS, Hanks balanced salt solution.

MATERIALS AND METHODS

Cell culture. H4-II-E-C3 hepatoma cells, derived from Reuber H-35 hepatoma cells (7), were a gift from McArdle Laboratory, Madison, Wisconsin. Cells were grown in 75 cm^2 Falcon tissue culture flasks in a humidified 5% CO_2, 95% air atmosphere at 37°.

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The growth medium was Williams E medium supplemented with 10% heat-inactivated fetal calf serum. Tissue culture reagents were purchased from Flow Laboratories, Rockville, Maryland. No antibiotics were used. Cells were removed for enumeration by trypsinization or with the aid of a rubber policeman and quantitated using a hemocytometer. Cell viability was monitored by trypan blue dye exclusion (0.5% trypan blue).

Materials. Methyl-^{3}H-deoxythymidine, 2, 8-^{3}H-guanosine, 5-^{3}H-uridine were obtained from NEN, 5-^{3}H-L-proline and 4, 5-^{3}H-L-leucine from Schwarz, Mann. Unlabeled nucleotides were purchased from Calbiochem. All other chemicals were reagent grade. Δ^9THC was kindly provided by Prof. L. S. Harris, Virginia Commonwealth University, Richmond, Virginia. This preparation was found to be homogeneous by thin layer chromatography (8). Precoated silica gel plates from Kontes/Quantum and polyethyleneimine (PEI) cellulose plates from E. M. Laboratories were used for thin layer chromatography.

Uptake studies. 5×10^5 cells in 5 ml medium were seeded in 6 cm plastic dishes (Flow Laboratories) and 16–18 hr were allowed for cell attachment. The cells were pre-incubated with different concentrations of Δ^9THC (10 μl in ethanol) as listed in the figure legends. Δ^9THC concentrations are always referred to as the amount of Δ^9THC added to the culture dishes. No corrections were made for non-specific absorption to the plastic dishes and for serum binding. The same amount of ethanol was added to control cultures as to those treated with Δ^9THC. Whenever using concentrations of Δ^9THC of 50 μM or higher, cells were exposed to the drug for no longer than 30 min. In dishes containing 40 μM Δ^9THC, no cells could be seen detached or floating around even after overnight exposure to the drug. After a 15 min preincubation period, radioactive nucleoside was added to a final concentration as indicated in the figure legends and the dishes were further incubated for varying intervals at 37°. Uptake of radioactive label was terminated by aspirating the medium, rinsing three times with 3 ml ice-cold HBSS and adding 0.5 ml of ice-cold 5% TCA. This manipulation took 15–20 sec. After 30 min at 4°, the TCA supernatant (TCA soluble fraction) was counted in Aquasol (NEN) with a counting efficiency of 45%. The TCA precipitate, which firmly adhered to the dishes, was washed three times with 3 ml ice-cold 5% TCA, two times with absolute methanol, air-dried, solubilized with 0.5 ml of a 1 M methylbenzethonium hydroxide solution in methanol at 65° for 15 min, transferred to scintillation vials with 3 ml of methanol and counted in PPO toluene (4 g PPO/l). Unless specified otherwise, all measurements were made in triplicate sister cultures and the averages were plotted. For measurements of amino acid uptake the medium was replaced with 2.5 ml of HBSS containing 10% fetal calf serum after the 16–18 hr incubation period.

Analysis of nucleotide pools. The TCA supernatant (0.5 ml) was washed three times with water-saturated ether. During this process no radioactivity was lost. A 100 μl aliquot of the ether-washed aqueous phase was spotted together with marker (thymidine, dTMP, dTDP, dTTP) on PEI cellulose thin layer sheets and developed in one dimension essentially as described by Randerath and Randerath (9) (3 min water, 3 min 0.3 M LiCl, 6 min 0.8 M LiCl, and up to 15 cm 1.4 M LiCl). After drying, carrier spots were marked under ultraviolet light, cut out and counted in PPO/toluene (4 g PPO/l) with a counting efficiency of 12%.

Preparation and assay of thymidine kinase. Three 75 cm^2 flasks, each containing 5×10^6 cells, were incubated with 50 μM Δ^9THC, (dissolved in 75% dimethylsulfoxide/water) for 3 hr. The final DMSO content of the incubation mixture was 0.75%. Out of six control flasks, three were incubated with the drug vehicle alone. After trypsinization the cells were washed with medium, followed by lysing buffer (10 mM Tris-HCl pH 7.6, 0.5 M KCl, 20 mM $MgCl_2$, 1 mM β-mercaptoethanol, 200 μM adenosine triphosphate). The packed cells were suspended in four volumes of lysing buffer and lysed by quick freezing and thawing (four times). The degree of lysis was monitored through a microscope to ascertain that all cells were completely lysed. The 100,000 g supernatant of the lysed cells was used as

a source of thymidine kinase. The standard assay mixture (0.1 ml) contained 100 mM Tris-HCl pH 7.8, 3.3 mM β-mercaptoethanol, 10 mM ATP, 20 mM $MgCl_2$, 50 μM thymidine (specific activity 0.5 Ci/mmole), and between 50 and 150 μg of protein. Under these conditions, enzyme activity was proportional to the amount of protein added and to the time of incubation (up to 25 min at 37°). The amount of phosphorylated thymidine produced was measured as described by Breitman (10). Protein was determined by the method of Lowry *et al.* (11).

RESULTS

Uptake studies. The final ethanol concentration in the medium (0.2% or less) did not inhibit cell growth or nucleoside uptake. Radioactive thymidine was taken up at a constant rate into TCA-insoluble material both in the presence or absence of Δ⁹THC (Fig. 1a). In the TCA-soluble fraction radioactivity accumulated and reached a plateau after 20 min. At this time apparently the intracellular nucleotide pools have equilibrated with exogenously added thymidine (Fig. 1b). Both uptake into TCA soluble material and incorporation were inhibited. A lineweaver-Burk plot indicated that Δ⁹THC is a non-competitive inhibitor of thymidine uptake (Fig. 2). The K_m was estimated to be 2.2 μM which is slightly higher than the values of around 0.5 μM reported for HeLa (12), 3T3 (13) and Novikoff rat hepatoma cells (14). Nahas *et al.* (5) have reported a K_m of 0.95 μM in cultured lymphocytes.

The effect of various Δ⁹THC concentrations on the uptake of several nucleosides and amino acids is seen in Fig. 3. Even at high concentrations (10^{-4} M), Δ⁹THC did not inhibit the uptake of uridine, L-proline, and L-leucine. Uptake of thymidine, adenosine, and guanosine was affected to the same extent by Δ⁹THC with half maximal inhibition at 70 μM Δ⁹THC, whereas the inhibition of cytidine uptake was more pronounced (see Fig. 3).

Analysis of thymidine nucleotide pools. After pulse labeling with ³H-thymidine the TCA-soluble material was analyzed by thin-layer chromatography. All radioactiv-

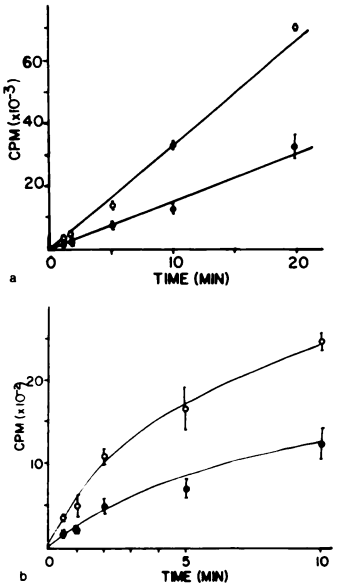

FIG. 1. *Time course of thymidine uptake by Reuber H-35 hepatoma cells*

Figure 1a represents TCA-insoluble radioactivity, fig. 1b TCA-soluble radioactivity. The final thymidine concentration was 1 μM (2 μCi/ml). O—O—O no Δ⁹THC present; ●—●—● in the presence of 70 μM Δ⁹THC. Experimental points indicate plus or minus one standard deviation. For more details refer to section on uptake studies.

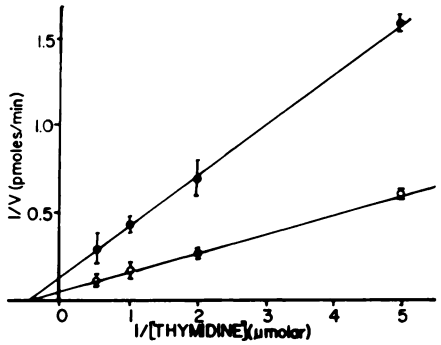

FIG. 2. *Lineweaver-Burke plot showing non-competitive inhibition of thymidine uptake into TCA-soluble material by 60 μM Δ⁹THC*

The initial rates were estimated from duplicate 1 min values. The dishes were supplemented with 0.2, 0.5, 1 and 2 μM ³H-thymidine (500 cpm/pmole). Uptake rates were calculated from the cpm per dish. O—O—O with Δ⁹THC, ●—●—● without Δ⁹THC. Points traced in figure indicate plus or minus one standard deviation.

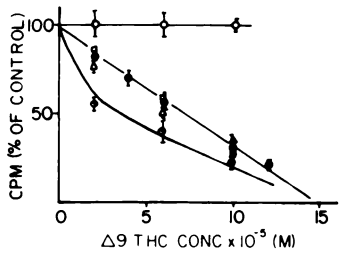

FIG. 3. *Inhibition of nucleoside and amino acid uptake by different concentrations of Δ⁹THC*

The cells were pulse labeled with the radioactive precursor for 30 min, the reaction was stopped as described in the text and the TCA-soluble radioactivity was measured. Results are expressed as % of control experiments, plus or minus one standard deviation, where the Δ⁹THC concentration was zero. The TCA-soluble radioactivity in cpm taken up by control cultures was: uridine 90,000; proline 29,000, leucine 10,200, thymidine 53,600, adenosine 752,000, cytidine 15,100, and guanosine 243,000. Symbols: —○—○— uridine (.0724 μM, 2 μCi/ml), L-proline (1.18 μM, 10 μCi/ml), and L-leucine (.002 μM, 4 μCi/ml). —●—●— thymidine (1 μM, 2 μCi/ml), □—□—□— adenosine (1 μM, 2 μCi/ml), Δ—Δ—Δ— guanosine (.336 μM, 2 μCi/ml), —⊙—⊙— cytidine (1 μM, 1 μCi/ml). The concentrations are final concentrations.

ity was recovered in the spots corresponding to thymidine, dTMP, dTDP and dTTP. No counts remained at the origin. Concentrations of 70 μM Δ⁹THC reduced the amount of label found in dTTP by about 50% (Fig. 4). The radioactivity contained in dTDP and dTMP was suppressed to the same extent (data not shown). Intracellular free thymidine levels were not affected by Δ⁹THC. The small amounts of thymidine found (ca. 100 cpm) did not increase with increasing pulse-lengths (data not shown).

Effect of Δ⁹THC on thymidine kinase activity. Lowered thymidine nucleotide pools might result from inhibition of thymidine kinase by Δ⁹THC. To confirm or discard this possibility the activity of thymidine kinase derived from Reuber hepatoma cells was measured. Neither Δ⁹THC nor the drug vehicle (final DMSO concentration in the lysate, 7.5%) inhibited the enzyme, as seen in Table 1. Preincubation of the cells for 3 hr with 50 μM Δ⁹THC prior to isolation of the enzyme resulted in no decrease of thymidine kinase activity. The specific activities of thymidine kinase shown in Table 1 were about forty times higher than those of similar preparations of the enzyme exposed to 100° for 2 min and to enzyme preparations repeatedly exposed to freezing and thawing.

DISCUSSION

Nahas *et al.* (5) demonstrated that the inhibition of thymidine incorporation by Δ⁹THC depends on the amount of serum present in the culture medium due to serum-binding of Δ⁹THC. Therefore it is likely that the actual free concentration of Δ⁹THC interacting with the cells is lower than indicated in the text or figures. The finding that Δ⁹THC did not inhibit uridine and leucine uptake (see Fig. 3) disagrees with previous reports. In studies performed with cultured lymphocytes, uridine and leucine uptake into TCA soluble material were inhibited to the same extent as thymidine uptake (5). Obviously, the results obtained from experiments with one cell line cannot necessarily be applied to other cell lines. This view is supported by other data. End *et al.* (15) showed that Δ⁹THC inhibited uptake of leucine and uridine in mouse neuroblastoma (NB2A) cells in tissue culture, whereas in rat glioma cells (C6) only leucine, but not uridine uptake, was inhibited. In addition, Δ⁹THC has been reported to have no effect on thymidine nucleotide pools in Lewis lung adenocarcinoma cells (3, 16).

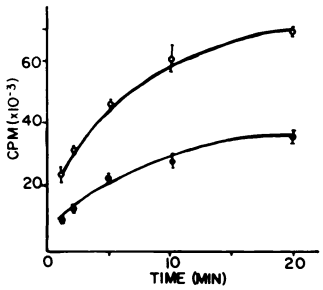

FIG. 4. *Intracellular radioactive dTPP levels as a function of time in the absence and presence of Δ⁹THC*

After labeling the cells with 1 μM thymidine (2 μCi/ml), the TCA soluble material was separated by thin-layer chromatography and the radioactivity found in the spot corresponding to dTTP was plotted. ○—○—○: dTTP in the absence of Δ⁹THC; —●—●—: dTTP in the presence of 70 μM Δ⁹THC. Points in figure plus or minus one standard deviation.

TABLE 1

Activity of thymidine kinase[a] prior to and after exposure to Δ⁹THC

Δ⁹THC-pretreated cells were incubated for 3 hr with 5×10^{-5} Δ⁹THC prior to isolation of enzyme; control cells received only solvent (75%); and no addition was made to naive control cells. A, no THC added; B, solvent added; C, 320 μM M Δ⁹THC.

Thymidine kinase derived from	A	B[b]	C	Statistical differences between[c]		
				A and B	B and C	A and C
Control cells	120 ± 4	118 ± 11	119 ± 1	N.S.	N.S.	N.S.
Naive control cells	119 ± 5	119 ± 1	120 ± 8	N.S.	N.S.	N.S.
Δ⁹THC-treated cells	119 ± 10	121 ± 14	119 ± 13	N.S.	N.S.	N.S.

[a] Expressed as pmoles phosphorylated thymidine per mg protein and per 20 min incubation at 37°.

[b] Assay mixture contained only solvent (10 μl 75% DMSO in a final volume of 0.1 ml). The final concentration of DMSO was 7.5%. Notice that in this assay, the DMSO concentration was 7.5% while in the incubation medium of the pretreated cells DMSO concentration was only 0.75% (refer to *materials and methods*).

[c] In all cases $p > 0.5$; refer to Snedecor, G. W., Statistical Methods, 5th ed., The Iowa University Press, p. 46.

Δ⁹THC inhibited uptake of thymidine into thymidine nucleotides and TCA insoluble material to the same extent, suggesting a common underlying mechanism. As Δ⁹THC decreased the amount of label found in dTTP, it is tempting to speculate that Δ⁹THC might inhibit DNA synthesis (measured as thymidine incorporation) by limiting the supply of dTTP. This view is supported by the finding that in HeLa S3 cells grown in thymidine free medium, exogenously added thymidine was incorporated into DNA almost immediately at full specific activity, blocking any further incorporation of de novo synthesized thymidine (17).

The inability to find any differences in the levels of intracellular pools of free thymidine in control and Δ⁹THC-treated cells can be explained by the loss of free intracellular thymidine by washing the cells to remove extracellular thymidine. Cass and Patterson (12) previously demonstrated that washing removes intracellular thymidine, but not thymidine nucleotides. It is a well known biochemical phenomenon that cells retain intracellular metabolites by phosphorylating them. Since cellular thymidine kinase was not affected by Δ⁹THC, even when the cells had been exposed to Δ⁹THC prior to isolation of the enzyme, we conclude that Δ⁹THC interfered with thymidine uptake in some step before the formation of dTMP. A hypothesis presented here is that membrane associated proteins involved in thymidine transport are inhibited by Δ⁹THC. It has been previously shown that Δ⁹THC does strongly inhibit lysolecithin acyl transferase and ($Na^+ + K^+$) dependent ATPase, which are membrane associated enzymes (18, 19). Further support for this hypothesis is obtained from the interpretation of the data presented in Fig. 2. The data, obtained using intact cells, may be ascribed to: (a) inhibition of an enzymatic process in the synthesis of nucleotides or (b) to the inhibition of a transport process at the membrane level, since both processes follow saturation kinetics. Having ruled out the first alternative by showing that thymidine kinase is not inhibited by Δ⁹THC, the second alternative remains as a plausible explanation for the decreased uptake of thymidine. Further investigations are in progress in this laboratory in the search for an answer to this question.

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