MOLECULAR PHARMACOLOGY, 13, 864-871

A Comparative Study of the Disposition of (−)-Δ^9-Tetrahydrocannabinol in Neuroblastoma and Glioma Cells in Tissue Culture: Relation to Cellular Impairment

DAVID W. END,[1] KENNETH THOURSEN, WILLIAM L. DEWEY, AND RICHARD A. CARCHMAN

Department of Pharmacology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

(Received September 27, 1976)
(Accepted April 19, 1977)

SUMMARY

END, DAVID W., THOURSEN, KENNETH, DEWEY, WILLIAM L. & CARCHMAN, RICHARD A. (1977) A comparative study of the disposition of (−)-Δ^9-tetrahydrocannabinol in neuroblastoma and glioma cells in tissue culture: relation to cellular impairment. *Mol. Pharmacol.*, 13, 864-871.

(−)-Δ^9-Tetrahydrocannabinol (Δ^9-THC) at a concentration of 100 μM irreversibily inhibited the proliferation of mouse neuroblastoma (NB2A) cells in tissue culture. Only a 60% reduction in rat glioma (C6) cell proliferation was observed at 100 μM Δ^9-THC, which could be reversed by removing the drug. Dose-dependent inhibition of protein, RNA, and DNA synthesis, accompanied by inhibition of precursor uptake, was observed in NB2A cells at 1, 10, and 100 μM Δ^9-THC, with the highest concentration of drug producing greater than 90% inhibition of DNA and RNA synthesis. Inhibition of DNA and RNA synthesis to less than 50% of control was observed only at 100 μM Δ^9-THC in C6 cells. The pattern of subcellular distribution of [^{3}H]Δ^9-THC in NB2A cells did not differ appreciably from the distribution of the radioactive drug in C6 cells. In both cell lines, a large amount of intracellularly bound [^{3}H]Δ^9-THC appeared in the crude nuclear fractions. NB2A cells displayed a 10-fold greater uptake of drug. This enhanced uptake of drug could be related to the greater sensitivity of NB2A cells to inhibition of growth and macromolecular synthesis by Δ^9-THC as compared with the C6 cells.

INTRODUCTION

Recent investigations have demonstrated that (−)-Δ^9-tetrahydrocannabinol and related cannabinoids can alter several basic cellular processes in mammalian cells *in vivo* and in tissue culture. Depression of the immune response following chronic exposure to cannabinoids has been observed in laboratory rodents and humans (1-3). This laboratory has previously reported on the antineoplastic activity of cannabinoids in murine tumors *in vivo* and *in vitro* (4-6). Inhibition of macromolecular synthesis and cell proliferation by micromolar concentrations of Δ^9-THC[2] has recently been demonstrated in numerous cell lines, including human lymphocytes, tetrahymena, slime mold (*D. discoideum*), and human neuroblastoma (7-10).

This work was supported in part by Grants DA-00490, CA-17840, and GM-07111 from the National Institutes of Health.

[1] Predoctoral Fellow of the National Science Foundation (5M-17622901).

[2] The abbreviation used is: Δ^9THC, (−)-Δ^9-tetrahydrocannabinol.

ISSN 0026-895X

Such a divergent array of Δ^9-THC-sensitive cells showing similar effects would suggest that a single mechanism may be involved. However, these investigations have been complicated by the preponderance of toxic biochemical alterations produced by this drug at high concentrations and by subsequent cell degeneration.

Our initial studies with rat glioma (C6) cells in tissue culture revealed that this cell line was relatively unaffected by concentrations of Δ^9-THC which severely impaired functions of mouse neuroblastoma (NB2A) cells. After characterizing the responses of these two neurally derived cells to Δ^9-THC, a comparative study of the disposition of [^{3}H]Δ^9-THC was conducted to determine whether there were binding sites unique to the NB2A cells, which could be related to the effects of the drug.

MATERIALS AND METHODS

Tissue culture. Rat glial (C6) and mouse neuroblastoma (NB2A) cells were obtained from the American Type Culture Collection. The NB2A cells were grown in Eagle's minimal essential medium containing Hanks' balanced salt solution and supplemented with 2% 5 × nonessential amino acids, 10% fetal calf serum, 2 mM glutamine, and penicillin-streptomycin (50 IU/ml–50 μg/ml). Rat C6 cells were maintained in Ham's F-10 medium supplemented with 12.5% horse serum, 2.5% fetal calf serum, glutamine (2 mM), and penicillin-streptomycin (50 IU/ml–50 μg/ml). Cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°. Tissue culture reagents were obtained either from Flow Laboratories or from Grand Island Biological Company. Under the above conditions the NB2A cells doubled in number every 19 hr, and the C6 cells, every 10 hr. Δ^9-THC was received from the National Institute of Drug Abuse.

Cell growth. Cells were transferred from dense cultures grown in 250-ml T-flasks (Falconware) to Petri dishes (60 mm) at a density of 5×10^4 cells/plate. Four hours after plating, Δ^9-THC (1, 10, or 100 μM) in ethanol or an equivalent volume (10 μl) of ethanol was added to each plate (3 ml of medium per plate). At 12-hr intervals the medium was removed and the cells were enumerated on a Coulter counter (model ZB_1). Medium and drug or drug vehicle were replenished daily.

Cell morphology. A 1–2-mg pellet of Δ^9-THC resin was placed in the center of 60-mm Petri dishes containing cultures of NB2A cells. Because of its hydrophobic nature, Δ^9-THC did not immediately go into solution. This allowed a transient diffusion gradient to form from the center to the periphery of the dishes. A piece of sterile glass equivalent to the size of the drug pellet was placed in control plates. Cell morphology was observed during a 2-hr period by means of a Nikon inverted phase microscope. Photomicrographs were made with a Polaroid attachment under a magnification of 200 ×.

Macromolecular synthesis. The cell cultures were refed twelve hours prior to the determination of uptake and incorporation of radiolabeled precursors. The cells were incubated with Δ^9-THC (1, 10, or 100 μM) or drug vehicle for 15 min prior to the addition of 1 μCi (10 μl) of either tritiated thymidine (specific activity 100 μCi/mg), uridine (specific activity 100 μCi/mg), or leucine (specific activity 200 μCi/mg) (New England Nuclear).

Following a 2-hr incubation with radiolabel, cellular uptake and incorporation of radiolabeled precursor into trichloracetic acid-soluble and -insoluble material was determined as previously described (4, 5). Each experimental point was determined in triplicate, and there was less than 10% variability within each experimental group.

Drug uptake. Confluent cell cultures in 60-mm Petri dishes were obtained as previously described, and [^{3}H]Δ^9-THC (National Institute of Drug Abuse; specific activity, 100 μCi/mg) was delivered in a 10-μl volume of ethanol to achieve a concentration of 50 μM labeled drug in 3 ml of medium. Uptake was terminated at 15 or 60 min by removing the medium and washing the cell monolayers twice with ice-cold phosphate-buffered NaCl. Cells were scraped into 3 ml of this buffer and collected by centrifugation at 2000 × g for

10 min at 4°. The pellet was digested in 2 ml of 1 N NAOH. A 0.5-ml aliquot was removed and neutralized with 1 N HCl before addition of 10 ml of Aquasol-2 scintillation fluor. Protein determinations (11) were performed on the remaining digest. For each experiment, plates were grown in parallel with the experimental groups for the determination of cell number.

Subcellular distribution of [^{3}H]Δ^9-THC. Confluent cell cultures (4–5 × 10^6 cells) were administered 50 μM [^{3}H]Δ^9-THC (specific activity, 100 μCi/mg). Following a 15-min incubation the medium was removed, and cell monolayers were washed with two 10-ml portions of ice-cold phosphate-buffered NaCl. Cells were scraped into 14 ml of ice-cold 0.32 M sucrose containing 10 μM $CaCl_2$ and homogenized in a ground glass Dounce apparatus. The homogenate was centrifuged at 1000 × *g* for 10 min to obtain a low-speed pellet, which was subsequently resuspended in 15 ml of 0.32 M sucrose and layered over 3 ml of 2.0 M sucrose for centrifugation (104,000 × *g* for 60 min) to separate nuclear debris from the crude nuclear pellet.

A mitochondrial fraction was obtained from the low-speed supernatant by centrifugation (11,400 × *g* for 20 min), and the pellet was washed once. The mitochondrial supernatant plus the 5 ml of wash were centrifuged at 104,000 × *g* for 60 min to pellet the microsomal fraction. Protein and radioactivity for each fraction were determined as previously described.

RESULTS

Cell growth. Figures 1 and 2 describe the effects of 1, 10, and 100 μM Δ^9-THC on the growth of NB2A and C6 cells over a 5-day period. Each point represents the average of duplicate plates. The ethanol vehicle did not produce a significant effect on cell proliferation in either cell line. In NB2A cultures, no significant retardation of growth was observed at 1 or 10 μM Δ^9-THC until 72 hr of exposure, at which time the cell number was reduced to approximately 75% of control. Concentrations of 100 μM Δ^9-THC were extremely toxic to NB2A cells, as evidenced by a rapid reduction in cell number, which decreased to less than 1.0% of control at the end of 5 days. These cells did not recover when 100 μM Δ^9-THC was removed on day 3.

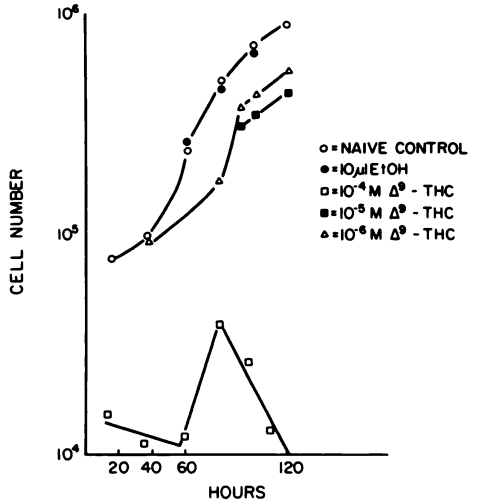

FIG. 1. *Effects of Δ^9-THC on proliferation of NB2A cells*

Cells incubated with Δ^9-THC (1–100 μM) or 10 μl of ethanol vehicle were compared with untreated control cultures. After 72 hr of exposure to 100 μM Δ^9-THC, drug was removed from the cell cultures. Each point represents the average of two plates.

No significant inhibition of C6 cell proliferation was observed at 1 or 10 μM Δ^9-THC. After 72 hr of incubation with 100 μM Δ^9-THC, the cell number was reduced to approximately 13% of control. When drug was removed from cultures previously exposed to 100 μM Δ^9-THC by refeeding plates in drug-free medium, C6 cells recovered to growth rates comparable to those in control cultures.

NB2A cell morphology. Figure 3 represents a composite of morphological changes observed in NB2A cells grown in the presence of Δ^9-THC resin. NB2A cells immediately adjacent to the Δ^9-THC pellet (Fig. 3A) appeared sparse, rounded, and extensively infiltrated with translucent vacuoles, a state indicative of cell death. In a broad band (Fig. 3B) interposed between the ring of dead cells and the periphery of the plates, the NB2A cells were differentiated, as shown by the appearance of long, axon-like processes projecting from the nucleated soma. At the periphery

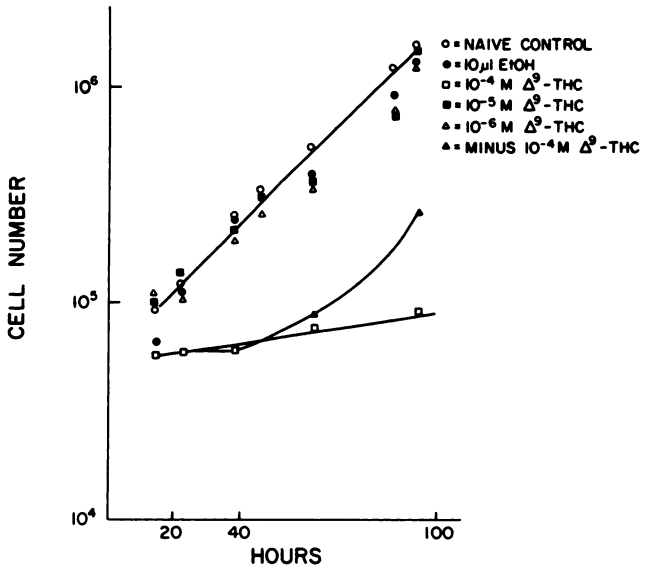

FIG. 2. *Effects of Δ⁹-THC on proliferation of C6 cells*

Cells incubated with Δ⁹-THC (1–100 μM) or 10 μl of ethanol vehicle were compared with untreated control cultures. After 72 hr of exposure to 100 μM Δ⁹-THC, drug was removed from the cell cultures. Each point represents the average of two plates.

of the plates (Fig. 3C), the cell population was predominantly rounded, with the axon-like processes absent. Cell densities were maximal (periphery), which supported the presumption that this rounded morphology reflected an undifferentiated, rapidly proliferating state. Control Plates (Fig. 3D) had mixed populations of differentiated and rounded cell types distributed randomly over the entire surface of the plates.

Macromolecular synthesis. Δ⁹-THC inhibited the incorporation of radiolabeled precursor into acid-precipitable material (DNA, RNA, and protein) in NB2A cells in a dose-dependent manner (Table 1). Thymidine incorporation appeared to be the most sensitive parameter tested; cells treated with 1 μM Δ⁹-THC had incorporation reduced to 54% of control. The cellular uptake of [³H]thymidine was similarly but less extensively inhibited. The cellular uptake and incorporation of [³H]uridine were equally inhibited by approximately 20% and 50% at 1 and 10 μM Δ⁹-THC, respectively. Tritiated uridine cellular uptake and incorporation were barely detectable at 100 μM drug (99% inhibition). Tritiated leucine uptake was more resistant to Δ⁹-THC, in that significant inhibition (62%) occurred only at 100 μM Δ⁹-THC, while leucine incorporation was inhibited 26% and 74% by 10 and 100 μM Δ⁹-THC, respectively.

NB2A cell number after 4 days of drug administration, expressed as a percentage of control, correlated quite closely with the dose-dependent inhibition of [³H]thymidine incorporation (DNA synthesis) by Δ⁹-THC 49%, 32%, and 7% vs. 54%, 32%, and 3%, respectively, for increasing concentrations of Δ⁹-THC (1, 10, and 100 μM).

Rat glioma (C6) cells, which appeared to be quite refractory to growth inhibition by Δ⁹-THC, were also more resistant than NB2A cells to inhibition of macromolecular synthesis by the drug. Only at 100 μM Δ⁹-THC was there inhibition of DNA synthesis (43% of control), while [³H]thymidine uptake was 80% of control (Table 2). Tritiated uridine uptake and incorporation were equally inhibited by the drug in a dose-dependent fashion. Δ⁹THC (1, 10, and 100 μM) reduced these parameters to 84%, 75%, and 43% of the controls. Tritiated leucine uptake and incorporation were un-

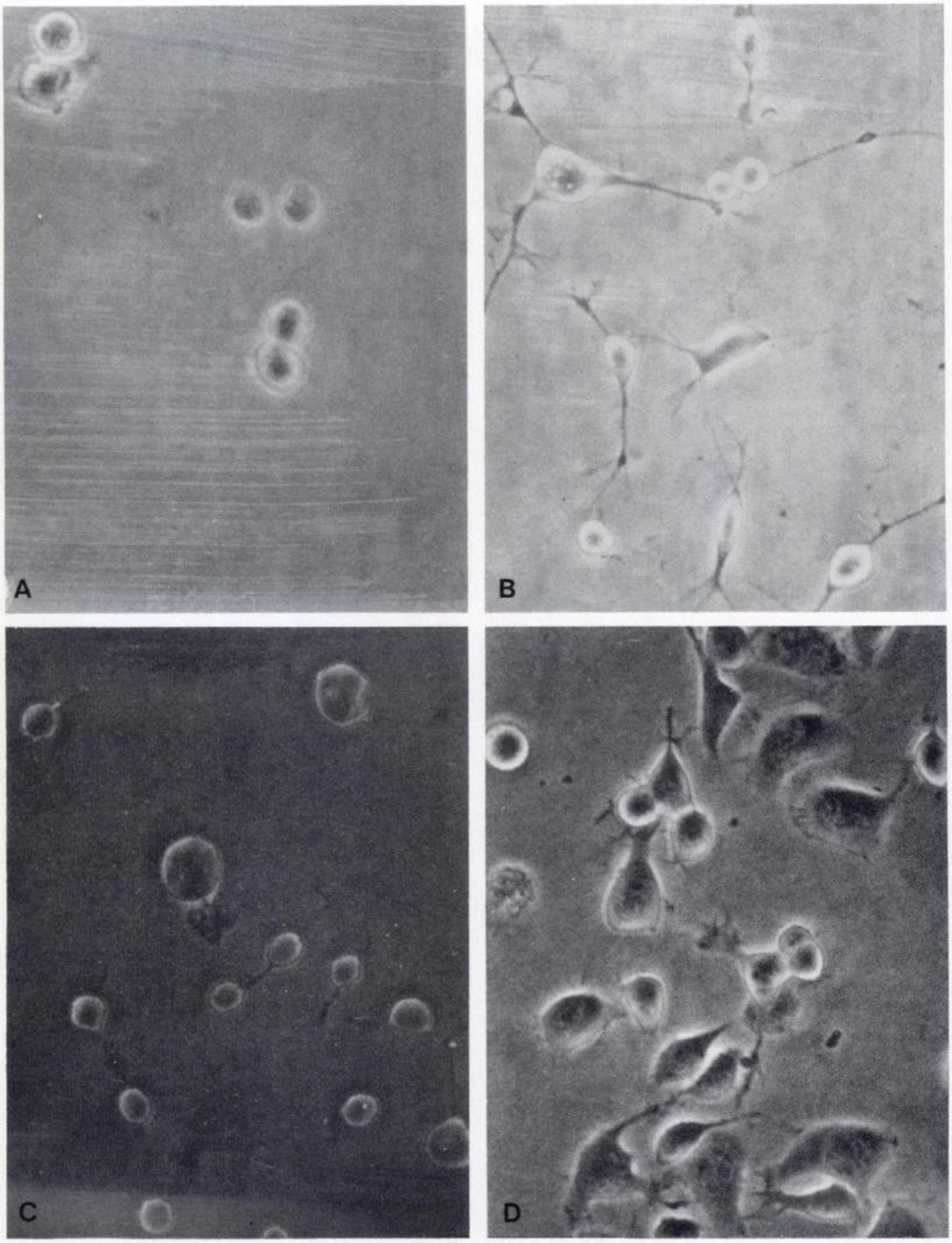

FIG. 3. *Morphological changes in NB2A cells induced by Δ^9-THC resin*

Shown are cells immediately adjacent to the Δ^9-THC pellet (A), 2 cm from the Δ^9-THC (B), and around the periphery of the 6-cm Petri dish (C), and control cells immediately adjacent to a sterile glass pellet (D). All ×200.

affected by Δ^9-THC (1–100 μM) (Table 2).

Subcellular distribution of [^{3}H]Δ^9-THC. Table 3 contains the results of our investigation into the subcellular distribution of [^{3}H]Δ^9-THC in NB2A and C6 cells, expressed as the percentage of label found in the homogenate and as tissue concentration of Δ^9-THC plus metabolites (micrograms per milligram of protein). In NB2A cells, 45% of the label appeared in the crude nuclear fraction. The concentration of [^{3}H]Δ^9-THC plus metabolites (0.78 μg/mg) was approximately twice that of the homogenate (0.40 μg/mg). The crude NB2A mitochondrial fraction showed a similar affinity for the drug, with concentrations of 0.75 μg/mg, which represented 13% of the total radioactivity. Accumulations of label elsewhere in the cells were not as striking, with 8.8% (0.25 μg/mg) in nuclear debris, 3.5% (0.34 μg/mg) in microsomes, and 8.8% (0.11 μg/mg) sus-

TABLE 1

Effects of Δ^9-THC on macromolecular synthesis in NB2A cells

Cells were incubated with Δ^9-TCH (1, 10, or 100 μM) and radiolabeled precursor for 2 hr prior to the determination of acid-soluble (uptake) and acid-precipitable (incorporation) radioactivity. Values are reported as percentages of ethanol-treated controls. Differences greater than 10% of control were significant at the $p < 0.05$ level ($n = 3$).

Δ^9-THC	Uptake			Incorporation		
	[^{3}H]Thymidine	[^{3}H]Leucine	[^{3}H]Uridine	[^{3}H]Thymidine	[^{3}H]Leucine	[^{3}H]Uridine
μM	*% control*			*% control*		
1	91	108	82	54	92	79
10	77	105	55	32	74	50
100	12	38	1	3	26	0.6

TABLE 2

Effects of Δ^9-THC on macromolecular synthesis in glioma cells

Experimental conditions were the same as in Table 1.

Δ^9-THC	Uptake			Incorporation		
	[^{3}H]Thymidine	[^{3}H]Leucine	[^{3}H]Uridine	[^{3}H]Thymidine	[^{3}H]Leucine	[^{3}H]Uridine
μM	*% control*			*% control*		
1	106	108	84	90	118	83
10	103	104	75	107	114	75
100	80	102	43	43	114	47

TABLE 3

Subcellular distribution of [^{3}H]Δ^9-THC in neurally derived tumor cells in tissue culture

Cells were incubated with 50 μM [^{3}H]Δ^9-THC for 15 min and fractionated as described under MATERIALS AND METHODS. Results are the means and standard errors of six determinations.

Cell fraction	NB2A		Glioma	
	Distribution	Concentration	Distribution	Concentration
	%	*μg/mg protein*	%	*μg/mg protein*
Homogenate		0.40 ± 0.01		0.065 ± 0.001
Nuclei	45.1 ± 1.8	0.78 ± 0.03	20.6 ± 2.1	0.079 ± 0.004
Nuclear debris	8.8 ± 0.6	0.25 ± 0.01	10.3 ± 1.2	0.029 ± 0.006
Mitochondria	13.1 ± 0.8	0.75 ± 0.05	10.5 ± 0.4	0.068 ± 0.002
Microsomes	3.5 ± 0.3	0.34 ± 0.03	7.6 ± 0.6	0.041 ± 0.003
104,000×*g* supernatant	8.8 ± 0.4	0.11 ± 0.02	28.7 ± 0.9	0.080 ± 0.008

pended in the 104,000 × *g* supernatant.

The C6 crude nuclear fraction contained 21% of the total bound [^{3}H]Δ^9-THC. This fraction of drug produced a concentration of 0.079 μg/mg, which was again greater than the 0.065 μg/mg concentration observed for the homogenate. Crude mitochondrial fractions showed no appreciable accumulation of drug (0.069 μg/mg, or 11% of the total bound label). Tritiated Δ^9-THC was also found in soluble cellular constituents, with 29% of the drug appearing in the 104,000 × *g* supernatant at a concentration of 0.08 μg/mg. Both microsomal and nuclear debris contained approximately 10% of the drug at concentrations of 0.041 and 0.029 μg/mg, respectively.

Uptake of [^{3}H]Δ^9-THC. NB2A cells took up 10 times more [^{3}H]Δ^9-THC than did C6 cells when incubated with 50 μM [^{3}H]Δ^9-THC for 15 and 60 min (Fig. 4). These results agree quite closely with the 10-fold differences in drug concentrations observed in the subcellular distribution studies (Table 3).

When C6 cells were incubated for 60 min in NB2A tissue culture medium containing 50 μM [^{3}H]Δ^9-THC, uptake of label was increased approximately 3-fold (Fig. 4). Conversely, if NB2A cells were transferred to C6 medium plus labeled drug, uptake was substantially decreased. To account for these medium dependent influences on the uptake of [^{3}H]Δ^9-THC, florisil was added to each of the culture media to precipitate the unbound drug. Aliquots of Florosil-treated media were assayed for radioactivity to measure the ratio of bound to free [^{3}H]Δ^9-THC. In C6 medium 82% of the added label was bound to medium constituents, while in NB2A medium only 43% of the label was bound.

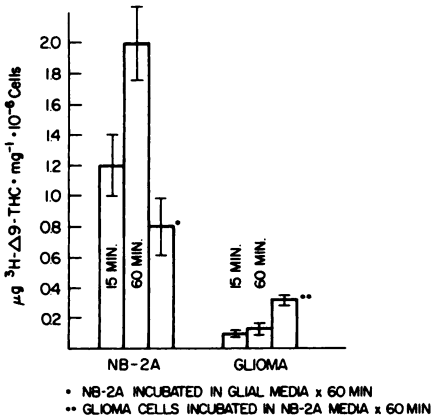

FIG. 4. *Uptake of [^{3}H]Δ^9-THC in drug-sensitive NB2A cells and drug-resistant C6 cells*

Cells were incubated with 50 μM [^{3}H]Δ^9-THC for 15 or 60 min. The means and standard errors of five determinations are shown.

DISCUSSION

Differential sensitivity to the growth-inhibitory effects of Δ^9-THC was studied in two cell lines. At 10 μM Δ^9-THC, both cell proliferation and DNA synthesis were inhibited by 70% of control in NB2A cells. Inhibition of RNA and protein synthesis (50% and 36%, respectively) accompanied these effects. These data agreed quite closely with the findings of Blevins and Regan (12) for mouse and human neuroblastoma cells exposed to Δ^9-THC in tissue culture. Rat glioma cells (C6) were resistant to the effects of 1 and 10 μM Δ^9-THC. Only at 100 μM Δ^9-THC were significant reductions in C6 proliferation and macromolecular synthesis observed. This differential sensitivity appeared to be specific for Δ^9-THC, inasmuch as C6 cells were more sensitive than NB2A cells inhibition of protein synthesis by cycloheximide.

To better characterize the mechanism of cytotoxicity of Δ^9-THC, a comparison of the disposition of labeled drug in these two cell lines was undertaken. The 10-fold greater uptake of [^{3}H]Δ^9-THC in NB2A cells could account for the enhanced sensitivity of these cells to the drug. Binding of [^{3}H]Δ^9-THC to medium constituents was shown to have a marked effect on the disposition of the drug in the two cell lines. In C6 medium, 82% of the labeled drug was bound (as opposed to 43% in NB2A medium), which restricted drug access to C6 cells, since when C6 cells were transferred to NB2A medium (which contained 3 times more free [^{3}H]Δ^9-THC), a 3-fold increase in [^{3}H]Δ^9-THC uptake was observed. This observation may explain the competitive interactions between medium serum concentration and inhibition of DNA synthesis by Δ^9-THC in lymphocytes reported by Nahas *et al.* (13).

However, when culture conditions were controlled, NB2A uptake of [^{3}H]Δ^9-THC still exceeded C6 drug uptake. This suggests that C6 cells may exclude or rapidly clear Δ^9-THC. Our data support the findings of Martin *et al.* (14) that white matter, which has its embryological origins in glial cells, accumulated less [^{3}H]Δ^9-THC than did neurally derived gray matter in the brains of dogs.

Subcellular fractionation studies revealed large accumulations of intracellularly bound [^{3}H]Δ^9-THC in crude nuclear fractions of NB2A (45%) and C6 (21%) cells. This is in agreement with the reports of Colburn *et al.* (15) and Martin *et al.* (14) that large amounts of labeled Δ^9-THC appeared in the crude nulcear fractions of mammalian brains.

This accumulation of drug in nuclear fractions, in addition to the close correlation of inhibition of DNA synthesis by Δ^9-THC with inhibition of cell proliferation in both cell lines, suggested that interference with DNA replication might be the primary event in the cytotoxicity of Δ^9-THC. The magnitude of inhibition may depend upon the amount of Δ^9-THC reaching the nucleus. The morphological differentiation observed for NB2A cells in the presence of Δ^9-THC lends further support to this hypothesis. Other DNA synthesis inhibitors, such as fluorodeoxyuridine, induce similar morphological changes (16). Furthermore, Huot (9) demonstrated that Δ^9-THC induced clumping of chromatin and other morphological changes in monkey kidney fibroblasts, and Friedman[3] has observed single strand breaks in liver DNA from mice treated with Δ^9-THC. Evidence is accumulating to suggest that Δ^9-THC may produce physical changes in the DNA template which prevent its replication and transcription.

Finally, the sensitivity of cells to Δ^9-THC appears to depend in part on the uptake of the drug by the cells. The differential uptake and sensitivity of the NB2A and C6 cells were also seen (data not shown) in Lewis lung adenocarcinoma cells and normal bone marrow cells, where the differences in sensitivity to Δ^9-THC [Lewis lung was more sensitive than bone marrow (6)] were mirrored by the larger uptake of drug by the Lewis lung adenocarcinoma cells.

[3] M. A. Friedman, personal communication.

REFERENCES

1. Nahas, G. G., Sucui-Foca, N., Armand, J. P. & Morishima, A. (1974) *Science*, **183**, 419–420.
2. Levy, J. A., Munson, A. E., Harris, L. S. & Dewey, W. L. (1975) *Fed. Proc.*, **34**, 782.
3. Nahas, G. G., Zagury, D., Schwartz, I. W. & Nagel, M. D. (1973) *Nature*, **243**, 407–408.
4. Carchman, R. A., Warner, W., White, A. C. & Harris, L. S. (1976) in *Marijuana, Chemistry, Biochemistry, and Cellular Effects* (Nahas, G. G., ed.), pp. 329–345, Springer, New York.
5. White, A. C., Munson, J. A., Munson, A. E. & Carchman, R. A. (1976) *J. Natl. Cancer. Inst.*, **56**, 655–658.
6. Carchman, R. A., Harris, L. S. & Munson, A. E. (1975) *Cancer Res.*, **36**, 95–100.
7. Zimmerman, A. M. & Zimmerman, S. B. (1976) in *Marijuana, Chemistry, Biochemistry, and Cellular Effects* (Nahas, G. G., ed.), pp. 195–206, Springer, New York.
8. Armand, J. P., Hsu, T. & Nahas, G. G. (1974) *Fed. Proc.*, **33**, 539.
9. Huot, J. (1976) in *Marijuana, Chemistry, Biochemistry, and Cellular Effects* (Nahas, G. G., ed.), pp. 307–311, Springer, New York.
11. Sutherland, E. W., Cori, C. F., Haynes, R. & Olson, N. S. (1949) *J. Biol. Chem.*, **180**, 825–837.
12. Blevins, R. D. & Regan, J. D. (1976) *Arch. Toxikol.*, **35**, 127–135.
13. Nahas, G., Desoize, B., Hsu, J. & Morishima, A. (1976) in *Marijuana, Chemistry, Biochemistry, and Cellular Effects* (Nahas, G. G., ed.), pp. 299–312, Springer, New York.
14. Martin, B. R., Dewey, W. L., Harris, L. S. & Beckner, J. S. (1975) *J. Pharmacol. Exp. Ther.*, **196**, 128–144.
15. Colburn, R. W., Ng, L. K. Y., Lemberger, L. & Kopin, I. (1974) *Biochem. Pharmacol.*, **23**, 873–877.
16. Klebe, R. J. & Ruddle, F. H. (1969) *J. Cell Biol.*, **43**, 69a.