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Cannabinoids induce incomplete maturation of cultured human leukemia cells

(Δ^9-tetrahydrocannabinol/marijuana/differentiation/promonocytes/ML-2 cells)

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ABSTRACT **Monocyte maturation markers were induced in cultured human myeloblastic ML-2 leukemia cells after treatment for 1–6 days with 0.03–30 μM Δ^9-tetrahydrocannabinol (THC), the major psychoactive component of marijuana. After a 2-day or longer treatment, 2- to 5-fold increases were found in the percentages of cells exhibiting reactivity with either the murine OKM1 monoclonal antibody or the Leu-M5 monoclonal antibody, staining positively for nonspecific esterase activity, and displaying a promonocyte morphology. The increases in these differentiation markers after treatment with 0.03–1 μM THC were dose dependent. At this dose range, THC did not cause an inhibition of cell growth. The THC-induced cell maturation was also characterized by specific changes in the patterns of newly synthesized proteins. Pronounced among these changes was an increase in the synthesis of at least 10 proteins that are found abundantly in monocytes. The THC-induced differentiation did not, however, result in cells with a highly developed mature monocyte phenotype; the THC-treated cells failed to exhibit other monocyte markers such as attachment to the surface of tissue culture dishes or morphological maturation beyond the promonocyte stage. However, treatment of these "incompletely" matured cells with either phorbol 12-myristate 13-acetate or 1α,25-dihydroxycholecalciferol, which are inducers of differentiation in myeloid leukemia cells (including ML-2 cells), produced cells with a mature monocyte morphology. Two other cannabinoids, cannabidiol and cannabinol, which were more cytotoxic than THC at comparable doses, also caused an increase in the expression of maturation markers, but at doses higher than those required for THC. The ML-2 cell system described here may be a useful tool for deciphering critical biochemical events that lead to the cannabinoid-induced "incomplete" cell differentiation of ML-2 cells and other related cell types. Findings obtained from this system may have important implications for studies of cannabinoid effects on normal human bone-marrow progenitor cells.**

Marijuana smoking or exposure of animals to the biologically active ingredients of marijuana, namely cannabinoids, including the most psychoactive component, Δ^9-tetrahydrocannabinol (THC) (1–5), causes aberrations in the function of macrophages and lymphocytes (6–10), cells that play a critical role in the immune response. These aberrations may result from changes in the maturation of bone-marrow progenitor cells that cause these progenitors to produce semimature cells with altered functional characteristics. This possibility could be substantiated by showing that the differentiation of cultured human bone marrow progenitor cells is modulated by cannabinoids. Unfortunately, the mixed cell populations that exist in human bone marrow make it difficult to study maturation events in distinct homogenous cell lineages. To circumvent this problem, we tested cannabinoids for their ability to induce cell differentiation in the human myeloblastic ML-2 leukemia cell line (11–13). This cell line was chosen because, in common with other related human leukemia cells (14–19), it displays useful markers of cell differentiation and, when treated with appropriate chemical inducers, exhibits a phenotype resembling that of mature blood cells (12, 13). Using these cells, we showed that THC and two related cannabinoids, cannabidiol (CBD) and cannabinol (CBN), induced the expression of monocyte maturation markers; these compounds, however, failed to bring about a terminal cell differentiation.

MATERIALS AND METHODS

Chemicals. THC, CBD, and CBN were obtained from the National Institute of Drug Abuse repository in Research Triangle Park, NC, and [^{35}S]methionine (1420 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham and phorbol 12-myristate 13-acetate (PMA) was from Cancer Research (Eden Prairie, MN). 1α,25-Dihydroxycholecalciferol [1,25-$(OH)_2D_3$] was a gift from M. Uskokovic (Hoffmann–La Roche). The cannabinoids were maintained in stock solution of 10–100 mg/ml of ethanol at −20°C.

Cell and Culture Conditions. The human myeloblastic ML-2 leukemia cells (11) were obtained from J. Minowada (Veterans Administration Medical Center, Hines, IL) and the human myeloblastic KG-1 cells (clone 2) from H. P. Koeffler (University of California, Los Angeles) (19). The human promyelocytic HL-205 cell clone (20) was isolated from the human leukemia HL-60 cells originally provided by R. C. Gallo (National Cancer Institute, Bethesda, MD) (15). Human monocytes were prepared from peripheral blood pooled from five individuals as described previously (21). The leukemia cells were inoculated into 60-mm Petri dishes (Falcon Plastics) at 1.5×10^5 cells per ml of growth medium (5 ml total). The growth medium consisted of RPMI 1640 medium supplemented with 20% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (GIBCO). The cultures were incubated at 37°C in an atmosphere of 8% CO_2 in air in a humidified incubator. The cannabinoids dissolved in ethanol were added to the cultures one day after initial cell seeding. PMA and 1,25-$(OH)_2D_3$ were dissolved in dimethyl sulfoxide. The final concentration of ethanol or dimethyl sulfoxide in the growth medium of control and treated cultures was 0.1%.

Labeling of Cells and Two-Dimensional Electrophoresis. One day after THC treatment of ML-2 cells incubated in 60-mm Petri dishes, 0.4-ml samples from each cell suspension

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Abbreviations: THC, Δ^9-tetrahydrocannabinol; CBD, cannabidiol; CBN, cannabinol; PMA, phorbol 12-myristate 13-acetate; 1,25-$(OH)_2D_3$, 1α,25-dihydroxycholecalciferol.

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were placed into a flat-bottomed multiwell plate. The cells in the wells were then labeled with [^{35}S]methionine (60 μCi/ml) and 18 hr later were harvested and solubilized in 50 μl of a solution containing 9 M urea, 4% Nonidet P-40, 2% (vol/vol) mercaptoethanol, and 2% ampholytes (pH 9–11; LKB) at a final pH of 9.5. Two-dimensional electrophoresis was performed with the 7 × 7 inch Iso-Dalt system (22) with a 1:1 mixture of Bio-Lyte pH 5–7 and pH 3–10 (Bio-Rad) used as ampholyte. The gels were then dried and autoradiographed on Kodak XAR-2 film for 1 week.

Reactivity with Murine Anti-Cell Surface Monoclonal Antibodies. The OKM1 monoclonal antibody, which detects a cell surface antigen common to human blood monocytes and granulocytes (23), was obtained from Ortho Diagnostic Systems. The Leu-M5 antibody, which detects an antigen specific to human monocytes/macrophages (24), was obtained from Becton Dickinson; another monoclonal antibody, B52.1.1 (25), which also detects a monocyte/macrophage surface antigen, was kindly supplied by G. Trinchieri (Wistar Institute of Anatomy and Biology, Philadelphia). Reactivity with these antibodies was detected by indirect immunofluorescence of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Coulter Immunology). The immunofluorescence was determined after 10^6 cells suspended in 200 μl of phosphate-buffered saline (PBS; Dulbecco's plus 0.1% sodium azide) were mixed with 5–20 μl of the monoclonal antibody and incubated for 30 min at 4°C. Afterward, the cells were washed twice with ice-cold PBS and resuspended in 100 μl of FITC-conjugated goat anti-mouse antibody for 30 min at 4°C. After two additional washes, the cell suspensions were mixed with a glycerol-based mounting medium and dropped on glass slides. The cells were then overlaid with a coverslip, sealed with nail polish, and examined for reactivity with the antibody. Each experimental point was based on an analysis of 200 cells from each of two separate preparations.

Nonspecific Esterase Activity. Nonspecific esterase activity was determined cytochemically (26). After centrifugation, the cells (in about 0.1 ml of medium) were spread on a glass microscope slide, air dried, and stained. The staining solution was prepared by mixing two solutions. The first contained 20 μl (40 mg) of the substrate α-naphthyl butyrate (liquid, N-8000; Sigma) dissolved in 4 ml of acetone. The second solution was prepared by mixing 0.4 ml of 4% pararosaniline (free base, P-7632; Sigma) in 2 M HCl with 0.4 ml of 4% sodium nitrite (crystalline, S-2252; Sigma) in distilled water after addition of 100 ml of 0.1 M sodium phosphate buffer, pH 7.5. Prior to staining, the combined solution was filtered and 10 drops of the filtrate were added to each slide. After 30 min at room temperature, the slides were rinsed with distilled water and counterstained with Mayer's hematoxylin (no. MHS-1; Sigma) for 10 min. The slides were rinsed in distilled water, then air dried overnight and mounted with glycerol.

Evaluation of Morphological Differentiation. For morphological evaluation of cell differentiation, control and treated ML-2 cells were spread on glass slides in the same manner as the cells were prepared for detecting nonspecific esterase activity, except that they were stained with Wright's stain (no. 840-100; Sigma). For each determination, 200–400 cells from at least two preparations were evaluated.

RESULTS

Expression of Monocyte Markers in Human Myeloblastic Leukemia Cells After Treatment with THC. During a six-day period in culture, 9–13% of control ML-2 cells reacted with the OKM1 monoclonal antibody, which detects a cell surface antigen common to mature granulocytes and monocytes (23), 19–38% of control cells reacted with the Leu-M5 monoclonal antibody, which detects a monocyte/macrophage-specific surface antigen (24), and more than 95% of control cells reacted with B52.1.1, another monocyte-specific monoclonal antibody (25). In addition to expressing these antigenic determinants, 15–17% of the control cells stained positively for nonspecific esterase activity, a characteristic of monocytes/macrophages (26); and 9–12% of the control cells displayed a promonocytic cell morphology with the remaining cells exhibiting the immature myeloblastic phenotype. Treatment of the ML-2 cells for four days with 0.03–30 μM THC resulted in an increase in the percentage of cells exhibiting monocytic maturation markers. More specifically, the percentage of cells reacting with either the OKM1 or Leu-M5 antibody increased by 2- to 4-fold; the percentage of cells staining positively for nonspecific esterase activity increased by 3- to 4-fold; and the percentage of cells exhibiting a promonocytic morphology increased by 2- to 5-fold (Fig. 1). Results obtained after either a 2- or a 6-day treatment with THC were similar to those obtained after a 4-day treatment, while those treated for only 1 day yielded results that were halfway between these and those of the control

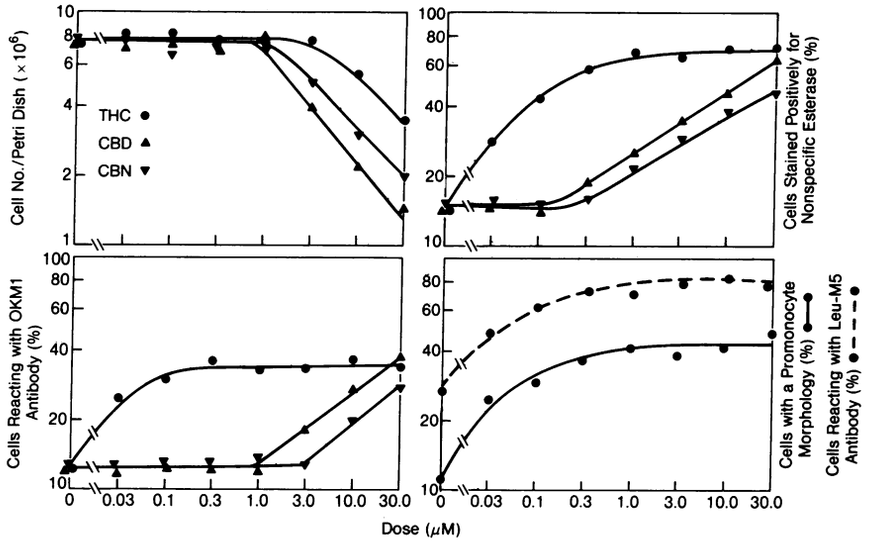

FIG. 1. Cell number and expression of cell differentiation markers in ML-2 cells treated for 4 days with THC (●), CBD (▲), or CBN (▼) at various concentrations.

cells (data not shown). The THC-induced increase in the expression of the differentiation markers was dose dependent up to about 1 μM (Fig. 1). Although the treated cells displayed an increase in reactivity with either the OKM1 or Leu-M5 antibody and an increase in staining for nonspecific esterase activity, they failed to attach to the surface of tissue culture dishes or display morphological maturation beyond the promonocytic stage, which are two other common characteristics of functionally mature monocytes and macrophages. Furthermore, induction of the maturation markers at doses between 0.03 and 3 μM THC was not coupled with a concomitant cessation of cell replication (Fig. 1), as is characteristic of blood cells that have attained functional maturity.

In addition to ML-2 cells, we included in our studies cells from another myeloblastic cell line, KG-1, as well as cells from the promyelocytic HL-205 cell line. As in ML-2 cells, THC induced in the KG-1 cells an incomplete cell maturation, which was even less pronounced than that in the ML-2 cells. Treatment for 6 days with 10 μM THC caused an increase in the percentage of KG-1 cells staining for nonspecific esterase activity and exhibiting a promonocyte morphology; these percentages were increased from <5% in the control to about 20% in the treated cultures. Again, as in ML-2 cells, control and THC-treated KG-1 cells did not exhibit a mature monocytic morphology, nor did they attach to the surface of tissue culture dishes, characteristics that are typical of functionally mature monocytes and macrophages. The human HL-205 cells, which unlike the ML-2 and KG-1 cells, are arrested at the more-mature promyelocytic stage, were not susceptible to induction of cell differentiation by THC.

These results indicate that THC treatment causes the ML-2 and, to a lesser degree, the KG-1 myeloblastic leukemia cells to attain a number of maturation markers associated with monocyte differentiation. However, this treatment does not induce a complete terminal maturation but rather induces an intermediate stage with a promonocyte phenotype.

Changes in the Synthesis of Monocyte Marker Proteins in ML-2 Cells After Treatment with THC. To further characterize the THC-induced cell maturation, we analyzed the pattern of newly synthesized proteins in either control or THC-treated ML-2 cells by means of two-dimensional gel electrophoresis. For comparison and as a reference, we included human peripheral blood monocytes. Treatment of the ML-2 cells with 1, 3, or 10 μM THC yielded increases and decreases in the synthesis of a substantial number of proteins. Among the proteins that increased after THC treatment were at least 10 (≈25–150 kDa) that are abundant in monocytes but were synthesized at low quantities in the control ML-2 cells (Fig. 2). Among the ML-2 proteins that exhibited a decrease or low level of synthesis after THC treatment were 5 (≈15–80 kDa)

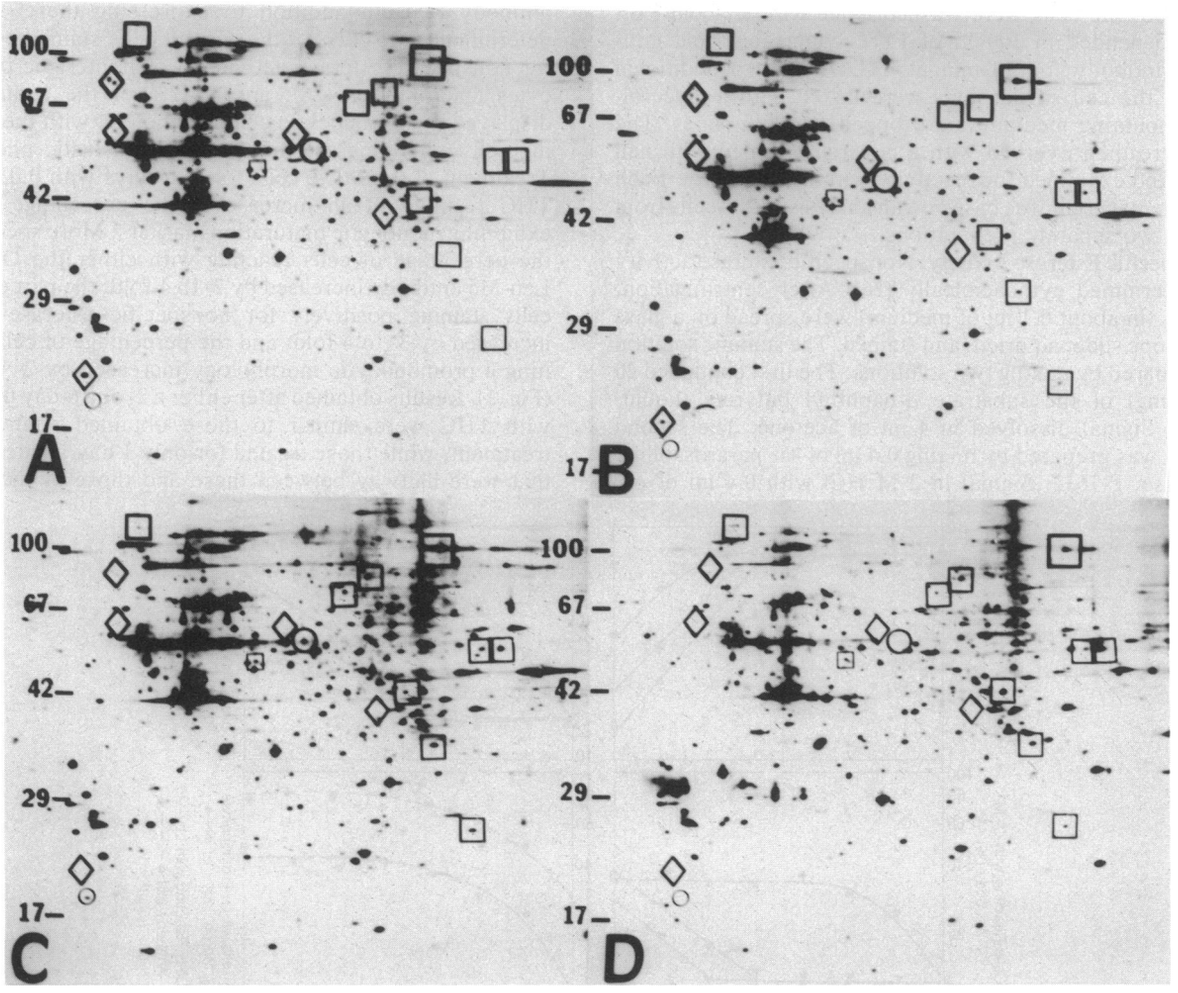

FIG. 2. Two-dimensional electrophoretic patterns of cells labeled with [^{35}S]methionine. (*A*) Control ML-2 cells; (*B* and *C*) ML-2 cells treated with either 1 μM (*B*) or 10 μM THC (*C*); (*D*) untreated peripheral blood monocytes. Each panel shows the same section of the respective two-dimensional electrophoretic protein pattern, with acidic proteins to the left and higher molecular masses at the top. The vertical scale shows approximate molecular masses in kilodaltons. ML-2 cell proteins that increase (□) or decrease (◇) as a result of THC treatment are indicated. Proteins that increase (○) as a result of THC treatment but are not detected in the monocytes are also indicated.

Table 1. Induction of cell differentiation markers by either PMA or 1,25-$(OH)_2D_3$ in ML-2 leukemia cells: Effect of pretreatment with THC as compared with untreated controls

Inducer	Conc., nM	Cells × 10^{-6} per Petri dish		Cells reacting with OKM1 antibody, %		Cell staining for nonspecific esterase, %		Cells exhibiting specific morphology, %					
								Myeloblast		Promonocyte		Monocyte	
		Control	THC	Control	THC	Control	THC	Control	THC	Control	THC	Control	THC
None	—	4.5	3.5	13	42	15	62	91	59	9	41	0	0
PMA	0.3	4.3	3.2	68	36	66	65	20	62	29	16	51	22
	1.0	0.5	0.4	88	≥95	≥95	≥95	11	30	17	25	72	45
	3.0	0.5	0.4	≥95	≥95	≥95	≥95	1	13	6	22	93	65
1,25-$(OH)_2D_3$	10.0	4.0	3.0	84	79	67	68	19	25	20	31	61	44
	30.0	4.0	2.8	≥95	≥95	72	82	17	21	17	14	66	65
	100.0	4.0	3.0	≥95	≥95	84	87	9	29	13	10	78	70

THC at 10 μM was added to the cultures 1 day after cell seeding, PMA and 1,25-$(OH)_2D_3$ were added 2 days later, and the experiments were terminated 4 days after the initial treatment with THC. Cells treated with PMA at doses above 0.3 nM attached to the surface of the tissue culture dishes.

that are also scarce in the monocytes (Fig. 2). A number of the newly synthesized proteins (≈20–50 kDa) did not correspond to any monocyte proteins (Fig. 2). These proteins may be associated with other effects of THC or represent differentiation proteins associated with a less-mature state. In addition to the THC-induced changes, an appreciable number of proteins found in monocytes were not detected in either control or THC-treated ML-2 cells.

These results indicate that THC can cause changes in protein synthesis in ML-2 cells that result in a protein pattern that resembles, although only in part, that of peripheral blood monocytes.

Expression of Monocyte Markers in ML-2 Cells After Treatment with Either CBD or CBN. In addition to THC, we treated the ML-2 cells with two other cannabinoids, CBD and CBN. Treatment of the ML-2 cells for 4 days with 3–30 μM CBD resulted in a dose-dependent increase in the percentage of cells reacting with the OKM1 antibody. Doses of CBD above 0.1 μM produced a similar increase in the percentage of cells staining positively for nonspecific esterase activity (Fig. 1) and in the percentage of cells reacting with the Leu-M5 antibody (data not shown). At doses above 1 μM, CBD also caused a dose-dependent decrease in cell growth (Fig. 1). The treated ML-2 cells did not, however, attach to the surface of Petri dishes. The CBN produced a similar response, although the doses required to achieve comparable cellular changes were about 2 to 3 times higher than those required for CBD (Fig. 1). Although the type of differentiation induced in the ML-2 cells by CBD and CBN resembled that induced by THC, the pattern of acquisition of the differentiation markers was different from that induced by THC (Fig. 1).

Induction of Cell Maturation by Either PMA or 1,25-$(OH)_2D_3$ in ML-2 Cells Pretreated with or Without THC. The previous experiments in this study indicated that THC induces an incomplete cell maturation in ML-2 cells. However, the same ML-2 cells can acquire a mature phenotype when treated with either PMA (13) or 1,25-$(OH)_2D_3$ (12), which are common inducers of cell differentiation. As a result, we tested these two agents for their ability to complete the differentiation process initiated by THC in the ML-2 cells. The results indicated that either PMA or 1,25-$(OH)_2D_3$, when added 2 days after THC treatment, could cause the ML-2 cells, which were induced only to the promonocyte stage by THC, to express a mature phenotype similar to that induced by PMA or 1,25-$(OH)_2D_3$ alone. This completion of differentiation was also indicated by the finding that neither the effect of PMA nor that of 1,25-$(OH)_2D_3$ was additive with that of THC. Moreover, pretreatment with 10 μM THC for 2 days reduced, to some degree, the ability of the lower dose of PMA (e.g., 0.3 nM) to complete the differentiation process, as indicated by the lower percentage of cells reacting with the OKM1 antibody and those achieving monocytic morphology (Table 1). The reducing effect of THC on PMA-induced cell differentiation was, however, not observed when ML-2 cells were treated for only 1 hr rather than 2 days prior to the addition of PMA (data not shown).

DISCUSSION

Marijuana smoking and direct exposure to cannabinoids, the biologically active components of marijuana, cause disturbances in the function of macrophages and both T and B lymphocytes (5), which are cells associated with the immune response. These dysfunctions may result from cannabinoid-induced incomplete or altered differentiation processes in blood-forming cells. To study such a possibility, we tested, *in vitro*, three biologically active ingredients of marijuana—namely, THC, CBD, and CBN—for their ability to induce cell differentiation in a human myeloblastic leukemia cell line designated ML-2 (11). Leukemia cells are believed to be blood cells blocked at an early stage of their cell maturation (14)—e.g., the ML-2 cells used in the present studies are halted at the myeloblastic stage with a small fraction of promonocyte cells normally present (12). Using this ML-2 cell system, we were able to show that THC, CBD, and CBN were capable of inducing maturation markers associated with monocyte differentiation. However, the pattern of marker acquisition after THC treatment was different from that observed with either CBD or CBN, suggesting that the mode of action of THC may be different from that of CBD and CBN.

Cell maturation with THC, the most psychoactive cannabinoid tested, as well as the most reactive in our system, was induced at a dose as low as 0.03 μM, which is within the range of concentrations found in the plasma of humans after the smoking of marijuana cigarettes (2). This low effective dose is also within the normal range of some circulating hormones. Indeed, recent studies have shown that some cannabinoids, in common with hormones, can bind to high-affinity specific cellular receptors (27). However, it is still unclear whether the biological activity of the cannabinoids is initiated as a result of their interaction with these receptors.

The THC-induced cell differentiation of the ML-2 cells did not result in cells with a highly developed mature phenotype, since the treated cells did not exhibit some markers prevalent in functionally mature monocytes/macrophages. Examples of such markers not displayed include cell attachment to the surface of tissue culture dishes, synthesis of a series of specific monocyte proteins (as detected by two-dimensional gel electrophoresis), the presence of morphologically mature monocytes, and cessation of cell growth. However, the ML-2

cells, whether untreated or induced for an "incomplete" cell maturation by THC, can acquire a mature phenotype that resembles that of monocytes/macrophages when treated with either PMA or 1,25-$(OH)_2D_3$, commonly used as inducers of a "complete" cell differentiation in myeloid cells, including ML-2 cells (12, 13). This completion of maturation was also indicated by the finding that, unlike in other studies with inducers of monocytic differentiation (25, 28, 29), neither the effect of PMA nor that of 1,25-$(OH)_2D_3$ was additive or synergistic with that of THC. Moreover, 2-day (but not 1-hr) pretreatment of the ML-2 cells with 10 μM THC reduced to some degree the ability of PMA at its lower dose (e.g., 0.3 nM) to complete the differentiation process, as indicated by the lower percentage of cells reacting with the OKM1 antibody and those achieving monocytic morphology. These results suggest that THC not only induces in ML-2 cells an "incomplete" monocytic differentiation but also induces in these cells conditions that reduce the transition of the treated cells from their "incomplete" state to the more mature monocytic/macrophage phenotype. More experiments are needed, however, to clarify whether THC-induced "incomplete" cell maturation is normal but incomplete or whether THC induces, in the ML-2 cells, an aberrant type of cell differentiation that involves within it, as the above experiments suggest, a block in the transition of the cells from an intermediate to the more advanced stage of maturation.

The cell system described here may be useful for deciphering critical biochemical events that lead to the cannabinoid-induced "incomplete" cell differentiation of ML-2 cells and related cell types. Findings obtained from this system may have important implications for studies of cannabinoid effects on normal human bone-marrow progenitor cells.

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