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Suppression by Cannabinoids of a Cloned Cell Line with Natural Killer Cell Activity (42676)

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Abstract. Preincubation of a cloned cell line with natural killer (NK) cell activity, as well as splenic mononuclear cells with either Δ^9-tetrahydrocannabinol (THC) or 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) suppressed NK cytolytic activity against YAC-1 target cells in a dose-dependent manner. THC was more inhibitory for cloned cells than 11-OH-THC and suppressed the lytic activity of these cells without reducing cell viability in the concentration range of 5 μg/ml (16 μ*M*) to 10 μg/ml (32 μ*M*). THC also inhibited proliferation of cloned NK cells, but this inhibitory effect was reversible in that extensive washing of cells following cannabinoid pretreatment eliminated the suppressive effect. Single-cell analysis revealed that THC did not inhibit the binding of cloned NK cells to target cells and further showed that NK cells freshly isolated from mouse spleen were restricted in killing capacity following binding to target cells. Therefore, THC and 11-OH-THC appear to directly inhibit NK cell cytolytic activity in a postbinding stage.

Natural killer (NK)[1] cells are considered to be the first line of defense against many tumors and viruses prior to immune sensitization of the host and possibly also an important source of immunoregulator substances in normal and pathological states (1). A large number of exogenous substances have been reported to affect NK activity, either *in vitro* or *in vivo* (2). We and others have reported that cannabinoids, including Δ^9-tetrahydrocannabinol (THC) and the 11-hydroxy metabolite (11-OH-THC), are suppressive for NK activity *in vitro* as well as *in vivo* (3–5). For example, it has been reported that cannabinoids, when injected into rats or mice, suppress NK activity and alter a variety of other activities which, in turn, may affect NK cells (3, 5). Cloned cells with NK activity have become available and have been studied extensively in terms of the nature and mechanism of NK activation and function (6). In this study, we have utilized cloned NK cells and found that these cells, when treated *in vitro* with either THC or 11-OH-THC, show a reduced ability to kill target tumor cells. In addition, these cannabinoids inhibited cloned NK cell proliferation in the presence of human interleukin 2 (IL-2), but not target cell binding. These studies support and extend our previous findings with purified NK cells (5) and provide evidence that cannabinoids directly affect NK cells suggesting the need for further investigation of the molecular mechanisms of suppression by cannabinoids.

Materials and Methods. *Marijuana components.* THC and 11-OH-THC dissolved in ethyl alcohol were provided by the Research Technology Branch, National Institute on Drug Abuse (Rockville, MD). For use, ethyl alcohol was evaporated with nitrogen gas and the cannabinoid resuspended in dimethyl sulfoxide (DMSO, Sigma Chemical, St. Louis, MO). The cannabinoids were diluted to the desired concentration in warm culture medium.

Preparation of effector cells. Spleen mononuclear cells from 6- to 8-week-old C3H/HeN mice (National Cancer Institute, Frederick, MD) were used as the source of freshly isolated NK cells. Large granular lymphocytes (LGL) were enriched using a modifica-

[1] Abbreviations used: THC, Δ^9-tetrahydrocannabinol; 11-OH-THC, 11-hydroxy-Δ^9-tetrahydrocannabinol; DMSO, dimethy sulfoxide; EtOH, ethyl alcohol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FCS, fetal calf serum; RPMI, Roswell Park Memorial Institute; IL-2, interleukin 2; NK, natural killer; LGL, larger granular lymphocytes.

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tion of the method previously reported (7). The tissue culture medium used in these studies was RPMI 1640 supplemented with 10% fetal calf serum (Hyclone, Logan, UT) 10 m*M* Hepes buffer, glutamine, and antibiotics. Briefly, spleen cells were incubated in plastic flasks for 2 hr and then nonadherent cells were collected and passed through a nylon wool column. Nylon wool column nonadherent cells were collected and centrifuged on a discontinuous Percoll (Pharmacia, Piscataway, NJ) density gradient. Cells recovered from fractions 2 and 3 (52.1 and 56.6% Percoll, respectively) were used as LGL. The cloned NK cell line, NK B61A2, which is IL-2 dependent, was derived from C57B1/6 mice as previously described (6).

Proliferation assay. Ten thousand cloned NK cells were incubated with various concentrations of THC for 22 hr in the presence of 100 units/ml of human recombinant IL-2 (kindly supplied by Dr. J. Farrar, Hoffman-LaRoche, Nutley, NJ) in a 96-well flat bottom microplate. During the last 18 hr of incubation the cultures were pulsed with 0.5 μCi of [^{3}H]thymidine. In some experiments, the NK clone was washed two times with Hanks' balanced salt solution to remove THC, before adding [^{3}H]thymidine. Radioactive thymidine incorporated by these cells was measured after collecting the cells on fiber filter mats and ^{3}H counting in a scintillation counter.

Cytotoxicity assay. Five hundred thousand splenic nonadherent cells, LGL, or cloned NK cells were incubated in 96-well round bottom microplates for 4 hr with various concentrations of THC, 11-OH-THC, or either DMSO or medium only. After preincubation with cannabinoids, 150 μl of supernatant fluid was removed and replaced with 150 μl of medium containing 5×10^3 ^{51}Cr-labeled YAC-1 target cells, prepared as previously described (8). The cells were mixed and incubated for 4 hr at 37°C in 5% CO_2 and 95% air. In some experiments, effector cells were washed with medium to remove THC before adding target cells. After the 4-hr cytolysis assay, 100 μl of supernatant fluid was removed and radioactivity measured using a gamma counter (5). All determinations were performed in triplicate. The percentage specific lysis was calculated as

$$\frac{\text{cpm experimental} - \text{cpm control}}{\text{cpm total release} - \text{cpm control}} \times 100.$$

Target binding and single-cell cytotoxicity assay. Equal numbers of effector and target cells were mixed and incubated in medium at 37°C for 10 min and then centrifuged at 250*g* for 5 min (9). The cell pellets were resuspended by 10 times gentle pipetting, a drop of the suspension was placed on a glass slide, a coverslip was used to spread the drop, and cells were examined microscopically. Total binding capacity of effector cells was calcuated as

$$\frac{\text{number of effector cells bound to targets}}{\text{total number of effector cells}} \times 100.$$

The remaining cell suspension was mixed with tissue culture medium supplemented with 1% agarose (type II, Sigma Chemical) in a 35-mm culture dish. One milliliter of culture medium was added to the agarose layer and incubated at 37°C in 5% CO_2 and 95% air for 3 hr. After incubation, culture medium was removed, 1 ml of 1% trypan blue was added to the culture preparations, and the cells were allowed to stain for 5 min at room temperature. Trypan blue was then removed, the cells were washed with medium, and the preparations were fixed with 0.2% formaldehyde and examined microscopically. Percentage maximal killing was calculated as

$$\frac{\text{number of conjugates with stained targets}}{\text{total number of conjugates}} \times 100.$$

The percentage NK was calculated as total binding capacity × percentage maximal killing.

Results. *Suppression of NK activity by cannabinoids.* Previously we reported (5) that THC and 11-OH-THC suppressed NK activity of splenocytes from C3H/HeJ mice (endotoxin hyporesponsive strain). In the present report, we show similar results with

the endotoxin responsive strain C3H/HeN (Fig. 1). As before, the 11-hydroxy metabolite appeared to be more inhibitory, effector cell viability was not effected by drug treatment, and suppression of the response occurred in the drug range of 10^{-5} *M*. The cytolytic activity of cloned NK cells was also suppressed by a 4-hr preincubation with either THC or 11-OH-THC (Fig. 2). In contrast to freshly isolated cells, however, the suppression was less especially that resulting from 11-OH-THC treatment. Preincubation of target cells with 0.8–2.5 μg THC/ml or the presence of THC during the 4-hr cytotoxicity assay did not affect target lysis by the NK cells (data not shown).

Suppression of proliferation of cloned NK cells. THC also inhibited DNA synthesis by cloned cells stimulated with IL-2. However, if the cells were cultured for 4 hr in the presence of THC and then washed extensively, the drug inhibitory effect was reversed (Fig. 3).

Single-cell analysis of THC suppression. Previously we reported (5) that THC appeared not to inhibit the binding of freshly isolated splenic NK cells to the target cells.

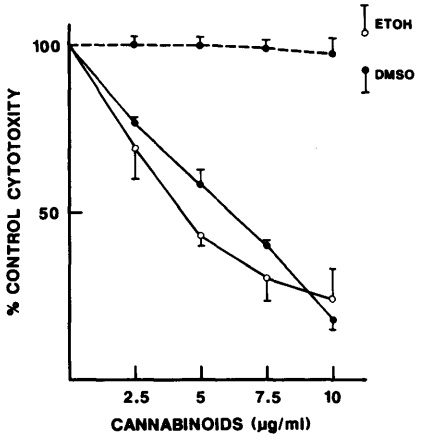

FIG. 1. Suppression of the cytolytic function of splenic mononuclear cells by cannabinoids. Cells were preincubated for 4 hr with either cannabinoids or drug vehicle, washed extensively by centrifugation, and tested for viability by dye exclusion (● - - - ●) and lytic activity against YAC-1 targets (○; ●). Lytic data expressed as means ± SD percentage of control specific lysis (35.8%) and obtained at an *E/T* ratio of 100/1. Data obtained from three experiments.

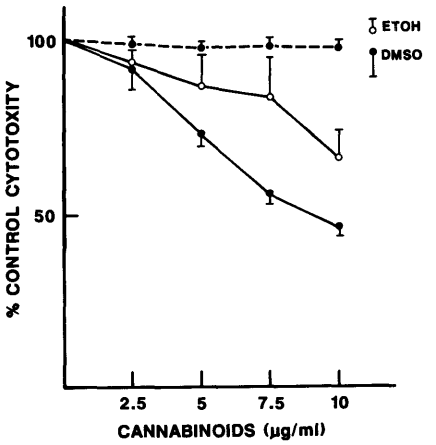

FIG. 2. Suppression of the cytolytic function of cloned cells (NK B61A2) by cannabinoids. Cells were treated with drugs and analyzed for cell viability (● - - - ●) and lytic activity (○; ●) as described in the legend to Fig. 1. Lytic data expressed as means ± SD percentage of control specific lysis (36.3%) and obtained at an *E/T* ratio of 20/1. Data obtained from three experiments.

Cloned NK cells also bound to target cells without any interference by drug treatment (data not shown). In the present report, using the single-cell cytotoxicity assay, this finding was extended to show that THC decreased the number of effector cells capable of killing susceptible target cells (Table I). Therefore, although binding is not diminished by either

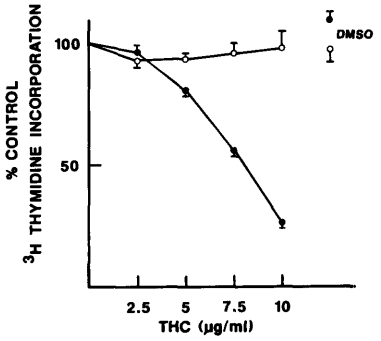

FIG. 3. Suppression of IL-2-induced proliferation of cloned cells (NK B61A2) by THC. Cells were treated with IL-2 (100 units/ml) and then either exposed for 4 hr to THC and then pulsed with [^{3}H]thymidine for 18 hr (●) or treated with THC, washed by centrifugation, and then pulsed with [^{3}H]thymidine (○). Data expressed as means ± SD percentage control proliferation (2477 ± 155 cpm). Data obtained from three experiments.

TABLE I. THE SINGLE-CELL CYTOXICITY ASSAY OF MURINE LARGE GRANULAR LYMPHOCYTES[a]

Treatment	Cell viability	Total binding capacity	Percentage maximal killing	Percentage NK	Cell viability × % NK	^{51}Cr release assay
Medium control	100 ± 0.5	14.3 ± 0.4	33.7 ± 2.3	100[b]	100	100
DMSO	101.2[c] ± 1.1	14.1 ± 0.3	31.9 ± 1.9	93.3	94.4	81.6[c]
THC 5 (μg/ml)	100.3 ± 0.8	14.5 ± 0.8	27.8 ± 0.1	83.6	83.9	51.9
THC 7.5 (μg/ml)	95.7 ± 1.2	13.2 ± 1.1	23.9 ± 1.9	65.5	62.6	38.6
THC 10 (μg/ml)	89.8 ± 2.0	13.6 ± 0.6	18.0 ± 2.0	50.8	45.5	20.9

[a] LGL (5×10^5/ml) were preincubated with THC for 4 hr and then washed and cytotoxicity against YAC-1 was measured by either single-cell cytotoxicity assay or standard ^{51}Cr release assay. (Data shown as mean ± SEM.)

[b] Calculated as total binding capacity × percentage maximal killing with experimental values expressed as a percentage of medium control value of 481.9.

[c] Expressed as mean percentage of medium control ± SEM.

splenic NK cells or cloned NK cells, it appears that THC treatment does reduce the killing capacity of cells which have bound to target cells.

Discussion. The major psychoactive marijuana component THC and the active metabolite 11-OH-THC have been reported to inhibit NK activity when administered either *in vitro* or *in vivo* (3–5). Although previous reports suggested that the cannabinoids were not killing the effector cells or preventing effector cell binding to susceptible targets, little information has been obtained concerning the molecular basis of the drug effects. NK activity appears to be present in heterogeneous cell populations making it difficult to evaluate the direct effect of cannabinoids on these cells. Even LGL purified from blood or spleen cell are still heterogeneous and are troublesome to prepare. Therefore, cannabinoid effects on cloned cells with NK activity were examined in the present study in order to develope a homogeneous population which would serve as a suitable model for studies involving the molecular basis of the cannabinoid effects. Both THC and 11-OH-THC suppressed cytolysis by cloned NK cells, similar to effects on splenic NK cells. However, cloned NK cells appeared to be slightly more resistant to suppression than spleen cells in that minimal suppressive concentrations were 5 and 2.6 μg/ml, respectively. This could be explained by differences in cell size because the extent of suppression may be related to cannabinoid density on the cell surface as previously suggested (10). The cloned NK cells are larger than freshly isolated LGL and therefore at a constant drug concentration these larger cells may have a lower surface density of drug molecules. It is also possible that either the metabolism or density of cell surface drug interactive sites of cloned NK cells may be different from freshly isolated LGL resulting in the slight difference in susceptibility to THC.

By using single-cell analysis, we confirmed directly that THC inhibited cytolytic function at a postbinding stage. It was also found that the cannabinoids inhibited proliferation of cloned NK cells stimulated with IL-2. The inhibition of proliferation was reversed by washing the cells following drug treatment suggesting that the reversible nature of THC effects on NK proliferation is similar to effects on T-lymphocyte proliferation (11). Since both cell types require IL-2 receptor binding and subsequent signal transduction events for proliferation it is possible that THC interferes with this IL-2 activation system. We are currently examining this possibility.

These data indicate that cannabinoids reversibly suppress both proliferation and cytolytic activity of cloned NK cells. Cannabinoids do not affect NK cell binding to target cells but suppress a postbinding stage of the cytolytic process. These findings provide evidence for direct inhibitory effects of both THC and 11-OH-THC on NK cells and that NK cell clones will be useful for further investigations into the molecular mechanism(s) of such suppression.

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