Differential Inhibition of RAW264.7 Macrophage Tumoricidal Activity by Δ^9Tetrahydrocannabinol (43926)

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Abstract. **Δ^9tetrahydrocannabinol (THC), the major psychoactive component of marijuana, has been shown to inhibit macrophage cell contact-dependent cytolysis of tumor cells. The purpose of this study was to determine whether THC inhibited macrophage cytolytic function by targeting selectively tumor necrosis factor (TNF)-dependent pathways versus L-arginine-dependent reactive nitrogen intermediates. An *in vitro* system employing RAW264.7 macrophage-like cells as effectors and TNF-sensitive mouse L929 fibroblasts or nitric oxide ($NO^{\cdot}$)-sensitive P815 mastocytoma cells as targets, was employed to assess the effect of THC on cytolysis. Macrophages were pretreated with THC or vehicle for 48 hr, subjected to multistep activation with 10 U/ml recombinant mouse γ-interferon (IFN-γ) plus 100 ng/ml LPS or to direct activation with 1 μg/ml LPS, and co-cultured with tumor cells in the presence or absence of THC. THC inhibited TNF-dependent killing by macrophages subjected to either multistep or direct activation. Decreased amounts of TNF-α were detected in medium of macrophage cultures treated with THC. In contrast, THC inhibited $NO^{\cdot}$-dependent cell contact killing only for macrophages subjected to direct activation. Decreased levels of NO_2^-, a stable degradation product of the short-lived and highly toxic effector molecule $NO^{\cdot}$, were produced by these macrophages. In addition, the effect of the enantiomeric pairs (−)CP55,940/(+)CP56,667 or (−)HU-210/(+)HU-211 on macrophage cell contact-dependent killing was assessed. Inhibition of macrophage tumoricidal activity against TNF-sensitive L929 cells was effected by both isomers of THC analogs. In contrast, both of the enantiomeric pairs had an effect on killing of $NO^{\cdot}$-sensitive P815 mastocytoma cells only for macrophages subjected to direct activation. These data suggest that cannabinoids inhibit macrophage cell contact-dependent killing of tumor cells by a noncannabinoid receptor-mediated mechanism. However, specific cytolytic pathways are inhibited differentially by cannabinoids depending on the activation stimuli to which macrophages are exposed.**

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Macrophages are important effector cells in the immune response in their capacity as antigen-presenting cells, as producers of positive and negative modulatory proteins (1, 2), and as cytotoxic effectors against tumor cells, protozoa, and virus-infected cells (3–5). In executing cytolytic functions, macrophages employ a number of intracellular and extracellular killing mechanisms (6). Macrophages can destroy microorganisms by phagocytosis, and subsequent intracellular exposure to hydrolytic enzymes, toxic oxygen intermediates, or L-arginine-derived toxic nitrogen intermediates. Activated macrophages can also kill target cells in a contact-dependent manner in which effectors first bind to the target and then secrete cytolytic molecules such as tumor necrosis factor (TNF), nitric oxide ($NO^{\cdot}$), cytolytic protease, and other molecules that destroy target cells (6–8).

Δ^9tetrahydrocannabinol (THC), the major psychoactive component of marijuana, is immunosuppressive

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in vivo and *in vitro* (9–14). THC has been shown to induce a variety of suppressive effects on macrophage functional activities. This cannabinoid alters macrophage morphology (15), suppresses macrophage protein expression in response to bacterial immunomodulators (16), and inhibits the production of tumor necrosis factor-α (TNF-α) (17, 18). We have demonstrated previously that THC inhibits tumoricidal, amoebicidal, and antiviral cell contact-dependent activities of Bacillus Calmétte-Guérin (BCG)-activated peritoneal macrophages (19). In these studies, conjugation of BCG-activated macrophages with target cells was not affected. Rather, THC apparently inhibited relatively late events associated with effector cell-target cell contact-dependent killing such as the synthesis, secretion, and/or delivery of molecules involved in microbicidal and tumoricidal activities.

The mechanism by which THC elicits its psychoactive and immunosuppressive effects is under investigation. The discovery of a cannabinoid receptor (CBR1) in rat and human brain, human testis, and murine splenocytes and the identification of a different cannabinoid receptor (CBR2) in the human promyelocytic leukemia cell line HL-60 suggest that THC may elicit its psychoactive and immunosuppressive effects through a cannabinoid receptor (20–24). However, since THC is a highly lipophilic molecule, one cannot rule out the possibility that THC also may alter cell function through noncannabinoid receptor-mediated mechanisms such as membrane perturbation (25).

Macrophages have been shown to kill tumor cells using both TNF- and L-arginine-dependent pathways (7, 8). Therefore, the purpose of this study was to determine whether THC inhibited macrophage cytolytic function by targeting selective macrophage killing pathways. An *in vitro* model system consisting of RAW264.7 macrophage-like cells as effectors and L929 mouse fibroblasts or P815 mastocytoma cells as targets was employed. These targets were used since L929 cells are TNF-sensitive and NO·-resistant, while the P815 cells are TNF-resistant and NO·-sensitive (26). RAW264.7 macrophages were also treated with pairs of THC analogs in order to elucidate whether inhibition of macrophage contact-dependent cytotoxicity involved a cannabinoid receptor-mediated mechanism.

Materials and Methods

Macrophage Cell Culture. The macrophage-like cell line RAW264.7 (TIB 71) was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Macrophages were grown in complete RPMI 1640 medium (supplemented with 10% heat-inactivated fetal bovine serum [FBS] ([Bio Whittaker, Walkersville, MD], 1% L-glutamine, 1% nonessential amino acids, 1% antibiotic/antimycotic [10,000 U/ml of penicillin, 25 μg/ml of amphotericin D, and 10,000 μg/ml of streptomycin], 25 m*M* Hepes [4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid], 1.5% sodium bicarbonate, and 1% minimal essential medium vitamins) at 37°C in a humidified 5% CO_2 atmosphere. Viability of macrophages was assessed by trypan blue exclusion. Total cell counts were performed using a hemacytometer. Cell preparations showing greater than 95% viability were employed in drug exposure studies.

Target Cell Culture. Mouse L929 fibroblasts (ATCC CCL1) and P815 mastocytoma cells (ATCC TIB 64) were used as target cells to measure the effect of THC on tumor necrosis factor (TNF)-dependent and L-arginine (NO·)-dependent macrophage cytolytic pathways, respectively. Cells were grown in complete RPMI at 37°C in a humidified 5% CO_2 atmosphere and were radiolabeled with 200 μCi of ^{51}Sodium chromate ($Na_2{}^{51}CrO_4$) (Amersham, Arlington Heights, IL) for 2 hr at 37°C. Labeled cells were suspended in complete RPMI and used in a cytotoxicity assay to measure macrophage contact-dependent cytolytic activity.

Preparation of THC and THC Analogs. THC was supplied by the National Institute on Drug Abuse, Rockville, MD. The THC analogs, (−)CP55,940 and (+)CP56,667 were supplied by Pfizer, Inc. (Groton, CT), and (−)HU-210 and (+)HU-211 were provided by Dr. Raphael Mechoulam (Hebrew University, Israel).

THC (stock solution of 100 mg/ml in 95% ethanol) and THC analogs (stock solution of 1 mg/ml in 95% ethanol) were diluted in complete RPMI to yield appropriate final concentrations for treatment of macrophage cultures.

Exposure of RAW264.7 Cell Cultures to THC, THC Analogs, and Activating Stimuli. Macrophages were seeded into 25 cm^2 flasks (Nunc, Naperville, IL) at a concentration of 1×10^6 cells/flask and were allowed to adhere overnight. Cells were then pretreated for 48 hr with THC, THC analog (10^{-7}–10^{-5} *M*), vehicle (0.38%, 0.26%, or 0.1% ethanol in complete RPMI), or placebo (complete RPMI only). This pretreatment regimen was selected based on previous studies in this laboratory which have shown this protocol to be optimal for THC-induced inhibition of macrophage cytolytic activities. Naive controls, consisting of both stimulated and unstimulated macrophages cultured in complete medium in the absence of ethanol, were included in preliminary cytotoxicity assays to determine whether the ethanol vehicle affected macrophage function. There were no observed differences in cytolytic activity between complete medium or vehicle-treated stimulated or unstimulated macrophages. During the last 4 hr of drug treatment, cells were activated using either a multistep activation procedure employing 10 U/ml recombinant murine γ-interferon

(IFN-γ) (Genzyme, Cambridge, MA) in combination with 100 ng/ml *Escherichia coli* lipopolysaccharide (LPS) (Serotype 055:B5) (Sigma Chemical Co., St. Louis, MO), or a direct activation protocol using a relatively high dose of LPS (1 μg/ml). Following drug pretreatment and activation with IFN-γ plus LPS or with LPS alone, macrophages were used in cytotoxicity assays.

Cytotoxicity Assay. RAW264.7 cells were harvested from flasks by scraping, suspended at 5×10^6 cells/ml in medium in the presence or absence of THC, THC analog, or vehicle and plated in 96-well U-bottom tissue culture plates in 100-μl aliquots (5×10^5 cells/well). Controls included macrophages plated in the presence of 12.5 μ*M* to 200 μ*M* N^G-monomethyl L-arginine (p-hydroxyazobenzene-p'sulfonate) (N^GMMA) (Calbiochem Corp., LaJolla, CA), an analog of L-arginine known to inhibit the production of NO· by activated macrophages, or of 250 U to 1000 U of rabbit anti-mouse TNF-α (Genzyme) or normal rabbit serum (NRS).

Radiolabeled L929 cells or P815 cells suspended in complete RPMI were added to monolayers of adherent macrophages in 96-well tissue culture plates to obtain a 5:1 effector:target (E:T) ratio. This ratio was selected because studies in this laboratory have demonstrated that it is optimal for RAW264.7 cell contact-dependent killing. Plates were centrifuged for 2 min (200 *g*), and incubated at 37°C in the presence of 5% CO_2. After 20 hr of co-culture, plates were centrifuged (500 *g*, 10 min) and 175 μl of supernatant fluid were harvested and counted in a gamma counter (Packard Instrument Company, Downers Grove, IL). The percent specific release of radiolabel from target cells was determined from the mean of four replicate wells for each experimental group using the following formula:

$$\% \text{ Cytolysis} = \frac{\text{Experimental Release} - \text{Spontaneous Release}}{\text{Maximum Release} - \text{Spontaneous Release}} \times 100\%.$$

Spontaneous release of radiolabel from target cells in the absence of macrophages was quantitated in complete RPMI, vehicle, THC, THC analog, NRS, rabbit anti-mouse TNF-α, or N^GMMA. Maximum release of radiolabel was determined by the addition of 150 μl of 1 *N* NaOH to target cells.

Macrophage-Conditioned Medium. RAW264.7 macrophages seeded into 25-cm^2 flasks were treated with THC (10^{-7}–10^{-5} *M*) or vehicle (0.1% ethanol) for 48 hr. Cells were treated with 10 U/ml IFN-γ in combination with 100 ng/ml LPS for the last 4 hr of the drug or vehicle treatment period. After the 4-hr IFN-γ/LPS activation period, conditioned medium (CM) was harvested (4 hr CM). Fresh medium containing THC or vehicle was added to the respective flasks, and 20 hr later the conditioned medium (20-hr CM) was harvested. The 4-hr CM should contain any initial burst of TNF or other effector molecules produced by macrophages in response to activating stimuli while the 20-hr CM is representative of medium containing effector molecules produced during the cytotoxicity assay. Both 4-hr CM and 20-hr CM were centrifuged for 10 min at 1000 *g* to remove cellular debris, aliquoted, and frozen at −80°C until assayed for the presence of TNF-α.

Measurement of TNF-α in Conditioned Medium. Levels of TNF-α in 4-hr CM and 20-hr CM were measured using the enzyme-linked immunosorbent assay (ELISA) Factor-Test mTNF-α (Genzyme). A standard calibration curve using recombinant murine TNF-α was employed to allow for extrapolation of levels of TNF-α in the conditioned medium. In addition, a cytotoxicity assay using radiolabeled L929 cells was performed to confirm the results of the ELISA assay and to measure TNF functional activity. Briefly, 1×10^5 [^{51}Cr]-labeled L929 cells were added to each well of a 96-well microtiter plate and incubated for 2 hr at 37°C to allow for cell adherence. Following removal of the medium, 150 μl of CM were added to the radiolabeled target cells in the presence of 1 μg/ml actinomycin D. Actinomycin D was added in order to prevent target cell repair following exposure to TNF-α. Rabbit anti-mouse TNF-α antibody (Genzyme) (1000 U), or an equivalent concentration of NRS, was added to control wells to confirm that the cytolytic activity of CM for L929 cells was due to TNF-α. Plates were centrifuged at 500 *g* for 2 min and were incubated at 37°C in the presence of 5% CO_2 for 20 hr. Following the incubation period, supernatants were harvested, the radioactivity present was quantitated in a gamma counter, and the percentage specific release of radiolabel was determined.

Measurement of Cell Surface-Associated TNF-α. Flow cytometry was employed to determine the effect of THC on the expression of surface-associated TNF-α. RAW264.7 cells seeded into 25-cm^2 flasks were treated for 48 hr with THC (10^{-5}–10^{-7} *M*) or vehicle (0.1% ethanol in complete RPMI). During the last 4 hr of the treatment regimen, cells were activated using either a multistep activation protocol employing IFN-γ in combination with LPS or with a direct activation protocol using LPS alone. After the 4-hr activation period, cells were harvested, washed twice with PBS, pH 7.3, containing 0.1% NaN_3 and 1% BSA, and assessed for viability using the trypan blue exclusion method. Cells were suspended in PBS/NaN_3/BSA at a concentration of 2×10^6/ml, and 100 μl of the cell suspension were incubated with murine IgG for 30 min

at 4°C to block sites for Fc receptors. Cells, then, were incubated for 30 min at 4°C with rabbit anti-mouse TNF-α (Genzyme) or NRS diluted 1:3200 in PBS/NaN_3/BSA, washed twice, and treated with a FITC-conjugated $(Fab')_2$ fragment with the specificity donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:200 in PBS/NaN_3 containing 1% donkey serum. Cells were washed twice in PBS/NaN_3 and fixed with 0.5% paraformaldehyde. Data on 2×10^5 cells were obtained using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) equipped with an Argon Laser emitting 15 mW at 488 nm. Ten thousand events were collected and were analyzed by a Hewlett-Packard 9000 computer (Portland, OR) using the Consort 30 program (Becton-Dickinson). Background controls for the secondary antibody, for nonspecific binding, and for activation were included in each experiment.

Measurement of NO_2^- Production. Nitrite (NO_2^-) concentration in macrophage-conditioned medium was assayed using the Griess reagent (27). Macrophage cytolytic activity correlates with the production of L-arginine-derived NO_2^-, which is a stable degradation product of the short-lived and highly toxic effector molecule NO• (28). Briefly, 1×10^5 macrophages, pre-treated with vehicle or THC for 48 hr, were plated in the presence or absence of N^GMMA (12.5–200 μ*M*). RAW264.7 macrophages were activated using either a multistep or direct activation protocol for 24 hr. Following activation, samples (100 μl) were removed from the conditioned medium and were incubated (room temperature, 10 min) with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2.5% H_3PO_4), a colorimetric indicator for the presence of NO_2^-. Absorbance at 550 nm was determined with a microplate reader (Molecular Devices Corp., Menlo Park, CA) and the concentration of NO_2^- was extrapolated using sodium nitrite as a standard.

Membrane Preparations for Radioligand Binding Assays. RAW264.7 cells, grown in roller bottle flasks (Costar) to a concentration of 3×10^9 cells/flask, were pelleted by centrifugation and resuspended in ice-cold assay buffer (320 m*M* sucrose, 50 m*M* Tris-HCl, pH 7.4, 2 m*M* EDTA, 5 m*M* $MgCl_2$). The cell suspension was dounce-homogenized in 30 ml of ice-cold assay buffer. The homogenate was centrifuged at 1600 *g* for 10 min at 4°C, the supernatant saved, and the pellet was processed twice more as described above. All three supernatants were combined and centrifuged at 39,000 *g* for 15 min. The resultant P_2 pellet was resuspended in 3 ml of a solution containing 50 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, and 3 m*M* $MgCl_2$. Frozen aliquots were stored at −80°C.

For brain membrane preparations, male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 200–250 g were decapitated and the brains rapidly removed. The whole brain was dissected free, homogenized in 30 ml of 0.32 *M* sucrose containing 2 m*M* EDTA and 5 m*M* $MgCl_2$. The homogenate was centrifuged at 1,600 *g* for 10 min, and the supernatant was saved. The pellet was processed twice more as described above. All three supernatants were combined and centrifuged at 39,000 *g* for 15 min. The resultant P_2 pellet was resuspended in 50 ml buffer (50 m*M* Tris-HCl, pH 7.0, 2 m*M* EDTA, 3 m*M* $MgCl_2$) and was incubated at 37°C for 10 min before centrifugation at 23,000 *g* for 10 min. The pellet was resuspended in 50 ml of 50 m*M* Tris-HCl/2 m*M* EDTA/3 m*M* $MgCl_2$ and incubated at 30°C for 40 min before centrifugation at 11,000 *g* for 15 min. The final pellet was resuspended in 10 ml of buffer containing 50 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, and 3 m*M* $MgCl_2$. A Bradford protein assay was performed on final membrane preparations and aliquots, then, were quickly frozen with liquid nitrogen and stored at −80°C.

Radioligand Binding Assays. The filtration procedure used for [^{3}H]-CP55,940 (Dupont, Boston, MA) binding was a modification of the centrifugation method described by Devane *et al.* (29). Briefly, the binding assay was performed in siliconized glass tubes containing 100 μl of radiolabeled ligand, 100 μl of competing unlabeled drug (1 μ*M* final concentration), 70 μg of membrane protein, and sufficient buffer (50 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 3 m*M* $MgCl_2$, 5 mg/ml BSA) to make a final volume of 1 ml. Tubes were incubated at 30°C for 1 hr. The reaction was stopped with the addition of 2 ml of ice-cold 50 m*M* Tris-HCl (pH 7.4) buffer containing 1 mg/ml BSA followed by rapid filtration through polyethyleneimide-treated 2.5 cm GF/C glass fiber filters (Whatman, Hillsboro, OR). The reaction tube was washed with 2 ml of buffer and filtered. The filters were washed twice with 4 ml of buffer and shaken for 1 hr in 10 ml of scintillation fluid. Radioactivity was quantitated using a Packard 2200CA liquid scintillation counter. Specific binding was defined as the difference between the binding that occurred in the presence and absence of 1 μ*M* unlabeled ligand. The data were represented in graphical form using the Sigmaplot 5.0 program (Jandel Scientific, San Rafael, CA).

Statistical Methods. Statistical analysis was performed using the Student's *t* test. A 95% confidence level was chosen for all experiments to determine the statistical significance for differences between paired values.

Results

Effect of THC on Macrophage Cell Contact-Dependent Tumoricidal Activity. A chromium release assay was used to assess the effect of THC on RAW264.7 macrophage-mediated killing of TNF-

sensitive versus NO·-sensitive target tumor cells. Macrophages cultured in the absence of activating signals elicited minimal cytolysis of target cells. Macrophages subjected to multistep activation with IFN-γ in concert with LPS demonstrated approximately 50% cytolysis of L929 cells and 61% cytolysis of P815 cells

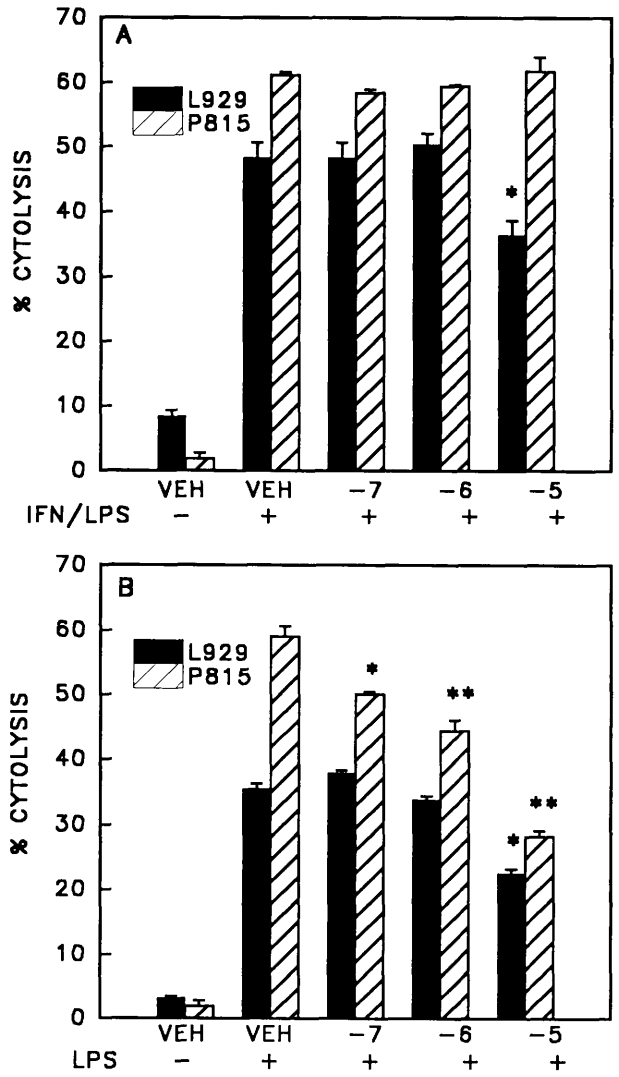

Figure 1. Inhibition of macrophage cell contact-dependent cytolysis by THC. RAW264.7 macrophages were treated *in vitro* for 48 hr with vehicle (0.1% ethanol in complete RPMI) or THC and were subjected to multistep activation or direct activation during the last 4 hr of the treatment period (A) with 10 U/ml IFN-γ plus 100 ng/ml LPS or (B) with 1 μg/ml LPS, respectively. Radiolabeled L929 cells or P815 cells were added to monolayers of macrophages at an E:T ratio of 5:1. Co-cultures were incubated for 20 hr at 37°C in the presence of vehicle or THC. VEH, 0.1% ethanol; −7, −6, and −5 represent 10^{-7}, 10^{-6}, and 10^{-5} *M* THC, respectively. Inclusion of 1000 U of rabbit anti-mouse TNF-α in medium during the assay resulted in an average of 10% cytolysis of L929 cells and 48% cytolysis of P815 cells, regardless of the activation protocol employed. Inclusion of 200 μ*M* N^GMMA in medium during the assay resulted in an average of 50% macrophage-mediated killing of L929 cells and 2% killing of P815 cells, regardless of the activation protocol employed. * and ** represent values significantly different ($P < 0.01$ and $P < 0.001$, respectively) when compared with the value obtained for activated, vehicle control macrophages.

(Fig. 1A). THC, at the highest concentration (i.e., 10^{-5} *M*), inhibited macrophage killing of TNF-sensitive L929 cells ($P < 0.01$). However, killing of NO·-sensitive P815 cells was not inhibited by THC at any concentration when the multistep activation protocol was employed.

A different pattern of cell contact-dependent killing was exhibited by THC-treated macrophages when a direct activation protocol (LPS alone) was employed (Fig. 1B). Macrophage killing of both TNF-sensitive L929 cells and NO·-sensitive P815 cells was inhibited by THC. However, killing of L929 cells was inhibited only at 10^{-5} M THC ($P < 0.01$) upon comparison with the vehicle control. In contrast, THC inhibited the killing of NO·-sensitive P815 cells in a dose-related fashion. A 52%, 27%, and 15% inhibition of killing was noted for macrophages treated with 10^{-5}, 10^{-6}, and 10^{-7} *M* THC, respectively.

Inclusion of 1000 units of rabbit anti-mouse TNF-α in medium during the chromium release assay inhibited macrophage-mediated killing of L929 cells (i.e., by greater than 50%) but not of P815 cells. Alternatively, the inclusion of 200 μ*M* N^GMMA in medium during the chromium release assay inhibited macrophage-mediated killing of NO·-sensitive P815 cells (i.e., greater than 50%) but not of L929 cells. These controls confirmed the specificity of L929 cells and P815 cells to TNF-mediated killing and NO·-mediated killing, respectively. Spontaneous release was determined using target cells incubated with vehicle or with THC in the absence of macrophages. THC, in the concentrations employed, did not elicit the release of radiolabel from target cells at levels above those noted for vehicle controls.

Effect of THC on the Secretion of TNF-α by RAW264.7 Macrophages in Response to IFN-γ/LPS Activation. Since THC inhibited cell contact-dependent killing of TNF-sensitive L929 cells, an ELISA was used to determine whether THC altered the capacity of macrophages to secrete TNF-α. Results of a representative experiment for measuring levels of TNF-α in 4-hr CM and 20-hr CM are shown in Table I. The concentration (ng/ml) of TNF-α in 4-hr CM was decreased 21% for macrophages subjected to 10^{-5} *M* THC treatment. Similarly, levels of TNF-α in 20-hr CM were decreased 34% upon treatment of macrophages with 10^{-5} *M* THC ($P < 0.05$). Similar results were obtained when a functional assay in which ^{51}Cr-labeled TNF-sensitive L929 cells were incubated in the presence of CM for 20 hr (data not shown). Rabbit anti-mouse TNF-α specifically neutralized the activity (i.e., greater than 50%) of the CM on ^{51}Cr-labeled L929 cells. Previous studies (18) have shown that the concentration of TNF-α in conditioned medium is decreased for macrophage cultures subjected to direct activation with LPS.

Table I. Effect of THC on Macrophage Secretion of TNF-α in Response to Multistep Activation with IFN-γ and LPS

Treatment[a]	IFN/LPS	Secretion of TNF-α (ng/ml TNF-α ± SD)	
		4-hr CM	20-hr CM
Vehicle	−	1.7 ± 0.6	<1
Vehicle	+	40.2 ± 1.0	25.5 ± 0.1
10^{-7} *M* THC	+	46.2 ± 2.4	29.4 ± 2.5
10^{-6} *M* THC	+	46.7 ± 1.3	26.2 ± 2.1
10^{-5} *M* THC	+	31.6 ± 1.0	16.9 ± 0.5[b]

[a] RAW264.7 macrophages were treated *in vitro* for 48 hr with vehicle (0.1% ethanol in complete RPMI) or THC and were activated during the last 4 hr of the treatment period with 10 U/ml IFN-γ plus 100 ng/ml LPS. CM was collected directly after the 4-hr activation period and fresh medium containing vehicle or THC was added to the macrophages for 20 hr. Both 4-hr CM and 20-hr CM were diluted 1:100 in complete RPMI and were assessed for the presence of TNF-α using a TNF-α specific ELISA.
[b] Value is significantly different ($P < 0.05$) from that obtained for activated, vehicle control macrophages.

Effect of THC on RAW264.7 Surface-Associated TNF-α. Flow cytometry was employed to determine the effect of THC on macrophage surface expression of TNF-α (Table II). After a 4 h activation period, macrophages subjected to direct activation (i.e., 1 μg/ml LPS) exhibited a greater expression of cell surface TNF-α than when a multistep activation protocol (i.e., IFN-γ plus LPS) was employed. Upon direct activation, a 2- to 3-fold increase in the percentage of macrophages (35%) expressing cell surface-associated TNF-α was observed upon comparison with the percentage of unactivated cells (14%) expressing surface-associated TNF-α. Treatment of macrophages with THC (10^{-5} *M*) did not greatly alter the percentage of cells expressing cell surface-associated TNF-α (i.e., 39%). Upon activation, the mean log fluorescence intensity of cells expressing surface-associated TNF-α did not exhibit a major difference when compared with that of unactivated cells (35 $\log_{10}$ vs 55 $\log_{10}$). In addition, THC did not have a major effect on mean log fluorescence intensity 26 $\log_{10}$). Activation with IFN-γ plus LPS elicited few cells (20%) expressing surface-associated TNF-α when compared with unactivated cells (14%). The macrophages which stained positive for surface-associated TNF-α exhibited similar levels of mean log fluorescence intensity (37 $\log_{10}$) upon comparison with unactivated cells (55 $\log_{10}$). In addition, THC did not have a major effect on mean log fluorescence intensity (40 $\log_{10}$).

Table II. Effect of THC on RAW264.7 Surface-Associated TNF-α

Treatment[a]	% Positive cells[b]	Mean log fluorescence
Vehicle	14	55 $\log_{10}$
Vehicle + LPS	35	35 $\log_{10}$
10^{-5} *M* THC + LPS	39	26 $\log_{10}$
Vehicle + IFN/LPS	20	37 $\log_{10}$
10^{-5} *M* THC + IFN/LPS	25	40 $\log_{10}$

[a] RAW264.7 macrophages were treated *in vitro* for 48 hr with vehicle (0.1% ethanol in complete RPMI) or THC and were activated during the last 4 hr of the treatment period with 10 U/ml IFN-γ plus 100 ng/ml LPS or with 1 μg/ml LPS. Cells were harvested and processed for flow cytometry as detailed in Materials and Methods.
[b] % Positive cells and mean log fluorescence intensity were determined using the Consort 30 program. % Positive cells for each experimental group was determined by subtracting the % binding of NRS to RAW264.7 macrophages from the % binding of rabbit anti-mouse TNF-α to RAW264.7 macrophages.

Effect of THC on Macrophage NO_2^- Production. THC inhibited cell contact-dependent killing of NO·-sensitive P815 target cells. Therefore, studies were undertaken to determine whether THC inhibited the production of NO_2^- by macrophages. Macrophages subjected to multistep activation with IFN-γ plus LPS produced 2-fold more NO_2^- than macrophages exposed to a direct activation with LPS alone (Table III). THC had no effect on levels of NO_2^- produced by macrophages subjected to multistep activation. In contrast, macrophages directly activated with LPS produced less NO_2^- when treated with 10^{-5} and 10^{-6} *M* THC ($P < 0.01$). Addition of 200 μ*M* N^GMMA to control macrophage cultures reduced the production of NO_2^- by 50%.

Effect of THC Analogs on Macrophage Cell Contact-Dependent Tumoricidal Activity. Macrophages were treated with the paired THC analogs, (−)HU-210/(+)HU-211 or (−)CP55,940/(+)CP 56,667, and were employed in a chromium release as-

Table III. Effect of THC on Nitrite Production by RAW264.7 Macrophages

Treatment[a]	Nitrite production (μ*M* NO_2^- ± SD)	
	IFN/LPS	LPS
Medium	29.3 ± 0.1	ND[b]
Vehicle	33.4 ± 0.1	17.5 ± 0.6
10^{-7} *M* THC	29.2 ± 1.8	15.9 ± 0.5
10^{-6} *M* THC	34.9 ± 1.6	11.9 ± 0.3[c]
10^{-5} *M* THC	28.6 ± 0.8	12.0 ± 0.4[c]

[a] Macrophages were plated in 96-well microtiter plates (1×10^5 cells/well) in vehicle (0.1% ethanol in complete RPMI) or THC for 48 hr in the presence or absence of N^GMMA (50–200 μ*M*) and were activated for 24 hr with either 10 U/ml IFN-γ plus 100 ng/ml LPS or with 1 μg/ml LPS. CM was harvested and added to an equal volume of the Griess reagent, the absorbance at 550 nm was determined, and the concentration of NO_2^- was calculated using sodium nitrite in complete RPMI as a standard. Cells treated neither with IFN-γ plus LPS nor with LPS produced <1 μM NO_2^- N^GMMA (200 μ*M*) inhibited the production of NO_2^- by 50%.
[b] Not determined.
[c] Values are significantly different ($P < 0.01$) when compared with those obtained for activated, vehicle control macrophages.

say to assess their capacity to lyse target tumor cells. Results of a representative experiment using (−)HU-210/(+)HU-211 are illustrated in Figures 2 and 3. (−)HU-210 and (+)HU-211 were equally effective in inhibiting cell contact-dependent cytolysis of L929 tumor cells by macrophages subjected to multistep

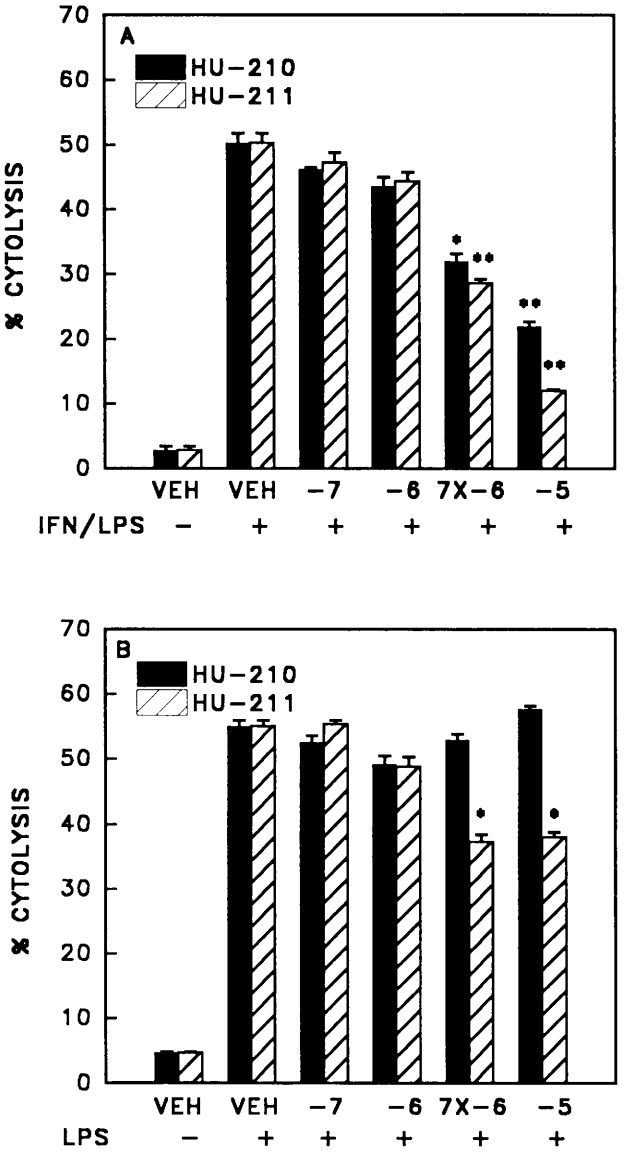

Figure 2. Effect of (−)HU-210 and (+)HU-211 on macrophage cell contact-dependent cytolysis of L929 cells. RAW264.7 macrophages were treated *in vitro* for 48 hr with vehicle or THC analogs. Macrophages were activated during the last 4 hr of the treatment period (A) with 10 U/ml IFN-γ plus 100 ng/ml LPS or (B) with 1 μg/ml LPS. Radiolabeled L929 cells were added to macrophage monolayers at an E:T ratio of 5:1. Effector and target cells were co-cultured for 20 hr at 37°C in the presence of vehicle, (−)HU-210, or (+)HU-211. Ethanol vehicle concentrations of THC analogs were as follows: 10^{-5} *M*, 0.38% ethanol; 7 × 10^{-6} *M*, 0.26% ethanol; and 10^{-6} and 10^{-7} *M*, 0.1% ethanol. Levels of cytolysis obtained for all vehicle control concentrations were equivalent. VEH, 0.38% ethanol; −7, −6, 7 × −6, and −5 represent 10^{-7} *M*, 10^{-6} *M*, 7 × 10^{-6} *M*, and 10^{-5} *M* analog, respectively. * and ** represent values significantly different ($P < 0.01$ and $P < 0.001$, respectively) from % cytolysis obtained from activated, vehicle control macrophages.

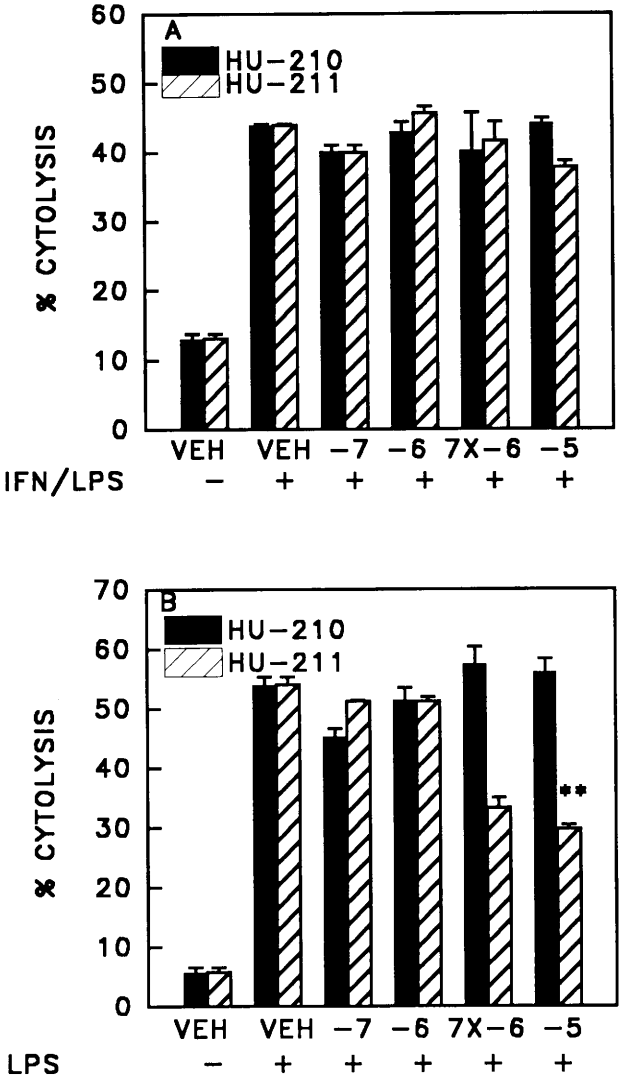

Figure 3. Effect of (−)HU-210 and (+)HU-211 on macrophage cell contact-dependent cytolysis of P815 Cells. RAW264.7 macrophages were treated *in vitro* for 48 hr with vehicle or THC analogs. Macrophages were activated during the last 4 hr of the treatment period (A) with 10 U/ml IFN-γ plus 100 ng/ml LPS or (B) with 1 μg/ml LPS. Radiolabeled P815 cells were added to macrophage monolayers at an E:T ratio of 5:1. Effector and target cells were co-cultured for 20 hr at 37°C in the presence of vehicle, (−)HU-210, or (+)HU-211. Ethanol vehicle concentrations of THC analogs were as follows: 10^{-5} *M*, 0.38% ethanol; 7 × 10^{-6} *M*, 0.26% ethanol; and 10^{-6} *M* and 10^{-7} *M*, 0.1% ethanol. Levels of cytolysis obtained for all vehicle control concentrations were equivalent. VEH, 0.38% ethanol; −7, −6, 7 × −6, and −5 represent 10^{-7} *M*, 10^{-6} *M*, 7 × 10^{-6} *M*, and 10^{-5} *M* analog, respectively. ** represents values significantly different ($P < 0.001$) from % cytolysis obtained from activated, vehicle control macrophages.

activation (Fig. 2A). Both analogs inhibited macrophage killing of TNF-sensitive L929 cells at 7 × 10^{-6} *M* and 10^{-5} *M*. However, when macrophages subjected to direct activation were employed as effectors, killing of L929 cells was inhibited only by (+)HU-211 at concentrations of 7 × 10^{-6} *M* and 10^{-5} *M* (Fig. 2B). Macrophages subjected to direct activation were impaired in their ability to kill P815 cells only by (+)HU-

211 at concentrations of 7×10^{-6} *M* and 10^{-5} *M* (Fig. 3B). Neither (−)HU-210 nor (+)HU-211 inhibited the ability of macrophages subjected to multistep activation to kill NO·-sensitive P815 cells (Fig. 3A).

Similar results were obtained when macrophages were treated with the stereoisomers (−)CP55,940/(+)CP56,667 (Fig. 4 and 5). Both (−)CP55,940 and (+)CP56,667 inhibited the ability of macrophages subjected to multistep activation to kill TNF-sensitive L929 cells (Fig. 4A), but had no effect on the ability of the macrophages to kill P815 cells (Fig. 5A). When macrophages treated with (−)CP55,940 and (+)CP56,667 were subjected to direct activation, both analogs inhibited the ability of macrophages to kill L929 cells (Fig. 4B) and P815 cells (Fig. 5B).

Binding of [^{3}H]CP55,940 to Rat Brain Membranes and RAW264.7 Membranes. Radioligand binding experiments revealed specific binding (45%–60%) of [^{3}H]CP55,940 for rat brain membrane preparations (Fig. 6A), which were used as a positive control. However, no specific binding of [^{3}H]CP55,940 to membrane preparations of RAW264.7 macrophage-like cells was demonstrated (Fig. 6B).

Discussion

Previous studies have shown that THC, the major psychoactive component in marijuana, inhibits macrophage functional activities (15–18), including cell contact-dependent cytolysis of tumor cells (19). Currently, it is not known whether one macrophage population is capable of exerting one or several cytolytic mechanisms simultaneously or if there exist distinct populations of macrophages, each of which exerts distinct cytolytic mechanisms. Macrophages can use both TNF- and L-arginine-dependent cytolytic mechanisms to kill target tumor cells. It has been demonstrated that TNF-α is preferentially cytotoxic or cytostatic for some tumor cells in tissue culture (8). This cytokine has been reported to induce both apoptotic and necrotic cell lysis (30). In addition to a soluble form of TNF-α, a membrane-associated form of TNF-α has been identified (31, 32). It has been postulated that membrane-associated TNF-α aids in killing target cells by either cell-to-cell contact or by local release of TNF-α (31).

Tumor cells also have been shown to be killed by nitric oxide. Nitric oxide is a highly toxic, short-lived molecule. In the macrophage, L-arginine is converted to NO· plus citrulline by an inducible cytosolic enzyme macrophage nitric oxide synthase (mac NOS). Under basal conditions, mac NOS mRNA accumulation is negligible, while multistep or direct activation greatly enhances mac NOS mRNA levels within a few hours (33). The principle action of the relatively large quantities of NO· generated by activated macrophages appears to be a cytostatic or a cytotoxic one depending on the target cell. The objective of the present study was to determine whether THC exerted a differential inhibitory effect on macrophage TNF-dependent ver-

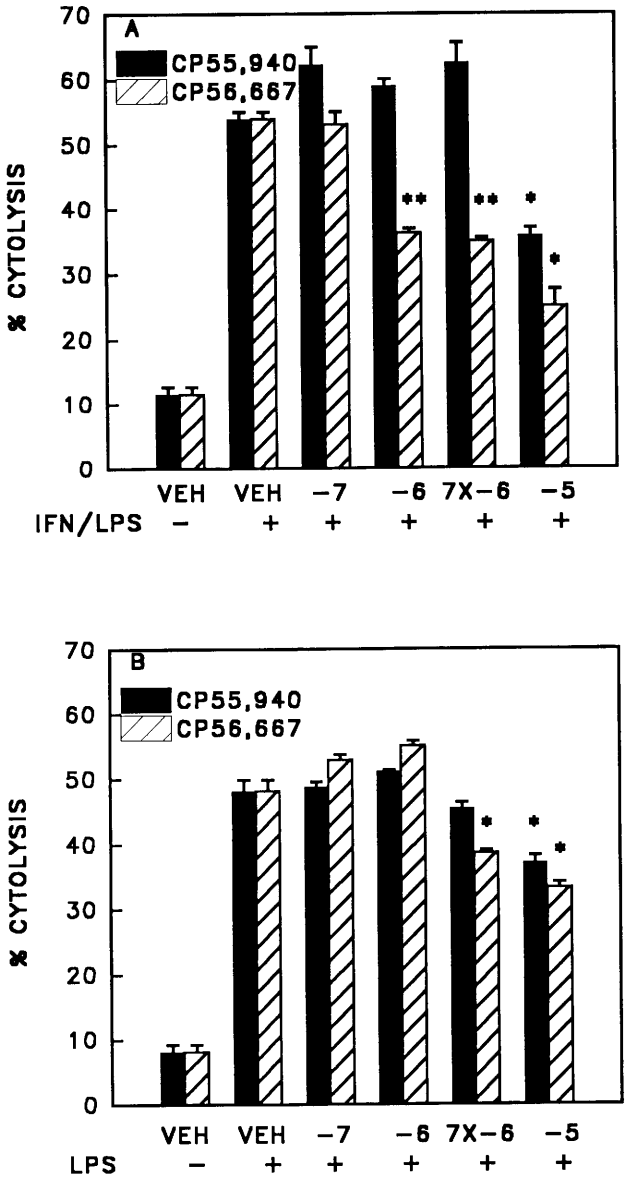

Figure 4. Effect of (−)CP55,940 and (+)CP56,667 on macrophage cell contact-dependent cytolysis of L929 Cells. RAW264.7 macrophages were treated *in vitro* for 48 hr with vehicle or THC analogs. Macrophages were activated during the last 4 hr of the treatment period (A) with 10 U/ml IFN-γ and 100 ng/ml LPS or (B) with 1 μg/ml LPS. Radiolabeled L929 cells were added to monolayers of macrophages at an E:T ratio of 5:1. Effector and target cells were co-incubated for 20 hr at 37°C in the presence of vehicle, (−)CP55,940, or (+)CP56,667. Ethanol vehicle concentrations of THC analogs were as follows: 10^{-5} *M*, 0.38% ethanol; 7×10^{-6} *M*, 0.26% ethanol; 10^{-6} and 10^{-7} *M*, 0.1% ethanol. Levels of cytolysis obtained for all vehicle control concentrations were equivalent. VEH, 0.38% ethanol; −7, −6, 7 × −6, and −5 represent 10^{-7} *M*, 10^{-6} *M*, 7×10^{-6} *M*, and 10^{-5} *M* analog, respectively. * and ** represent values which are significantly different ($P < 0.01$ and $P < 0.001$, respectively) from % cytolysis obtained from activated, vehicle control macrophages.

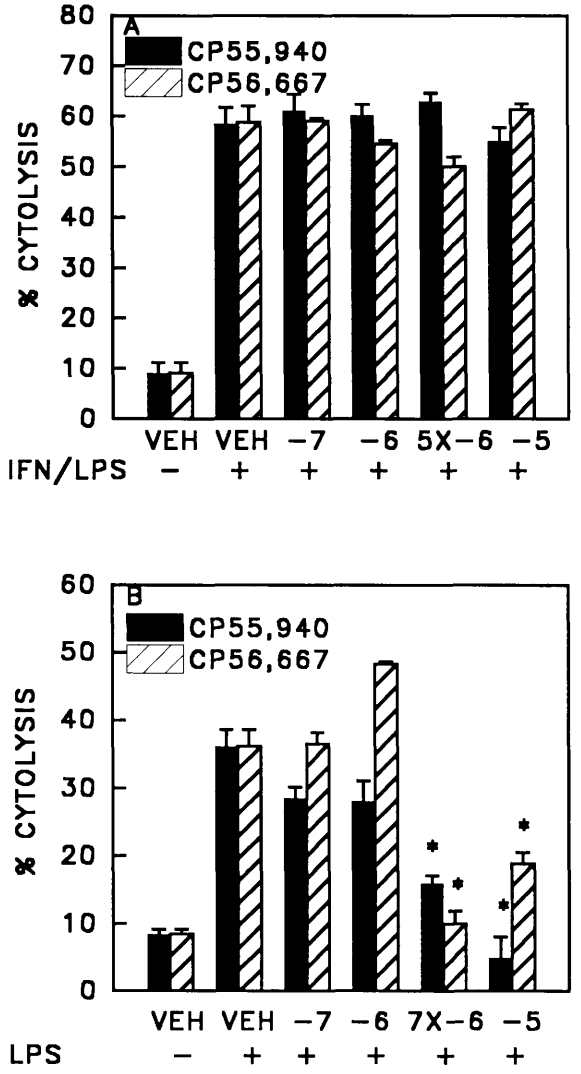

Figure 5. Effect of (−)CP55,940 and (+)CP56,667 on macrophage cell contact-dependent cytolysis of P815 Cells. RAW264.7 macrophages were treated *in vitro* for 48 hr with vehicle or THC analogs. Macrophages were activated during the last 4 hr of the treatment period (A) with 10 U/ml IFN-γ and 100 ng/ml LPS or (B) with 1 μg/ml LPS. Radiolabeled P815 cells were added to monolayers of macrophages at an E:T ratio of 5:1. Effector and target cells were co-incubated for 20 hr at 37°C in the presence of vehicle, (−)CP55,940 or (+)CP56,667. Ethanol vehicle concentrations of THC analogs were as follows: 10^{-5} *M*, 0.38 ethanol; 7×10^{-6} *M*, 0.26% ethanol; 10^{-6} and 10^{-7} *M*, 0.1% ethanol. Levels of cytolysis obtained for all vehicle control concentrations were equivalent. VEH, 0.38% ethanol; −7, −6, 7 × −6, and −5 represent 10^{-7} *M*, 10^{-6} *M*, 7×10^{-6} *M*, and 10^{-5} *M* analog, respectively. * represents values which are significantly different ($P < 0.01$) from % cytolysis obtained from activated, vehicle control macrophages.

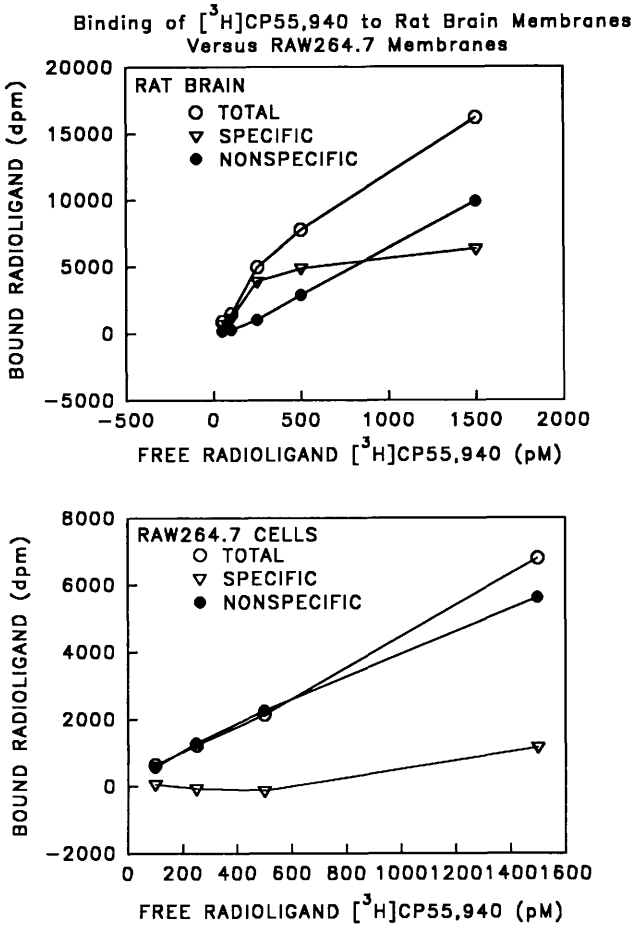

Figure 6. Binding of [^{3}H]CP55,940 to rat brain membranes versus RAW264.7 membranes. The filtration procedure was used for [^{3}H]CP55,940 binding to rat brain membranes (A) and RAW264.7 membranes (B). The binding assay was performed in silanized glass tubes containing 100 μl of radiolabeled ligand, 100 μl of competing unlabeled drug (1 μ*M* final concentration), 70 μg membrane protein, and sufficient buffer (50 μ*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 3 m*M* $MgCl_2$, 5 mg/ml BSA) to make a final volume of 1 ml. Tubes were incubated at 30°C for 1 hr. The reaction was stopped with the addition of 2 ml of ice-cold 50 m*M* Tris-HCl (pH 7.4) buffer containing 1 mg/ml BSA followed by rapid filtration through polyethyleneimide-treated 2.5 cm GF/C glass-fiber filters. Specific binding was defined as the difference between the binding that occurred in the presence and absence of 1 μ*M* unlabeled ligand.

sus L-arginine-dependent cytolytic pathways. RAW264.7 macrophage-like cells were used as effectors for cell contact-dependent cytolytic studies because these cells produce and secrete both TNF and NO·.

THC, at the highest drug concentration (i.e., 10^{-5} *M*), impaired RAW264.7 macrophage-like cell killing of TNF-sensitive L929 tumor target cells. This inhibition was effected regardless of whether macrophages were subjected to either direct activation with a relatively high dose of LPS or to multistep activation with IFN-γ in concert with a relatively low dose of LPS. Addition of rabbit anti-TNF-α to macrophage-L929 cell co-cultures during the chromium release assay resulted in inhibition of killing of L929 cells but had no effect on killing of P815 cells. These observations indicate that in the *in vitro* model system used, TNF-α is the principal mediator of L929 cytolysis. In addition, direct measurement of TNF-α using an ELISA assay indicated that macrophages subjected to multistep activation in concert with a relatively high concentration of THC (i.e., 10^{-5} *M*) elicited less TNF-α into the

culture medium. These results are in agreement with those of Fischer-Stenger *et al.* (18), who demonstrated that levels of TNF-α in cultures of RAW264.7 macrophages subjected to direct activation with 1 μg/ml LPS and exposed to 10^{-5} *M* THC yielded less TNF-α. In this earlier study, it was shown also that THC had no effect on the synthesis of the 26-kDa presecretory form of TNF-α. However, cleavage of the presecretory form to the 17-kDa secretory form of TNF-α was inhibited, accounting for the decreased levels of extracellular TNF-α.

Both direct and multistep activation of macrophages result in the release of TNF-α. However, the kinetics of release of this monokine have been shown to differ depending on the activation protocol employed (34). Direct activation results in a rapid burst of TNF-α (34, 35). This production can be inhibited by the simultaneous addition of actinomycin D, indicating that a new mRNA is rapidly induced (36). RAW264.7 macrophages exposed to 1 μg/ml LPS elicit maximal yields of extracellular TNF-α by 2–4 hr poststimulation (35). In contrast, the kinetics of TNF-α expression after multistep activation are slower (34). However, upon multistep activation with optimal concentrations of IFN-γ and LPS, similar or greater yields of TNF-α can be obtained over a longer period of time (37). Thus, shorter periods of time are required to induce the production of TNF-α using LPS versus IFN-γ in combination with LPS (34).

In addition to an extracellular 17-kDa secreted form of TNF-α, a 26-kDa form of TNF-α has been identified on the surface of macrophages (31, 32). It has been postulated that this cell surface-associated TNF-α may either be in a transmembrane arrangement or, alternatively, be associated with its cellular receptor (31, 35, 38). In order to determine whether THC affected the expression of cell-surface TNF-α, flow cytometry experiments were performed. A greater percentage of macrophages expressed surface TNF-α at 4 hr following direct activation with LPS than when subjected to multistep activation with IFN-γ plus LPS. These relative levels of surface TNF are in agreement with those recorded for secreted TNF and reflect the differential kinetics of TNF production following direct versus multistep activation of macrophages. THC did not decrease the percentage of cells that expressed cell surface-associated TNF-α regardless of the activation protocol used. In addition, the mean log fluorescence intensity of activated cells, or of THC-treated cells expressing surface-associated TNF-α, did not differ from unactivated cells. However, in the present study, only a small percentage of RAW264.7 macrophages was induced to express surface-associated TNF-α. Thus, relatively small changes in levels of TNF-α cell surface expression may not have been recognized. These results on cell surface expression of TNF-α are in agreement with those of Jue *et al.* (35), who estimated that RAW264.7 macrophages subjected to direct activation with 1 μg/ml LPS expressed less than 1% of TNF-α in the form of a membrane-bound precursor at any one point in time. In addition, these investigators reported that fixed RAW264.7 cells which had been activated with LPS exhibited a very low level of cytolytic activity when co-incubated with TNF-sensitive L929 cells. These observations suggest that cell surface-associated TNF-α may play a relatively minor role in effector cell-target cell contact-dependent cytotoxicity. Rather, a concentration gradient of secreted TNF may be established in the surrounding environment of the activated macrophage, thereby rendering attached target cells more susceptible to cytolysis. Thus, the present data suggest that exposure of macrophages to THC results in lower levels of secretory TNF with the consequence of decreased cell contact-dependent killing of TNF-sensitive tumor cells.

THC exhibited a different pattern of inhibition of cell contact-dependent cytolysis when NO•-sensitive P815 mastocytoma cells were employed as targets. The cannabinoid had no effect on killing of P815 cells by macrophages subjected to multistep activation with IFN-γ and LPS. However, RAW264.7 cells exposed to THC and subjected to direct activation with relatively high concentrations of LPS (i.e., 1 μg/ml), exhibited a dose-related impairment of cell contact-dependent cytolysis of P815 cells. Addition of N^GMMA, an analog of L-arginine known to inhibit the production of NO• by activated macrophages, during the chromium release assay inhibited macrophage-mediated killing of P815 cells but not of L929 cells. These data verify that in this *in vitro* effector cell:target cell system, NO• is a principal mediator of P815 cytolysis. Nitrite production by RAW264.7 macrophages, as measured in culture supernatants using the Griess reagent, paralleled the data obtained for cell contact-dependent killing of P815 target cells. That is, THC had no effect on NO_2^- production by macrophages subjected to multistep activation. In contrast, levels of NO_2^- in conditioned medium collected from macrophage cultures subjected to direct activation exhibited a drug dose-related decrease. These results are in agreement with reports that macrophages subjected to multistep activation produce greater levels of NO_2^- than macrophages subjected to a direct activation (33, 39). Indeed, in this study, 2-fold greater levels of NO_2^- were produced in cultures of macrophages subjected to multistep activation when compared with those subjected to direct activation. Thus, the lack of drug-induced inhibition of killing of P815 cells by macrophages subjected to multistep activation may be a consequence of the higher levels of NO• produced by macrophages as

measured by the presence of NO_2^- in macrophage-conditioned medium.

THC is a highly lipophilic molecule (25). It has been proposed that many of the cellular effects elicited by this molecule are a consequence of membrane perturbation which results either in physical disruption of cellular membranes (40) or in alteration of cell membrane fluidity (25, 41). Recently, a cannabinoid receptor (CBR1) has been identified in human and rat brain which is expressed also in human testis and murine splenocytes (20–23). In addition, a distinct cannabinoid receptor (CBR2) has been identified in the human promyelocytic leukemia cell line HL-60 and in rat spleen (24). Both cannabinoid receptors are G-protein-coupled receptors which have seven transmembrane domains. CBR1 has been identified as a G-inhibitory protein-coupled receptor (20). These observations suggest the existence of cannabinoid receptor subtypes and that THC may elicit its psychoactive and immunosuppressive effects through a cannabinoid receptor. Stereoselectivity is a crucial criterion for identification of receptor-mediated drug action. Therefore, RAW264.7 macrophages were treated with enantiomeric pairs of THC analogs in order to determine whether a cannabinoid receptor was involved in THC-mediated inhibition of macrophage cell contact-dependent tumoricidal activities. The THC analog pairs (−)CP55,940/(+)CP56,667 and (−)HU-210/(+)HU-211 were employed to determine whether the inhibitory effects of THC on macrophage tumoricidal activities were stereoselective. These analogs were developed based on their pharmacological effects. The negative isomers are psychoactive and bind to the CBR1 cannabinoid receptor while positive isomers are not psychoactive and exhibit low affinity for this receptor (42–47). Thus, use of these stereoisomer pairs could provide insight concerning the role of a cannabinoid receptor in macrophage contact-dependent killing of tumor cells. If RAW264.7 cells expressed a receptor similar to the CBR1, then the negative isomers should elicit an inhibitory effect on tumor cell killing while the positive isomers should have no effect. Alternatively, if the inhibition of tumoricidal activity was elicited through a noncannabinoid receptor-mediated mechanism, then both analogs would be expected to elicit similar inhibitory effects. However, Munro *et al.* (24) have demonstrated binding of both cannabinol (CBN), a weakly psychoactive cannabinoid, and (−)CP55,940 to the peripheral cannabinoid receptor CBR2.

In the present study, depending on the activation protocol employed, inhibition of macrophage tumoricidal activities was effected by both isomers of THC analogs, and in some cases, the greatest inhibition was caused by the positive isomers. Membrane preparations of RAW264.7 macrophages did not exhibit specific binding for [^{3}H]CP55,940 which has been shown to bind to both CBR1 and CBR2 (20, 24), indicating that CBR1 expression either is absent in RAW264.7 macrophage-like cells or, if expressed, is at levels not detectable by radioligand binding. Studies are in progress, using highly sensitive *in situ* polymerase chain reaction, to establish the presence or absence of a CBR1 in these cells. The observation that greater inhibition in tumoricidal activity was mediated by the positive isomers may reflect preferential membrane perturbation elicited by the (+)enantiomers. Recent studies have shown that +HU-211, a nonpsychotropic cannabinoid, blocks the N-methyl-D-aspartate (NMDA) receptor induced uptake of $^{45}Ca^{2+}$ ions into primary rat forebrain cell cultures (48). It was suggested that HU-211 inhibited the influx of calcium ions across the NMDA receptor-linked ion channel. A similar alteration of calcium fluxes may occur in RAW264.7 macrophages which have been exposed to HU-211.

The results of this study indicate that THC inhibits both TNF- and NO·-mediated killing by activated macrophages. However, THC differentially affected macrophage cell contact-dependent cytolytic pathways depending on whether multistep activation versus direct activation was employed. However, relatively high concentrations of THC and THC analogs were required to inhibit macrophage killing. Studies are in progress to further define the kinetics of cannabinoid-mediated inhibition of RAW264.7 macrophage-like cell cytolytic activities and to establish the extent to which a cannabinoid receptor plays a role in macrophage-mediated contact-dependent killing of target cells.

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