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Δ^9-Tetrahydrocannabinol Inhibition of Tumor Necrosis Factor-α: Suppression of Post-translational Events[1]

K. FISCHER-STENGER,[2] D. A. DOVE PETTIT[3] and G. A. CABRAL

Department of Microbiology and Immunology, Medical College of Virginia/Virginia Commonwealth University, Richmond, Virginia

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ABSTRACT

Δ^9-Tetrahydrocannabinol (Δ^9-THC), the major psychoactive component of marijuana, has been shown to suppress macrophage soluble cytolytic activity. The purpose of this study was to determine whether Δ^9-THC inhibited this function by affecting tumor necrosis factor-α (TNF-α). The RAW264.7 macrophage cell line was used as an *in vitro* bacterial lipopolysaccharide-inducible system for production of TNF-α. Macrophage-conditioned medium of RAW264.7 macrophages treated with Δ^9-THC was shown to be deficient in tumoricidal activity. Immunoprecipitation experiments demonstrated that the macrophage-conditioned medium of cultures treated with drug contained lower levels of TNF-α. Northern analysis indicated that Δ^9-THC had no effect on the levels of TNF-α messenger RNA. However, radiolabel pulsing and pulse-chase experiments revealed that the intracellular conversion of the 26-kD presecreted form of TNF-α to the 17-kD secreted form was inhibited by the drug. These results indicate that Δ^9-THC suppresses soluble macrophage tumoricidal activity, at least in part, by decreasing the intracellular conversion of presecretory TNF-α to its 17-kD secretory form.

Δ^9-THC is the major psychoactive component of marijuana. It is widely acknowledged that this compound induces a variety of suppressive and inhibitory effects on the host immune response, including effects on macrophage function (Blanchard *et al.*, 1986; Cabral *et al.*, 1986; Cabral *et al.*, 1987a; Cabral and Vasquez, 1991a). Macrophages are important in nonspecific host resistance to viruses and microbial pathogens and serve as central regulators of specific immune responses (Solbach *et al.*, 1991). Macrophages also interact with other cells of the immune system that play a role in specific cell-mediated and humoral responses.

It has been demonstrated that exposure of mouse peritoneal exudate cells to graded doses of Δ^9-THC suppresses the ability of these cells to spread and to phagocytize yeast particles *in vitro* (Lopez-Cepero *et al.*, 1986; Specter *et al.*, 1991; Tang *et al.*, 1992). Δ^9-THC also has been shown to inhibit macrophage cell contact-dependent cytolytic activities and to deplete soluble tumoricidal activity found in supernatants from activated macrophages treated with drug *in vivo* (Burnette-Curley *et al.*, 1993; Cabral and Vasquez, 1991b). Furthermore, Δ^9-THC has been reported to alter the expression of macrophage proteins associated with the activated state (Cabral and Mishkin, 1989; Mishkin and Cabral, 1987) and to limit the release of cytokines by activated macrophages (Nakano *et al.*, 1992; Watzl *et al.*, 1991; Zheng *et al.*, 1992). These findings suggest that Δ^9-THC may alter macrophage functional competence, at least in part, by suppressing the expression of macrophage effector molecules.

Cytolytic factors are produced and secreted into the culture medium when macrophages are activated and triggered with bacterial LPS (Adams and Hamilton, 1984; Hamilton and Adams, 1987; Hamilton *et al.*, 1986). The release of TNF-α by activated macrophages after stimulation with endotoxin is a major mechanism of tumor cell killing (Beutler and Cerami, 1986; Carswell *et al.*, 1975; Gifford and Flick, 1988; Klostergaard, 1987). In the present study, the RAW264.7 macrophage-like cell line, which produces TNF-α after triggering with LPS, was used as an *in vitro* model to assess the effect of Δ^9-THC on the expression and secretion of this cytolytic factor.

Materials and Methods

Cell lines. The RAW264.7 mouse macrophage cell line (TIB 71, American Type Culture Collection, Rockville, MD) and the L929 mouse fibroblast cell line (CCL-1, American Type Culture Collection) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, McLean, VA), 1.5% sodium bicarbonate, 25 mM HEPES buffer, 1% L-glutamine, 1% minimal essential medium vitamins, 1% nonessential amino acids, 100 units/ml of penicillin and 100 μg/ml of streptomycin. The cells were maintained

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ABBREVIATIONS: Δ^9-THC, Δ^9-tetrahydrocannabinol; TNF-α, tumor necrosis factor-α; MϕCM, macrophage-conditioned medium; LPS, lipopolysaccharide; SDS, sodium dodecyl sulfate; GPD, glyceraldehyde 3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis.

at 37°C in 5% CO_2 in 75-cm^2 tissue culture flasks and were subcultured twice weekly.

Antiserum. Polyclonal rabbit antimouse TNF-α antiserum (neutralizing) was purchased from Genzyme (Boston, MA) and was used in the immunoprecipitation studies.

Drug treatment. Δ^9-THC (formula weight = 316 Daltons) was obtained from the National Institute on Drug Abuse (Rockville, MD). The drug was prepared from a stock solution of 100 mg/ml in 95% ethanol. The stock was appropriately diluted first in ethanol and then in complete RPMI-1640 medium such that 10 μl from the respective stock solutions were added per milliliter of medium to yield final concentrations of 10^{-7} M, 10^{-6} M and 10^{-5} M Δ^9-THC in 0.1% ethanol. The vehicle consisted of medium containing 0.1% ethanol. RAW264.7 macrophages were grown to 50% confluence in T_{25}-cm^2 or T_{150}-cm^2 tissue culture flasks and were exposed to Δ^9-THC. Preliminary studies were performed to establish the time kinetics for the optimal drug-induced suppression of RAW264.7-mediated soluble cytolytic activity. Incubation of the macrophages with the drug for 48 hr before exposure to bacterial LPS resulted in maximal inhibition of soluble tumoricidal activity against murine L929 tumor cells.

MϕCM. Confluent RAW264.7 macrophage monolayers in T_{25}-cm^2 tissue culture flasks were exposed to medium, vehicle or Δ^9-THC for 48 hr and were triggered for either 4 or 24 hr with 1 μg/ml of LPS (*Escherichia coli* 055:B5 LPS, Sigma, St. Louis, MO) to induce production of TNF-α. After LPS triggering, MϕCM was collected, centrifuged at 1000 × *g* for 10 min to remove cellular debris, aliquoted and stored at −70°C until assayed for TNF-α.

Cytotoxicity assays. Cytotoxicity assays were performed in sterile, U-bottom, 96-well microtiter plates (Costar, Cambridge, MA). Briefly, L929 target cells were suspended in 1 ml of RPMI-1640 supplemented as before and were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL) per 1×10^7 cells for 3 hr. The cells were washed three times with warm medium, were resuspended in RPMI and were plated at a concentration of 1×10^5 cells/well. Plates containing the target cells were centrifuged at 500 × *g* for 2 min and were incubated at 37°C for 2 hr to allow for cell adherence. After removal of medium, 150 μl of MϕCM were added to the radiolabeled target cells. 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. After the incubation period, the plates were centrifuged at 1000 × *g* for 10 min, the supernatants were harvested and the radioactivity present was determined in a gamma counter. The results were reported as percentage of specific release according to the following formula: experimental release − spontaneous release/total release − spontaneous release × 100. The total releasable ^{51}Cr was obtained by treating radiolabeled target cells with 1 N NaOH.

Statistical analysis was performed on all data by using Student's *t* test. A 99% confidence level was chosen for all experiments to determine the statistical significance for differences between paired values of percent specific release.

Metabolic labeling. RAW264.7 macrophages in T_{25}-cm^2 tissue culture flasks were exposed to Δ^9-THC or vehicle for 48 hr. Cells, then, were triggered with 1 μg/ml of LPS for a total of 4 hr at 37°C (Jue *et al.*, 1990). After the first 2.5 hr of treatment with LPS, the cells were washed twice with warm methionine-free RPMI supplemented with L-glutamine and were incubated for 30 min in the same medium containing LPS. The medium then was replaced with 2 ml of fresh methionine-free medium containing LPS and 150 μCi Tran (^{35}S)-label (methionine and cysteine; ICN Biomedicals, Costa Mesa, CA) and the cells were incubated for an additional hour. This protocol allowed for a 1-hr radiolabeling pulse. The supernatants (2 ml) were removed and the cells were washed twice with cold phosphate-buffered saline. Then the cells were treated with 2 ml of lysis buffer (50 mM Tris HCl, pH 7.4; 0.5% Nonidet P40; 0.15 M NaCl; 0.02% sodium azide; 5 mM EDTA; 0.1 mM phenylmethanesulfonyl fluoride; 20 μg/ml of soybean trypsin inhibitor; 5 μM leupeptin; and 5 μM antipain) for 10 min. Cell lysates and supernatants were clarified by centrifugation. Then, 200 μl of 10× lysis buffer was added to the supernatants.

For radiolabel time pulse experiments, drug- or vehicle-treated macrophages were triggered with LPS for a total of 4 to 8 hr. Replicate cultures were subjected to 1-hr pulses with 150 μCi Tran (^{35}S)-label from 3 to 4, 4 to 5, 5 to 6, 6 to 7 and 7 to 8 hr postinitiation of LPS triggering. After each pulsing period, the culture supernatants were harvested and treated with lysis buffer as previously described.

For pulse-chase experiments, drug- or vehicle-treated macrophages were triggered with LPS for a total of 4 hr. The macrophages were pulsed with 150 μCi Tran (^{35}S)-label during the last hour of the triggering period (0 chase time). The chase period was initiated by washing the remaining cells twice with complete RPMI-1640 followed by addition of radiolabel-free complete RPMI-1640 containing LPS. The cell pellets and culture supernatants were harvested at 0, 1, 2, 8 and 20 hr postchase and the supernatants were treated with lysis buffer as described earlier.

Trichloroacetic acid-precipitable counts were calculated for supernatants and cell pellets from all radiolabeling experiments using Skatron filters (Sterling, VA). The supernatants and lysates were aliquoted and frozen at −20°C until assayed by immunoprecipitation and SDS-PAGE.

Immunoprecipitation and gel electrophoresis. Aliquots (500 μl) of the cell lysates and the supernatants from the metabolically radiolabeled RAW264.7 macrophages, containing a constant number of trichloroacetic acid-precipitable counts, were incubated with 10 μl of a 50% suspension of protein A Sepharose CL-4B (Pharmacia LKB Biotechnology, Piscataway, NJ) at 22°C for 1 hr to remove any nonspecific association with the protein A Sepharose. The Sepharose beads were removed by centrifugation and 40 μl of a 1:10 dilution of rabbit anti-TNF-α antiserum (neutralizing) were added. After incubating for 16 hr at 4°C, 80 μl of 50% protein A Sepharose bead suspension were added and the mixture was incubated for another 2 hr at 4°C. All incubations were performed with gentle agitation. The Sepharose beads were washed twice each with lysis buffer, with phosphate-buffered saline containing 0.05% sodium azide and with 0.1% SDS. The washed beads were combined with 50 μl of 2× sample solubilization buffer (125 mM Tris HCl, pH 6.9; 4% SDS; 20% glycerol; and 10% 2-mercaptoethanol), heated for 5 min at 90°C and clarified by centrifugation. The samples were separated in a 15% 0.75-mm polyacrylamide gel at 15 mA/gel. The [^{14}C]-labeled protein molecular weight standards (molecular weight range, 14.3–200.0 kD; GIBCO/BRL, Gaithersburg, MD) were included in each gel analysis. Gels were fixed in 40% methanol and 10% acetic acid, treated with EN^{3}HANCE autoradiography enhancer (NEN Research Products, Boston, MA), dried and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at −70°C.

RNA isolation and northern blot analysis. RAW264.7 macrophages in T_{150}-cm^2 tissue culture flasks were exposed to Δ^9-THC or vehicle for 48 hr and were triggered with 1 μg/ml of LPS for 2, 4, 6, 12 or 24 hr in the presence of vehicle or drug. The total cellular RNA was isolated from macrophages by the guanidinium isothiocyanate/cesium chloride method (Chirgwin *et al.*, 1977). The purified RNA was separated by electrophoresis in a 1% agarose-formaldehyde gel followed by blot transfer onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membranes were baked for 2 hr at 80°C in a vacuum oven and prehybridization was carried out for 4 hr at 37°C in 50% formamide, 5× SSC (20× SSC = 3.0 M NaCl, 0.3 M $Na_3C_6H_5O_4$, pH 7.0), 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll (Sigma), 0.1 M NaH_2PO_4 and 20 μg/ml of sheared and denatured salmon sperm DNA. Then the membranes were hybridized for 18 hr at 37°C in hybridization buffer (prehybridization buffer containing 5% dextran sulfate and the nick-translated TNF-α complementary DNA) and were washed at 37°C in 2× SSC/0.2% SDS (four times for 5 min each), in 2× SSC/0.2% SDS (20 min) and in 0.5× SSC/0.2% SDS (20 min). Autoradiography was carried out at −70°C using XRP-5 film (Eastman Kodak) and intensifying screens. The quality and equivalent loading of RNA was confirmed by ethidium bromide staining of the gel before northern transfer. In addition, the membranes were stripped for 15 min at 95°C in 0.01× SSC/0.01% SDS and were rehybridized with a ^{32}P-labeled GPD complementary DNA to ensure that equivalent amounts of RNA were transferred to each lane.

Probes. The plasmid containing the full-length mouse TNF-α complementary DNA was provided by Dr. Bruce Beutler (University of Texas Southwestern, Medical Center at Dallas, Dallas, TX; Caput *et al.*, 1986). The clone was digested with BamHI and PstI. The resulting fragments were separated using gel electrophoresis and a 1.6-kilobase fragment containing TNF-α complementary DNA was excised, eluted (Centrilutor, Amicon, Beverly, MA) and purified using the Glassmax system (GIBCO/BRL). The clone containing the GPD complementary DNA was digested with PstI (Thompson *et al.*, 1986). The fragments were also separated by gel electrophoresis and a 1.3-kilobase fragment containing GPD complementary DNA was obtained as described. The TNF-α complementary DNA and the GPD complementary DNA were ^{32}P-labeled by means of nick translation (GIBCO/BRL) and were used as probes in the hybridization experiments.

Image analysis. Autoradiograms were analyzed in a Shimadzu CS9000U dual-wavelength flying-spot scanner (Kyoto, Japan) interfaced to a WIN 286 computer (Win Laboratories, Fairfax, VA) using a Quantascan two-dimensional analysis binary format and American Standard Code for Information Interchange (ASCII) conversion software (Shimadzu). The ASCII-converted files were analyzed further on a VAXcluster Computer System (Digital Equipment, Richmond, VA) using SAS/GRAPH software (SAS Institute, Cary, NC). SAS/GRAPH-analyzed data were subjected to final analysis using a WIN 386 computer utilizing QGEL analysis software (HBI, Saddlebrook, NJ).

Results

Effect of Δ^9-THC on MϕCM cytolytic activity. Δ^9-THC was shown to inhibit in a dose-related fashion the cytolytic activity of LPS-triggered RAW264.7 cells (fig. 1). The MϕCM obtained from macrophage cultures inoculated with placebo (*i.e.*, medium) or with vehicle, as expected, elicited minimal cytolytic activity against murine L929 fibroblasts. Maximal cytolytic activity (*i.e.*, 25–30%) was recorded for MϕCM obtained from placebo or vehicle-treated cultures receiving LPS for 24 hr. Conditioned-medium from macrophage cultures exposed to 10^{-5} M Δ^9-THC produced little (< 5%) cytolytic activity against L929 tumor cells.

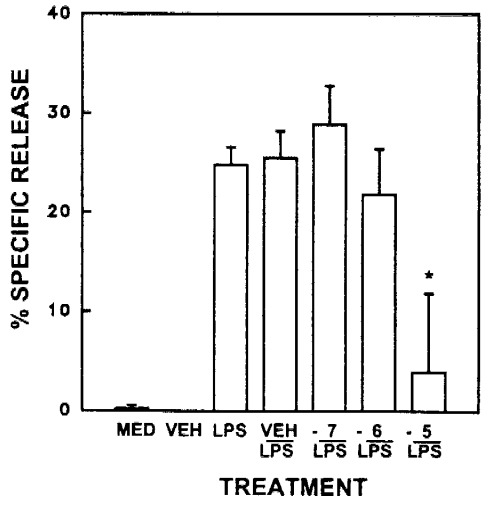

Fig. 1. Δ^9-THC inhibition of LPS-inducible cytolytic activity in MϕCM. RAW264.7 macrophage-like cells were exposed to medium, vehicle or Δ^9-THC at various concentrations for 48 hr and then were maintained in the presence or absence of LPS for 24 hr. Then MϕCM was assessed for cytolytic activity by a cytotoxicity assay using ^{51}Cr-labeled L929 tumor cells as targets. The percent specific release represents the release of the radiolabel from target cells ± S.D. from four wells from one of four representative experiments. Minimal cytolytic activity was detected in MϕCM of cultures not treated with LPS. By contrast, maximal cytolytic activity was recorded for cultures treated with LPS. Δ^9-THC was shown to exert a dose-related inhibition of cytolytic activity in MϕCM from cultures treated with LPS. Inhibition was shown to be significant at $P < .01$ for MϕCM exposed to 10^{-5} M Δ^9-THC. MED = medium; VEH = vehicle; −7, −6 and −5 represent the respective molar concentrations of Δ^9-THC.

TNF-α production by RAW264.7 macrophages. Cytolytic activity in MϕCM directed against L929 fibroblasts has been shown to be mediated primarily by TNF-α (Higuchi *et al.*, 1990). To confirm that triggering RAW264.7 cells with 1 μg/ml of LPS for 4 hr induced the macrophages to produce and secrete TNF-α in our *in vitro* system, MϕCM from Tran (^{35}S)-labeled cultures were subjected to immunoprecipitation with anti-TNF-α antiserum and were analyzed by SDS-PAGE and autoradiography. This time period for LPS induction of TNF-α was selected because it has been shown to be optimal for eliciting this cytokine from RAW264.7 macrophages (Beutler *et al.*, 1985). No TNF-α was detected in MϕCM in the absence of LPS triggering (fig. 2). However, after triggering with LPS, a 17-kD protein, which represents the mature secreted form of TNF-α, plus multimers ranging in relative molecular weight from 17 to 26 kD were immunoprecipitated with the anti-TNF-α antibody.

Effect of Δ^9-THC on production of TNF-α. Immunoprecipitation of RAW264.7 macrophage lysates and culture supernatants was performed to determine the effect of Δ^9-THC on TNF-α production. RAW264.7 cells were triggered with LPS for 4 hr. Immunoprecipitates were subjected to SDS-PAGE and autoradiography and the volume density of precipitated protein bands was evaluated by image analysis. Maximal inhibition was recorded for cultures maintained in 10^{-5} M Δ^9-THC (fig. 3A). Medium from LPS-triggered macrophage cultures treated with 10^{-5} M drug contained 54% of the amount of the 17-kD TNF-α species that was found in medium of LPS-triggered cultures maintained in the vehicle. In addition, decreased levels of intermediate multimer species, ranging in relative molecular weight from the 26-kD presecreted form of TNF-α to the 17-kD secreted form, were observed in supernatants compared with those from the LPS-triggered vehicle control.

Analysis of immunoprecipitates of cell lysates demonstrated that Δ^9-THC had no major effect on the total level of the 26-

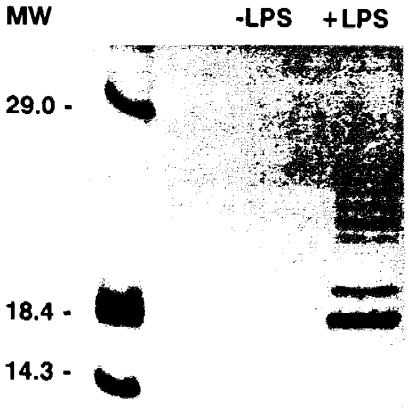

Fig. 2. Immunoprecipitation analysis of MϕCM of RAW264.7 cells treated with LPS. Macrophages were maintained in the presence or absence of LPS for 4 hr and were pulsed with Tran (^{35}S)-label during the last hour. Supernatants were immunoprecipitated with anti-TNF-α antiserum and were analyzed by SDS-PAGE and fluorography. The numbers along the left margin represent relative molecular weights in kiloDaltons. These results confirmed that triggering of RAW264.7 cells with 1 μg/ml of LPS elicited TNF-α into the MϕCM.

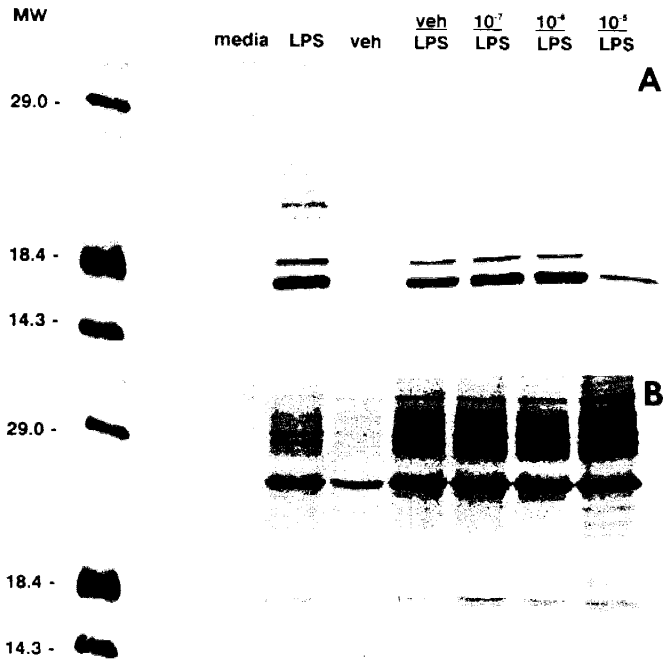

Fig. 3. Effect of Δ⁹-THC on production of TNF-α. RAW264.7 cells were treated with medium, vehicle or Δ⁹-THC for 48 hr. Then the cells were maintained in the presence or absence of 1 μg/ml of LPS for 4 hr. Cultures were labeled with Tran (³⁵S)-label for the last hour. Supernatants and cell lysates then were subjected to immunoprecipitation with anti-TNF-α antiserum, analytical SDS-PAGE and fluorography. A) Supernatants from RAW264.7 cells. Note that treatment of cultures with 10^{-5} M Δ⁹-THC resulted in decreased levels of the 17-kD secretory form of TNF-α in MϕCM. B) Cell lysates of RAW264.7 cells. Δ⁹-THC had no major effect on the expression of the 26-kD presecretory form of TNF-α. Note, however, that treatment of cultures with 10^{-5} M Δ⁹-THC resulted in accumulation of intermediate multimers ranging from the 26-kD presecretory form to the 17-kD secretory form of TNF-α.

kD TNF-α presecretion form (fig. 3B). However, accumulation of multimers, ranging in relative molecular weight from 26 to 17 kD, was observed in immunoprecipitates of lysates of RAW264.7 cells that were exposed to 10^{-5} M drug.

To confirm that Δ⁹-THC affected the production of the 17-kD secreted form of TNF-α, western blot analysis was performed on the supernatants and lysates of pellets of RAW264.7 macrophages after triggering with LPS for 4 or 24 hr. Western blot analysis confirmed the results obtained by immunoprecipitation in that a drug dose-related decrease in the amount of the secreted form of TNF-α was observed in macrophage cultures triggered with LPS for 4 or 24 hr (data not shown).

Effect of Δ⁹-THC on TNF-α messenger RNA. The immunoprecipitation and western blot data indicated that Δ⁹-THC altered levels of the 17-kD secreted form of TNF-α but did not affect the intracellular levels of the 26-kD presecreted form. These data suggested that the drug did not affect the levels of TNF-α-specific message in RAW264.7 cells. To confirm that Δ⁹-THC did not decrease levels of TNF-α messenger RNA, northern analysis was performed on total cellular RNA from RAW264.7 macrophages triggered with LPS from 2 to 24 hr. The volume densities of complementary DNA TNF-α and complementary DNA GPD-hybridized messenger RNA bands were evaluated by image analysis and volume density units of TNF-α messenger RNA were standardized *versus* the volume density units of the GPD messenger RNA control (fig. 4). Peak levels of TNF-α messenger RNA were detected in vehicle-treated RAW264.7 cells from 2 to 6 hr in response to LPS (fig. 4A). Maximal levels of TNF-α messenger RNA were recorded 4 hr after LPS triggering (*e.g.*, 3778 volume density units). Levels of messenger RNA declined after this period such that, by 24 hr postexposure to LPS, only residual levels of TNF-α messenger RNA were detected (*e.g.*, 150 volume density units; fig. 4B). A similar profile of TNF-α messenger RNA expression was recorded for drug-treated cells. Δ⁹-THC did not inhibit maximal levels of total TNF-α messenger RNA from RAW264.7 macrophages triggered with LPS from 2 to 6 hr. Levels of messenger RNA comparable to those of vehicle-treated cells, also, were recorded at 12 hr. However, less TNF-α messenger RNA was detected in drug-treated cells triggered with LPS for 24 hr. The level at 24 hr was 5-fold less that of vehicle-treated cells (*i.e.*, 150 *versus* 31 volume density units).

Effect of Δ⁹-THC on TNF-α secretion. Radiolabel time pulse experiments were performed to determine whether Δ⁹-THC affected the rate of secretion of TNF-α. The cultures were pulsed for 1-hr periods ranging from 3 to 7 hr after triggering with LPS and the supernatants were subjected to immunoprecipitation, SDS-PAGE and fluorography (fig. 5A). Relatively high levels of the 17-kD secretory form of TNF-α were detected in the supernatants from vehicle-treated RAW264.7 cells immediately after the 1-hr radiolabeling pulse (*i.e.*, 3–4 hr after initiation of LPS triggering). Substantial but lesser amounts of secretory TNF-α were detected 4 to 5 hr postinitiation of LPS triggering. Secretory TNF-α was found to reach a plateau to minimal levels for the 1-hr pulsing periods subsequent to 4 to 5 hr after initiation of LPS triggering. These results indicate that maximal rates of TNF-α secretion occur at, or before, 3 to 4 hr after initiation of LPS triggering. By contrast, lesser amounts of the 17-kD secretory form of TNF-α were observed 3 to 4 hr and 4 to 5 hr postinitiation of LPS triggering in culture supernatants of RAW264.7 cells maintained in 10^{-5} M Δ⁹-THC. Peak amounts of secretory TNF-α were detected in these supernatants 6 to 7 hr after initiation of LPS triggering (fig. 5B). Secretory TNF-α levels were found to reach a plateau to a minimal amount after the 6- to 7-hr pulsing period. These results indicated a decreased rate of release of TNF-α from drug-treated cells.

Effect of Δ⁹-THC on TNF-α processing. The decreased rate of release of TNF-α from drug-treated cells could have resulted from drug-induced defects in secretion of this cytokine. Alternatively, a decreased release of TNF-α may have been a consequence of drug-induced dysfunction in cleavage of the 26-kD presecretory form to the 17-kD secretory form. To discriminate between these two possibilities, pulse-chase experiments were performed to define the intracellular and extracellular fate of TNF-α. RAW264.7 cells were triggered with LPS for up to 24 hr. At 3 hr post-triggering, cultures were pulsed for 1 hr with Tran (³⁵S)-label and maintained in radiolabel-free medium after the pulse. Supernatants and cell lysates were evaluated at discrete periods postchase. Autoradiograms revealed that, for LPS-triggered, vehicle-treated RAW264.7 cells, the 17-kD secretory form of TNF-α was present at relatively high amounts in supernatants immediately after the 1-hr pulsing period (*i.e.*, 0 hr postchase; fig. 6A). Lesser amounts of radiolabeled secretory TNF-α were found at 1 and 2 hr postchase. At 8 and 20 hr postchase, little radiolabeled 17-kD TNF-α was detected in the culture supernatants (fig. 6B). These data indicate that, for vehicle-treated cells, newly synthesized presecretory TNF-α was rapidly processed and secreted into the culture superna-

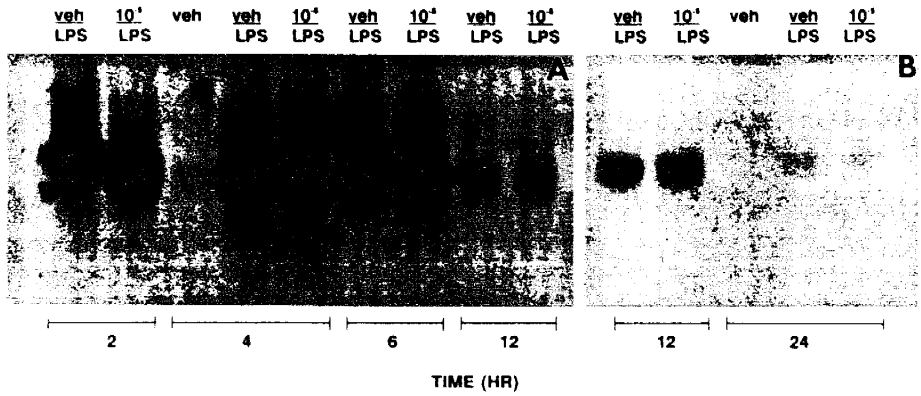

Fig. 4. Effect of Δ^9-THC on RAW264.7 TNF-α messenger RNA. Northern analysis using a mouse TNF-α complementary DNA probe was performed on total cellular RNA from macrophages pretreated with drug or vehicle for 48 hr and triggered with LPS for 2 to 24 hr in the presence or absence of the drug. A) Northern analysis of total RNA of macrophages after 2 to 12 hr treatment with LPS. Peak levels of TNF-α messenger RNA were detected in vehicle-treated cells from 2 to 6 hr. B) Extended exposure of a northern blot of total RNA of macrophages after 12 and 24 hr of treatment with LPS. At 24 hr, only residual levels of TNF-α messenger RNA were detected. Δ^9-THC did not suppress levels of TNF-α messenger RNA from RAW264.7 macrophages triggered with LPS from 2 to 12 hr. However, less TNF-α messenger RNA was detected in drug-treated cells at 24 hr.

tants. The bulk of the newly processed TNF-α was secreted from RAW264.7 cells within 1 hr postchase. By contrast, a lesser amount of 17-kD TNF-α (less than 50% of the level recorded for vehicle-treated cells) was detected immediately after the radiolabel pulse period in the supernatants of RAW264.7 cultures, which were triggered with LPS and maintained in 10^{-5} M drug. However, persistently higher levels of secretory TNF-α were detected in culture supernatants of LPS-triggered, drug-treated cells throughout the postchase period. As late as 20 hr postchase, considerably more 17-kD TNF-α was detected in the supernatants of drug-treated cells compared with that in the supernatants of vehicle-treated controls. These results indicate that exposure of LPS-triggered RAW264.7 cells to Δ^9-THC either results in a slower rate of secretion of the 17-kD form of TNF-α or inhibits processing of the 26-kD presecretory form to the 17-kD secretory species.

To differentiate between impaired secretion of TNF-α and dysfunctional processing of the presecretory form, cell lysates corresponding to each supernatant from the pulse-chase experiments were evaluated. Little of the radiolabeled 26-kD presecretory form of TNF-α was detected in the cell lysates of LPS-triggered, vehicle-treated cells immediately after the 1-hr pulsing period (*i.e.*, 0 hr postchase). These results indicate that TNF-α was rapidly cleaved and secreted into the extracellular medium (fig. 7A). By contrast, relatively high amounts of the 26-kD presecretory form of TNF-α were detected in lysates of LPS-triggered, drug-treated cells. At 20 hr postchase, a considerable amount of the 26-kD species, in addition to multimer forms ranging in relative molecular weight ranging from 26 to 17 kD, was detected in cell lysates of drug-treated cells (fig. 7B). These results indicate that processing of the 26-kD presecretory form of TNF-α to the 17-kD secretory form is impaired in drug-treated cells, which results in less TNF-α being secreted.

Discussion

Δ^9-THC, the major psychoactive component of marijuana, has been shown to alter macrophage function (Burnette-Curley *et al.*, 1993; Cabral and Vasquez, 1991a; Cabral and Vasquez, 1991b; Specter *et al.*, 1991). Exposure to Δ^9-THC decreases activated macrophage cytolytic activity (Burnette-Curley *et al.*, 1993; Cabral and Vasquez, 1991b). However, the mechanism by which the drug affects this activity remains to be defined. Different macrophage cytolytic mechanisms are responsible for the cytolysis of specific target cells (LeBlanc *et al.*, 1990; Nacy *et al.*, 1984; Wing *et al.*, 1977). Therefore, it is possible that Δ^9-THC may alter several cytolytic mechanisms exhibited by activated macrophages. The present study was designed to determine whether Δ^9-THC inhibited soluble macrophage cytolytic activity by altering TNF-α production. The drug doses used in this study were within physiologically achievable concentrations in humans. Because RAW264.7 macrophages express and secrete TNF-α into the culture medium after triggering by LPS, these cells were utilized as *in vitro* models for defining the effects of Δ^9-THC on the synthesis, processing and extracellular release of this cytolytic molecule.

MϕCM from LPS-triggered, drug-treated macrophages exhibited a decreased capacity to lyse murine L929 tumor target cells. The cytolytic activity was affected in a dose-related fashion. These results are in agreement with the findings of previous studies that demonstrated that both *in vivo* and *in vitro* exposure to Δ^9-THC results in inhibition of bacterial immunomodulator-induced macrophage cytolytic activity against various target cells (Burnette-Curley *et al.*, 1993; Cabral and Vasquez, 1991b). L929 tumor cells are highly sensitive to TNF-α-mediated cytolysis but are relatively resistant to alternate macrophage-mediated killing pathways, *i.e.*, arginine-dependent nitric oxide killing (Higuchi *et al.*, 1990). In addition, we demonstrated that, although Δ^9-THC suppresses macrophage-mediated killing of L929 tumor cells, which are sensitive to TNF-α, it has little effect on P815 mastocytoma cells, which are relatively resistant to TNF-α (D. Burnette-Curley, personal observation). Thus, these data suggest that the decreased cytolysis recorded for MϕCM from LPS-triggered, drug-treated macrophage cultures is a result, at least in part, of decreased expression and/or release of TNF-α. Nevertheless, because macrophages elicit a variety of molecules with cytolytic potential, other factors in addition to TNF-α may be targeted by Δ^9-THC.

To confirm the functional data that indicated that Δ^9-THC inhibited TNF-α-mediated killing, polyvalent anti-TNF-α antiserum was used in immunoprecipitation and western immunoblot analyses. Initial experiments centered on examina-

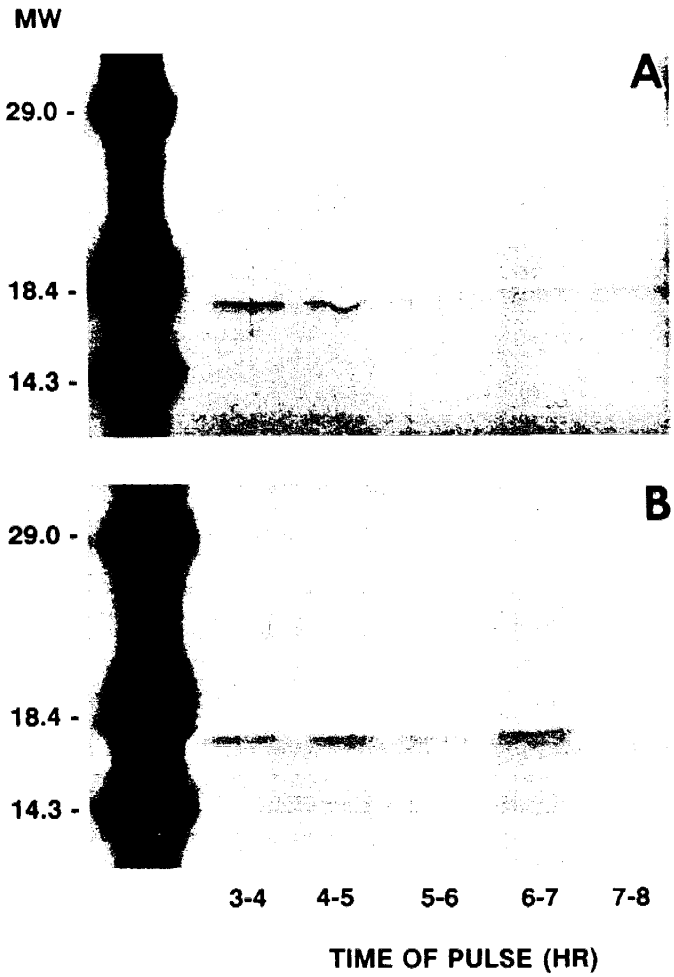

Fig. 5. Immunoprecipitation analysis of RAW264.7 cultures subjected to radiolabel pulsing. Macrophages were maintained in the presence of the drug or vehicle for 48 hr. Then the cells were triggered with LPS for up to 8 hr in the presence or absence of the drug. The cultures were pulsed with Tran (^{35}S)-label for 1-hr periods from 3 to 7 hr postinitiation of LPS triggering. The supernatants were harvested immediately after each 1-hr pulsing period and were subjected to immunoprecipitation, analytical SDS-PAGE and fluorography. The time periods indicate the time of the 1-hr radiolabel pulse after initiation of LPS triggering. A) Supernatants of LPS-triggered, vehicle-treated RAW264.7 cells. Peak levels of the 17-kD secretory TNF-α were detected 3 to 4 hr after initiation of LPS triggering. B) Supernatants of LPS-triggered, 10^{-5} M Δ⁹-THC-treated cells. Peak levels of secretory TNF-α were detected 6 to 7 hr after initiation of LPS triggering. Note that lower, but persistently elevated, levels of TNF-α were maintained for each pulsing period from 3 to 7 hr postinitiation of LPS triggering.

tion of the effect of Δ⁹-THC on TNF-α production after a 4-hr triggering period with LPS because this regimen has been reported to be optimal for the elaboration of this cytokine (Jue *et al.*, 1990). Δ⁹-THC was shown to decrease the amount of TNF-α that was secreted from RAW264.7 cells. Maximal inhibition of TNF-α was recorded for the highest drug dose used. MϕCM from LPS-triggered macrophages maintained in 10^{-5} M drug contained 50% less of the 17-kD, nonglycosylated, secreted species compared with that from vehicle controls. In addition, lesser amounts of the glycosylated multimer forms of the secreted species were present in the MϕCM.

The decreased levels of the secreted form of TNF-α could have resulted from decreased expression of TNF-α messenger RNA in response to LPS. Alternatively, Δ⁹-THC could have affected the stability of the TNF-α message. Northern blot analysis using a ^{32}P-labeled TNF-α complementary DNA revealed that the majority of the TNF-α message was elicited during an initial burst from 2 to 6 hr after triggering with LPS. Δ⁹-THC was shown to have little effect on the amount of message that was expressed in response to LPS. Although nuclear run-off experiments were not performed to measure rates of transcription, these results suggest that Δ⁹-THC had no effect on TNF-α transcriptional events. Thus, the drug exerted its effects on TNF-α at a post-transcriptional stage. These results are supported by the observation that cyclosporin, a potent immunosuppressive drug, inhibits TNF-α activity in the supernatants of drug-treated macrophages but does not affect TNF-α messenger RNA expression (Nguyen *et al.*, 1990).

TNF-α is translated as a 26-kD precursor (Gifford and Flick, 1988; Jue *et al.*, 1990). Some of these precursor molecules are glycosylated before cleavage and secretion into the culture medium (Jue *et al.*, 1990). Nonglycosylated TNF-α (17 kD) is secreted along with the glycosylated forms (Jue *et al.*, 1990). Initial immunoprecipitation studies using culture supernatants indicated that, after a 4-hr LPS triggering period, less TNF-α was present in MϕCM from drug-treated RAW264.7 cells. This lower level of secretory TNF-α could represent the consequence of a general shutdown in secretion. Alternatively, drug exposure could have resulted in a decreased rate of TNF-α secretion from affected macrophages. Thus, to define the effect of Δ⁹-THC on the kinetics of TNF-α secretion, radiolabel pulsing experiments followed by immunoprecipitation were performed. TNF-α was secreted in an initial burst which occurred at, and/or before, 4 hr after initiation of LPS triggering in macrophages not treated with the drug. This burst tapered off after the 4- to 5-hr pulse period, such that release of TNF-α occurred at minimal levels thereafter. By contrast, maximal levels of TNF-α secretion were recorded 6 to 7 hr postinitiation of LPS triggering for cultures maintained with the drug. In addition, although the majority of TNF-α was secreted in an initial relatively short burst from vehicle-treated cells, lower but persistently elevated levels were maintained from drug-treated cells throughout each 1-hr radiolabel pulsing period and peaked 6 to 7 hr postinitiation of LPS triggering. Image analysis indicated that the sum yield of secretory TNF-α released from drug-treated cells throughout the 3- to 7-hr plateau period was approximately 50% that recorded for vehicle-treated cells for the same period. These results indicate that Δ⁹-THC did not shut down secretion but resulted in diminished levels of secretory TNF-α elicited at a slower rate from macrophages.

The decreased rate of secretion of TNF-α could result from drug-induced impairment of TNF-α release from macrophages. Alternatively, Δ⁹-THC could affect the synthesis and/or processing of the 26-kD presecreted form of TNF-α. To determine whether Δ⁹-THC affected the synthesis and/or processing of TNF-α, pulse-chase experiments followed by immunoprecipitation analysis were performed. The secretory form of TNF-α (*i.e.*, 17 kD) was readily detected immediately after the radiolabel pulse in the culture supernatant of LPS-triggered, vehicle-treated cells. Little TNF-α was detected in supernatants for postchase periods ranging from 1 to 20 hr. Examination of cell lysates demonstrated that little of the 26-kD intracellular form was present. These results indicate that in vehicle-treated, LPS-triggered RAW264.7 cells, the 26-kD form of TNF-α is rapidly cleaved into the 17-kD species and secreted. By contrast, pulse-chase experiments indicated that, for drug-treated

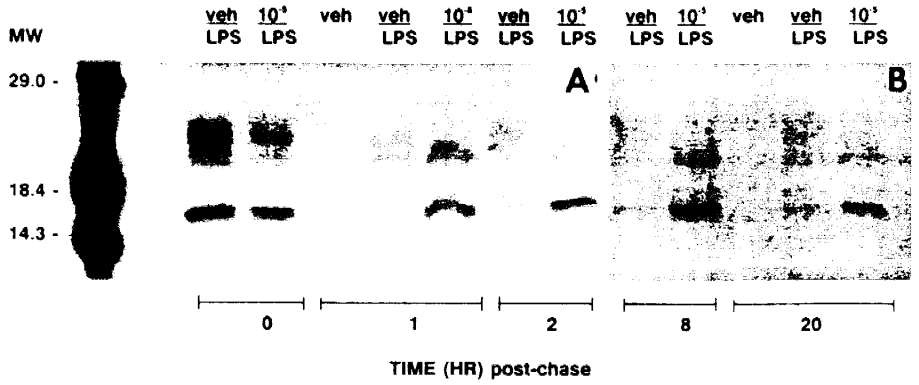

Fig. 6. Immunoprecipitation analysis of MϕCM of RAW264.7 cells subjected to radiolabel pulse-chase experiments. Macrophages were maintained in the presence of the drug or vehicle for 48 hr. Then the cells were triggered with LPS for up to 24 hr. At 3-hr post-triggering, the cultures were pulsed for 1 hr with Tran (^{35}S)-label and chased with label-free medium after the pulse. The supernatants were harvested at the indicated times postchase. Then the supernatants were subjected to immunoprecipitation, analytical SDS-PAGE and fluorography. The time periods indicate the time postchase with the radiolabel. A) A burst of labeled secretory TNF-α was elicited from vehicle-treated macrophages immediately after the 1-hr pulsing period (*i.e.*, 0 time postchase). Residual amounts were elicited thereafter. A,B) By contrast, a lower, but persistent, level of secretory TNF-α was recorded for drug-treated cells from 0 to 20 hr postchase.

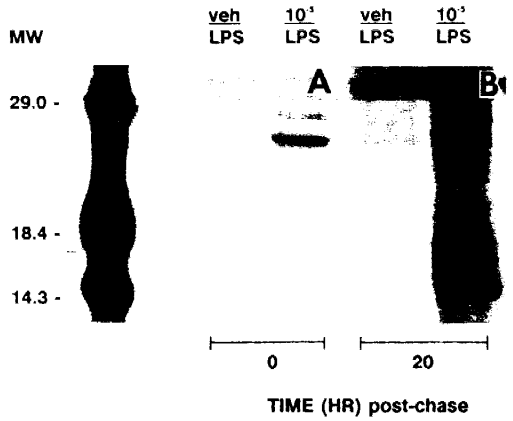

Fig. 7. Immunoprecipitation analysis of lysates of RAW264.7 cells subjected to radiolabel pulse-chase experiments. Macrophages were maintained in the presence of the drug or vehicle for 48 hr. Then the cells were triggered with LPS for up to 24 hr. At 3-hr post-triggering, the cultures were pulsed for 1 hr with Tran (^{35}S)-label and maintained in radiolabel-free medium after the pulse. Then the cell lysates were subjected to immunoprecipitation, analytical SDS-PAGE and fluorography. The time periods indicate the time postchase with the radiolabel. A) At 0-hr postchase (*i.e.*, 1-hr postpulse), little of the radiolabeled 26-kD presecretion form of TNF-α remains within the vehicle-treated cells. By contrast, relatively high levels of presecretory TNF-α remained within the drug-treated cells. B) At 20-hr postchase (*i.e.*, 21-hr postpulse), little of the 26-kD presecretory form of TNF-α was found within the vehicle-treated cells. However, considerable presecretory TNF-α remained within the drug-treated cells. In addition, intermediates ranging in relative molecular weight from 26 to 17 kD, representing apparently incompletely cleaved presecretory TNF-α, were detected within the drug-treated cells.

cells, less TNF-α was secreted into the culture medium immediately after the 1-hr pulsing period. Indeed, approximately, one-half the amount recorded for vehicle-treated cultures was detected in drug-treated cultures. Furthermore, little radiolabeled TNF-α was detected at subsequent periods postchase in culture supernatants of vehicle-treated cultures. By contrast, the 17-kD secretory form was detectable in culture supernatants of drug-treated cultures throughout the postchase period. Examination of lysates of the corresponding pellets from drug-treated cultures after the 1-hr pulsing period demonstrated that relatively high levels of presecretory TNF-α were retained within macrophages. Even at 20-hr postchase point, considerable presecretory TNF-α was detected in lysates of drug-treated cells. Furthermore, multimeric forms, ranging in relative molecular weight from 26 to 17 kD, were detected within macrophages. These results indicate that Δ^9-THC does not inhibit the synthesis of the 26-kD presecretory form of TNF-α. Rather, the lower levels of TNF-α elicited by drug-treated cells are primarily a consequence of dysfunctional cleavage of the 26-kD species.

The inhibition in the cleavage of presecretory TNF-α was not caused by a drug-induced toxicological response because generalized inhibition of macromolecular synthesis did not occur. That is, Δ^9-THC did not alter the kinetics of TNF-α messenger RNA expression in response to LPS. Furthermore, microscopic examination revealed no overt cytopathologic findings in drug-treated cultures. The mechanism by which Δ^9-THC affects processing of the presecreted form of TNF-α remains to be defined. However, the cannabinoid is highly lipophilic (Wing *et al.*, 1985) and has been shown to disrupt cellular membranes (Cabral *et al.*, 1987b). Perturbation of cellular membranes by Δ^9-THC has been postulated as one modality by which the drug alters protein synthesis (Cabral and Mishkin, 1989) and post-translational events of gene products, including glycoproteins (Mishkin and Cabral, 1987).

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Send reprint requests to: G. A. Cabral, Medical College of Virginia/VCU, Box 678, MCV Station, Richmond, VA 23298–0678.