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# Suppression of human monocyte interleukin-1β production by ajulemic acid, a nonpsychoactive cannabinoid

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**Abstract**

Oral administration of ajulemic acid (AjA), a cannabinoid acid devoid of psychoactivity, reduces joint tissue damage in rats with adjuvant arthritis. Because interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) are central to the progression of inflammation and joint tissue injury in patients with rheumatoid arthritis, we investigated human monocyte IL-1β and TNFα responses after the addition of AjA to cells *in vitro*. Peripheral blood and synovial fluid monocytes (PBM and SFM) were isolated from healthy subjects and patients with inflammatory arthritis, respectively, treated with AjA (0–30 μM) *in vitro*, and then stimulated with lipopolysaccharide. Cells were harvested for mRNA, and supernatants were collected for cytokine assay. Addition of AjA to PBM and SFM *in vitro* reduced both steady-state levels of IL-1β mRNA and secretion of IL-1β in a concentration-dependent manner. Suppression was maximal (50.4%) at 10 μM AjA ($P < 0.05$ vs untreated controls, $N = 7$). AjA did not influence TNFα gene expression in or secretion from PBM. Reduction of IL-1β by AjA may help explain the therapeutic effects of AjA in the animal model of arthritis. Development of nonpsychoactive therapeutically useful synthetic analogs of *Cannabis* constituents, such as AjA, may help resolve the ongoing debate about the use of marijuana as medicine.


*Keywords:* Ajulemic acid; Interleukin-1β; Monocytes; Inflammation; Cannabinoids

## 1. Introduction

The *Cannabis* plant has been a source of medicinal preparations since the earliest written records on pharmacobotany. The Chinese emperor Shen-nung (c. 2000 BC) wrote about the many biological effects of *Cannabis*, including its ability to ''undo rheumatism'' [1]. A major obstacle to broad acceptance of cannabinoids as therapeutic agents is their potent psychoactive effects. A class of cannabinoids, the THC acids, first described in 1972 [2], shows promise as therapeutic agents that are free of cannabimimetic central nervous system activity. These cannabinoid acids, metabolites of THC, the psychoactive agent of *Cannabis*, do not produce behavioral changes in humans at doses several times greater than THC doses given to the same volunteers [3]. The term cannabinoid acid includes all the carboxylic acid metabolites of the cannabinoids, and their synthetic analogs. THC-11-oic acid (Fig. 1), the principal metabolite in this series, is effective in animal models of inflammation and pain at oral doses of 20–40 mg/kg [4–6]. However, a molecule more potent than THC-11-oic acid is needed for clinical use. It has been known for some time that specific modifications of the pentyl side chain of THC increase its potency [7]. In particular, extending the chain length to seven carbons and introducing branching close to the ring lead to compounds with potencies that are 50–100 times greater than THC [7].

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*Abbreviations:* AjA, ajulemic acid; CBD, cannabidiol; IL-1β, interleukin-1β; LPS, lipopolysaccharide; PBM, peripheral blood monocytes; SFM, synovial fluid monocytes; THC, tetrahydrocannabinol; and TNFα, tumor necrosis factor-α.

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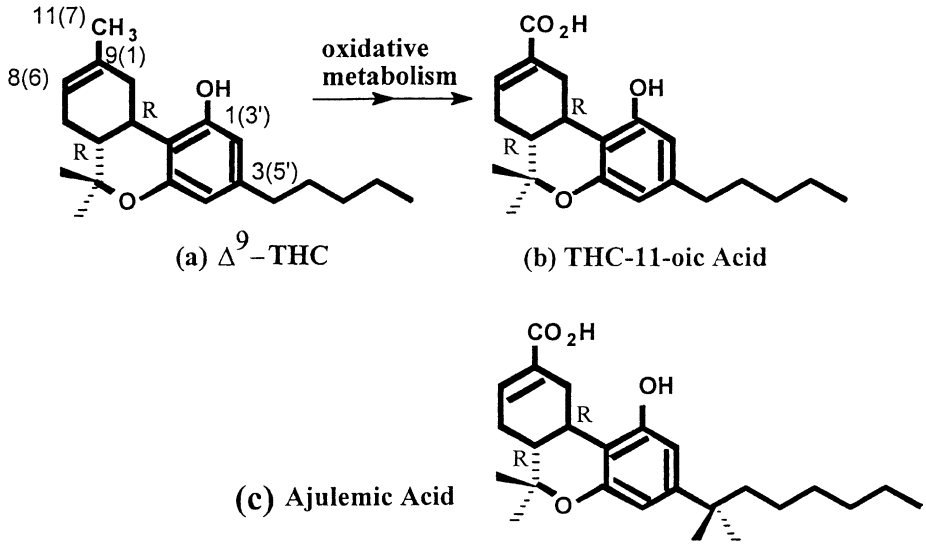


Fig. 1. Cannabinoid structures. (a) $\Delta^9$-THC is the principal component of *Cannabis* that is responsible for its mood-altering effects. (b) THC-11-oic acid is a major nonpsychoactive metabolite of THC in most species including humans. (c) Ajulemic acid is a nonpsychoactive synthetic analog of THC-11-oic acid.

This strategy was employed in designing the structure of 1′,1′-dimethylheptyl-THC-11-oic acid [trivial name ajulemic acid (AjA)] [8]. This analog (AjA; Fig. 1) of THC-11-oic acid is a potent anti-inflammatory and analgesic agent in several animal models [9]. In rats, AjA is equipotent to morphine as an analgesic but has a longer duration of action [10]. AjA also suppresses the activities of 5-lipoxygenase and cyclooxygenase but, unlike the nonsteroidal anti-inflammatory drugs currently used, AjA is not ulcerogenic [10]. Moreover, pharmacologically meaningful physical dependence was not evident in rats treated with doses up to 40 mg/kg/day of AjA. In addition, AjA is not psychoactive; indeed, AjA suppresses THC-induced catalepsy in mice [9]. We have shown [11] that the oral administration of AjA at a dose of 0.1 mg/kg three times weekly reduces significantly the severity of adjuvant-induced polyarthritis in rats, although periarticular inflammation did occur in treated rats. Histomorphological evaluation of the joints suggested that synovial inflammation also occurred in AjA-treated animals, but that it did not progress to cartilage degradation, bone erosion, and distortion of joint architecture (crippling), as was observed in rats given a placebo.

Although a variety of mediators contribute to inflammatory responses, it seems clear that the actions of IL-1β and TNFα are central to the progression of joint tissue injury in patients with rheumatoid arthritis [12]. Blockade of TNFα with neutralizing antibodies or soluble receptors, and blockade of IL-1β action by the administration of the interleukin-1 receptor antagonist (IL-1ra) reduce joint swelling and pain of patients with rheumatoid arthritis [13]. Indeed, IL-1β is a potential target of therapeutic intervention in many conditions characterized by inflammation and altered immune responses [14]. Therefore, we investigated monocyte IL-1β and TNFα responses after the addition of AjA to human cells *in vitro*. We report here that AjA suppresses IL-1β production by activated human peripheral blood and synovial fluid monocytes.

## 2. Materials and methods

### 2.1. Materials

AjA was obtained from Organix. Its purity was monitored by high pressure liquid chromatography and compared with material synthesized previously [8]. The sample was 97% chemically pure, and was 99% chirally pure in the *R*,*R* enantiomer. AjA was dissolved in DMSO and added to wells in 5-μL volumes. The concentration of DMSO remained constant at 0.5%, a concentration that did not impair monocyte viability during a 72-hr exposure. LPS was obtained from the Sigma Chemical Co.

### 2.2. Cell isolation and stimulation

Mononuclear cells (PBMC) were isolated from peripheral blood of healthy volunteers using Ficoll-Hypaque separation. Volunteers included 2 males, ages 23 and 50, and 5 females, ages 23–45. PBM were isolated by adherence in 24-well tissue culture plates. Adherent cells were washed, and cells were maintained overnight at 37°, 5% $CO_2$, in RPMI 1640 2% autologous serum. Cells were washed again, medium was replaced, and experiments were performed with the rested cells [15]. SFM were isolated in a similar manner from 3 patients with inflammatory arthritis. These included 2 females (ages 44 and 59) with rheumatoid arthritis, and 1 male with inflammatory arthritis of unknown cause. Written informed consent was obtained from all volunteers and patients. The experimental protocol was

approved by the University of Massachusetts Medical School Human Subjects Review Committee (Docket No. H-9941). Cells were maintained overnight at 37°, 5% $CO_2$, in RPMI with 2% autologous serum; nonadherent cells were removed, and experiments were performed with the rested monocytes [15]. Cells were treated with or without AjA for 60 min, and then were stimulated with 10 ng/mL of LPS for 4 hr (for mRNA determination in cells) or 18 hr (for cytokine assay in supernatants).

### 2.3. ELISA

Mature, 17-kDa IL-1β and TNFα were measured in supernatants with ELISA kits (R&D Systems). The IL-1β antibody cross-reacts 10–15% with pro-IL-1β. Our previous studies [16] indicated that only about 2% of IL-1β secreted from LPS-stimulated monocytes is pro-IL-1β. The sensitivities of the IL-1β and TNFα assays are 1.0 and 4.4 pg/mL, respectively.

### 2.4. Measurement of cytokine mRNA by the hybridization/colorimetric assay

Quantikine mRNA (R&D Systems) is a colorimetric microplate method used to quantify cytokine-specific mRNA at low levels [17]. RNA samples were hybridized with mRNA-specific biotin-labeled "capture" probes and digoxigenin-labeled "detection" probes in a microtiter plate. After the hybridization reaction was complete, samples were transferred to a streptavidin-coated microtiter plate, and the RNA/probe complex was captured. Unbound material was washed away, and anti-digoxigenin alkaline phosphatase conjugate was added. Unbound conjugate was washed away, and substrate solution was added, followed by the addition of an amplifier solution. Development of color is in proportion to the amount of pro-IL-1β or TNFα mRNA in the original sample. The reaction was stopped, and the color intensity was measured with a standard plate reader at 490 nm. The minimum detectable level was 1.8 amol/mL.

### 2.5. Stability of IL-1β mRNA

To investigate whether steady-state levels of IL-1β mRNA were altered by changing mRNA stability, new transcription was arrested by addition to the cells of 5 μg/mL of actinomycin D 4 hr after stimulation. Steady-state mRNA levels were assayed at intervals to determine the decay of mRNA. IL-1β mRNA was measured by the hybridization/colorimetric assay. Half-life of mRNA was determined by log scale linear regression analysis.

### 2.6. Statistical analysis

Data were analyzed and compared by Student's paired *t*-test, and by ANOVA.

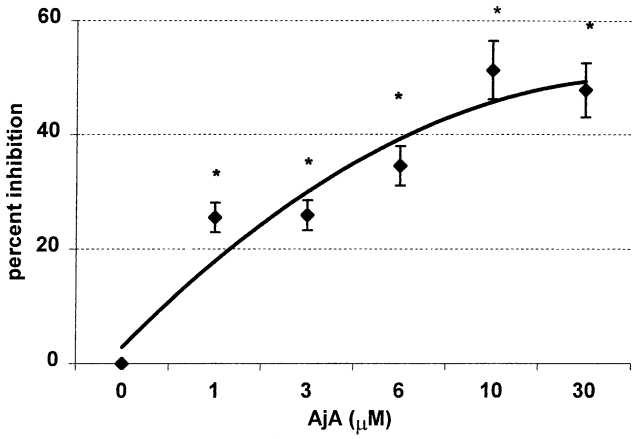


Fig. 2. Effect of AjA on the secretion of IL-1β from stimulated PBM. Cells ($2 \times 10^6$) from three healthy volunteers were incubated with AjA at the indicated concentrations for 60 min, and then were stimulated for 18 hr with 10 ng/mL of LPS. The amount of IL-1β in the supernatant was measured by ELISA; triplicate determinations were made on each sample. Error bars are SD of the means. The untreated LPS-stimulated control cells released $4132 \pm 193$ pg/mL of IL-1β (mean ± SD; N = 3). Key: (*) $P < 0.05$ vs untreated controls.

## 3. Results

### 3.1. Effect of AjA on IL-1β secretion from stimulated PBM

The concentration–response curve for suppression of IL-1β is shown in Fig. 2. In these experiments, the maximum suppression of IL-1β release occurred at 10 μM. Cells from different donors did exhibit some difference in sensitivity to AjA. In a series of experiments, 10 μM AjA reduced IL-1β release from LPS-stimulated cells by $50.4 \pm 8.8\%$ (mean ± SEM; N = 7; $P < 0.05$ vs untreated control cells).

### 3.2. Effect of AjA on gene expression in stimulated PBM

A representative experiment illustrating the concentration-dependent reduction by AjA of steady-state IL-1β mRNA is shown in Fig. 3. In a series of four experiments,

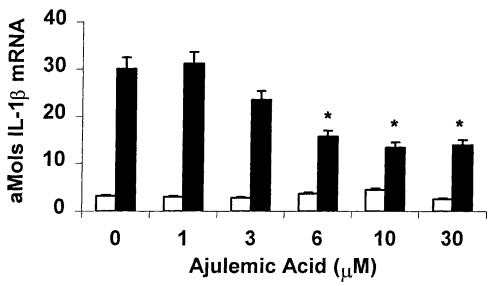


Fig. 3. Effect of AjA on IL-1β mRNA in stimulated PBM. Cells ($2 \times 10^6$/mL) were incubated for 60 min with AjA, and then were stimulated for 4 hr with 10 ng/mL of LPS. Steady-state levels of mRNA were quantified by a hybridization/colorimetric assay. Values are means ± SD of triplicate samples. Key: (*) $P < 0.05$ vs untreated control cells.

10 μM AjA reduced steady-state pro-IL-1β mRNA levels by 46.7 ± 8.9% (mean ± SD; $P < 0.05$ vs untreated control cells).

### 3.3. IL-1β mRNA stability in the presence of AjA

To determine whether AjA influenced the stability of LPS-induced IL-1β mRNA, actinomycin D was added to cells 4 hr after LPS stimulation to stop further transcription. AjA did not accelerate or retard IL-1β mRNA degradation (Fig. 4). The $T_{1/2}$ value of mRNA decay for the representative experiment shown in Fig. 4 was 3.5 hr in untreated control cells, whereas $T_{1/2}$ was 2.4 hr in AjA-treated cells. In a series of four experiments, the $T_{1/2}$ for untreated control cells was 3.8 ± 0.8 hr (mean ± SD), and $T_{1/2}$ for the AjA-treated cells was 3.0 ± 1.1 hr, a difference that was not statistically significant.

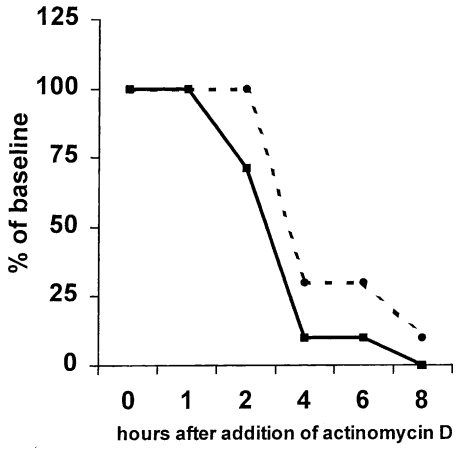


Fig. 4. Effect of AjA on the rate of decay of IL-1β mRNA. PBM ($2 \times 10^6$/mL) were stimulated with 10 ng/mL of LPS in the absence (broken line) or presence (solid line) of 10 μM AjA. New transcription was stopped by the addition of 5 μg/mL of actinomycin D 4 hr after LPS stimulation. Relative steady-state mRNA levels for IL-1β were measured at intervals thereafter. Half-life ($T_{1/2}$) for untreated control cells (●- - -●) was 3.5 hr, and $T_{1/2}$ for AjA-treated cells (■—■) was 2.4 hr. IL-1β mRNA half-lives were determined by regression analysis. Values are means of triplicate samples that did not differ by more than 8%. This figure illustrates one experiment representative of three other similar experiments (see "Section 3").

### 3.4. Effect of AjA on TNFα production by stimulated PBM

AjA at concentrations from 0.1 to 30 μM did not change the secretion of TNFα from stimulated human PBM (Fig. 5). The secretion of TNFα was reduced 25.8 ± 29.2% by 10 μM AjA (mean ± SD; $P > 0.1$; N = 7). TNFα mRNA responses to AjA were not consistent (data not shown). The mean change of TNFα mRNA at 10 μM AjA was a decrease of 10.3 ± 59.3% (mean ± SD; N = 3; not significantly different from untreated control cells).

### 3.5. Studies of SFM

Addition of AjA reduced TNFα secretion significantly from one of seven PBM samples tested. Therefore, we tested the effect of AjA on cytokine production by activated SFM *in vitro*. In three experiments, AjA reduced steady-state levels of IL-1β mRNA in these cells (Fig. 6A). AjA did not alter TNFα mRNA in a consistent manner (data not shown). AjA also reduced secretion of both IL-1β and TNFα from these cells (Fig. 6B). However, AjA was more effective in reducing IL-1β than TNFα secretion. At 10 μM, AjA reduced IL-1β secretion 71 ± 24% (mean ± SD) and reduced TNFα secretion 33 ± 14% from LPS-stimulated SFM from patients with inflammatory arthritis.

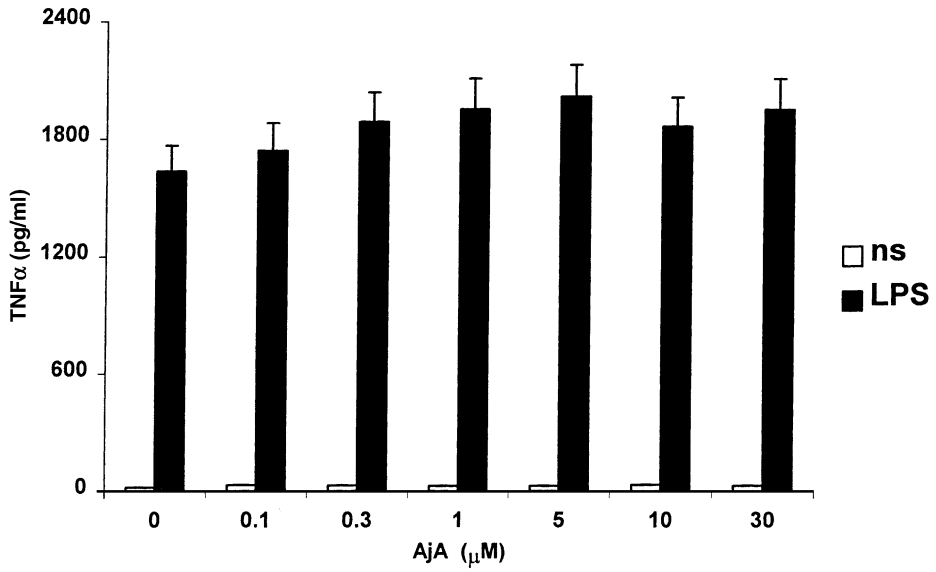


Fig. 5. Effect of AjA on the secretion of TNFα from stimulated PBM. The experimental procedure and methods were described in the legend for Fig. 2. LPS: cells were stimulated for 18 hr with 10 ng/mL of LPS; ns: unstimulated cells. Error bars are SD for four experiments.

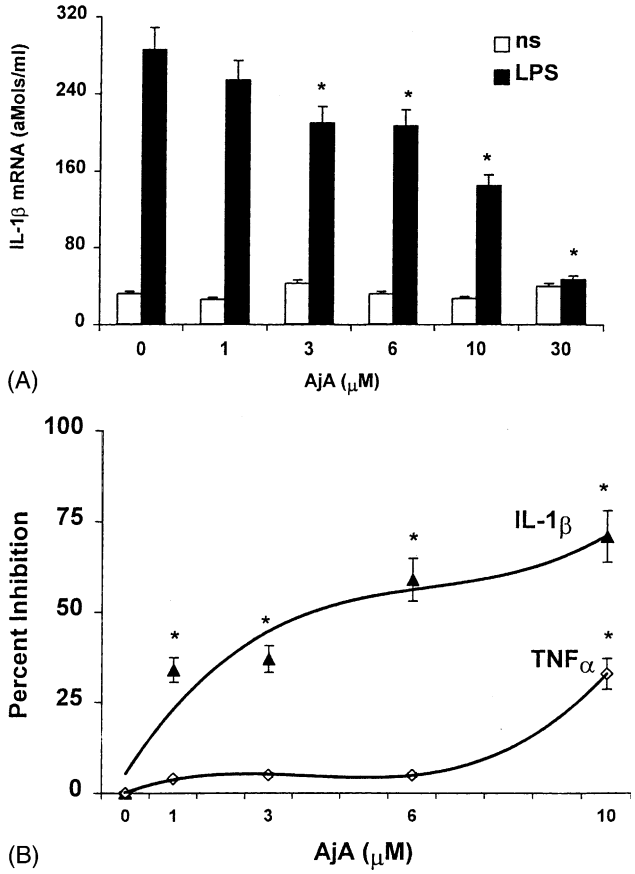


Fig. 6. Effect of AjA on the production and release of IL-1β from SFM. SFM ($2 \times 10^6$/mL) were treated for 60 min with AjA, and then were stimulated with 10 ng/mL of LPS. The experimental protocol was as in Figs. 2 and 3. (A) IL-1β mRNA in unstimulated (ns) cells or in cells stimulated (LPS) for 4 hr with 10 ng/mL of LPS. (B) Cytokine secretion measured in supernatants after 18 hr of LPS stimulation. The untreated, LPS-stimulated control cells released 1029 ± 256 pg/mL of IL-1β. Key: (*) $P < 0.05$ vs untreated control cells. Values for both panels are means ± SD, N = 3.

### 3.6. Comparison of AjA with other cannabinoids

Other cannabinoids also influence cytokine production [18]. CBD, the major nonpsychoactive component of marijuana, suppresses TNFα production by synovial cells from mice with collagen-induced arthritis [19]. Therefore, it was of interest to compare the effects of AjA on IL-1β production with THC, THC-11-oic acid, and CBD. The concentration-dependent response for THC was similar to that for AjA (Fig. 7). Indeed, the $EC_{50}$ for THC was lower than for AjA. Surprisingly, both THC-11-oic acid and CBD increased the secretion of IL-1β from stimulated PBM (data not shown).

## 4. Discussion

Results of experiments presented here indicate that the addition of AjA to human PBM *in vitro* suppresses the production of IL-1β by activated cells. In contrast, AjA does not have an appreciable effect on TNFα production by activated PBM. AjA reduced the steady-state levels of IL-1β mRNA and the subsequent secretion from activated PBM of IL-1β. The reduction in *IL-1β* gene expression is not due to altered mRNA stability.

The effect of AjA on cytokine release from cells closer to the joints of patients with inflammatory arthritis was somewhat different. When added to SFM *in vitro*, AjA reduced the secretion of both IL-1β and TNFα from these cells. However, the effect of AjA on IL-1β secretion was more robust than its effect on TNFα secretion. In addition, the reduction of IL-1β mRNA levels in SFM was consistently significant, whereas the influence of AjA on TNFα mRNA was variable and not statistically significant.

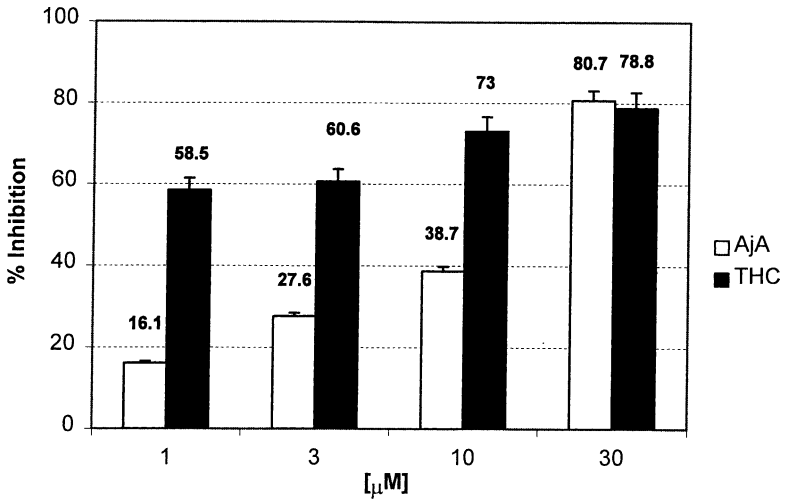


Fig. 7. Comparison of AjA and THC effects on IL-1β secretion from human PBM. Cells ($2 \times 10^6$/mL) were incubated for 60 min with test agents, and then were stimulated for 18 hr with 10 ng/mL of LPS. IL-1β in the supernatant was measured by ELISA. Values are means ± SD for triplicate samples. Unstimulated cells released 82.4 ± 4.1 pg/mL of IL-1β. Stimulated cells released 626 ± 3.0 pg/mL. $P < 0.05$ vs untreated control cells for all conditions except for 1 μM AjA. Results are representative of two similar experiments.

We did not attempt to obtain information about receptor involvement in the effects of AjA reported here. Cannabinoid acids, including AjA, exhibit only modest affinity for either CB1 or CB2, the two known cannabinoid receptors [20–22]. Although possible, it is unlikely that the effects of AjA are mediated by either of these receptors. Little evidence exists for a third member of the family of heteromeric G protein-coupled cannabinoid receptors. Of course it is possible that other, perhaps intracellular, high-affinity binding sites mediate some actions of AjA.

Other cannabinoids also block the production of inflammatory cytokines [18]. Therefore, we compared the effects of AjA on IL-1β production with that of other cannabinoids known to have anti-inflammatory properties. The concentration-dependent response of AjA was similar to that of THC, although the $EC_{50}$ value for THC was somewhat lower. In contrast, CBD and THC-11-oic acid both increased secretion of IL-1β. Thus, the anti-inflammatory effects of these cannabinoids are likely due to different mechanisms. THC also reduces IL-1 secretion from THP-1 cells differentiated by exposure to phorbol myristate acetate and then stimulated with LPS [23]. In addition, treatment of a murine model of arthritis with CBD, the major nonpsychoactive constituent of *Cannabis sativa*, blocks the inflammatory infiltrate and progression of joint tissue damage [19]. Synovial cells in culture from the knees of animals treated with CBD exhibit reduced spontaneous release of TNFα compared with cells from untreated control mice. The authors also showed that administration of CBD to C57/BL mice blocked LPS-induced increases in serum TNFα. It may be stated fairly that the effects of cannabinoids on cytokine production and activity are complex, and, in turn, the manner in which cytokines influence immune responses is equally complex.

Major advances in the treatment of rheumatoid arthritis have been made recently because of the development of agents that block the actions of IL-1β [24] and TNFα [25,26]. Animal models continue to be instructive in determining the relation of inflammatory cytokines to joint tissue injury. Results of our own animal study [11] in which AjA reduced clinical inflammation (joint redness and swelling) only modestly, but prevented joint cartilage and bone damage, may be explained by the differential effects of AjA on IL-1β and TNFα presented here. In a series of experiments, van den Berg and his colleagues [27] reported that blockade or elimination of IL-1β does not prevent joint swelling, but it is effective in reducing joint cartilage degradation and bone erosion. In contrast, blockade or elimination of TNFα prevents joint swelling but not cartilage and bone damage [27]. Using two murine models of arthritis, Campbell and colleagues [28] showed that TNF is important but not obligatory for the development of acute inflammatory and chronic autoimmune arthritis. A dual role (pro-inflammatory and immunosuppressive) for TNF has been proposed in several experimental models of autoimmune disease [29]. These animal models may provide answers to why IL-1 or TNF blockade is not successful in all patients with rheumatoid arthritis.

Clarification of the mechanisms whereby cannabinoids alter cytokine production should help shed light on the potential therapeutic effects of nonpsychoactive cannabinoids. This effort will be very important now that psychoactivity can be separated from the medicinal properties of cannabinoids. Synthetic nonpsychoactive analogs, such as AjA may provide an alternative to *Cannabis* preparations or pure THC (Marinol®) for suppression of chronic pain and inflammation. The data reported here provide further support for the idea that cannabinoid acid metabolites are useful template molecules for the creation of synthetic analogs with potential clinical value [9].

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