



The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis

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The therapeutic potential of cannabidiol (CBD), the major nonpsychoactive component of cannabis, was explored in murine collagen-induced arthritis (CIA). CIA was elicited by immunizing DBA/1 mice with type II collagen (CII) in complete Freund's adjuvant. The CII used was either bovine or murine, resulting in classical acute CIA or in chronic relapsing CIA, respectively. CBD was administered after onset of clinical symptoms, and in both models of arthritis the treatment effectively blocked progression of arthritis. CBD was equally effective when administered i.p. or orally. The dose dependency showed a bell-shaped curve, with an optimal effect at 5 mg/kg per day i.p. or 25 mg/kg per day orally. Clinical improvement was associated with protection of the joints against severe damage. *Ex vivo*, draining lymph node cells from CBD-treated mice showed a diminished CII-specific proliferation and IFN- γ production, as well as a decreased release of tumor necrosis factor by knee synovial cells. *In vitro* effects of CBD included a dose-dependent suppression of lymphocyte proliferation, both mitogen-stimulated and antigen-specific, and the blockade of the Zymosan-triggered reactive oxygen burst by peritoneal granulocytes. It also was found that CBD administration was capable of blocking the lipopolysaccharide-induced rise in serum tumor necrosis factor in C57/BL mice. Taken together, these data show that CBD, through its combined immunosuppressive and anti-inflammatory actions, has a potent anti-arthritic effect in CIA.

Cannabidiol (CBD) is one of the major components of *Cannabis sativa*, marijuana (1). Marijuana contains approximately 80 constituents, termed cannabinoids (2, 3). CBD is not psychoactive, unlike the other major component of cannabis, Δ^9 -tetrahydrocannabinol (Δ^9 THC) (4, 5). A vast literature documents the immune modulating effects of cannabinoids, *in vivo* and *in vitro*, mainly of Δ^9 THC and synthetic analogues such as CP55,940 (reviewed in ref. 6). A nonexhaustive list of *in vitro* effects includes inhibition of the proliferative responses of T lymphocytes (7), inhibition of cytotoxic T cell activity (8), suppression of macrophage function and antigen presentation (9, 10), and inhibition of NO production by macrophages (11). Reports on the *in vitro* effects of CBD on immune cells are scarce and include the modulation of tumor necrosis factor (TNF), IL-1, and IFN- γ by human peripheral blood mononuclear cells (12, 13) and the suppression of chemokine production by a human B cell line (14). These potentially anti-inflammatory properties of CBD, together with the lack of psychotropic effect and low toxicity (15), prompted us to test the potential of CBD as a therapeutic agent in collagen-induced arthritis (CIA).

CIA, a murine model for rheumatoid arthritis (RA), is elicited by immunizing DBA/1 mice with type II collagen (CII) in complete Freund's adjuvant (16). The immune response to CII involves both humoral and cellular mechanisms (17, 18), and the cellular response is T helper 1-mediated (19). CIA is characterized by rapid onset of clinical joint inflammation, resulting in destruction of joint tissues and cartilage/bone erosions. Suppression of the inflammatory process by blocking TNF with mAbs has proven an effective treatment of CIA (20, 21), and

these findings led to the successful use of TNF blockade in multiple phase I, II, and III clinical trials with RA patients (reviewed in ref. 22), thus validating the predictive value of CIA as a model for RA. In the present study, we report that CBD has a beneficial therapeutic action on established CIA, and we explore its mode of action.

Materials and Methods

Purification of CBD. CBD was purified from hashish as reported (23). Its purity was established on the basis of melting point (66–67°), optical rotation ($\alpha_D = 125^\circ$), and single peak on gas chromatography (23).

Induction and Monitoring of Heterologous CIA. Bovine CII was purified from hyaline cartilage (21). Male DBA/1 mice (8–12 weeks old) were immunized with 100 μ g of CII emulsified in complete Freund's adjuvant (Difco) by intradermal injection at the base of the tail. From day 15 after immunization onward, mice were examined daily for onset of clinical arthritis. Assessment of arthritis included monitoring of clinical scores where 0 = normal; 1 = slight swelling and erythema; 2 = pronounced edema; 3 = joint rigidity. Each limb was graded, resulting in a maximal clinical score of 12 per animal. The arthritis was monitored over 10 days, after which the mice were killed (21).

Induction and Monitoring of Homologous CIA. Mouse CII was purified from sternal cartilage from female DBA/1 mice, as described for bovine CII. For the chronic experiments, 6-week-old mice were immunized with mouse CII (100 μ g) in complete Freund's adjuvant. The animals were boosted 15 days later with 100 μ g CII i.p. From day 30 after immunization onward, 80% of the mice developed a chronic relapsing arthritis, which was monitored for 5 weeks as described above.

Administration of CBD. CBD treatment commenced at the first clinical signs of arthritis and was administered i.p. daily until day 10 of arthritis. The CBD concentrations used were 20 mg/kg ($n = 12$), 10 mg/kg ($n = 17$), 5 mg/kg ($n = 15$), and 2.5 mg/kg ($n = 9$). CBD was dissolved in ethanol/cremophor (Sigma) (1:1, vol/vol) and further diluted in saline, so that the final solution was ethanol/cremophor/saline (1:1:18). Mice injected with ve-

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Abbreviations: CBD, cannabidiol; CIA, collagen-induced arthritis; CII, type II collagen; LNC, lymph node cell; TNF, tumor necrosis factor; RA, rheumatoid arthritis; LPS, lipopolysaccharide.

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hicle alone (ethanol/cremophor in saline) served as controls ($n = 23$).

For the oral treatment protocol, CBD was dissolved in olive oil and administered by oral gavage, daily, from the onset of arthritis for 10 days. The doses used were 10 mg/kg, 25 mg/kg, and 50 mg/kg ($n = 6$ per group). Control mice were fed olive oil ($n = 6$).

For the chronic experiments, mice were treated from the first symptoms of arthritis for 5 weeks. For the i.p. route, CBD was injected daily at 10 mg/kg ($n = 7$) or 5 mg/kg ($n = 7$). Again, mice injected with vehicle alone served as controls ($n = 7$). For the oral route, the treatment was administered daily (Monday to Friday) at a dose of 25 mg/kg ($n = 6$) and control mice were fed olive oil ($n = 6$).

Histological Analysis. At the end of each experiment, hind paws were removed postmortem, fixed in formalin, and decalcified in 5% EDTA. The paws were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Arthritic changes in the foot joints were scored as mild (mild synovial hyperplasia), moderate (pannus formation and erosions limited to the cartilage-pannus junction), or severe (extended bone and cartilage erosions with loss of joint architecture). All assessments were performed by an observer blinded to the treatment received.

Lipopolysaccharide (LPS) Induction of Serum TNF in Mice. Female C57BL/6 mice were injected i.p. with a sublethal dose of LPS (100 μ g, *Escherichia coli* O55:B5, Difco). CBD was injected simultaneously, either i.p. or s.c., at a dose of 10 mg/kg. Ninety minutes later, the mice were bled and serum TNF levels were determined by bioassay (24).

Reactive Oxygen Intermediate Production by Mouse Granulocytes. C57BL/6 mice were injected i.p. with 1.5 ml thioglycollate (Difco), and 18 h later the cells were harvested by sterile lavage with PBS. The cells were washed and resuspended in Hanks' balanced salt solution without phenol red, and 0.5 ml of the cell suspension was added into luminometer tubes. CBD (dissolved in ethanol) was added at this point at a final concentration of 6 μ g/ml. Finally, 10 μ l Luminol (Sigma) and 30 μ l Zymosan (Sigma) were added, and the chemiluminescence was measured immediately in a luminometer (Biolumate LB 95, Berhold, Wildbad, Germany).

Preparation of CBD for *in Vitro* Experiments. CBD was dissolved in ethanol at a stock concentration of 10 mg/ml and stored at 4°C for up to 2 months. CBD stock was further diluted in warm medium immediately before use. All tissue culture media, vehicle control, and CBD preparations were shown to contain less than 0.1 unit/ml endotoxin, as assessed by the chromogenic Limulus Amebocyte Lysate assay (BioWhittaker). At the end of all *in vitro* experiments described below, viability of the cells was assessed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test (25).

Splenic Lymphocyte Culture. Pooled spleens from 10-week-old DBA/1 mice were pushed through a sieve and a single cell suspension was prepared. The cells were washed, layered over a Lympholyte-M density gradient (Cedarlane Laboratories), and spun at 2,000 rpm for 45 min. The buffy coat containing lymphocytes was washed three times and then plated at 2×10^5 cells/100 μ l per well in complete medium comprising DMEM supplemented with 10% heat-inactivated FCS, 1% glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2×10^{-5} M 2-mercaptoethanol. Cells were stimulated with 5 μ g/ml Con A in the presence of 0–10 μ g/ml CBD. After 72 h, cells were pulsed with 0.5 μ Ci/well [3 H]thymidine (Amersham Pharmacia)

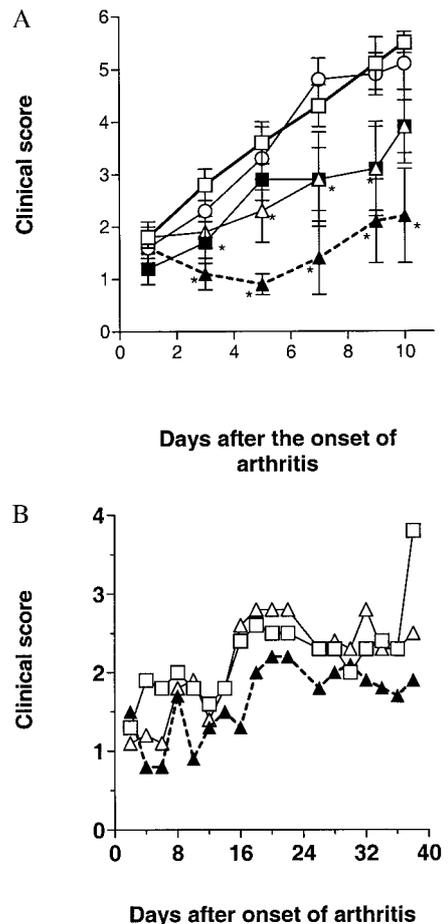


Fig. 1. From the first clinical signs of arthritis, mice were treated daily with CBD, i.p. at the following doses: 20 mg/kg (■), 10 mg/kg (△), 5 mg/kg (▲), or 2.5 mg/kg (○). Mice treated with the vehicle alone served as controls (□). Each point is the mean of n mice \pm SEM. (A) Shown is the clinical score over 10 days of classical CIA in three pooled experiments (controls, $n = 23$; CBD 20 mg/kg, $n = 17$; CBD 10 mg/kg, $n = 15$; CBD 5 mg/kg, $n = 15$; CBD 2.5 mg/kg, $n = 9$). * denotes a P value < 0.05 (Mann–Whitney U test). (B) An experiment in chronic relapsing homologous CIA, where control, $n = 6$; CBD 10 mg/kg, $n = 6$, and CBD 5 mg/kg, $n = 6$. The area under the curve was 38.4 for the control group, 37.3 for the 10 mg/kg group, and 28.9 for the 5 mg/kg group (not significant).

overnight, harvested, and assessed for incorporation of radioactivity.

Draining Lymph Node Cell (LNC) Culture. Mice (controls or CBD-treated) were killed at day 3 after disease onset, and inguinal LNCs were cultured as described (26). Cells were cultured with or without bovine CII (50 μ g/ml) in Tris-buffered saline, pH 7. Supernatants were collected after 72 h and stored at -20°C until cytokine measurement. Alternatively, after 72 h, cells were pulsed with [3 H]thymidine overnight and assessed for incorporation of radioactivity.

Culture of Murine Synovial Cells. Mice (control mice or CBD-treated) were killed at day 10 of arthritis and the knee joints were removed. Synovial cell cultures were performed as described (27). Briefly, synovial membranes were excised under a dissecting microscope and digested with 1 mg/ml collagenase A and 0.15 mg/ml DNase type IV in the presence of 33 μ g/ml polymyxin B. The cells then were washed extensively and cultured in 96-well plates at a density of 2×10^6 cells/ml (100

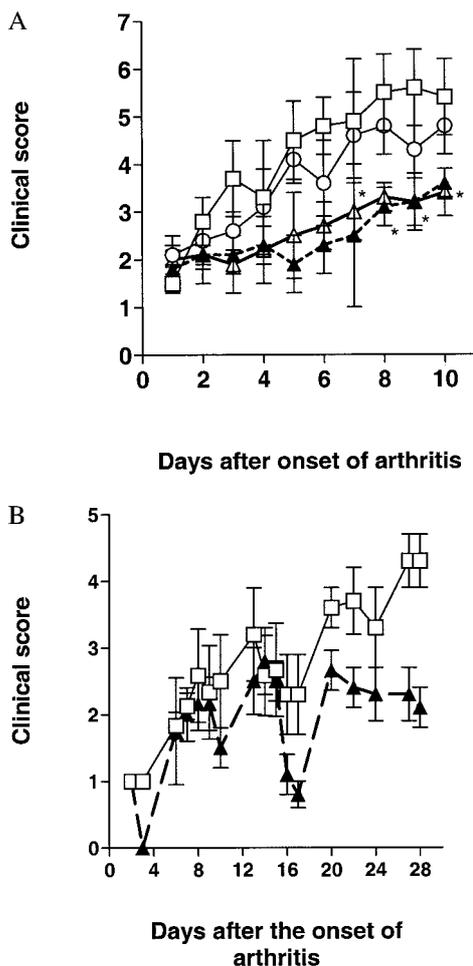


Fig. 2. From the first clinical signs of arthritis, mice were given CBD by oral gavage, at the following doses: 50 mg/kg (Δ), 25 mg/kg (\blacktriangle), or 10 mg/kg (\circ). Mice treated with olive oil served as controls (\square). Each point is the mean of n mice \pm SEM. (A) Shown is the clinical score over 10 days of classical CIA (controls, $n = 6$; CBD 50 mg/kg, $n = 6$; CBD 25 mg/kg, $n = 6$; CBD 10 mg/kg, $n = 6$). * denotes a P value < 0.05 (Mann-Whitney U test). (B) An experiment in chronic relapsing homologous CIA, where control, $n = 6$ and CBD 25 mg/kg, $n = 6$. The area under the curve was 72.3 for the control group and 49.7 for the CBD-treated group ($P < 0.05$, Mann-Whitney U).

μ l/well) in complete medium with or without CBD at specified concentrations. Supernatants were collected after 24 h and stored at -20°C until measured for TNF.

Cytokine Assays. For determination of bioactive TNF levels, an assay was performed by using the WEHI 164 cell line (28), as described (29), or BALB/c CL7 cells, as described (24). IFN- γ levels were measured by sandwich ELISA. The capture/detection antibody pair used was R4-6A2 (obtained from the American Type Culture Collection, courtesy of J. Abrams) and hamster mAb 1222-00 (Genzyme).

Results

Systemic Administration of CBD Has a Dose-Dependent Therapeutic Effect on CIA. CBD at the doses of both 20 mg/kg per day and 10 mg/kg per day had a slight therapeutic effect on CIA, whereas the lower dose of 5 mg/kg caused an optimal suppression of disease (Fig. 1A). The therapeutic action of CBD was lost when the dose was further lowered to 2.5 mg/kg per day. The dose-dependent effects of CBD were confirmed in the homologous CIA model, a chronic relapsing arthritis with

Table 1. Histology of the hind feet in acute bovine CIA

Arthritic changes	Intraperitoneal, $n = 20/\text{group}$		Oral, $n = 12/\text{group}$		
	Control	CBD, 5 mg/kg	Control	CBD, 25 mg/kg	CBD, 50 mg/kg
Normal	0%	34%*	0%	0%	0%
Mild	31%	26%	20%	41%	34%
Moderate	31%	20%	5%	34%	16%
Severe	38%	20%	75%	25%**	50%

Histological findings at the end of the experiments in classical CIA. The results are shown as the percentage of all feet studied that were given a specified score. *, $P = 0.0083$; **, $P = 0.0373$ (Fisher's exact test).

a clinical pattern that more closely resembles human disease. Thus, the clinical score in these mice typically goes up and down for several weeks. Overall, the arthritis is chronic relapsing and progressive (30, 31). It was found that 5 mg/kg i.p. CBD was optimal in suppressing the arthritis (Fig. 1B). The area under the curve, which reflects overall disease severity over 28 days, was 38.4 in the controls and 37.3 in the 10 mg/kg group and was reduced to 28.9 in the 5 mg/kg-treated group. CBD treatment caused no obvious side effects in these mice.

Oral Administration of CBD Has an Equally Potent Therapeutic Effect on Established Arthritis.

Daily oral gavage of CBD immediately after onset of arthritis resulted in suppression of acute CIA (Fig. 2A). The optimal dose was 25 mg/kg, although the higher dose of 50 mg/kg worked almost as well. The 25 mg/kg dose was used in a chronic experiment in homologous CIA and was shown to effectively suppress progression of disease over a study period of 4 weeks (Fig. 2B). The area under the curve was reduced from 72.3 in the controls to 49.7 in the treated animals ($P < 0.05$).

Effect of CBD on Joint Damage. Joints in the hind paws of control mice and mice treated with CBD were assessed for hyperplasia and destruction. Table 1 shows the results in acute CIA. First, in the i.p. experiments none of the control mice had normal feet, whereas 34% of the feet in mice treated with 5 mg/kg CBD were completely protected. Sixty nine percent of all of the feet in the control mice were moderately or severely affected (31% and 38%, respectively), whereas in mice treated with 5 mg/kg CBD this was lowered to 40% (20% in each category). For the oral treatment protocol, it was found that 25 mg/kg was the optimal dose. Although no normal feet were found in this treatment group, only 25% were severely affected, as compared with 75% in the controls and 50% in the 50 mg/kg group (Table 1). Thus, the histological findings confirm that CBD at an i.p. dose of 5 mg/kg or an oral dose of 25 mg/kg has an optimal therapeutic effect on acute CIA. Those optimal doses, when tested in the chronic model, gave a good protection against histological

Table 2. Histology of the hind feet in chronic homologous CIA

Arthritic changes	Intraperitoneal, $n = 12/\text{group}$		Oral, $n = 12/\text{group}$	
	Control	CBD, 5 mg/kg	Control	CBD, 25 mg/kg
Normal	0%	30%	0%	36%**
Mild	25%	50%	20%	28%
Moderate	65%	10%*	80%	36%**
Severe	10%	10%	0%	0%

Histological findings at the end of the experiments in chronic homologous CIA. The results are shown as the percentage of all feet studied that were given a specified score. *, $P = 0.0373$; **, $P = 0.0312$ (Fisher's exact test).

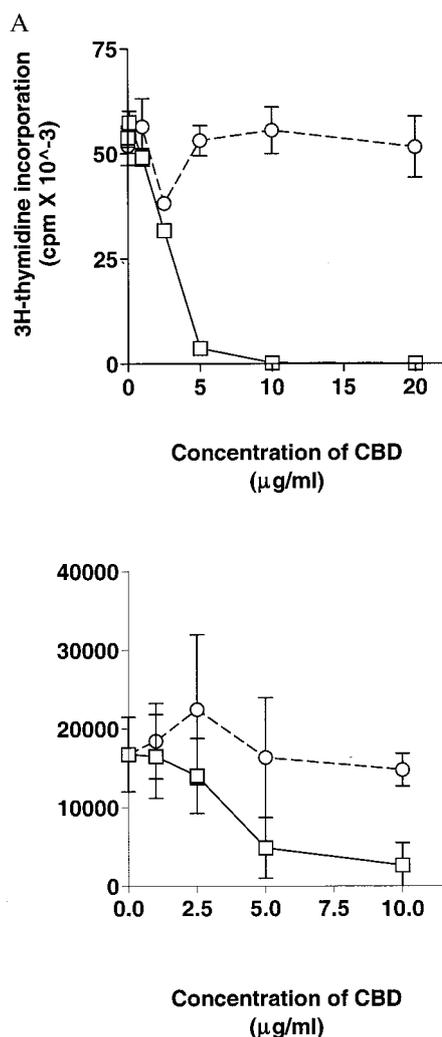


Fig. 4. (A) The effect of CBD on Con A-induced proliferation of splenic lymphocytes. (B) The effect of CBD on CII-specific proliferation of LNCs from arthritic mice. CBD was added *in vitro* at the concentrations shown for 72 h (□). For the control cultures, the vehicle (ethanol) was used at corresponding dilutions (○). Each point represents the mean ± SD of triplicate cultures. A is a representative experiment of five experiments and B of three experiments.

tion for the bell-shaped dose dependency, but such behavior has been repeatedly described for cannabinoids (32).

CBD was found to exert a potent immunosuppressive effect both *in vivo* and *in vitro*. LNCs from mice treated with CBD showed a diminished CII-specific proliferation and markedly

Table 4. CBD inhibits Zymosan-induced release of reactive oxygen intermediates by mouse peritoneal granulocytes

	Chemiluminescence peak
Granulocytes	300
+ Zymosan	1,868
+ Zymosan + CBD simultaneously	1,024 (45%)
+ Zymosan + CBD pretreatment	157 (92%)

Thioglycollate-elicited peritoneal granulocytes from C57BL/6 mice were stimulated with Zymosan in the absence or presence of CBD, 6 μg/ml. CBD was either added simultaneously with the Zymosan or the cells were pretreated with CBD for 1 h before stimulation. Production of reactive oxygen intermediates was measured by chemiluminescence. Percentage inhibition is shown in brackets. One representative experiment of four is shown.

Table 5. LPS-induced rise in serum TNF is blocked by simultaneous administration of CBD

	Control, LPS	CBD i.p.	CBD s.c.
Serum TNF (S50)	3,572 ± 892	679 ± 321	377 ± 85

Mice were injected with LPS, 100 μg, i.p. CBD was administered simultaneously, either i.p. or s.c. Mice were bled 90 min later, and TNF was measured by bioassay. Results are the mean ± SD of n = 13 per group.

diminished IFN-γ release (Table 3). In independent *in vitro* experiments, it was found that CBD suppressed the CII-specific proliferation of LNCs from arthritic mice in a dose-dependent manner, and it also suppressed Con A-induced proliferation of purified lymphocytes.

Synovial cells from mice that had been treated with an optimal dose of CBD (5 mg/kg per day i.p. for 10 days) released significantly less TNF when cultured *in vitro* than synovial cells from control animals (Fig. 3). This finding suggests that the therapeutic actions of CBD include the suppression of TNF-α, a proinflammatory cytokine known to be a major mediator of arthritis (22). This was corroborated by the finding that CBD, when injected i.p. or s.c. at a concentration of 10 mg/kg, blocked LPS-induced serum TNF in C57BL/6 mice (Table 5). Nevertheless, we could not find suppression of TNF release by arthritic synovial cells when CBD was added *in vitro* (not shown), nor could we demonstrate in multiple attempts that CBD suppressed TNF release by mouse bone marrow-derived macrophages or RAW cells (data not shown). This discrepancy between *in vivo* and *in vitro* results suggests that the TNF suppression, which is observed *in vivo* after administration of CBD, might be mediated by an active metabolite of CBD. Another possibility is that the decreased TNF expression *in vivo* is an indirect consequence of a suppressed T helper 1 response.

Thus, the anti-arthritis potency of CBD seems to be the result of a combination of immunosuppression, especially of a T helper 1 response and an anti-inflammatory action by way of reducing TNF in the synovium, a combination that has proven successful in the past when anti-IL-12 and anti-TNF were combined to treat CIA (33). Apart from these major effects, we also have demonstrated other *in vitro* anti-inflammatory actions of CBD that may contribute to its anti-arthritis potency, such as the inhibition of the release of reactive oxygen species by Zymosan-stimulated neutrophils (Table 4 and ref. 34). We also observed the blockade of NO production by peritoneal macrophages (not shown), as reported in the literature (11).

Cannabis has a long history as a medicinal preparation, mainly for properties such as analgesia, antiemesis, ocular hypotension, and anticonvulsion (reviewed in ref. 35). Recent research *in vitro* and in animal models has led to increasing evidence that cannabinoids are also important modulators of the immune system (6) and thus could have a role in the treatment of chronic inflammatory diseases, were the development of clinical trials not hampered by legal obstacles. It is therefore important to find out whether nonpsychoactive cannabinoids are suitable for treating chronic inflammatory disease. A recent report describes the effect of a nonpsychoactive synthetic derivative from tetrahydrocannabinol (THC), dimethylheptyl-THC-11-oic acid, in adjuvant arthritis in rats (36). The authors found that the compound reduced the severity of arthritis when administered from immunization onward (i.e., in a preventive protocol). The present study shows that CBD, a natural constituent of marijuana, is effective as an anti-arthritis therapeutic in established CIA. Its efficacy when given orally renders it an attractive candidate for the treatment of RA. The experiments in the chronic CIA model show that prolonged treatment with CBD does not induce tolerance, a phenomenon often observed with

cannabinoids (37, 38). Moreover, clinical trials with CBD have been conducted in humans with epilepsy (39) and Huntington's disease (40), and it was found that chronic oral administration of CBD, up to 10 mg/kg per day for 6 weeks, had no side effects. Interestingly, one paper describes that feeding 300 or 600 mg CBD to healthy human volunteers resulted in elevated plasma cortisol levels (41), yet another factor that may contribute to its anti-inflammatory/immunosuppressive actions. All of this suggests that CBD may be valuable in the treatment of other chronic inflammatory diseases as well. Indeed, preliminary studies indicate that CBD is able to delay and attenuate experimental allergic encephalomyelitis in mice (R.G. and H. Ovidia, unpublished observations) as well as inflammatory bowel disease in IL-10 knockout mice (T. Sheinin and M.F., unpublished observations).

The results presented here leave a number of questions to elucidate in the future. First, is CBD solely responsible for the anti-arthritis effects *in vivo* or is there an active metabolite

involved? Second, via which receptor does CBD exert its effects? Two receptors for cannabinoids have been identified, the brain receptor, CB1 (42), and the peripheral cannabinoid receptor, CB2 (43), which is present on T and B lymphocytes, natural killer cells, and macrophages (6). The affinity of CBD for the cannabinoid receptors is very low, lower than that of the other cannabinoids (43, 44). The possibility that CBD, because of its lipophilicity interferes with cell membranes, thus altering their functions, or that a metabolite acts on the CB2 receptor, cannot be ruled out. These questions should be the subject of future studies.

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