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A Novel Synthetic, Nonpsychoactive Cannabinoid Acid (HU-320) With Antiinflammatory Properties in Murine Collagen-Induced Arthritis

Percy F. Sumariwalla,[1] Ruth Gallily,[2] Susanna Tchilibon,[2] Ester Fride,[3] Raphael Mechoulam,[2] and Marc Feldmann[1]

Objective. **To explore the antiarthritic potential of a novel synthetic cannabinoid acid, Hebrew University–320 (HU-320), in the DBA/1 mouse model of arthritis, and to investigate in vitro antiinflammatory and immunosuppressive effects of HU-320 on macrophages and lymphocytes.**

Methods. **DBA/1 mice were immunized with bovine type II collagen (CII) to induce arthritis and then injected intraperitoneally daily with HU-320. The effects of treatment on arthritic changes in hind feet were assessed clinically and histologically, and draining lymph node responses to CII were assayed. Murine splenic and human blood lymphocytes were cultured to study the effect of HU-320 on polyclonal mitogenic stimulation. Macrophage cultures were set up to evaluate in vitro effects of HU-320 on production of tumor necrosis factor α (TNFα) and reactive oxygen intermediates (ROIs). The effect of HU-320 administration on lipopolysaccharide-induced serum TNF levels was assayed using C57BL/6 mice. Bioactive TNF production was measured using BALB/c clone 7 target cells. Evaluation of HU-320 psychoactivity was performed using established laboratory tests on Sabra mice.**

Results. **Systemic daily administration of 1 and 2 mg/kg HU-320 ameliorated established CII-induced arthritis. Hind foot joints of treated mice were protected from pathologic damage. CII-specific and polyclonal responses of murine and human lymphocytes were down-modulated. HU-320 inhibited production of TNF from mouse macrophages and of ROIs from RAW 264.7 cells and suppressed the rise in serum TNF level following endotoxin challenge. HU-320 administration yielded no adverse psychotropic effects in mice.**

Conclusion. **Our studies show that the novel synthetic cannabinoid acid HU-320 has strong antiinflammatory and immunosuppressive properties while demonstrating no psychoactive effects. The profound suppressive effects on cellular immune responses and on the production of proinflammatory mediators all indicate its usefulness as a novel nonpsychoactive, synthetic antiinflammatory product.**

Rheumatoid arthritis (RA) is an inflammatory polyarthritis affecting 0.5–1% of the human population depending on age and sex, leading to joint destruction, deformity, loss of function, and reduced quality of life (1). Its symptoms (pain, stiffness) and signs (swelling, tenderness) are mediated by a complex interplay of cells and molecules. Key molecules include tumor necrosis factor α (TNFα) and interleukin-1 (IL-1); important cells include macrophages, T lymphocytes, fibroblast-like synoviocytes, endothelial cells, dendritic cells, and plasma cells (2–4). The molecular mediator whose importance is best documented in RA is TNF. This is because neutralization of TNF either with the use of a monoclonal antibody (e.g., infliximab) or with TNF receptor (p75) IgG Fc fusion protein (e.g., etanercept) has demonstrated marked therapeutic effects in a very large number of RA patients treated (>300,000), even patients with late-stage RA that has been resistant to conventional treatments (5–8). The TNF in the syno-

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[1]Percy F. Sumariwalla, PhD, Marc Feldmann, MBBS, PhD: Kennedy Institute of Rheumatology, Imperial College London, London, UK; [2]Ruth Gallily, PhD, Susanna Tchilibon, MSc, Raphael Mechoulam, PhD: Hebrew University Medical Faculty, Jerusalem, Israel; [3]Ester Fride, PhD: College of Judea and Samaria, Ariel, Israel.

Address correspondence and reprint requests to Marc Feldmann, PhD, Kennedy Institute of Rheumatology, Imperial College London, Charing Cross Campus, Arthritis Research Campaign Building, 1 Aspenlea Road, Hammersmith, London W6 8LH, UK. E-mail: m.feldmann@imperial.ac.uk.

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vium is chiefly produced by the macrophages, and its regulation is T cell dependent (4,9).

Collagen-induced arthritis (CIA) is a mouse model of arthritis which resembles RA in several pathologic features and in its response to therapy with biologic agents (10–12). Heterologous CIA induced using bovine type II collagen (CII), for example, exhibits an acute single phase of inflammation and has well-characterized cellular and humoral immune responses, but fails to exhibit the recrudescence and remission phases of human RA (11,12). The T cell response in CIA (as in RA) is characterized by a preponderance of the CD4+ Th1 subset of cells (13). The CIA model has been used as a test bed for screening novel chemical compounds and biologic therapies, thus helping the search for new therapeutic agents for arthritis (14). The successful use of anti-TNF reagents to ameliorate CIA in established disease served to demonstrate the central nature of TNF in the inflammatory response in arthritis, as well as to validate the usefulness of the CIA model as a test bed for therapeutics used after disease onset (15,16).

The marijuana plant *(Cannabis sativa)* has been used for millennia to alleviate pain and symptoms associated with several diseases. Approximately 60 chemicals unique to the plant have been isolated (17,18). Most abundant among them are the main psychoactive ingredient of the plant, delta-9-tetrahydrocannabinol (Δ^9-THC) (19), and cannabidiol (CBD) (20), with no demonstrable psychoactivity (21,22). An exhaustive body of literature describes the in vitro and in vivo immunomodulating effects of Δ^9-THC (23). In addition to its antiinflammatory properties, CBD has been reported to have anticonvulsive, antinausea, antianxiety, and possibly antischizophrenic properties (24–28). There are reports of the in vitro suppressive effects of CBD on down-modulating the release of TNF, IL-1, and interferon-γ (IFNγ) from human peripheral blood cells (24,29). Recently, our group showed that CBD has profound therapeutic potential in ameliorating the disease manifestations of CIA. In addition, it suppressed T cell responses and production of TNF and IFNγ. More importantly, it decreased the release of bioactive TNF from synovial cells isolated from arthritic knee joints of treated mice (30), establishing a mechanism for its beneficial effect.

Potentially promising novel antiinflammatory therapeutics should preferably be devoid of psychotropic effects, such as those observed with Δ^9-THC. It has been documented that Δ^9-THC (see Figure 1), the major psychoactive component of *Cannabis*, as well as its

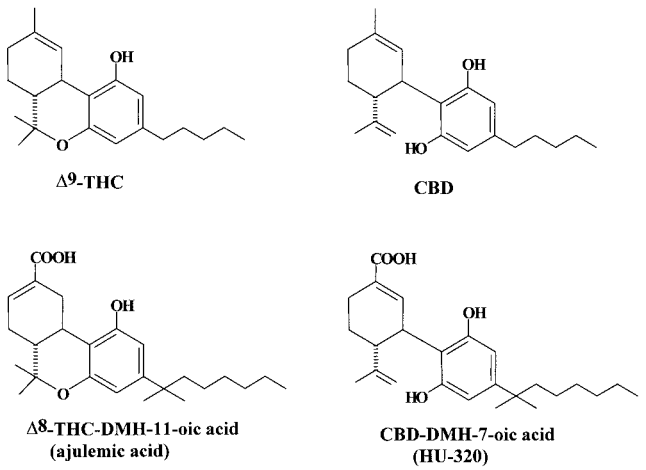

Figure 1. Chemical structures of cannabinoids. All structures were drawn using ChemWindows software, version 6.0. $\triangle^9$-THC = delta-9-tetrahydrocannabinol; CBD = cannabidiol; $\triangle^8$-THC-DMH-11-oic acid (ajulemic acid) = dimethylheptyl (DMH) homolog of Δ^8-THC-11-oic acid; CBD-DMH-7-oic acid (Hebrew University–320 [HU-320]) = DMH homolog of CBD-7-oic acid.

isomer Δ^8-THC and many of the metabolites of both, bind to the cannabinoid receptor CB1, the expression of which is seen on neural cells (31). Our group at Hebrew University has reported the synthesis of the dimethylheptyl (DMH) homolog of Δ^8-THC-11-oic acid (named ajulemic acid [CT-3]) (Figure 1) (32). CT-3 has been found to have potent antiinflammatory properties (32,33); however, it binds to CB1 (31) and has been shown to cause sedation in mice (34). Therefore, the use of this potentially antiinflammatory, nonetheless psychotropic, agent in the clinical scenario of patients presenting with chronic inflammatory conditions would present problems.

On the other hand, the nonpsychoactive CBD (Figure 1) does not bind to the cannabinoid receptors CB1 or CB2, which are differentially expressed on peripheral immune cells (35). Our research interest in the use of CBD as an antiinflammatory drug stemmed largely from its reported nonpsychotropic behavioral effects on laboratory mice. We have indeed reported that the binding of CBD and many of its metabolites and homologs to CB1 and CB2 is negligible (35). We therefore assumed that analogs of CBD that bind with very low affinity to the cannabinoid receptors (35) would be nonpsychoactive and nonsedative in vivo.

We have also shown that CBD inhibits fatty acid amide hydrolase, an enzyme that hydrolyzes the endogenous cannabinoid anandamide and also effectively blocks the cellular uptake of anandamide. These two

combined effects ultimately result in enhancement of the levels of anandamide outside the cell, and this in turn might mediate some of the pharmacologic effects of CBD (35).

Hebrew University–320 (HU-320 [CBD-DMH-7-oic acid]) (Figure 1) has very low binding affinity with both cannabinoid receptors (35). This finding prompted us to evaluate any adverse psychotropic activity of HU-320 on laboratory mice, as well as to evaluate the potential antiinflammatory/immunosuppressive properties of HU-320 in CIA and other in vitro cell culture systems, in light of the promising studies done earlier with the parent molecule, CBD.

The tetrad of tests routinely employed for behavioral studies comprised the ring immobility (catalepsy) test; the open field test, which measures horizontal (ambulation/locomotor) and vertical (rearing) activity; a test for hypothermia (a measure of the change in body temperature or BT [Δ°C]); and a test for antinociception (hot plate latency test for analgesia) (36–38). Martin et al have shown previously that, when administered individually, these assays are not selective for any particular class of compounds. However, when collectively performed and evaluated together, these tests "have proven to be highly predictive of cannabinoids" (37) and serve as useful prognosis valuators for their potential psychotropic status (39).

Our findings in this study indicate that HU-320 has a disease-ameliorating action on established CIA. This is likely to be due to its potent antiinflammatory and immunosuppressive properties exhibited in vitro. We also demonstrate here that in vivo administration of HU-320 fails to elicit any psychotropic behavioral responses in standard laboratory tests conducted on mice.

MATERIALS AND METHODS

Preparation of HU-320 solutions for in vitro and in vivo studies. For in vitro studies, 1 mg HU-320 was dissolved prior to all experiments in 50 or 100 μl 100% ethanol (analytical grade reagent; Hayman, London, UK). The stock solution was appropriately diluted in culture medium to yield final culture concentrations ranging from 0.01 μg/ml to 10 μg/ml.

For in vivo administration to mice in studies conducted for determining serum TNF levels, HU-320 was dissolved in absolute ethanol and cremophor-EL (Sigma, Poole, UK) (1/1 [volume/volume]) and further diluted using 0.85% physiologic saline (1/1/18 [v/v/v]) to yield doses of 0.5 and 5 mg/kg body weight. The lipopolysaccharide (LPS) preparation used in this study was *Escherichia coli* O55:B5-phenol extract (Difco, Detroit, MI). HU-320 solutions were always prepared fresh in small batches before every experiment.

Induction and assessment of arthritis. Bovine CII was purified from bovine articular cartilage obtained from the femoral heads of 2–3-month-old calves as previously described by Miller (40). Purified bovine CII was dissolved at 4 mg/ml in 0.1*M* glacial acetic acid and stored frozen at –70°C. Ten-week-old male DBA/1-Ola/Hsd mice (H-2^q; Harlan Laboratories UK, Blackthorn, Bicester, UK) were immunized by a single intradermal injection of bovine CII (200 μg) emulsified with Freund's complete adjuvant (1/1 [v/v]; Difco) at the base of the tail.

Day 1 of arthritis was considered to be the time of appearance of the first visible signs of edema and/or erythema in any of the paws. The average day of arthritis onset was postimmunization day 21. The clinical assessment of arthritis was based on a scoring system in which 0 = normal paws; 1 = slight swelling and redness at the base of the paws; 1.5 = swelling and redness progressively enveloping the digits; 2 = frank redness and swelling; and 2.5 = pronounced redness and swelling accompanied by limb immobility. Each limb was graded, resulting in a maximum possible clinical score of 10 per animal. The paw thickness was recorded for hind paws in all mice for 10 days using calipers (least detectable measure = 0.1 mm; Kroeplin, Röhm GB, Kingston-upon-Thames, UK). All arthritic mice were randomly distributed in various treatment groups and were monitored and treated daily throughout the 10-day period of heterologous CIA, after which they were humanely euthanized.

Administration of HU-320 for clinical treatment studies. HU-320 was administered intraperitoneally (IP) to arthritic mice from day 1 of disease at doses of 0.5, 1, and 2 mg/kg body weight (average weight of a mouse = 25 gm) daily for 10 days. A mixture of cremophor-EL, absolute ethanol, and phosphate buffered saline (pH 7.2; BDH Laboratory Supplies, Poole, UK) (1/1/18 [v/v/v]) was administered to a group of arthritic mice as the vehicle control. At the end of treatment, mice were humanely euthanized and their feet sent for histologic study. All clinical treatment studies with mice had received the prior approval of the local ethical review process committee, which followed the Declaration of Helsinki principles.

Histologic analysis. At the end of the experiments, hind feet of mice from different treatment and control groups were dissected, fixed in 4.5% buffered formalin, decalcified in 5.5% EDTA, embedded in paraffin wax blocks, sectioned to 5-μm thickness on a microtome, and stained using Mayer's modification of Harris's hematoxylin and eosin method. Arthritic changes in the distal interphalangeal, proximal interphalangeal, first metatarsal medial cuneiform, and metatarsophalangeal joints were assessed using a scoring system in which 0 = normal joint architecture; 1 = mild changes, synovitis, and pannus front with few discrete cartilage focal erosions; 2 = moderate changes, accompanying chondrocyte denucleation, loss of large areas of cartilage, eroding pannus front, and synovial hyperplasia with infiltrating mononuclear cells and polymorphonuclear cells; and 3 = total destruction of joint architecture. An observer blinded to the treatments received performed histologic assessment of all joints.

Antigen-specific response of draining lymph node cells. Arthritic mice (n = 5, day 7 of arthritis) were euthanized, and draining inguinal lymph node cell cultures were set up as

described previously (41). Cells were stimulated with bovine CII (50 μg/ml) in Tris buffered saline, pH 7.0, in the presence of HU-320 (0.01–2.5 μg/ml). Appropriate vehicle (absolute ethanol) dilutions were also incorporated. Seventy-two hours later, the cells were pulsed with 0.5 μCi of ^{3}H-thymidine (Amersham Pharmacia, Buckinghamshire, UK). The following day, cells were harvested using a cell harvester (Skatron Instruments, Cox Scientific, Northhantshire, UK), and the amount of radioactivity incorporated was assessed using a liquid scintillation counter (Wallac 1205 Betaplate; Perkin-Elmer, Milton Keynes, UK).

Separation and mitogenic stimulation of murine splenic lymphocytes. Murine splenic lymphocytes were prepared as previously described from a single-cell suspension of pooled spleens of 8–10-week-old female DBA/1 mice (30). The "enriched" splenic lymphocytes were plated at 2×10^5/100 μl in a 96-well plate (Life Technologies, Paisley, UK) using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 m*M* L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from PAA Laboratories, Somerset, Buckinghamshire, UK) and 5×10^{-5}*M* β-mercaptoethanol (Sigma). The cells were stimulated with 5 μg/ml concanavalin A (Con A; Sigma). HU-320 and absolute ethanol (vehicle) were added to cultures at various concentrations. The cells were pulsed 72 hours later, and incorporation of radioactivity was assessed as described above.

Separation and mitogenic stimulation of human peripheral blood lymphocytes (HPBLs). HPBLs were obtained by achieving cell separation, in a J6-MC elutriator (Beckman, Buckinghamshire, UK), of a density gradient–separated buffy coat containing mononuclear cells from single-donor plateletpheresis residues purchased from the North London Blood Transfusion Service (Colindale, UK) (42). A pool of "enriched" lymphocyte fractions (average purity $\geq$93% as assessed by flow cytometry) was used for setting up cultures. Cells were cultured in supplemented RPMI 1640 (PAA Laboratories) with 10% FCS at a density of 2×10^5/100 μl, then stimulated with 5 μg/ml phytohemagglutinin (PHA; Sigma) for 48 hours. HU-320 and absolute ethanol (vehicle) were added to cultures at various concentrations. The cells were pulsed after incubation, and incorporation of radioactivity was assessed as described above.

In parallel experiments, supernatants from human lymphocytes treated with PHA and HU-320 or with PHA and vehicle were assayed for human IFNγ. At the end of all in vitro culture experiments, cell viability was assessed with an MTT test (Methyl Thiazol Tetrazolium test assay; Sigma) (43).

Culture and treatment protocols for murine macrophages. *Primary peritoneal macrophages.* Peritoneal exudate macrophages were harvested from 8-week-old naive female C57BL/6 mice (H-2^b; Harlan Laboratories UK) injected IP with 1.5 ml of a 3% thioglycollate–fluid broth medium (Difco) and cultured essentially as described previously (44). The cells were plated (1.2×10^5/well) in 96-microwell flat-bottomed plates (Nunc, Roskilde, Denmark), allowed to adhere at 37°C for 2 hours, and thereafter rinsed and further incubated for 24 hours in 5% FCS containing DMEM supplemented with the antibiotics penicillin (100 units/ml) and streptomycin (100 μg/ml). Spent culture medium was replaced with fresh medium before in vitro treatments were carried out.

RAW 264.7 macrophage cell line. RAW 264.7 cells are a monocyte/macrophage cell line derived from BALB/c mice (45) and obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured using DMEM with 5% FCS and all supplements. Before cell treatment, the spent culture medium was replaced with fresh growth medium.

Various concentrations of HU-320 and vehicle (diluted ethanol) were added to the peritoneal macrophages and to the RAW 264.7 cells, followed by 1 μg/ml LPS. The macrophages were cultured at 37°C in a humid atmosphere with 5% CO_2 for 24 hours. Supernatant fluids from treated macrophages were harvested and kept at −20°C until bioassayed for TNF levels using a BALB/c clone 7 (CL7) target cell assay. The viability of the macrophages was determined by erythrosin B or trypan blue dye exclusion test (Sigma).

Murine serum TNF levels. Circulating bioactive TNF was assayed in the sera of 9-week-old female C57BL/6 mice terminally bled after 90 minutes of challenge with a sublethal dose of LPS diluted in saline (100 μg/100 μl per mouse IP). Mice in the treated group received HU-320 IP at a dose of 0.5 or 5 mg/kg body weight immediately after LPS challenge. TNF levels were determined by bioassay using BALB/c CL7 target cells.

TNF determination by bioassay. Macrophage culture supernatant fluids (Sups) were assayed for TNF levels as described previously, with the use of BALB/c CL7 target cells (46). Briefly, CL7 cells (10^4/100 μl) were plated, and diluted test Sups were added on the next day with actinomycin D (2 μg/ml; Sigma) and incubated for 20 hours. The cells were stained with 2% crystal violet, rinsed, and dried. Destruction of the target monolayer was evaluated colorimetrically by measuring the absorbance (λ_{max} = 550 nm) of stained cells with an MR700 microplate reader (Dynatech, Farmingdale, NY). The TNF titer was expressed in S_{50} units, defined as the reciprocal of the dilution of test Sups required to destroy 50% of the target cells. Calculations were performed with a logit transformation computer program.

Assay for reactive oxygen intermediates (ROIs) by zymosan-stimulated RAW 264.7 cells. RAW 264.7 cells were washed twice and resuspended at a density of 10^6/ml in Hanks' balanced salt solution (HBSS; PAA Laboratories) without phenol red. The cells were distributed in plastic luminometer tubes (0.5×10^6/0.5 ml). HU-320 (dissolved in ethanol and diluted in HBSS) was added to the test samples (2.5, 5, or 10 μg/ml), followed by the addition of 10 μl luminol (Sigma) and 30 μl zymosan (Sigma) to all tubes, and the chemiluminescence peak was recorded immediately using a luminometer (Biolumate LB 9500T; Berthold, Wildbad, Germany).

Behavioral laboratory tests for evaluation of HU-320 psychoactivity. Eight-week-old female Sabra strain mice (Harlan Laboratories, Jerusalem, Israel) were maintained at constant ambient temperature (20–22°C) over a 12-hour light/dark phase cycle. All behavioral studies on mice were performed during the light phase. Between 30 minutes and 60 minutes before conducting behavioral tests, HU-320, CT-3, and Δ^9-THC were freshly prepared in vehicle (ethanol/cremophor-EL/0.85% physiologic saline, 1/1/18 [v/v/v]) and administered IP to mice at a dose of 20 mg/kg body weight. The time intervals had been previously shown to yield maximal effects (60 minutes for

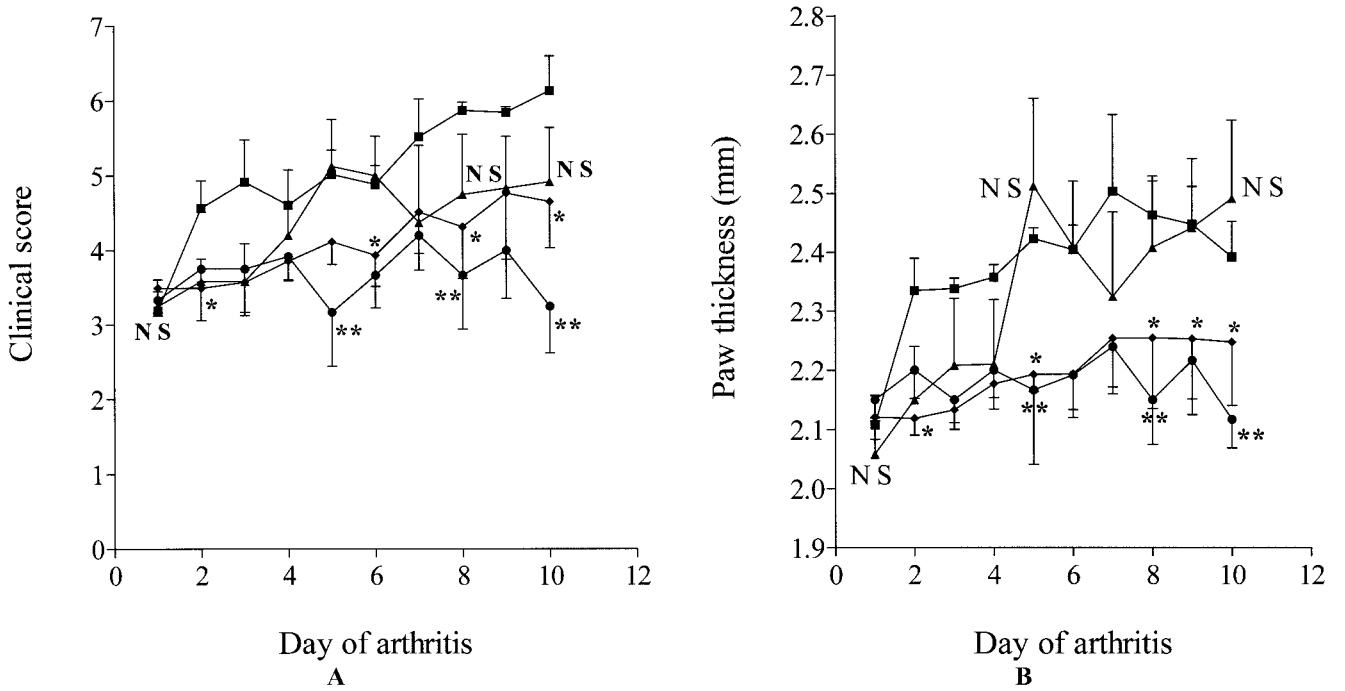

Figure 2. Amelioration of established collagen-induced arthritis (CIA) by intraperitoneal administration of HU-320. **A,** Clinical score profile and **B,** paw thickness measurement over a 10-day period of acute CIA in which mice treated with HU-320 at 0.5 mg/kg (triangles; n = 6), 1 mg/kg (diamonds; n = 19), or 2 mg/kg (circles; n = 6) were compared with vehicle-treated controls (squares; n = 19). Values are the mean and SEM. $* = P < 0.01$; $** = P < 0.001$ versus vehicle-treated control group, by one-way analysis of variance with Tukey's posttest for multiple comparisons. NS = not significant (see Figure 1 for other definitions).

Δ^9-THC and HU-320, 30 minutes for CT-3, and 30 minutes or 60 minutes for vehicle). A group of mice injected with vehicle alone served as controls.

Four consecutive observations were performed on each mouse based on standard well-established laboratory procedures for behavioral studies (38). Briefly, each mouse was observed in an open field (20 × 30 cm divided into 12 squares of equal size). Horizontal (ambulation) and vertical (rearing) locomotor activities were measured for 8 minutes and were represented as the number of crosses/rears made by the mouse in the field. Immediately thereafter, "freezing" of activity (catalepsy) was measured as immobility on an elevated metallic ring (5.5-cm diameter) for up to 240 seconds and expressed as the time (in seconds) mice stayed motionless. BT (rectal temperature) was measured immediately before injection (BT_1) and again after the ring test (BT_2) with a telethermometer (Yellow Springs Instruments, Yellow Springs, OH). Hypothermia was expressed as $\Delta°C = BT_2 - BT_1$. Finally, analgesia on a hot plate maintained at 54°C was measured as the latency period (in seconds) until the first hind paw lick occurred (the maximum period allowed for conducting this test was 45 seconds). In addition to the 4 measures of central nervous system (CNS)–mediated activities, one additional measure of autonomic motor system–regulated peripheral activity under conscious control from signals originating from the cerebral cortex (i.e., intestinal motility of fecal matter, measured for 8 minutes as defecation in an open field) was assayed in Sabra mice.

Each experiment performed used 4–6 mice per group. Two such experiments were performed, and comparable data were obtained from both. Pooled data from 9–11 mice per group were used for all calculations. Results were normalized in relation to the vehicle-injected controls using well-established formulas for normalization of data in behavioral studies to evaluate psychoactivity of cannabinoids. The formulas used were as follows: for ambulation, rearing, and intestinal motility in an open field, $[1 - (V - E)/V] \times 100$ (%); for immobility on an elevated metallic ring, $[(E - V)/(240 - V)] \times 100$ (%); and, for hot plate analgesia, $[(E - V)/(45 - V)] \times 100$ (%), where V = the reading from the vehicle-treated mouse and E = the reading from the experimental compound–treated mouse. Hypothermia was expressed as the raw data (change in body temperature), since each mouse used was its own control.

Statistical analysis. Data were analyzed using either the GraphPad Prism version 3.0 software package or the GraphPad InStat version 3.01 software package (GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) with Tukey's posttest for multiple comparisons was used to compare HU-320 treatment groups with the vehicle-treated group for both clinical score and paw thickness assessments. The statistical significance of histology

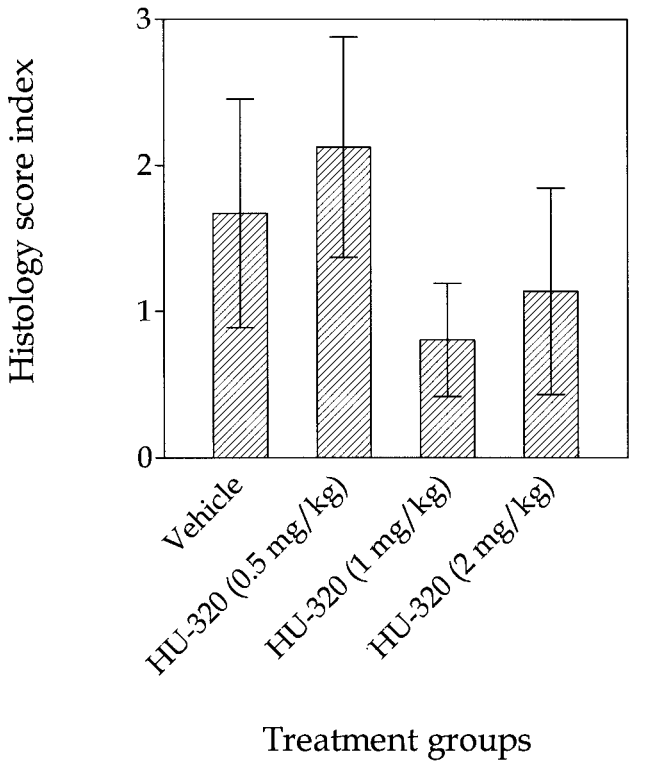

Figure 3. Histology score index of hind foot joints of mice from different treatment groups. On day 10 of arthritis, paws were obtained postmortem, fixed, sectioned, and stained with hematoxylin and eosin for histologic assessment of joints. Bars show the mean ± SD (n = 6 mice per group). The index was calculated as the total additive blinded histology score (given to every single hind foot joint scored using the specific scoring system as outlined in Materials and Methods) divided by the total number of joints scored per mouse. Statistical significance of data was calculated using the nonparametric Kruskal-Wallis analysis of variance with the assumption that the data analyzed were independent of each other and with the application of Dunn's posttest to account for multiple comparisons. The means were found to vary significantly ($P = 0.023$) when all 4 groups were compared with each other.

data was assessed using a 4 × 4 contingency table applying the chi-square test for trend to account for multiple comparisons, while the nonparametric Kruskal-Wallis ANOVA was used for the evaluation of statistical significance of the differences in the histology score index between various treatment groups. Dunn's posttest was applied to account for multiple comparisons. Student's unpaired 2-tailed *t*-test with 95% confidence intervals was applied to determine the statistical significance of all in vitro parametrically distributed data comparisons. One-way ANOVA with Newman-Keuls posttest was applied to compare data observations on individual behavioral tests on mice between treatment groups. *P* values less than or equal to 0.05 were considered significant for all data comparisons.

RESULTS

Reduction of clinical severity of CIA by systemic administration of HU-320. HU-320 was administered IP daily to DBA/1 mice after onset of clinical signs of arthritis, at doses of 0.5, 1, and 2 mg/kg. HU-320 treatment reduced clinical scores and spread of arthritis to uninvolved limbs (Figure 2A). HU-320 administration at 1 and 2 mg/kg had very similar therapeutic profiles, while the lower dose of 0.5 mg/kg failed to effectively suppress disease. On day 10 of disease, the mean ± SEM clinical score in the vehicle-treated control group was 6.13 ± 0.47, while the score in the 1 mg/kg–treated group was 4.66 ± 0.62 (a 24% reduction) ($P < 0.01$ by one-way ANOVA), and that in the 2 mg/kg–treated group was 3.25 ± 0.63 (a 47% reduction) ($P < 0.001$ by one-way ANOVA). Interestingly, the therapeutic efficacy of HU-320 did not improve when doses >2 mg/kg were administered. Instead, these higher doses (5 and 10 mg/kg) yielded no further significant reduction in clinical scores compared with that achieved with 2 mg/kg (data not shown).

A similar therapeutic profile of HU-320 was seen when severity of arthritis was evaluated by paw thickness measurements. A statistically significant reduction of paw thickness of hind feet was noted (Figure 2B). On day 10 of disease, the mean ± SEM paw thickness in the vehicle-treated group was 2.39 ± 0.06 mm, which was reduced to 2.12 ± 0.05 mm upon treatment with 2 mg/kg of HU-320 ($P < 0.001$ by one-way ANOVA with Tukey's posttest).

Oral administration of HU-320 also suppressed all clinical parameters of the disease, as was seen in our

Table 1. Degree of joint erosions and pathologic damage accompanying arthritic disease after treatment with HU-320*

Treatment group	Joint inflammation/erosions, %				No. of joints screened
	Normal	Mild	Moderate	Severe	
Vehicle	14	29	23	34	35
HU-320					
0.5 mg/kg	8	25	–	67	12
1 mg/kg	38	44	16	3	32
2 mg/kg	38	32	11	19	37

* On day 10 of arthritic disease, mice in different treatment groups were humanely euthanized and hind feet were sent for sectioning and hematoxylin and eosin staining. The degree of joint inflammation and erosions was scored in a blinded manner using a well-established scoring system as outlined in Materials and Methods. Values are percentages of screened joints within each category of inflammation and erosions, by treatment group. Data were statistically analyzed using a 4 × 4 contingency table applying the chi-square test for trend to account for multiple comparisons. A significant linear trend ($P = 0.0022$) toward joint protection and reduction of pathologic damage to joint architecture was seen with increasing doses of HU-320.

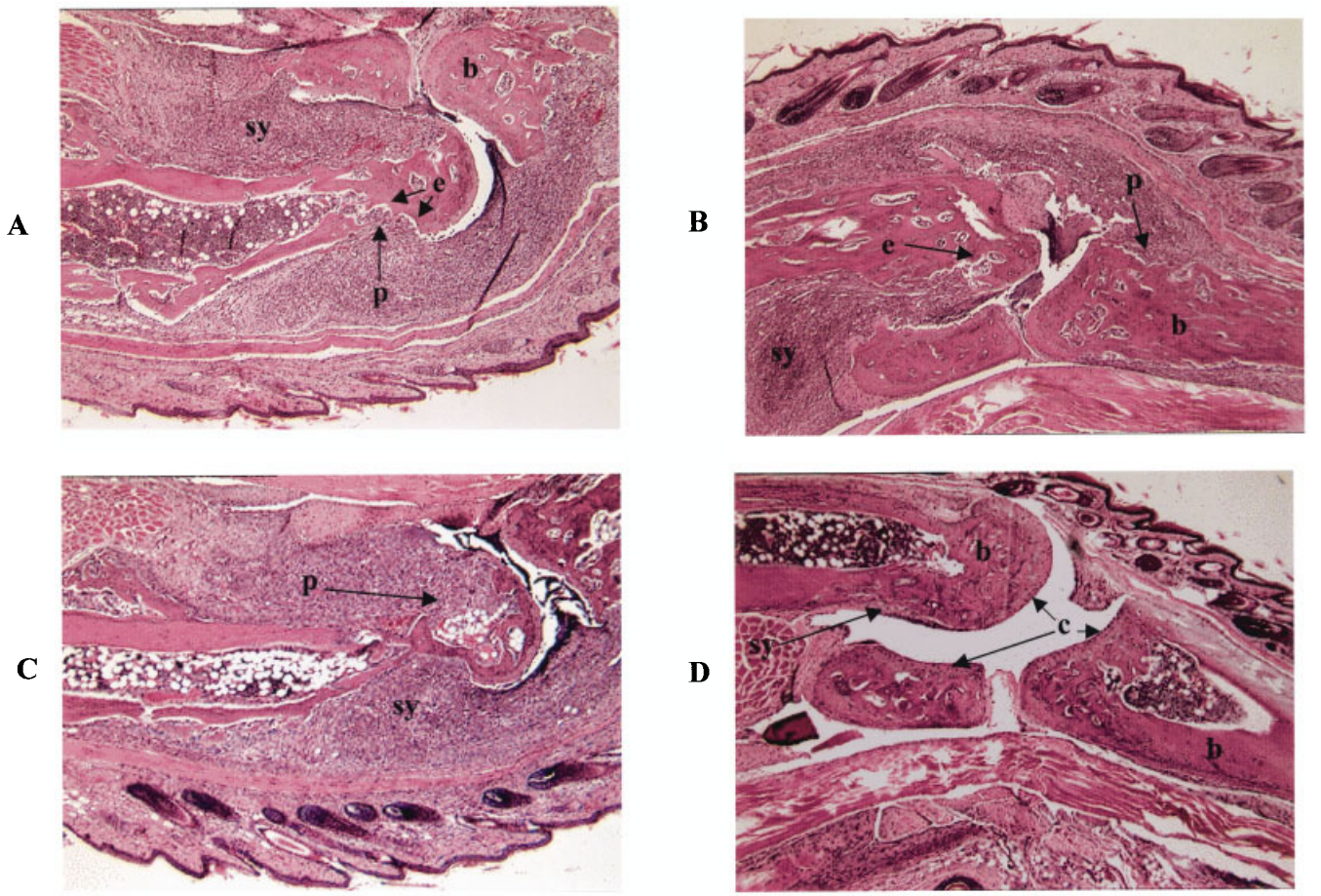

Figure 4. Image analysis photographs of proximal phalangeal joints of mouse hind feet. **A,** Joint from untreated control mouse with collagen-induced arthritis, showing severe synovial hyperplasia leading to bone and cartilage erosions. **B,** Joint from vehicle-treated mouse, showing acute destructive inflammatory arthritis and severe chondrolysis and subchondral bone damage by an inflammatory pannus tissue. **C,** Joint from mouse injected intraperitoneally (IP) with HU-320 (0.5 mg/kg/day), showing severe destructive inflammatory features of synovial infiltrate consisting mainly of neutrophils and mononuclear cells. **D,** A protected joint from a mouse injected IP with HU-320 (1 mg/kg/day), exhibiting a clear joint space, no damage to cartilage and bone, and well-demarcated and stained chondrocytes with an uninflamed synovium at the cartilage–bone junction. All sections shown are representative images exhibiting the maximum blinded histologic score for each group. Five micrometer–thick sections were stained with hematoxylin and eosin. **sy** = synovial hyperplasia; **p** = pannus; **e** = erosions; **b** = bone; **c** = cartilage. (Original magnification × 40.)

preliminary experiment. The effect of abrogating established arthritis upon daily oral application of HU-320 was best seen at the dose of 40 mg/kg (data not shown).

Treatment with HU-320 protects against pathologic joint damage. Figure 3 represents the histology score index profile of all hind foot joints of mice that had been treated with different modalities. The distal, proximal, first metatarsal, and tarsal joints were all individually assessed (in a blinded manner) for arthritic changes involving synovial hyperplasia and destruction of the normal joint architecture, and a histology score index was calculated for each treatment group (Figure 3). The median histology score indices of the different treatment groups were found to vary significantly when the groups were compared ($P = 0.023$ by Kruskal-Wallis ANOVA with Dunn's posttest to correct for multiple comparisons).

A significant trend ($P = 0.0022$ by chi-square test for trend) toward joint protection and reduction of pathologic damage to joint architecture was seen with increasing doses of HU-320 (Table 1). This trend was calculated by applying a 4 × 4 contingency table to account for multiple comparisons. As shown in Table 1, there was a reduction in the number of severely affected joints (1 of 32 joints screened [3%])

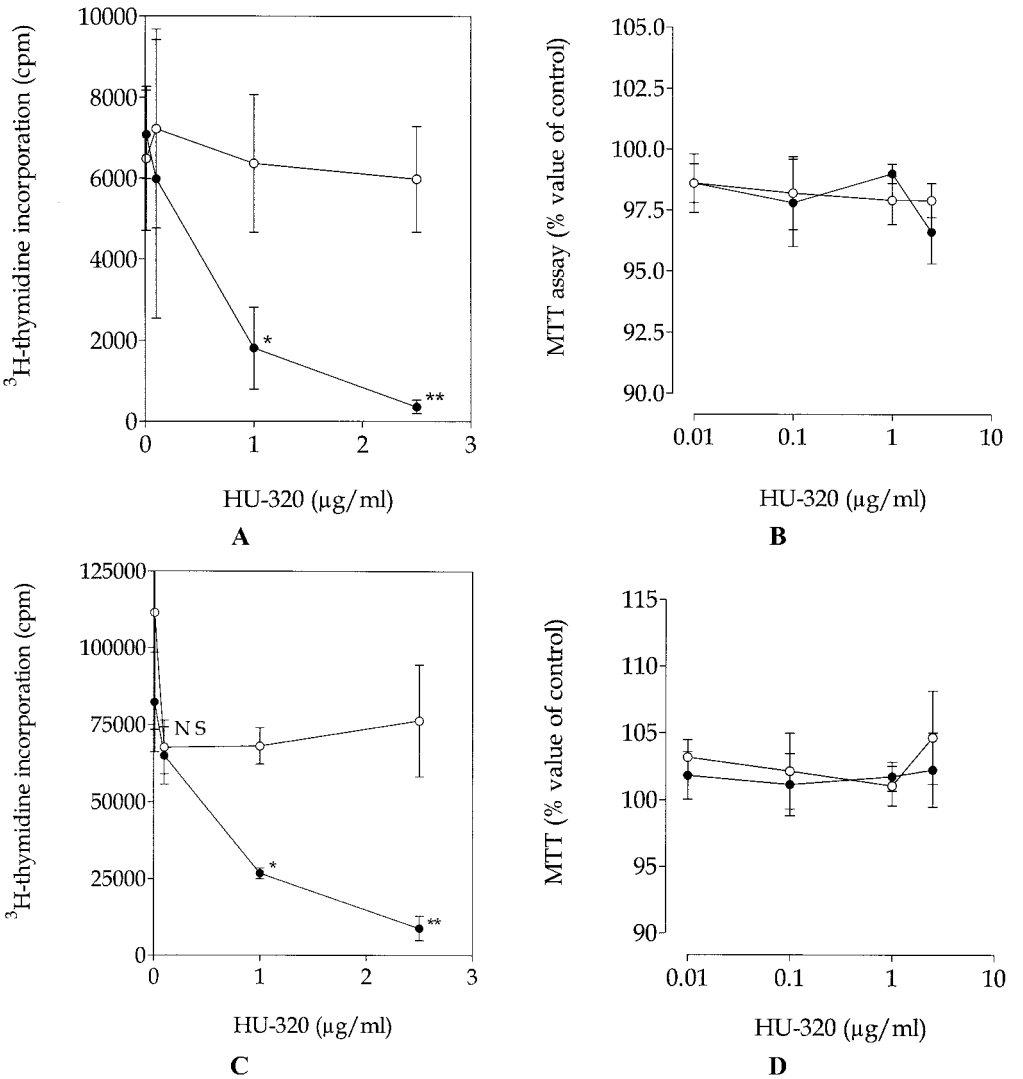

Figure 5. A, HU-320–induced suppression of an antigen (bovine type II collagen [CII])–specific response of pooled lymph node cell cultures from mice on day 7 of CIA (n = 5). **B,** Viability data (MTT assay) of pooled murine lymph node cells from day 7 CIA mice (n = 5) stimulated with bovine CII in vitro and treated with HU-320 or vehicle. **C,** HU-320–induced suppression of the response to a polyclonal mitogen (concanavalin A [Con A]) by pooled "enriched" splenic lymphocytes from nonimmunized female DBA/1 mice (n = 4). **D,** Viability data (MTT assay) of pooled "enriched" splenic lymphocytes from nonimmunized female DBA/1 mice (n = 4) stimulated with a polyclonal mitogen (Con A) in vitro and treated with HU-320 or vehicle. HU-320 (solid circles) was added in vitro at the concentrations shown for a period of 72 hours along with either bovine CII (50 μg/ml) or Con A (5 μg/ml) as stimulus. Vehicle (absolute ethanol) (open circles) was added to control cell cultures set up alongside at the corresponding dilutions. Radiolabeled (^{3}H) thymidine was added to each well, and incorporation was measured after an overnight period of incubation. In parallel cultures, MTT reagent was added 72 hours after stimuli and treatments, and the viability of cells was assayed spectrophotometrically at the wavelength of 574 nm. Values in **A** and **C** are the mean ± SEM from 3 experiments. Results of viability assays (**B** and **D**) are expressed as the mean ± SEM percentage value (of control cell cultures with stimulus alone) from 3 experiments. *P* values were calculated using Student's unpaired 2-tailed *t*-test with 95% confidence intervals. * = $P < 0.05$; ** = $P < 0.005$ versus control cell cultures. See Figure 2 for other definitions.

in mice treated with 1 mg/kg HU-320 compared with the vehicle-treated control group (12 of 35 joints screened [34%]).

A modest increase in the number of normal joints was seen in both 1 mg/kg (12 of 32 joints screened [38%]) and 2 mg/kg (14 of 37 joints screened [38%]) HU-320–treated groups of mice versus the vehicle-treated group of mice (5 of 35 joints screened [14%]). It should thus be noted that IP treatment of arthritic mice with HU-320 at the doses of 1 and 2 mg/kg offered a certain degree of protection to the joints against pathologic damage brought about by the ongoing disease process. A lower dose of treatment (0.5 mg/kg) failed to protect joints (Figure 3 and Table 1). This result was concordant with the fact that HU-320 treatment at 0.5 mg/kg showed poor clinical efficacy in ameliorating disease signs in our murine model of arthritis.

Image analysis photographs of the proximal phalangeal joints of untreated control mice with CIA and vehicle-treated mice (Figures 4A and B) showed a severe degree of cartilage and bone erosions accompanied by high numbers of infiltrating synovial cells in the inflamed synovium. In contrast, a representative proximal phalangeal joint treated with HU-320 at 1 mg/kg was completely protected from pathologic damage (Figure 4D). The lower dose of administration of HU-320 (0.5 mg/kg) failed to confer protection to the joint (Figure 4C).

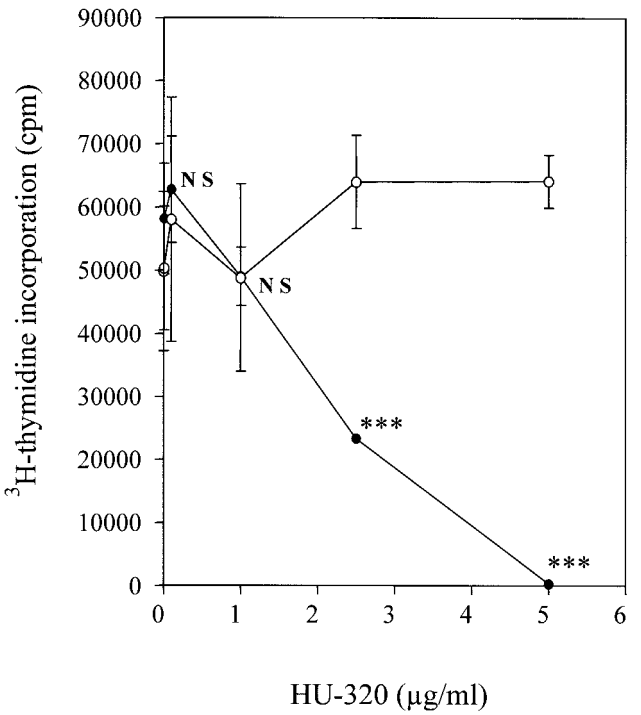

Figure 6. Suppression of the response to a polyclonal mitogen (phytohemagglutinin [PHA]) by human peripheral blood lymphocytes from a single donor. HU-320 (solid circles) was added in vitro at the concentrations shown for a period of 48 hours along with 5 μg/ml PHA. Vehicle (absolute ethanol) (open circles) was added to control cell cultures set up alongside at the corresponding dilutions. Values are the mean ± SD of quadruplicate cultures. *P* values were calculated using Student's unpaired 2-tailed *t*-test with 95% confidence intervals. *** = $P < 0.0005$ versus control cell cultures. NS = not significant.

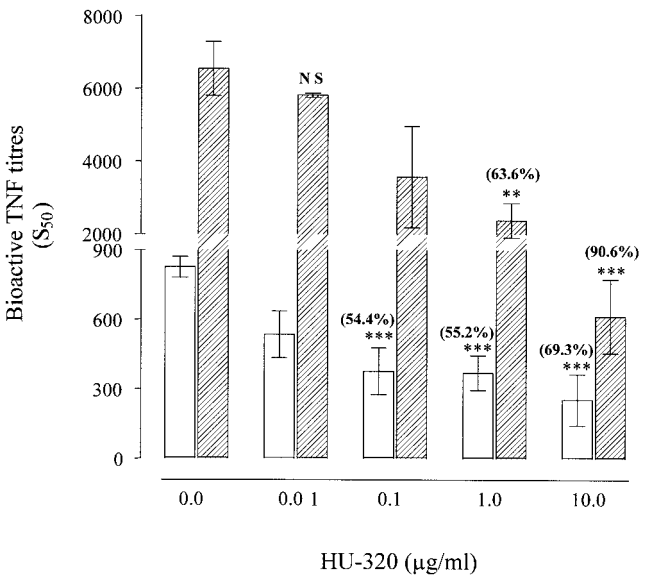

Figure 7. Suppression of bioactive tumor necrosis factor (TNF) production from thioglycollate-elicited peritoneal macrophages (open bars) and RAW 264.7 cells (hatched bars) upon in vitro addition of HU-320. The TNF titer was expressed in S_{50} units, defined as the reciprocal of the dilution of test supernatant fluids required to destroy 50% of the BALB/c clone 7 target cells (see Materials and Methods). HU-320 was added to cultures at the indicated concentrations, while 1 μg/ml lipopolysaccharide was used as a stimulus. Bars show the mean ± SD from 3 pooled experiments. Values in parentheses represent the percentage inhibition of TNF production compared with control cell cultures. *P* values were calculated using Student's unpaired 2-tailed *t*-test with 95% confidence intervals. ** = $P < 0.005$; *** = $P < 0.0005$ versus control cell cultures. NS = not significant.

Suppression of antigen-specific and polyclonal murine lymphocyte responses by in vitro addition of HU-320. Addition of HU-320 in vitro to lymphocytes harvested from draining lymph nodes of arthritic DBA/1 mice suppressed their antigen (CII)–specific responses (Figure 5A). In the presence of 1 μg/ml and 2.5 μg/ml HU-320, 71.6% and 93.9% suppression of CII-specific responses, respectively, was noted (Figure 5A). Diluted ethanol (at the corresponding concentration values used for HU-320) was incorporated as

Table 2. Effect of HU-320 on zymosan-induced release of ROIs from RAW 264.7 cells*

Treatment	Chemiluminescence peak, mean ± SEM†	Inhibition, %
Cells + zymosan (control)	9,391 ± 1,027	Referent
Cells + HU-320 (2.5 μg/ml) + zymosan	5,376 ± 255‡	43
Cells + HU-320 (5.0 μg/ml) + zymosan	2,140 ± 441‡	77
Cells + HU-320 (10 μg/ml) + zymosan	689 ± 199‡	93

* RAW 264.7 cells were stimulated with zymosan in the presence or absence of HU-320 as indicated. Production of reactive oxygen intermediates (ROIs) was measured by chemiluminescence using a luminometer. *P* values were calculated by Student's unpaired 2-tailed *t*-test with 95% confidence intervals.
† From 3 experiments.
‡ $P < 0.001$ versus cells stimulated with zymosan alone.

vehicle control. There was no loss in cell viability with these doses of HU-320 as judged by MTT assay (Figure 5B).

Splenic lymphocyte responses to Con A were also similarly suppressed (Figure 5C). We observed a 60.7% suppression of thymidine incorporation at the dose of 1 μg/ml HU-320, while 88.5% suppression at 2.5 μg/ml HU-320 was noted (Figure 5C). Diluted ethanol (at the corresponding concentration values used for HU-320) was incorporated as vehicle control. There was no loss in cell viability with these doses of HU-320 as judged by MTT assay (Figure 5D).

Table 3. Effect of in vivo adminstration of HU-320 on the LPS-induced rise in serum TNF levels*

		LPS + HU-320–injected mice	
	LPS-injected mice (n = 12)	0.5 mg/kg HU-320 (n = 5)	5 mg/kg HU-320 (n = 7)
Serum bioactive TNF titer, mean ± SD S_{50} units†	353.6 ± 215.4	43.7 ± 31.5	241.9 ± 167.3
Inhibition, %‡	–	87.6	31.6

* Mice were injected intraperitoneally (IP) with 100 μg lipopolysaccharide (LPS) diluted in saline, and HU-320 diluted in vehicle was administered immediately thereafter at the indicated doses. Control mice were injected with LPS diluted in vehicle. Ninety minutes later, mice were terminally bled using a humane protocol for exsanguinations. Levels of bioactive tumor necrosis factor (TNF) were measured from individual serum samples. Serum samples from mice injected IP with LPS diluted in either saline or vehicle gave comparable bioactive TNF titers.
† Defined as the reciprocal of the dilution of test supernatant fluids required to destroy 50% of the BALB/c clone 7 target cells (see Materials and Methods).
‡ Calculated from serum samples of control mice injected with vehicle-diluted LPS.

Suppression of mitogenic response of HPBLs by HU-320 treatment. HPBL cultures were stimulated in vitro with PHA in the presence or absence of graded concentrations of HU-320. HU-320 inhibited mitogenic response by 53% and 100% at the doses of 2.5 μg/ml and 5 μg/ml, respectively (Figure 6). Ethanol diluted in medium was used as the vehicle control and showed no significant suppression of mitogenic response at the corresponding concentrations (Figure 6). Inhibition of IFNγ production by 47% was seen in the supernatants of PHA-stimulated cells treated with 5 μg/ml HU-320 for 48 hours (data not shown).

Inhibition of TNF production from murine macrophages in vitro by HU-320. We studied the effect of HU-320 on TNF production by thioglycollate-elicited peritoneal macrophages (TGMφ) from C57BL/6 mice and by a mouse cell line (RAW 264.7). As shown in Figure 7, addition of 10 μg/ml HU-320 caused 69.3% and 90.6% inhibition of TNF production from TGMφ and RAW 264.7 cells, respectively ($P < 0.0005$ in both cases versus control cultures, by Student's *t*-test). This inhibition was dose dependent. Even at 1 μg/ml HU-320, 55.2% inhibition in TGMφ ($P < 0.0005$) and 63.6% inhibition in RAW 264.7 cells ($P < 0.005$) was seen. All cells were viable at the end of experiments, as determined by erythrosin B dye exclusion test.

Suppression of ROI production from RAW 264.7 cells by HU-320. RAW 264.7 cells release a burst of ROIs upon stimulation with zymosan. This release of ROIs was measured as a chemiluminescence peak using a luminometer. Treatment of RAW 264.7 cells with 2.5, 5, and 10 μg/ml HU-320 prior to the addition of zymosan markedly suppressed release of ROIs from this murine monocyte/macrophage cell line, by as much as 93% in a concentration-dependent manner (Table 2).

Rise in LPS-induced serum TNF levels blocked by systemic administration of HU-320. C57BL/6 mice were injected IP either with 100 μg LPS diluted in saline or with 100 μg LPS followed immediately by HU-320 (0.5 mg/kg or 5 mg/kg). Using a bioassay, TNF levels were measured from individual serum samples collected 90 minutes after systemic administration of the challenging dose of LPS or LPS + HU-320. As shown in Table 3, administration of HU-320 at the lower dose of 0.5 mg/kg resulted in ~88% suppression of serum TNF levels.

Lack of adverse effects on CNS-mediated functions in mice with HU-320 treatment. Sabra strain female mice were injected (20 mg/kg IP) with HU-320,

Table 4. No adverse effects of HU-320 administration on central nervous system–mediated voluntary and involuntary functions*

Assay	Δ^9-THC	HU-320	CT-3
Central activity			
Ambulation	66	88	53
Rearing	11†	81	16†
Catalepsy	40†	9	35†
Analgesia	31	1	36
Hypothermia, Δ°C ($BT_2 - BT_1$)	−2.2†	−0.33	−2.7†
Peripheral activity			
Intestinal motility	0‡	0‡	11‡

* Except where indicated otherwise, values are standardized percentages with respect to vehicle-injected control mice. Central and peripheral effects of Δ^9-THC (δ-9-tetrahydrocannabinol), HU-320, and CT-3 (ajulemic acid [the dimethylheptyl homolog of Δ^8-THC-11-oic acid]) were evaluated using 4 standard laboratory tests for assay of pharmacologic activity of cannabinoids (ambulation/rearing, catalepsy, analgesia, and hypothermia [measured as a change in body temperature [BT]) (see Materials and Methods). One additional test for intestinal motility measured the effects of cannabinoids on peripheral voluntary function. Results have been normalized in relation to vehicle-injected controls using well-established formulas for normalization of data in behavioral studies to evaluate psychoactivity of cannabinoids (see Materials and Methods). The combined data (mean ± SEM) for vehicle-injected control mice (n = 11) were as follows: 213 ± 24 squares crossed (ambulation), 98 ± 12 rears (rearing), 5.4 ± 9.0 seconds (catalepsy), 15.8 ± 2.1 seconds (analgesia), −0.5 ± 0.14°C (hypothermia), and 3.6 ± 0.81 fecal pellets (intestinal mobility). *P* values were calculated by one-way analysis of variance with Newman-Keuls posttest.
† $P < 0.05$ versus vehicle-treated control group.
‡ $P < 0.01$ versus vehicle-treated control group.

CT-3, Δ^9-THC, or vehicle alone (controls) 30–60 minutes before 4 standard laboratory evaluation tests were conducted for psychoactive effects of drugs on the CNS. As shown in Table 4, administration of HU-320 had no significant effect on any CNS-mediated functions of ambulation, immobility, change in body temperature, and antinociception. However, CT-3 and Δ^9-THC had significant effects on CNS-mediated functions, indicating strong cannabimimetic activity of these compounds. HU-320 was found to be essentially psychoinactive (Table 4), except for influencing intestinal motility (an additional test performed as an index of CNS-mediated peripheral voluntary reflex).

DISCUSSION

We report the use of a synthetic cannabinoid acid, Hebrew University–320 (HU-320), a putative metabolite of a homolog of cannabidiol (CBD), as an antiarthritic therapeutic agent in CIA. We have previously documented that CBD has antiarthritic potency in established murine CIA (30). In the present study, we also found in vitro immunosuppressive effects of HU-320 on several important immunoregulatory cell types and their proinflammatory mediators, as well as the lack of THC-like psychotropic activity in mice.

In the initial experiments, HU-320 was administered IP to DBA/1 mice after onset of clinical signs of arthritis. We found that HU-320 suppressed clinical scores and paw thickness of arthritic mice at doses of 1 and 2 mg/kg. A lower dose (0.5 mg/kg) failed to abrogate clinical parameters of the disease. The dose of 1 mg/kg HU-320 offered a significant degree of protection against joint damage. Histologic evidence of this was seen as a reduction in synovial hyperplasia due to a reduction in numbers of infiltrating immune cells, such as monocytes, macrophages, neutrophils, and lymphocytes compared with control groups. Thus, HU-320 protects the joint from cartilage loss and subchondral bone damage, possibly as a consequence of controlling both the influx of immune cells at the site of disease and the ongoing inflammation.

Addition of HU-320 in vitro to several important cell types down-modulates the release of key mediators of arthritis such as TNF, IFNγ, and ROIs (2). Our additional finding, that systemic administration of 0.5 mg/kg HU-320 immediately following endotoxin challenge in mice effectively arrested the rise in serum TNF levels, supports our hypothesis that clinical abrogation of disease by HU-320 is brought about in part by the down-modulation of TNF production. However, higher doses of HU-320 were found to be ineffective in reducing endotoxin-induced TNF production, suggesting a possible "bell-shaped" dose-response curve, as reported for CBD and other cannabinoids (30,47). The absence of a similar bell-shaped curve for HU-320 in CIA, and the fact that higher doses were required for controlling disease, may be related to the chronic nature of ongoing established inflammation in the CIA model versus the acute nature of endotoxic shock, and may also be related to the importance of other cell types in the pathology of CIA.

Our preliminary experiments have shown that oral administration of HU-320 also effectively suppressed the clinical signs of established arthritis in the DBA/1 CIA model, as was previously described for CBD (30). Maximum oral efficacy was seen at the highest administered dose of 40 mg/kg (data not shown). We thus believe that the efficacy of HU-320 in treating established CIA, both systemically and orally, renders it an attractive candidate for the treatment of RA and other chronic inflammatory diseases which also involve

cytokine (TNF) up-regulation, such as Crohn's disease and psoriasis (3).

In our study, we also demonstrate that HU-320 exerts in vitro antiinflammatory/immunosuppressive actions on lymphocytes and macrophages, which play an important role as effector/regulatory cells in an immune response (48). When added in vitro to antigen (bovine CII)–specific primed lymph node cells, HU-320 suppressed their proliferative response to CII in a dose-dependent manner, thereby suggesting that HU-320 exerts its antiarthritic effects in our CIA model by suppressing the cellular immune response to CII. HU-320 exhibited strong immunosuppressive effects on murine and human "enriched" lymphocytes when it was added to mitogen-induced lymphocyte transformation assays in vitro. However, differences were seen in the levels of suppression of responses, and these differences could be attributed to different types of lymphocytes used in these assays.

The effector arm of the immune response was also affected, since we observed that in vitro addition of HU-320 to PHA-stimulated human lymphocytes markedly diminished the release of IFNγ by these cells (data not shown). However, we could not demonstrate a similar suppression of IFNγ release from CII-stimulated lymph node cells from DBA/1 mice. This discrepancy in the results of IFNγ release from "purified" lymphocytes obtained from two different species (mouse and human) remains unexplained at present, but it may be due to the different stimuli used (i.e., antigen versus mitogen).

Macrophages as well as neutrophils serve as phagocytes and proinflammatory cells at sites of infection and inflammation. TNF and reactive oxygen species form an important component for the maintenance of innate immunity and protection in animals, but in excess these soluble mediators can induce pathology (49,50). We demonstrated that in vitro addition of HU-320 to primary murine macrophage cultures (TGMφ), as well as to a murine monocyte/macrophage line (RAW 264.7 cells), significantly suppressed the release of bioactive TNF. Zymosan-induced release of destructive oxygen species by RAW 264.7 cells was also significantly blocked in the presence of HU-320. Taken together, these results indicate active antiinflammatory actions of HU-320 on macrophages. However, we could not demonstrate, in multiple attempts, the blockade of nitric oxide production by either TGMφ or RAW 264.7 cells, unlike reported results with other cannabinoids (51). The possibility that some of the inhibitory effects of HU-320 were due to the vehicle used (ethanol) was ruled out by the fact that the vehicle alone showed no significant inhibitory effect in any of the in vivo or in vitro assays carried out.

CBD and Δ^9-THC are neuroprotective antioxidants due to their phenolic ring structure (52). Since both CT-3 and HU-320 have the same phenolic ring structure, it is reasonable to assume that they would also function as antioxidants, although no data are available at present. It has been shown that antioxidants attenuate acute TNF-mediated toxicity in a rat model of brain injury (53). HU-320 could have antioxidant properties, as seen from our data showing reductions in TNF and ROI levels from stimulated RAW 264.7 cells. Another cannabinoid, HU-211, has already been well documented as a novel TNF inhibitor protecting against proinflammatory cytokine–mediated cerebral damage following closed head injuries (54).

It is known that HU-320 has very low ability to bind to either of the cannabinoid receptors, CB1 or CB2 (35). The receptor CB1 is expressed on cells of the CNS, while CB2 serves as a peripheral receptor, being expressed on cells of the immune system and, to a lesser stoichiometric ratio, on cells at nerve endings (23). We have demonstrated in the present study that HU-320 exerts no THC-like activity in laboratory mice, which supports the observation that HU-320 has very low potency for binding to CB1 (35). Another nonpsychoactive, antiinflammatory cannabinoid, HU-308, has been shown to reduce cAMP levels upon forskolin stimulation in only CB2-transfected cells and not in CB1-expressing cells (55). In the present study, we have seen a similar suppression of proliferative ability and TNF production by murine lymphocytes/macrophages in the presence of HU-320; however, the binding of HU-320 to CB2 is reported to be low.

Recently, Zurier et al have reported antiarthritic and joint-protective effects of CT-3 in adjuvant-induced arthritis in rats (56). However, possible psychoactive effects in rats were not fully evaluated in that study, although CT-3 has been reported to cause sedation in mice (34). In order to further clarify this point, particularly since CT-3 binds to CB1, we investigated the activity of this compound in the tetrad of assays specific for cannabinoids. Our results indicate that the activity of CT-3 in the tetrad parallels that of Δ^9-THC. In contrast, HU-320 is inactive in the tetrad.

The exact mechanism by which CBD or HU-320 functions as an antiinflammatory agent is not yet fully understood. It has been known that CBD stimulates the vanilloid receptor VR1 and also inhibits the uptake of anandamide, a property not exhibited by HU-320 (35). HU-320 has not yet been tested for its ability to stimu-

late VR1, and it is possible that CBD and HU-320 bind to one of the numerous putative (but yet unidentified) novel cannabinoid receptors, which have been assumed to exist (57).

Our present study thus shows that the synthetic cannabinoid HU-320 is an effective antiarthritic agent in established murine CIA, with effects resembling those reported for CBD (30). Part of this antiarthritic potency of HU-320 could be attributed to its abilities to downmodulate TNF production and to suppress antigen (CII)–specific cellular immune responses. The immunosuppressive ability of HU-320 extends to macrophages and lymphocytes. Suppression of TNF, ROI, and IFNγ responses reveals additional antiinflammatory properties of HU-320.

Our novel findings about the potent antiinflammatory and immunosuppressive properties of this synthetic cannabinoid acid suggest clinical utility and gain further strength from the results of our additional studies showing that administration of HU-320 to mice does not have any adverse psychoactive behavioral effects on CNS-mediated voluntary and involuntary functions. We thus believe that the antiarthritic potency of HU-320, mediated through its immunosuppressive and antiinflammatory capabilities, renders it a promising therapy, an oral agent that might control arthritis and other chronic inflammatory diseases.

Like other cannabinoids, HU-320 is lipophilic and chemically stable at room temperature. We prepared HU-320 solutions fresh in our studies, just before assaying in various systems. At present nothing is known about the toxicity of HU-320, but since it is a major metabolite of a homolog of CBD, which is known to be relatively nontoxic, it is very unlikely that HU-320 would be toxic. However, subsequent studies are needed to document this prior to therapeutic trials in humans.

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