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(54) Title: USE OF CANNABINOIDS AS ANTI-INFLAMMATORY AGENTS

(57) Abstract

The application relates to the identification that cannabinoids, such as cannabidiol can be used to treat inflammatory diseases. Cannabinoids for use in treating inflammatory diseases, methods of treating inflammatory diseases and cannabinoids in combination with pharmaceutically acceptable carriers are claimed.

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USE OF CANNABINOIDS AS ANTI-INFLAMMATORY AGENTS

This application relates to anti-inflammatory agents, and in particular to the use of cannabinoids for the treatment of inflammatory diseases such as rheumatoid arthritis, multiple sclerosis and Crohn's Disease, and to medicinal preparations containing cannabinoids.

Cannabis sativa, commonly known as marijuana, has been used for several years for its medicinal effects, including antipyretic and analgesic properties. Approximately 80 cannabis constituents, termed cannabinoids, naturally occur as 21 carbon atom compounds of cannabis and analogues of such compounds and their metabolites [Mechoulam, In "Marijuna Chemistry, Metabolism and Clinical effects, Academic Press, New York (1973), pages 1-99].

The major psychoactive component of marijuana is Delta-9-tetrahydrocannabinoid (THC), which has been widely studied. Studies have shown that THC affects growth, development and reproductive activity [Pharmacol Rev. 38 (1986), pages 1-18 and 151-178; Marihuana, Pharmacological Aspects of Drug Dependence, Springer Verlag (1996), pages 83-158]. Studies in mice have shown that THC suppresses antibody formation against sheep red blood cells and causes changes in cytokine production. In vitro studies, however, have shown that THC may suppress or enhance (depending on dosage) the production of various cytokines such as IL-1, IL-6 and TNFα by leukocytic cells.

Cannabidiol (CBD) is present in most cannabis preparations (hashish, marijuana, ganja) in higher concentrations than THC. Cannabidiol was first isolated in 1940 by Todd and Adams [J. Amer. Chem. Soc., 62, 2194 (1940), J. Chem. Soc., 649 (1940)]. Its structure was elucidated by Mechoulam and Shvo in 1963 [Tetrahedron, 19 (1963), page 2073]. Its absolute stereochemistry was determined in 1967 [Tet. Lett., 1109-1111 (1967)]. The synthesis of cannabidiol in its racemic form and its natural form were reported in the 1960's [J. Amer. Chem. Soc., 87, 3273-3275 (1965), Helv. Chim. Acta, 50 719-723 (1967)].

Cannabidiol has no psychotropic (cannabimimetic activity) and does not bind either the brain or the peripheral receptors, CB1 and CB2 respectively [Science 169, 611-612 (1970); "Marijuana/cannabinoids: neurobiology and neurophysiology", ed. L. Murphy and A. Bartke, CRC Press, Boca Raton, 1-33 (1992)]. Cannabidiol has, however, been observed to have anticonvulsant effects [Pharmacol, 124, 141-146 (1982)]. Cannabidiol has also been effective in animal models predictive of antipsychotic activity, and has been found to have antipsychotic effects in the case of schizophrenia [Psychopharmacol., 104, 260-264 (1991); J. Clin. Psychiatry, 56, 485-486 (1995)].

Cannabidiol has sporadically been studied for its immunomodulatory effects in vivo and in vitro. Smith et al [Proc. Soc. Exp. Bio Med. 214 (1997), pages 69-75] demonstrated that BALB/C mice injected with cannabidiol did not show significant change in the level of

mRNA of IL-1, IL-6 and TNFα. At an 8 mg/kg dose of cannabidiol, the mortality of mice sublethally injected with Legionella was not affected.

Preliminary studies by Formukong et al [Inflammation, 12, 361-371 (1988)] showed that cannabidiol inhibited PBQ-induced writhing in mice when given orally at doses up to 10 mg/kg. Cannabidiol was also shown to reduce TPA-induced erythema, which is dependent upon prostaglandin release, in mice when applied topically.

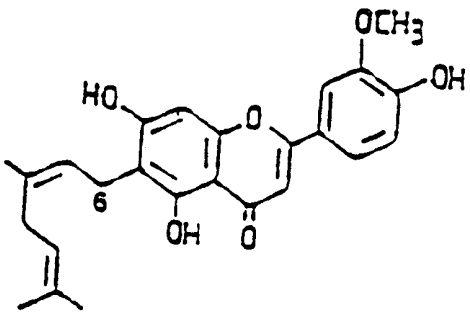
In an in vitro study, Coffey et al [Biochem. Pharmacol, 52 (1996), pages 743-51] demonstrated that THC and cannabidiol inhibited nitric oxide (NO) produced by mouse peritoneal macrophages activated by LPS and IFNγ. Watzl et al [Drugs of Abuse, Immunity and Immunodeficiency, Plenum Press, New York, 63-70 (1991)] studies in vitro the effects of THC and cannabidiol on secretions of IL-1, IL-2, IL-6, TNFα and IFNγ by human leukocytes following activation by mitrogen. They found that both cannabinoids in low concentrations increase IFNγ production, whereas in high concentrations (5-24 μg/ml) completely blocked IFNγ synthesis, and cannabidiol decreased both IL-1 and TNFα production and did not affect IL-2 secretion.

The inventors have now unexpectedly found that cannabinoids may be used to treat inflammatory diseases, such as rheumatoid arthritis and Crohn's disease. Inflammatory diseases involve the complex interaction between several components such as Interleukins (IL-1, IL-6 and IL-8), TNF-α and various mediators such as nitric oxide, ROI and PGE_2.

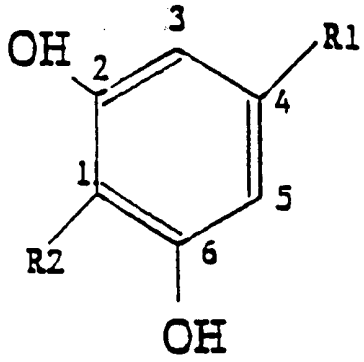
Cannabinoids have been found by the inventors to act as anti-inflammatory agents in vivo.

Accordingly, a first aspect of the invention provides use of one or more cannabinoids as an anti-inflammatory agent.

Preferably, the cannabinoid is an isolated cannabinoid such as cannflavone-2 (formula I) or a cannabinoid having the general formula II.



Formula I



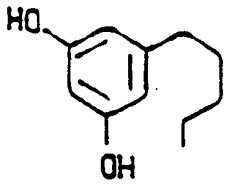
Formula II

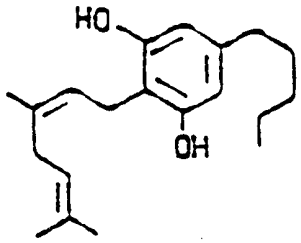
where:

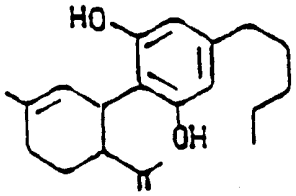
R1 is a straight or branched chain saturated or unsaturated alkyl having preferably 2 to 6 carbon atoms, especially 5 carbon atoms;

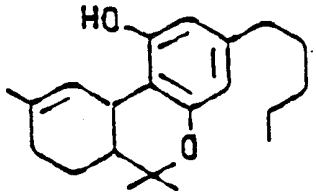
R2 is H or a saturated or unsaturated straight, branched or cyclic hydrocarbon group, or forms a substituted or unsubstituted cyclic ether with the O atom at the sixth position.

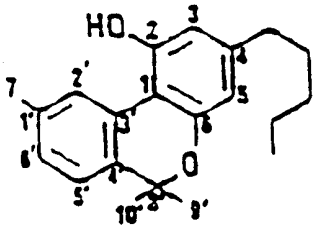
Especially preferred cannabinoids are:

olivetol  Formula III

cannabigerol  Formula IV

cannabidiol  Formula V

tetrahydrocannabinol  Formula VI

or cannabinol  Formula VII

The term isolated is intended to include a naturally occurring cannabinoid which has been purified from a natural source or one which has been chemically synthesised.

Preferably the cannabinoid is used as an anti-inflammatory agent against inflammatory diseases, especially rheumatoid arthritis or Crohn's Disease, sarcoidosis, asthma, Alzheimer s disease, multiple sclerosis, Psoriasis, ulcerative colitis, osteoarthritis or spondyloarthropathy (e.g. ankylosing spondylitis).

The invention also provides a method of treating a patient suffering from an inflammatory disease comprising the step of administering to the patient a pharmaceutically acceptable amount of a cannabinoid.

The cannabinoid is preferably as defined above.

The patient is preferably a mammal such as a human.

Cannabinoids may be used separately or as mixtures of two or more cannabinoids. They may be combined with one or more pharmaceutically acceptable compounds such as carriers.

The invention also provides the use of one or more cannabinoids as previously defined in the manufacture of a medicament to treat inflammatory diseases.

A further aspect of the invention provides a method of treating an inflammatory disease comprising the step of administering to a patient one or more cannabinoids as previously defined. The cannabinoids may for example be applied orally, intramuscularly, subcutaneously, intradermally, intravenously, by nasal spray or topically.

As a general proposition, the total pharmaceutically effective amount of cannabinoid administered will be in the range of 1 μg/kg/day to 50 mg/kg/day of patient body weight,

preferably 2.5 to 10 mg/kg/day especially 5 mg/kg/day.

Accordingly, the invention also relates to medicinal preparations, including topical formulations, capsules, tablets and/or injectable formulations, containing one or more cannabinoids as previously defined for use as anti-inflammatory agents.

Preferably the cannabinoids, according to any previous aspect of the invention, are used or combined with one or more known anti-inflammatory compounds, especially anti-rheumatoid arthritis compounds, such as methotrexate. This allows advantageous properties of the cannabinoids to be combined with known properties of the known compound(s).

The invention will now be described by way of example only with reference to the figures in which:

Figure 1 shows the clinical scores for mice treated with CBD (cannabidiol). Using the Mann-Whitney U-test for comparison of non-parametric data, the following p-values were obtained when comparing treated mice with control mice: for 20 mg/kg, $p < 0.05$ at day 3, day 7 and day 9; for 10 mg/kg. $p < 0.05$ for days 3, 5, 7 and 9; for 5 mg/kg. $p = 0.0004$ at day 3, $p = 0.0096$ at day 5; $p = 0.0269$ at day 7, and $p = 0.0285$ at day 9.

Figure 2 shows the effect of CBD on paw thickness. For 10 mg/kg. $p = 0.004$ at day 3 and $p = 0.0145$ at day 5; for 5 mg/kg. $p = 0.0001$ at day 3 and $p<0.0001$ at days 5, 7, 9

and 10.

Figure 3 shows histological data for treating mice with CBD as described in the examples. The hind paws were assessed as normal, mildly affected or severely destroyed.

Figure 4 shows dose-dependent effects of CBD in a chronic CIA model. From the first signs of joint swelling mice were treated 3 times a week over a 5 week period with CBD, 5 mg/kg or 10 mg/kg i.p. Control mice were treated with vehicle alone, as described in Materials and Methods. Results are expressed as a mean of 6 mice. The AUC for the control group is 38.4, and for the 5 mg/kg group 28.9.

Figure 5 shows the effect of oral feeding of CBD. From the first signs of arthritis mice were treated daily over a 10 day period with CBD at the concentrations mentioned. The drug was administered by oral gavage. Control mice were fed vehicle (olive oil) along, as described in Materials and Methods. Results are expressed as a mean +/- SEM. The 25mg/kg group was significantly better than the control group from day 5 onwards ($p=0.0411$).

Figure 6 shows the effect of oral feeding of CBD on chronic CIA. Mice were fed 25 mg/kg CBD. Controls were fed vehicle (olive oil).

Figure 7 shows the effects of CBD on experimental autoimmune encephalomylitis.

Two groups of 6 SJL/J female mice were injected with mouse spinal cord homogentate to induce EAE. The treatment began at the induction of the disease (day 0) and continued once a day for 14 days. CBD was injected i.p. at a dose of 10 mg/kg. The control group was left untreated.

Figure 8 shows that CBD reduces serum TNFα levels after LPS stimulation. Female C57BL/6 mice were injected ip (intraperitonally) with 100 μg LPS along with CBD ip or subcutaneously (s.c.) 200 μg/mouse (10 mg/kg). After 90 min. the mice were bled and serum TNFα level was determined by bioassay.

Figure 9 shows the effect of CBD on response of lymphocytes to Mitogens.

Spleen cells (1 x 10^6/well) from either BALB/c (Figure 9a) or C57BL/b (Figure 9b) mice were incubated in flat-bottomed microplates for 2 days with medium, 3μg/ml ConA or 50μg/ml LPS, in the presence of the indicated concentrations of CBD. Cultures were pulsed with ^{3}H-thymidine and harvested 6 hours later.

Figure 10 shows the effect of CBD on mixed leukocyte reaction.

Spleen cells (1 x 10^6/well) from either BALB/c mice were incubated in flat-bottomed microplates for 3 days with an equal number of irradiated syngeneic or allogeneic (B6) splenocytes, in the presence of the indicated concentrations of CBD. Cultures were pulsed with ^{3}H-thymidine and harvested 18 hours later.

Figure 11 shows the effect of CBD on cell mediated cytotoxicity.

Spleen cells (1.25 x 10^6/ml) from B6 (H-2^b) mice were incubated for 5 days with an equal number of irradiated BALB/c (H-2^d) splenocytes, in the presence of the indicated concentrations of CBD (mixed leukocyes cultures, MLC). Cells harvested from MLC were tested for their cytotoxic activity against ^{51}Cr-labeled P815 (H-2^d)lymphoma cell line. Cytotoxic activity is given in LU/10^6 cells (see Materials and Methods).

Example 1

Effect of CBD on TNFα production by Thioglycollate-induced macrophages

Thioglycollate-elicited peritoneal macrophages from C57BL/6 mice were activated with LPS and IFNγ. Effects of different concentrations of CBD on the production TNFα was studied. IFN-γ and IFN-α were purchased from Boehringer Mannheim, Germany. The results are shown in Table 1.

TABLE 1

Effect of CBD on TNFα production by Thioglycollate induced macrophages.

A) ACTIVATION BY LPS 1 μg/ml

CELLS & AGENT	6 h	% INHIBITION	24 h	% INHIBITION
0	0.4		9	
LPS	596		277	
LPS + CBD 6 μg/ml	290	51	34	88
LPS + CBD 4 μg/ml	271	54	36	87
LPS + CBD 2 μg/ml	543	9	90	67

B) ACTIVATION BY LPS 1 μg /ml + IFNγ 10 U/ml

CELLS & AGENT	6h	% INHIBITION	24h	% INHIBITION
0	0.4		9	
LPS + IFNγ	716		2664	
LPS + IFNγ 6 CBD μg/ml	548	24	207	92
LPS + IFNγ 4 CBD μg/ml	478	33	437	84
LPS + IFNγ 2 CBD μg/ml	744	0	4578	enhanced 72%

Thioglycolate-elicited peritoneal macrophages from C57BL/6 mice.

Table 1 shows that CBD inhibits TNFα production. Low concentrations of CBD appear to enhance TNFα production.

Example 2

The Effects of CBD on Nitric Oxide Production was also studied

Results are shown in Table 2.

TABLE 2

Effect of CBD on nitric oxide (NO) generation by Thioglycollate induced macrophages.

A) ACTIVATION BY LPS (1 μg/ml)

NO (nM)*

CELLS & AGENT	24 h	% INHIBITION	48 h**	% INHIBITION
CONTROL	0.1		0.3	
LPS 1 μg/ml	5.4		7.3	
LPS 1 μg/ml + CBD 8	0.1	99	0.4	95
LPS 1 μg/ml + CBD 6	0.1	99	2.1	71
LPS 1 μg/ml + CBD 4	0.5	90	4.6	37
LPS 1 μg/ml + CBD 2	3.7	32	6.7	9

B) Activation by LPS 1μg/ml + IFNγ 10 U/ml

NO (nM)*

CELLS & AGENT	24 h	% INHIBITION	48 h**	% INHIBITION
LPS + IFNγ	13.9		20.5	
LPS + IFNγ + CBD 8	0.2	99	3.1	85
LPS + IFNγ + CBD 6	5.5	61	18.1	11.5
LPS + IFNγ + CBD 4	6.9	51	21.3	- (↑ 4%)
LPS + IFNγ + CBD 2	12	14	25.4	- (↑ 24%)

* Assayed by Griess reagent

** After 48 hr the Mϕ cultured with 8 μg/ml CBD were only 70% viable

Once again, low concentrations of CBD appear to activate nitric oxide production, whilst higher concentrations inhibit nitric oxide production.

Example 3

In vitro effects on human peripheral blood mononuclear cells

Preparation of CBD for in vitro experiments

CBD was dissolved in ethanol/DMSO. The ethanol was subsequently evaporated by means of a SpeedVac, and the CBD was resuspended in warm medium at a stock concentration

of 1mg/ml.

Culture of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from the whole blood of healthy donors by Ficoll Hypaque gradient. They were cultured at $2x10^5$ cells/ml in 96- well microtitre plates (200µ/well) and incubated for 6 hours with a dose range of CBD (from µg/ml). After this 6 hour pretreatment period, the cells were stimulated with either LPS from Salmonella typhimirium, 10 ng/ml, for 24 hours (for TNF and IL-1β) or with PHA, 5 µg/ml, for 72 hours (for IFNγ) Viability of the PBMC was assessed with the MTT test.

RESULTS

In vitro effects of CBD on cytokine release by cultured cells

Table 3 summarizes the effects of CBD on activated human PBMC. Interestingly, it was found that the lower concentrations of CBD (0.1 to 5 µg/ml) significantly suppressed the release of the LPS-induced proinflammatory cytokines TNFα and IL-1β, whereas the higher concentrations increased their release. This finding was reproducible and is important in view of the fact that we also found a bell-shaped effect *in vivo* when treating arthritic mice with CBD. The highest dose of 20 mg/kg i.p. was not capable of ameliorating arthritis (Fig. 1 and 2).

Table 3

	TNF α (pg/ml)	IL-1β (pg/ ml)	IFNγ (pg/ml)
Cells only	27 ± 2	40 ± 0	82 ± 12
Cells + stimulus in vehicle	8889 ± 195	1408 ± 165	1881 ± 114
CBD 0.1μg/ ml	2959 ± 434	621 ± 82	2062 ± 316
CBD 1μg/ ml	2503 ± 181	671 ± 74	1082 ± 75
CBD 2.5μg/ ml	3071 ± 296	630 ± 81	1171 ± 138
CBD 5μg/ ml	4152 ± 499	908 ± 99	791 ± 121
CBD 10μg/ ml	10,964 ± 1714	1575 ± 335	150 ± 43
CBD 20μg/ ml	15,071 ± 2594	2292 ± 251	ND
CBD 30μg/ ml	20,824 ± 1046	4158 ± 313	ND

Table 3

Human PBMC were cultured and stimulated with or without CBD, as described in Materials and Methods. The stimulus for TNF and IL-1 production was LPS, the stimulus for IFNγ production was PHA. The results are the mean of triplicate wells ±SEM. ND= not done.

Example 4

In Vivo Studies on the Effect of CBD

Induction and Monitoring of Collagen Induced Arthritis

Bovine type II collagen (CII) was purified from hyaline cartilage, as described [Williams, 1992#18]. Male DBA/1 mice (8-12 weeks old) were immunized with 100μg of CII emulsified in complete Freund's adjuvant CFA (Difco, Detroit, MI) by intradermal injection at the base of the tail. From day 15 after immunization onwards, mice were

examined daily for onset of CIA using two clinical parameters: paw swelling and clinical score [Williams, PNAS, Vol. 89, pages 97848]. Paw swelling was assessed by measuring the thickness of the affected hind paws with 0-10mm callipers (Kroeplin, Schluchtern, Germany). For the clinical score, 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 sacrificed.

For the chronic experiments, 6 weeks old mice were immunized with mouse CII (100 μg CII i.d. = intradermal). From day 30 after immunization onwards, the mice developed a chronic relapsing arthritis, which was monitored for 5 weeks, in the same way as described above.

Administration of Cannabidiol

Cannabidiol (CBD) treatment commenced at the onset of disease and was administered i.p. daily until day 10 of arthritis in the acute arthritis model with bovine CII. The CBD concentrations used were 20 mg/kg (n = 17), 5 mg/kg (n = 15), and 2.5 mg/kg (n = 9). CBD was dissolved in ethanol/cremophor (Sigma Chemical Co., Poole, UK) (1/1, v/v) and further diluted in saline. Mice injected with vehicle alone (ethanol/cremophor in saline) served as controls (n = 23).

For the chronic experiment with mouse CII, mice were treated from the first symptoms of

arthritis every other day, for 5 weeks. For the i.p. route CBD was injected at doses of 10 mg/kg (n=7) and 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 at a dose of 25 mg/kg (n = 6) and control mice were fed olive oil (n = 6).

For the oral treatment protocol in the acute CIA model, 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), corresponding to 2, 5, and 10 mg/kg i.p., respectively. Control mice were fed olive oil (n=6).

Histological Analysis

Hind paws and knee joints were removed *post mortem* on the tenth day of arthritis, fixed in formalin and decalcified in 5% EDTA. Paws and knees were then embedded in paraffin, sectioned and stained with haematoxylin and eosin. Arthritic changes in the ankle, the metatarsophalangeal joints, the proximal interphalangeal and the distal interphalangeal joints were scored blindly as *mild* (mild synovial hyperplasia), *moderate* (pannus formulation and erosions limited to the cartilage-pannus junction), or *severe* (= extended bone and cartilage erosions with loss of joint architecture).

Results

CBD has a dose-dependent therapeutic effect on CIA

CBD at the doses of both 20 mg/kg and 10 mg/kg had a slight therapeutic effect on CIA, especially on the clinical score (Fig. 1). The beneficial effect of 10 mg/kg seemed better than that of 20 mg/kg, particularly during the first few days of treatment (Fig. 1). It was therefore decided to lower the dose of CBD to 5 mg/kg. This concentration caused a dramatic suppression of ongoing CIA, as assessed by both the clinical score (Fig. 1) and the paw thickness (Fig. 2). The therapeutic action of CBD was lost by further lowering the concentration to 2.5 mg/kg (Fig. 1 and 2). At this low dose, CBD was found to have no effect at all on the progression of clinical arthritis, as assessed by clinical score and paw thickness (Fig. 1 and Fig. 2).

The dose-dependent effects of CBD were confirmed in the chronic CIA model (Fig. 4). It was found that 5mg/kg was optimal in suppressing the arthritis. The area under the curve (AUC) was 28.9, as compared to 38.4 in the control group. 10 mg/kg was less effective than 5 mg/kg.

Oral feeding of CBD has a similar therapeutic effect on established and chronic arthritis

Daily oral gavage of CBD after onset of arthritis resulted in an adequate suppression of the arthritis (Fig. 5). Again, 25 mg/kg (which corresponds to 5 mg/kg i.p.) was the optimal dose.

Figure 6 shows that oral feeding of 25 mg/kg CBD resulted in suppression of the

progression of chronic CIA. The area under the curve (AUC) was reduced from 72.3 in the controls to 49.7 in the treated animals.

Histological data confirm the clinical results

Joints in the hind paws of control mice and mice treated with CBD, 5 mg/kg and 10 mg/kg, were assessed blindly for hyperplasia and destruction. In the control mice, no normal joints were found, whereas 11% of the joints in mice treated with 10 mg/kg CBD and 33% of the joints in mice treated with 5 mg/kg CBD were completely protected (Fig. 3). 69% of all joints in the control mice were moderately or severely affected. In mice treated with 5 mg/kg CBD, this was lowered to 42%. Thus, the histological findings confirm the clinical results that CBD, at 5 mg/kg/day, has a marked therapeutic effect on CIA.

Example 5

Cannabidiol suppression of autoimmune encephalomyelitis (EAE) in S3L mice

The effect of cannabidiol on EAE was studied. EAE resembles the disease state of human multiple sclerosis (MS) and acute disseminating encephalomyelitis.

The methods used were based upon those used by Lehmann D et al, J. Neuroimmunology, Vol. 50, pages 35-42, 1994.

Animals

6-12-week-old female SJL/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed under standard conditions in top filtered cages. All animals were fed a regular diet and given acidified water without antibiotics.

Antigens

Mouse spinal cord homogenate (MSCH) was obtained as follows. Spinal cords from 3-10 month-old mice of various strains were obtained by insufflation, MSCH was prepared by homogenization in PBS (1:1 v/v). The homogenate was lyophilized, reconstituted in PBS to a concentration of 100 mg/ml (dry weight) and stored at -20°C until used.

Tuberculin purified protein derivative (PPD) was obtained from Statens Serum Institute, Copenhagen, Denmark.

Induction and clinical evaluation of EAE

Induction of acute EAE in mice was based on a modification of Bernard's procedure (Bernard et al., 1976). Briefly, equal volumes of MSCH (100 mg/ml in PBS) and CFA enriched with *Mycobacterium tuberculosis* H37Ra (6 mg/ml) (Difco Laboratories, Detroit, MI) were emulsified. The emulsion (50-100 µl) was administered s.c. (subcutaneously) into the four footpads of each mouse. Immediately thereafter and 2 days later, mice were injected i.v. (intraveneously) with pertussigen. All animals were examined daily for signs of disease. The first clinical indications appeared on day 9-11 post immunization and were scored according to the following six point scale: 0, no abnormality; 1, mild tail

weakness; 2, tail paralysis; 3, tail paralysis and hind leg paresis; 4, hind leg paralysis or mild forelimb weakness; 5, quadriplegia or moribund state; 6, death.

Mice were treated with cannabidiol at a dose of 10 mg/kg. The results are shown in Figure 7 and Table 4.

Table 4

	CONTROL	CBD
Incidence	4/6	2/6
Duration (days)	4.66	2.16
Mean maximum score	2	1

The results show that cannabidiol markedly suppresses EAE in mice.

Example 6

The effect of CBD on serum TNFα levels

Figure 8 indicates that CBD at 10 mg/kg decreases serum TNFα production in LPS challenged mice.

Example 7

The effect of Cannabidiol on T and B cell proliferation and function

Mice and tumor cell-lines and medium

Female mice (aged 8-12 weeks) of strains C57BL/6 (B6, H-2^b) and BALB/c (H-2^d) were purchased from Harlan, Jerusalem, and maintained under specific pathogen-free (SPF) conditions in the animal facilities of the Hebrew University Medical School, Jerusalem, in accordance with the Hebrew University guidelines, DMEM (Biological Industries, Beit Haemek, Israel) was supplemented with 1mM sodium pyruvate, 10mM HEPES buffer, 0.5mM asparagine-HCl, 0.03mM filic acid, 0.5mM L-aspartic acid, 5 x 10^{-5}M 2-mercaptoethanol, 2mM glutamine, antifiotics and 10% FCS (complete DMEM).

Mitogen-induced cell proliferation

Spleen cells, at a final concentration of 5 x 10^6 cells/ml, were cultured in triplicate wells of flat-bottom microtiter plates (Nunc, Denmark) in medium alone, 2.5 μg/ml concanavalin A (ConA, Biomakor, Israel) or 50 μg/ml lipopolysaccharide (LPS, Difco). The final volume was 200 μl/well. Following two days of incubation at 37°C, in an 8% CO_2-in-air incubator (as in all other procedures described here), 1 μCi of ^{3}H-thymidine was added to each well. Cells were harvested 6 h later, with a Tomtec (USA) cell harvester and counted in a MicroBeta scintillation counter (Wallac, Finland).

Mixed Leukocyte Reaction (MLR)

Spleen cells (1 x 10^6/well) were co-cultured in triplicate wells of flat-bottom microtiter

plates (Nunc), with an equal number of irradiated (25 cGy) syngeneic or allogeneic splenocytes in a final volume of 200 µl/well. After 3-days incubation, the cells were labelled with ^{3}H-thymidine (1 µCi/well) and harvested, following an additional incubation of 18 h, as described above.

Mixed Leukocyte culture (MLC)

MHC-restricted CTL were activated in MLC by co-culturing 2.5 x 10^{6} responding spleen cells for 5 days with an equal number of irradiated (25 Gy) allogeneic splenocytes in 2 ml/well of complete DMEM in 24-well plates (Costar).

Cell mediated cytotoxicity

Cytotoxic assays were performed as described previously (Leshem *et al*, 1999). Briefly, effector cells were serially diluted (threefold) in triplicate wells of conical-bottom microplates (Nunc) and mixed with washed ^{51}Cr-labeled target cells in a final 200 µl volume to make 4-6 effector target cell ratios. Microplates were centrifuged (70 x g, 2 min.) and incubated for 4 h. They were then centrifuged at 200 x g for 5 min. and the supernatants (150 µl) were counted in an automatic c-counter (LKB-Wallac, Finland). Percent of specific cytotoxic activity was calculated according to the formula: [(experimental cpm - background cpm)/(maximal cpm - background cpm) x 100]. Lytic units (LU), were drawn from the cytotoxicity measured at 4-6 E:T cell ratios. One 1 LU is defined as the number of effector cells causing lysis of 30% target cells (Leshem and Brass, 1998).

Figures 9a and 9b show that CBD decreases the response of BALB/C splenocytes and C57BL/b splenocytes respectively to challenge by Concanavalin A (ConA) and LPS.

The effect of CBD on MLR and cell mediated cytotoxicity is shown in Figures 10 and 11 respectively. A slight decrease in ^{3}H-thymidine uptake was observed above 4 µg/ml CBD. Figure 11 shows that low concentrations of CBD increase cytotoxicity, above approximately 1 µg/ml CBD though, a decrease in cytotoxicity was observed.

Example 8

Reactive oxygen intermediation (ROI) production by granulocytes is inhibited by CBD

Thioglycollate-elicited granulocytes were harvested from C57BL/6 mice by sterile lavage with PBS 18 hrs. after intraperitoneal injection with 1.5 ml thioglycollate medium (1.5 ml in 3% solution). The cells were washed and resuspended at 5 x 10^5 cells/ml in Hanks' buffered salt solution without phenol red, and distributed at 0.5 ml/tube into luminometer plastic tubes. CBD dissolved in ethanol and medium at concentration of 6 µg/ml was added to some tubes and finally luminol 10 µl and zymosan 30 µl was added for 0, 1 or 2 hours. The tube was inserted into luminometer (Biolumate LB 95 oot Berhold Wildbad Germany) which had been prewarmed to 37°C. The granulocyte luminol-enhanced chemiluminescence response to zymosan was considered as the positive control.

All cells were viable at the end of the experiment. CBD inhibited 45-92% of the chemiluminescence peak observed.

Table 5

ROI production by Granulocytes from C57BL/6 mice checked by chemiluminescence

Treatment	Chemiluminescence computer peak	% inhibition
Granulocytes (control)	300	
Granulocytes + Zymzan	1868	
Granulocytes + Zymozan + CBD 6µg/ml (0h)	1024	45
Granulocytes + Zymozan + CBD 5µg/ml (1h)	157	92
Granulocytes + Zymozan + CBD 6µg/ml (2h)	235	87

The granulocyte cells were pretreated with CBD 6µg/ml for 0-2 hours before performing the ROI test. After 1-2 hours of CBD treatment, the cells were about 100% available.

Example 9

The effect of CBD on TNF and cytokine release from rheumatoid synovial cells.

Culture of human rheumatoid synovial cells

Synovial membrane tissue was obtained from a patient fulfilling the revised American College of Rheumatology criteria for rheumatoid arthritis who underwent joint replacement

surgery. Synovial cell cultures were prepared as described. Briefly, synovial membrane tissue was digested with collagenase type A (1 mg/ml) and DNAase I (0.15 mg/ml) in RPMI 1640 containing 5% FCS for 2 hours at 37°C. The digested tissue was pushed through a 200 μm^2-nylon mesh and cultured at 10^6 cells/ml/well in RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine, and antibiotics in 24-well culture plates at 37°C in 5% CO_2 for 48 hours in complete medium with or without CBD at specified concentrations.

Culture of murine synovial cells

DBA/1 mice which had been immunized with bovine CII in CFA to induce CIA, as discussed above, were sacrificed at day 10 of arthritis and the knee joints were removed. Synovial cell cultures were performed as previously described. Briefly, synovial membranes were excised under a dissecting microscope and digested with collagenase A (1 mg/ml) (Boehringer-Mannheim) and DNAase type IV (0.15 mg/ml) (Sigma, Dorset, UK) at 37°C for 20 minutes, in the presence of polymyxin B (33 µg/ml) (Sigma). The cells were then washed extensively and cultured in 96-well plates at a density of 2 x 10^6 cells/ml (100µl/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 cytokines.

CBD suppresses spontaneous TNF release by synovium taken from arthritic animals

Synovial cells from arthritic mice at day 10 are known to spontaneously produce large amounts of TNF when cultured *in vitro*. It was found that CBD, when added to the *in vitro*

cultures, exerted a dose-dependent suppression of TNF release (Table 6).

The effect of CBD on cytokine release by human rheumatoid synovium

Similarly, rheumatoid synovial cells spontaneously produce cytokines when cultured *in vitro*. Table 7 shows the effects of CBD on the release of several cytokines, as measured by ELISA. We found a dose-dependent inhibition of IL-6, IL-8, IL-10 and IL-11. In this first initial experiment TNFa was not suppressed which is discordant with the murine results. With restricted number of human synovial cells in this sample, the optimal dose for inhibiting TNFα may have been missed.

Table 6

Mouse synovial cells TNFα is inhibited by CBD.

	O.D.	TNF (pg/ml)
Synovial cells (SC)	0.183±0.003	>1000
SC + vehicle	0.181±0.004	>1000
CBD 1μg/ml	0.190±0.003	>1000
CBD 2.5μg/ml	0.193±0.004	>1000
CBD 5 μg/ml	0.422±0.251	100
CBD 10 μg/ml	0.922±0.103	2
CBD 20 μg/ml	1.152±0.117	<0.01
CBD 50 μg/ml	1.163±0.119	<0.01

Well cytotoxicity assay was used.

Table 7

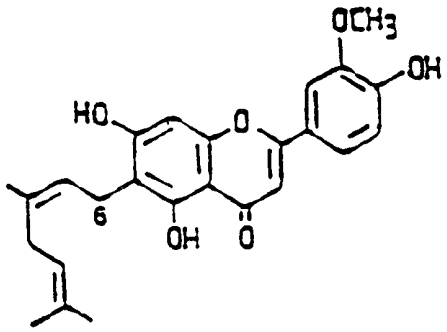
Human Rheumatoid Synovial cell cytokine production is regulated by CBD.

Drug Dose	TNF pg/ml		IL-6 ng/ml		IL-8 ng/ml		IL-10 pg/ml		IL-11 pg/ml	
	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
	2064	184	304	87	175	78	256	54	2139	70
NIL	2271	121	125	26	87	34	52	12	100	1
20ug/ml	2089	783	326	51	183	61	249	30	2059	216
1ug/ml	1963	225	281	43	174	25	273	33	2151	168
0.1ug/ml										
Control (vehicle)	1960	94	258	105	165	53	256	24	2110	78
NIL	1818	78	322	49	224	79	272	27	1884	51
20ug/ml	1656	319	337	70	173	43	223	5	2110	90
1ug/ml	1916	54	310	21	178	48	222	19	1984	168
0.1ug/ml										

CLAIMS:

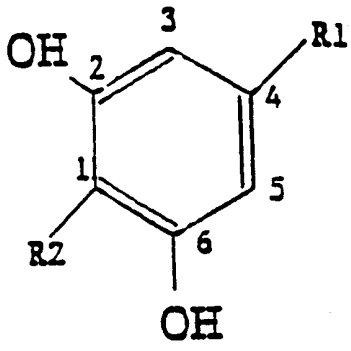
1. A cannabinoid for use in treating inflammatory diseases.

2. A cannabinoid according to claim 1, selected from cannflavone-2 (formula I).



Formula I

and a cannabinoid having general formula II



Formula II

where:

R1 is a straight or branched chain saturated or unsaturated alkyl having preferably 2 to 6 carbon atoms, especially 5 carbon atoms;

R2 is H or a saturated or unsaturated straight, branched or cyclic hydrocarbon group, or

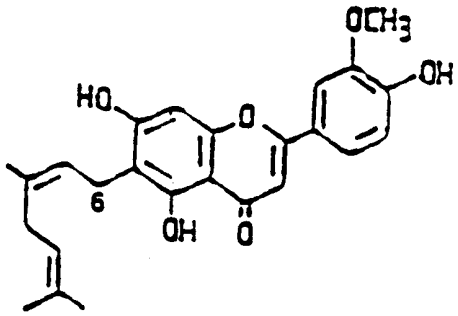
forms a substituted or unsubstituted cyclic either with the O atom at the sixth position.

3. A cannabinoids according to claim 1 or claim 2, wherein the cannabinoid is cannabidiol.

4. In combination, a cannabinoid according to any preceding claim with a second antiinflammatory compound.

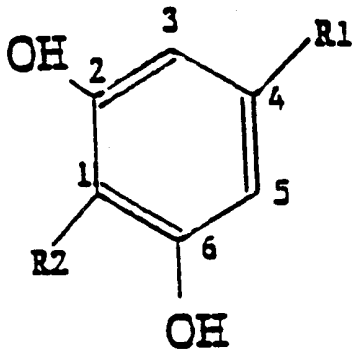
5. A method of treating a patient suffering from an inflammatory disease comprising the step of administering to said patient a pharmaceutically effective amount of an cannabinoid.

6. A method according to claim 5, wherein the cannabinoid is selected from a cannflavone-2 (formula I)



Formula I

and a cannabinoid have general formula II



Formula II

where:

R1 is a straight or branched chain saturated or unsaturated alkyl having preferably 2 to 6 carbon atoms, especially 5 carbon atoms;

R2 is H or a saturated or unsaturated straight, branched or cyclic hydrocarbon group, or forms a substituted or unsubstituted cyclic ether with the O atom at the sixth position.

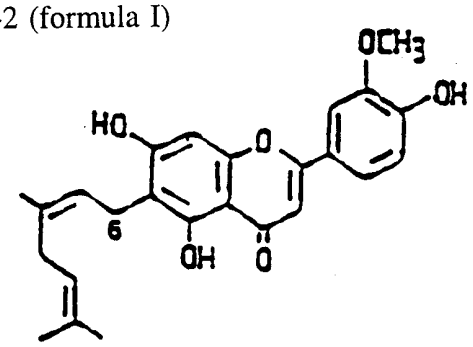
7. A method according to claim 6, wherein the cannabinoid is cannabidiol.

8. A method according to claim 6, wherein the inflammatory disease is selected from rheumatoid arthritis, Chrohn's disease, sarcoidosis, Alzheimer's disease, multiple sclerosis, asthma, psoriasis, ulcerative colitis, osteoarthritis and spondyloarthropathy.

9. A method according to claim 6, wherein the cannabinoid is combined with the use of a second antiinflammatory compound.

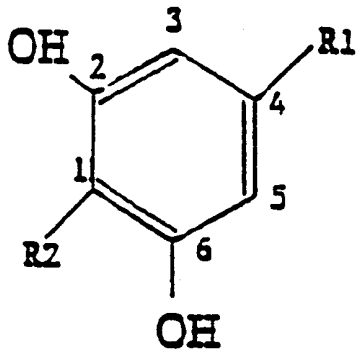
10. A cannabinoid in combination with a pharmaceutically acceptable carrier.

11. A cannabinoid according to claim 10, wherein the cannabinoid is selected from a cannflavone-2 (formula I)



Formula I

and a cannabinoid having general formula II



Formula II

where:

R1 is a straight or branched chain saturated or unsaturated alkyl having preferably 2 to 6 carbon atoms, especially 5 carbon atoms;

R2 is H or a saturated or unsaturated straight, branched or cyclic hydrocarbon group, or forms a substituted or unsubstituted cyclic ether with the O atom at the sixth position.

12. A cannabinoid according to claim 10 or 11, wherein the cannabinoid is cannabidiol.

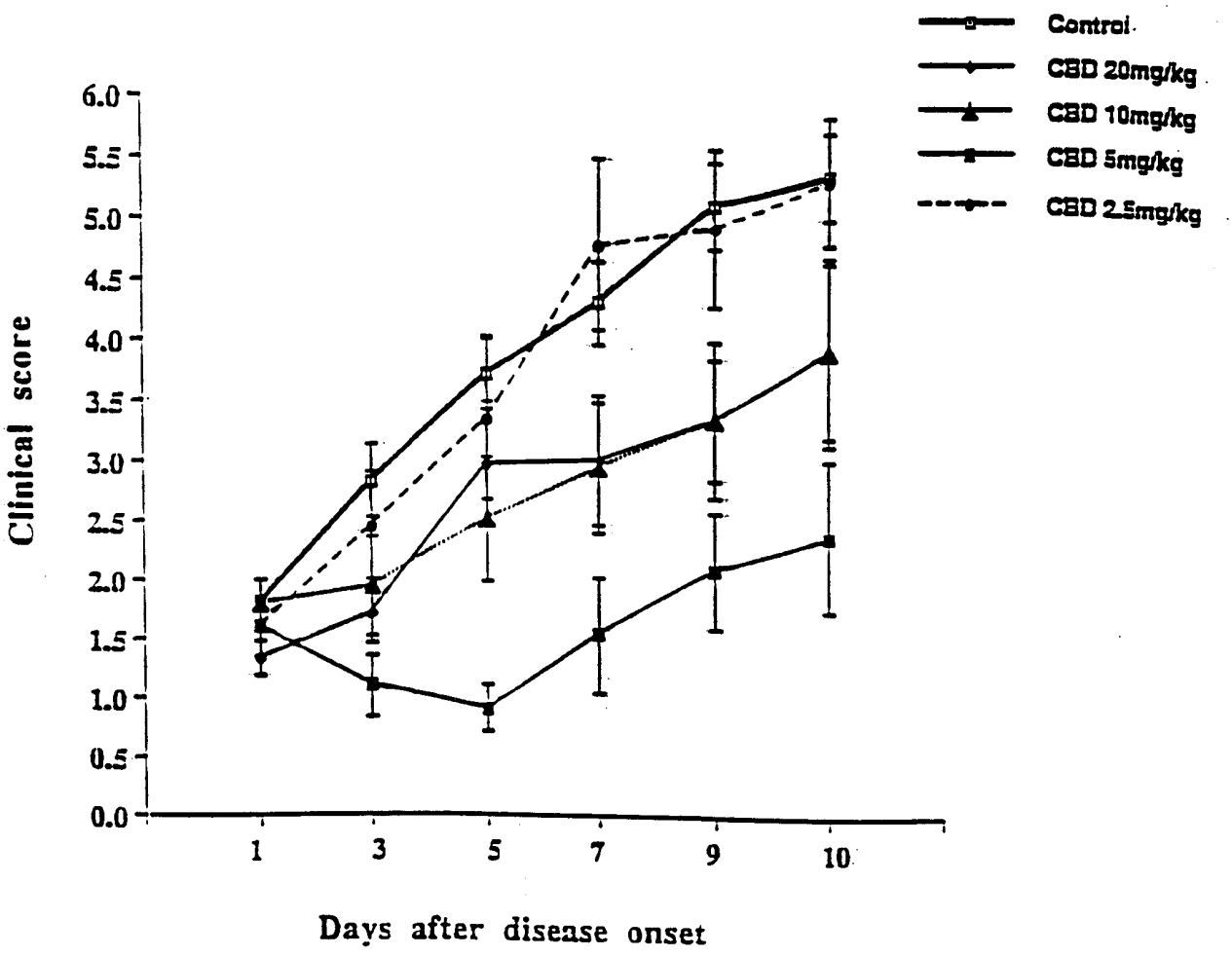

Control
CBD 20mg/kg
CBD 10mg/kg
CBD 5mg/kg
CBD 2.5mg/kg
Clinical score
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
3
5
7
9
10
Days after disease onset

FIGURE 1

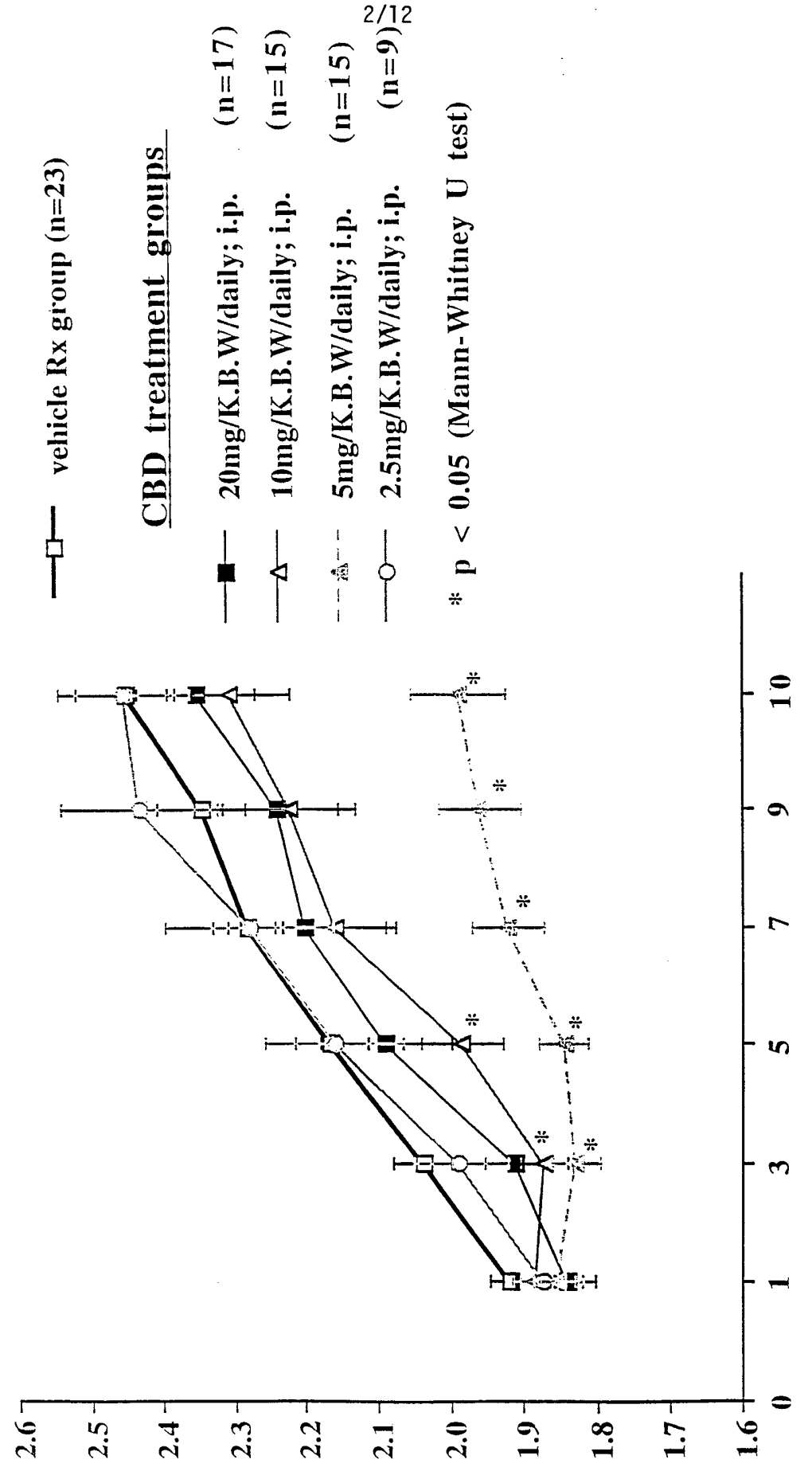

Effect of CBD Rx on CIA in DBA/1 mice
vehicle Rx group (n=23)
CBD treatment groups
20mg/K.B.W/daily; i.p. (n=17)
10mg/K.B.W/daily; i.p. (n=15)
5mg/K.B.W/daily; i.p. (n=15)
2.5mg/K.B.W/daily; i.p. (n=9)
* p < 0.05 (Mann-Whitney U test)
Mean paw-swelling (mm)
Days after the onset of arthritis
2.6
2.5
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
0
1
3
5
7
9
10

FIGURE 2

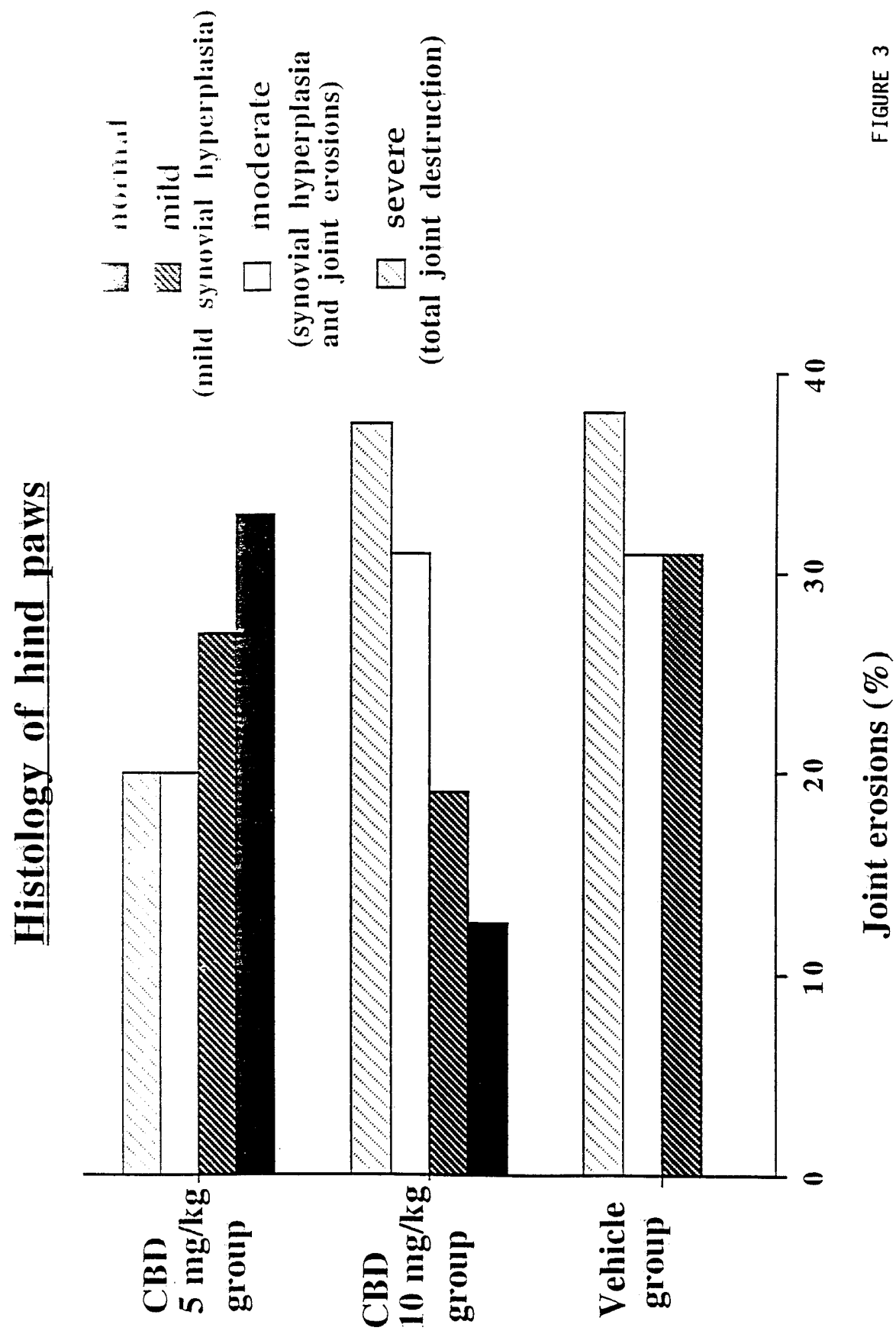

Histology of hind paws
normal
mild
(mild synovial hyperplasia)
moderate
(synovial hyperplasia and joint erosions)
severe
(total joint destruction)
CBD 5 mg/kg group
CBD 10 mg/kg group
Vehicle group
0
10
20
30
40
Joint erosions (%)

FIGURE 3

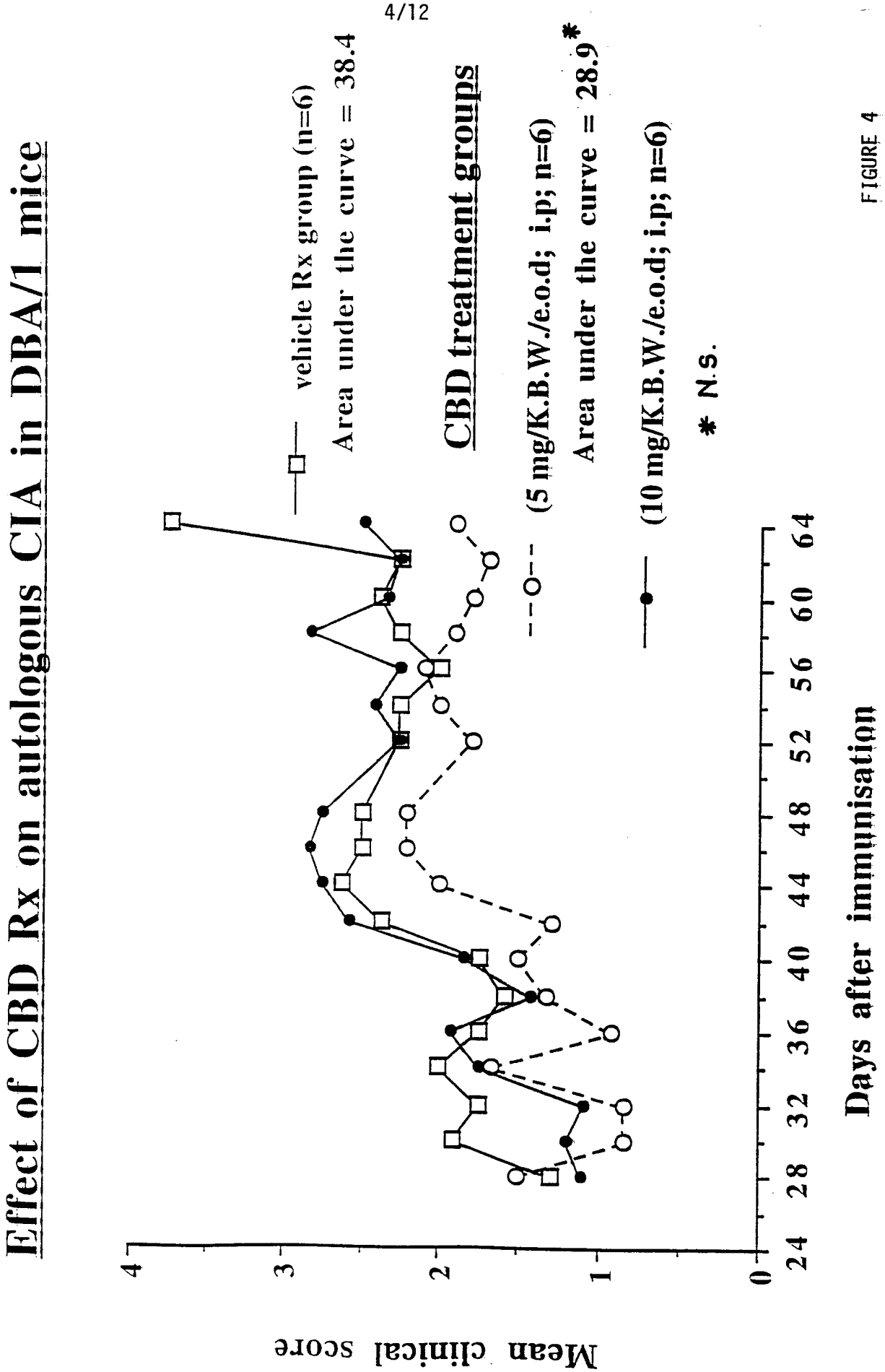

Effect of CBD Rx on autologous CIA in DBA/1 mice
vehicle Rx group (n=6)
Area under the curve = 38.4
CBD treatment groups
(5 mg/K.B.W./e.o.d; i.p; n=6)
Area under the curve = 28.9*
(10 mg/K.B.W./e.o.d; i.p; n=6)
* N.S.
Mean clinical score
Days after immunisation
0
1
2
3
4
24 28 32 36 40 44 48 52 56 60 64

FIGURE 4

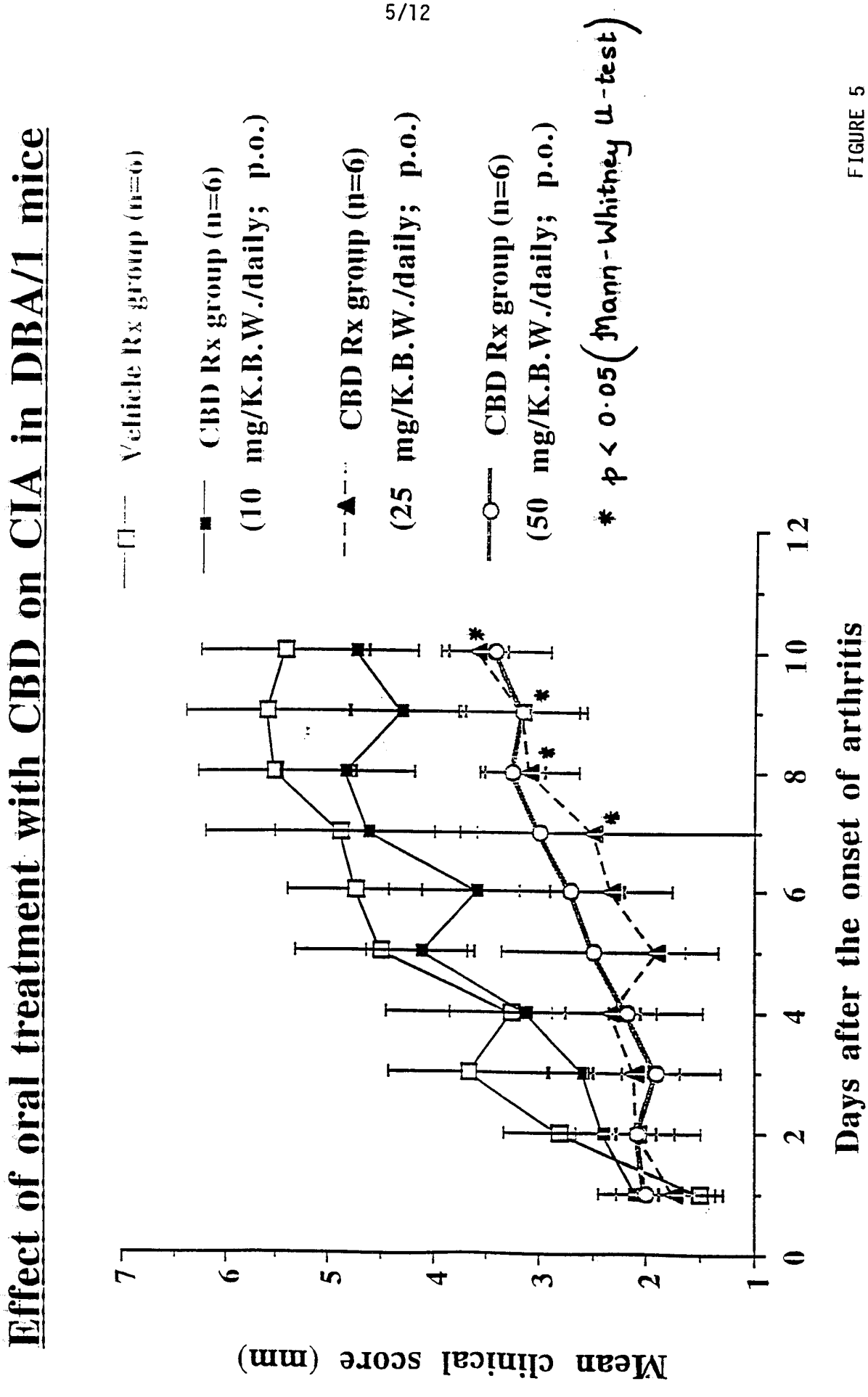

Effect of oral treatment with CBD on CIA in DBA/1 mice
—□— Vehicle Rx group (n=6)
—■— CBD Rx group (n=6)
(10 mg/K.B.W./daily; p.o.)
--▲-- CBD Rx group (n=6)
(25 mg/K.B.W./daily; p.o.)
—○— CBD Rx group (n=6)
(50 mg/K.B.W./daily; p.o.)
* p < 0·05 (Mann-Whitney U-test)
Mean clinical score (mm)
Days after the onset of arthritis
0
2
4
6
8
10
12
1
2
3
4
5
6
7

FIGURE 5

FIGURE 6

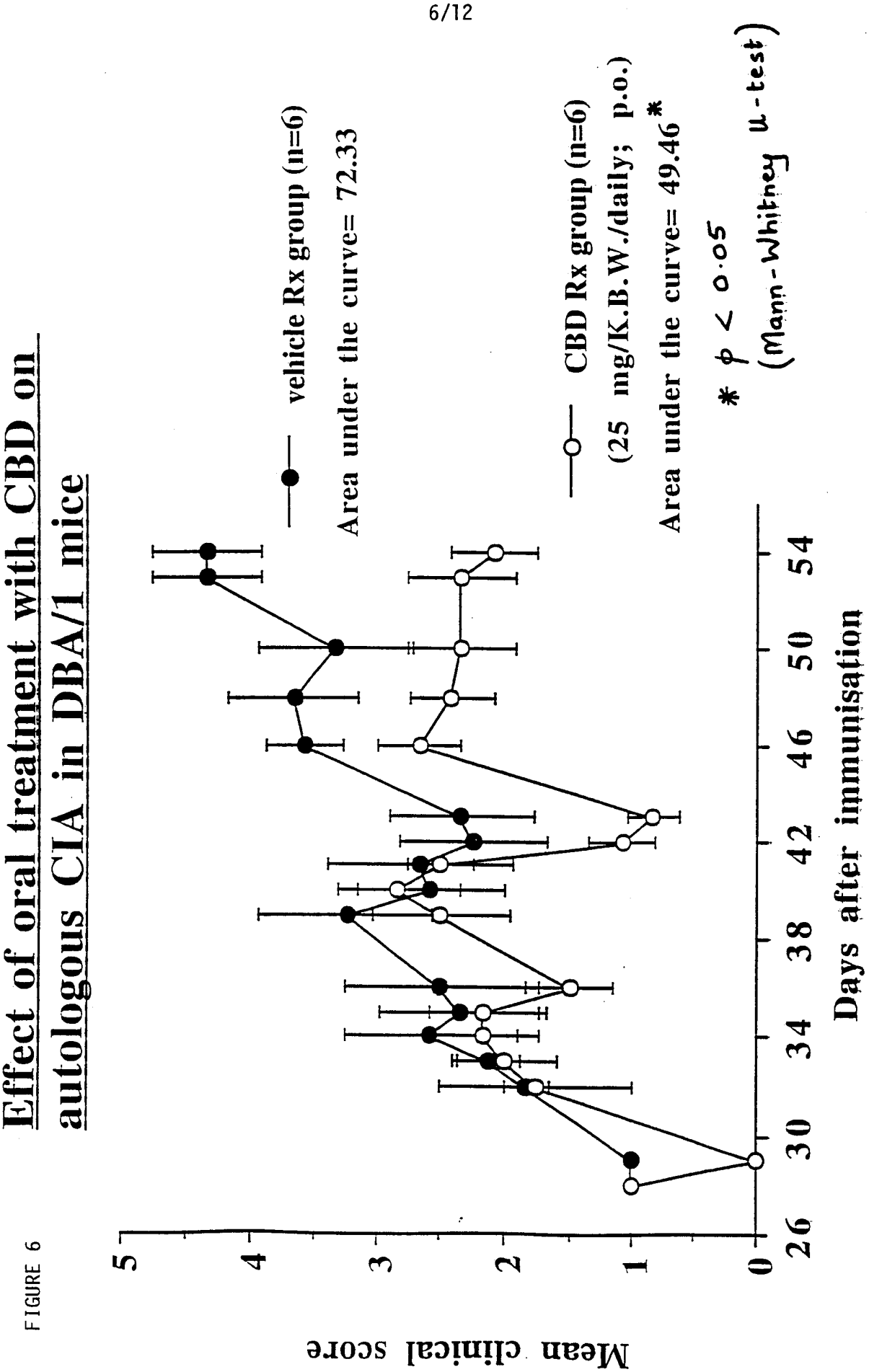

Effect of oral treatment with CBD on autologous CIA in DBA/1 mice
vehicle Rx group (n=6)
Area under the curve= 72.33
CBD Rx group (n=6)
(25 mg/K.B.W./daily; p.o.)
Area under the curve= 49.46*
* p < 0.05
(Mann-Whitney u-test)
Mean clinical score
Days after immunisation
0
1
2
3
4
5
26
30
34
38
42
46
50
54

Figure 7

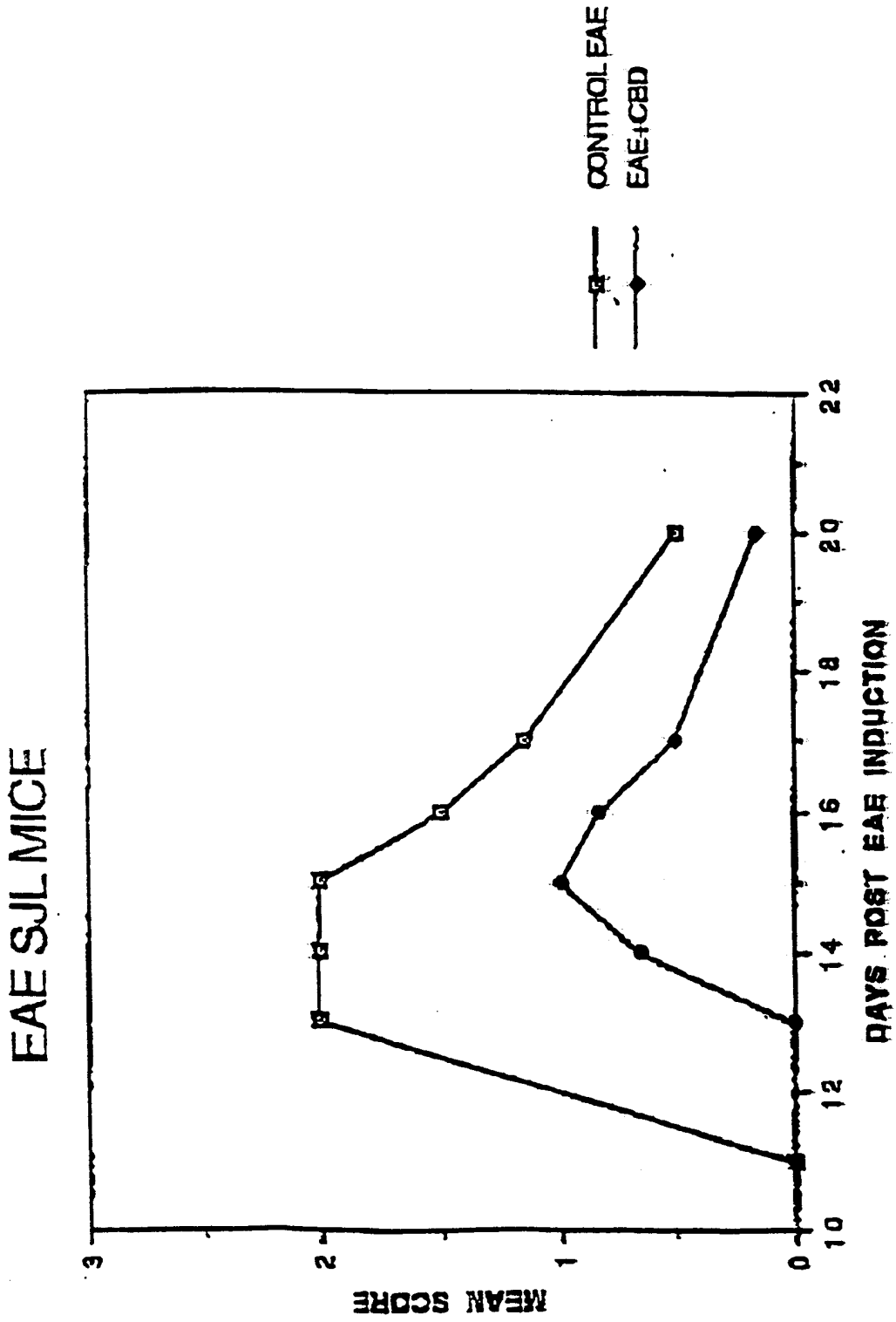
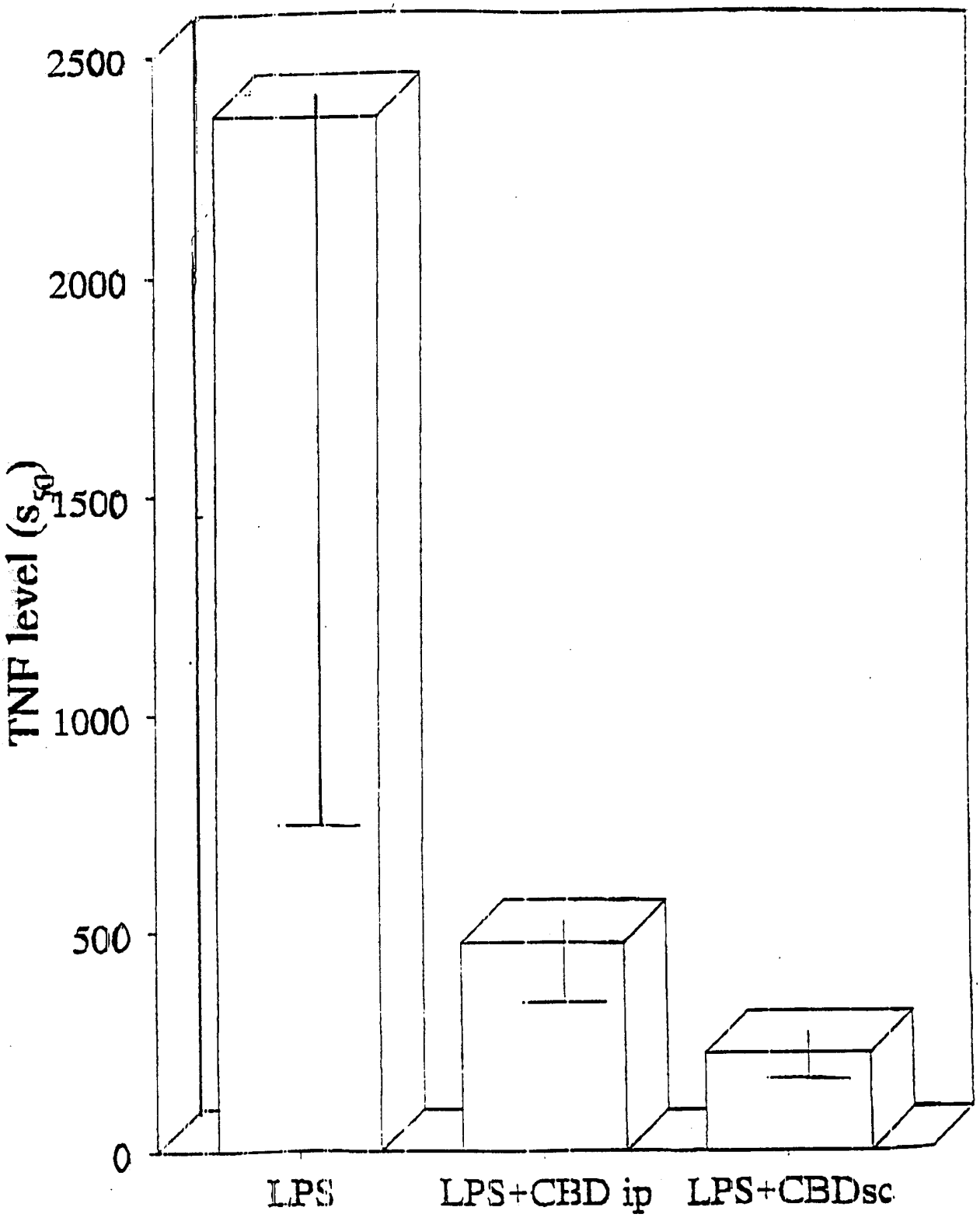

EAE SJL MICE
CONTROL EAE
EAE+CBD
MEAN SCORE
0
1
2
3
10
12
14
16
18
20
22
DAYS POST EAE INDUCTION

FIGURE 8


2500
2000
1500
1000
500
0
TNF level (s_{50})
LPS
LPS+CBD ip
LPS+CBDsc

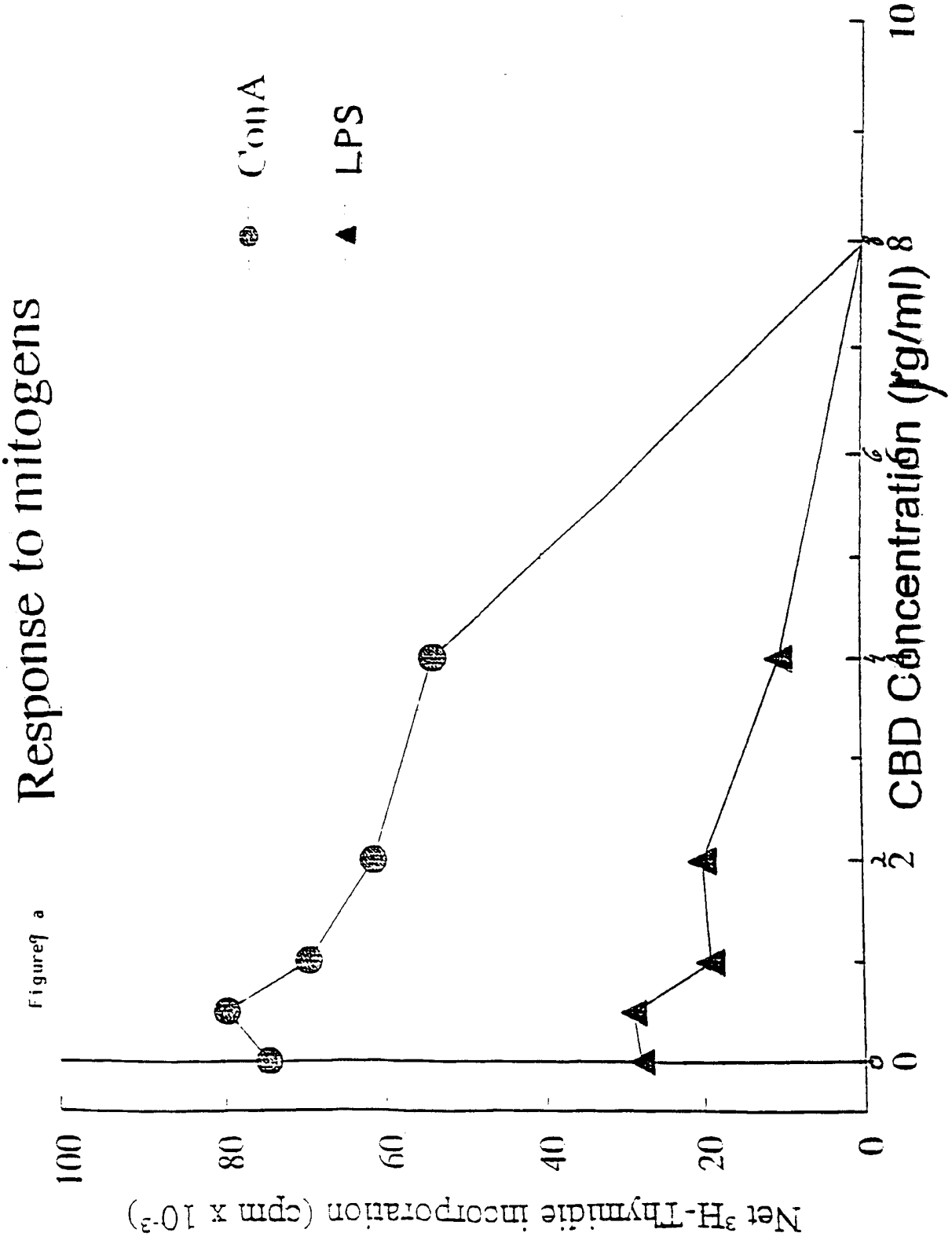

Response to mitogens
ConA
LPS
Net ³H-Thymidie incorporation (cpm x 10⁻³)
CBD Concentration (µg/ml)
0
20
40
60
80
100
0
2
4
6
8
10

Figure 9a

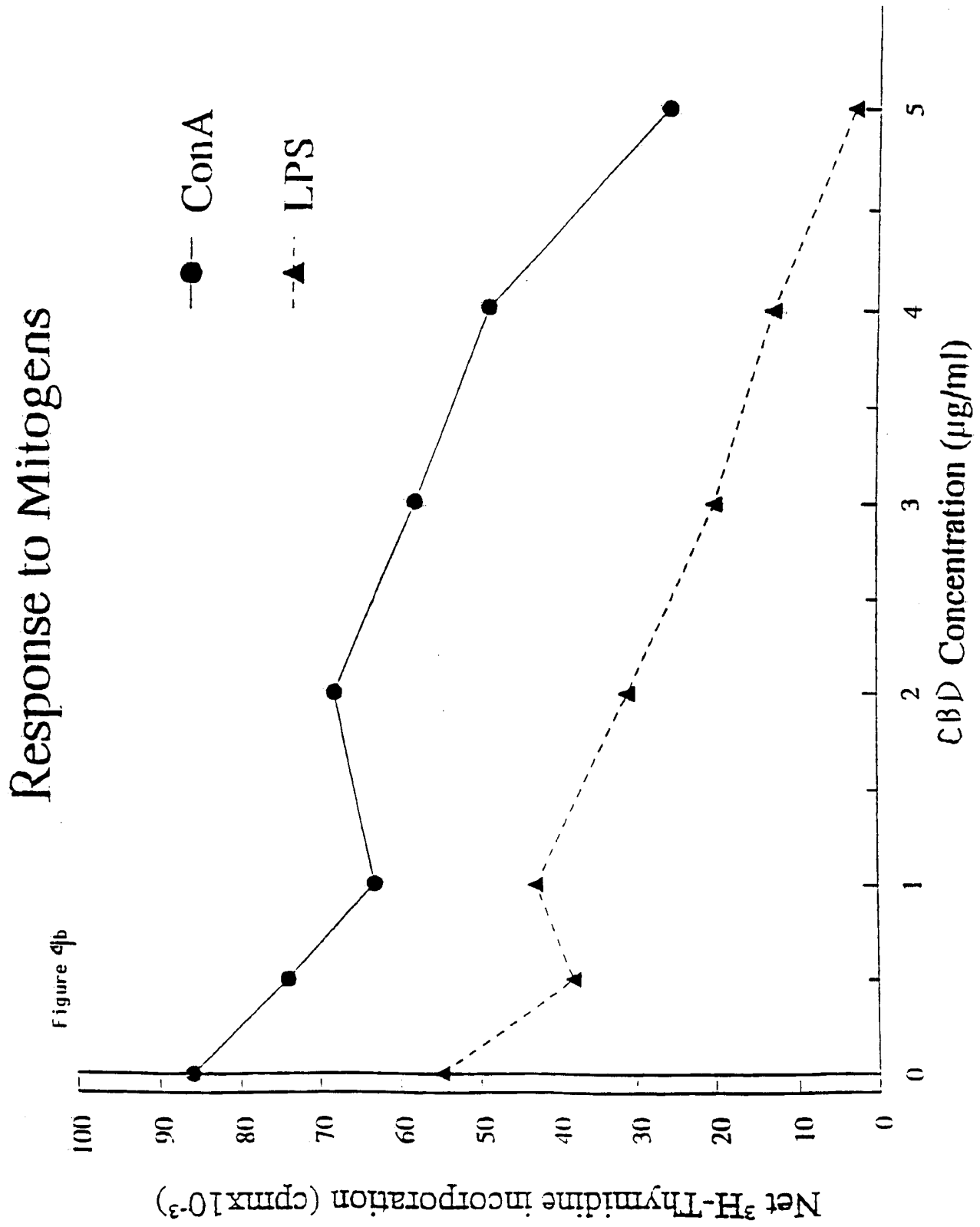

Response to Mitogens
ConA
LPS
Net ³H-Thymidine incorporation (cpmx10-³)
CBD Concentration (µg/ml)
0
10
20
30
40
50
60
70
80
90
100
0
1
2
3
4
5

Figure 4b

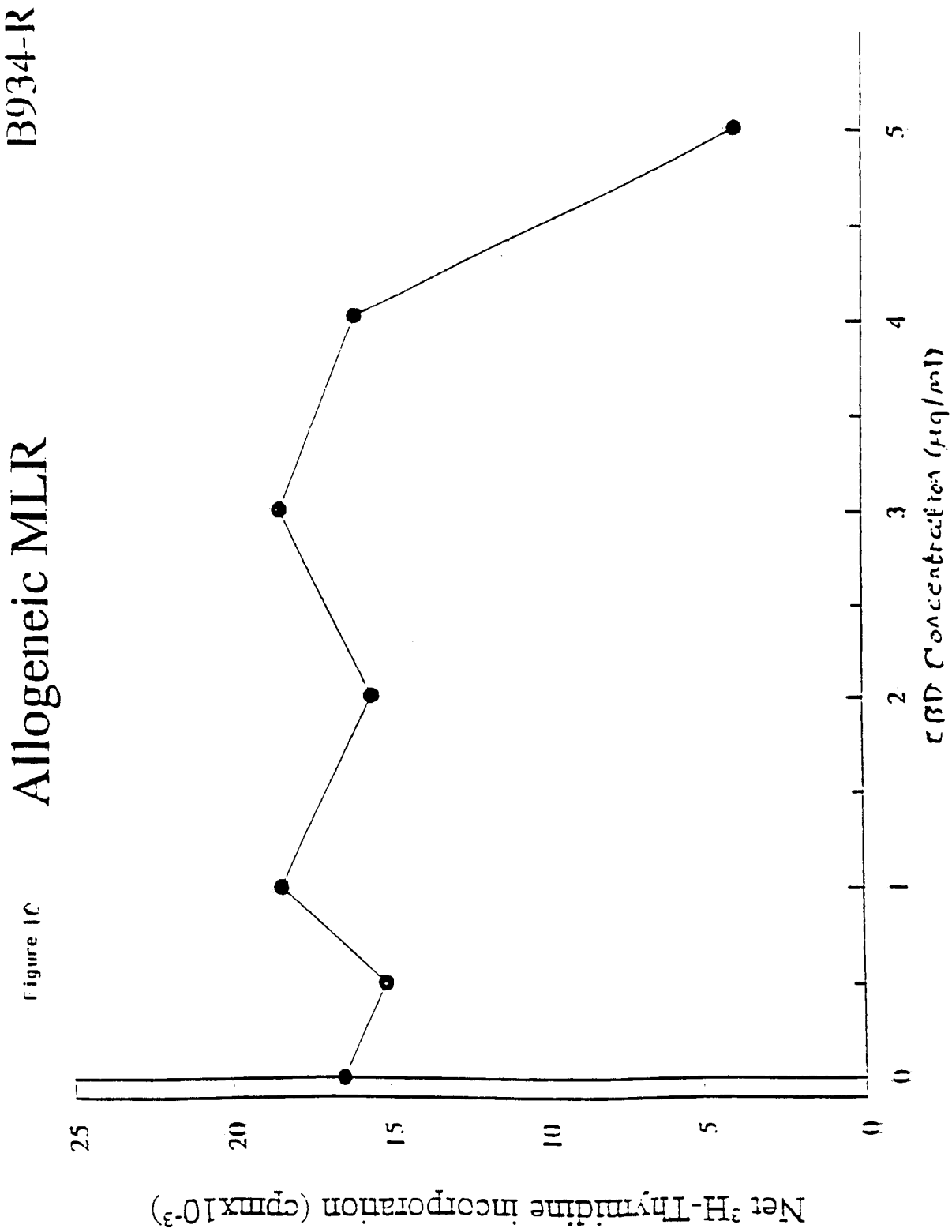
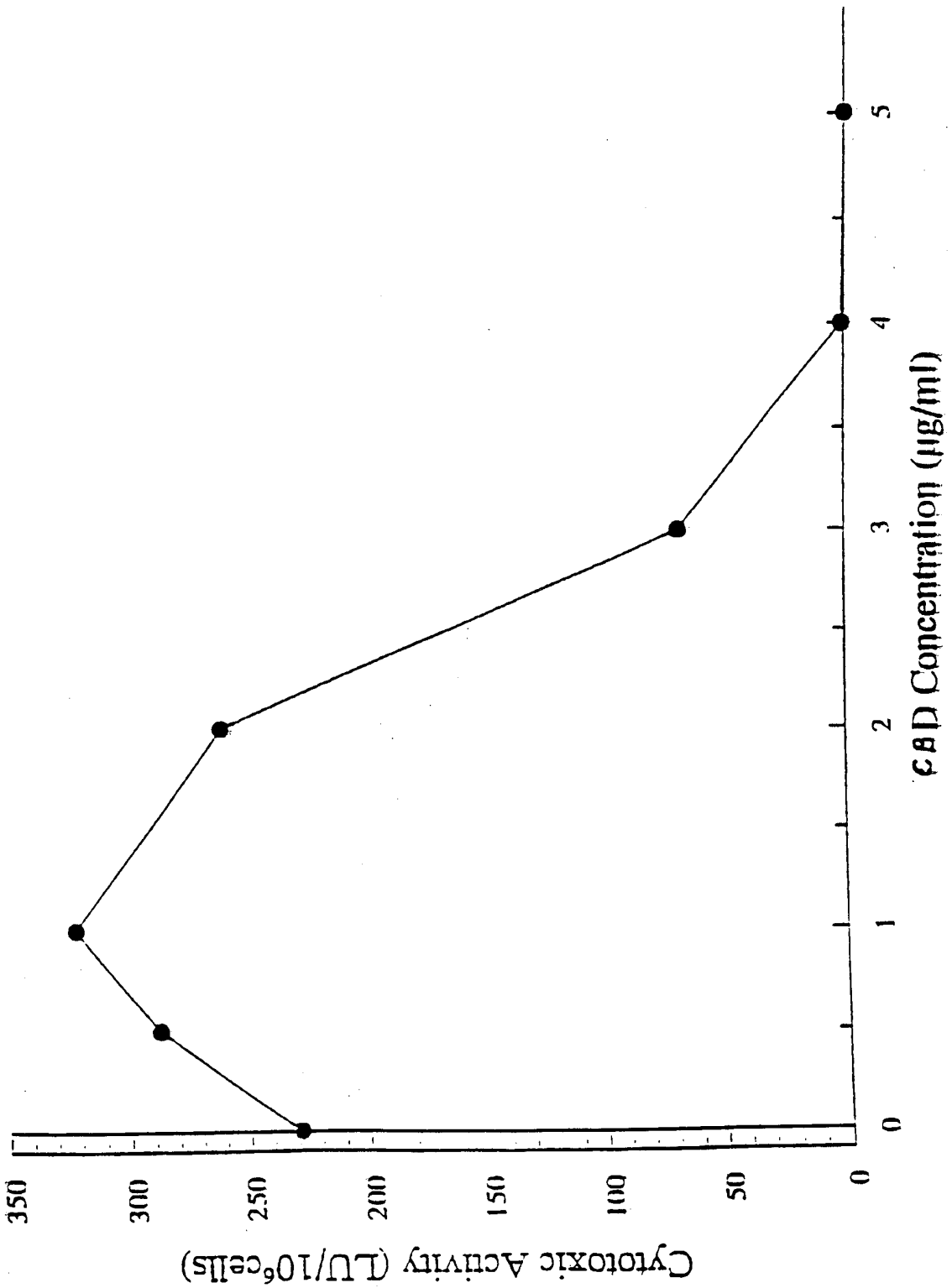

B9934-R
Allogeneic MLR
Net ³H-Thymidine incorporation (cpmx10⁻³)
25
20
15
10
5
0
0
1
2
3
4
5
CBD Concentration (μg/ml)

Figure 10

Figure 11 Allogeneic Cytotoxicity B934-R



INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/GB 99/01140

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/35 A61K31/05

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BARRETT ML ET AL: "Cannflavin A and B, prenylated flavones from Cannabis sativa L." EXPERIENTIA, APR 15 1986, 42 (4) P452-3, XP002114248 SWITZERLAND the whole document ---	1,2,5,6, 8,10,11
X	BARRETT ML ET AL: "Isolation from Cannabis sativa L. of cannflavin--a novel inhibitor of prostaglandin production." BIOCHEM PHARMACOL, JUN 1 1985, 34 (11) P2019-24, XP002114249 ENGLAND the whole document ---	1,2,5,6, 8,10,11

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 September 1999	16/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Mair, J

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Int ıtional Application No
PCT/GB 99/01140

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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X	US 4 973 603 A (BURSTEIN) 27 November 1990 (1990-11-27) the whole document especially column 1, line 48 & 51	1,2,5,6, 8,10,11

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INTERNATIONAL SEARCH REPORT

:ernational application No.
PCT/GB 99/01140

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 5-9
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 5-9 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ıtional Application No
PCT/GB 99/01140

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