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Protection Against Septic Shock and Suppression of Tumor Necrosis Factor α and Nitric Oxide Production by Dexanabinol (HU-211), a Nonpsychotropic Cannabinoid

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ABSTRACT

Dexanabinol, HU-211, a synthetic cannabinoid devoid of psychotropic effects, improves neurological outcome in models of brain trauma, ischemia and meningitis. Recently, HU-211 was found to inhibit brain tumor necrosis factor (TNFα) production after head injury. In the present study, we demonstrate the ability of HU-211 to suppress TNFα production and to rescue mice and rats from endotoxic shock after LPS (*Escherichia coli* 055:B5) inoculation. In BALB/c mice, a dose of 10 mg/kg LPS, injected i.p., caused 57% and 100% mortality, at 24 and 48 hr, respectively. HU-211, administered i.p. 30 min before lipopolysaccharide (LPS), reduced lethality to 9 and 67% at these time points ($P < .05$). When coinjected with D-galactoseamine (i.p.), LPS was 100% lethal within 24 hr, whereas eight hourly injections of HU-211 caused mortality of C57BL/6 mice to drop to 10% ($P < .001$). Administration of LPS to Sprague-Dawley rats resulted in a 30% reduction in the mean arterial blood pressure within 30 min, which persisted for 3 hr. HU-211, given 2 to 3 min before LPS, completely abolished the typical hypotensive response. Furthermore, the drug also markedly suppressed *in vitro* TNFα production and nitric oxide generation (by >90%) by both murine peritoneal macrophages and rat alveolar macrophage cell line exposed to LPS. HU-211 may, therefore, have therapeutic implications in the treatment of TNFα-mediated pathologies.

Tumor necrosis factor is a pleiotropic cytokine that is involved in the pathogenesis of various immune mediated processes. It is the key mediator in septic shock (Tracey, 1991) and is involved in the pathogenesis of inflammatory diseases such as multiple sclerosis, inflammatory bowel disease, rheumatoid arthritis and cachexia (Tracey and Cerami, 1994). The cytokine is released mainly by mononuclear phagocytic cells in response to injection of LPS (an endotoxin derived from Gram-negative bacteria), into experimental animals (Waage, 1992). Administration of TNFα to rodents is fatal, causing hemorrhage and ischemic necrosis of the gastrointestinal tract, hemorrhages and leukocyte infiltration in the lung, acute tubular necrosis of the kidney, metabolic acidosis, hypermetabolism, hypotension and increased pituitary and stress hormone production (Beutler and Cerami, 1988; Tracey *et al.*, 1986; Bauss *et al.*, 1987 Remick *et al.*, 1987; Benveniste, 1992). These pathological manifestations are similar to those induced by endotoxin. The administration of hrTNFα to humans causes similar toxic effects (Tracey *et al.*, 1986). In addition, TNFα affects the central nervous system and results in fever, sickness behavior, anorexia, sympathetic discharge and stimulation of pituitary hormones (for review, see Rothwell and Hopkins, 1995). Although circulating TNFα and other cytokines are known to be transported into the brain and to affect its function (Watkins *et al.*, 1995), some cytokines (TNFα, IL-1, IL-6) are also synthesized in the injured brain, mainly by microglia, but also by neurons, astrocytes and endothelial cells (Brenner *et al.*, 1993; Sawada *et al.*, 1989; Woodroofe *et al.*, 1991; Mier *et al.*, 1992; Spangelo *et al.*, 1990). Septic shock is now considered a systemic inflammatory response caused not only by Gram-negative and Gram-positive bacteria but also by noninfectious disorders such as ischemia and trauma (Tracey and Cerami, 1994; Rothwell and Hopkins, 1995; Glauser *et al.*, 1994). It is still a major cause of death in intensive care units, and attempts are being made to block the generation of TNFα or to inhibit its action on target cells, either by specific antibodies against various components of TNFα, soluble receptors or by inter-

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ABBREVIATIONS: CHI, closed head injury; D-GALN, D-galactosamine; FCS, fetal calf serum; HU-211, dexanabinol [(+)-(3*S*,4*S*)-7-hydroxy Δ^6-tetrahydrocannabinol-1,1-dimethylheptyl]; LPS, lipopolysaccharide; MABP, mean arterial blood pressure; NMDA, N-methyl-D-aspartate; NO, nitric oxide; TNFα, tumor necrosis factor; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; IL, interleukin.

ference with its intracellular signaling. For example, tyrphostins, which inhibit tyrosine kinase and block tyrosine phosphorylation of a $p42^{MAPK}$ protein, inhibit LPS-induced NO and TNFα production. These agents also protect mice against LPS-induced lethal toxicity (Novogrodsky *et al.*, 1994). In addition, pyridinyl immidazoles have been shown to inhibit cytokine production *in vitro* and to attenuate inflammatory reactions *in vivo* (Lee *et al.*, 1993; Reedy *et al.*, 1994).

We recently demonstrated in an experimental rat model that the synthetic cannabinoid Dexanabinol (HU-211) inhibits the production of TNFα in brain tissue after CHI (Shohami *et al.*, 1996). The compound does not bind to the cannabinoid receptor and does not cause cannabimimetic effects (Feigenbaum *et al.*, 1989), but exhibits pharmacological properties characteristic of NMDA-receptor antagonists. This cannabinoid stereospecifically blocks the NMDA-receptor by interacting with a site close to but distinct from that of noncompetitive NMDA-receptor antagonists and from the recognition sites of glutamate, glycine and polyamines (Nadler *et al.*, 1993a). In primary rat forebrain cultures, HU-211 attenuated $^{45}Ca^{++}$ influx through the NMDA receptor channel (Nadler *et al.*, 1993b) and prevented NMDA receptor-mediated neuronal cell death (Eshhar *et al.*, 1993). In rat and gerbil models of ischemia, HU-211 improved the neurological status, protected the blood-brain barrier and reduced neuronal death in the CA_1 region of the hippocampus (Bar-Joseph *et al.*, 1994; Belayev *et al.*, 1995a, b). In an experimental rat model of CHI, HU-211 improved the recovery of motor and memory functions, reduced the breakdown of the blood-brain barrier, attenuated the development of cerebral edema and the accumulation of $^{45}Ca^{++}$ (Shohami *et al.*, 1993, 1995; Nadler *et al.*, 1995). In addition, coadministration of HU-211 with antibiotics in experimental bacterial meningitis resulted in a better outcome than after treatment with antibiotics alone (Bass *et al.*, 1996), perhaps by interrupting the production of toxic mediators during bacteriolysis. After showing that HU-211 inhibits production of TNFα in the brain we designed the present study to investigate whether HU-211 could protect rodents from endotoxic shock and suppress TNFα production *in vivo* after LPS inoculation. In addition, we studied the *in vitro* effect of HU-211 on TNFα and NO production by macrophages after their activation with LPS. It has been shown that hypersecretion of glucocorticoids plays a crucial role in protecting animals from endotoxic shock (Dantzer *et al.*, 1996), and that natural and synthetic cannabinoids affect adrenocortical function (Eldridge and Landfield, 1990). We have therefore determined the effect of HU-211 on the secretion of glucocorticoids after LPS administration. We found that although application of HU-211 markedly inhibits TNFα production *in vivo* and *in vitro* and protects mice from endotoxic shock, it does not affect secretion of glucocorticoids.

Materials and Methods

Drug Treatment

HU-211 (Pharmos Corp. Rehovot, Israel) was studied in a variety of experimental paradigms, both *in vivo* (at a dose range of 1.25–14 mg/kg, depending on species and route of administration) and *in vitro* (at a dose range of 1–50 μM) (for review, Shohami *et al.*, 1996). Similar dose ranges were selected in the present study. HU-211 was dissolved in cosolvent Cremophor EL/ethanol (1:1) (50 mg/ml) and diluted (1:20) in saline before injection to yield a dose of 4 to 10 mg/kg. Control mice or rats were injected with an equivalent volume of the vehicle. For the *in vitro* experiments, HU-211 was dissolved in DMSO (20 mg/ml) and diluted in medium before administration. The LPS preparation used in this study was *Escherichia coli*, 055:B5, phenol extraction (Difco, Detroit, MI).

Effect of HU-211 on TNFα Levels in Serum of LPS-Treated Mice

Female C57BL/6 mice (9–12 weeks old, weighing 20–27 g) were injected i.p. with 5 mg/kg LPS, along with HU-211 (10 mg/kg). After 90 min they were bled, and serum TNFα activity (titer) was bioassayed.

Effect of HU-211 on Mouse Endotoxic Lethality

BALB/c male mice were injected i.p. with HU-211 (10 mg/kg, $n = 12$) or vehicle ($n = 14$), followed 30 min later by an i.v. injection of LPS (10 mg/kg). Mortality was recorded once daily for 2 days. To sensitize mice to LPS toxicity, and to induce 100% mortality within 24 hr, C57BL/6 female mice ($n = 20$) were coadministered LPS (0.5 μg/kg) and D-GALN (900 μg/kg), according to the protocol of Lehmann *et al.* (1987). Half of the mice served as controls, and half were treated within 5 min of injection with HU-211 (7.5 mg/kg i.v.), followed by eight additional injections, once every hour. Survival was followed for up to 7 days.

Effect of HU-211 on LPS-Induced Cardiovascular Alterations in the Rat

Male Sprague-Dawley rats (Harlan, Jerusalem, Israel) were anesthetized with halothane in 70:30 nitrous oxide/oxygen (4% for induction and 1% for maintenance, with use of a face mask). The right femoral artery was connected with a polyethylene (PE 50) tube to a computerized physiograph *via* a pressure transducer (XTT Vigo, Oxnard, CA). Rectal temperature was recorded and maintained at 37–38°C with a heating lamp. After 10 to 15 min of base-line recording, rats were i.v. administered saline, vehicle (cosolvent, Cremophor EL/ethanol) (1:1) or HU-211 (4 mg/kg). Within 2 to 5 min, an i.v. injection of LPS (15 mg/kg) was given, and the heart rate and blood pressure were recorded for the next 4 hr. The hematocrit was measured before drug administration, and 1, 2 and 3 hr later. The dosage of LPS in this study and the experimental protocol were based on previous reports (Terashita *et al.*, 1992; Xu *et al.*, 1992) and on our preliminary studies, which show that this dose leads to significant hypotensive response in the rat.

Macrophage Cells and Cultures

Peritoneal exudate macrophages. Peritoneal exudate macrophages were harvested from female C57BL/6 mice pretreated with thioglycollate, and cultured essentially as described previously (Avron and Gallily 1995). The cells were layered (1.2×10^5/well) in 96 flat-bottomed microwell plates (Nunc, Roskilde, Denmark), rinsed and incubated for 24 hr in DMEM supplemented with 5% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml).

RAW 264.7 cells. RAW 264.7 cells were obtained from ATCC (Rockville, MD) and maintained in RPMI-1640 supplemented with 10% FCS and antibiotics. Before cell treatment, the macrophage medium was replaced with fresh growth medium (DMEM + 5% FCS).

Various concentrations of Dexanabinol (HU-211), as well as vehicle-diluted DMSO, were added to the peritoneal macrophages and to the RAW 264.7 cells, followed by 1 μg/ml LPS. The macrophages were cultivated at 37°C in a humid atmosphere with 5% CO_2 for 6 hr. Supernatant fluids from the control and HU-211-treated macrophages were harvested and kept at −20°C until assayed for TNFα levels. The viability of the macrophages was determined by erythrosin B exclusion (Hibbs *et al.*, 1987).

Rat alveolar macrophage cell line (NR8383). A rat alveolar macrophage cell line (NR8383) was obtained from the University of Texas (San Antonio, TX). The cells were grown in tissue culture flasks containing F12 medium supplemented with antibiotics and 10% FCS. Cells (10^6/ml) were then cultivated in 24-well plates and incubated with 10 ng/ml LPS, with or without HU-211 for 18 hr to assess the accumulation of nitrites in the supernatant fluid, and for 2 hr to evaluate the accumulation of specific TNFα mRNA.

TNFα Determination by Cytotoxicity Assay

Macrophage culture supernatant fluids were assayed for TNFα levels as described previously (Brenner *et al.*, 1993), with use of BALB/c CL.7 as target cells. CL.7 cells (10^4) were plated per microwell in 100 μl DMEM containing 5% FCS. The next day, 3-fold dilutions of test Sups were added to the target cells, followed by actinomycin D (2 μg/ml, Sigma Chemical Co. St. Louis, MO). The cultures were incubated for 18 to 20 hr, stained with 2% crystal violet, rinsed and dried. Destruction of the target monolayer was evaluated by measuring the absorbance of stained cells at 550 nm with a MR700 microplate reader (Dynatech, Farmingdale, NY). The TNFα titer was expressed in S_{50} units, defined as the reciprocal of the dilution of test Sup required to destroy 50% of the target cells. Calculations were performed with a logit transformation computer program.

Nitric Oxide Determination

Nitric oxide generation was determined by measuring the accumulated nitrite in the supernatants, as described previously (Hibbs *et al.*, 1987). An equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthalene diamine HCl, 2% H_3PO_4), was added to the tested supernatant. After 10 min incubation at room temperature, color production was measured at 550 nm with the aid of an ELISA reader (MR 700, Dynatech), and calculated according to a standard curve.

Corticosterone Determination

Rats were maintained and serum corticosterone was determined as described previously (Weidenfeld *et al.*, 1989). Rats were decapitated at different times (0–10 hr) after LPS administration, their trunk blood collected and the sera kept at −40°C until assayed.

Northern Blot Analysis

Total RNA was extracted as described (Chirgwin *et al.*, 1979). NR8383 cells (untreated or treated with LPS and HU-211) were homogenized in guanidine thiocyanate buffer and then centrifuged through CsCl. RNA was denatured in glyoxal and subjected to electrophoresis on a 1.5% agarose gel in 10 mM sodium phosphate buffer. The RNA was transferred to a nylon-based membrane (GeneScreen, Dupont-New England Nuclear, Boston, MA), and hybridized with TNFα and β-actin nick-translated probes. The DNA probes used for hybridization with rat TNFα and rat β-actin mRNAs were prepared from amplimer sets (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer's instructions. The membranes were subjected to autoradiography with Kodak XAR-5 film at −70°C in the presence of an intensifying screen. Quantification of relative mRNA levels of autoradiograms was determined by densitometry.

Statistical Analysis

Percent survival of the LPS-infected mice was recorded at 24 or 48 hr. The effect of HU-211 was evaluated by the Fisher's exact probability test. Serum TNF levels are expressed as S_{50}, and comparisons between HU-211-treated and nontreated animals were made by the Student t test. $P < .05$ was considered significant.

Results

HU-211 protects mice in a model of endotoxic shock. The protective effect of HU-211 on mouse survival was determined after administration of LPS alone, or of LPS in combination with D-GALN. At a dose of 10 mg/kg, LPS alone caused 57% and 100% mortality at 24 and 48 hr, respectively. The administration of HU-211 (10 mg/kg) 30 min before LPS, reduced mortality to 9 and 67% at 24 and 48 hr, respectively ($P < .05$, fig. 1A). The combination of LPS (0.5 μg/kg) and D-GALN (900 μg/kg) was chosen to cause 100% mortality within less than 24 hr after the combined treatment (Lehmann *et al.*, 1987). This treatment was lethal within 24 hr in 100% of the mice, whereas coadministration of HU-211 (7.5 mg/kg) with these agents, followed by eight hourly injections of HU-211, reduced mortality at 24 hr to 10%, $P < .001$ (fig. 1B). No further mortality was observed for up to 7 days.

HU-211 abolishes hypotension after LPS administration to rats. Hypotension and intravascular dehydration caused by increased electrolytes and water shifts are among the early responses and complications in septic shock

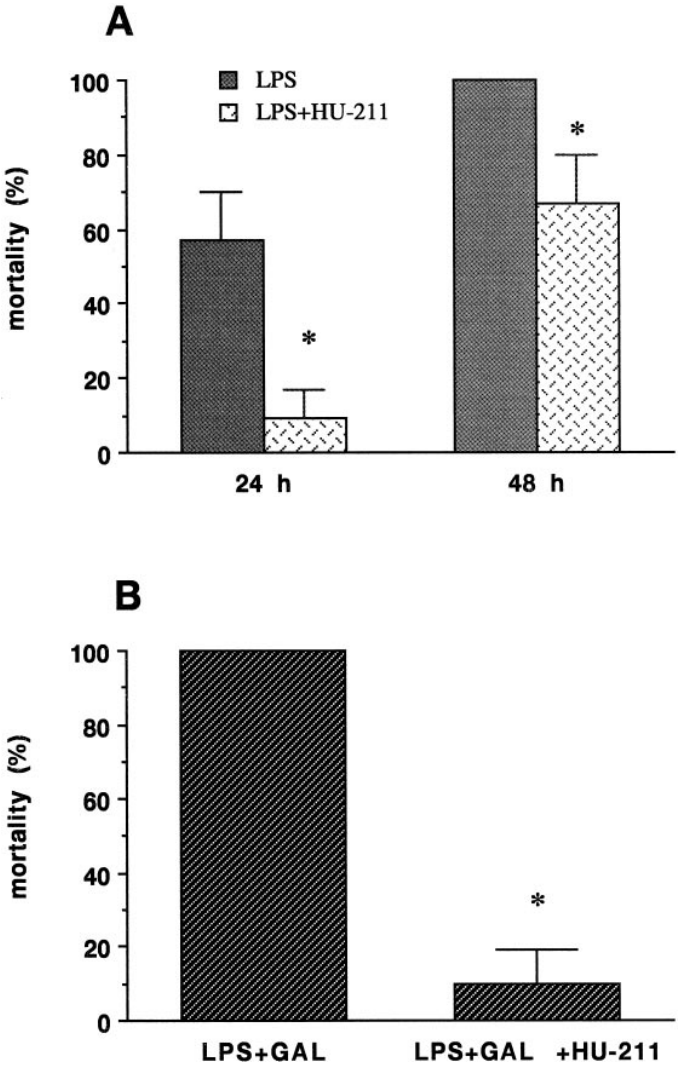

Fig. 1. Effect of HU-211 on endotoxin lethality in mice. (A) BALB/c male mice were administered i.p. with HU-211 (10 mg/kg, $n = 12$) or vehicle ($n = 14$), followed 30 min later by i.v. injection of LPS (10 mg/kg). Mortality was recorded once daily for 2 days. (B) C57BL/6 female mice ($n = 20$) were coadministered LPS (10 ng/mouse) and D-GALN (GAL) (18 μg/mouse). Ten mice served as controls, and the remainder were treated within 5 min of injection with HU-211 (7.5 mg/kg i.v. b.wt.), followed by eight hourly injections. Survival was recorded for up to 24 hr. Data are expressed as percent mortality (± S.E.M.). *$P < .01$ Fisher's exact probability test.

(Tracey, 1991). We, therefore, investigated the effect of HU-211 on LPS-induced hypotension and hematocrit changes in the rat. Base-line recording of blood pressure and hematocrit revealed no significant effect of saline, vehicle or HU-211 on either of these physiological parameters. Within 30 min of the administration of LPS (15 mg/kg) there was a 30% reduction in MABP, which persisted for 3 hr (fig. 2A). HU-211 (4 mg/kg i.v.), given 2 to 3 min before LPS, completely abolished the typical hypotensive response. Slight and transient, yet not significant, hypertension was observed in these animals during the first 10 to 15 min, but at 30 min after LPS administration, MABP reverted to its normal level, and remained constant throughout the 3-hr follow-up period. At 1 to 3 hr after LPS administration there was a 10% increase in hematocrit, but HU-211 totally abolished this response (fig. 2B).

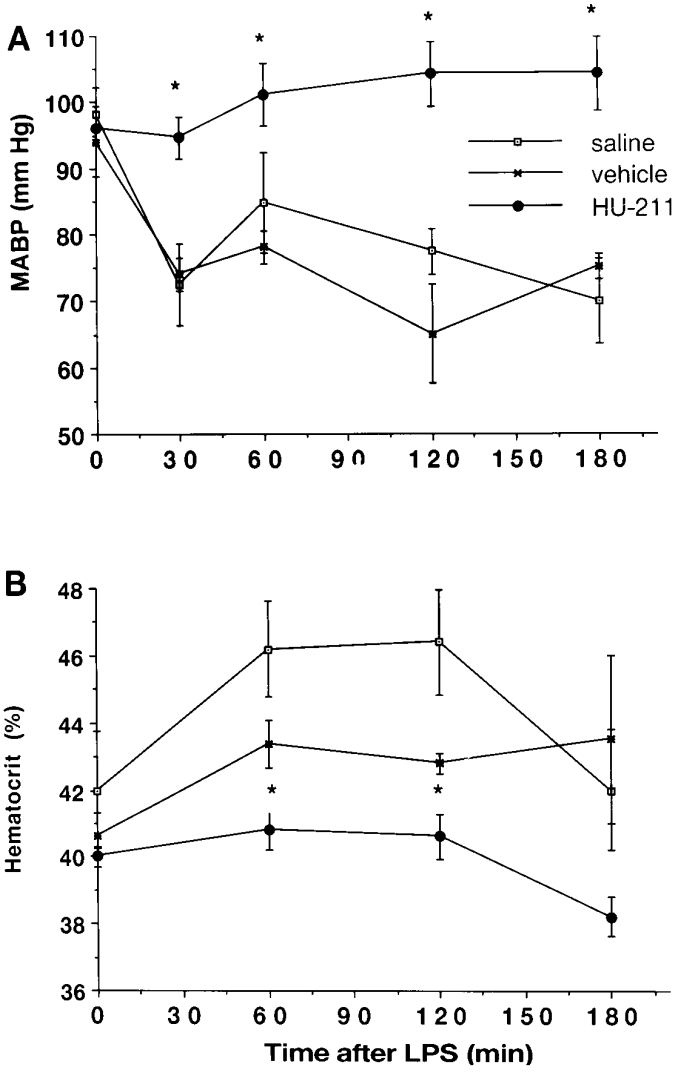

Fig. 2. Effect of HU-211 on LPS-induced cardiovascular alterations in the rat. (A) Effect on hypotension: Sprague-Dawley rats (Harlan, Jerusalem, Israel) were anesthetized with halothane, and their right femoral artery was connected (with a polyethylene PE-50 tube) to a computerized physiograph. After 10 to 15 min of base-line recording, rats were injected i.v. with saline, vehicle (cosolvent Cremophor EL/ethanol) or HU-211 (4 mg/kg). Within 2 to 5 min, an i.v. injection of LPS (15 mg/kg) was given, and heart rate and blood pressure were recorded for the next 3 hr. (B) Hematocrit was measured before drug administration, and after 1, 2 and 3 hr.

HU-211 inhibits TNFα production after LPS administration. Because TNFα is considered the primary mediator of LPS toxicity, we examined the effect of HU-211 on the bioactivity and protein concentration of TNFα in LPS-treated mice. TNFα serum activity (S_{50}) was undetectable in untreated mice, or in mice treated with HU-211 alone. The TNFα levels rose to a peak of S_{50} =1238 $\pm$ 80 within 1.5 hr post LPS injection. Coadministration of HU-211 with LPS suppressed the rise in serum TNFα level to S_{50} = 300 $\pm$ 32 (75% inhibition) (fig. 3A). In correlation with the TNFα bioactivity, the TNFα serum protein level, as assessed by ELISA, rose, peaking at 8051 $\pm$ 468 pg/ml 1.5 hr after LPS inoculation, and dropped to 3512 $\pm$ 169 pg/ml (55% inhibition) upon coadministration of HU-211 (fig. 3B).

Effect of HU-211 on TNFα production by murine macrophages *in vitro*. Because macrophages are the main producers of TNFα after activation with LPS, we examined the effect of HU-211 on the generation of TNFα by murine peritoneal macrophages *in vitro*. As can be seen in figure 4B, HU-211 markedly suppressed TNFα production. The addition of 20.8 μM HU-211 to macrophage cultures resulted in the highest suppression (92% after 6 hr), 10.4 μM, caused 59% inhibition after 6 hr. The viability of the control macrophages and after DMSO and HU-211 treatment was 90 to

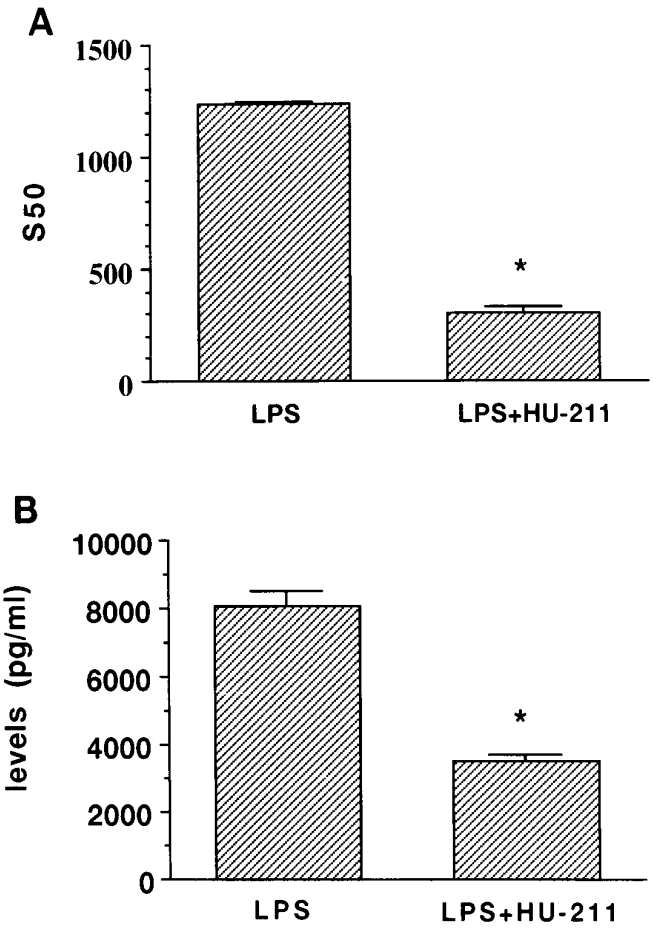

Fig. 3. Effect of HU-211 on serum TNFα activity (A) and levels (B) of LPS-treated animals. Female C57BL/6 mice were injected i.p. with 100 μg LPS, along with HU-211 (200 μg/mouse). After 90 min they were bled, and serum TNFα activity was determined by bioassay (A) and by ELISA (B). Basal levels of TNF in untreated or HU-211-treated mice were below the level of detection.

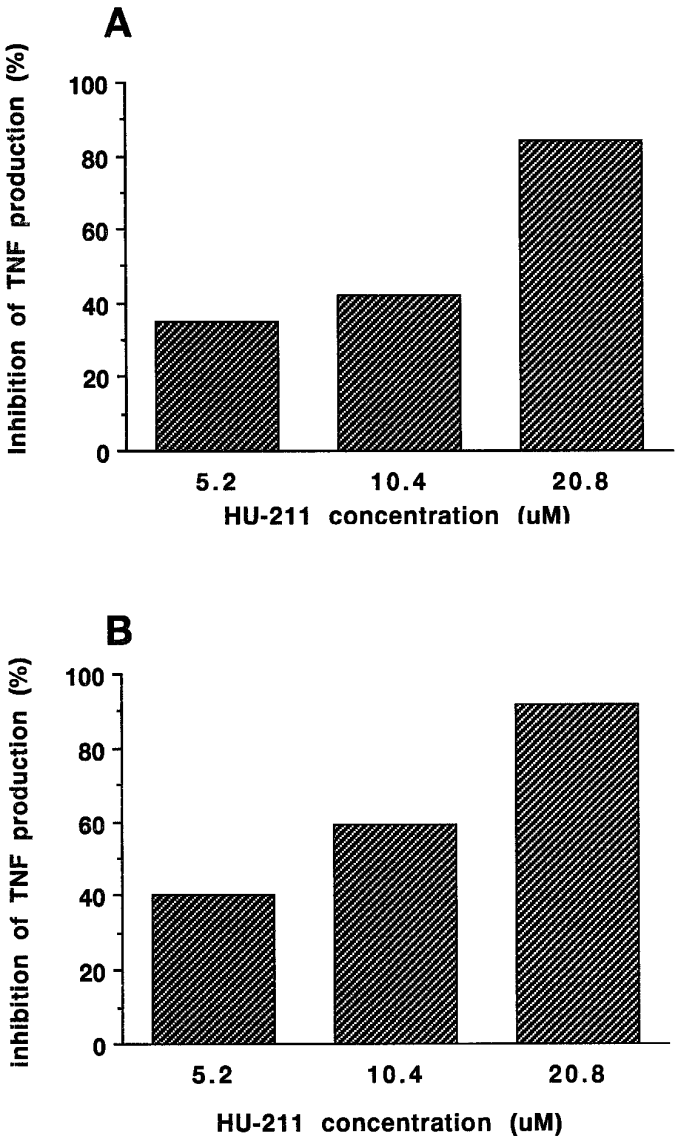

Fig. 4. Inhibition of TNFα production by RAW 264.7 (A) and TGMϕ (B). Cells were exposed to LPS (1 μg/ml) in the presence of 5.2, 10.4 or 20.8 μM HU-211 for 6 hr. Supernatant TNFα levels were determined by bioassay, and the percent inhibition was calculated. S_{50} of TNFα produced by RAW 264.7 was 4829 and that of TGMϕ was 3940, after stimulation with 1 μg/ml LPS.

99%. RAW 264.7 cells also produced TNFα upon activation with LPS. The addition of 20.8, 10.4 or 5.2 μM HU-211 suppressed TNFα production by 84, 41 and 35%, respectively (fig. 4A).

Effect of HU-211 on TNFα mRNA. Normally, rat macrophage NR 8383 cells do not express TNFα mRNA unless the cells are activated by LPS. These cells are highly sensitive to minute amounts of LPS and in our experiments there was a slight accumulation of TNFα mRNA in the untreated control cells (fig. 5, lane 1), probably because of a slight contamination of the medium with LPS. Cells incubated with LPS (10 ng/ml) showed a marked increase in TNFα gene expression after 2 hr (lane 2). In the presence of HU-211 (2.6 and 13 μM) the LPS response was completely inhibited (lanes 5 and 6, respectively). When administered alone, the drug also inhibited the basal (or the LPS contamination-induced expression of TNF; lanes 3 and 4).

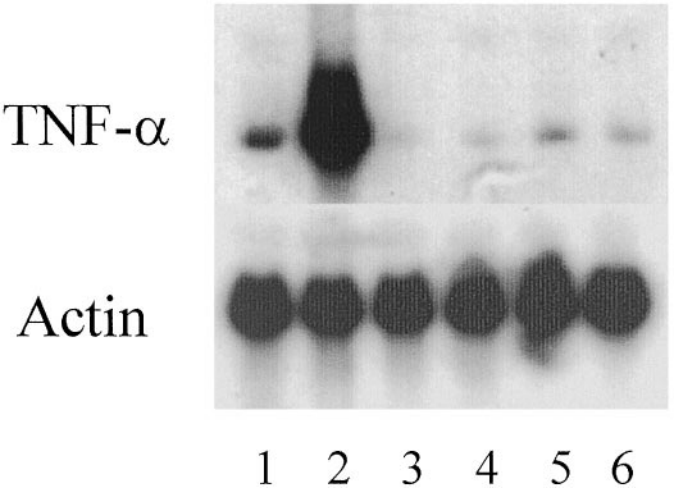

Fig. 5. Effect of HU-211 on TNFα mRNA levels extracted from NR8383 cells 2 hr after the addition of LPS to the medium. A representative of two individual experiments. Lane 1, −LPS, −HU-211; lane 2, +LPS, −HU-211; lane 3, −LPS, 2.6 μM HU-211; lane 4, −LPS, 13 μM HU-211; lane 5, +LPS, 2.6 μM HU-211; lane 6, +LPS, 13 μM HU-211.

Effect of HU-211 on NO generation by rat alveolar macrophage cell line, NR 8383. To further explore the suppressive influence of HU-211 on macrophage functions involved in endotoxic shock syndrome, we examined the drug's effect on NO production (as nitrite accumulation) by LPS-activated NR8383 cells. As seen in table 1, HU-211 at concentrations of 5 and 1 μg/ml suppressed LPS-induced nitrite accumulation by 86 and 74%, respectively, and under basal conditions, by 57 and 29%, respectively.

Effect of HU-211 on serum corticosterone level after LPS injection. To test whether the inhibitory effect of HU-211 on TNF production is mediated by glucocorticoids, its influence on LPS-induced production of corticosterone was studied. As seen in table 2, when injected into LPS-treated rats, HU-211 (5 mg/kg) had no effect on serum corticosterone levels.

Discussion

After establishing the beneficial effects of HU-211 in brain trauma (Shohami *et al.*, 1993, 1995), ischemia (Bar-Joseph *et al.,* 1994; Belayev *et al.*, 1995a, b) and pneumococcal meningitis (Bass *et al.*, 1996), in which TNFα is considered to be one

TABLE 1

NO production by NR8383 macrophage cells treated with LPS and HU-211

A rat alveolar macrophage cell line (NR8383, 10^6/ml) was cultivated in 24-well plates and incubated with 10 ng/ml LPS (055:B5), with or without HU-211, for 18 hr to evaluate the accumulation of nitrites in the supernatant. NO generation was determined by measuring the accumulated nitrite in the supernatants, according to the Griess reaction as described. The results are mean ± S.D. of a representative experiment of four individual experiments, each carried out in triplicates. Analysis of variance performed on the three groups treated with LPS yielded $P < .0001$. * $P < .001$ *vs.* LPS alone, by Bonferroni test.

Treatment	Nitrite (ng/10^6 cells/ml ± S.D.)
None	14 ± 4
HU-211, 2.6 μM	10 ± 4
HU-211, 13 μM	6 ± 2
LPS, 10 μg/ml	483 ± 35
LPS, 10 μg/ml + HU-211, 2.6 μM	125 ± 23*
LPS, 10 μg/ml + HU-211, 13 μM	67 ± 12*

TABLE 2

The effect of HU-211 on rat serum corticosterone level after LPS inoculation

Rats were injected with LPS (250 μg/kg) or saline together with HU-211 (5 mg/kg) or its vehicle, and decapitated 2 to 10 hr later. Trunk blood was collected, serum was separated and CS levels were assayed (n = 6 rats/group).

Time Postinjection (hr)	Serum Corticosterone (μg/dl)			
	Vehicle	Vehicle + HU-211 (5 mg/kg)	LPS	LPS + HU-211 (5 mg/kg)
0	2.1 ± 0.4	2.5 ± 0.3	3.1 ± 0.8	3.6 ± 0.8
1	3.6 ± 0.6	4.1 ± 0.7	10 ± 1.2	12 ± 1.5
2	2.5 ± 0.4	5.1 ± 0.8	35 ± 2.6	38 ± 2.1
4	3.2 ± 0.6	4.8 ± 0.4	45 ± 2.5	47 ± 3.1
10	9.6 ± 0.8	10.8 ± 2.4	13 ± 1.2	12 ± 1.3

of the deleterious mediators, we demonstrated in the present study the protective effects of HU-211 in mouse and rat models of septic shock, in which TNFα is the key destructive mediator. We also showed the inhibition of LPS-stimulated production of TNFα and NO in macrophages by HU-211.

The mechanism(s) by which HU-211 exerts its anti-TNF effect on macrophages is still not elucidated. Burnette-Curley and Cabral (1995) who studied the effect of HU-211 and its stereoisomer, HU-210, on macrophage cell contact-dependent tumoricidal activity suggested that the anti-TNF effect of cannabinoids (and their analogs) is not mediated only *via* the cannabinoid receptor. The high lipid solubility of HU-211, and that of other cannabinoids, may promote their partitioning into cell membranes and thus disrupt membrane proteins function and modify cellular function by interaction with intracellular elements. The involvement of the NMDA receptor in inhibiting cytolysis of TNF-sensitive tumor cells by cannabinoids may also be excluded, because HU-211 is ~3 orders of magnitude more potent at inhibiting this receptor than its enantiomer, HU-210 (Feigenbaum *et al.*, 1989; Nadler *et al.*, 1993a). Cannabinoid treatment does not always lead to inhibition of cytokine levels. Klein *et al.* (1995) reports that δ-9-tetrahydrocannabinol increases, rather than decreases IL-1 and TNFα production by mouse peritoneal macrophages. The mechanism by which these agents affect TNFα production, and particularly the role of other receptors, needs further investigation.

Activation of macrophages by LPS results in rapid induction of TNFα mRNA and in the release of TNFα by cleavage of the 26-kDa presecretory form to the 17-kDa secretory form. Mechanisms of inhibition of TNFα release may therefore involve either suppression of mRNA expression [*e.g.,* the methylxanthine derivative, pentoxyfilline (Doherty *et al.*, 1991)], acceleration of mRNA degradation [*e.g.,* thalidomide (Moreira *et al.*, 1993)] or suppression of the processing of the precursor protein to the 17-kDa secretory form. Our present findings demonstrate that, in LPS-stimulated macrophages, HU-211 inhibits TNFα gene expression by a mechanism which still needs to be determined. We have recently demonstrated, in a CHI model in the rat, that HU-211 inhibits the production of TNFα and that the inhibition appears to be post-transcriptional (Shohami *et al.*, 1997). This apparent discrepancy may be caused by the different stimuli used in the two studies, LPS and mechanical trauma, and is now under investigation.

NO is known to be generated by the inducible enzyme nitric oxide synthase in macrophages, endothelial cells and smooth muscle cells, although its exact role in septic shock is not clear. Its production could contribute to the hypotension typically seen in endotoxic shock. Indeed blocking the activity of NO synthase in animals proved beneficial in some studies (Wolfe and Dasta, 1995; Teale and Atkinson, 1992). The results of our investigation show that HU-211 suppresses the generation of both TNFα and NO and that the inhibition correlates with the compound's ability to protect rodents from endotoxic shock.

Cytokine synthesis is down-regulated by glucocorticoids. Pretreatment with glucocorticoids attenuates the effects of LPS, whereas adrenalectomy or administration of glucocorticoid receptor antagonists (*e.g.,* RU-38486) enhances the sensitivity to LPS-induced septic shock (Dantzer *et al.*, 1996). Therefore, we tested whether the protective effect of HU-211 is mediated by changes in adrenocortical function. As expected, LPS induced a 10- to 15-fold increase in serum corticosterone levels within 2 to 4 hr; however, HU-211 did not affect this response (table 2). This suggests that the protective influence exerted by HU-211 in endotoxic shock does not involve changes in corticosterone secretion. This finding also rules out the possibility that HU-211 interacts with LPS and neutralizes it at the peritoneal cavity. After LPS and HU-211 administration, corticosterone is elevated, whereas TNFα is inhibited, which supports our hypothesis of a specific inhibition of the LPS-induced TNFα production by HU-211. Adrenalectomized rats treated with LPS could not be rescued by HU-211 (unpublished data). It is conceivable that glucocorticoids play a permissive role in the protective mechanism of HU-211 during endotoxic shock, namely, they attenuate the production of cytokines and/or NO in response to LPS (Dantzer *et al.*, 1996).

To date, no specific pharmacological agents are used in the management of septic shock. Although several compounds have been suggested to inhibit TNFα activity in various animal models (Glauser *et al.*, 1994), they have not been introduced into clinical practice. The results of the present *in vivo* studies, in two experimental models using two species, suggest that HU-211 may have important clinical implications. It is noteworthy that dexanabinol, HU-211, was tested in human volunteers in phase I clinical trial, and is now under phase II clinical trial for severe head injury. Thus, this novel drug appears to be a promising candidate for the treatment in the nontreatable and devastating TNFα-mediated diseases.

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