

Effects of Cannabinoid Receptor Agonist and Antagonist Ligands on Production of Inflammatory Cytokines and Anti-Inflammatory Interleukin-10 in Endotoxemic Mice

SIDNEY R. SMITH, CAROL TERMINELLI, and GEORGETTA DENHARDT

Department of Immunology Schering-Plough Research Institute, Kenilworth, New Jersey

Accepted for publication December 10, 1999 This paper is available online at <http://www.jpet.org>

ABSTRACT

Previous studies have shown that mice primed with *Corynebacterium parvum* produce higher levels of inflammatory cytokines than unprimed mice upon challenge with lipopolysaccharide (LPS). Herein, we describe experiments in which two cannabinoid (CB) agonists, WIN 55212-2 [(*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-*de*]1,4-benzoxazin-6-yl](1-naphthyl)methanone} and HU-210 [(–)-11-hydroxy- Δ^8 tetrahydrocannabinol-dimethylheptyl], were examined for their effects on LPS-induced cytokines in *C. parvum*-primed and unprimed mice. These agonists have been reported to bind selectively to the CB2 and CB1 receptor subtypes, respectively. WIN 55212-2 (3.1–50 mg/kg i.p.) and HU-210 (0.05–0.4 mg/kg i.p.) decreased serum tumor necrosis factor- α and interleukin-12 (IL-12) and increased IL-10 when administered to mice before LPS. The drugs also protected *C. parvum* mice (but not unprimed mice) against the lethal effects of LPS. The protection afforded to *C. parvum* mice could not be attributed to the higher levels of IL-10 present in

these mice after agonist treatment. The WIN 55212-2- and HU-210-mediated changes in the responsiveness of mice to LPS were antagonized by SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride], a selective CB1 receptor antagonist, but not by SR144528 [*N*-[(1*S*)-*endo*-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide], a selective antagonist at the CB2 receptor. Therefore, both CB agonists modulated LPS responses through the CB1 receptor. Surprisingly, SR141716A itself modulated cytokine responses in a manner identical with that of WIN 55212-2 and HU-210 when administered alone to mice. The agonist-like effects of SR141716A, which were more striking in unprimed than in primed mice, suggested that the antagonist also could function as a partial agonist at the CB1 receptor. Our findings indicate a role for the CB1 receptor subtype in cytokine modulation by CB ligands.

Psychoactive cannabinoids (CBs) have a variety of pharmacological effects that are evoked through interactions with central or peripheral CB receptors. Δ^9 -Tetrahydrocannabinol (Δ^9 -THC), the principal psychoactive component of marijuana, has been the focal point of the majority of investigations aimed at elucidating the biological properties of CB agonists (Dewey, 1986). The central effects that occur after in vivo exposure to Δ^9 -THC include euphoria, hypokinesia, hypothermia, antinociception, and catalepsy. Such effects are thought to be mediated via a CB receptor subtype that has been designated as CB1 and is regionally distributed in brain tissue (Herkenham et al., 1990). CB1 receptors are present at high densities in the forebrain and cerebellum consistent

with the observed effects of CBs on cognition and movement. Several CB agonists that mimic the central effects of Δ^9 -THC (cannabimimetic compounds) have been developed and include CP 55940, a bicyclic analog of Δ^9 -THC (Johnson and Melvin, 1986) and WIN 55212-2 [(*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-*de*]1,4-benzoxazin-6-yl](1-naphthyl)methanone], an aminoalkylindole (Martin et al. 1991; Pacheco et al., 1991).

One of the major advances in CB research has been the development of a potent and selective antagonist of the CB1 receptor, SR 141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride]. This compound was found to block the hypokinetic, hypothermic, cataleptic, and antinociceptive effects of Δ^9 -THC and WIN 55212-2 in mice and rats (Rinaldi-

Received for publication October 5, 1999.

ABBREVIATIONS: CB, cannabinoid; THC, tetrahydrocannabinol; CP 55940, [(–)-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]4-[3-hydroxypropyl]cyclohexan-1-ol]; WIN 55212-2, [(*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-*de*]1,4-benzoxazin-6-yl](1-naphthyl)methanone]; SR141716A, [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride]; CNS, central nervous system; GPCR, G protein-coupled receptor; SR144528, [*N*-[(1*S*)-*endo*-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide]; LPS, lipopolysaccharide; HU-210, [(–)-11-hydroxy- Δ^8 tetrahydrocannabinol-dimethylheptyl]; TNF- α , tumor necrosis factor- α ; IL, interleukin; HU-211, [(+)-(3*S*,4*S*)-7-hydroxy Δ^6 -tetrahydrocannabinol-1,1-dimethylheptyl].

Carmona et al., 1994, 1995; Compton et al., 1996; Reche et al., 1996). In addition, studies with SR141716A have provided evidence for the presence of CB1 receptors in peripheral tissues as well as in the central nervous system (CNS). The existence of functional CB1 receptors in peripheral tissues has been most convincingly demonstrated in a rat hemorrhagic shock model in which anandamide, an endogenous CB agonist, was found to elicit a hypotensive response when administered exogenously to urethane-anesthetized rats (Wagner et al., 1997). The hypotension caused by endogenous and exogenous anandamide could be blocked by SR141716A, indicating the involvement of the CB1 receptor subtype in the response (Varga et al., 1995; Lake et al., 1997). Macrophages from hypotensive rats were shown to be cellular sources of endogenous anandamide, and capable of transferring the hypotensive response into normotensive recipients. The successful transfer of the hypotensive response appeared to require the presence of activated CB1 receptors in the peripheral tissues of the normotensive recipients because the cells did not transfer hypotension into recipients that had been treated with SR141716A (Wagner et al., 1997).

It is the CB2 receptor subtype, however, that has been defined as the peripheral CB receptor, primarily because CB2 mRNA expression has been detected mainly in cells of the immune system (Derocq et al., 1995; Galiegue et al., 1995; Carayon et al., 1998). Like the CB1 receptor subtype, the CB2 receptor is a member of the G protein-coupled receptor (GPCR) family and on stimulation causes inhibition of adenyl cyclase. A potent, selective, and orally active antagonist of the CB2 receptor, SR144528 [*N*-(1*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide was recently identified and shown to have a 700-fold higher affinity for the CB2 receptor than for the CB1 receptor (Rinaldi-Carmona et al., 1998).

In the present study, we have examined the *in vivo* effects of CB receptor agonists on cytokine production after the induction of endotoxemia in *C. parvum*-primed (Smith et al., 1993) and unprimed mice. We also have used the highly selective CB receptor antagonists SR141716A and SR144528 in an attempt to identify the receptor subtype(s) through which CBs mediate their modulatory effects on lipopolysaccharide (LPS)-induced cytokines.

Experimental Procedures

Mice and Materials. Specific pathogen free BDF1 male mice, 8 to 12 weeks old (Jackson Laboratories, Bar Harbor, ME), were used in this study. *Corynebacterium parvum* (*Propionibacterium acnes*) cells were obtained from the American Type Culture Collection (Rockville, MD). LPS (*Escherichia coli* serotype 055:B5) was purchased from Difco (Detroit, MI). WIN 55212-2 was purchased from Research Biochemicals International (Natick, MA) and HU-210 [(−)-11-hydroxy-Δ⁸ tetrahydrocannabinol-dimethylheptyl] from Biomol (Plymouth Meeting, PA). SR141716A and SR144528 were synthesized according to methods published by Sanofi Recherche (France).

Priming with *C. parvum* and Induction of Lethal Endotoxemia in Primed and Unprimed (Normal) Mice. For priming with *C. parvum*, BDF1 mice were injected *i.v.* with 0.5 mg of heat-sacrificed cells of American Type Culture Collection strain 11827 as previously described (Smith et al., 1996). Endotoxemia was induced by challenging the mice with LPS (20 μg) by the *i.v.* route 1 week

after priming. LPS (1600 μg/mouse) was administered to unprimed BDF1 by the *i.p.* route.

Drug Treatment. WIN 55212-2 and SR141716A were prepared in a methylcellulose vehicle and HU-210 in dimethyl sulfoxide/Tween for administration by the *i.p.* route. SR144528 was administered *p.o.* in dimethyl sulfoxide/Tween 20. SR141716A and SR144528 were dissolved in 1:1:18 emulphor/ethanol/saline for *i.c.v.* administration into conscious mice (Haley and McCormick, 1957).

Cytokines in Sera. Enzyme-linked immunosorbent assay kits from Genzyme (Cambridge, MA) with a sensitivity of 15 pg/ml were used to quantify the levels of tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), and IL-12 in sera from endotoxemic mice. In some experiments, TNF-α and IL-10 levels were measured with enzyme-linked immunosorbent assays in which monoclonal anti-TNF-α (hamster clone TN3-19.12) and anti-IL-10 (rat clone JES5-2A5) antibodies from Pharmingen (San Diego, CA) were paired with biotinylated goat polyclonal antibodies from R&D Systems (Minneapolis, MN).

Data Analysis. Data were analyzed statistically by the unpaired *t* test of the Graph PAD prism statistical program. *P* values < .05 were considered as statistically significant.

Results

Effects of WIN 55212-2 and HU-210 on LPS-Induced Serum Cytokines in *C. parvum*-Primed and Unprimed Mice. In the present study, WIN 55212-2 and HU-210 were used as prototype CB agonists to determine any effects of such compounds on cytokine production in endotoxemic mice. In competitive-binding experiments with Chinese hamster ovary cells stably expressing human CB1 or CB2 receptors, it has been shown that WIN 55212-2 binds with higher affinity to CB2 receptors than to CB1 receptors, whereas HU-210 is more selective for the CB1 receptor (Felder et al., 1995). To determine the effects of WIN 55212-2 on LPS-induced serum cytokine responses, the drug was administered *i.p.* to *C. parvum* mice 1 h before an LPS challenge. The levels of inflammatory cytokines were measured at the time of their maximum postchallenge levels; 1.5 h for TNF-α and 3 h for IL-12. In addition to inflammatory cytokines, substantial levels of anti-inflammatory IL-10 appear in the serum subsequent to LPS and levels of this cytokine were measured at 1.5 h, 3 h, or at both timepoints.

When administered to *C. parvum* mice 1 h before LPS, WIN 55212-2 (3.125–50 mg/kg) caused a dose-related decrease in the TNF-α (Fig. 1A) and IL-12 (Fig. 1B) responses. The ED₅₀ values (with 95% CL) were 7.5 (2.2–25.7) and 2.4 (0.14–39.8) mg/kg for the suppression of TNF-α and IL-12, respectively. The interferon-γ, IL-1, and IL-6 responses also were reduced by this treatment (data not shown). In contrast to the reduction in circulating inflammatory cytokines, IL-10 levels in WIN 55212-2-treated mice were elevated compared with those of vehicle-treated control mice (Fig. 1C). There was no change in the magnitude of the IL-10 response when assessed at 1.5 and 3 h in mice treated with 3.1 and 12.5 mg/kg WIN 55212-2. However, the circulating levels of IL-10 increased substantially during this interval in mice given a 50-mg/kg dose of the agonist. WIN 55212-2 had similar effects on LPS-induced cytokines in unprimed mice (Fig. 2). The drug caused a significant reduction in TNF-α (Fig. 2A) and IL-12 (Fig. 2B) and a significant elevation in IL-10 levels (Fig. 2C) when administered to unprimed mice at doses of 12.5 and 50 mg/kg. Circulating cytokines could not be de-

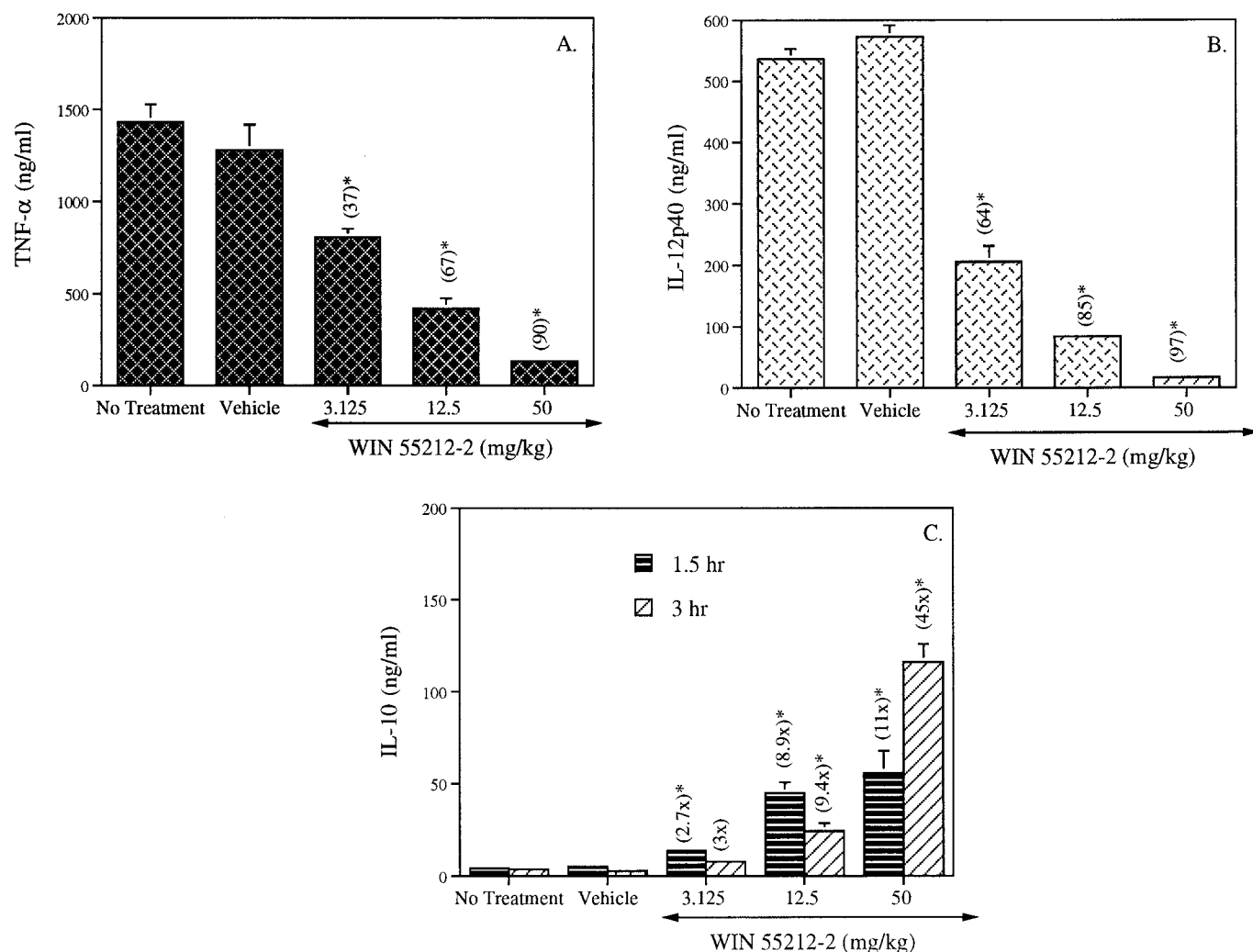


Fig. 1. The modulatory effects of WIN 55212-2 on LPS-induced cytokines in *C. parvum*-primed mice. WIN 55212-2 was administered i.p. to the mice 1 h before challenge with LPS. The circulating levels of TNF- α (A) and IL-12 (B) were measured 1.5 and 3 h after the LPS challenge, respectively. IL-10 levels (C) were measured at both timepoints. The values shown are the mean $\pm$ S.E. of six mice per group. The numbers in parentheses indicate the percentage of inhibition of TNF- α and IL-12 and the fold increase in IL-10 compared with the responses of vehicle (methylcellulose)-treated control mice. The results shown for this experiment are typical of those obtained in four separate experiments. Significantly different from the response of mice treated with vehicle, * $P < .01$.

tected in *C. parvum* or unprimed mice in the absence of LPS whether they were treated with WIN 55212-2 or not. The central effects (e.g., hypokinesia and catalepsy) that are typically seen with psychoactive CBs were observed at all dose levels in both primed and unprimed mice shortly after treatment with WIN 55212-2 and were still evident at the time of the LPS challenge.

HU-210 is a potent CB agonist that is structurally related to (-)- Δ^9 THC and exhibits selectivity for the CB1 receptor subtype over CB2 (Felder et al., 1995). When given to *C. parvum* mice 1 h before LPS, HU-210 (0.1 mg/kg i.p.) produced changes in cytokine responses that were qualitatively similar to those caused by WIN 55212-2. As shown in Fig. 3, A and B, HU-210 caused a 55 and 87% suppression of the TNF- α (Fig. 3A) and IL-12 (Fig. 3B) responses, respectively, and a 4- to 6-fold elevation in IL-10 (Fig. 3C). The CNS changes (hypokinesia and catalepsy) that occurred at cytokine-modulating doses of HU-210 also were similar to those seen in WIN 55212-2-treated mice. However, these changes were somewhat delayed in onset

and of longer duration compared with those caused by WIN 55212-2.

Antagonism of WIN 55212-2- and HU-210-Mediated Changes in Cytokine Responses by SR141716A.

SR141716A is a highly selective, potent, and orally active CB antagonist that has a high affinity for the CB1 receptor ($K_i = 2$ nM) and a low affinity for the CB2 receptor ($K_i > 1000$ nM) (Rinaldi-Carmona et al., 1995). The drug has been shown to antagonize the classical pharmacological responses (hypothermia, antinociception, ring-immobility) elicited by CB receptor agonists, consistent with its affinity for the brain (CB1) receptor. To determine its ability to antagonize the modulatory effects of WIN 55212-2 and HU-210 on LPS-induced cytokine responses, SR141716A was administered to mice 1 h before treatment with WIN 55212-2 or HU-210. The mice were challenged with LPS 1 h after the administration of the agonists (2 h after exposure to SR141716A). Serum cytokine levels were determined as described above.

When administered to *C. parvum* mice 1 h before treat-

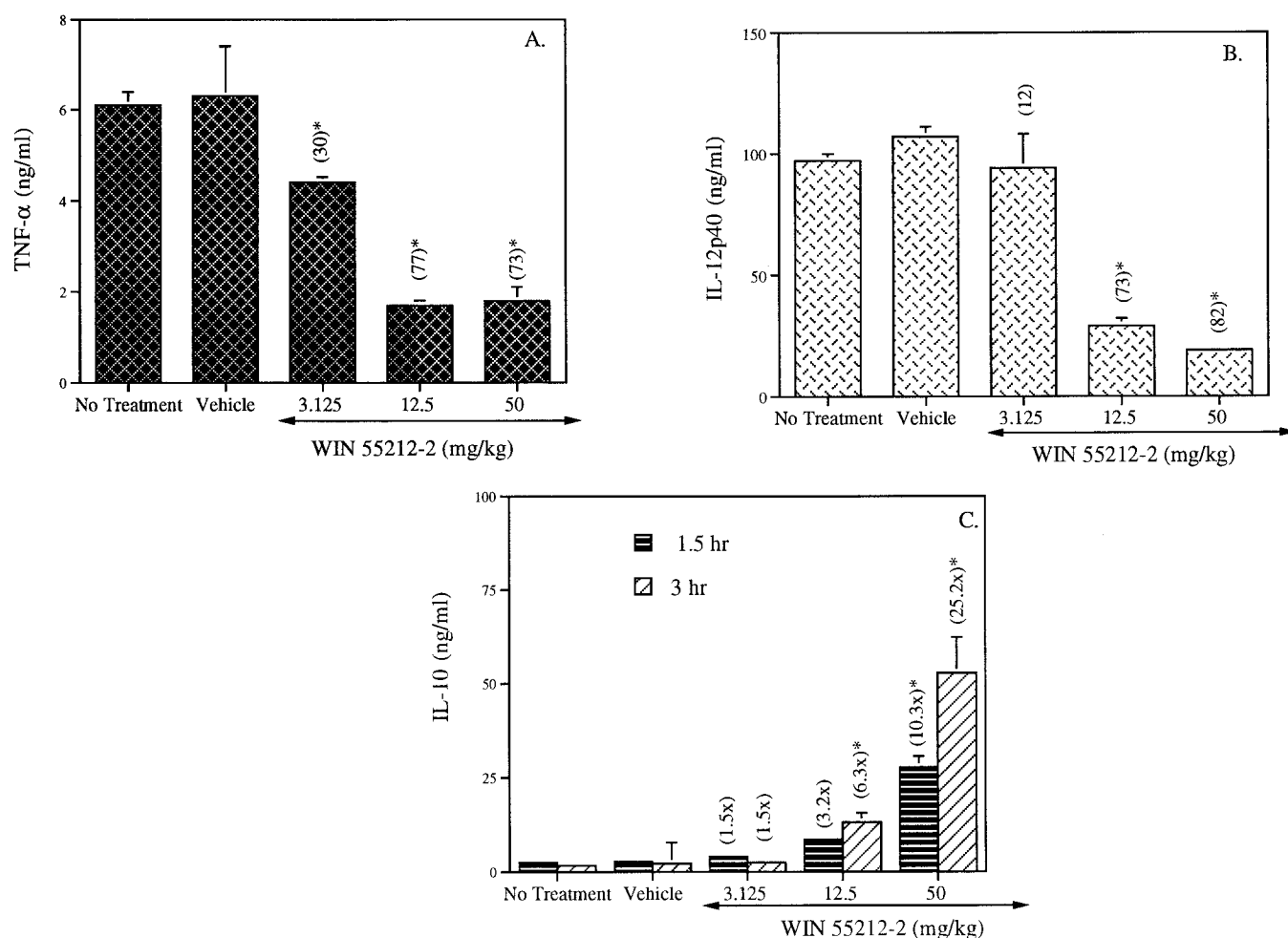


Fig. 2. The reduction in circulating TNF- α (A) and IL-12 (B) and elevation in circulating IL-10 (C) in unprimed (normal) BDF1 mice treated with WIN 55212-2, 1 h before challenge with LPS. The values shown are the mean $\pm$ S.E. of six mice per group. Significantly different from mice treated with vehicle, * $P < .01$.

ment with WIN 55212-2 (3.1, 12.5, or 50 mg/kg i.p.), SR141716A (25 and 100 mg/kg i.p.) was found to block completely the central effects evoked by the agonist (data not shown). The SR141716A-treated animals did not exhibit hypokinesia or catalepsy up to 4 h after treatment with WIN 55212-2. When the mice were given a lower dose (6.3 mg/kg) of SR141716A before the WIN 55212-2 treatment, the antagonist was only effective in preventing the CNS effects caused by the 3.1-mg/kg dose of the agonist. CNS changes were clearly evident although relatively mild in mice given a 6.3-mg/kg dose of the antagonist followed by 12.5- and 50-mg/kg doses of the agonist.

In parallel with its antagonism of the WIN 55212-2-mediated CNS effects, SR141716A was found to prevent the agonist-induced changes in LPS-induced cytokine responses. Surprisingly however, when administered alone to *C. parvum* mice before LPS, the antagonist itself caused slight but statistically significant changes in the cytokine responses. Thus, in the experiment shown in Fig. 4, the antagonist inhibited the TNF- α response by 36 and 38% (Fig. 4A) and caused a 1.7- and 5.6-fold increase in the IL-10 response (Fig. 4B) at doses of 25 and 100 mg/kg, respectively. Despite these agonist-like effects on cytokine responses, it was still possible

to detect the antagonistic activity of SR141716A on cytokine modulation by WIN 55212-2. As shown in Fig. 5, there was a 37% reduction in the TNF- α response when SR141716A (100 mg/kg i.p.) was given alone to *C. parvum* mice 2 h before LPS (Fig. 5A). This response was decreased by 56, 63, and 94%, respectively, when WIN 55212-2 was administered alone to mice at doses of 3.125, 12.5, and 50 mg/kg (Fig. 5B). The magnitude of the inhibited TNF- α response in mice treated with both antagonist and agonist (Fig. 5C) never exceeded that obtained in mice treated with the antagonist alone (Fig. 5A). Thus, the CB1 antagonist was able to block completely the suppressive effects of WIN 55212-2 on LPS-induced TNF- α . Similarly, the antagonism by SR141716A on the WIN 55212-2-mediated increase in circulating IL-10 also was evident, despite the ability of the antagonist itself to cause increases in the levels of this cytokine. As shown in Fig. 5D, SR141716A caused a 4.6-fold elevation in the IL-10 response when administered alone to mice. Treatment with WIN 55212-2 alone at doses of 3.1, 12.5, and 50 mg/kg resulted in 1.4-, 16.5-, and 39.7-fold increases in IL-10, respectively (Fig. 5E). The increases were 3.9-, 6.3-, and 7.2-fold in mice given both the antagonist and agonist (Fig. 5F), indicating strong antagonism by the CB1 selective antagonist of the WIN

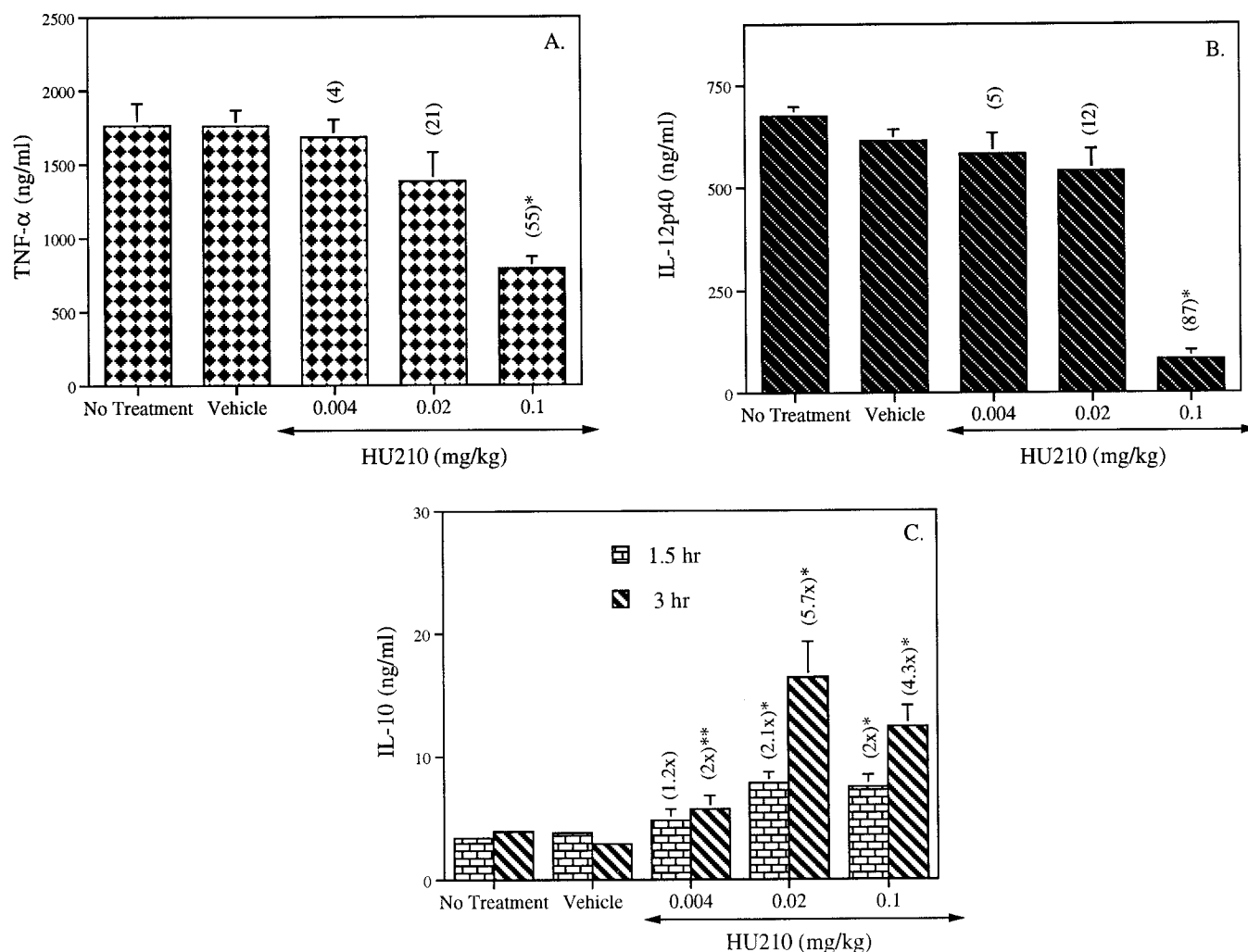


Fig. 3. The modulatory effects of HU-210 on LPS-induced serum TNF- α (A), IL-12 (B), and IL-10 (C) in *C. parvum*-primed mice. HU-210 was administered i.p. to the mice 1 h before challenge with LPS. Data were pooled from two identical experiments and the values shown are the mean $\pm$ S.E. of 12 mice per group. Significantly different from mice treated with vehicle, ** $P < .05$ and * $P < .01$.

55212-2-mediated enhancement in IL-10. SR 141716A also was found to exhibit a similar antagonistic activity toward cytokine modulation by HU-210 (data not shown). The ability of SR141716A to antagonize cytokine modulation caused by WIN 55212-2 and HU-210 strongly suggests that such effects of the CB agonists occur through interactions with the CB1 receptor.

Further evidence for the involvement of the CB1 receptor subtype in the pharmacological actions of WIN 55212-2 on LPS-induced cytokine responses is shown in Fig. 6. In this experiment, SR141716A was administered to mice at a dose of 6.3 mg/kg 1 h before treatment with WIN 55212-2. As described above, this dose of the antagonist was only partially effective in blocking the central effects of WIN 55212-2 when the agonist was administered to mice at a dose of 12.5 mg/kg. Partial antagonism of cytokine modulation also was observed when the 6.3-mg/kg dose of SR141716A was given to mice before treatment with WIN 55212-2. In the experiment shown in Fig. 6B, WIN 55212-2 alone reduced the TNF- α levels by 39, 75, and 87% when administered to the mice at doses of 3.1, 12.5, and 50 mg/kg, respectively. These

same doses of WIN 55212-2 decreased TNF- α by 21, 43, and 67%, respectively, in mice that were given SR141716A before the agonist (Fig. 6C). The decrease in TNF- α inhibition from 39% in mice treated with the agonist alone to 21% after treatment with both antagonist and agonist represented complete (100%) antagonism because this level of inhibition (22%) also was observed in mice that only received the antagonist (Fig. 6A). Therefore, at an agonist/antagonist dose ratio of 1:2, there was no inhibition of TNF- α by WIN 55212-2. As the dose of the agonist was increased relative to that of the antagonist (agonist/antagonist ratios of 2:1 and 8:1), the antagonism by SR141716A became diminished, but its activity was never reversed completely by the agonist treatment. The antagonism by SR141716A of the WIN 55212-2-mediated elevation in IL-10 was similarly affected by increasing doses of the agonist. As shown in Fig. 6E, WIN 55212-2 caused a 7.6- and 26-fold enhancement of the LPS-induced IL-10 response when administered alone to mice at doses of 12.5 and 50 mg/kg, respectively. The antagonist blocked completely the enhancement caused by the 12.5-mg/kg dose of agonist, but only exhibited a partial antago-

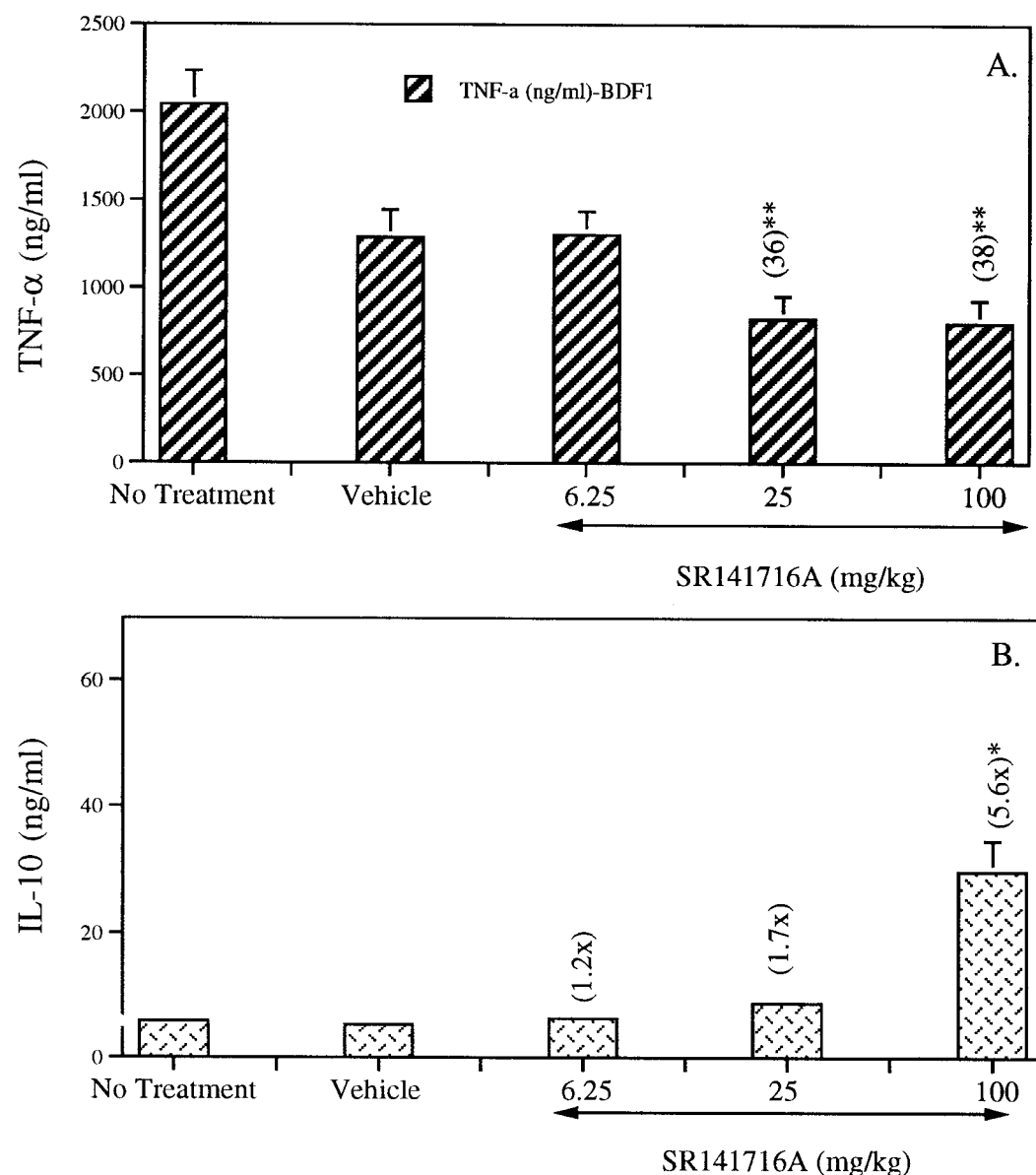


Fig. 4. The modulatory effects of the CB1 selective antagonist SR141716A on LPS-induced serum TNF- α (A) and IL-10 (B) in *C. parvum*-primed mice. Serum TNF- α and IL-10 were measured 1.5 and 3 h after the LPS challenge, respectively. SR141716A was administered to mice by the i.p. route 2 h before challenge with LPS. The values shown represent the mean $\pm$ S.E. of six mice per group. Significantly different from mice treated with vehicle, ** $P < .05$ and * $P < .01$.

nism of the increase caused by the 50-mg/kg dose. (Fig. 6F). Thus, the antagonistic activity of this lower dose of SR141716A on the WIN 55212-2 inhibited TNF- α and elevated IL-10 responses could be diminished by increasing the dose of agonist relative to that of the antagonist. These results give further support to the hypothesis that cytokine modulation by CB agonists occurs through interactions with the CB1 receptor.

Direct Effects of SR141716A on Cytokine Responses in Unprimed (Normal) Mice. The LPS-induced TNF- α response in unprimed mice is considerably weaker than that in *C. parvum* mice and SR141716A itself was found to have a strong modulating effect on this response in unprimed mice. As shown in Fig. 7A, SR141716A inhibited the TNF- α response by 50% or more when administered alone to unprimed mice at doses of 6.25 to 100 mg/kg. The antagonist also caused a significant elevation in the IL-10 response at a dose of 25 mg/kg (Fig. 7B). Thus, in unprimed mice, the CB1

antagonist had relatively strong agonist-like effects on LPS-induced cytokines, resembling WIN 55212-2 and HU-210 in its actions on these responses. The cytokine-modulating effects of SR141716A in unprimed mice provided more definitive evidence for the ability of the CB1 receptor antagonist to exhibit partial agonism at the CB1 receptor. In fact, this relatively strong partial agonism exhibited by SR141716A precluded an investigation of its ability to antagonize cytokine modulation by WIN 55212-2 and HU-210 in unprimed mice.

Failure of CB2 Selective Antagonist SR144528 to Block Cytokine Modulation by WIN 55212-2. As indicated above, published data have suggested that WIN 55212-2 is 19-fold more selective for the CB2 receptor than for the CB1 receptor. Therefore, SR144528 a potent, selective, and orally active antagonist for the CB2 receptor was used to determine any contribution of the CB2 receptor subtype to the modulatory effects that had been observed

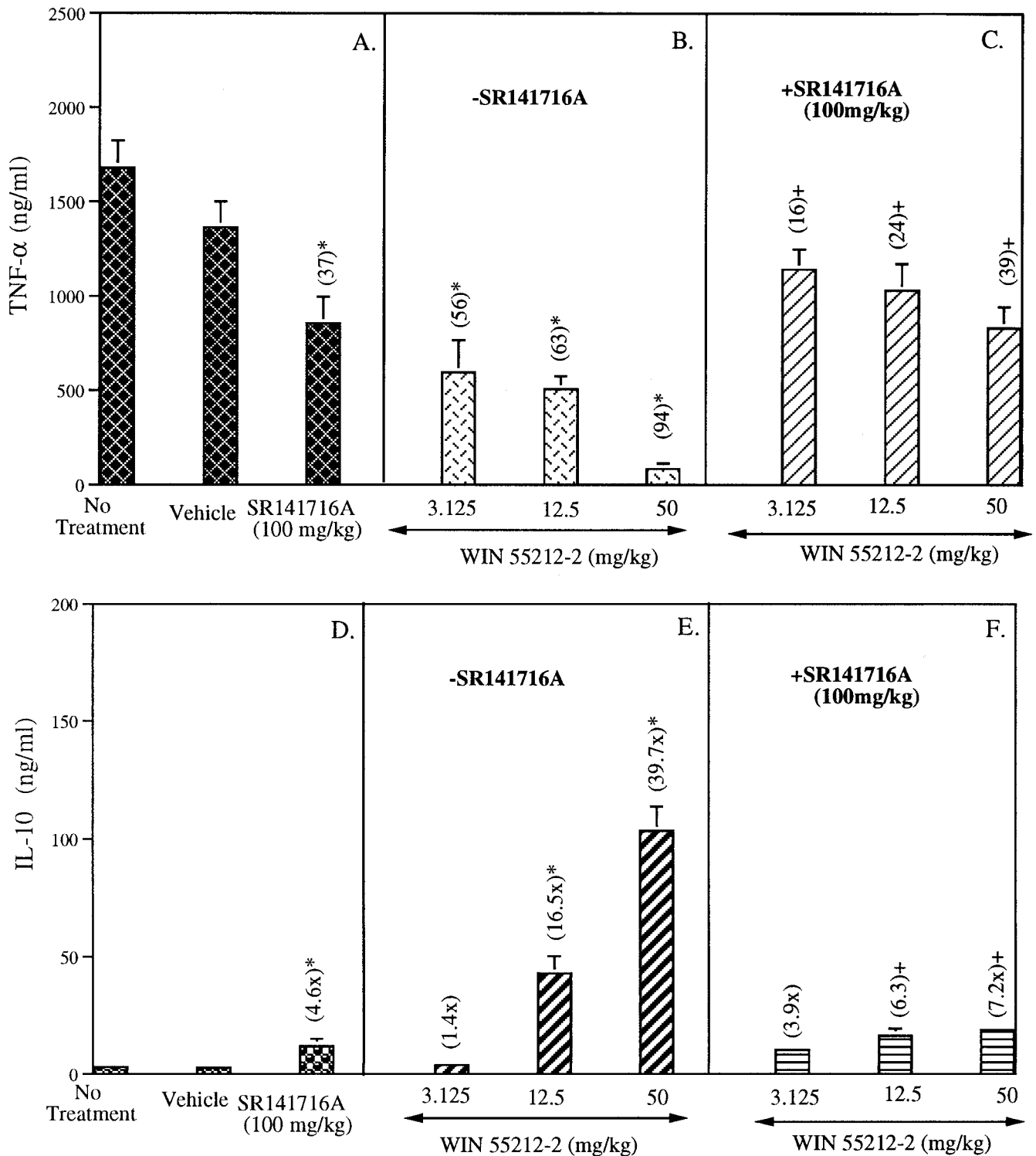


Fig. 5. Antagonism of WIN 55212-2-modulated cytokine responses in *C. parvum* mice by the CB1 antagonist SR141716A. Mice were treated i.p. with WIN 55212-2 at doses of 3.125, 12.5, or 50 mg/kg 1 h before LPS. SR141716A was administered to mice at a dose of 100 mg/kg 2 h before LPS (1 h before WIN 55212-2). Serum TNF- α and IL-10 were measured 1.5 and 3 h after the LPS challenge, respectively. The TNF- α and IL-10 responses in mice treated with SR141716A alone are shown in A and D, in mice treated with WIN 55212-2 alone in B and E, and in mice treated with SR141716A + WIN 55212-2 in C and F. The values shown represent the mean $\pm$ S.E. of six mice per group. Significantly different from mice treated with vehicle, * $P < .01$. Significantly different from the response in mice treated with WIN 55212-2 alone, + $P < .01$.

with WIN 55212-2 on LPS-induced serum cytokines. SR144528 inhibits the binding of the bispecific CB agonist CP 55940 to mouse spleen (CB2 receptor) with an oral ED₅₀ of 0.35 mg/kg and does not inhibit the binding of CP

55940 to mouse brain (CB1 receptor) at doses up to 10 mg/kg (Rinaldi-Carmona et al., 1998). In the experiment shown in Fig. 8, SR144528 (10 mg/kg) was administered orally to *C. parvum* mice 1 h before treatment with WIN

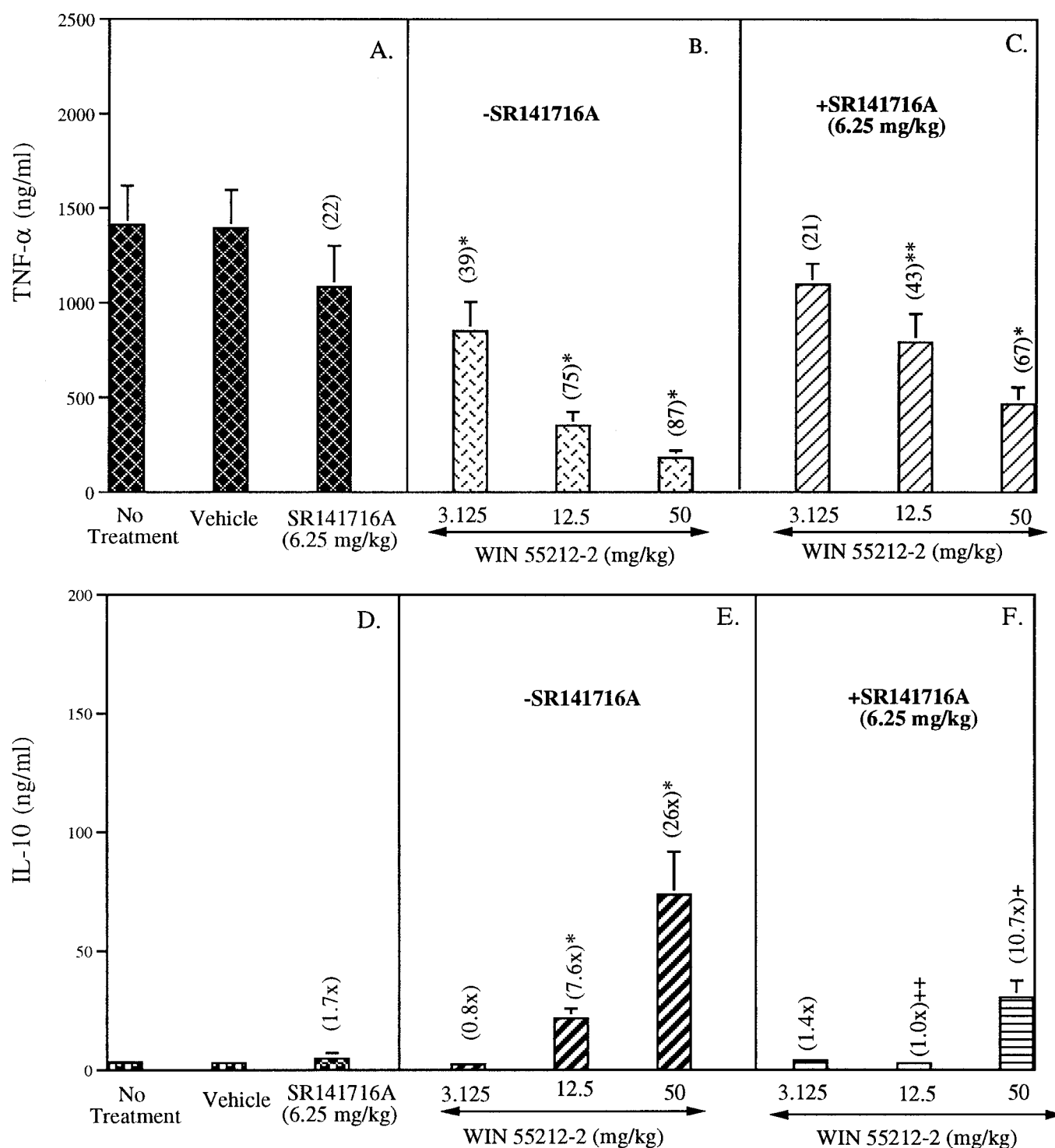


Fig. 6. Ability of WIN 55212-2 to reverse the antagonism by a low dose of SR141716A (6.3 mg/kg) of its modulatory effects on LPS-induced cytokines. SR141716A was administered to the mice at a dose of 6.25 mg/kg 2 h before LPS (1 h before WIN 55212-2). The doses of WIN 55212-2 were 3.125, 12.5, or 50 mg/kg. Serum TNF- α and IL-10 were measured 1.5 and 3 h after the LPS challenge, respectively. The TNF- α and IL-10 responses in mice treated with SR141716A alone are shown in A and D, in mice treated with WIN 55212-2 alone in B and E, and in mice treated with SR141716A + WIN 55212-2 in C and F. Values represent the mean $\pm$ S.E. of six mice per group. Significantly different from the responses in mice treated with vehicle, ** P < .05 and * P < .01. Significantly different from the response caused by the same dose of WIN 55212-2 alone, ++ P < .05 and + P < .01.

55212-2 (50 mg/kg) and 2 h before an LPS challenge. There was no evidence of antagonism by SR144528 of the WIN 55212-2-inhibited TNF- α (Fig. 8A) or WIN 55212-2-elevated IL-10 (Fig. 8B) responses. Therefore, the results

obtained with the selective CB1 and CB2 receptor antagonists clearly indicated that cytokine modulation by WIN 55212-2 occurred through CB1 despite its demonstrated selectivity for CB2 in in vitro studies (Felder et al., 1995).

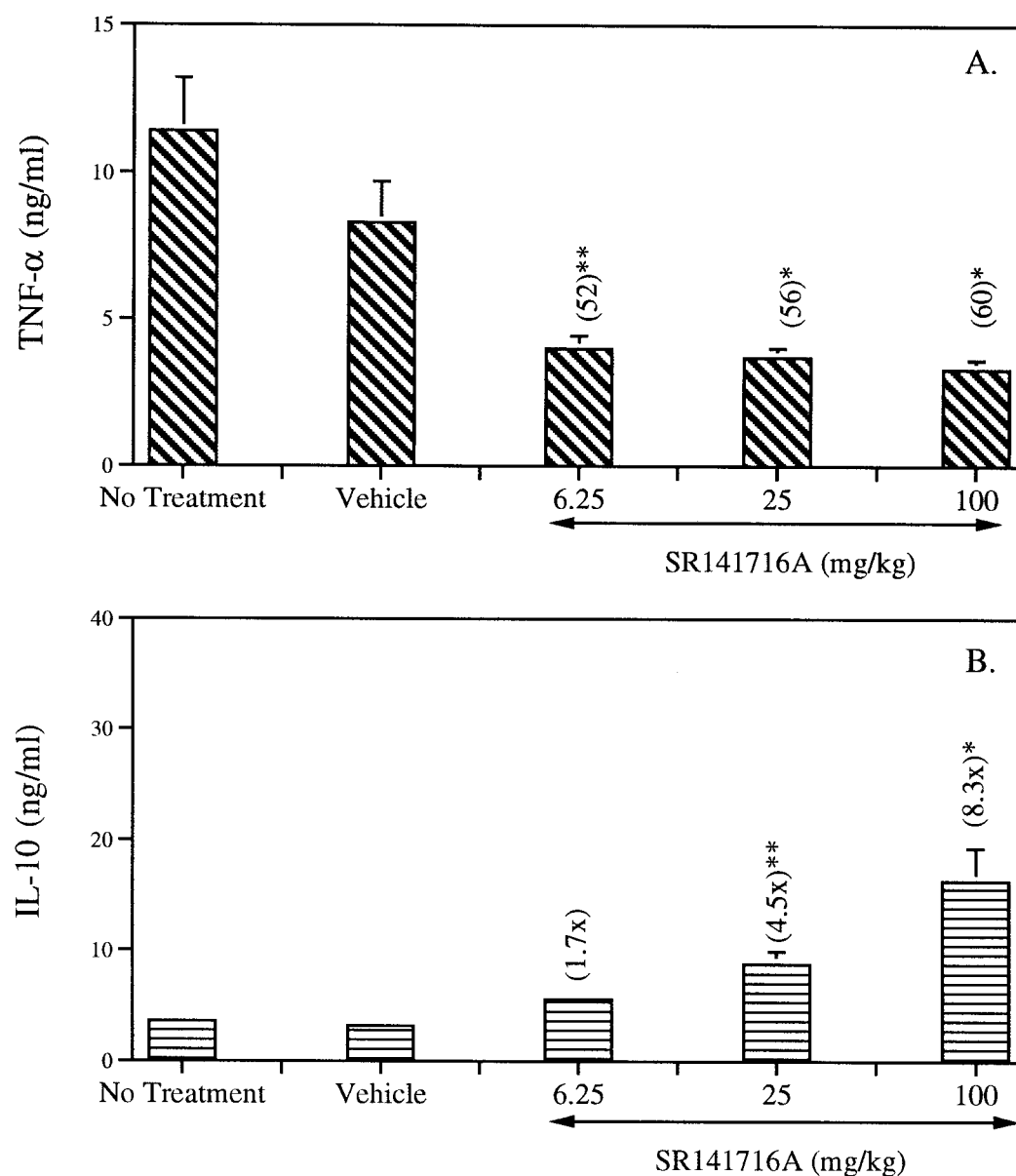


Fig. 7. The modulatory effects of SR141716A on the LPS-induced TNF- α (A) and IL-10 (B) responses in unprimed mice. SR141716A was administered to mice by the i.p. route 2 h before LPS. Serum TNF- α and IL-10 were measured 1.5 and 3 h after the LPS challenge, respectively. The values represent the mean $\pm$ S.E. of six mice per group. Significantly different from the responses in mice treated with vehicle, ** $P < .05$ and * $P < .01$.

As expected from its failure to antagonize cytokine modulation by WIN 55212-2, SR144528 also was found to have no effect on cytokine modulation by HU-210 (data not shown).

Effects of Centrally Administered SR141716A on LPS-Induced Cytokines in *C. parvum* Mice. In the previous experiments, CB receptor agonists and antagonists were administered systemically to mice by the i.p. route. The CB1 receptors that appear to be involved in the cytokine modulation by CB agents have been detected in some peripheral tissues as well as in the CNS. Therefore, it was of interest to determine whether cytokine modulation by systemically administered CBs occurred through interactions with peripheral or central CB1 receptors. When injected alone by the i.c.v. route, SR141716A (100 μ g) caused a modest reduction (36%) in the LPS-induced serum TNF- α response (Fig. 9A) as was observed when the drug was admin-

istered alone to *C. parvum* mice by the i.p. route. The response was reduced by 49 and 83% in mice treated with 12.5 and 50 mg/kg WIN 55212-2 (Fig. 9B). The dose-related suppression of TNF- α caused by i.p. treatment with WIN 55212-2 (Fig. 9B) was partially antagonized by centrally administered SR141716A as evidenced by a decrease in the level of inhibition (29 and 60%) in antagonist-treated mice (Fig. 9C). The elevation in IL-10 caused by the WIN 55212-2 treatment (Fig. 9E) was blocked by central SR141716A (Fig. 9F). Although central SR141716A did not prevent the CNS changes caused by WIN 55212-2, the symptoms were not nearly as severe as in mice that only received the agonist. SR141716A also antagonized cytokine modulation by HU-210 when administered centrally to *C. parvum* mice (Fig. 10). There was complete antagonism of both the inhibition of TNF- α (Fig. 10, B and C) and the enhancement of serum IL-10 (Fig. 10, E and F) by HU-210. The CNS effects pro-

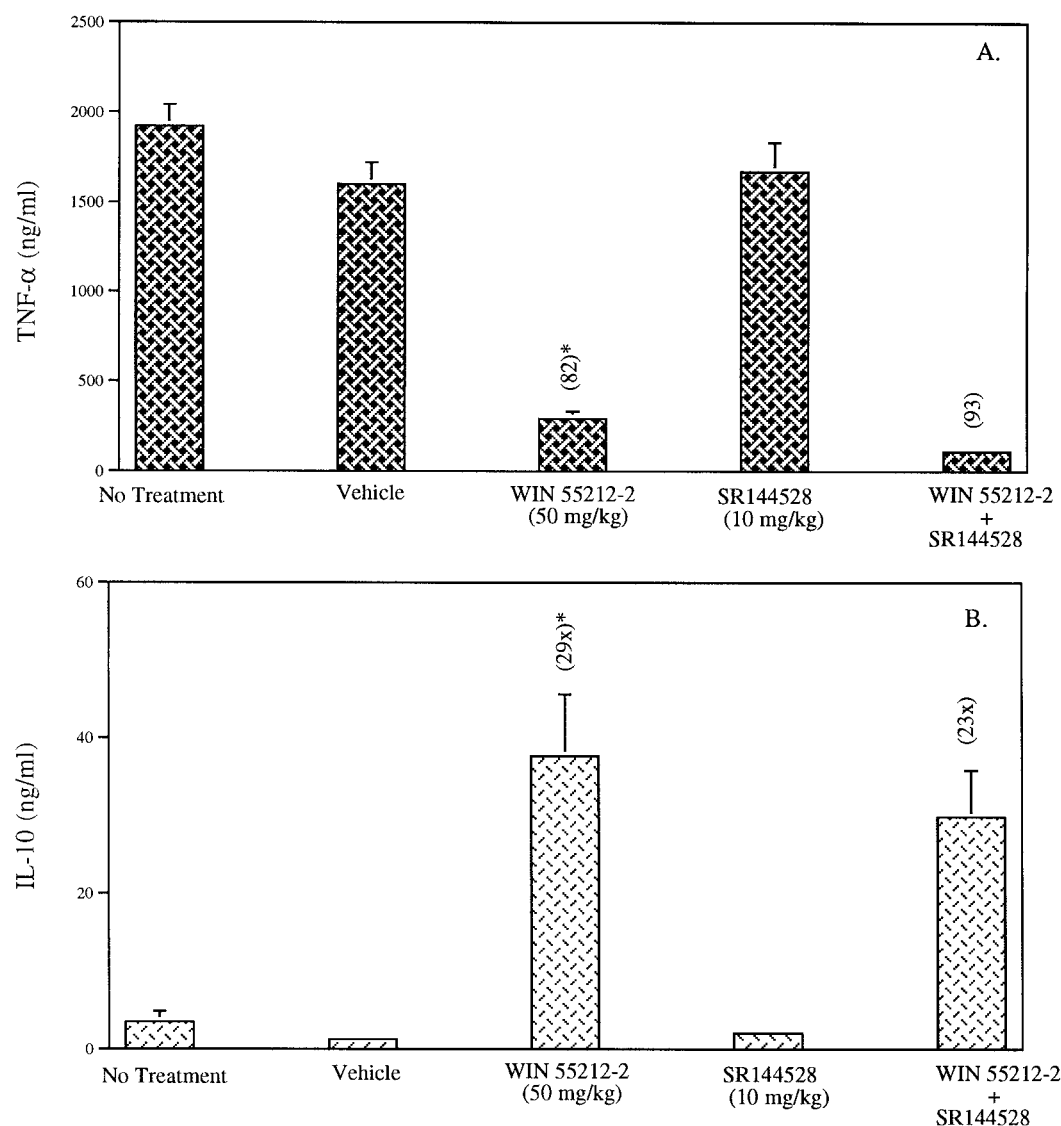


Fig. 8. WIN 55212-2-mediated modulation of LPS-induced TNF- α (A) and IL-10 (B) in *C. parvum*-primed mice pretreated with the CB2 antagonist SR144528. SR 144528 (10 mg/kg) was administered orally to the mice 1 h before treatment with WIN 55212-2 (50 mg/kg) and 2 h before the LPS challenge. Serum TNF- α and IL-10 were measured 1.5 and 3 h after the LPS challenge, respectively. Data were pooled from three individual experiments and the values represent the mean $\pm$ S.E. of 18 mice per group. Significantly different from the response in mice treated with vehicle, * $P < .01$.

duced by the 0.05-mg/kg dose of HU-210 were blocked completely by central SR141716A, but there was only a delay in the appearance of the more severe CNS symptoms evoked by higher doses of the agonist.

Effects of WIN 55212-2 and HU-210 on LPS Lethality. HU-210 (0.4 mg/kg i.p.; Fig. 11, A and B) and WIN 55212-2 (12.5 and 50 mg/kg i.p.; Fig. 11, C and D) protected *C. parvum* mice (but not unprimed mice) from the lethal effects of LPS. It was possible that the protection afforded to primed mice by these agonists reflected the higher levels of anti-inflammatory IL-10 that were produced by *C. parvum* mice after agonist treatment. To explore this possibility, the mice were injected with an anti-IL-10 monoclonal antibody 24 h before treatment with WIN 55212-2 or HU-210 and challenge with LPS. The anti-IL-10 monoclonal antibody had no effect on the rate of survival of agonist-treated mice, indicating that IL-10 was not solely responsible for the protection afforded to mice by the CB agonists (data not shown).

Discussion

LPS-stimulated cytokine production in mouse models of endotoxemia provides a convenient system in which to evaluate drugs that have the ability to modulate the complex network of cytokines that is involved in inflammation and immunity. The prototypical CB agonist Δ^9 -THC has been studied extensively for its modulatory effects on cytokine production in vitro, but until recently there was little information relative to its effects on cytokine responses in vivo (Klein et al., 1995; Berdyshev et al., 1998). In a recent publication, Δ^9 -THC and WIN 55212-2 were investigated for their effects on LPS-induced bronchopulmonary inflammation in mice (Berdyshev et al., 1998). Both drugs were found to cause a dose-related decrease in TNF- α levels in bronchoalveolar lavage fluids, an effect that was accompanied by a moderate reduction in neutrophil recruitment. Whether these effects resulted from the specific activation of CB2 or CB1 receptors was not determined.

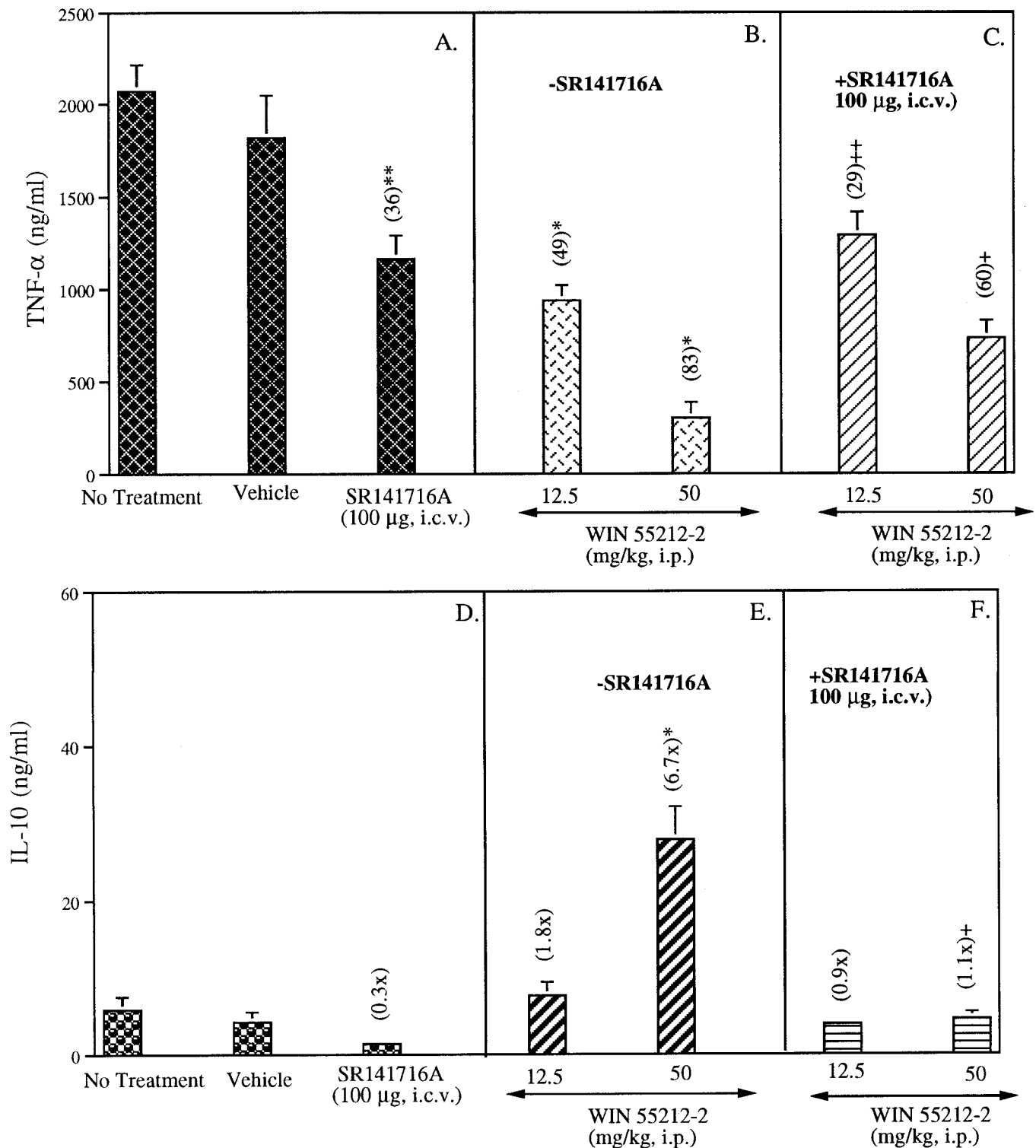


Fig. 9. Antagonism of WIN 55212-2-modulated cytokine responses in *C. parvum* mice pretreated centrally with the CB1 antagonist SR141716A (100 μg i.c.v.). WIN 55212-2 (12.5 and 50 mg/kg) was administered to mice by the i.p. route 1 h after treatment with SR141716A and 1 h before LPS. The TNF-α and IL-10 responses in mice treated with SR141716A alone are shown in A and D, in mice treated with WIN 55212-2 alone in B and E, and in mice treated with SR141716A + WIN 55212-2 in C and F. The values shown represent the mean ± S.E. of six mice per group. Significantly different from mice treated with vehicle, ** $P < .05$ and * $P < .01$. Significantly different from mice treated with WIN 55212-2 alone, ++ $P < .05$ and + $P < .01$.

The results of the present study clearly indicate that LPS-induced cytokines in *C. parvum*-primed and unprimed mice can be modulated by CB agonists through activation of the CB1 receptor. The decreases in serum TNF-α and IL-12 as

well as the increase in serum IL-10 that occurred in agonist-treated mice could be blocked by SR141716A, a highly selective CB1 receptor antagonist, but not by SR144528, a highly selective CB2 receptor antagonist. Thus, the results of our

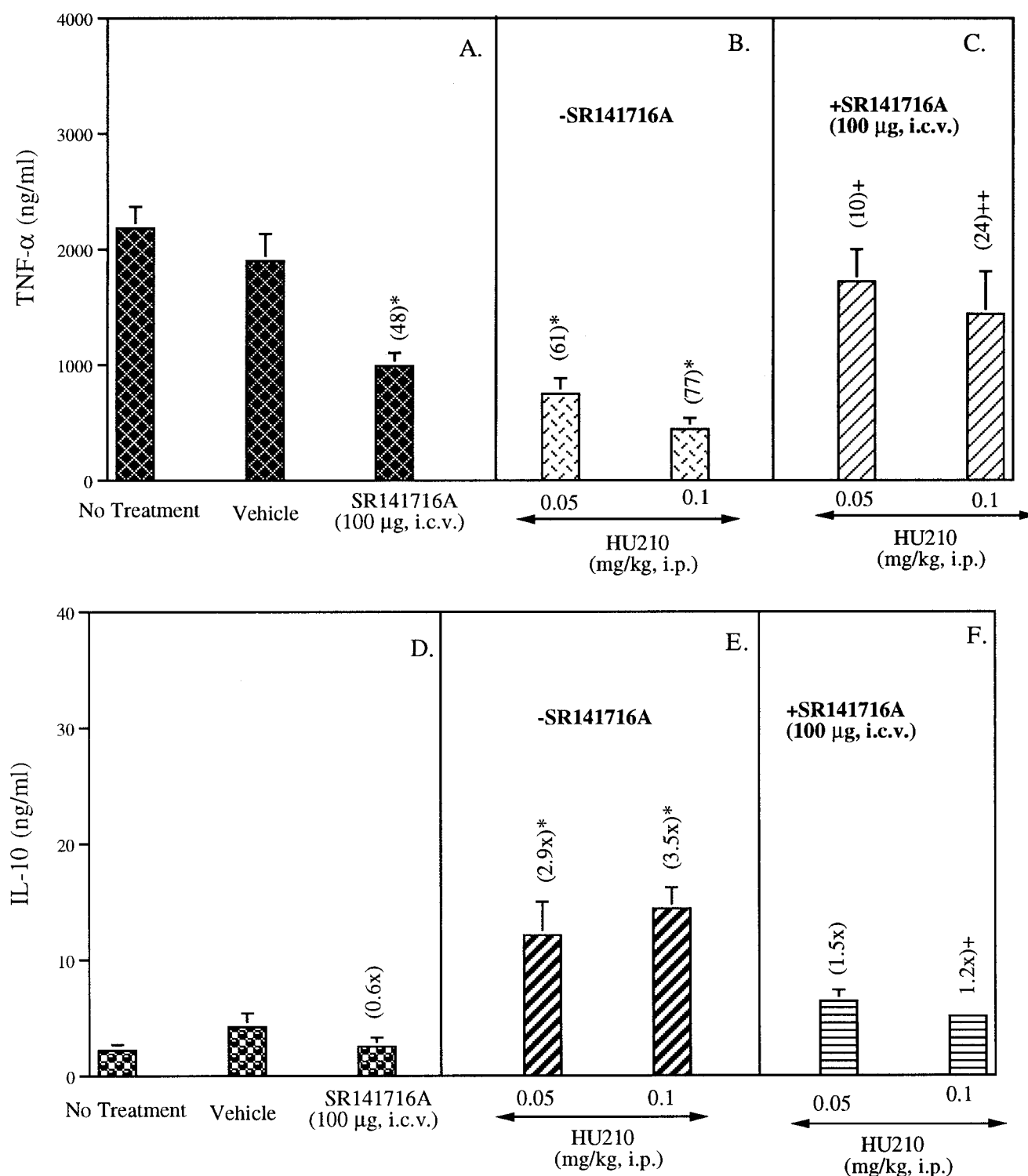


Fig. 10. Antagonism of HU-210-modulated cytokine responses in *C. parvum* mice pretreated centrally with the CB1 antagonist SR141716A (100 µg i.c.v.). HU-210 (0.05 and 0.1 mg/kg) was administered to mice by the i.p. route 1 h after treatment with SR141716A and 1 h before LPS. The TNF-α and IL-10 responses in mice treated with SR141716A alone are shown in A and D, in mice treated with HU-210 alone in B and E, and in mice treated with SR141716A + HU-210 in C and F. The values shown represent the mean ± S.E. of six mice per group. Significantly different from mice treated with vehicle, * $P < .01$. Significantly different from mice treated with WIN 55212-2 alone, ++ $P < .05$ and + $P < .001$.

studies on cytokine modulation by CB agonists in mouse endotoxemia strongly suggest that such effects occur through interactions with the CB1 receptor. To our knowledge, this is the first indication of a role for the CB1 receptor in cytokine modulation by CB receptor agonists.

Assuming a role for the CB1 receptor subtype in cytokine modulation by CB agonists in endotoxemic mice, such effects could occur through central CB1 receptors, through CB1 receptors present in peripheral tissues, or through both central and peripheral receptors. That anti-inflam-

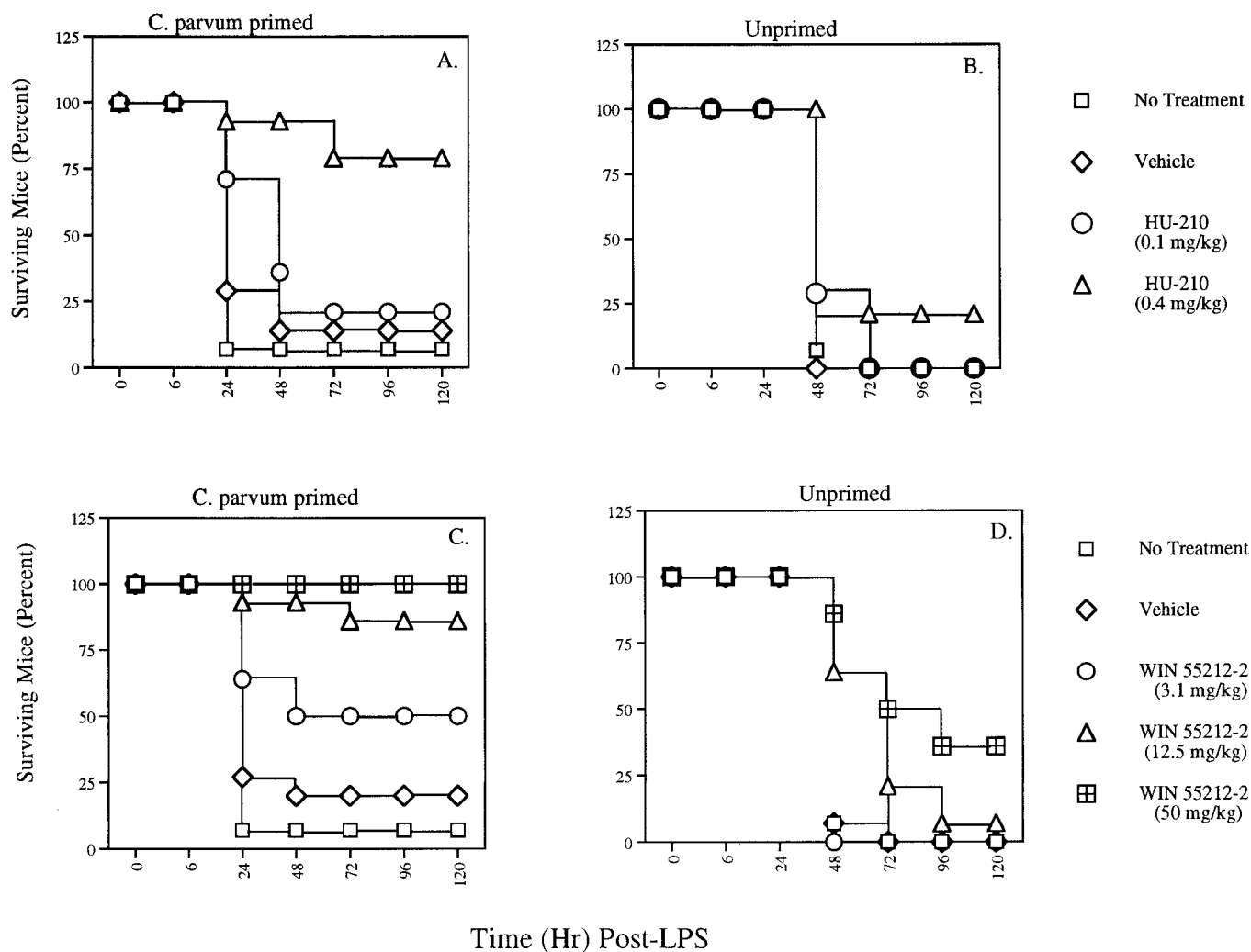


Fig. 11. LPS lethality in mice treated with HU-210 (A and B) and WIN 55212-2 (C and D). The CB agonists were administered to mice by the i.p. route 1 h before LPS and lethality monitored over a 5-day interval. A and C, *C. parvum*-primed mice; B and D, unprimed mice.

matory effects can evolve from the interaction of CB agonists with peripheral CB1 receptors was shown in a recent study where peripheral, but not systemic anandamide was found to inhibit carrageenan-induced paw edema in rats (Richardson et al., 1998). Although we cannot formally exclude a role for peripheral CB1 receptors in cytokine modulation by WIN 55212-2 and HU-210, the following observations suggest the likely involvement of the central CB1 receptor. First, the agonist-induced changes in cytokine production were generally accompanied by central effects such as catalepsy and hypokinesia, indicating that doses of the drugs that modulate cytokine responses are similar to those that produce changes in the CNS. Second, there was a similar level of antagonism of both the cytokine modulatory and CNS effects of WIN 55212-2 and HU-210 when mice were given SR141716A before the agonists. Thus, when the CNS changes caused by the CB agonists were only partially antagonized by SR141716A, there was also partial antagonism of their modulatory effects on cytokine responses. Similarly, when SR141716A was found to completely antagonize the CNS changes caused by the CB agonists, there was complete antagonism

of agonist-modulated cytokine responses as well. Third, the antagonistic activity of a relatively low dose of SR141716A toward both the behavioral changes and cytokine modulation caused by CB agonist treatment could be reversed by increasing the dose of agonist relative to that of the antagonist. These latter observations suggest that the effects of the CB agonists and the selective CB1 receptor antagonist occur through interactions with the same receptor subtype. Fourth, cytokine modulation also is observed when CB agonists are administered centrally to mice at doses that are inactive when given by the i.p. route (data not shown). Together, these observations would seem to support the contention that central CB1 receptors are involved in cytokine modulation by CB agonists. However, the possibility still remains that although the CNS changes are evoked through the central CB1 receptor, cytokine modulation by CB agonists may occur through central, peripheral, or both central and peripheral CB1 receptors.

As shown herein with WIN 55212-2 and HU-210, HU-211, the nonpsychotropic stereoisomer of HU-210, also was reported to down-regulate TNF- α levels in mice undergoing

septic shock (Gallily et al., 1997). However, in that study, there was no indication of the CB receptor subtype that might be involved in cytokine modulation by the agonist. Given the structural similarities between HU-210 and HU-211, there is a distinct possibility that the effects seen with HU-211 also may have occurred through interactions with the CB1 receptor.

The mechanism by which CB agonists produce their modulatory effects on LPS-induced cytokines is unclear. The potential role of glucocorticoids as proximal mediators of these effects was examined by Gallily et al. (1997) in their studies with HU-211 in endotoxemic mice and rats. They observed no increase by HU-211 in the levels of corticosterone that were produced when rats were given an LPS challenge. Similarly, we have been unable to detect increases in serum corticosterone levels in mice treated with WIN 55212-2 and HU-210 (S.R.S., C.T., and G.D., unpublished data). Furthermore, when steroids are administered exogenously to mice before LPS, there is either no change or a slight diminution in the LPS-induced IL-10 response. This is in contrast to the augmented IL-10 response that is seen after treatment with CB agonists. Thus, endogenously produced glucocorticoids would appear to have no role in the cytokine modulatory effects of CB agonists.

An unexpected finding in the present study was the ability of SR141716A itself to modulate LPS-induced cytokine responses. Its effects on inflammatory cytokine responses and anti-inflammatory IL-10 were qualitatively similar to those of the CB agonists WIN 55212-2 and HU-210. Therefore, at a functional level, SR141716A appeared to be a partial agonist of LPS-induced cytokine responses in both *C. parvum*-primed and unprimed mice. The CB1 selective antagonist exhibited partial agonism when administered to mice alone at doses that were found to completely antagonize the cytokine modulatory activities of WIN 55212-2 and HU-210. The phenomenon of partial agonism, which is exhibited by some antagonists at GPCRs, is less well understood than the phenomenon of inverse agonism that is being observed with increasing frequency. In fact, SR141716A has been reported to exhibit inverse agonism in vitro in increasing the twitch contraction amplitude in the mouse-field-stimulated bladder and guinea pig mesenteric plexus-longitudinal muscle, and in vivo in causing hyperalgesia in a mouse model of thermal pain (Pertwee et al., 1996; Pertwee and Fernando, 1996; Richardson et al., 1997). According to present dogma, an equilibrium exists between active and ground (inactive) states of GPCRs. Inverse agonists are thought to drive the conformation of GPCRs toward the inactive or ground state, whereas full agonists have the opposite effect. The similarities in the modulatory effects of SR141716A and the CB agonists WIN 55212-2 and HU-210 on LPS-induced cytokine responses suggest that both types of ligands have the ability to drive the CB receptor toward a more activated state. Therefore, cytokine modulation by SR141716A appears to be a result of partial agonism rather than inverse agonism at the CB1 receptor.

We do not believe that agonists at the CB1 receptor can be easily developed as anti-inflammatory or immunomodulatory drugs not only because of their central effects but also because they are likely to cause hypotension even in normotensive individuals through interactions with peripheral CB1 receptors. However, the results presented herein raise the

possibility of discovering novel CB1 antagonists that are more potent than SR141716A in exhibiting partial agonism of cytokine responses when bound to the CB1 receptor. Theoretically, such antagonists should largely be devoid of the central effects exhibited by agonists interacting with the brain CB1 receptor and indeed could have utility as anti-inflammatory and/or immunomodulatory drugs. Our studies further suggest that until a dominant role for the CB2 receptor subtype in immune modulation by CBs can be convincingly demonstrated in vivo, it would be prudent to consider the potential involvement of the CB1 receptor in any pharmacological effect that is seen with CB agonists in peripheral tissues and organs.

Acknowledgments

We thank Dr. Robert West for helpful discussions on the biochemistry of GPCRs.

References

- Berdyshev E, Boichot E, Corbel M, Germain N and Lagente V (1998) Effects of cannabinoid receptor ligands on LPS-induced pulmonary inflammation in mice. *Pharmacol Lett* **63**:125–129.
- Dewey WL (1986) Cannabinoid pharmacology. *Pharmacol Rev* **38**:151–178.
- Deroocq J-M, Segui M, Marchand J and Le Fur G (1995) Cannabinoids enhance human B-cell growth at low nanomolar concentrations. *FEBS Lett* **369**:177–182.
- Carayon P, Marchand J, Dussosoy D, Deroocq J-M, Jbilo O, Bord A, Bouaboula M, Galiegue S, Mondiere P, Penarier G, Le Fur G, Defrance C and Casellas P (1998) Modulation and functional involvement of CB2 peripheral cannabinoid receptors during B-cell differentiation. *Blood* **92**:3605–3615.
- Compton DR, Aceto MD, Lowe J and Martin BR (1996) In vivo characterization of a specific cannabinoid receptor antagonist (SR141716A). Inhibition of delta 9-tetrahydrocannabinol-induced responses and agonist activity. *J Pharmacol Exp Ther* **277**:586–594.
- Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, Lai Y, Ma A and Mitchell RL (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol Pharmacol* **48**:443–450.
- Galiegue S, Mary S, Marchand J, Dussosoy D, Carriere D, Carayon P, Bouaboula M, Shire D, Le Fur G and Casellas P (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* **232**:54–61.
- Gallily R, Yamin A, Waksman Y, Ovadia H, Weidenfeld J, Bar-Joseph A, Biegon A, Mechoulam R and Shohami E (1997) Protection against septic shock and suppression of tumor necrosis factor α and nitric oxide production by Dexamabinol (HU-211), a nonpsychotropic cannabinoid. *J Pharmacol Exp Ther* **283**:918–924.
- Haley TJ and McCormick WG (1957) Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *Br J Pharmacol* **12**:12–15.
- Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, DeCosta BR and Rice KC (1990) Cannabinoid receptor localization in brain. *Proc Natl Acad Sci USA* **87**:1932–1936.
- Johnson MR and Melvin LS (1986) The discovery of nonclassical cannabinoid analogs. In *Cannabinoids as Therapeutic Agents* (Mechoulam R ed) pp 121–145, CRC Press, Boca Raton, FL.
- Klein TW, Newton C, Zhu W, Daaka Y and Friedman H (1995) Δ^9 -tetrahydrocannabinol cytokines and immunity to *Legionella pneumophila*. *Proc Soc Exp Biol Med* **209**:205–212.
- Lake KD, Martin BR, Kunos G and Vara K (1997) Cardiovascular effects of anandamide in anesthetized and conscious normotensive and hypertensive rats. *Hypertension* **29**:1204–1210.
- Martin BR, Compton DR, Thomas BF, Prescott WR, Little PJ, Razdan RK, Johnson MR, Melvin LS, Mechoulam R and Ward SJ (1991) Behavioral, biochemical and molecular modeling evaluations of cannabinoid analogs. *Pharmacol Biochem Behav* **40**:471–478.
- Pacheco MA, Childers SR, Arnold R, Casiano F and Ward SJ (1991) Aminoalkylindoles: Actions on specific G-protein-linked receptors. *Pharmacol Exp Ther* **257**:170–183.
- Pertwee RG and Fernando SR (1996) Evidence for the presence of cannabinoid CB1 receptors in mouse urinary bladder. *Br J Pharmacol* **118**:2053–2058.
- Pertwee RG, Fernando SR, Nash JE and Coutts AA (1996) Further evidence of cannabinoid CB1 receptors in guinea-pig small intestine. *Br J Pharmacol* **118**:2199–2205.
- Reche I, Fuentes JA and Ruiz-Gayo M (1996) A role for central cannabinoid and opioid systems in peripheral delta 9-tetrahydrocannabinol-induced analgesia in mice. *Eur J Pharmacol* **301**:75–81.
- Richardson JD, Aanonsen L and Hargreaves KM (1997) SR141716A, a cannabinoid receptor antagonist produces hyperalgesia in untreated mice. *Eur J Pharmacol* **319**:R3–R4.
- Richardson JD, Kilo S and Hargreaves KM (1998) Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB1 receptors. *Pain* **75**:111–119.
- Rinaldi-Carmona M, Barth F, Heaulme M, Shire D, Calandra B, Congy C, Martinez

- S, Maruani J, Neliar G, Caput D, Ferrara P, Soubrie P, Breliere J-C and Le Fur G (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett* **350**:240–244.
- Rinaldi-Carmona M, Barth F, Heaulme M, Alonso R, Shire D, Calandra B, Congy C, Soubrie P, Breliere J-C and LeFur G (1995) Biochemical and pharmacological characterization of SR141716A, the first potent and selective cannabinoid receptor antagonist. *Life Sci* **56**:1941–1947.
- Rinaldi-Carmona M, Barth F., Millan J, Derocq J-M, Casellas P Congy C, Oustric D, Sarran M, Bouaboula M, Calandra B, Porttier M, Shire D, Breliere J-C and Le Fur G (1998) SR1442528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J Pharmacol Exp Ther* **284**:644–650.
- Smith SR, Calzetta A, Bankowski J, Kenworthy-Bott L and Terminelli C (1993) Lipopolysaccharide-induced cytokine and mortality in mice treated with *Corynebacterium parvum*. *J Leukoc Biol* **54**:23–29.
- Smith SR, Terminelli C, Denhardt G, Narula S and Thorbecke GJ (1996) Administration of interleukin-10 at the time of priming protects *Corynebacterium parvum*-primed mice against LPS-and TNF- α -induced lethality. *Cell Immunol* **173**:207–214.
- Varga K, Lake KD, Martin BR and Kunos G (1995) Novel antagonist implicates the CB1 cannabinoid receptor in the hypotensive action of anadamide. *Eur J Pharmacol* **278**:279–283.
- Wagner JA, Varga K, Ellis EF, Rzigalinski BA, Martin BR and Kunos G (1997) Activation of peripheral cannabinoid receptors in haemorrhagic shock. *Nature (Lond)* **390**:518–521.

Send reprint requests to: Sidney R. Smith, Department of Immunology, Schering-Plough Research Institute, 2015 Galloping Hill Rd., Kenilworth, NJ 07033. E-mail: sidney.smith@spcorp.com
