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The anti-inflammatory activities of cannabinoid receptor ligands in mouse peritonitis models

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Abstract

In this report, we describe experiments in which cannabinoid receptor ligands were evaluated for effects on the development of a peritoneal inflammation when elicited in mice with thioglycollate broth or staphylococcus enterotoxin A. The cannabinoid receptor agonists [(−)-11-hydoxy-Δ^8 tetrahydrocannabinol-dimethylheptyl] (HU-210) and {(*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl[pyrrolo[1,2,3-de]1,4-benzoxazin-6-yl](1-naphthalenyl) methanone} (WIN 55212-2) blocked the migration of neutrophils into the peritoneal cavity in response to these inflammatory stimuli. This effect was caused by a delay in the production of the neutrophil chemoattractants, KC and macrophage inflammatory protein-2. HU-210 and WIN 55212-2 blocked neutrophil chemokines and neutrophil migration whether administered subcutaneously (s.c.) or intracerebroventricularly (i.c.v.). Their modulatory effects on the inflammation were antagonized by centrally administered [*N*-(piperdin-1-yl)-5-(4-chloropheny)-1-(2,4-dichloropheny)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride] (SR141716A), a selective cannabinoid CB_1 receptor antagonist. This latter observation, and the ability of the cannabinoid receptor agonists to suppress the peritoneal inflammation at relatively low doses when administered i.c.v., indicated a role for central cannabinoid CB_1 receptors in the anti-inflammatory activities of HU-210 and WIN 55212-2. The cannabinoid receptor agonists had no effect on monocyte migration elicited by thioglycollate, despite their ability to suppress monocyte chemotactic protein-1 levels in lavage fluids. The cannabinoid CB_2 receptor antagonist, {*N*-[(1*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]5-(4-choro-3 methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide} (SR144528) inhibited the peritoneal inflammation in a manner analogous to that of HU-210 and WIN 55212-2 when administered i.c.v., but it did not appear to act through central cannabinoid CB_1 receptors. The present results add to the body of literature indicating that cannabinoid receptor ligands have diverse anti-inflammatory properties.

Keywords: Peritonitis; Thioglycollate; Staphylococcus enterotoxin A; Chemokine; Cannabinoid receptor agonist; Cannabinoid receptor antagonist

1. Introduction

In earlier reports, we described the ability of the non-selective cannabinoid receptor agonists, [(−)-11-hydoxy-Δ^8tetrahydrocannabinol-dimethylheptyl] (HU-210) and {(*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl [pyrrolo[1,2,3-de]1,4-benzoxazin-6-yl](1-naphthalenyl) methanone} (WIN 55212-2) to modulate cytokine responses in endotoxemic mice (Smith et al., 2000, 2001). These agents were shown to inhibit the production of inflammatory cytokines (tumor necrosis factor α and interleukin-12) and to increase the production of anti-inflammatory interleukin-10 elicited by endotoxin. The modulatory effects of the drugs on cytokine responses were blocked by [*N*-(piperdin-1-yl)-5-(4-chloropheny)-1-(2,4-dichloropheny)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride] (SR141716A), a highly selective cannabinoid CB_1 receptor antagonist, but not by {*N*-[(1*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]5-(4-choro-3 methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide} (SR144528), a selective cannabinoid CB_2 receptor antagonist. These observations indicated that cytokine modulation by the cannabinoid receptor agonists occurred through activation of cannabinoid CB_1 receptors. Furthermore, the experimental evidence pointed to a role for central rather than peripheral cannabinoid CB_1 receptors in cytokine modulation by the cannabinoid receptor agonists. First, the modulation of endotoxin-induced cytokines by HU-210 and WIN 55212-2 was always accompanied by the central nervous system (CNS) changes (hypomobility, catalepsy and hypothermia) that are typically observed when central cannabinoid CB_1 receptors are activated by psychoactive cannabinoid recep-

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tor agonists. Second, the cannabinoid receptor agonists were able to modulate cytokines at lower doses when administered intracerebroventricularly (i.c.v.) than when given intraperitoneally (i.p.). Third, the changes in cytokines and behavior that occurred when HU-210 and WIN 55212-2 were administered to mice centrally or peripherally could be blocked by injecting SR141716A, i.c.v. While cytokine responses appeared to be modulated through central cannabinoid receptors, other reports have demonstrated a role for peripheral cannabinoid CB_1 receptors in the anti-inflammatory effects of cannabinoid receptor agonists, (Richardson et al., 1998; Jaggar et al., 1998).

There are some cannabinoid receptor agonists that have been shown to produce their anti-inflammatory effects through activation of cannabinoid CB_2 receptors which are found primarily in peripheral cells and tissues. Thus, phosphatidylethanolamine, an agonist with a reported selectivity for the cannabinoid CB_2 receptor, was shown to inhibit carrageenan-induced hyperalgesia and hindpaw edema in rats (Mazzari et al., 1996). Inflammatory products released from mast cells were thought to be the direct mediators of the carrageenan-induced inflammation and it was suggested that phosphatidylethanolamine inhibited the release of such products by binding to mast cell cannabinoid CB_2 receptors. More recently, HU-308 a selective cannabinoid CB_2 receptor agonist was reported to have anti-inflammatory and analgesic properties that were antagonized by SR144528 (Hanus et al., 1999).

There has also been considerable interest in the effects of cannabinoid receptor agonists on the adhesive and migratory properties of inflammatory cells. Δ^9-tetrahydrocannabinol was shown to inhibit macrophage spreading and phagocytosis of yeast when added to macrophage cultures prepared from human peripheral blood (Specter et al., 1991). In addition, peritoneal cells from mice orally treated with a group of synthetic nonpsychotropic carboxy derivatives of Δ^9-tetrahydrocannabinol exhibited reduced adhesion when added to tissue culture dishes indicating that the anti-adhesive properties of cannabinoids are not restricted to psychoactive cannabinoids (Burstein et al., 1992). Peritoneal macrophages exposed in vitro or in vivo to a non-selective cannabinoid receptor agonist, CP55,940, exhibited a reduced capacity for spontaneous and induced migration in vitro. The involvement of cannabinoid CB_1 or CB_2 receptors in the inhibitory effects of CP55,940, depended upon whether cells were exposed to the drug in vitro or in vivo (Sacerdote et al., 2000).

Cannabinoid receptor agonists have also been reported to stimulate cell migration. HU-210, WIN 55212-2 and the endocannabinoid, anandamide, were reported to induce the migration of human embryonic kidney 293 cells in modified Boyden chambers when the cells were stably transfected with cannabinoid CB_1 receptors (Song and Zhong, 2000). Cell migration was blocked by SR141716A, indicating an obligatory role for the transfected cannabinoid CB_1 receptors in the response.

In our continuing efforts to understand the roles of the cannabinoid receptor subtypes in the anti-inflammatory activities of cannabinoid compounds, we have studied the effects of cannabinoid receptor agonists on leukocyte migration in models of peritoneal inflammation in mice. We show that HU-210 and WIN 55212-2 can block neutrophil accumulation in the peritoneal cavity in response to thioglycollate or staphylococcus enterotoxin A. As with cytokine modulation, the inhibitory effects of the cannabinoid receptor agonists on neutrophil migration appeared to involve central cannabinoid CB_1 receptors. Neutrophil migration was also blocked by central SR144528, but not through the same receptors.

2. Materials and methods

2.1. Mice

Specific pathogen-free Balb/c female mice, 6–8 weeks old, were used in these experiments and were purchased from The Jackson Laboratory (Bar Harbor, ME). Cannabinoid CB_1 receptor knockout mice (Zimmer et al., 1999) were obtained from Dr. Andreas Zimmer and bred at Charles River (Boston, MA). The knockout mice (females, 20–25 g) were developed on a C57Bl/6 background and female C57Bl/6 of equivalent weight were used as wild-type controls.

2.2. Reagents

Thioglycollate broth was purchased from Difco Laboratories (Detroit, MI) and staphylococcus enterotoxin A from Sigma (St. Louis, MO).

2.3. Induction of peritoneal inflammation (peritonitis)

Mice were injected i.p. with 1 ml of a 3% solution of thioglycollate broth or staphyloccocus enterotoxin A (generally 16 μg/mouse) in 0.5 ml phosphate-buffered saline (PBS). In general, the peritoneal cavities were lavaged with 3 ml Hanks balanced salt solution (HBSS, Ca^{2+}, Mg^{2+} free) 4 h after thioglycollate and 2 h after staphylococcus enterotoxin A.

2.4. Drugs

WIN 55212-2 and its *S*(−) enantiomer (WIN 55212-3) were purchased from Sigma and HU-210 was purchased from BIOMOL (Plymouth Meeting, PA). SR141716A and SR144528 were synthesized according to methods published by Sanofi Recherche (France). WIN 55212-2, WIN 55112-3 and SR141716A were suspended in methylcellu-

lose and HU-210 was solubilized in a vehicle containing dimethyl sulfoxide,Tween 80 and phosphate buffered saline for injection by the subcutaneous route. The concentrations of dimethyl sulfoxide and Tween 80 never exceeded 2%. SR144528 was suspended in the same vehicle for oral (p.o.) administration. HU-210, WIN 55212-2, SR141716A and SR144528 were dissolved in 1:1:18 emulphor/ethanol/saline for intracerebroventricular (i.c.v.) administration into conscious mice (Haley and McCormick, 1957). This vehicle was used because it was found to have no effect on the behavior of mice as shown in a previous report (Welch et al., 1995).

2.5. Leukocyte counts

Total leukocyte counts in lavage fluids were obtained by Coulter counting and differential counts were performed on Wright–Giemsa stained cytosmears.

2.6. Measurement of monocyte chemotactic protein-1, KC and macrophage inflammatory protein-2 in lavage fluids

The levels of monocyte chemotactic protein-1, KC and macrophage inflammatory protein-2 in exudates harvested from the peritoneal cavities of mice were quantified by Enzyme-linked immunosorbant assay (ELISAs, R&D Systems, Minneapolis, MN). The detection limit for each ELISA was < 2 pg/ml.

2.7. Data analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnetts post-hoc test or by Student's *t*-test using the Prism 2.0 statistical program. *P* values < 0.05 were considered as statistically significant.

3. Results

3.1. Thioglycollate-induced peritonitis

In the experiments in which a peritonitis was elicited with thioglycollate broth, the total leukocyte counts in peritoneal exudates were 7–25 fold higher than those in exudates from mice injected with phosphate buffered saline. The increase in total leukocytes observed 4–24 h after thioglycollate, was caused by an influx of neutrophils which was followed by a large influx of monocytes into the peritoneal cavity between 24 and 72 h (data not shown). This pattern of neutrophil and monocyte migration is typical of a thioglycollate-induced peritonitis. The chemokines, KC, macrophage inflammatory protein-2 and monocyte chemotactic protein-1 were detected in peritoneal exudate fluids harvested from mice shortly after the injection of thioglycollate, reaching maximum levels 2 to 4 h after the induction of the peritonitis. Chemokine levels declined rapidly thereafter and were barely detectable in exudates harvested from mice 8 h after thioglycollate (data not shown). The early appearance of chemokines in inflammatory exudates was an indication that they were most likely produced by cells resident in the cavity, and not by those that migrated into the cavity in response to thioglycollate.

3.2. Effects of cannabinoid receptor agonists on thioglycollate-induced peritonitis

To determine the effects of cannabinoid receptor agonists on the thioglycollate-induced peritoneal inflammation, mice were treated with various doses of HU-210 or WIN 55212-2 by the s.c. route, simultaneously with the injection of thioglycollate (Fig. 1). Peritoneal exudates were harvested at 4 h to determine the effects of the drugs on cell migration and chemokine production. The number of neutrophils in peritoneal exudates from mice treated with HU-210 (0.4 mg/kg, s.c.) were reduced by 78% when compared to exudates from vehicle-treated control mice (Fig. 1A). HU-210 reduced the levels of monocyte chemotactic protein-1 in lavage fluids by 77% (Fig. 1B), but did not block the influx of monocytes into the peritoneal cavity (Fig. 1A), despite its ability to inhibit production of the chemokine. WIN 55212-2, a cannabinoid agonist that has been reported to have a greater selectivity for the CB_2 receptor than HU-210 (Felder et al.,1995), also inhibited neutrophil recruitment and monocyte chemotactic protein-1 production in mice with a thioglycollate-induced peritonitis. There was a dose-dependent reduction in total leukocyte and neutrophil counts (Fig. 1C) and the levels of monocyte chemotactic protein-1 (Fig. 1D) in 4 h exudates from WIN 55212-2-treated mice. In addition to monocyte chemotactic protein-1, the levels of KC and macrophage inflammatory protein-2 were also quantified in 4 h peritoneal exudates. However, as discussed below, monocyte chemotactic protein-1 was the only chemokine with decreased levels in the exudates obtained from HU-210- and WIN 55212-2-treated mice at this timepoint. WIN 55213-3, the inactive enantiomer of WIN 55212-2 had no effect on neutrophil counts or on monocyte chemotactic protein-1 levels in peritoneal exudates.

To identify the cannabinoid receptor subtype that was involved in these effects of the cannabinoid receptor agonists, selective cannabinoid CB_1 (SR141716A) and CB_2 (144528) receptor antagonists were administered to mice 1 h before treatment with HU-210 or WIN 55212-2. SR141716A (100 mg/kg, s.c.), exhibited a partial antagonism (approximately 50%) of the inhibitory effects of HU-210 on the total leukocyte, neutrophil and monocyte chemotactic protein-1 responses (Fig. 2A and B). The

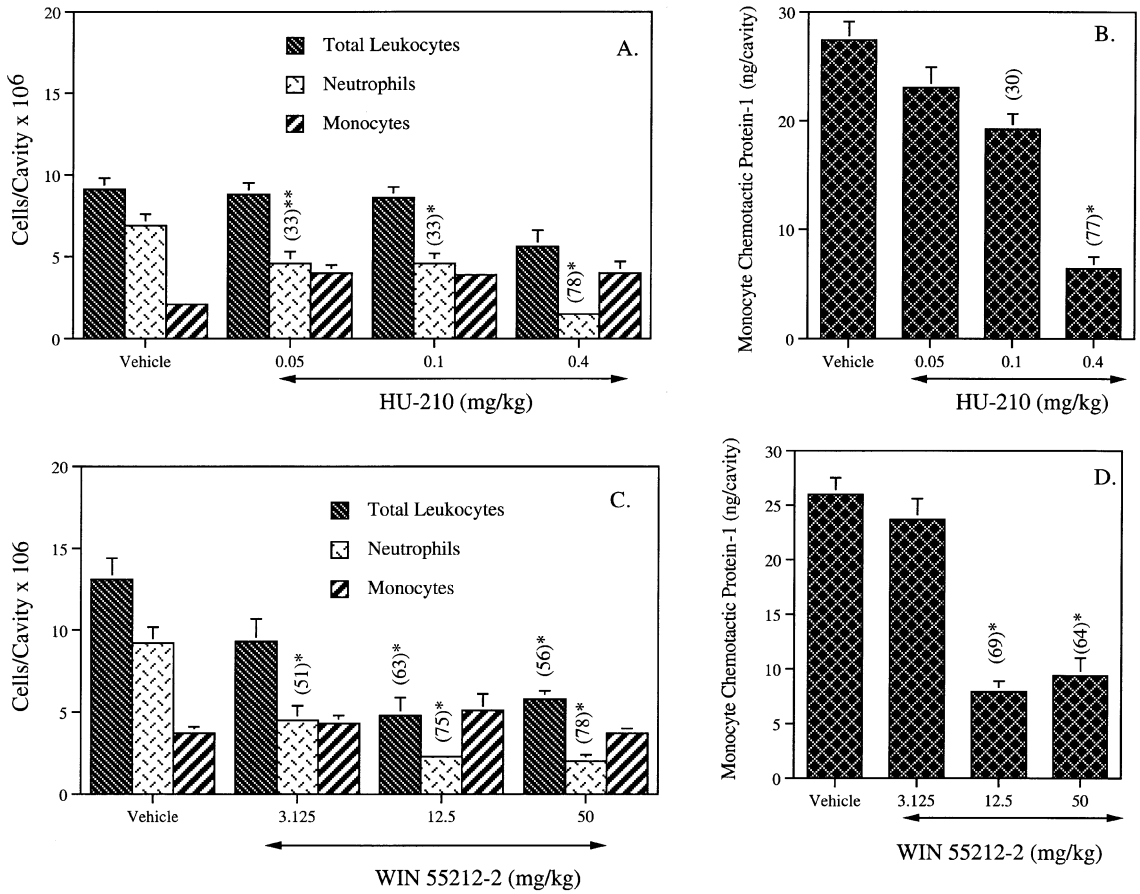

Fig. 1. The dose-dependent inhibitory effects of HU-210 and WIN 55212-2 on cell migration and monocyte chemotactic protein-1 production in mice with a thioglycollate elicited peritonitis. Thioglycollate broth (1 ml) was injected into the peritoneal cavity and exudates harvested by lavage 4 h later. The total cell counts of lavage fluids were obtained by Coulter counting and differential counts were performed on Wright–Giemsa stained cytospin preparations to obtain numbers of neutrophils and monocytes (A and C). The levels of monocyte chemotactic protein-1 in lavage fluids (B and D), were determined by ELISA. The values presented are the mean ± S.E.M. of five mice per treatment group. The number in the parenthesis is the percent decrease in the response when compared to that of vehicle-treated control group. Significantly different from vehicle control group, $^{**}P < 0.05$ and $^{*}P < 0.01$.

cannabinoid CB_1 receptor antagonist appeared to exhibit a somewhat stronger antagonism of the WIN 55212-2 inhibited responses (Fig. 3A and B). SR144528 did not antagonize the inhibitory effects of HU-210 or WIN 55212-2 on the peritoneal inflammation when administered orally to mice at a dose of 10 mg/kg (data not shown). SR144528 was reported to block the binding of a bispecific cannabinoid receptor agonist to cannabinoid CB_2 receptors in mouse spleen with an oral ED_{50} of 0.35 mg/kg (Rinaldi-Carmona et al., 1998). Therefore, the 10 mg/kg dose should have been sufficient to completely prevent cannabinoid receptor agonists from binding to that receptor.

Although the levels of monocyte chemotactic protein-1 were reduced in 4 h peritoneal exudates harvested from cannabinoid receptor agonist-treated mice, the levels of the neutrophil chemoattractants, KC and macrophage inflammatory protein-1 were elevated at this timepoint as compared to the levels in exudates from control mice. This finding was at variance with the decreased numbers of neutrophils found in peritoneal exudates from HU-210 and WIN 55212-2-treated mice. However, as shown in Fig. 4, the levels of KC (Fig. 4A) and macrophage inflammatory protein-2 (Fig. 4B) were decreased by 75% and 50%, respectively in peritoneal exudates harvested from WIN 55212-2-treated mice 1–2 h after thioglycollate. Therefore, the drug did have a suppressive effect on the production of neutrophil chemoattractants, just prior to the peak influx of neutrophils at 4 h. At the 4 h timepoint, the levels of KC and macrophage inflammatory protein-2 in exudates from WIN 55212-2-treated mice, were comparable to the maximum levels detected at 2 h in control exudates. The levels of monocyte chemotactic protein-1 peaked at 4 h in control exudates and were significantly lower at this timepoint in exudates from WIN 55212-2-treated mice (Fig. 4C). However, in 8 h exudates from WIN 55212-2-treated mice, the levels of the chemokine were equivalent to those present at 4 h in exudates from control mice. Thus, the suppressive effects of the drug on the production of both neutrophil and monocyte chemokines were very transient. It is obvious from these data why monocyte chemotactic protein-1

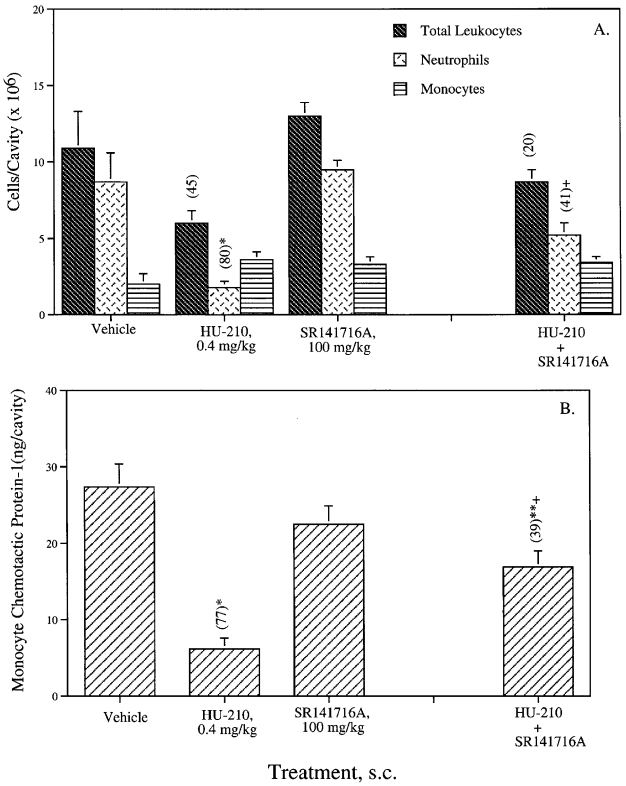

Fig. 2. The inhibitory effects of the cannabinoid receptor agonist HU-210 on neutrophil migration and monocyte chemotactic protein-1 production in mice with thioglycollate-induced peritonitis are partially reversed by SR141716A, a selective canabinoid CB_1 receptor antagonist. SR141716A (100 mg/kg, s.c.) was injected into mice 1 hr before treatment with HU-210 (0.4 mg/kg, s.c.) and induction of the peritonitis. Peritoneal exudates were harvested by lavage 4 h after thioglycollate. The cellular content of exudates is shown in (A) and the levels of monocyte chemotactic protein-1 in (B). The values represent the mean ± S.E.M. of five mice per treatment group. The percent decrease in the response when compared to that of the vehicle-treated control group is shown in the parenthesis. Significantly different from the responses in vehicle-treated control mice, $^{*}P < 0.01$ and $^{**}P < 0.05$. Significantly different from the responses of mice treated with HU-210 alone, $+P < 0.01$.

levels and not those of the neutrophil chemoattractants were used to monitor the suppressive effects of the cannabinoid receptor agonists on chemokine responses in 4 h exudates. HU-210 also caused a delay in the appearance of chemokines in peritoneal exudates (data not shown).

3.3. Antagonism of the anti-inflammatory effects of WIN 55212-2 by centrally administered SR141716A

The ability of SR141716A to antagonize the inhibitory effects of HU-210 (Fig. 2) and WIN 55212-2 (Fig. 3) on thioglycollate-induced cell migration and monocyte chemotactic protein-1 production suggested that the anti-inflammatory effects of the cannabinoid receptor agonists were elicited through cannabinoid CB_1 receptors. Since such receptors are found in the CNS as well as in the periphery, it was of interest to determine whether the anti-inflammatory activities of cannabinoid receptor agonists on the peritoneal inflammation were produced through central or peripheral receptors. To distinguish between these possibilities, SR141716A (100 μg) was injected into mice by the i.c.v. route 1 h prior to treatment with the cannabinoid receptor agonists and induction of the peritoneal inflammation. This dose of SR141716A was previously shown to be effective in blocking the cytokine modulatory effects of peripherally administered HU-210 and WIN 55212-2 (Smith et al., 2000). The results of a representative experiment are shown in Fig. 5. When administered centrally to mice, SR141716A again exhibited a partial antagonism of the inhibited neutrophil migration and monocyte chemotactic protein-1 production caused by HU-210 and WIN 55212-2 when given subcutaneously. Thus, in the experiment shown in Fig. 5, the neutrophil response was suppressed by 72% in mice treated with HU-210 alone and by 30% in mice treated with both HU-210 and SR141716A (Fig. 5A). The response was inhibited by 89% in mice treated with WIN 55212-2, alone and by 57% in mice treated with WIN 55212-2 and

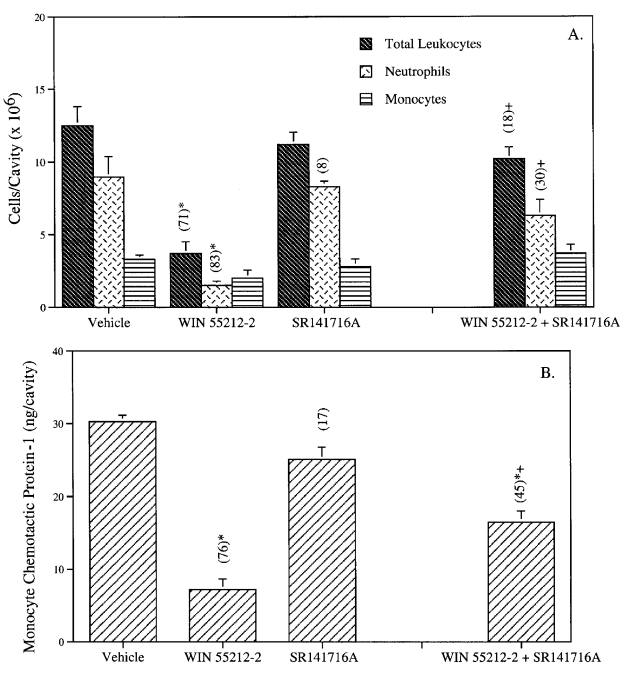

Fig. 3. The reduction in total leukocytes and neutrophils (A) and the levels of monocyte chemotactic protein-1 (B) in thioglycollate-induced peritoneal exudates from mice treated with WIN 55212-2 (50 mg/kg, s.c.). The drug was administered to mice immediately before thioglycollate and peritoneal exudates harvested by lavage 4 h later. SR141716A (100 mg/kg, s.c.) was administered to a group of mice, 1 h before treatment with WIN 55212-2. The values shown are the mean ± S.E.M. of five mice per treatment group. Significantly different from the responses in vehicle-treated control mice, $^{*}P < 0.01$. Significantly different from the responses in mice treated with HU-210 alone, $+P < 0.01$.

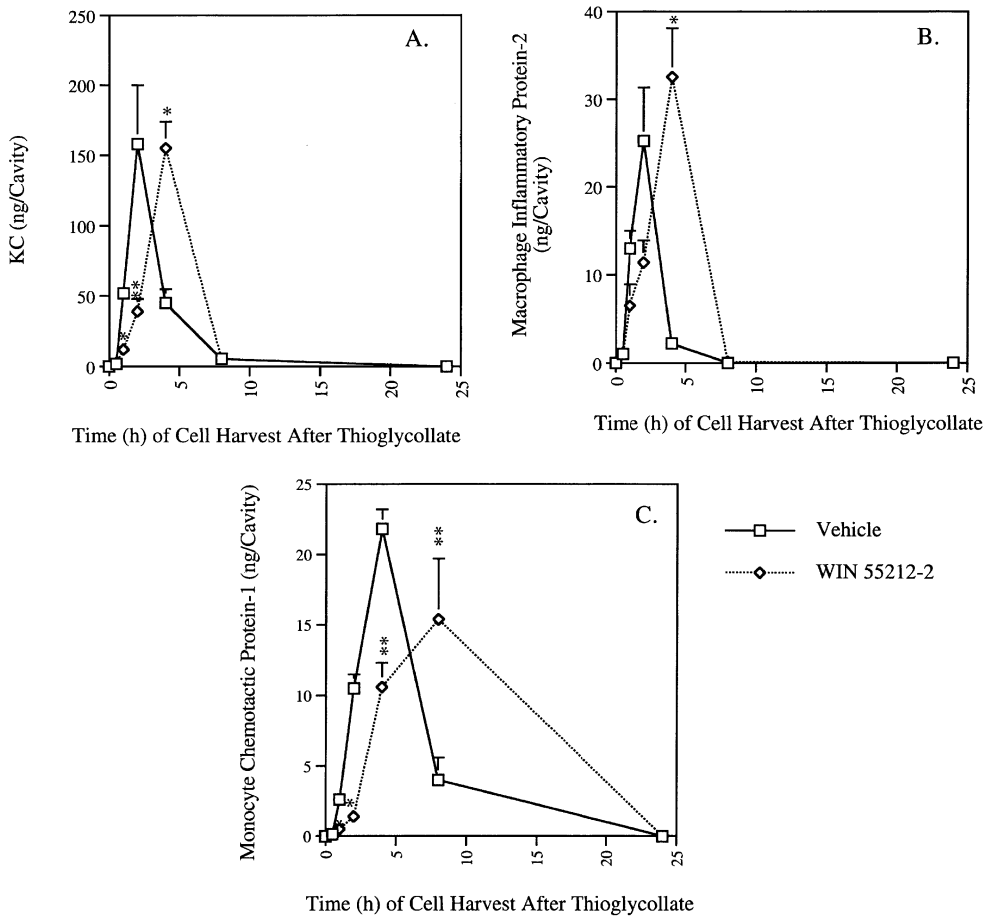

Fig. 4. Time course of the appearance of KC (A), macrophage inflammatory protein-2 (B) and monocyte chemotactic protein-1 (C) in peritoneal exudates harvested from control and WIN 55212-2-treated mice at various times after the injection of thioglycollate. WIN 55212-2 (50 mg/kg, s.c.) was administered to mice immediately before thioglycollate. The values presented are the mean ± S.E.M. of five mice per treatment group. Significantly different from the chemokine levels in exudates from vehicle treated control mice, $^{*}P < 0.01$ and $^{**}P < 0.05$.

SR141716A (Fig. 5C). The inhibitory effects of HU-210 and WIN 55212 on monocyte chemotactic protein-1 production were also partially antagonized by SR141716A (Fig. 5B and D). This partial antagonism of the anti-inflammatory activities of HU-210 and WIN 55212-2 by centrally administered SR141716A indicated some involvement of central cannabinoid CB_1 receptors in these effects.

It is also evident from the results presented in Fig. 5, that SR144528 did not antagonize the inhibitory effects of HU-210 and WIN 55212-2 on neutrophil migration and monocyte chemotactic protein-1 production. The responses were inhibited to the same extent in the presence and absence of the cannabinoid CB_2 receptor antagonist. However, SR144528 did cause a 50% reduction in the number of infiltrating neutrophils (Fig. 5A and C) and levels of monocyte chemotactic protein-1 (Fig. 5B and D) when injected alone into mice. SR141716A did not antagonize these effects of SR144528 and in fact, the levels of inhibition achieved with a combination of the antagonists suggested that the effects of the two agents were somewhat complementary. SR144528 did not cause the behavioral changes that are typically seen when cannabinoid receptor agonists interact with the central CB_1 receptor. Thus, in contrast to HU-210 and WIN 55212-2, there was no evidence of a role for central cannabinoid CB_1 receptors in the anti-inflammatory effects of SR144528. As previously shown for its cytokine modulatory effects (Smith et al., 2001), SR144528 was only effective when administered i.c.v. and did not cause changes in neutrophils or monocyte chemotactic protein-1 when administered systemically to mice.

3.4. Inhibition of thioglycollate-induced neutrophil migration and monocyte chemotactic protein-1 production by centrally administered HU-210 and WIN 55212-2

The most compelling evidence for the involvement of central cannabinoid CB_1 receptors in the inhibitory effects of HU-210 and WIN 55212-2 on the thioglycollate-in-

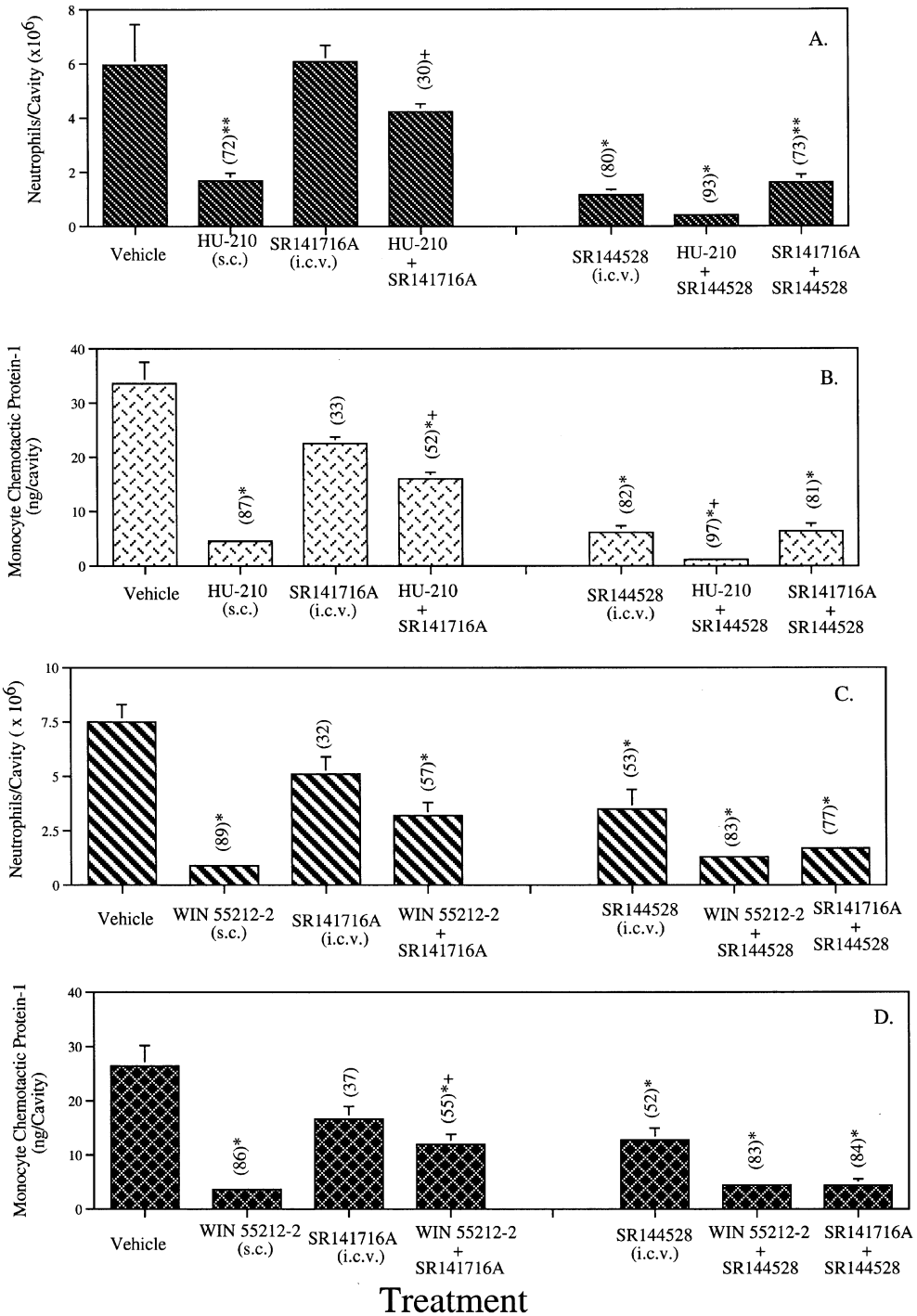

Fig. 5. Partial reversal of the inhibitory effects of peripherally administered HU-210 (0.4 mg/kg, s.c.) and WIN 55212-2 (50 mg/kg, s.c.) on cell migration and monocyte chemotactic protein-1 production by central SR141716A (100 μg, i.c.v). Neutrophil responses are shown in (A) and (C) and monocyte chemotactic protein-1 levels in (B) and (D). SR144528 (100 μg, i.c.v.) did not reverse the anti-inflammatory activities of the cannabinoid receptor agonists. Significantly different from the responses in vehicle-treated control mice, $^{*}P$, 0.01, P, 0.05. Significantly different from the responses in mice treated with HU-210 or WIN 55212-2 alone, $+P < 0.01$.

duced peritoneal inflammation was obtained in experiments where the cannabinoid receptor agonists were injected into mice by the i.c.v. route (Fig. 6). The drugs were found to block neutrophil migration (Fig. 6A and C) and monocyte chemotactic protein-1 production (Fig. 6B and D) in a dose-related manner. The levels of inhibition caused by 100 μg WIN 55212-2 (approximately 4 mg/kg) and 2.5 μg HU-210 (approximately 100 μg/kg) were

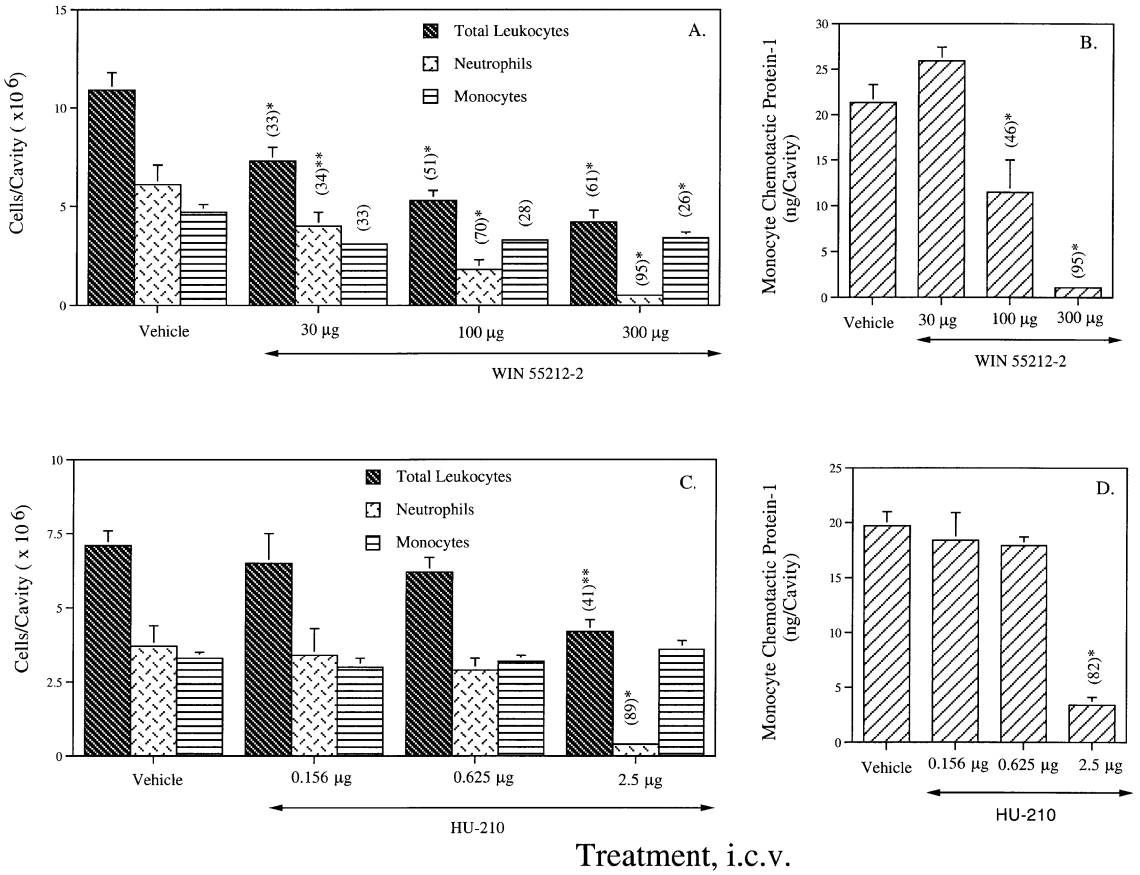

Fig. 6. The dose-dependent inhibitory effects of WIN 55212-2 and HU-210 on neutrophil migration (A and C) and monocyte chemotactic protein production (B and D) when administered to mice, i.c.v., at the time of the induction of a peritonitis with thioglycollate. The values shown are the mean ± S.E.M. of five mice per treatment group. Significantly different from the responses in vehicle-treated control mice, $^{*}P$, 0.01, $^{**}P < 0.05$.

comparable to those obtained when much higher doses of the drugs were administered to mice by the s.c. route. Thus, the findings with both cannabinoid receptor agonists and the cannabinoid CB_1 receptor antagonist strongly suggest that the anti-inflammatory activities of HU-210 and WIN 55212-2 are mediated through the central cannabinoid CB_1 receptor.

3.5. The monocytic component of the thioglycollate-induced peritoneal inflammation is unaffected by cannabinoid receptor agonist treatment

As indicated above, there were similar numbers of monocytes in 4 h peritoneal exudates from control and cannabinoid receptor agonist-treated mice despite the ability of HU-210 and WIN 55212-2 to markedly inhibit the production of monocyte chemotactic factor-1. To confirm the inability of HU-210 and WIN 55212-2 to affect monocyte accumulation, the drugs were administered to mice daily for 2–3 days after thioglycollate so that exposure to the drugs coincided with the peak of the monocyte response. This therapeutic regimen had no effect on monocyte accumulation in the peritoneal cavity when this response was assessed 4–72 h after thioglycollate (data not shown).

3.6. Comparative effects of cannabinoid agonists on thioglycollate-induced peritonitis in wild-type and cannabinoid CB_1 receptor deficient mice

As shown in Fig. 7, HU-210 and WIN 55212-2 strongly inhibited neutrophil migration and monocyte chemotactic protein-1 production in wild-type mice, but not in cannabinoid CB_1 receptor knockout mice. The drugs were administered by the s.c. route in these experiments in order to ascertain the involvement of receptors in the periphery as well as in the CNS. In the experiment shown, the total number of leukocytes was reduced by 51% and the number of neutrophils by 74% in exudates harvested from WIN 55212-2 treated wild-type mice (Fig. 7A). The monocyte chemotactic protein-1 levels in exudates were reduced by 64% (Fig. 7B). These components of the peritoneal inflammation in knockout mice were unaffected by treatment with WIN 55212-2. HU-210 also suppressed the neutrophil (68%, Fig. 7C) and monocyte chemotactic protein-1 (44%,

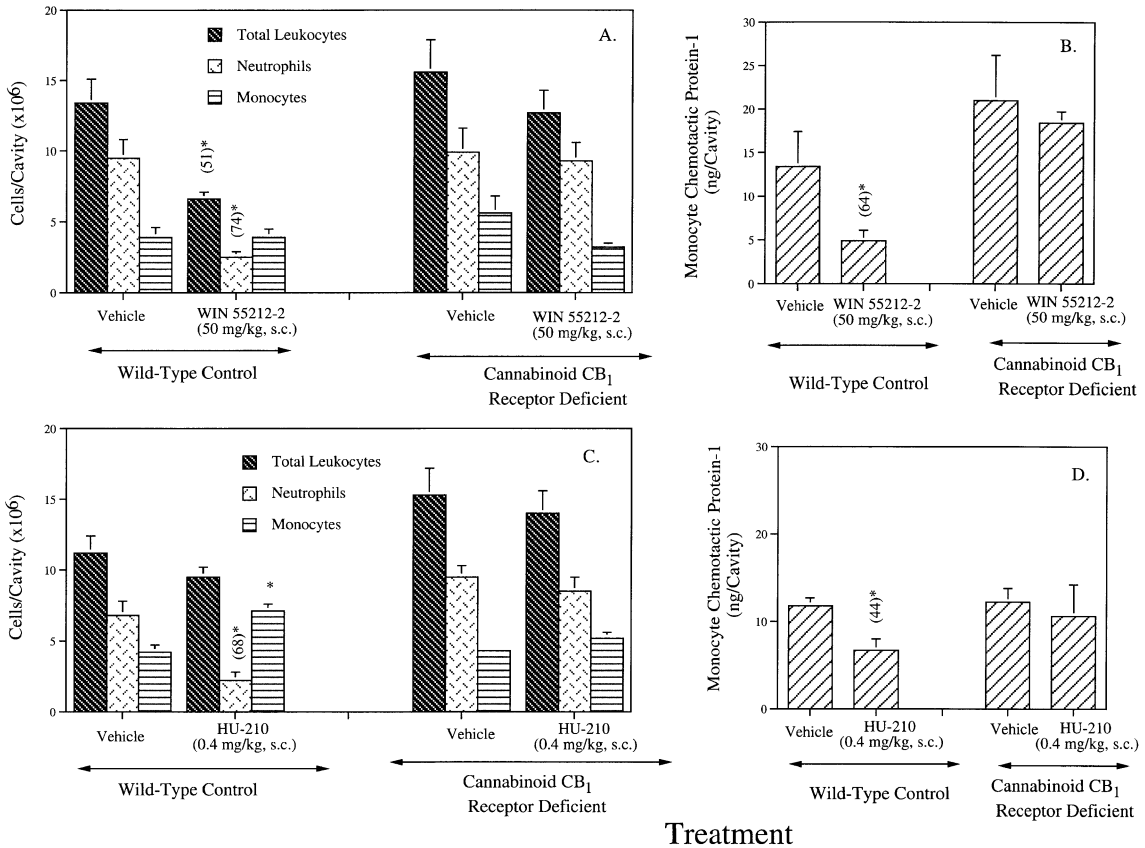

Fig. 7. The failure of WIN 55212-2 (50 mg/kg, s.c.) and HU-210 (0.4 mg/kg, s.c.) to inhibit neutrophil migration (A and C) and monocyte chemotactic protein-1 production (B and D) in cannabinoid CB_1 receptor KO mice with a thioglycollate-induced peritonitis. Significantly different from the responses in vehicle-treated control mice, $^{*}P < 0.01$.

Fig. 7D) responses in wild-type mice, but not in cannabinoid CB_1 receptor knockout mice. The failure of the cannabinoid receptor agonists to alter the inflammatory response in cannabinoid CB_1 receptor knockout mice suggested that their effects were produced through cannabinoid CB_1 receptors and that peripheral cannabinoid CB_2 receptors had no role in their anti-inflammatory activities.

3.7. Effects of HU-210 and WIN 55212-2 on a staphylococcus enterotoxin A-induced peritonitis

In view of the selective inhibitory effects of HU-210 and WIN 55212-2 on neutrophil migration in a peritonitis induced by thioglycollate where both neutrophils and monocytes migrate into the peritoneal cavity, it was of interest to determine whether similar effects on neutrophils could be observed in other peritonitis models. Staphylococcus enterotoxin A is one of several staphylococcal enterotoxins that have been used to induce an experimental enterotoxemia in primates in which there is an increase in neutrophils in the circulation 24 h after the treatment (Zehavi-Willner et al., 1984). The enterotoxin has also been shown to cause a dose-dependent increase in neutrophils when injected into the peritoneal cavities of mice, an effect that persists for at least 24 h (De Souza and Ribeiro-DaSilva, 1998). These latter results were confirmed in the present study, where an increase in neutrophil counts was observed within 2 h of an injection of staphylococcus enterotoxin A (16–64 μg) into the peritoneal cavity of Balb/c mice. An experiment representative of three in which the time course of the appearance of neutrophils and chemokines in peritoneal exudates from staphylococcus enterotoxin A-treated mice was determined is shown in Fig. 8. Neutrophils were present in exudates harvested 1 h after the enterotoxin injection and substantial numbers of the cells were still present in the peritoneal cavity at 24 h (Fig. 8A). The enterotoxin also triggered an early production of chemokines, with peak responses ocurring 1–2 h after induction of the peritonitis (Fig. 8B,C and D).

The ability of HU-210 and WIN 55212-2 to inhibit neutrophil migration when given to mice by the s.c. or i.c.v. routes was evident in this model as well. The suppressive effects of the drugs on the peritoneal inflammation when administered to mice by the i.c.v. route at the

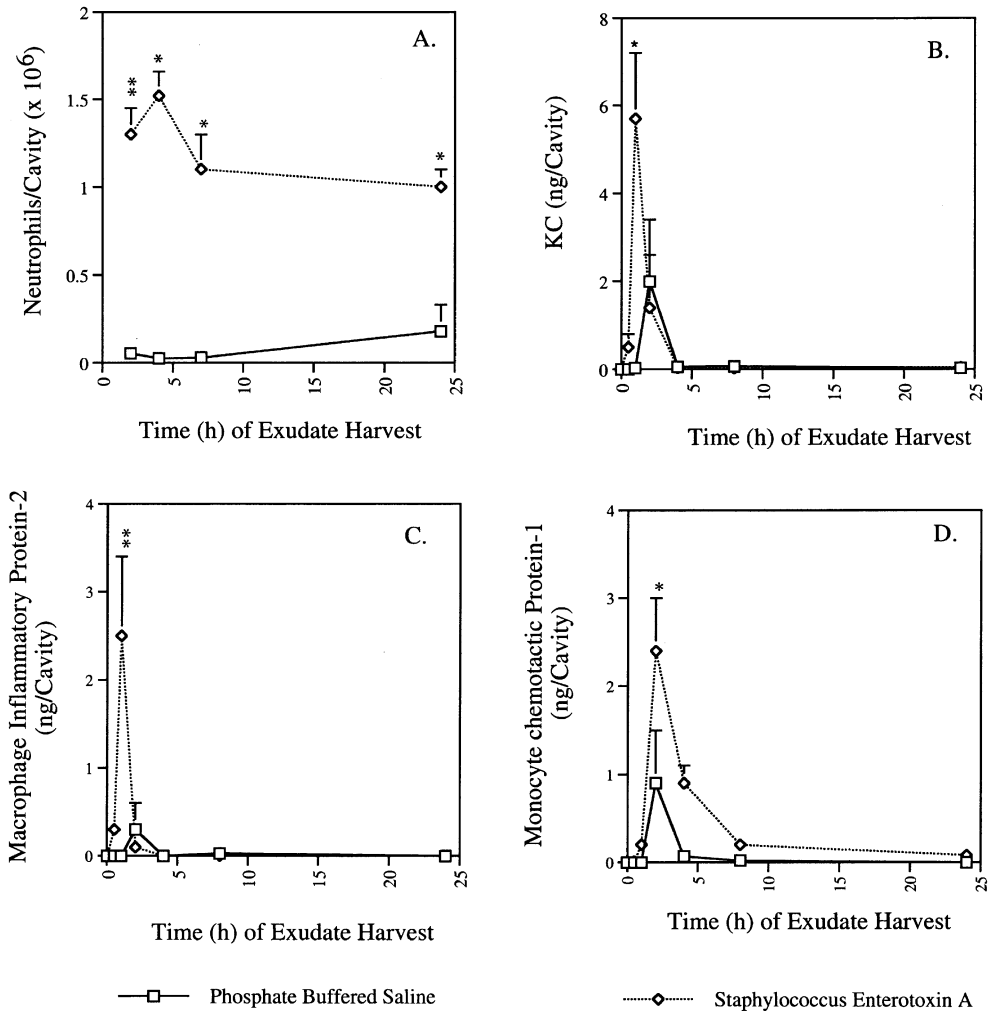

Fig. 8. Time course of the appearance of the neutrophils (A), KC (B) macrophage inflammatory protein-2 (C) and monocyte chemotactic protein-1 (D) in peritoneal exudates from mice injected i.p. with staphylococcus enterotoxin A (16 μg/mouse). Control mice received phosphate buffered saline, i.p. The values shown are the mean ± S.E.M. of five mice per treatment group. Significantly different from the response in mice injected with phosphate buffered saline, $^{*}P < 0.01$, $^{**}P < 0.05$.

time of the induction of the peritonitis is shown in Fig. 9. The doses administered to mice in this experiment were those found to be strongly effective in inhibiting neutrophil migration and monocyte chemotactic protein-1 production in the thioglycollate-induced peritonitis model. Both HU-210 (0.625 μg) and WIN 55212-2 (100 μg) caused a significant inhibition of the enterotoxin-induced peritoneal neutrophil (Fig. 9A) and monocyte chemotactic protein-1 (Fig. 9B) responses. As in the thioglycollate peritonitis model, SR141716A exhibited a more pronounced antagonism towards the anti-inflammatory effects of WIN 55212-2 than those of HU-210. Thus, in the experiment shown in Fig. 9, WIN 55212-2 caused a 97% reduction in the staphylococcus enterotoxin A-induced neutrophil response, an effect that was completely prevented by simultaneous treatment with SR141716A (Fig. 9A). The neutrophil influx was inhibited by 67% following treatment with HU-210 alone and by 48% in mice treated with both HU-210 and SR141716A. The antagonism by SR141716A of the HU-210- and WIN 55212-2-mediated inhibition of monocyte chemotatic protein-1 production paralleled that observed in the neutrophil response. The marked reduction (98%) in monocyte chemotactic protein-1 caused by treatment with WIN 55212-2 was blocked completely by SR141716A, because the levels in exudates from mice treated with WIN 55212-2 and SR141716A were equivalent to those in exudates harvested from mice treated with the cannabinoid receptor antagonist alone (35%). The monocyte chemotactic protein-1 levels in exudates from mice treated with HU-210 alone and in combination with SR141716A were decreased by 91% and 59%, respectively, again indicating a partial antagonism of the HU-210-mediated effect. As in the thioglycollate-induced peritonitis, there was no antagonism of the anti-inflammatory effects of HU-210 and WIN 55212-2 by SR144528. However, the drug did exhibit a capacity to block neutrophil

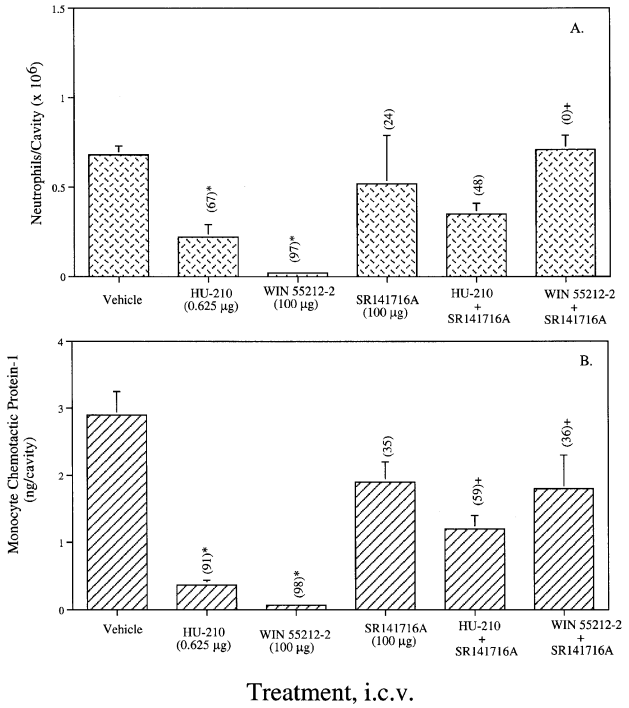

Fig. 9. Suppression of staphylococcus enterotoxin A-induced neutrophil migration (A) and monocyte chemotactic protein-1 production (B) by centrally administered HU-210 (0.625 μg, i.c.v.) and WIN 55212-2 (100 μg, i.c.v.). The ability of SR141716 (100 μg, i.c.v.) to block the inhibitory effects of the cannabinoid receptor agonists is also shown. The cannabinoid CB_1 receptor antagonist was administered to mice simultaneously with HU-210 or WIN 55212-2. The values presented represent the mean ± S.E.M. of five mice per treatment group. Significantly different from the responses in vehicle-treated control mice, $^{*}P < 0.01$. Significantly different from the responses in mice treated with HU-210 or WIN 55212-2 alone, $+P < 0.01$.

migration and monocyte chemotactic protein-1 production in this model as well, when injected alone into mice by the i.c.v. route. (data not shown).

4. Discussion

In this study, we have characterized the anti-inflammatory activities of two non-selective cannabinoid receptor agonists in mouse peritonitis models. Both HU-210 and WIN 55212-2 were found to block an influx of neutrophils into the peritoneal cavity following an intraperitoneal injection of thioglycollate or staphylococcus enterotoxin A. The attenuation of neutrophil migration by HU-210 and WIN 55212-2 appeared to involve central cannabinoid CB_1 receptors because the effect was observed whether the cannabinoid receptor agonists were administered centrally or peripherally. Furthermore, the ability of HU-210 and WIN 55212-2 to inhibit neutrophil recruitment was antagonized by SR141716A, a selective cannabinoid CB_1 receptor antagonist, but not by SR144528, a selective cannabinoid CB_2 receptor antagonist. These effects of the cannabinoid receptor agonists on neutrophil migration appeared to be due to an early suppression of KC and macrophage inflammatory protein-2 when cells resident in the peritoneal cavity were exposed to thioglycollate or staphylococcus enterotoxin A. There is also a large influx of monocytes into the peritoneal cavity in a thioglycollate-induced peritonitis. Monocyte chemotactic protein-1 was detected in exudates harvested from control mice 2–4 h after thioglycollate, but not in exudates harvested at the peak of the monocyte response (24–48 h). This suggests that the early production of the chemokine is sufficient to trigger monocyte migration. The transient appearance of monocyte chemotactic protein-1 in peritoneal exudates from thioglycollate, zymosan and endotoxin injected mice has been described (Aujuebor et al., 1998, 1999). Although monocyte chemotactic protein-1 levels were reduced in 4 h exudates harvested from WIN 55212-2 and HU-210-treated mice, there was never any indication of a depressed monocyte response. Therefore, monocyte chemotactic protein-1 may not be the dominant monocyte chemoattractant in this model and other monocyte chemotactic factors may be produced just prior to and/or during the peak monocyte response. Other chemokines known to recruit monocytes to sites of inflammation include regulated upon activation, normal T cell expressed and secreted (RANTES), the macrophage inflammatory proteins (MIP-1α and MIP-1β) and interferon-γ-inducible protein 10 (IP-10). However, HU-210 and WIN 55212-2 did not alter monocyte accumulation in the peritoneal cavity even when treatment was given prior to as well as during the peak of the monocyte response. Thus, it appears unlikely that the cannabinoid receptor agonists have the capacity to alter monocyte accumulation by suppressing the production of monocyte chemoattractants including those that might be produced during the peak monocyte response.

It has been reported that zymosan-induced neutrophil accumulation and monocyte chemotactic protein-1 production are greatly diminished when resident mast cells have been depleted from the peritoneal cavity by treatment with compound 48/80 (Aujuebor et al., 1999). This indicated a role for resident mast cells in neutrophil accumulation and monocyte chemotactic protein-1 production in zymosan-induced peritonitis. It was also reported that the depletion of peritoneal mast cells had no effect on the accumulation of neutrophils in the peritonitis caused by thioglycollate. Although neutrophil responses to thioglycollate were shown to be unaffected by mast cell depletion (Aujuebor et al., 1999), the levels of neutrophil and monocyte chemoattractants in exudates were not assessed. However, it is unlikely that KC and macrophage inflammatory protein-2 are derived from mast cells since even a transient decrease in neutrophil chemoattractants seems to result in a reduced neutrophil infiltrate as was observed here. A similar effect should have been observed upon mast cell depletion if these cells are the sources of KC and macrophage inflammatory protein-2 which appear to be the chemokines that

direct neutrophil migration in this model. Nevertheless, mast cells cannot be completely excluded as potential sources of the chemokines in the absence of comparative data on their levels in exudates from wild-type and mast cell deficient mice. The appearance of monocyte chemotactic protein-1, KC and macrophage inflammatory protein-2 in peritoneal exudates shortly after the injection of thioglycollate and before inflammatory cells began to infiltrate the cavity suggests that they are produced by a resident peritoneal cell(s). It is likely that both of the neutrophil chemoattractants originate from the same resident cell because they exhibit an identical time course of appearance in thioglycollate-induced exudates from untreated mice as well as in exudates from mice treated with cannabinoid receptor agonists where their peak production is somewhat delayed. Substantial levels of monocyte chemotactic protein-1 are also present in peritoneal exudates at the time of the peak production of KC and macrophage inflammatory protein-2. Therefore, it is distinctly possible that all three chemokines are derived from the same resident cell. Aside from mast cells, potential sources of the chemokines in thioglycollate peritonitis include resident macrophages (Aujuebor et al., 1999), endothelial cells (Rollins et al., 1990) and mesothelial cells (Antony et al., 1995; Topley et al., 1996).

As in previous studies of cytokine modulation (Smith et al., 2001), there are two observations in the present work which suggest that the inhibitory effects of HU-210 and WIN 55212-2 on chemokine production and neutrophil migration are mediated through central cannabinoid CB_1 receptors. First, when the drugs were administered by the i.c.v. route, the doses required to inhibit neutrophil chemoattractants and neutrophil migration were considerably lower than the effective subcutaneous doses. Second, when HU-210 and WIN 55212-2 were administered to mice by the s.c. route, the cannabinoid CB_1 receptor antagonist, SR141716A, was able to block the effects of the cannabinoid receptor agonists, on the peritoneal inflammation when given centrally or peripherally to mice. The antagonism by centrally administered SR141716A occurred at relatively low doses that failed to produce such effects when the drug was administered peripherally (s.c.). Together, these findings strongly suggest that the inhibitory effects of the cannabinoid receptor agonists on the peritoneal inflammation were mediated through the central cannabinoid CB_1 receptor.

The ability of HU-210 and WIN 55212-2 to block neutrophil migration in peritonitis models as demonstrated here is in contrast to the effects that were observed in vitro when the drugs were cultured with human embryonic kidney cells transfected with the human cannabinoid CB_1 receptor gene (Song and Zhong, 2000). Both agents were found to cause a concentration-dependent migration of the transfected cells, an effect that was also shown to be antagonized by SR141716A. In attempting to reconcile the differences between the in vitro and in vivo effects of HU-210 and Win 55212-2, it should be emphasized that in the present in vivo study, their inhibitory effects on neutrophil migration appeared to result primarily from their capacity to delay the production of neutrophil chemoattractants. This indirect effect on neutrophil migration in vivo is in contrast to the direct stimulatory effects that were shown when the cannabinoid receptor agonists were added in vitro to cells transfected with the cannabinoid CB1 receptor. It was suggested that the cannabinoid receptor agonist-induced migration of cannabinoid CB1 receptor transfected cells in vitro involved the activation of a mitogen-activated protein kinase (Song and Zhong, 2000). HU-210 was observed to have no effect on MIP-2-induced neutrophil chemotaxis in vitro (Kulkarni-Narla et al., 2001). This suggests that while HU-210 can inhibit the production of MIP-2, it does not block neutrophil migration in response to the chemokine. It should be noted that the in vivo data presented here are consistent with those obtained in a model of pulmonary inflammation in which WIN 55212-2 was shown to block neutrophil recruitment to the lungs of mice in response to inhaled lipopolysaccharide, (Berdyshev et al., 1998).

SR144528, the highly selective cannabinoid CB_2 receptor antagonist, was able to inhibit neutrophil migration and chemokine production when administered centrally to mice with a peritoneal inflammation that was elicited with thioglycollate or staphylococcus enterotoxin A. As with cytokine modulation (Smith et al., 2001), there was no reversal of the inhibitory effects of central SR144528 on neutrophil responses by SR141716A, the selective cannabinoid CB_1 receptor antagonist. These results give further evidence of the ability of SR144528 to alter inflammatory responses through activation of a central receptor that differs from the central cannabinoid CB_1 receptor. We have obtained some preliminary evidence in cannabinoid CB_1/CB_2 receptor double knockout mice which suggests that the anti-inflammatory activities of central SR144528 do not involve either the cannabinoid CB_1 or CB_2 receptor subtypes.

In summary, the results of this study indicate that cannabinoid receptor agonists can block neutrophil migration in mouse peritonitis models by delaying the production of the chemotactic factors KC and macrophage inflammatory protein-2. The ability of the HU-210 and WIN 55212-2 to cause such changes when given to mice centrally or peripherally and the antagonism of these effects by centrally administered SR141716A suggests that their anti-inflammatory activities result from the activation of central cannabinoid CB_1 receptors. The selective cannabinoid CB_2 receptor antagonist SR144528 produces similar anti-inflammatory effects when administered centrally to mice, but central cannabinoid CB_1 receptors do not appear to be involved. The mechanism(s) by which these various cannabinoid receptor ligands produce their effects through interactions with centrally located receptors is under intense investigation.

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