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Relative involvement of cannabinoid CB_1 and CB_2 receptors in the Δ^9-tetrahydrocannabinol-induced inhibition of natural killer activity

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Abstract

We demonstrated that in vivo administration of Δ^9-tetrahydrocannabinol in mice (15 mg/kg s.c.) significantly inhibited natural killer cell (NK) cytolytic activity without affecting Concanavalin A (ConA)-induced splenocyte proliferation. Moreover, we investigated the effect of in vivo pretreatment with cannabinoid receptor antagonists, namely, the selective cannabinoid CB_1 receptor antagonist SR 141716 [*N*-piperidin-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide] and the selective cannabinoid CB_2 receptor antagonist SR 144528 {*N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide}, on Δ^9-tetrahydrocannabinol-induced inhibition of NK cytolytic activity. Both antagonists partially reversed the Δ^9-tetrahydrocannabinol inhibition of NK cytolytic activity, although the cannabinoid CB_1 receptor antagonist was more effective than the cannabinoid CB_2 receptor antagonist. The parallel measurement of interferon γ and interleukin 2 levels revealed that Δ^9-tetrahydrocannabinol significantly reduced (about 70%) the former cytokine without affecting the latter. Cannabinoid CB_1 and CB_2 receptor antagonists completely reversed the interferon γ reduction induced by Δ^9-tetrahydrocannabinol. Our results indicate that both types of cannabinoid receptors are involved in the complex network mediating NK cytolytic activity.

Keywords: Δ^9-Tetrahydrocannabinol; SR 141716; SR 144528; Natural killer cell cytolytic activity; Interferon γ

1. Introduction

Besides their psychotropic effects, cannabinoids have been widely described as influencing immune function (Adams and Martin, 1996). Their mechanisms of action are still questioned, although the recent identification of specific functional cannabinoid receptors on immune cells suggests that cannabinoid immunological effects could be mediated at least partly by a highly specific receptor-associated mechanism. This is supported by the expression of the cannabinoid CB_1 receptor in the immune system (Parolaro, 1999) and the cloning of a second cannabinoid receptor, designated CB_2 (Munro et al., 1993), that is mainly present in cells of immune origin.

To date, no specific cannabinoid agonist that can discriminate between cannabinoid receptor subtypes has been described, and thus all the immunological studies have been performed using cannabinoid receptor agonists that bind both cannabinoid CB_1 and CB_2 receptors. In contrast, a selective cannabinoid CB_1 receptor antagonist, SR 141716, has been described and used to determine the role of the cannabinoid CB_1 receptor in cannabinoid-induced immunomodulation. The recent development of the first potent and specific antagonist for the cannabinoid CB_2 receptor, SR 144528 (Rinaldi-Carmona et al., 1998), provides a new tool to better discriminate the relative involvement of cannabinoid CB_1 and CB_2 receptors in the immunomodulatory effect of cannabinoids. We report here the effect of in vivo pretreatment with cannabinoid CB_1 and CB_2 receptor antagonists in mice on the immunosuppression induced by a psychotropic dose of Δ^9-tetrahydrocannabinol, as investigated by measuring splenocyte proliferation, natural killer cell (NK) cytolytic activity and cytokine production.

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2. Material and methods

2.1. Animals

Male Swiss mice (Charles River, Calco, Italy), 20–25 g body weight were used, fed on a pellet diet (Altromin-Rieper, Bolzano, Italy) with water ad libitum. Environmental conditions were standardized (22 ± 2°C, 60% humidity and 12 h artificial lighting per day).

2.2. Drugs

Δ^9-Tetrahydrocannabinol (a generous gift from National Institute of Drug Abuse, USA) was dissolved in 1:1:18 Cremophor (Sigma), ethanol, saline and injected s.c. at a dose of 15 mg/kg in a volume of 0.1 ml/10 g of body weight. As previously reported (Massi et al., 1998), this dose possesses a psychotropic action, as demonstrated by the presence of a full analgesic effect. The cannabinoid CB_2 receptor antagonist SR144528 {*N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide} was dissolved in 1% Tween-80, 2% dimethyl sulfoxide (DMSO) and 97% distilled water and administered p.o. at a dose of 10 mg/kg in a volume of 0.2 ml/10 g of body weight. According to kinetic parameters of SR144528 binding after in vivo administration (Rinaldi-Carmona et al., 1998), the antagonist was injected 90 min before Δ^9-tetrahydrocannabinol and mice were killed 1 h after the cannabinoid injection. The cannabinoid CB_1 receptor antagonist SR141716 [*N*-piperidin-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide] was suspended in 10% Tween-80, 20% DMSO and 70% distilled water and injected i.p. at the dose of 3 mg/kg in a volume of 0.1 ml/10 g of body weight. The compound was administered 30 min before Δ^9-tetrahydrocannabinol and mice were killed 1 h after the cannabinoid injection. Both the SR compounds were a generous gift from Dr. M. Mossé and Dr. F. Barth, Sanofi Recherche, Montpellier, France.

2.3. Collection of cells

When the mice were killed 1 h after the injection of Δ^9-tetrahydrocannabinol, the spleen was aseptically removed and cells were teased out using 20-gauge sterile needles through an incision made in the spleen cuticle (Manfredi et al., 1993) into Dulbecco's phosphate-buffered Saline (PBS) and washed twice with the same buffer. The cells were counted in Trypan blue and used for immunological testing.

2.4. Splenocyte proliferation

Microcultures of 4×10^6 splenocytes were set up in RPMI 1640, 2×10^{-5} M 2-mercaptoethanol, 10% fetal calf serum ± Concanavalin A (ConA: 2.5 and 5 μg/ml) in 96-well microtiter plates. After a 48-h incubation at 37°C, 1 μCi of [^{3}H]thymidine (specific activity 2 Ci/mmol, Amersham, UK) was added to all cultures. Cells were harvested 18 h later by an automated cells harvester (Flow Laboratories, Norway) and radioactivity was measured in a liquid scintillation counter (Packard, Downers Grove, IL, USA). Background values, i.e., [^{3}H]thymidine incorporation of unstimulated cells, were subtracted from mitogen-induced proliferation data (Manfredi et al.,1993). The two ConA concentrations were selected to achieve submaximal and maximal stimulation of proliferation. Proliferation is expressed as the mean of triplicate counts per minute (cpm) for the samples from each mouse.

2.5. NK activity of spleen lymphocytes

NK activity was evaluated in a 4-h [^{51}Cr] release assay. Briefly, 5×10^6 YAC-1 tumor cells, derived from a Moloney leukemia virus-induced lymphoma in A/SN mice and used as the target cell line, were labeled by incubation with 100 μCi of [^{51}Cr]sodium chromate (Amersham, UK, specific activity 250–500 mCi/mg chromium) in 0.2 ml of RPMI 1640 + 10% fetal calf serum for 1 h at 37°C under a 5% CO_2–95% air atmosphere. After three washes, the YAC-1 cells were suspended in RPMI with 10% fetal calf serum at a concentration of 10^5 cells/ml. [^{51}Cr]-labeled YAC-1 (10^4 cells/well) was incubated with splenic lymphocytes (effector cells) in 96-well microtiter plates at effector:target cell ratios (E:T) of 100:1 and 200:1. Each E:T ratio was tested in triplicate. After a 4-h incubation at 37°C in 5% CO_2–95% air atmosphere, plates were centrifuged at $400 \times g$ for 5 min, and 100 μl of supernatant was removed from each well and counted in a Packard γ-counter. Maximum [^{51}Cr] release and spontaneous release were determined in wells containing 3 M HCl or medium, respectively. Specific [^{51}Cr] release (specific lysis) was calculated according to the following formula: $100 \times$ [(experimental release − spontaneous release)/(maximal release − spontaneous release)].

2.6. Measurement of interleukin 2 and interferon γ level

Spleen cells from mice were collected, adjusted to 4×10^6 per ml in RPMI 1640, 2×10^{-5} M β-mercaptoethanol, 10% fetal calf serum, and incubated for 24 h with and without 10 μg/ml of ConA. The concentration of ConA was chosen after a series of titrations as being optimal for cytokine production. The supernatants of the cultures were collected and stored at −80°C until tested.

Interleukin 2 was determined in 24-h supernatants with a mouse cytokine enzyme-linked immunosorbent assay (ELISA) protocol, as standardized by Pharmingen (San Diego, CA, USA). Briefly, the anti-interleukin 2 capture monoclonal antibody (Pharmingen) was adsorbed onto a polystyrene 96-well plate and the interleukin 2 in the

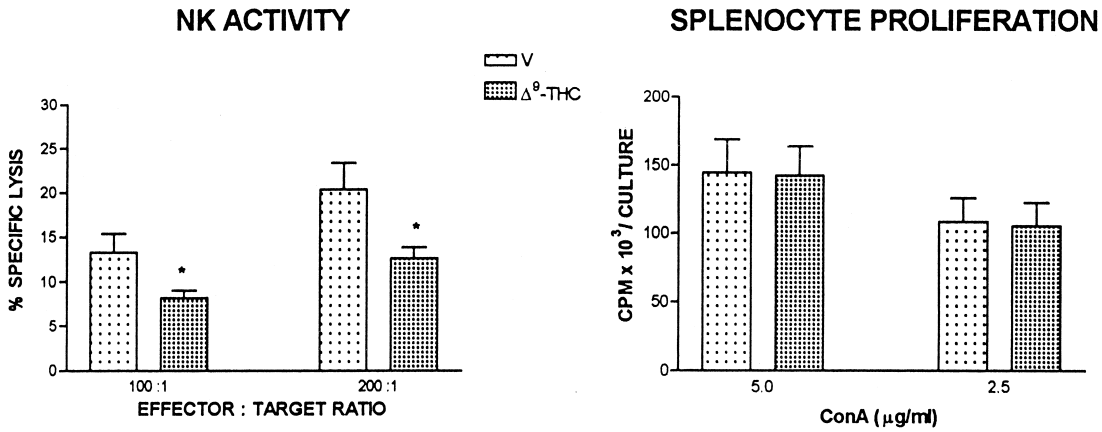

Fig. 1. NK cytolytic activity and splenocyte proliferation in response to ConA in mice after an acute injection of Δ^9-tetrahydrocannabinol (Δ^9-THC, 15 mg/kg s.c.). Mean ± S.E.M. for at least six mice. $^*P < 0.05$ vs. vehicle (Bonferroni's test).

sample was bound to the antibody-coated wells. The biotinylated anti interleukin 2 detecting monoclonal antibody (Pharmingen) was added to bind the interleukin 2 captured by the first antibody. After a wash step, avidin-peroxidase (Sigma) was added to the wells to bind the biotynilated detecting antibody and finally 2,2′-azino-bis (3-ethylbenzathiazoline-6-sulfonic acid, ABTS, Sigma) substrate was added. A colored product was formed in proportion to the amount of interleukin 2 present in the sample, and was measured at O.D. 405 nm.

Interferon γ was measured in 24-h supernatants using the same ELISA protocol outlined above except that anti-interferon γ capture and detecting antibody (Pharmingen) were used at 2 and 1 μg/ml, respectively.

2.7. Statistical analysis

Data are presented as means ± standard error. Data on ConA-induced proliferation, NK activity and cytokine production were evaluated by one-way or two-way analysis of variance (ANOVA), followed by Bonferroni's test for multiple comparisons.

3. Results

Fig. 1 shows the effects of acute s.c. injection of Δ^9-tetrahydrocannabinol on the cytotoxic activity of NK cells and on ConA-induced splenocyte proliferation. The cannabinoid significantly reduced the cytolytic NK activity at both considered effector:target cell ratios (38%). In contrast, Δ^9-tetrahydrocannabinol did not affect the proliferative response of splenocytes at the two tested mitogen concentrations.

In order to assess the relative involvement of cannabinoid CB_1 and CB_2 receptors in the inhibition of NK

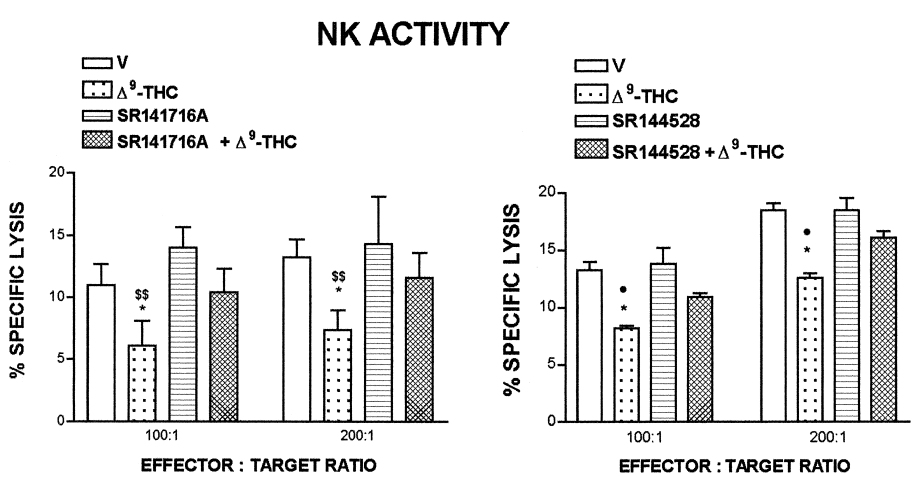

Fig. 2. Effect of SR 141716 (3 mg/kg i.p., 30 min before Δ^9-tetrahydrocannabinol, and SR 144528 (10 mg/kg p.o., 90 min before Δ^9-tetrahydrocannabinol) pretreatment on the inhibition of NK cytolytic activity induced by Δ^9-tetrahydrocannabinol (Δ^9-THC, 15 mg/kg, s.c.). Mean ± S.E.M. for at least six mice. $^*P < 0.05$ vs. vehicle; $\$\$P < 0.01$ vs SR 141716A; ● $P < 0.05$ vs. SR 144528 (Bonferroni's test).

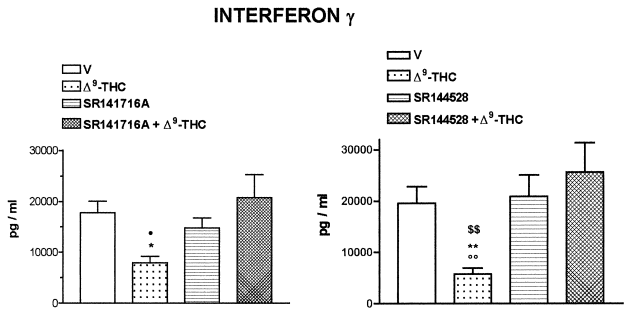

Fig. 3. Effect of SR 141716 (3 mg/kg i.p., 30 min before Δ^9-tetrahydrocannabinol) and SR 144528 (10 mg/kg p.o., 90 min before Δ^9-tetrahydrocannabinol) pretreatment on the inhibition of interferon γ release induced by Δ^9-tetrahydrocannabinol (Δ^9-THC, 15 mg/kg, s.c.). Mean ± S.E.M. for at least six mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. vehicle; ● $P < 0.05$ vs. SR 141716A + Δ^9-tetrahydrocannabinol; \$\$$P < 0.01$ vs. SR 144528; $^{\circ\circ}P < 0.01$ vs. SR 144528 + Δ^9-tetrahydrocannabinol (Bonferroni's test).

cytolytic activity, mice were in vivo pretreated with SR 141716A, the selective cannabinoid CB_1 receptor antagonist, and SR 144528, the selective cannabinoid CB_2 receptor antagonist. As shown in Fig. 2, the cannabinoid receptor antagonists by themselves did not affect cytolytic NK activity, but they attenuated the NK inhibition induced by Δ^9-tetrahydrocannabinol. The recovery induced by cannabinoid CB_1 receptor antagonist was 88% and 72% for effector:target cell ratios of 100:1 and 200:1, respectively, as compared to the activity of the Δ^9-tetrahydrocannabinol-treated group. In the same manner, the pretreatment with cannabinoid antagonist induced a recovery of 46% and 45% for 100:1 and 200:1 effector:target cell ratios, respectively.

Finally, both cannabinoid receptor antagonists were tested for their ability to antagonize the Δ^9-tetrahydrocannabinol-induced inhibition of cytokine production by evaluating interleukin 2 and interferon γ levels. These cytokines were chosen since we have previously demonstrated (Massi et al., 1998) their involvement in Δ^9-tetrahydrocannabinol-induced immunosuppression. As shown in Fig. 3, in vivo injection of Δ^9-tetrahydrocannabinol significantly reduced interferon γ production in activated splenocyte cultures (72%) while it did not affect interleukin 2 production (data not shown). The in vivo pretreatment with both cannabinoid receptor antagonists completely reversed the cannabinoid-induced inhibition of interferon γ production (Fig. 3).

4. Discussion

Our findings indicate that Δ^9-tetrahydrocannabinol, at an acute dose that induces significant analgesia, causes a strong reduction in NK cytolytic activity without affecting splenocyte proliferation. In agreement with the functional results, the level of interferon γ, one of the most important cytokines mediating NK cytolytic function, was reduced by about 72%. In contrast, interleukin 2, a cytokine mainly involved in regulating proliferation, was not affected. These data agree with those of previously reported in vitro and in vivo studies (Klein et al.,1998; Parolaro, 1999) demonstrating that Δ^9-tetrahydrocannabinol or synthetic cannabinoid receptor agonists reduce splenic NK cytolytic activity in a concentration-dependent manner. In the same time, the parallel reduction we observed in the interferon γ level suggests that marijuana acutely could depress the defense against tumors and microbes, considering the important role played by this cytokine in cell-mediated immune mechanisms. However, the lack of any effect on splenocyte proliferation and interleukin 2 level under our experimental conditions indicates that acutely administered Δ^9-tetrahydrocannabinol only partially affects the immune response and does cause a severe impairment. The results of in vitro studies on the effects of Δ^9-tetrahydrocannabinol on the proliferation of mouse splenocytes and interleukin 2 production are conflicting, with responses being both depressed, unaffected or even increased depending on the cannabinoid agonist concentrations (Klein et al., 1998; Parolaro, 1999). The data available for in vivo models are very sparse. Patrini et al. (1997) reported that in rats an acute i.p. injection of the synthetic cannabinoid receptor compound CP-55,940 {((−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol)}, at a dose inducing analgesia, significantly reduced the splenocyte proliferative response to phytohemagglutinin, while in other reports (Barghava et al., 1996; Massi et al., 1998) Δ^9-tetrahydrocannabinol at doses inducing analgesia in mice did not alter the splenocyte proliferative response. The chosen dose, the different receptor affinity of the two compounds and, finally, the different species could account for the different results. In line with Barghava et al. (1996) and Massi et al. (1998), our present data indicate that Δ^9-tetrahydrocannabinol, at an acute dose inducing significant analgesia, did not cause any significant change in splenocyte proliferation, although we cannot exclude that a higher dose and/or a longer exposure to the drug could result in a significant alteration. Concerning interleukin 2 levels, Barghava et al. (1996) found a reduction in its amount after Δ^9-tetrahydrocannabinol chronic treatment, thus we can suppose that the disagreement with our data could be related to acute vs. chronic injection.

When injected into mice both cannabinoid CB_1 and CB_2 receptor antagonists attenuated the Δ^9-tetrahydrocannabinol-induced suppression of NK cytolytic activity and interferon γ level, suggesting a role for cannabinoid receptors in these effects. However, the NK cytolytic activity was only partially reversed by pretreatment with the two cannabinoid receptor antagonists while the interferon γ level was completely restored. In our opinion, this different sensitivity to the cannabinoid receptor antagonists can be explained by considering the complex biochemical

network involved in the regulation of NK cytolytic activity. Although the interferon γ level plays a pivotal role, the action of other mediators could account for the residual inhibition of NK cytolytic activity.

Another interesting point is the greater effect of the cannabinoid CB_1 vs. CB_2 receptor antagonist in reversing the NK cytolytic activity inhibition induced by Δ^9-tetrahydrocannabinol. Thus, it appears that despite the more sustained expression of cannabinoid CB_2 receptor than cannabinoid CB_1 receptor in immune cells (Galiègue et al., 1995), the latter is more involved in the modulation of NK function. As already hypothesized (Klein et al., 1998), cannabinoid receptor expression is related to the stage of differentiation and therefore possibly related to cell function. Although in resting splenocytes the level of cannabinoid CB_2 receptor expression is higher than that of cannabinoid CB_1 receptor expression, their stimulation could induce a shift in the proportion of each receptor subtype, thus making the cannabinoid CB_1 receptor of functional importance in immune modulation.

Further dose–response experiments are now in progress to better compare the potency of cannabinoid CB_1 vs. CB_2 receptor antagonist in decreasing the Δ^9-tetrahydrocannabinol-induced inhibition of NK cytolytic activity but, although preliminary, our results indicate that, at least in splenocytes, both subtypes of cannabinoid receptors are involved in the modulation of NK cytolytic activity and interferon γ production.

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