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CANNABINOID RECEPTORS AND THE CYTOKINE NETWORK

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INTRODUCTION

Marijuana components, especially Δ^9-tetrahydrocannabinol (THC), have been shown to modulate immune function in both *in vivo* and *in vitro* paradigms[1]. However, the impact of drug-induced immunomodulation on host resistance to infection is uncertain and only a few studies have been reported coupling animal infection models and drug treatment. The molecular mechanisms of drug-induced immunomodulation are also unknown. An endogenous cannabinoid system has been described over the past few years composed of receptors and ligands[2] and evidence suggests that cells of the immune system express these receptors[3–5]. However, the precise role of these receptors in drug-induced immunomodulation is not clear.

Several years ago we reported on an animal infection model that was modulated by THC injection[6,7]. The drug, when injected one day before a primary infection with *Legionella pneumophila*, inhibited mice from developing a protective Th1-regulated cell-mediated immunity to a challenge infection three weeks later. We also reported that THC treatment of splenocyte cultures favored the development of Th2 activity over Th1 activity (Figure 1). In the present report, we present evidence that suggests the cannabinoid-induced skewing toward Th2 activity is mediated by cannabinoid receptor 2 (CB2) rather than receptor 1 (CB1).

METHODS

Animals and Drugs

Female BALB/c mice were purchased (National Cancer Institute-Harlan, Frederick, MD) and used at 8 to 9 weeks of age. They were housed and cared for in our accredited

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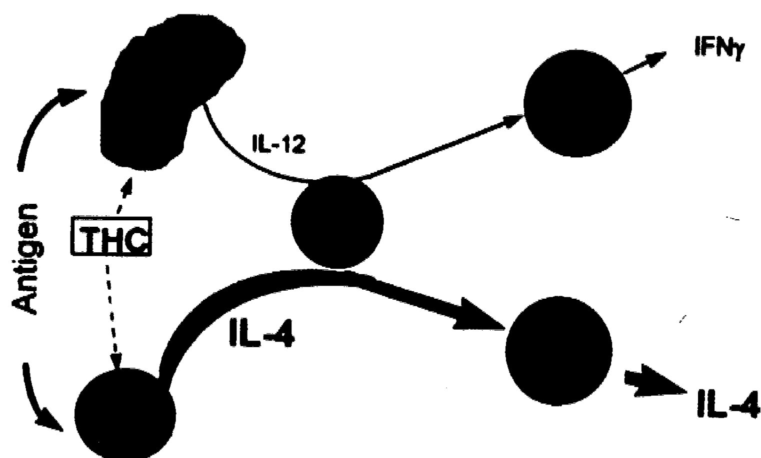

Figure 1. THC treatment of splenocytes causes an increase in Th2 activity and a decrease in Th1activity. Treatment of splenocytes containing macrophages (MΘ) and T cells (T?) with THC and antigens or mitogens causes the preferential development of Th2 cells secreting IL-4 from Th0. Development of Th1 cells secreting IFNγ was attenuated.

animal facility (AAALAC). THC was obtained from the Research Technology Branch of NIDA and was prepared as previously described[7]. The other cannabinoid agonists used were: CP55,940 (gift of Pfizer Central Research, Dr. Saul Kadin); WIN55,212-2 and WIN55,212-3 (purchased from RBI, Natrick, MA); and JWH-015 and JWH-051 (gift from John Huffman, Clemson University). All were dissolved to working concentrations in dimethylsulfoxide (DMSO, Sigma Chemical, St. Louis). The CB1 antagonist, SR141716A, was a gift of Sanofi Recherche, Dr. M. Mosse, and was also dissolved in DMSO to working concentrations. Pertussis toxin was obtained from Sigma Chemical.

Splenocyte Cultures

Single cell suspensions of mouse splenocytes were prepared and cultured as previously described[7]. Stimulating these cells with pokeweed mitogen (PWM) induces them in culture to produce and secrete detectable amounts of Th1 and Th2 cytokines such as IFNγ and IL-4/IL-10, respectively. Splenocytes (3×10^6/ml) were cultured in 24-well plates for 72 hours with PWM (10 μg/ml) as well as the various agonists and the antagonist. Supernatants were collected and tested for cytokines by ELISA.

Cytokine ELISAs

Cytokine levels were determined using sandwich ELISAs with antibody pairs (Pharmingen ,San Diego, CA) for each cytokine as previously described[7]. EIA plates (Costar, Cambridge, MA) were coated with the capture anti-murine cytokine overnight at 4°C. The plates were blocked for 30 min with 150 μl of PBS plus 0.5% BSA and 0.05% Tween 20. Supernatants from lymphocyte cultures or serial dilutions of standards were added for 1 hour. Biotinylated detecting antibody was added for 1 hour, followed by streptavidin-alkaline phosphatase (Southern Biotechnology, Birmingham, AL) for 30 min. The plates were washed between additions with 3–5 changes of nanopure water. Following addition of the substrate (1 mg/ml of p-nitrophenyl phosphate in diethanolamine buffer: 49 mg/l of

$MgCl_2$, 96 ml/l of diethanolamine, pH 9.8), plates were developed for 15–45 min. The capture antibodies, standards, and detection antibodies were used at the following concentrations: IFNγ, 4 μg/ml, 50 ng/ml, 2 μg/ml; IL-4, 2 μg/ml, 10 ng/ml, 1 μg/ml; and IL-10, 10 μg/ml, 50 ng/ml, 2 μg/ml.

RESULTS

THC Treatment Decreases Th1 Cytokines and Increases Th2 Cytokines

THC injection into mice had been shown to alter the balance of Th1 and Th2 activity developing in response to an infection with *Legionella pneumophila*[7]. We tested if this drug effect could be shown to occur in an *in vitro* system involving normal splenocytes activated by mitogens such as pokeweed mitogen. Splenocytes were cultured for 72 hours with mitogen only or mitogen and 3 or 5 μg/ml of THC and the supernatants harvested and tested for the level of the Th1 cytokine IFNγ and the Th2 cytokines IL-4 and IL-10. Figure 2 shows that THC treatment decreased in a dose-dependent manner the production of IFNγ (Th1) but increased IL-4 and IL-10 production (Th2).

The CB1 Antagonist, SR141716A, Does Not Attenuate the THC Effects on Cytokine Production

There are two cannabinoid receptors described to date in mice. The first termed CB1 is believed to be expressed in the brain and periphery[8] while the second termed CB2 is expressed primarily in the periphery[3]. A CB1 selective antagonist, SR141716A, has been described and used in studies to implicate a role of CB1 in cell function[9]. Since both CB1 and CB2 can serve as receptors for THC, we wanted to test if either was involved in the drug effects on cytokine production. Splenocyte cultures were incubated with combinations of the antagonist, mitogen, and THC and the cytokine levels measured by ELISA. Figure 3 shows that the suppressive effect of THC on IFNγ (Th1) was not attenuated by the CB1 antagonist suggesting that CB1 was not involved in mediating the drug effect. Similar results were obtained following analysis of IL-4 levels (data not shown).

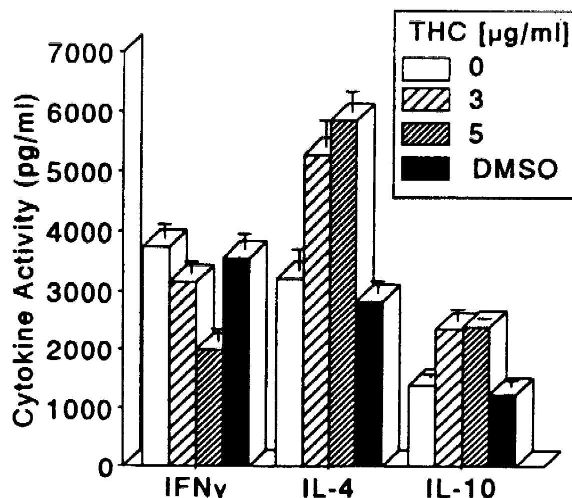

Figure 2. THC treatment of splenocytes causes a decrease in IFNγ production and an increase in IL-4 and IL-10. Splenocytes were cultured with pokeweed mitogen (10 μg/ml) alone or pokeweed mitogen and either several concentrations of THC or drug diluent, DMSO. The cultures were harvested after 72 hours and the supernatant levels of cytokines determined by ELISA.

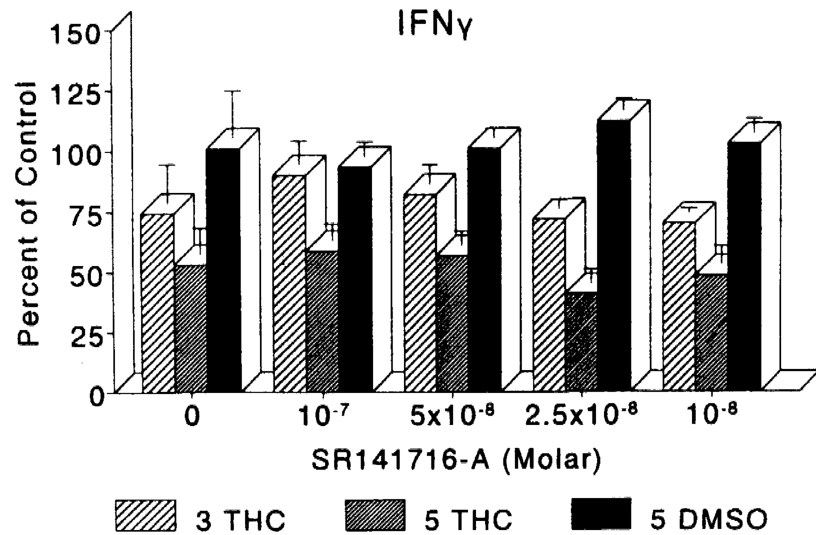

Figure 3. The CB1 antagonist, SR141716A, has no effect on THC-induced suppression of IFNγ. Splenocyte cultures were treating with increasing concentrations of SR141716A and then stimulated and analyzed for IFNγ as in Figure 2.

Pertussis Toxin Treatment Attenuates the THC Effect on IL-4 Production

CB1 and CB2 are G protein-coupled receptors linked to the pertussis toxin sensitive G protein, G_i[10,11]. To analyze whether or not the THC effect on cytokines was linked to a mechanism inhibited by G_i, splenocytes were treated with pertussis toxin and then challenged with mitogen in the presence of THC. Figure 4 shows that pertussis toxin treatment attenuated the THC enhancement of IL-4 suggesting that this effect was mediated through a pertussis toxin sensitive pathway such as associated with CB1 or CB2. However, in other studies, pertussis toxin had no effect on the drug-induced suppression of IFNγ production (data not shown).

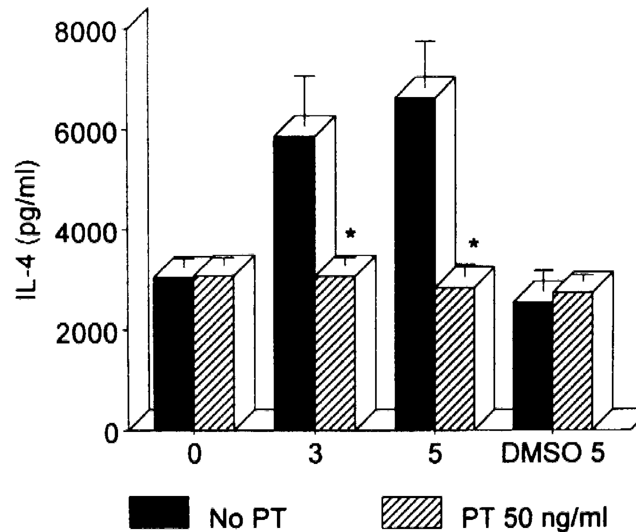

Figure 4. Pertussis toxin attenuates the THC effect on IL-4 production. Splenocyte cultures were preincubated for 24 hours with medium or pertussis toxin. The cultures were then stimulated and analyzed by ELISA for IL-4 as in Figure 2.

The CB2 Selective Agonist, JWH-051, Increases IL-4 Production

The above studies with SR141716A and pertussis toxin suggested that the cannabinoid effect on IL-4 but not IFNγ was mediated by a cannabinoid receptor, possibly CB2. Receptor agonists of different structures have affinities and potencies for cannabinoid receptors greater than THC. For example, the synthetic analogue of THC, CP55,940 has been shown to be more potent than THC[12] while another analogue, WIN55,212-2, has a lower affinity than THC for mouse CB2[13] and JWH-051 has a very high affinity for CB2[14]. To see if these different agonists displayed different potencies for enhancing IL-4, splenocyte cultures were stimulated with mitogen and treated with increasing concentrations of THC, CP55,940, WIN55,212-2, WIN55,212-3, JWH-015, and JWH-051. Figure 5 shows that the CP and WIN compounds were relatively inactive in increasing IL-4 when compared to THC. However, JWH-051 but not JWH-015, was at least as active as THC if not more in enhancing the splenocyte production of the Th2 associated cytokine, IL-4 (Figure 6).

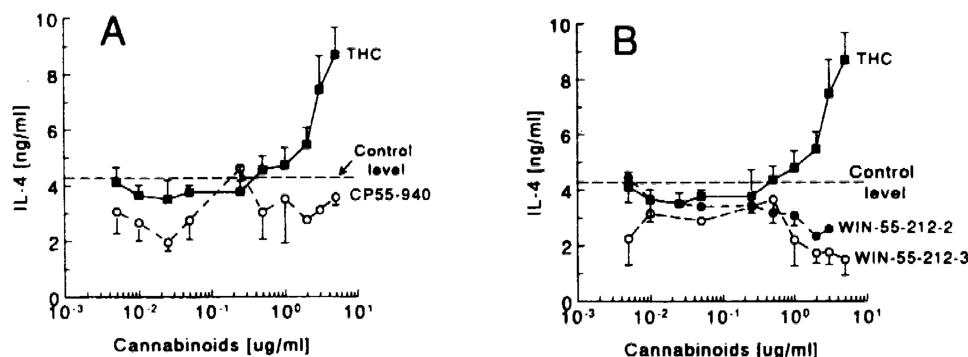

Figure 5. The agonists CP55,940, WIN55,212-2, and WIN55,212-3 are less active than THC at increasing IL-4. Splenocyte cultures were treated with mitogen and increasing concentrations of either THC or CP55,940 (A) or THC and the WIN compounds (B). Supernatants were analyzed after 72 hours by ELISA for IL-4.

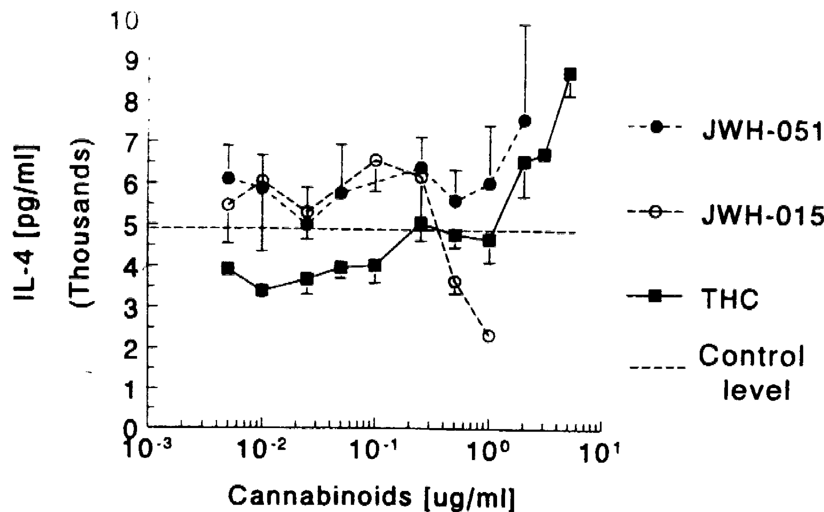

Figure 6. The agonist JWH-051 is active at increasing IL-4. Splenocyte cultures were treated with mitogen and either THC, JWH-051, or JWH-015. After 72 hours, supernatants were harvested and analyzed for IL-4 by ELISA.

DISCUSSION

The molecular mechanisms by which marijuana cannabinoids modulate immune cell function are poorly understood. Furthermore, the role of cannabinoid receptors in these effects is not known even though immune cells appear to have receptor activity[1]. It is becoming clear that cannabinoids modulate cytokine production and we report here that THC can directly act on lymphocyte cultures to alter the production of Th1 and Th2 cytokines. Drug treatment causes a shift toward a Th2 type activity and away from Th1 activity. This type of shift could have severe consequences for someone fighting an infection that is inhibited by Th1 immunity[15].

In addition to showing a THC-induced shift toward Th2 immunity, we also conducted studies to determine the role of cannabinoid receptors in the effect. The first studies were done with the CB1 antagonist, SR141716A, which has been shown to bind with high affinity to CB1 but not CB2[9], to inhibit a variety *of in vivo* and *in vitro* activities of cannabinoids, and to function as an inverse agonist[16]. Addition of the SR141716A to splenocyte cultures was shown to have no effect on THC-induced cytokine shift suggesting that CB1 receptors were not involved. Various studies have confirmed that CB2 is expressed primarily in the periphery including cells of the immune system[17,18] and that cannabinoid effects on immune cell function are at least partly mediated through this receptor[4]. Unfortunately, a CB2 antagonist is not currently available. However, in other systems, cannabinoid receptor involvement has been suggested by studies using pertussis toxin. This protein is an A-B toxin that catalyzes the ADP-ribosylation of a component of the G_i complex rendering the complex inactive. Receptor/ligand systems such as CB1 and CB2 that utilize G_i can be suppressed by prior treatment with pertussis toxin and so we tested if the toxin would inhibit the THC effect on Th cytokine production. Our results showed that toxin treatment of splenocyte cultures attenuated the cannabinoid-induced increase of IL-4 production. This data combined with the SR1417161A data suggest that a G_i-regulated receptor system such as the cannabinoid receptor system is involved in the upregulation of Th2 immunity. However, since the SR141716A compound did not inhibit the response, the role CB1 receptor subtype in this effect appears to be less likely than that of CB2. It is also possible that THC is working through some mechanism other than cannabinoid receptors which in turn is activating G_i leading to an increase in IL-4 production, however, this mechanism is currently not known and G_i is not linked to an upregulation of IL-4 because pertussis toxin had no effect on the cytokine response to PWM.

Another way to examine the role of CB2 in the drug effect on IL-4 production, is the use of various receptor agonists which appear to have varying affinities for CB1 and CB2. For example, THC and CP55,940 have higher affinities for mouse CB2 than does WIN55,212-2[13]. On the other hand, JWH-051 has a much higher affinity for human CB2 than do other agonists[14]. Although the structure/activity profiles of various agonists for CB1 and CB2 have not been done for the mouse receptors, we wondered if some of these agonists might have varying effects in the IL-4 production paradigm. We showed that THC and JWH-051 were observed to be more potent than CP55,940 and the WIN compounds in modulating IL-4 suggesting that one receptor subtype (e.g. CB2) was more involved in the response than the other. The agonist activity profiles and receptor affinities for mouse CB1 and 2 require further definition and study. However, our data suggest that the structure/activity profiles for each receptor are different and that one receptor subtype such as CB2 may be more involved in the modulation of IL-4 production.

SUMMARY

Splenocyte cultures from BALB/c mice were treated with THC and mitogen and shown to produce less Th1 cytokine, IFNγ, and more Th2 cytokines, IL-4 and IL-10. This suggested that drug treatment caused a shift in the development of Th1 and Th2 cells. In studies designed to look at molecular mechanisms, the CB1 antagonist, SR141716A, did not attenuate the THC enhancement of IL-4 production, but pertussis toxin attenuated the drug effect and the CB2 agonist, JWH-051, increased IL-4 production similar to THC. These results suggest that cannabinoids may increase Th2 development and IL-4 production in cultured immune cells through the activity of the CB2 receptor subtype. Studies are currently in progress to further define the molecular and cellular mechanisms involved.

ACKOWLEDGMENT

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