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Anandamide and Δ9-Tetrahydrocannabinol Directly Inhibit Cells of the Immune System via CB₂ Receptors

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Abstract

This study shows that two cannabinoids, Δ^9 -tetrahydrocannabinol (THC) and anandamide, induce dose related immunosuppression in both the primary and secondary in vitro plaque-forming cell assays of antibody formation. The immunosuppression induced by both compounds could be blocked by SR144528, an antagonist specific for the CB₂ receptor, but not by SR141716, a CB₁ antagonist. These studies are novel in that they show that both anadamide and THC are active in the nanomolar to picomolar (for anandamide) range in these assays of immune function, and that both mediate their effects directly on cells of the immune system through the CB₂ receptor.

1. INTRODUCTION

Tetrahydrocannabinol (THC) has been shown to have marked effects on various parameters of immune function including inhibition of in vitro antibody formation by mouse spleen cells (Nahas et al., 1973; Kaminski et al., 1992), suppression of lymphocyte proliferation driven by mitogens or IL-2 responses (Friedman et al., 1991; Kawakami et al., 1988), inhibition of cytotoxic T cell and NK cell activity (Klein et al., 1991), inhibition of macrophage antigen processing of certain proteins (McCoy et al., 1999) and inhibition of macrophage secretion of the proinflammatory cytokine TNF- α by a post-translational mechanism (Fischer-Stenger et al., 1993; Zheng and Specter,1996). Evidence indicates that THC polarizes immune responses towards a Th2 phenotype (Lu et al., 2006).

Cannabinoid research has seen marked advances with the cloning of two cannabinoid receptors designated, CB_1 and CB_2 (Matsuda et al., 1990; Munro et al., 1993). The receptors are unevenly distributed in neural versus immune tissues, with mRNA for CB_1 expressed preferentially in the brain and other neural tissues, and to a lesser extent in peripheral immune tissues (Schatz et al., 1997; Galiegue et al., 1995), whereas CB_2 is found primarily in cells of the immune system (Munro et al., 1993; Schatz et al., 1997), but not neurons. Microglia in the brain have been reported to express low levels of CB_1 constitutively, but to up-regulate CB_2 when activated (Cabral and Marciano-Cabral,2005). An important area of investigation is the

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dissection of whether CB_1 or CB_2 receptors mediate various effects of Δ^9 -THC on the immune system.

Discovery of endogenous ligands for the cannabinoid receptors, including anandamide (Devane et al., 1992), has increased awareness of the potential importance of cannabinoids in homeostatic processes, including immune function. There are few studies showing direct effects of endogenous cannabinoids on CB_1 or CB_2 receptors in the immune system. Anandamide has been reported to stimulate growth of hematopoietic cell lines by a cannabinoid receptor-independent pathway (Derocq et al., 1998), to induce apoptosis in a macrophage cell line via vanilloid receptors (Maccarrone et al., 2000), and to induce apoptosis in dendritic cells via CB_1 and CB_2 receptors (Do et al., 2004). Joseph et al showed that anandamide and a CB_2 specific agonist (JWH 133), but not a CB_1 specific agonist (DEA), could inhibit the chemotactic response of human $CD8^+$ T cells to a chemokine (Joseph et al., 2004). Cannabinol, Δ^9 -THC and CP55940 are reported to enhance IL-2 levels in an EL4 T cell line stimulated by phorbol ester plus a calcium ionophore, which was not blocked by a CB_2 antagonist (Jan et al., 2002).

It has been shown previously that THC is immunosuppressive in the primary mouse plaque-forming cell assays for antibody formation, and based on use of stereospecific cannabinoid compounds, it was concluded that the effects were cannabinoid receptor-mediated (Kaminski et al., 1992). However, whether the effects were mediated by CB_1 or CB_2 was not determined. Development of CB_1 and CB_2 selective antagonists (Rinaldi-Carmona et al., 1994; Rinaldi-Carmona et al., 1998) has provided new tools to determine which of the cannabinoid receptors mediates plaque-forming cell immunosuppression. The current study was undertaken to determine if THC induces immunosuppression in the primary and secondary PFC assay in vitro via CB_1 or CB_2 receptors, and to test the immunomodulatory activity of anandamide in those assays. We show that both THC and anandamide induce dose-dependent immunosuppression of in vitro primary and secondary antibody formation via the CB_2 receptor.

2. MATERIALS AND METHODS

Mice

Six-week-old C3HeB/FeJ female mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in sterilized cages with mouse chow and water provided ad libitum. All mice were acclimated for a minimum of 1 week prior to being used in experiments.

Compounds

The cannabinoid agonists, Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and anandamide, and the cannabinoid antagonists, SR141716 and SR144528, were provided by The National Institute on Drug Abuse (NIDA, Rockville, MD). Δ^9 -THC was supplied as a solution of 200 mg/ml in absolute ethanol. Anandamide was supplied as a solution of 10 mg/ml in absolute ethanol. The cannabinoid antagonists were supplied as crystals. Each agonist was dissolved to a final concentration of 4 mg/ml in absolute ethanol. All absolute ethanol solutions were diluted 25% to yield 75% ethanol solutions using sterile, nonpyrogenic water (Abbott Laboratories). These solutions were then added dropwise to the medium used for plaque-forming cell assay, which is a supplemented RPMI-1640 base, to the desired concentration for stock solutions (2 × 10^{-5} M) and stored at -80° C.

Primary in vitro antibody response

Mice were sacrificed and their spleens aseptically removed. Immune function was assessed using an in vitro plaque-forming cell (PFC) assay which measures the capacity of spleen cells to mount a primary antibody response to sheep red blood cells (SRBCs). A single cell

suspension of spleen cells was obtained by pushing the spleen through nylon mesh bags in RPMI supplemented with 5% fetal bovine serum (FBS). Red blood cells were lysed by hypotonic shock with sterile water. Cells were washed twice and resuspended in tissue culture medium consisting of RPMI supplemented with 10% FBS, 2mM L-glutamine, 50 μg/ml gentamicin, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 µg/ml of adenosine, uridine, cytosine, and guanosine, and 0.05 mM of 2-mercaptoethanol. The cells were counted and resuspended to 1.0×10^7 cells/ml and dispensed into flat-bottom 24-well tissue culture plates (Corning Costar). Sheep red blood cells (Rockland, Gilbertsville, PA) were washed three times in sterile saline and 3.5×10^6 cells were added to each well in 50 µl. To test the effect of cannabinoids, THC and anandamide were added in graded concentrations to the cultures in a volume of 50 µl. To determine the effects of cannabinoid antagonists, SR141716 or SR144528, at a concentration of 10⁻⁶ M, was added to spleen cultures in a volume of 50 μl 2 hr before the addition of either of the two cannabinoid agonists. Control cultures were untreated or received either antagonists alone or equal volumes of vehicle for either agonists and/or antagonists. Cultures were incubated in an atmosphere consisting of 7% O₂, 10% CO₂, and 83% N₂ at 37 °C. Twenty-four hours later, cultures were fed with a nutrient cocktail consisting of RPMI supplemented with approximately 30% FBS, 5.5 mM L-glutamine, 3 mM each of essential amino acids and nonessential amino acids, 5.5 mg/ml dextrose, 0.61% sodium bicarbonate, and 40 µg/ml each of adenosine, uridine, cytosine, and guanosine. Cultures were incubated for 5 days. On day 5, cells were harvested, washed in RPMI, and the number of direct PFCs (cells producing IgM antibodies against SRBCs) quantitated using the Cunningham modification of the Jerne hemolytic plaque assay (Cunningham and Szenberg, 1968), using guinea pig serum as the source of complement (Rockland). Data from the PFC assay were calculated as the mean number of PFC/10⁷ cells. For each dose, a minimum of triplicate cultures was tested. For untreated spleen cells, at least nine replicate wells were used to establish the normal, baseline response level. Results are expressed as a Suppression Index, where untreated spleen cells are given a value of 1.00 (100%), and responses of cultures receiving treatment with cannabinoids are calculated as:

 $SI = \frac{\text{mean \# PFC / well of treated cultures}}{\text{mean \#PFC untreated cultures}}$

Secondary in vitro antibody response

Mice were immunized in vivo i.p. using 10% SRBC in 0.2 ml of phosphate-buffered saline, 14 days before spleen harvest (Eisenstein et al., 1995). The spleens of the SRBC-immunized mice were removed and processed in the same manner as for the primary PFC assay outlined above. Experiments were repeated three times, and a minimum of triplicate cultures was tested for each dose.

Statistics

All Suppression Index data were transformed to normalized ranks to accommodate non-normality of the data. Between group differences were tested by ANOVA using vertical group comparisons at each dose. The Least Significant Difference method for multiple comparisons was used in some cases for horizontal and vertical comparisons between groups and doses. Significance of differences between dose response curves for different treatments was determined using nonlinear curve fitting methods. Differences between vehicle or antagonists alone compared with untreated cultures (SI= 1.0) were tested based on 95% confidence intervals.

3. RESULTS

Δ9-THC is immunosuppressive in a primary PFC assay via the CB₂ receptor

To determine the effects of THC on in vitro immune responses, mouse spleen cells were treated with the cannabinoid in a primary PFC assay. Figure 1 shows THC gave a dose-dependent immunosuppression curve, with maximal suppression observed between 10^{-6} and 10^{-9} M. The vehicle diluted similarly to the doses of the cannabinoid was not suppressive. Vehicle at a 2-fold concentration, to account for the amount present in cultures containing both an agonist and an antagonist, was also not suppressive. Pretreatment with the CB₂ antagonist, SR144528, completely blocked the immunosuppression. Pretreatment with the CB₁ antagonist, SR 141716, not only did not block the THC-mediated immunosuppression, it potentiated it, by maintaining the immunosuppression over a broader range of doses than the agonist alone.

Δ9-THC is immunosuppressive in a secondary PFC assay via the CB₂ receptor

Similar assays were carried out using the secondary PFC assay in which animals were primed in vivo with sheep red blood cells 14 days prior to harvest of spleen cells. Immunosuppression was again observed by THC over a broad range of doses from 10^{-7} to 10^{-10} M (Fig. 2). Vehicle at the highest concentration used or at 2X did not significantly suppress PFC responses. The CB₂ antagonist completely blocked the immunosuppression induced by THC, whereas the CB₁ antagonist had no effect. These results show that the CB₂ receptor mediates immunosuppression caused by Δ^9 -THC in both the primary and the secondary PFC assay.

Anandamide-mediated immunosuppression in primary and secondary PFC assays

The endogenous cannabinoid agonist, anandamide, was also examined for its effects on in vitro immune responses in both the primary (Fig. 3) and the secondary (Fig. 4) PFC assays. In the primary assay, anandamide resulted in a dose-response curve with maximal suppression occurring between 10^{-7} and 10^{-12} M. When used in the secondary assay (Fig. 4), anandamide yielded a dose-response curve in which suppression was observed at concentrations between 10^{-7} and 10^{-12} M. Cannabinoid receptor-specific antagonists were used to determine which cannabinoid receptors were responsible for mediating the suppressive effect of anandamide. Treatment of the primary (Fig. 3) splenocyte cultures with the CB₁ antagonist, SR141716, caused a slight reversal of suppression, but cultures were still significantly suppressed when compared to controls. In the secondary PFC assay (Fig. 4), the CB₁ antagonist gave partial reversal of suppression at 10^{-7} and 10^{-9} M, but overall, did not reverse the effect of anandamide. In contrast, pre-treatment of splenocyte cultures in both the primary and the secondary PFC assays with SR144528, the CB₂ antagonist, completely blocked the suppression induced by anandamide. These results show that anandamide mediates immunosuppression via the CB2 receptor in both the primary and the secondary PFC assays.

4. DISCUSSION

These studies clearly show that both Δ^9 -THC and anandamide are immunosuppressive when added to mouse spleen cells in vitro in both primary and secondary plaque-forming cell assays, and that both the exogenous and the endogenous compound act through CB₂ receptors in both assays. Both compounds give dose response curves in both assays. However, the shapes of the dose-response curves differed. In the primary and secondary assay, Δ^9 -THC produced dose-response curves with maximal suppression between 10^{-6} and 10^{-10} M. Anadamide gave suppression over a wider range of doses in the primary PFC, with strong suppression still evident at 10^{-12} M. These findings are of interest, as the participation of CB₁ versus CB₂, or other receptors, in cannabinoid-mediated immunomodulation is still incompletely defined. The present studies provide a clear example of CB₂-mediated effects. These results represent the

first demonstration of which we are aware of an effect of anandamide on the immune system via CB₂ receptors.

Comparison of the results for THC and anandamide in the in vitro PFC assays tested in the present experiments show overall consonance. Both the phytocannabinoid and the endocannabinoid were immunosuppressive in the $10^{-6}\,\mathrm{M}$ to $10^{-9}\,\mathrm{M}$ dose range, and the suppression was completely reversed in the primary and secondary PFC assays by the CB₂ antagonist, SR144528, although anandamide was suppressive over a wider range of doses. Others have also observed that Δ^9 -THC and anandamide have similar effects on release of IL-6, nitric oxide, and PGE₂ from a lipopolysaccharide-stimulated macrophage cell line (Chang et al., 2001). Thus, although anadamide has a short half-life within the cell and is easily biodegradable after endocytosis, compared to Δ^9 -THC (Cravatt et al., 1996), these two cannbinoids exhibit overall similarities in their behavior in this in vitro system using cells of the immune system.

The observations presented in this paper support a role for the CB_2 receptor in *in vitro* antibody formation to sheep red blood cells. The conclusion is consonant with information on expression of cannabinoid receptors in the immune system. It has been reported that CB_2 receptor mRNA is present in all three cell types needed for the plaque-forming cell assay, B cells, T cells and macrophages (Newton et al., 1998). Message for CB_2 can be up- or down-regulated in T-cells depending on the activation conditions (Daaka et al., 1996). Similarly, mouse peritoneal macrophages have been shown to express only CB_2 mRNA, with the level of expression a function of activation state (Carlisle et al., 2002). Quiescent peritoneal exudate cells expressed no CB_2 mRNA, but upon activation, CB_2 mRNA was evident, as was receptor protein, the latter detected using a rabbit antiserum to the receptor (Carlisle et al., 2002).

There are only a few studies documenting which receptor mediates alterations in immune function induced by cannabinoids. Inhibition of IL-2 release from phytohemagglutininstimulated human peripheral blood mononuclear cells was found to be mediated by CB₂ using receptor selective cannabinoid agonists and antagonists (Ihenetu et al., 2003). It was also concluded that CB2 mediated inhibition of IL-2 secretion induced by THC in mouse T-cells stimulated with anti-CD3 in the presence of peritoneal macrophages (Buckley et al., 2000). CB₂ was shown to mediate the inhibition of antigen processing and presentation to T cells of the protein, lysozyme, by a macrophage hybridoma (McCoy et al., 1999). Joseph et al observed that a synthetic CB₂ agonist could block the effect of a chemokine on human CD8⁺ T cells (Joseph et al., 2004). In contrast to these immunosuppressive effects, exposure of promyelocytic cell line HL-60 transfected with the CB₂ receptor and stimulated with a synthetic cannabinoid agonist resulted in up-regulation of chemokine and TNF-α production (Jbilo et al., 1999; Derocq et al., 2000). There is one report of activity of CB₁ in the immune system. Daaka et al found that CB_1 mediated Δ^9 -THC up-regulation of the alpha chain of the IL-2 receptor in an NK cell line via activation of NF-kB (Daaka et al., 1997). We show that a CB₁ antagonist potentiated the suppressive activity of THC.

The results presented in this paper contrast with a previous report where no suppression was found for anandamide in the primary plaque-forming cell assay using concentrations of 10 and 25 uM, doses which were not tested in the present experiments (Lee et al., 1995). Differences in the results obtained in the two studies could be due to the concentration differences or to differences in mouse strains. We used C3HeB/FeJ mice and the previous study used B6C3F1 mice. Prior work testing effects of opioids in the *in vitro* plaque-forming cell assay has demonstrated strong mouse strain differences (Eisenstein et al., 1995). Conceptually, it is reasonable that anandamide could have an effect on cells of the immune system, as it has been shown to bind to transfected COS cells via CB₂ receptors (Munro et al., 1993).

One seemingly disparate observation obtained in the present studies is the potentiation of the suppressive effect of Δ^9 -THC by SR141716 in the primary plaque-forming cell response (Fig. 1). It is noteworthy that in Figure 1, the potentiation of suppression was greatest at ultra-low concentrations. A similar effect was not seen in the secondary PFC assay (Fig. 2). When anandamide was the agonist, SR141716 actually partially antagonized its action (Fig. 3), an effect opposite of that seen with Δ^9 -THC. SR141716 has been reported to have some anomalous effects, including behaving as an inverse agonist at the CB₁ receptor, particularly when used in conjunction with CB₁ agonists (Sim-Selley et al., 2001). However, in the immune system, SR141716 is reported to have agonist-like effects in inducing cytokines in animals not receiving another cannabinoid agonist (Smith et al., 2000). Three hypotheses have been put forth to explain the agonist properties of SR141716: 1) the possible presence of two sites on the CB₁ receptor for ligand binding (Sim-Selley et al., 2001); 2) the existence of the CB₁ receptor in two different conformational states (Pertwee, 2003; Pertwee, 2005a); or antagonism of endogenous endocannabinoids (Pertwee, 2005b). An explanation for the effects observed with SR141716 in the present study is not easily forthcoming, as the outcome was different when Δ^9 -THC and anandamide were used as the agonists.

The current studies did not address which cells are targeted by the cannabinoids, and whether THC and anandamide both affect the same cells. It is well established that THC has effects on macrophages (Fischer-Stenger et al., 1993; Zheng and Specter,1996) and on dendritic cells (Do et al., 2004), but whether the drugs exert their immunosuppressive effects via macrophage or dendritic targets, or whether they directly affect function of T and B cells in the plaqueforming cell system is not known. Future experiments could dissect the cellular targets by selective depletion of T cells, B cells, macrophages or dendritic cells before exposure to the cannabinoid, followed by extensive washing and add-back of normal cells of the depleted subset which was not exposed to drug.

In regard to dose of drug, in the current work we were able to demonstrate robust effects on the immune system in the nanomolar range for THC and in the picomolar range for anadamide, in contrast to some of the published studies (Kaminski et al., 1992; Shivers et al., 1994). Our success with lower doses may be attributable to use of ethanol, rather than DMSO, to solubilize THC. We did not compare ethanol and DMSO in the present studies, but such a comparison would be worthwhile.

These studies demonstrate that the major psychoactive component of marijuana, $\Delta^9 THC$, is immunosuppressive in the immune system via the CB_2 receptor. Further, the endogenous cannabinoid, anandamide, is also immunosuppressive via the CB_2 receptor. These observations have implications for pharmacologic intervention to block actions of these compounds on the immune system where CB_2 predominates, with minimal effects on neural parameters that are mediated by CB_1 .

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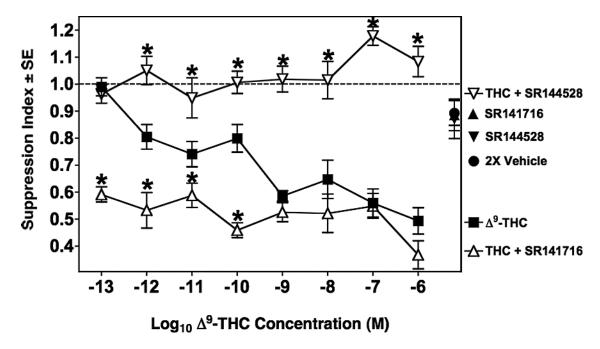


Fig. 1. Δ^9 -THC suppresses the primary plaque-forming cell response via CB2 receptors. A dose titration of THC, with or without a CB1 or CB2 antagonist, was carried out using spleen cells in a primary PFC assay. Each experiment was repeated 3 or more times, with triplicate wells for each dose. *p< 0.05 vs. THC alone. Values for vehicle or antagonists alone are not significantly different from 1.0.

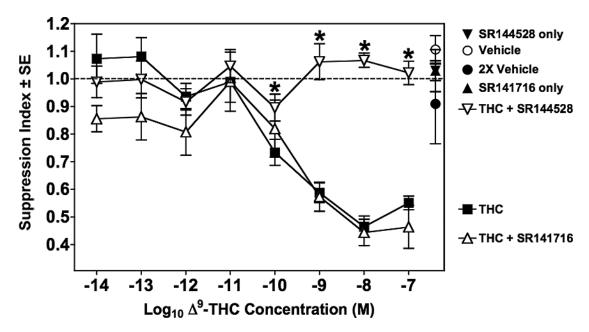


Fig. 2. Δ^9 -THC suppresses the secondary plaque-forming cell response via CB2 receptors. A dose titration of THC, with or without a CB1 or CB2 antagonist, was carried out using spleen cells in a secondary PFC assay. Each experiment was repeated 3 times, with triplicate wells for each dose. *p< 0.05 vs. THC alone. Values for vehicle or antagonists alone are not significantly different from 1.0.

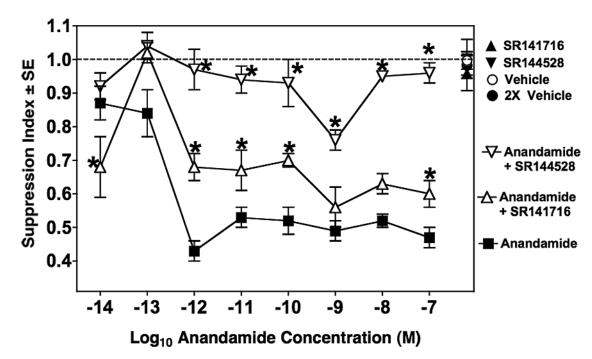


Fig. 3. Anandamide suppresses the primary plaque-forming cell response via CB2 receptors. A dose titration of anandamide, with or without a CB1 or CB2 antagonist, was carried out using spleen cells in a primary PFC assay. Each experiment was repeated three or more times, with triplicate wells for each dose. Anandamide plus CB1 antagonist was significantly different from control cultures. *p< 0.05 vs. anandamide alone. Values for vehicle or antagonists alone are not significantly different from 1.0.

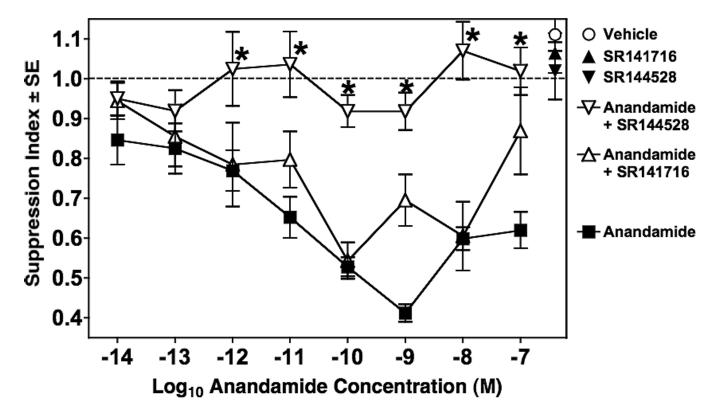


Fig. 4. Anandamide suppresses the secondary plaque-forming cell response via CB2 receptors. A dose titration of anandamide, with or without a CB1 or CB2 antagonist, was carried out using spleen cells in a secondary PFC assay. Each experiment was done two or more times, with triplicate wells for each dose. *p< 0.05 vs. anandamide alone. Values for vehicle or antagonists alone are not significantly different from 1.0.