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THE ROLE OF MACROPHAGES IN THC-INDUCED ALTERATION OF THE CYTOKINE NETWORK

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1. ABSTRACT

Delta-9-tetrahydrocannabinol (THC) has been shown to decrease Th1 responses (cell mediated immunity) while increasing Th2 responses (humoral immunity), both in vitro and in vivo. The addition of THC to murine splenocytes stimulated for 72 hrs with pokeweed mitogen (PWM) increased the detection of IL-4 and IL-10, cytokines associated with Th2 responses, and decreased IFNγ, IL-15 and IL-12, cytokines associated with Th1 responses. To investigate the cellular molecular basis for these effects of THC, cell depletion studies were performed. Removal of macrophages from the cell cultures eliminated the increase in IL-4 activity. This effect was observed when the macrophages were depleted during the first few hours but not after 24 hrs. Thus, it appears that the macrophages were producing an early factor responsible for the IL-4 increase. To examine the direct effect of THC on macrophages, peritoneal macrophages were cultured with various stimulators in the presence of THC. It was found that THC suppressed IL-12, IL-15 and IL-6 and increased IL-1α, IL-1β, and TNFα in all of the stimulated cultures. IL-12 and IL-15 are known to induce IFNγ production from T cells and NK cells and to be involved in the induction a Th1 response, while IL-1 is considered a growth factor for Th2 cells. Thus, the effects of THC on macrophages reflects the basic observation that THC decreases Th1 responses and increases Th2 responses. The data obtained adds to the understanding of the THC-induced Th1/Th2 shift, but the mechanisms still must be determined. However, the results of these studies do suggest that, in addition to lymphocytes, THC affects macrophages in splenocyte cultures and that the macrophages are involved in the alternation of the cytokines. Furthermore, the data demonstrate the diversity of the immunomodulatory effects THC exerts.

Drugs of Abuse, Immunomodulation, and AIDS, edited by Friedman *et al.*
Plenum Press, New York, 1998.

2. INTRODUCTION

Infection of mice with a sublethal concentration of *Legionella pneumophila*, an intracellular bacterium, causes an acute infection which mice survive because of their innate immune responses to *L. pneumophila*. TNFα and IFNγ have been demonstrated to be important during this time (1). Also during this time, the mice develop a strong cell-mediated (Th1) immune response which protects the mice from subsequent *L. pneumophila* infections (2). Development of Th1 responses is crucial for the mice to obtain an acquired immunity to *L. pneumophila* without which the mice will not survive a secondary lethal infection. We reported that the presence of delta-9-tetrahydrocannabinol (THC) during the early immune events following the intitial infection causes a shift from the protective Th1 immune responses, indicated by high levels of *L. pneumophila*-induced IFNγ production and anti-*L. pneumophila* IgG_{2a}, to Th2 responses, indicated by production of anti-*L. pneumophila* IgG_1 antibodies and a reduced antigen-specific IFNγ response (2). An antigen-specific IL-4 response was not detected; however, the presence of IgG_1 antibodies is indicative of an IL-4 response. IL-4 has been shown to be required for isotype switching to IgG_1 (2). The THC-treated mice also showed increased mortality to a challenge infection, further verifying the deficiency of the Th1 responses. The THC did not appear to affect the innate responses significantly enough to interfere with survival. In the present study, the possible mechanisms involved in the THC-induced shift in the development of Th1 to Th2 immunity were examined using in an vitro system of murine splenocytes and THC.

3. METHODS AND MATERIALS

3.1. Animals

BALB/c female mice (NCI—Charles River, Bethesda, MD) were used at 8 wks of age. They were housed in groups of 8–10 in plastic mouse cages with wire mesh lids, using NIH approved animal procedures. The mice were fed commercial mouse chow and water ad libitum.

3.2. THC

Delta-9-THC was obtained from the Research Technology Branch, National Institute on Drug Abuse. The cannabinoid was diluted in dimethylsulfoxide (DMSO, 20 mg/ml), prior to further dilution in warm RPMI (200 μg/ml).

3.3. Cell Preparation

Single spleen cell suspensions were prepared with a Stomacher 80 Lab Blender (Techmar, Cincinnati, OH) and the erythrocytes lysed by ammonium chloride. Macrophages were collected either as peritoneal resident macrophages or as peritoneal-elicited macrophages following injection of the mice with 3 ml of thioglycollate broth 4 days earlier. The macrophages (10^6 macrophages/well) were adhered in 24 well plates for 2 hrs to remove nonadherent cells (50% of total cells for resident and <5% for elicited cells). All cultures were performed in 10% fetal calf serum medium supplemented with penicillin-streptomycin. The splenocytes (3×10^6 cells/well) were cultured in 24 well plates for 72 hrs with PWM (10 μg/ml). The macrophages were stimulated with either killed *L. pneu-*

mophila (10^8 bacteria/ml), avirulent *L. pneumophila* (10^7 bacteria/ml) or LPS (10 μg/ml). The supernatants were harvested at 24 or 72 hrs and assayed for various cytokines.

3.4. Cytokine ELISAs

All cytokine levels were detected by sandwich ELISA techniques. Medium-bind 96-well Costar EIA plates (Cambridge, MA) were coated with 50 μl of a capture anti-murine cytokine (2–10 μg/ml) in PBS for 2 hrs at 37°C or overnight at 4°C. The plates were blocked for 30 min with 150 μl of blocking buffer. Supernatants from the cultures or serial dilutions of murine cytokine standard were added for 2 hr. In most cases, a biotinylated anti-murine cytokine was added in 50 μl (1–3 μg/ml) for 1 hr, followed by streptavidin-alkaline phosphatase (1:1000, 50 μl, Southern Biotechnology, Birmingham, AL) for 30 min. After 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) was added, plates were allowed to develop for 15–45 min. The plates were washed between additions with 3–5 changes of water. Units were calculated from the standard curve, which was performed for each plate. IL-6, IL-4, and IFNγ ELISAs were set up by this protocol using antibody pairs from Pharmingen (San Diego, CA), and 0.5% BSA, 0.05% Tween-20-PBS as blocking buffer. IL-1α was assayed using hamster anti-IL-1α as capture antibody and rabbit anti-IL-1α as detecting antibody (Genzyme), and alkaline phosphatase labeled goat anti-rabbit Ig (Southern Biotech). IL-12p40/p70 and IL-10 were assayed with Pharmigen pairs, but with 0.1 M $NaHCO_3$, pH 8.2, for the capture antibody, and 3% BSA-PBS as blocking buffer. IL-15 ELISAs were performed with Pharmingen antibody pairs, but with 0.1 M Na_2HPO_4 (pH 9.2), for the capture antibody and 1% BSA-PBS as the blocking buffer. Unfortunately, the unavailability of a IL-15 standard necessitated the expression of IL-15 data in relative units, which are equal to the $OD_{450} \times 1000$. TNFα and IL-1β were performed using DuoSet kits from Genzyme. IL-12, IL-15, IL-10, IL-1β, and TNFα ELISAs used horseradish peroxidase as the linked enzyme and Sigma's TMB substrate system.

4. RESULTS

As illustrated in Figure 1, THC decreased IFNγ production and increased the production of IL-4 and IL-10 in the splenocyte cultures stimulated with PWM (10 μg/ml) for 72 hrs. This effect was dependent upon the dose of THC, which had the greatest effect at 5

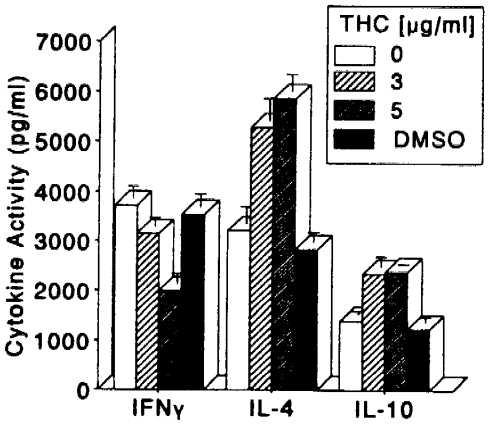

Figure 1. THC decreased IFNγ production and increased IL-4 and IL-10 in splenocyte cultures stimulated with PWM (10 μg/ml) for 72 hrs. THC (3 or 5 μg/ml) or the equivalent DMSO concentration was added at time 0. The data represent mean ± SE for 3 or 5 experiments.

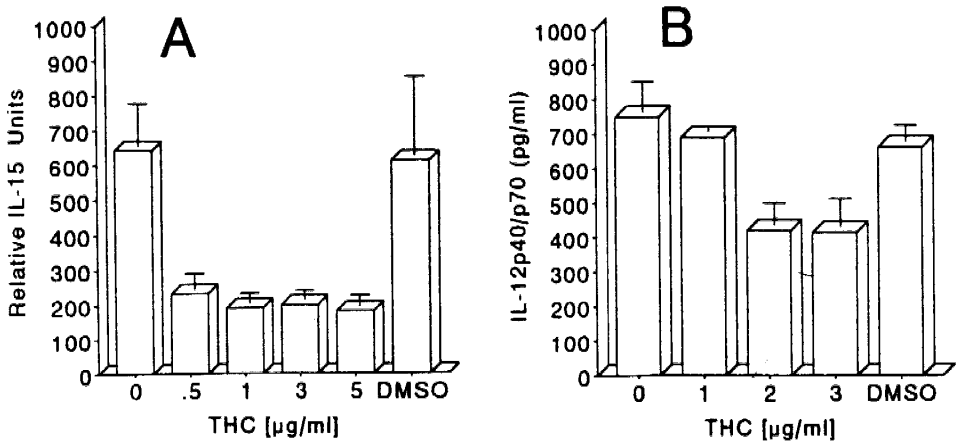

Figure 2. THC decreased IL-15 (A) and IL-12p40/p70 (B) production in splenocyte cultures stimulated with PWM for 72 hrs. THC or the equivalent DMSO concentration was added at time 0. The data represent mean ± SD for 3 experiments.

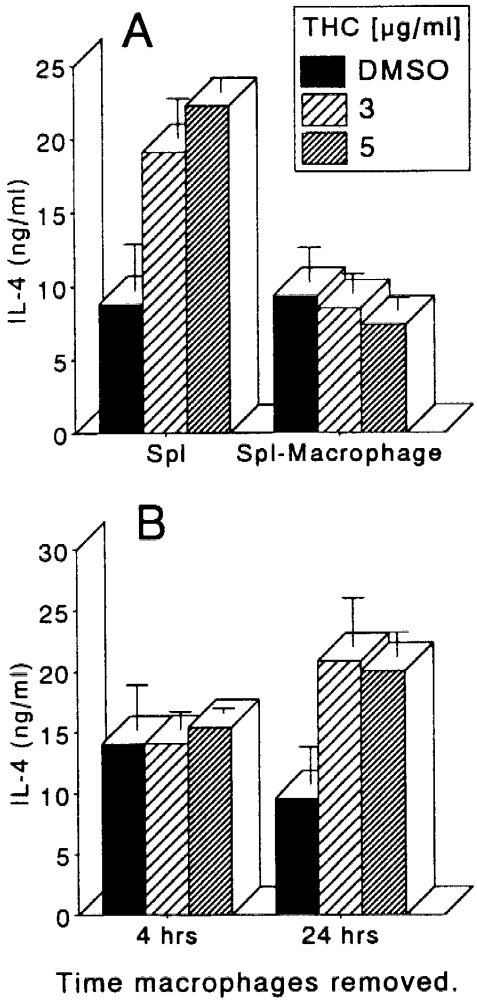

Figure 3. Depletion of macrophages attentuated the THC-induced IL-4 increase in PWM-stimulated 72 hr splenocyte cultures in a time dependent manner. In (A), cultures were either total spleen cells (Spl) or nonadhered spleen cells (Spl-macrophages), where macrophages were adhered out prior to the start of the incubations. In (B), total splenocyte cultures were set up and adhered cells removed at 4 or 24 hrs into the 72 hrs incubation. The data represent mean ± SD for 3 experiments.

ug/ml. In the same culture system, THC was observed to decrease IL-15 (Figure 2A) and IL-12 p40/p70 (Figure 2B) produced in response to PWM. The production of IL-15 was affected with as low as 0.5 μg/ml of THC.

When adhered cells were removed from these PWM-stimulated splenocyte cultures prior to the start of cultures, the elevated IL-4 levels were no longer evident (Figure 3A). This effect was time dependent. If the adhered cells were removed from the cultures at 4 hrs, the THC-induced IL-4 enhancement was not observed; however, when the cells were removed at 24 hrs, the IL-4 elevation was evident (Figure 3B). Therefore, it appears that adherent splenic cells were important for the THC-induce increase of IL-4 levels.

Because the majority of the splenic adhered cells are macrophages, resident and thioglycollate-elicited peritoneal macrophages were examined for THC-induced effects. THC was found to decrease production of IL-12 p40/p70 by thioglycollate-elicited macrophage cultures following 24 hr stimulation with either avirulent *Legionella pneumophila* (10^7/ml) or killed *L. pneumophila* (10^8/ml; Figure 4A). THC also decreased IL-6 production in these cultures (Figure 4B).

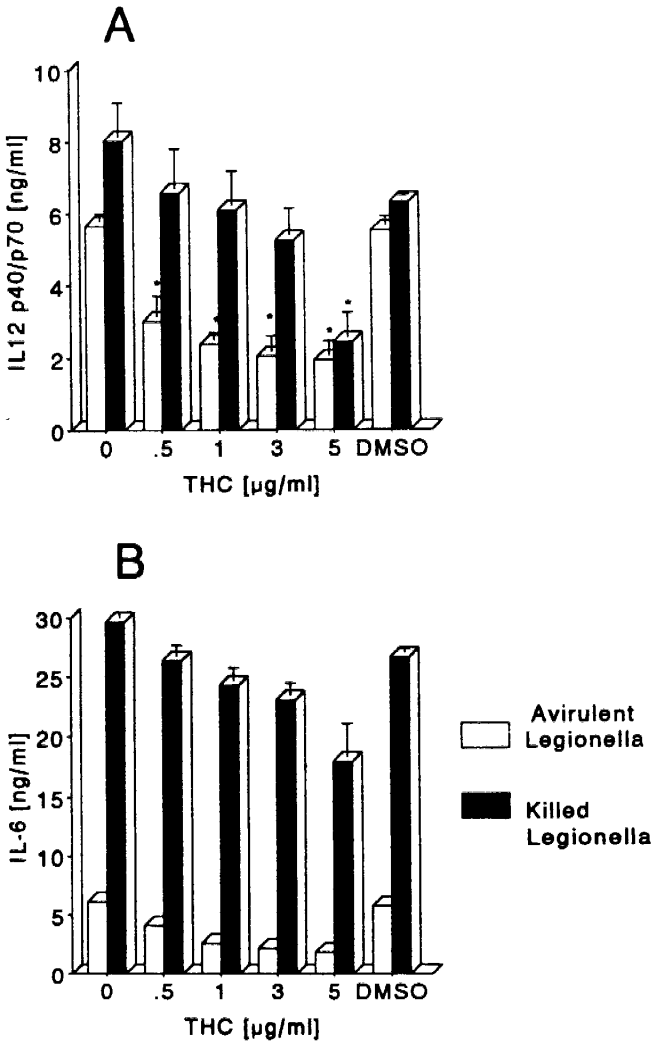

Figure 4. THC decreased production of IL-12p40/p70 (A) and IL-6 (B) by macrophages stimulated with killed or avirulent *L. pneumophila*. Thioglycollate-elicited macrophages stimulated with killed (10^8/ml) or avirulent (10^7/ml) *L. pneumophila* for 24 hrs. The data represent mean ± SD for 3 experiments.

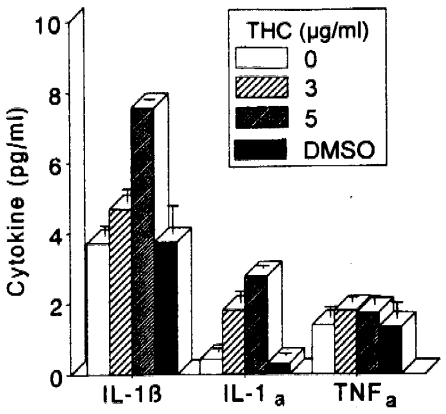

Figure 5. THC increased the levels of IL-1 and TNFa in macrophage cultures. Resident macrophage cultures were stimulated with *E. coli* LPS (10 µg/ml) for 24 hrs with either THC or equivalent DMSO concentration. The data represent mean ± SD for 3 experiments.

Using resident macrophage cultures stimulated with LPS (10 µg/ml) for 24 hrs, the effect of THC on production of other cytokines was also examined. In contrast to the suppressive effects of THC on IL-12 and IL-6, THC increased the levels of IL-1α and β and TNFα in supernatants of these cultures (Figure 5). The increase by THC was not in response to the different stimulator because IL-6 and IL-12 were also suppressed in LPS-stimulated macrophage cultures (data not shown). The macrophage data demonstrate the diversity of the immunomodulatory effects of THC.

5. DISCUSSION

Results of this investigation extend previous studies from our laboratories concerning THC-induced decreased protective immunity to *L. pneumophila*, by inducing a shift in the development of protective Th1 responses to detrimental Th2 responses (2). T-helper cells develop into 2 distinct effector cells, which results in the development of either cell-mediated immunity (Th1) or humoral immunity (Th2) (4). Different cytokines are associated with Th1 and Th2 responses both in terms of the Th cell development and in terms of the cytokines produced by the Th1/Th2 cells. IL-12 and IL-4 are the pivotal cytokines during Th1 and Th2 cell development, respectively, with IL-4 dominating over IL-12 (5). The primary cytokines produced by Th1 cells are IFNγ and TNFβ and by Th2 cells, IL-4 and IL-10 (5). Many of the cytokines produced by Th1/Th2 cells are inhibitory on the activities of the other Th cell. For example, IL-10 has been associated with downregulation of several Th1 responses (6).

In the present study, the presence of THC in PWM-stimulated splenocyte cultures increased in the supernatants the levels of IL-4 and IL-10, and decreased IFNγ, IL-12, and IL-15. It was observed that the THC-induced increase in IL-4 levels was related to presence of adhered splenocytes in these cultures, because their removal early in the cultures eliminated the elevated response. Therefore, it appears that the adhered cells were producing some factor involved in the increase. The majority of the adherent splenic cells are macrophages and macrophages have been shown to produce a variety of cytokines. Macrophages are also thought to be pivotal in the development of Th1/Th2 cells as sources of IL-12 and other cytokines, and as antigen-presenting cells (7). In addition to proinflammatory cytokines, TNFα, IL-6, IL-1α and β, macrophages produce IL-12 and IL-15. Thus, to study adhered cell involvement in this effect, THC was added to cultures of resident or thioglycollate-elicited macrophages stimulated with different stimuli. THC induced increased levels

of TNFα, IL-1α and β, while decreasing IL-12 and IL-6. No IL-15 was detected in the macrophage cultures (data not shown). Production of TNFα by macrophages has been shown to increase production of IL-10 by T cells (8). Therefore, increased TNFα from macrophages could result in increased IL-10 levels which in turn could decrease the development of Th1 responses. Additionally, IL-12 has been demonstrated to inhibit IL-4 synthesis (9). The decreased IL-12 levels could have caused the IL-4 production to increase; although, we do not believe this mechanism is involved in the IL-4 increases in vitro. The nonadhered cells, without macrophages to produce IL-12, did not have an increased IL-4. However, it is possible that the observed decreases in IL-12 could have more of a role in the THC-induced Th1 to Th2 shift in vivo. The high levels of IL-1 could also be involved in the increased Th2 responses. Th2 cells have been demonstrated to have IL-1 type 1 receptors and to respond to IL-1 as a growth factor (10,11). On the other hand, THC appears to decrease Th1 responses. IL-12 and IL-15 are known to be involved in induction of IFNγ from NK and γδ cells (12,13), which are involved in the innate immune responses to *L. pneumophila*. IFNγ is also synergistic with IL-12 in the induction of Th1 cells (14). Therefore, the THC-induced decreases in IL-12 and IL-15 levels could result in reduced Th1 development. The effect of THC on in vitro cultures agrees with the basic in vivo observation that THC induced a shift in the development of Th1 to Th2 immunity. However, which of these effects, the increased Th2 or the decreased Th1 responses, occurs first, or if it is a combination of the two is not known. Additionally, whether any or all of these in vitro observed effects are involved in the in vivo Th1 shift is also unknown at this time. The data does suggest, however, that macrophages are involved, and that THC is modulating these cells. Therefore, these issues will continue to be examined to define the mechanisms of the immunomodulatory effects of THC on the development of and the cytokine production by helper T cells and on macrophages. It is important to investigate these possible mechanisms in the light of increasing calls for the medicinal use of marijuana in AIDS and cancer patients.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Grant DA03646 from the National Institute on Drug Abuse.

REFERENCES

1. Yamamoto Y, Klein TW, Newton CA, Widen R, Friedman H. 1988. Differing macrophage and lymphocyte roles in resistance to *Legionella pneumophila*. J. Immunol. 89:584–589.
2. Newton CA, Klein TW, Friedman H. 1994. Secondary immunity to *Legionella pneumophila* and Th1 activity are suppressed by delta-9-tetrahydrocannabinol injection. Infect. Immun. 62:4015–4020.
3. Noben-Trauth N, Kropf P, Muller I. 1996. Susceptibility to *Leishmania major* infection in Interleukin-4-deficient mice. Science. 271:987–990.
4. Mosmann TR, Coffman RL. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. 7:145–173.
5. Seder RA, Paul WE. 1994. Acquisition of lymphokine-producing phenotype by CD4+ T cells. Annu. Rev. Immunol. 12:635–673.
6. Moore KW, O'Garra A, Malefyt R de W, Vieira P, Mosmann TR. 1993. Interleukin-10. Annu. Rev. Immunol. 11:165–190.
7. Constant SL, Bottomly K. 1997. Induction of Th1 and Th2 CD4+ T cells responses: The alternative approaches. Annu. Rev. Immunol. 15:297–322.
8. Daftarian PM, Kumar A, Kryworuchko M, Diaz-Mitoma F. 1996. IL-10 production is enhanced in human T cells by IL-12 and IL-6 and in monocytes by Tumor Necrosis Factor-α. J. Immunol. 157:12–20.

9. DeKruff RH, Fang Y, Wolf SF, Umetsu DT. 1995. IL-12 inhibits IL-4 synthesis in keyhole limpet hemocyanin-primed CD4+ T cells through an effect on antigen-presenting cells. J. Immunol. 154:2578–2587.
10. Greenbaum LA, Horowitz JB Woods, Pasqualinin T, Reich E-P, Bottomly K. 1988. Autocrine growth of CD4+ Tcells-differential effects of IL-1 on helper and inflammatory T cells. J. Immunol. 140:1555–1560.
11. Lictman AH, Chin J, Schmidt JA, Abbas AK. 1988. Role of interleukin 1 in the activation of T lymphocytes. Pros. Natl. Acad. Sci. USA. 85:9699–9703.
12. Trinchieri G Gerosa F. 1996. Immunoregulation by interleukin-12. J. Leuko. Biol. 59:505- 511.
13. Nishimura H, Hiromatsu K, Kobyashi N, Grabstein KH, Paxton R, Sugamura K, Bluestone JA, Yoshikai Y. 1996. IL-15 is a novel growth factor for murine γδ T cells induced by *Salmonella* infection. J. Immunol. 156:663–669.
14. Mosmann TR, Subash S. 1996. The expanding universe of T-cell subsets: Th1, Th2, and more. Immunol. Today. 17:138–146.