JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2009, 60, Suppl 3, 99-103
www.jpp.krakow.pl

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CANNABIDIOL-INDUCED LYMPHOPENIA DOES NOT INVOLVE NKT AND NK CELLS

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The major non-psychoactive compound of cannabis plant, cannabidiol, has been reported to be a promising therapeutic agent for many inflammatory, autoimmune and neurodegenerative diseases. In spite of growing interest in therapeutic use of cannabidiol very little is known about its influence on the immune system. Present study aimed to evaluate lymphocyte subsets distribution in peripheral blood after repeated, systemic administration of cannabidiol. Adult male Wistar rats received intraperitoneal injections of vehicle or cannabidiol at dose of 2.5 or 5 mg/kg/day, for 14 consecutive days. Blood samples were collected one hour after the last injection. Three-color immunofluorescent antibody staining procedure (CD3-FITC/CD45RA-PC7/CD161A-APC and CD3-FITC/CD4-PC7/CD8-APC) was used for determination of T, B, NK, NKT, T helper, and T cytotoxic lymphocyte subsets. Total leukocyte number and percentage numbers of leukocyte subpopulations were also assessed. Administration of cannabidiol at dose of 5 mg/kg caused a significant decrease in total leukocyte number and a significant fall in total numbers of T, B, and both T helper and T cytotoxic lymphocyte subsets. This immunosuppressive effect did not affect the total numbers of NK and NKT cells that are responsible for the primary, nonspecific antiviral and antitumor immune response. In contrast, administration of cannabidiol at dose of 2.5 mg/kg increased the total and percentage NKT cells numbers, and the percentage number of NK cells. The results suggest that repeated treatment with cannabidiol inhibits specific immunity by reduction of T, B, T cytotoxic, and T helper cell numbers, and may enhance nonspecific antiviral and antitumor immune response related to NK and NKT cells.

Key words: *cannabidiol, immunity, lymphocyte subsets, lymphopenia, nonspecific immune response, NK cells, rat, peripheral blood*

INTRODUCTION

Therapeutic properties of *Cannabis sativa*, Marihuana have been recognized for millennia. Recently, as endocannabinoid system is becoming more understood, attention has focused on natural (endo- and phyto-cannabinoids) and synthetic cannabinoids or cannabinergic ligands as safe therapeutic agents in treatment of many diseases (1). Cannabis contains over 70 different cannabinoids (2) and abundant literature describes immunomodulatory properties of major psychoactive Cannabis compound – delta-9-tetrahydrocannabinol (THC) (3-5). Cannabidiol (CBD) is a major, nonpsychoactive constituent of *Cannabis.* Recent studies have revealed a number of promising pharmacological properties exhibited by CBD, such as anti-inflammatory and anti-autoimmune effects, as well as neuroprotective actions, analgetic, anxiolytic and antipsychotic properties and anticancer potential (6-8). Because of lack of psychotropic effects and low toxicity of CBD (9, 10), there is a growing interest in application of CBD in therapy of various diseases. However, in spite of wide therapeutic potential of CBD, mechanisms underlying its therapeutic effectiveness remain unclear.

There are surprisingly few reports of effects of CBD on the immune system (4). Very few studies have been concerned with influence of CBD on immune parameters in humans or animal models. Most of research focused on cytokine production and majority of studies used *in vitro* cell cultures. CBD, both *in vivo* and *in vitro*, was reported to decrease TNF-alpha (TNF-α) and IFN-gamma (IFN-γ) production (7, 11). Recently, based on *in vivo* studies, CBD has been postulated to decrease production of pro-inflammatory cytokines associated with Th1 immune response (such as TNF-α, IFN-γ or IL-12) and to increase in production of anti-inflammatory cytokines associated with Th2 response (such as IL-4 or IL-10) (12, 13). There are also contrary reports concerning cytokine production, depending on used doses and experimental design (4, 7, 14).

Taking into account that CBD may act *via* various receptors (15-20) present at many different cell types in whole organism, and that it also interacts with other endogenous cannabinoids and modulates their effects, it is difficult to assume that the effects observed *in vitro* will be consistent with those observed in physiological conditions. We are not aware of literature reporting on the *in vivo* effects of CBD administration on peripheral blood lymphocyte subsets distribution or function. Therefore, in order to examine how CBD affects the immune system, we aimed to determine the effects of repeated, systemic administration of CBD on peripheral blood lymphocyte T (both CD4+ and CD8+), B, NK (natural killer), and NKT subsets distribution.

We were particularly interested which lymphocyte subset could be responsible for anti-inflammatory and autoimmune properties of CBD. We focused on T helper (Th, T CD4+) and T

cytotoxic (Tc, T CD8+) lymphocytes suggested to be involved in progression of several autoimmune diseases and in which CBD occurs to be effective treatment. We were also interested how lymphocyte subset and other leukocyte populations distribution relates to therapeutic effectiveness of different doses of CBD in animal models of various diseases. The results may help to understand therapeutic actions of CBD and immune background of diseases in which CBD is therapeutically effective and may also uncover other potentially useful properties of this non-psychoactive cannabinoid.

MATERIALS AND METHODS

Animals

Adult male Wistar rats, purchased from a licensed breeder, and weighing 250 ± 20 g at the beginning of the experimental procedure, were used. Animals were caged in groups of 4, with free access to food and water. Animal room was maintained at 22°C, under 12 h light/12 h dark illumination cycle (on 6am/off 6pm). For 7 days the rats were handled and adapted to the presence of the experimenter to minimalize stress evoked by experimental procedures. The animals were divided randomly into three groups: I - receiving CBD at dose of 2.5 mg/kg/day (n=9); II – receiving CBD at dose of 5 mg/kg/day (n=9); III – receiving vehicle (n=8).

Drugs administration and experimental procedure

CBD (*Cannabidiol*, Lipomed, Arlesheim, Switzerland) was dissolved in cremophor (Sigma), ethanol, saline vehicle (in proportion 1:1:18), which was also used in a control group (21). For 14 consecutive days animals were receiving intraperitoneal injections of vehicle or CBD at doses of 2.5 or 5 mg/kg/day. Doses were based on recent literature, therapeutic effectiveness of CBD in animal models, and our preliminary experiments. All solutions were administered in volume of 1 ml/kg and were prepared immediately before the injections.

Blood sampling

Blood samples in volume of 0.5 ml were collected by cardiac puncture, under halothane anesthesia (*Narkotane* Zentiva, Prague, Czech Republic) and according to our standard procedure (22), one hour after the last injection.

Measured parameters

In every blood sample distribution of lymphocyte subsets was determined by flow cytometry using three color immunofluorescent antibody staining procedure (CD3-FITC/CD45RA-PC7/CD161A-APC and CD3-FITC/CD4-PC7/CD8-APC) for determination of T (CD3+), B (CD3$^-$ CD45RA+), NK (CD3$^-$ CD16.1a+), NKT (CD3+ CD16.1a+), as well as T helper (CD3+CD4+) and T cytotoxic (CD3+CD8+) lymphocyte subsets. Total leukocyte number was assessed by hematocytometer (Baker System 9120 CP, Biochem Immunosytems). Percentage numbers of leukocyte populations: lymphocytes, neutrophils, eosinophils, and monocytes were determined by morphological method (May-Grunwald and Giemsa staining). The total number of each leukocyte subset was calculated as total leukocyte number × percentage of individual leukocyte subset.

Statistical analysis

Data were analyzed with one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) *post hoc*

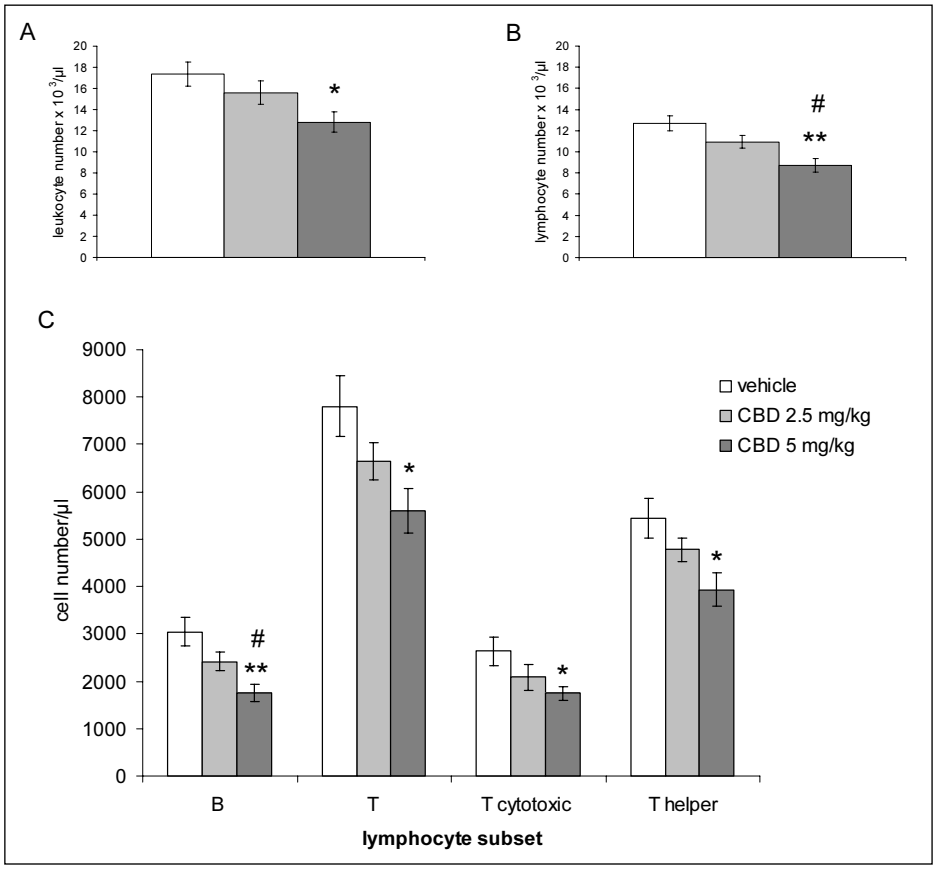

Fig. 1. Dose-dependent, proportional fall in lymphocyte subset numbers following repeated CBD treatment. Significant decrease in total leukocyte (insert A), lymphocyte (insert B), and peripheral blood B, T, T cytotoxic and T helper lymphocyte subsets (insert C) total numbers following administration of CBD at dose of 5 mg/kg. One-way ANOVA followed by Student-Newman-Keuls test: **p<0.01 and *p<0.05 vs control, #p<0.05 vs dose of 2.5 mg/kg.

test. Results are presented as Mean ± SEM and statistical significance threshold is $p<0.05$.

The principles for the care and use of laboratory animals in research, as outlined by the Local Ethical Committee, were strictly followed and all the protocols were reviewed and approved by the Committee. All efforts were made to minimize animals' discomfort and the number of animals used.

RESULTS

Intraperitoneal administration of CBD at dose of 5 mg/kg/day for 14 consecutive days resulted in significantly decreased total leukocyte number in peripheral blood (*Fig. 1A*). This effect was mainly due to dose-dependent fall in total lymphocyte number, significant in group receiving CBD at dose of 5 mg/kg compared with control (*Fig. 1B*). This decrease in total lymphocyte number was significant also in comparison to group receiving CBD at lower dose of 2.5 mg/kg.

Lymphopenic effect caused by administration of CBD at dose of 5 mg/kg involved all measured lymphocyte subsets besides NK and NKT cells responsible for primary, nonspecific antiviral and antitumor immune response. While total numbers of B, T, T helper and T cytotoxic lymphocytes were significantly decreased (*Fig. 1C*), total number of NK and NKT cells remained unaffected in spite of significant lymphopenia produced by administration of CBD at dose of 5 mg/kg (*Fig. 2*).

In contrast, dose of 2.5 mg/kg not only did not cause any significant lymphopenic effects but it induced increase in total and percentage NKT cells number and percentage number of NK cells (*Fig. 2* and *3*). Administration of CBD at dose of 2.5 mg/kg produced over two-fold increase in NKT cells total number in comparison to vehicle treated rats (*Fig. 2A*). NK cells total number was also slightly increased in CBD treated rats, but this effect did not reach level of statistical significance (*Fig. 2A*). However, NK and NKT cells total number in rats treated with CBD at dose of 2.5 mg/kg was significantly elevated in comparison to rats receiving dose of 5 mg/kg, which in case of NK cells fell below baseline, indicating that administered doses of CBD exerted bidirectional effects on NK cell numbers (*Fig 2A*). Fall in peripheral blood lymphocyte subset numbers observed as result of CBD administration at dose of 5 mg/kg was proportional, thus no change in percentage distribution of B, T, Tc and Th cells in lymphocyte population was observed. However, administration of CBD at dose of 2.5 mg/kg produced significant increase in both NK and NKT cells percentage numbers in total lymphocyte population (*Fig. 2B*). NK cells percentage number in population of peripheral blood lymphocytes was increased also in comparison to group receiving dose of 5 mg/kg (*Fig. 2B*). NKT cells percentage number in group receiving dose of 2.5 mg/kg was elevated almost three-fold in comparison to controls (*Fig. 2B, Fig. 3*).

CBD administration also resulted in significant elevation of eosinophil percentage number in total leukocyte population which was increased from 2.0 ± 0.4% in controls to 3.5 ± 0.4% ($p<0.05$) after administration of CBD at dose of 5 mg/kg. CBD administration did not affect neutrophil number.

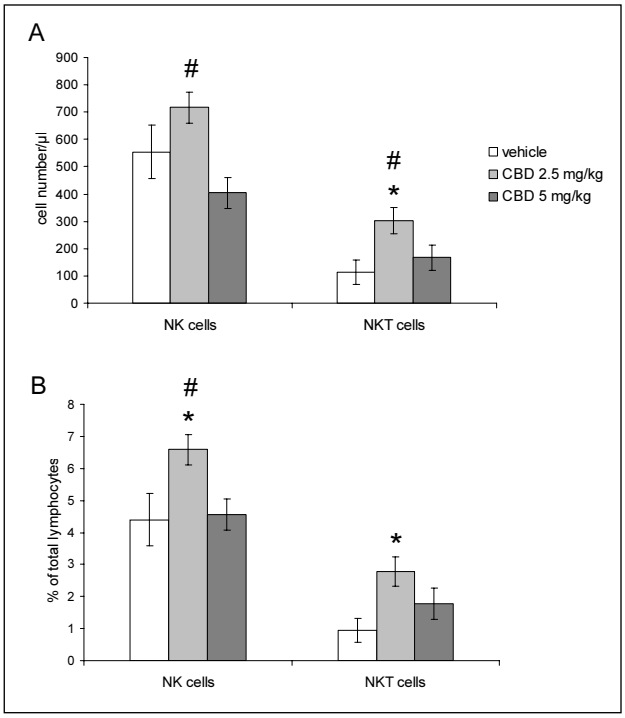

Fig. 2. Significant increase in total (insert A) and percentage (insert B) NKT cells number and percentage number of NK cells following repeated CBD administration at dose of 2.5 mg/kg. Bidirectional effect of different doses of CBD on the NK cells numbers is shown. One-way ANOVA, followed by Student-Newman-Keuls test: *$p<0.05$ vs control; #$p<0.05$ vs dose of 5 mg/kg.

DISCUSSION

Our experiments revealed that systemic, repeated for 14 days administration of CBD, at relatively low doses of 2.5 mg/kg or 5 mg/kg/day, produced bidirectional effects on lymphocyte subset distribution in peripheral blood of rats. Administration of CBD

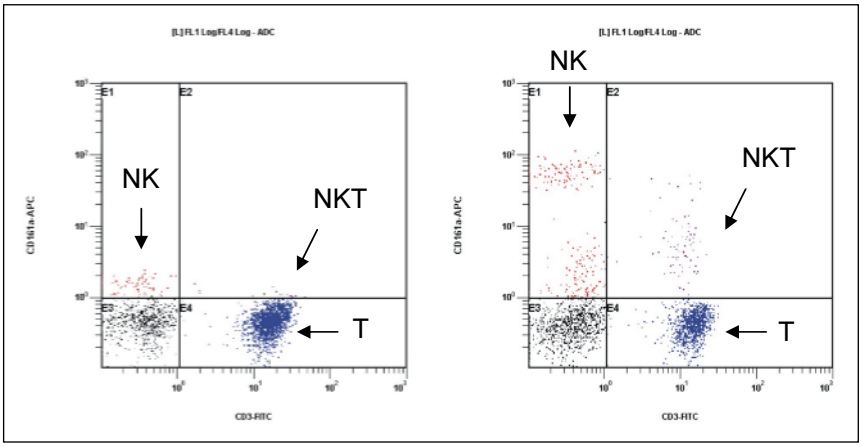

Fig. 3. Cytometric analysis of peripheral blood NK, NKT and T cells of representative rats receiving vehicle (left cytogram) and cannabidiol at dose of 2.5 mg/kg (right cytogram). Right cytogram shows increased, in comparison to rat receiving vehicle, NKT cell population and population of NK cells with elevated, in comparison to control rat, expression of CD16.1a marker (characteristic for NK subpopulation) as result of repeated treatment of CBD at dose of 2.5 mg/kg.

at dose of 5 mg/kg resulted in clearly immunosuppressive (lymphopenic) effects on total leukocyte number and B, T, Tc and Th lymphocyte subsets, but this lymphopenic effect did not include NK and NKT cells. In contrast, CBD administered at dose of 2.5 mg/kg did not produce significant lymphopenia, but resulted in increase of NKT cells total number and percentage numbers of NK and NKT cells.

Our findings suggest that anti-inflammatory and anti-autoimmune properties of CBD may be due to suppression of both humoral and cellular, specific immune response associated equally with B, T, T helper and T cytotoxic lymphocytes, which was expressed as proportional fall in those lymphocyte subset numbers in peripheral blood.

NK cells are major component of primary, nonspecific antiviral and antitumor immune response and their activity may be an important factor associated with risk of development of neoplastic changes (23). Also, number of NKT cells in circulation has been recently suggested to be important in cancer patients prognosis (24). While number of NK and NKT cells may explain some antitumor effects exhibited by CBD, to confirm enhancement of antitumor action by CBD *via* NK and NKT cells further studies of NK cells cytotoxic activity should be performed.

Lymphopenic effects produced by CBD was observed after administration of CBD at dose of 5 mg/kg - dose which seems to be most effective in number of animal models of inflammatory and neurodegenerative diseases (11, 13, 25). CBD administered i.p. at dose of 5 mg/kg was found to block the progression of collagen-induced arthritis – a murine model of rheumatoid arthritis (11), while dose of 2.5 mg/kg was ineffective and in our study failed to produce significant lymphopenia. Moreover, the same lymphopenic dose of 5 mg/kg was found to reduce incidence of diabetes in NOD mice (autoimmune disease mediated mainly by Tc and Th cells), by decreasing damage of pancreatic islets (13). CBD, at dose of 20 mg/kg p.o. which corresponds to about 4 mg/kg i.p, was also found to be effective in attenuating hyperalgesia in rat models of neuropathic and inflammatory pain (11, 26, 27). CBD at dose of 5 mg/kg was reported to be maximally effective dose in preventing cerebral ischemia in gerbils (25), but the mechanism of its effectiveness is not clear (28). Thus, our study confirms previous findings and suggests that dose of 5 mg/kg is optimal by producing lymphopenic effect in rodents, and could be effective in treatment of inflammatory diseases (11, 13).

Most recent literature indicates that CBD, *in vivo*, produces shift from Th1 to Th2 immune response which is expressed as decrease in production of pro-inflammatory cytokines TNF-α, IFN-γ, IL-12, IL-6, and increase in production of cytokines associated with immunosupression like IL-4 or IL-10 (12, 13). This shift from Th1 to Th2 immune response is suggested to be responsible for anti-inflammatory properties of CBD. However, this issue is not clear yet, since opposite findings were reported as well (4, 14). Sacerdote *et al*. have found decrease of macrophage IL-10 production and increase in IL-12 production, which suggest pro- rather than anti-inflammatory action of CBD on macrophage function. IL-12 is also strongly associated with NK cells function. Our findings, therefore, may provide at least partial explanations of changes in cytokines production observed after administration of CBD.

It is has been established that cannabinoids usually exert most of their effects in inverted U shaped dose-response curve (8, 25). Indeed, in our study, as result of CBD administration at 2.5 mg/kg dose, we observed increase in NK and NKT cells numbers, and administration of a dose of 5 mg/kg, in spite of lymphopenia, resulted in significantly increased percentage of eosinophils in peripheral blood. This may suggest that CBD at small doses may stimulate primary, nonspecific immunity, while inhibiting specific immune response associated with B and T (both CD4+ and CD8+) lymphocytes and high risk of autoimmune response. Furthermore, mechanisms of CBD anti-tumor action have not been yet identified, so our findings that CBD has potential to increase NK and NKT cells number are promising and this issue deserves focus in further studies.

Present data suggest that CBD may exert its anti-inflammatory and anti-autoimmune effects not only by suppressing Th1 immune response and stimulating Th2-dependent response, as it has been recently postulated, but it may also act by suppression of specific immunity associated with B, T, Tc and Th lymphocytes and by enhancement of nonspecific immune response associated with NK and NKT cells. Furthermore, taking into account that there is wide range of therapeutically effective doses of CBD (10 to 1500 mg/day, in humans, p.o.), administration of doses examined in our study should produce very similar or even the same effects. The results suggest that CBD effects are very dose-sensitive and precise dose may be critical to obtain efficient therapeutic effects.

Acknowledgements: This work was supported by Polish Ministry of Science and Higher Education Grant N N303 394036.

Conflict of interest statement: None declared.

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Received: November 6, 2008
Accepted: February 2, 2009

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