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STUDIES ON IMMUNOTOXIC POTENTIAL OF METHAMPHETAMINE (MA) IN Balb/C MICE

II. The Functional Alteration of Effector Cells in Immune System

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ABSTRACT : *Several populations of lymphocytes possess receptors for autonomic neurotransmitter, which make lymphocytes susceptible to autonomic stimulation. This study was to evaluate the functional alternation of effector cells of the immune system. Female Balb/C mice, 15-20 g, were injected with MA subcutaneously under various conditions. Mixed lymphocyte reaction (MLR) showed certain T cell subsets were affected by MA. The level of interleukin-2 (IL-2) production was inhibited due to a defect in expression of the IL-2 receptor. In mice injected with 20 mg MA/kg, 1 day before assay, phagocytosis of peritoneal macrophages showed 14.07 ± 3%, which was similar degree to 5 mg MA/kg treatment for 4 consecutive days. Phagocytosis was almost recovered to that of control after 4 day in 20 mg/kg injected mice. Maximum inhibition of plaque forming cell (PFC) occurred when MA was given early, indicating the inductive time point of antibody production was affected. The cortisol level increased in the MA treated group (0.05, 0.20, and 0. 08 μg/dl for control, low, and high dose-MA treated mice, respectively). Based on these results, MA has general suppression effects on the immune systems by functional alteration of effector cells. Considering the increment of serum cortisol levels, MA partially impacts the neuroendocrine system to lead to failure of immune response.*

Key Word : *Mathamphetamine, Mixed lymphocyte reaction (MLR), IL-2, Suppresser cell, Phagocytosis, Plaque Forming Cell (PFC), Cortisol.*

I. INTRODUCTION

The field of neural-immune interactions has developed rapidly after the discovery of autonomic nerve fibers in specific compartments of both primary and secondary lymphoid organs (Felten *et al.*, 1985, 1987).

The specific receptors for many neuroendocrine factors have been identified in immunocompetent cells, and these include ACTH (Weigent *et al.*, 1987), vasoactive intestinal peptide (VIP) (Danek *et al.*, 1983), substance P (Payan *et al.*, 1984), prolactin (Bernton *et al.*, 1988), Steroid hormone (Su *et al.*, 1988), catecholamine (Ovadia *et al.*, 1987). In vivo studies have shown that the communication between the neuroendocrine and the immune system is bi-directional. Changes of sympathetic tonus lead to changes in immune parameters, on the other hand, Stimmlation of the immune system leads to change in central and peripheral sympathetic activity. Furthermore, several study have shown that lymphocytes can synthesize biologically active neuroendocrine peptide hormone and that they have receptors for the same hormones (Blalock *et al.*, 1981; Smith *et al.*, 1983; Goetzl *et al.*, 1985; Johnson *et al.*, 1982).

At present, no studies have been demonstrated the relationsrip between MA abuse and immune function. Only Manuel *et al.*, (1991) have demonstrated that amphetamine showed inhibition of T cell proliferation to mitogen. Based on previous study, the purpose of this study was to evaluate the mechanism of funtional alteration of immune response in the MA treated mice.

II. MATERIALS AND METHODS

1. Animals

Female Balb/C mice were obtained from KRICT and used the bred weighing 15 to 20 g. The animals

were randomly distributed in polypropylene cages on sawdust bedding (β-chip) with tap water and commercial rodent chow pellet (Sam yang) available *ad libitum*.

2. MA Treatment

MA was obtained from Korean authorities and dissolved in saline prior to use. Mice were injected subcutaneously for 7 or 14 consecutive day at 0.5, or 5 or 20 mg MA/kg, according to the various experimental schedules, while control mice received saline as a vehicle.

3. Spleen Cell Suspension

Splenocyte suspensions were prepared by gently squeezing the organs in cold RPMI 1640 (Gibco) and passing the cells though a nylon filter with a pore sized 53 μm (Spectrum, USA). Following red blood cell lysis by hypotonic shock and wash, cells were resuspended in complete culture media. The culture medium was RPMI 1640 supplemented with gentamycin sulfate (100 mg/L), L-glutamine (2 mM), Herpes buffer (10 mM), and 10% fetal calf serum (Gibco). Cell numbers were determined with a haemocytometer and viability was measured by exclusion of trypan blue (Leslie *et al.*, 1989).

4. Mixed Lymphocyte Reaction (MLR)

To 50 μl, 2×10^5 splenocyte/well of experimental mice, 50 μl of the same number of C57BL/6 splenocyte (allogeneic stimulator) and 100 μl of media were added in 96 well round bottomed plates (Nunc). Splenocytes (responder) of experimental mice were adjusted to 4×10^6/ml. Splenocytes (stimulator) from C57BL/6 ($H\text{-}2^q$) were adjusted to 2×10^7/ml, incubated at 37°C, 40 min with mitomycin C (Sigma) at 25 ug/ml and adjusted 4×10^6/ml. Responder cells were cultured alone or with stimulator cells in triplicate for 96 hr and pulsed with 3H-thymidine (0.5 μCi/well in 25 ul). At 18 hr, cells were harvested and the incorporated radioactivity was determined (Hohn *et al.*, 1989).

5. Interleukin-2 (IL-2) Production

On day 8, obtained splenocyte, 2×10^6/ml, incubated with 10 μg/ml Con A at 37°C in 5% CO_2 incubator for 48 hr. The supernatant was harvested and stored at -20°C in aliquots until assay. Supernatant, 50 ul, and diluted 1/2 or 1/5 supernatant, respectively, were put in a 96 well flat-bottomed well plate in triplicate. CTLL cell at 1×10^5 cell/ml were added to each well. They were incubated at 37°C in 5% CO_2 in air for 24 hr, pulsed-labeled with 1 μCi/well of ^{3}H-thymidine for 24 hr and the uptake measured (Gillis *et al.*, 1978).

6. Expression of high affinity IL-2 Receptor

To prepare the blast cells, 2.5 ml of 8×10^6/ml splenocyte, 500 μl of 400 μg/ml Con A and 7.0 ml of complete media were put in a 25 cm^2 tissue culture flask (Falcon). After 72 hr of culture, cells were washed with media containing 100 mM a-methylmannoside. 50 μl of adjusted Con A blast cell were cultured in 96 well round bottomed culture plate containing 50 μl of 0.125, 0.5, 2, 8 U/ml recombinant IL-2 standard (5×10^5 U/mg, KIST). The proliferation assayed by ^{3}H-thymidine uptake of 0.5 μCi/well for 6 hr and radioactivity was measured (Anderson *et al.*, 1979).

7. Phagocytosis

Mice were injected with 20 mg MA/kg on day 1 or day 4, or injected for 4 consecutive days with daily 5 mg MA/kg. On the 5th day, one % (v/v) SRBC was prepared in PBS. 0.5 ml SRBC suspension was injected into the peritoneal cavity of the mouse. PBS was given intraperitoneally, 30 min later and peritoneal cells were harvested. After being incubated at 37°C, 15 min on the slide, the cells were stained with Wright-Giemsa. The percentage of phagocytosis (the percentage of macrophage ingesting SRBC) was calculated.

8. Splenic IgM Antibody

Animals were immunized with 0.2 ml of 10^9 Sheep red blood cell (SRBC) intravenously 4 day prior to assay. Mice were injected with MA at 5 MA mg/kg on day 1, 2, 3, and 4, respectively. On the 5th day, to 50 μl, 4×10^7 cell/ml, of splenocyte suspension, 100 μl of guinea pig complement (Gibco) diluted to 1:5 with PBS, 100 μl of SRBC (2×10^9/ml), and 500 μl of medi-

um were added. The well-mixed suspension was placed in microchamber, sealed with wax, and incubated at 37°C for 1 hr. The number of plaques was counted (Cunningham *et al.*, 1968).

9. Serum Cortisol Level

Serum cortisol level was measured by fluorometric enzyme immunoassay. Blood was drawn from the retro-orbital venous plexus by capillary tube and serum was collected. The cortisol level was measured by Stratus flourometric enzyme immunoassay. 20 μl samples, premixed with buffer containing 8-anilino-1-naphthaline sulfonic acid and conjugate containing calf intestinal alkaline phosphatase linked to cortisol, were mixed well and reacted to plastic tabs containing rabbit anti-cortisol serum. Subsequently, substrate was added and unbound enzyme-labeled cortisol was washed. The front surface fluorescence of the tab was read by Stratus fluorometric analyzer (Bazter Healthcare Co., U.S.A.).

10. Statistical Analysis

The data were analyzed using paired Student's t-test and considered significant at $p<0.05$.

III. RESULTS

1. Mixed Lymphocyte Reaction

The mixture of lymphocytes from two genetically different mice was carried out to measure the function of T cell recognition of lymphocyte. The stimulation indices of control, 0.5, and 5 mg MA/kg treated mice were 22.96±3.62, 15.14±1.09, and 8.67±1.36, respectively (Fig. 1), indicating the function of T cell recognition decreased.

2. IL-2 Production and Expression of High Affinity IL-2 Receptor

The responsiveness of CTLL-2 cells to standard γIL-2 was initiated at 0.125 U/ml. The maximum proliferation was shown at 4 U IL-2/ml and declined at 8 U IL-2/ml concentration. The level of IL-2 produced by splenocytes from 5 mg MA/kg treated animals following Con A stimulation was inhibited and also inhibited in 1/2 or 1/4 diluted supernatant (Fig. 2). IL-2 receptor were rapidly expressed on T cells after interaction with antigen or mitogen. The proliferation of blast cells was increased according to rising gam-

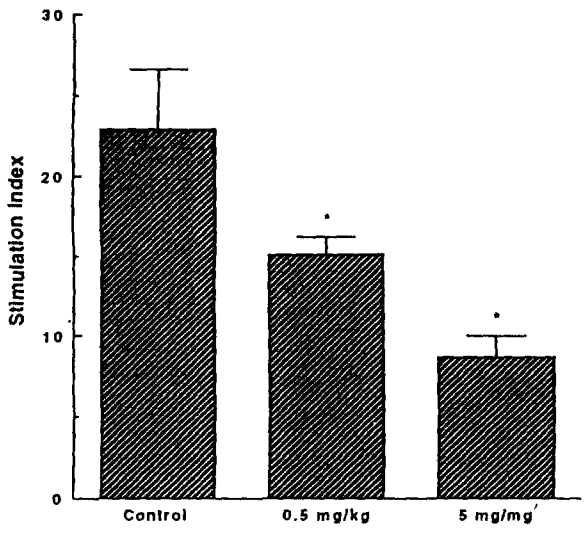

Fig. 1. Effect of methamphetamine (MA) on mixed lymhocyte reaction from Balb/C mice injected for 14 days (mean±SD, n=3). On day 15, responder cells were prepared from the splenocytes of control and treated Balb/C mice ($H\text{-}2^b$) and stimulator cells prepared from the splenocytes of C57BL/6 ($H\text{-}2^d$) were treated with mitomycin C before addition to the culture. Values were presented as a stimulation index (cpm responder + stimulator/cpm). * Significantly different from control; * $P<0.05$.

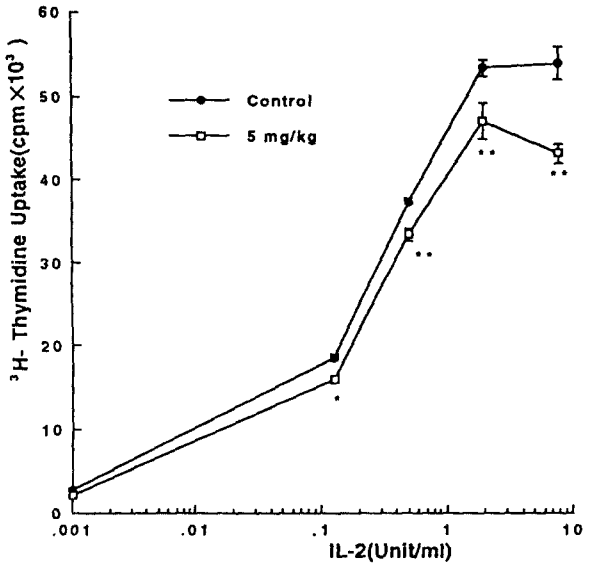

Fig. 2. Effect of methamphetamine on IL-2 production of supernatant from Con A-induced splenocyte from Balb/C injected at 5 mg MA/kg for 7 days (mean±SD, n=3). On day 8. splenocytes (2×10^6/ml) were cultured in complete media containing Con A (10 ug/ml). Culture supernatants were harvested at 48 hr and diluted 1/2 or 1/4, and tested for production of IL-2 by CTLL-2 cells. *Significantly different from control; *$p<0.05$.

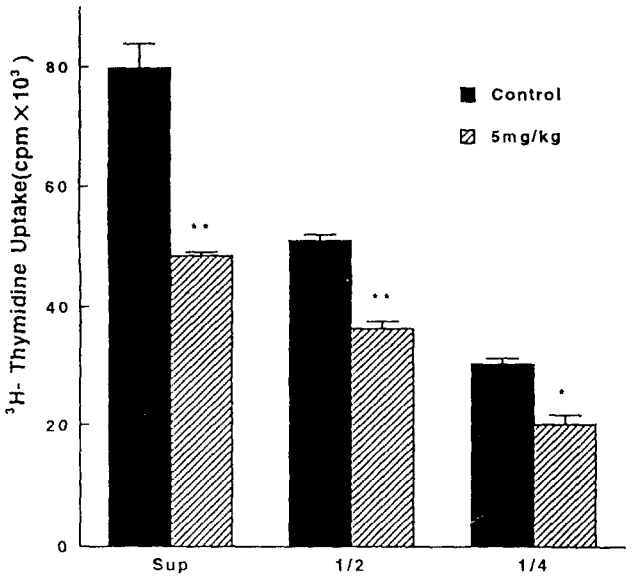

Fig. 3. Effect of methamphetamine on the expression of high affinity IL-2 receptor from Con A-induced Blast of Balb/c mice injected at 5 mg MA/kg for 7 days (mean± SD, n=3). On day 8, Con A induced splenoblasts were examined the responsiveness to exogenous IL-2 (0.125, 0.5, 2, 8 U/ml). *,**Significantly different from control; *p<0.05; **p<0.01.

ma IL-2 concentration. The peak proliferation responses were at 2 U/ml IL-2 concentration in both the MA treated and control groups, but the degree of response was decreased in MA-treated group (Fig. 3). The failure of this process may result from the failure of triggering the resting T cell to enter the activated state by antigen or mitogen.

3. Phagocytosis

The phagocytic percentage of control was 25.27±5.25% (Table 1). Daily injection of 5 mg MA/kg for 4 consecutive days suppressed ingestion of SRBC (16.02±4.24%). In mice injected with 20 mg MA/kg on day 4, 24 hr before the assay, the phagocytic percentage was 13.95±4.01%, which was decreased by 45% to controls. However the phagocytosis of 20 mg MA/kg injected mice on day 1 was recovered almost to that of control.

4. Splenic IgM Antibody Response

Groups 1, 2, 3, and 4 were immunized on the 1st day and were given MA at the same dosage on days 1, 2, 3, and 4, respectively. On 5 day, experiment was carried out. The maximum inhibition of PFC occurred if MA was given on the same day of immunization (Fig. 4). The effects of inhibition gradually disappeared with increased time intervals between immunization and the administration of MA.

Table 1. Effect of methamphetamine on phagocytosis from Balb/C mice.

		20 mg MA/kg		
	Control	5 mg MA /kg×4	Day 1	Day 4
(n=4/group)	25.27± 5.25	16.02± 4.24*	22.01± 4.72	13.95± 4.01**

Mice were injected with MA at 20 mg/kg on day 1 or day 4. Also mice were injected for 4 consecutive days with a daily dosage of 5 mg/kg. On day 5, 0.2 ml of 1% (v/v) SRBC suspenison was injected into the peritoneal cavity of the mouse. Thirty minutes later, peritoneal macrophages were obtained and counted phagocytized cells. The values were calculated as the percentage of macrophage ingesting SRBC. *, **Significantly different from control; *p<0.05, **p<0.01.

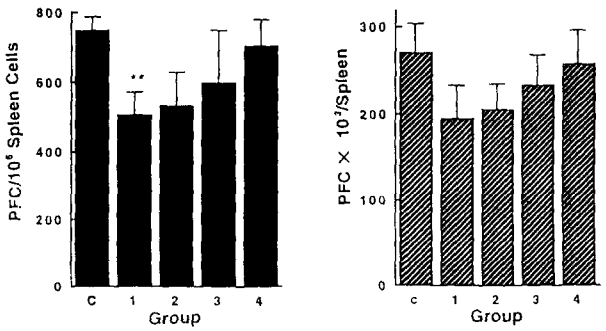

Fig. 4. Effect of methamphetamine on direct IgM plague forming cell (PFC) response from Balb/C mice injected at 5 mg MA/kg on day 1, 2, 3 and 4, respectively (mean± SD, n=3). The control (C) group was injected with saline on day 1. On day 1, 0.2 ml of 10^9 sheep RBC was injected ip to all mice. The PFC assay was performed on day 5. **Significantly different from control; **p<0.01.

5. Serum Cortisol Levels

Cortisol levels of the two control group were not detectble in control group for the reason that the sensitivity of the Stratus cortisol assay was 0.1 μg/dl (Fig. 5). Other two control levels were 0.1 μg/dl. The mean values of control, 0.5 and 5 mg MA/kg treated groups were 0.05, 0.08, and 0.15 μg/dl, respectively. The increments of increasing serum cortisol level of two treated groups were about 4- and 8.5 fold, respectively, to that of control.

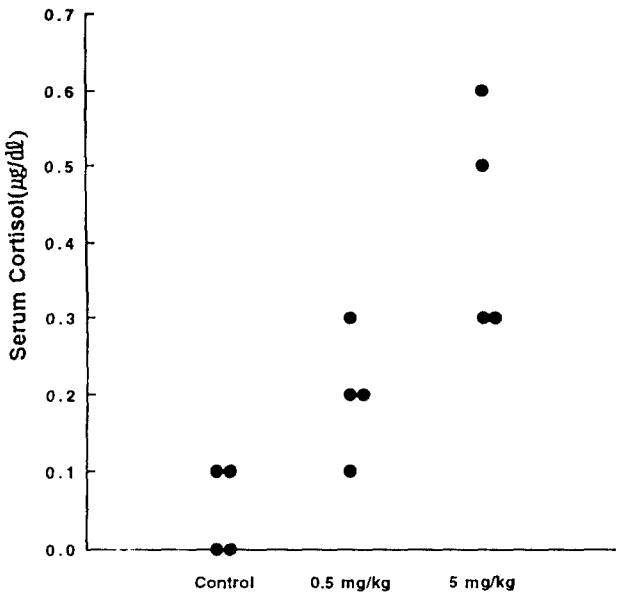

Fig. 5. Effect of methamphetamine on serum cortisol level from Balb/C mice. The blood was collected beginning at 10:00 hr on day 15. Serum cortisol was measured by fluorometric enzyme immunoassay. Each dot represents the serum cortisol level in each mouse.

IV. DISCUSSION

Although systemic research in psychoneuroimmunology is relatively new, the range of the phenomena that bear on the brain and immune system is quite broad (Ader *et al.*, 1987; Madden *et al.*, 1995). Receptors for many hormones and neurotransmitters including catecholamines and acetylcholine, have been detected on the surface of lymphocytes (Paut, 1987). A growing body of evidence indicated that communication from the nerve system to the immune system may occur via innervation of lymphoid tissues, neurohormones and endocrine hormones. The similarity in chemical structure between MA and catecholamine is significant for the understanding of the sympathomimetic properties of MA (Bowman, 1980).

In light of the preferential effects of MA on T cell for assessing cell-mediated immunity, MLR was utilized. As observed in Fig. 1, stimulation index of treated groups reduced MLR in a dose dependent manner. The ability of splenocytes to undergo MLRs has been reported to be a sensitive assay for CD4 activity. This may suggest that certain T lymphocyte subsets are apparently affected. A decreased Th/Ts cell ratio after mental stress, or injection of epinephrine has been demonstrated (Crary *et al.*, 1983; Landmann *et al.*, 1984). In different experimental systems, a variety of cell types, including T cells (Zubler *et al.*, 1980), B cell (Katz, *et al.*, 1974), and macrophage (Oehler, *et al.*, 1977) have been cited as the mediators of suppression. To elucidate this hypothesis, flow cytometric analysis of lymphocytes subpopulation is needed in future studies.

One week following MA exposure, production of IL-2, a regulatory cytokine involved in the generation of both cell-mediated and humoral immune response decreased. IL-2 production is confined to CD^{4+} T cells of the Th1 phenotype. IL-2 exerts a pleiotropic effect to lymphocytes via specific receptors. The α chain of IL-2 receptor, bound IL-2 with a low affinity ($K_d=10^{-8}$ M), whereas the β chain bound IL-2 with an intermediate affinity ($K_d=10^{-9}$ M) (Thomas, 1989). It appears that the combination of these two chains form the high affinity receptor. The resting cells do not express high affinity IL-2 receptors but receptors are rapidly expressed on T cells after interaction with antigen or mitogen. Since the overall functional response of the lymphocyte was decreased in MA treated mice, changes of IL-2 and its receptor were more or less expected.

The macrophage plays a critical role in the T cell dependent antibody response in that it functions to process and present antigen to the T cell. MA affects the ability of macrophage to ingest SRBC. The inhibitory effect was higher 24 hr after MA injection, but decreased with time. Most antigens, including SRBC, require the assistance of both macrophages and Th cells to present antigens to B cells. Maximum inhibition of PFC occurred when MA was given early to immunized mice, which suggests that the inductive phase of antibody production is the target of MA action. Cocaine, a psychostimulant, also suppressed the antibody response to SRBC and enhanced generation of suppresser T cells (Bagasra, *et al.*, 1989). On the contrary, Havas *et al.*, (1987) found that cocaine treatment in female mice resulted in an slight increase of antibody responses.

The level of cortisol increased in a dose dependent manner. Glucocorticosteroids (GCS) are potent immunosuppressive agents under both experimental and clinical conditions (Cupps *et al.*, 1982). Experimental evidence in mice has been presented to suggest that GCS-induced lymphocytopenia may, in part, be due to a consequence of an acute redis-

tribution of recirculating lymphocytes to certain lymphoid tissues. The observation by some results demonstrated that far more immunopotent T cells can be sequestered into the bone marrow shortly following steroid treatment from normal animals. These results are in good agreement with the decrease in leukocyte count in MA treated mice in 14 days of consecutive injection studies.

Based on these study, MA has general suppressive effects on the immune system by functional alteration of effector cells vital to immune response. Considering the increment of serum cortisol levels, it is postulated that MA is partially impacted by the neuroendocrine system to lead to failure of immune response. To provide evidence in relationship between neurotransmitter and immunity, and what subpopulation of lymphocytes are pivotal in changing the immune response of MA treated mice, further studies should be conducted to examine catecolamine levels and change in lymphacyte subset.

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