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Preventive Effects of Melatonin on the Cell-Mediated Immunotoxicity of Cadmium in ICR Mice

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ABSTRACT *: To investigate the preventive effects of melatonin (MLT) on the immunotoxicity of cadmium acetate[$Cd(AC)_2$] in ICR mice, MLT(10, 50 mg/kg as cadmium) were orally administered to mice once a day (5 : 00, PM) for 28 consecutive days. Cadmium (Cd) test solution was also administered at 25 mg/kg of cadmium through the same route 2hr after administration of MLT daily. Mice were immunized and challenged with sheep red blood cells (SRBC). Immune functions evaluated were delayed type hypersensitivity (DTH) response, mitogenic response and flow cytometry analysis. The results of these studies were summarized as follows; DTH response was abnormally increased in mice treated with Cd alone. DTH response was normally depressed in mice treated with Cd plus MLT along with the increase of MLT doses. The mitogenic response of splenic T cell to Con A and that of B cells to LPS was remarkably increased by MLT treatment as compared with treatment of Cd alone. Splenic $CD4^+$ cells were significantly increased by MLT treatment, as compared with treatment of Cd alone. In case of CD 8^+ cells, the slight increase was observed in MLT treatment. Splenic T cells and B cells were significantly increased by MLT treatment as compared with treatment with Cd alone. These results suggest that MLT has significant preventive effects on the immunotoxic status induced by Cd exposure.*

Key Words *: Melatonin (MLT), Cadmium (Cd), Immunotoxicity*

I. INTRODUCTION

The immune system is highly integrated and complex network of cells that produce specific and nonspecific factors regulating its overall activity (Jerne, 1974). Both positive and negative regulating factors are produced by specific subpopulations of lymphoid and myeloid cells (Gershon *et al.*, 1981). Therefore, agents that are toxic to the immune system may act via numerous direct or indirect mechanisms in producing their untoward effects. Many agents have previously been shown to exert deleterious effects on the immune system (Dean *et al.*, 1982; 1985). MLT nocturnally produced by the pineal gland is chemically expressed during darkness in the organism. This lipid soluble and low molecular weight molecule, which is structurally related to serotonin, is able to cross membranes and the blood brain barrier and is present at night in all biological fluids (Reiter, 1991). Maestroni and Conti (1990) showed that physiological concentrations of MLT stimulated $CD4^+$ T cells *in vitro* to release mediators, which competed with the specific binding of ^{3}H-naloxane to mouse brain and thymus membranes. Chronic administration of MLT enhanced the antibody response via induction of T helper cell activity, augmented mitogenic T and B cell responses, induced higher levels of interferon-γ(IFN-γ) and interlukin-2(IL-2) in splenocytes (Caroleo *et al.*, 1992; 1994; Sze *et al.*, 1993), and enhanced the antigen presentation by splenic macrophages to T cells by increasing the expression of MHC class molecules and the production of IL-1 and TNF-α (Pioli, Caroleo, Nistico & Doria, 1993). Lissoni *et al.* (1993) and Maestroni (1995) postulated that two of the main target cells for MLT activity on immune functions in humans might be T lymphocytes and

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macrophages. On the other hand, Cd has been widely used in industry, as an anticorrosive agent for steel, iron, brass and other alloys, and as a stabilizer for paint, pigments, batteries and plastics (Marcus *et al.*, 1977; John, 1982; Frank and Jerome, 1980; Goyer, 1986). Especially, Kim *et al.* (1998) reported that the total cadmium intake via market and traditional food and cigarette smoking was 136.6 μg/week. Cd-induced toxicity to specific organs, including the immune system, has been extensively studied. Effects of Cd on immune system include reports of both immunosuppression and immune enhancement. Cell-mediated immunity was more consistently shown to be depressed (Descotes, 1992). Suppression of mitogenic response of spleen cells to Con A, PHA, and LPS was also found to be time-dependent. Spleen cell surface marker expression (Mac-1, Lyt-1, Lyt-2 and 14.8) was altered in response to Cd treatments, but these changes did not appear to be correlated with the humoral immunity or mitogen-induced proliferation data (Buchiel *et al.*, 1987). High doses of Cd are known to be toxic to the immune system of many species (Koller, 1980). The mechanism(s) by which Cd produce its effects on the immune system is (are) unclear. Numerous studies have demonstrated immunotoxic effects of high doses of Cd given by the oral route of exposure (Koller, 1973; Koller *et al.*, 1976; Koller & Kovacic, 1984; Koller & Brauner, 1977; Blakely & Archer, 1981, 1982; Lawrens 1981a,b). That is, Cd has biphasic activity for the immune functions. The purpose of these studies was to determine whether MLT restores the cell-mediated immune functions by subcronic exposure of Cd. The *in vivo* lymphoid assays such as DTH, mitogenic response and splenic proportion of $CD4^+$ and $CD8^+$ cells using flow cytometry analysis were performed using spleen cells from Cd-treated mice in order to analysis a cell-mediated immunocompetence.

II. MATERIALS AND METHODS

1. Experimental animals

ICR outbred male mice aged 7 weeks old were maintained under a 12 hr light cycle (hour 6, light on; hour 18, light off) with free access to food and water and at a temperature of $23\pm1^{\circ}C$ in a fully air-condition cabinet. Mice were housed in polycarbonate cage fitted with filter tops on aspen bedding chips. The health of mice was monitored at regular intervals using sentinel animals that were examined at necropsy for gross pathology and histopathology. The mice were also examined for bacterial and viral infections via serologic testing performed by microbiological associates (Bethesda, MO, USA). All mice divided into five groups (five per cage), and MLT (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in absolute ethanol 1 m*l* and then distilled water. MLT (10 or 50 mg/kg) was orally administered to mice daily for 28 consecutive days. Cadmium as $Cd(AC)_2$ (Sigma Chemical Co., St. Louis, MO, USA) was prepared and also administered at 25 mg/kg with same route 2 hrs later the administration of MLT. The normal mice were given vehicle. In order to account for prevention of MLT to immunosuppression of Cd-treated groups, five groups such as normal (vehicle) group, MLT (10 mg/kg), MLT (10 and 50 mg/kg) plus Cd-treated groups and Cd-treated group were studied. The all routes of administration were oral, and the treatment was continued for four weeks.

2. Immunization

SRBC collected from single female sheep were kept at $4^{\circ}C$ in sterile Alsever's solution (Gibco Co., Grand Island, NY, USA). SRBC were washed three times in phosphate buffered saline (PBS, Gibco Co., Grand Island, NY, USA, pH 7.2) after centrifugation at $400\times g$ for 10 min and diluted to provide a desired concentration by hemacytometer count. All experimental mice were immunized by intraperitoneal (i.p.) injection of 0.1 m*l* SRBC suspension (1×10^8 cells/m*l*) 5 days prior to each assay as described by Lake and Reed (1976). To assess the delayed type hypersensitivity (DTH), separate groups of mice were challenged by subcutaneous (s.c.) injection of 0.05 m*l* of SRBC suspension (2×10^9 cell/m*l*) into the left hind footpad (LHFP) of mice 4 days after immunization.

3. Preparation of spleen cell suspension

ICR mice were killed by cervical dislocation and their spleens were removed aseptically. Cell suspensions from the spleens were prepared in complete medium (RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine) by the modified method of Mishell *et al.* (1980). In brief, the suspensions were then minced, and gently squeezed into fragments between the frosted ends of two sterile microscope slides in cold complete medium. The cell suspension was washed three times by centrifugation and finally suspended in cold complete medium. Cell viability was determined by trypan blue exclusion test. Cell viability always exceeded 95% as determined by counting in hemacytometer chambers.

4. Delayed type hypersensitivity

Four days after immunization, footpad reaction was elicited with a challenge of 10^8 SRBC in 0.05 ml into the LHFP and PBS into right hind footpad (RHFT). The footpad reaction was evaluated 24 hr after challenge by measuring the increase in thickness with a dial gauge caliper (Mitutoyo Mfg. Co., Ltd., Japan) displayed in 0.01 mm gradation, as described by Titus and Chiller (1981) and Henningen *et al.* (1984). The percentage increase was calculated as $(T_{24}-T_0)\times 100$ per T_0, where T_0 is the thickness of RHFP injected with PBS and T_{24} is the thickness of LHFP 24 hr after challenge.

5. Mitogen-induced splenocyte proliferation

This test was performed using the modified method described by Buttke *et al.* (1993). Splenocyte (2×10^5 cells/well) were incubated in 96 well flat-bottomed tissue culture plates (Falcon No. 3072) in 0.1 ml culture media with mitogens. Mitogens were added to the cultures to give final concentrations of 4 μg/ml for Con A (Sigma Chemical Co., St. Louis, MO, USA) and 20 μg/ml for LPS (Sigma Chemical Co., St. Louis, MO, USA), because these concentrations of mitogens produced optimum stimulation of the incubated lymphocytes. The cultures were incubated at 37°C under 5% CO_2-air for 72 hrs, 20 μl/well of combined 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetra-zolium (MTS, Promega Co., Medison, WI, USA)/phenazine methosulfate (PMS, Sigma Chemical Co., St. Louis, MO, USA) solution was added into each well of the 96 well assay plate containing the samples. After 1 hr at 37°C in a humidified 5% CO_2 atmosphere, the absorbance at 490 nm was recorded using an ELISA microplate reader.

6. Flow cytometry analysis

To stain T cells, monoclonal antibodies were used as described by Pelletier *et al.* (1987). Each spleen was removed and mashed. Spleen cells were made to the free cells with trypsin (Sigma chemical Co., St. Louis, MO, USA), and stained with either one of fluorescein isothiocyanate (FITC) conjugated anti-mouse YTS 177.9 for T-helper/inducer ($CD4^+$) cells, with phycoerythrin (PE) conjugated anti-mouse Lyt-2 for T-suppressor/cytotoxic ($CD8^+$) cells, FITC conjugated anti-mouse MCA 500F for T cell (CD3), and PE conjugated anti-mouse MCA 1439 for B cell (CD19). IgG2a (PE) and IgG2a (FITC) were used as negative control (Serotec Ltd., Oxford, UK). Briefly, appropriate volume of each monoclonal antibody (Mab) and RPMI-1640 (Gibco Co., Grand Island, NY, USA) were added in tube and mixed thoroughly. To these mixture, 50 μl of cell suspension (2×10^7 cells/ml) was added and reacted in dark condition for 40 min. Then, these reactants were washed two times with PBS and fixed with 1% paraformaldehyde (Sigma chemical Co., St. Louis, MO, USA) in PBS. Finally the ratios of T cell and B cell subpopulations and $CD4^+$ and $CD8^+$ cells were measured by flow cytometry (Becton-Dickinson, Mountain View, CA, USA).

7. Statistical analysis

Statistical significance of the differences among groups was examined at a 5% level of significance by Student's t-test (Diem and Lentner, 1975). All data are expressed in terms of mean$\pm$standard deviation (S.D.).

III. RESULTS

1. Delayed type hypersensitivity

Table 1 shows the effects of MLT on DTH response in Cd-treated mice. DTH response was remarkably increased in the mice treated with Cd alone as compared with those in normal mice, but the increase in DTH response was significantly suppressed to normal value in the mice treated with Cd and MLT ($P<0.01$).

2. Mitogen-induced splenocyte proliferation

T cell proliferation to Con A and B cell proliferation to LPS were observed in Cd-treated mice (Table 2). B cell proliferation to LPS was decreased in the mice treated with Cd alone as compared with those in normal mice. The treatment of MLT and Cd increased T and B cell proliferation to mitogen depressed by treatment of Cd alone along with increase of MLT doses. Especially, in case of treatment of MLT alone and MLT (50 mg/kg) plus Cd, mitogenic response to Con A was significantly increased ($P<0.001$).

3. Flow cytometry analysis

Spleen cells obtained from treated ICR mice were analyzed by flow cytometry for their expression of cell surface antigens detected using monoclonal antibody (Table 3 and 4). Data was expressed as the total number of antigen positive cells obtained from the spleen, using the percentage of positive

Table 1. Preventive effects of melatonin on the DTH in cadmium-treated ICR mice

Treatment	Dose (Oral) Cd (mg/kg) or MLT (mg/kg)	FPSI
Normal (Vehicle)	–	20.93±2.12
Control (Cd)	25	30.00±1.62$^{\#\#}$
MLT	10	22.57±1.87**
Cd+MLT	25+10	27.82±1.88
Cd+MLT	25+50	25.76±1.87**

MLT: melatonin, Cd: cadmium. MLT was given to mice 2 hr before oral administration with 25 mg/kg of Cd by same route everyday for 28 days at a dose of 10 mg/kg or 50 mg/kg. Mice were challenged s.c. with 10^8 SRBC on left hind footpad 4 days after first immunization. Footpad swelling index (FPSI)=$[(T_{24}-T_0)/T_0]\times 100$, where T_0 is the right hind footpad thickness challenged with PBS, and T_{24} is left hind footpad thickness 24 hrs after challenge. Each value represents the mean±S.D. of results obtained from 10 mice. Shaded asterisks denote a significant difference compared with control group (**P <0.01), Opensquares denote a significant difference between normal and control group ($^{\#\#}P<0.01$).

Table 2. Preventive effects of melatonin on the mitogen-induced proliferation of splenocytes in cadmium-treated ICR mice

Treatment	Dose (Oral) Cd (mg/kg) or MLT (mg/kg)	Con A (4 μg/ml) Optical density	LPS (20 μg/ml) Optical density
Normal (Vehicle)	–	0.247±0.007	1.353±0.074
Control (Cd)	25	0.184±0.007$^{\#\#}$	1.302±0.025
MLT	10	0.457±0.013***	1.837±0.103**
Cd+MLT	25+10	0.251±0.014*	1.458±0.022**
Cd+MLT	25+50	0.272±0.011**	1.631±0.038**

Con A: concanavalin A, LPS: lipopolysaccharide. MLT was given to mice 2 hr before oral administration with 25 mg/kg of Cd(as cadmium acetate) by same route daily for 28 days at a dose of 10 mg/kg or 50 mg/kg. Each value represents the mean±S.D. of results obtained from 10 mice. Other legends and methods are the same as those in Table 1. Shaded asterisks denote a significant difference compared with control group (***P<0.001, **P <0.01, *P<0.05), Opensquares denote a significant difference between normal and control group ($^{\#\#}P<0.01$).

Table 3. Preventive effects of melatonin on the proportion of the splenic $CD4^+$ cells & $CD8^+$ cells in cadmium-treated ICR mice

Treatment	Dose (Oral) Cd (mg/kg) or MLT (mg/kg)	No. of Cells/Spleen ($\times 10^7$)			
		Total cells	$CD4^+$ cells	$CD8^+$ cells	$CD4^+/CD8^+$
Normal (Vehicle)	–	25.29±1.23	6.37±0.12	2.10±0.10	3.03±0.11
Control (Cd)	25	28.52±0.85	1.37±0.07$^{\#\#}$	0.91±0.05	1.51±0.09$^{\#\#}$
MLT	10	30.56±1.26	6.54±0.98	2.60±0.06	2.51±0.22**
Cd+MLT	25+10	29.02±0.98	4.50±0.56	2.58±0.08	1.74±0.07*
Cd+MLT	25+50	32.50±1.56	6.13±0.36	2.83±0.04	2.17±0.15**

MLT was given to mice 2 hr before oral administration with 25 mg/kg of Cd(as cadmium acetate) by same route daily for 28 days at a dose of 10 mg/kg or 50 mg/kg. Each value represents the mean±S.D. of results obtained from 10 mice. Other legends and methods are the same as those in Table 1. Shaded asterisks denote a significant difference compared with control group (**P<0.01, *P<0.05), Opensquares denote a significant difference between normal and control group ($^{\#\#}P<0.01$).

Table 4. Preventive effects of melatonin on the proportion of the splenic T cells & B cells in cadmium-treated ICR mice

Treatment	Dose (Oral) Cd (mg/kg) or MLT (mg/kg)	No. of Cells/Spleen ($\times 10^7$)			
		Total cells	T cells	B cells	T/B
Normal (Vehicle)	–	25.29±1.23	9.31±0.67	9.51±0.78	0.98±0.04
Control (Cd)	25	28.52±0.85	5.93±0.45	4.93±1.02	1.20±0.06##
MLT	10	30.56±1.26	10.91±0.85	7.70±0.45	1.42±0.06*
Cd+MLT	25+10	29.02±0.98	11.14±0.57	6.15±0.26	1.81±0.03**
Cd+MLT	25+50	32.5±1.56	22.36±0.32	8.79±0.34	2.54±0.05**

MLT was given to mice 2 hr before oral administration with 25 mg/kg of Cd(as cadmium acetate) by same route daily for 28 days at a dose of 10 mg/kg or 50 mg/kg. Each value represents the mean±S.D. of results obtained from 10 mice. Other legends and methods are the same as those in Table 1. Shaded asterisks denote a significant difference compared with control group (**P<0.01, *P<0.05), Opensquares denote a significant difference between normal and control group (##P<0.01).

cells for each mouse multiplied by the total number of recovered spleen cells. The percentage of $CD4^+$ cells and $CD8^+$ cells were decreased by treatment of with Cd alone as compared with normal mice, and $CD4^+$ cells and $CD8^+$ cells were slightly increased by treatment of MLT alone, and MLT restores $CD4^+$ cells decreased by treatment of Cd (Table 3). Similarly, MLT restored both T and B cells significantly decreased by Cd.

IV. DISCUSSION

In this study, the administrative doses of MLT and Cd were chosen according to LD_{50} and the references, and initial dose ranging studies were performed to determine the optimal oral dose of MLT for prevention of immunosuppression. Many immunotoxicological studies on Cd have reported that this metal, at high concentrations, generally has immunosuppressive activities (Vos, 1977; Koller, 1980). On the other hand, the first evidence of functional relationships between the pineal gland and the immune system was reported by Csaba and coworkers (1968; 1975). DTH-response for assessing *in vivo* cell-mediated immune status was remarkably increased in ICR mice treated with Cd alone, in contrast to previous observation in B6C3F1 mice exposed to Cd, was normally restored by MLT treatment. It is reported that at least two separate subpopulations of T helper (Th) cells exist in the Cd-treated mouse, Th_1 cells, which secret IL-2 and IFN-γ and Th_2 cells, which secret IL-4 and IL-5 (Mosmann *et al.*, 1986). Further studies by this group have shown that only Th_1 cells are involved in mediating DTH response (Cher and Mosmann, 1987). MLT partially restored the cell-mediated DTH (Pierpaoli and Regelson, 1994). These reports were supported by the results of in this study that MLT may block an abnormal increase of DTH response and Th_1 cells produced by Cd. Burchiel *et al.* (1987) reported that suppression of the mitogenic response of spleen cells to Con A, PHA and LPS was also found to be time dependent. These reports were supported by the results of this study in which MLT significantly restored mitogenic response to Con A and LPS suppressed by Cd. In addition, our results on the mitogenic response are in agreement with previous reports, in which MLT treatment *in vivo* significantly increased mitogen-induced lymphocyte proliferation (Mocchegiani *et al.*, 1994) and induction of IL-2 expression by Con A in young and old mice (Caroleo *et al.*, 1994). It is well known that functionally distinct subpopulations of lymphoid and myeloid cells exist in the bone marrow, spleen and peripheral blood. The patterns of reactivity of the Lyt-1, Lyt-2 and 55-7.2 monoclonal antibodies have been previously described by Ledbetter & Herzenberg (1979). Based upon the total number of antigen positive cells, the Lyt-1 to Lyt-2 ratio (an index of T helper to T suppressor activity) was altered by Cd. In contrast to previous results of Burchiel *et al.* (1987), these results demonstrate that changes in cell surface markers on discrete subpopulations of lymphoid cells present in the spleen of Cd-exposed mice may correlate with alterations in the cell-mediated immune functions of these cells. The number of splenic T cells and B cells were decreased in Balb/c mice exposed to Cd (Ilback *et al.*, 1994). MLT is able to activate human

Th_1 lymphocytes by increasing the production of IL-2 and IFN-γ *in vitro*, and to activate $CD4^+$ cells, but Th_2 cells appear not to be affected by MLT (Garcia-Maurino *et al.*, 1997). These previous results were supported by those in this study, which MLT restored the number of $CD4^+$ cells, $CD8^+$ cells, T cells and B cells decreased by treatment of Cd alone. On the other hand, Gonalez-Haba *et al.* (1995) reported that binding of 2-[^{125}I]iodomelatonin by peripheral blood T cells was 10-fold higher than that seen in B lymphocytes, and that within T cells binding of MLT was mostly found in $CD4^+$ cells rather than in $CD8^+$ cells. These previous results are supported by the results of this study which $CD4^+$ cells to $CD8^+$ cells ratio ($CD4^+/CD8^+$) and T cells to B cells ratio decreased by treatment with Cd alone, was restored along with increase of MLT dose. In conclusion, MLT significantly seemed to restore the cell-mediated immunotoxic status induced by Cd.

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