Melatonin Fails to Modulate Immune Parameters Influenced by Calorie Restriction in Aging Fischer 344 Rats

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The aim of this study was to determine if long-term treatment with melatonin (MEL), a purported anti-aging agent, was as effective as calorie restriction (CR) in modulating immune parameters in aging Fischer 344 male rats. Splenic lymphocytes were isolated from 17-month-old rats that, beginning at 6 weeks of age, were treated with MEL (4 or 16 μg/ml in drinking water) and from 17-month-old rats fed *ad libitum* (AL) or rats fed a CR diet (55% of AL intake). The number of splenic T cell populations and T cell subsets was measured by flow cytometry, the proliferative response of splenocytes to Concanavalin A (Con A) and lipopolysaccharide (LPS) was measured by [^{3}H]thymidine incorporation, and the induction of cytokine production (IL-2 and IFN-γ) was measured by ELISA assay. In addition, the level of the natural killer (NK) cell activity was assessed by fluorimetric assay. CR rats had a higher number of lymphocytes expressing the naïve T cell marker (CD3 OX22) than AL rats ($P < 0.05$). CR rats also showed greater induction of proliferative response, IL-2 and IFN-γ levels following Con A simulation, and NK cell activity than AL rats ($P < 0.05$). MEL-treated rats did not differ from AL rats in any of these parameters or in any other measurement. These results indicate that MEL treatment is unable to modulate immune function in a manner comparable with that of CR. [Exp Biol Med Vol. 227(3):201–207, 2002]

Key words: melatonin; caloric restriction; immunomodulation, aging; rat

Melatonin (MEL) is a chemical mediator produced mainly in the pineal gland, although other organs have been demonstrated to have the enzymatic machinery for its synthesis. The classical effects of MEL relate to the control of the circadian rhythms and regulation of the hypothalamopituitary glandal axis, but other actions have also been reported (1–10). The most recently described property of MEL is its antioxidant capability. For example, it can scavenge hydroxyl free radial and peroxynitrite (11–13). Exogenous chronic MEL treatment has been reported to extend life span in mice and rats (14, 15). In addition, some evidence suggests that MEL may be immunostimulatory (reviewed in Ref. 10). MEL treatment has been reported to enhance mitogen-induced lymphocyte proliferation (16, 17), to stimulate natural killer (NK) cell activity (18), to increase *in vivo* and *in vitro* antibody response (9, 16), and to augment antigen presentation by macrophages (19). In addition, MEL administration was reported to reverse thymus involution in mice and to restore the proliferative response of thymocytes to mitogenic lectins in normal mice and in the mice that were immunodepressed by acute restraint stress or by corticosterone treatment (20). In other studies, however, MEL administration *in vivo* or *in vitro* was ineffective in modulating immune function in mice (21, 22), rats (23), or humans (24).

In laboratory rodents, a chronic decrease in caloric intake can extend the life span, postpone the onset, and lower the incidence of age-associated diseases (reviewed in Refs. 25–27). Caloric restriction (CR) delays the age-related decline of various physiological systems, including the immune system. For example, immune parameters such as mitogen-induced lymphocyte proliferation and IL-2 production (28), T cell-mediated cytotoxicity (29), and NK cell activity (30) increase with CR. Whether MEL treatment is equally robust as an immunoenhancer or in life span extension is far less clear. The present study, part of a larger investigation on the effect of MEL on lifespan and other age-related traits, investigates the effect on immune func-

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tion of long-term MEL treatment in comparison with CR, a well-established means of retarding aging of immune function. Our results demonstrate that long-term MEL treatment at doses bracketing those previously used (14, 15) did not mimic the stimulatory effect of CR on mitogen-induced proliferation, IL-2, IFN-γ, or NK activity in aging rats.

Materials and Methods

Animals. All animals were maintained in the UTHSCSA Laboratory Animal resources, an AAALAC approved facility. All procedures and experiments involving the use of the rats in this study were approved by the Institutional Animal Care and Use Committee and are consistent with the National Institutes of Health *Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Education*, the *Guide for the Care and Use of Laboratory Animals*, and the *Animal Welfare Act*. The presence of murine viral antibodies (Sendai, Reo-3, GP-VII, PVM, KRU, H-1, SDA, LCM, and Adeno) and mycoplasma antibodies was monitored regularly from sentinel animals kept in open cages and exposed to bedding of all of the animals in the study. All tests for pathogenic organisms were negative throughout the course of the study.

Male Fischer 344 rats (specific pathogen free) were purchased from Charles River Laboratories (Boston). The animals were part of a comprehensive study of the effect of MEL and CR on longevity, age-related pathology, and immune function. The rats were housed two per cage in microisolator caging with a 14:10-hr light:dark cycle and were fed Harlan Teklad Irradiated Laboratory Diet. At 5 weeks of age, the rats were randomly assigned to one of four groups. Dietary and MEL treatments were begun at 6 weeks. CR rats were given daily food rations amounting to 55% of the average daily food consumption of the *ad libitum* (AL) group (based on the intake of the previous week). AL rats were given food *ad libitum*. AL and CR rats were given acidified water (pH 2.5) containing dimethyl sulfoxide (DMSO), the vehicle used to dissolve MEL, at 100 μl/l. The two remaining groups were supplemented with MEL in the drinking water at 4 and 16 μg/ml, respectively.

MEL Treatment. MEL and DMSO were purchased from Sigma (St. Louis, MO). MEL (4 and 16 μg, respectively, for the two MEL groups) was dissolved in 100 μl of DMSO and was added to 1 liter of acidified water and mixed thoroughly. The lower dose was chosen because it was reported to reduce mortality in aged rats (15) and a similar dose regimen was reported to extend life span in several strains of mice (14). All water bottles, including those containing the MEL stock solutions, were prepared fresh twice weekly, and bottles containing melatonin were wrapped in aluminum foil to eliminate light exposure. Bottles were changed regularly (Tuesdays and Fridays) between 0600 and 0830 hr.

Blood Sampling and Plasma Separation. To assess the levels of MEL in the four experimental groups, blood was collected from a sample of rats at 6 months of age, 4.5 months after the onset of experimental treatments. Blood was collected by nicking the tail of rats at four different times of the day: two times during the light period (1500 and 1900 hr) and two times during the dark period (0100 and 0400 hr; lights off from 2100–0700 hr). Blood was collected from a rat within 2–3 minutes of cage disturbance to ensure against stress-related effects. Nighttime sampling was conducted under a red light. Plasma was separated by centrifugation of the blood samples at 3500 rpm for 15 min at 40°C and was stored at −70°C until assay.

Extraction of Plasma MEL. Plasma MEL was extracted by dichloromethane (31). Thirty milliliters of each plasma sample was extracted into 10 volumes of dichloromethane by gentle vortex. After centrifugation at 1500 rpm for 10 min at 40°C, the organic phase was collected and dried by vacuum aspiration. The residue was reconstituted for radioimmunoassay (RIA) in 260 μl of the RIA buffer (0.1% gelatin, 0.1 *M* Tricine, and 0.9% NaCl, pH 8.0). This buffer extract was incubated at 4°C overnight before proceeding to RIA. Spiking aliquots of rat plasma with 2000 cpm of iodinated tracer in 20 μl of assay buffer and incubating at 4°C overnight prior to extraction tested extraction efficiency. Recovery of iodinated melatonin averaged 85%–86%. Assay results were not corrected for percentage of recovery. Parallelism of aliquots of extracted plasma samples was demonstrated against the standard curve using in this assay (data not shown).

RIA for MEL. The MEL concentrations in extracted samples were measured by adaptation of a direct RIA commercially available with antibodies from Stockgrand (School of Biological Sciences, University of Surrey, Guilford, Surrey, UK), which had been previously described (31). Other than the primary antibody: rabbit anti-melatonin antiserum (Stockgrand no. R/R/19540-16876) and the secondary antibody: donkey anti-rabbit IgG (Stockgrand no. SAB/D/07), 2-[125I]iodomelatonin (2200 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). MEL, normal rabbit serum, and other chemicals were all from Sigma Chemical Co. (St. Louis, MO).

To perform this assay, a standard curve ranging from 2.5–500 pg/ml was constructed in the RIA buffer by sequential 2- or 2.5-fold dilutions of a MEL standard stock in 20% ethanol. One hundred microliters of each extracted sample and standard in duplicate was incubated at 40°C overnight (15–18 hr) with 100 μl of the primary antibody (diluted 1:100,000) and 10,000 cpm of iodinated tracer in 100 μl of the RIA buffer. The antibody-bound and -free fractions were separated by adding into the reaction mixture the second antibody (diluted 1:15), normal rabbit serum, and 6% polyethyleneglycol. After incubation at 40°C for 4 hr, the radioactivity in the precipitate was quantified using a Packard Cobra II auto-gamma counter. The sensitivity of the assay was 2.5 pg/ml. The intra- and interassay coefficients of variations were 3.8% and 12.0%, respectively using a pooled extracted plasma sample that had a mean value

of 37.9 pg/ml. Samples from different ages, treatments, and times of the day were included in each assay.

Lymphocyte Subpopulation Measurement. The spleen lymphocytes phenotype was evaluated by a standard one-color or two-color immunofluorescent antibody staining procedure as we previously described (32). Briefly, an aliquot (1–2 million) of freshly isolated cells was washed with FACS buffer (PBS with 5% FCS and 0.1% sodium azide) and was stained with the following antibodies: murine monoclonal antibodies (MAb); fluorescein isothiocyanate (FITC)-conjugated anti-rat CD3; phycoerythrin (PE) anti-rat CD4 (clone W3/25); FITC-conjugated anti-rat CD8 (clone OX8), and anti-mouse NKR-P1A antibody. The expression of naive marker (OX22) (33) was evaluated by using the FITC-conjugated OX22 Mab. All antibodies were obtained from PharMingen (San Diego, CA). Cells were incubated with antibody for 30 min at 4°C and were then washed three times with FACS buffer. Cells were analyzed with a flow cytometer (FACScan, Becton Dickinson Immunocytometry Systems, Mountain View, CA). The samples were gated using forward versus 90-degree light scatter to excluded granulocytes and monocytes from the splenocytes population. For each test sample, 10,000 cells were analyzed using Lysys II software (Becton Dickinson).

Lymphocyte Culture and Proliferation Assay. Spleens were removed aseptically and single cell suspensions were prepared as we previously described (34). Erythrocytes in the blood and spleen samples were removed using Lympholyte-R (Accurate Chemical and Scientific Corporation, Westbury, NY). Cells were resuspended in RPMI 1640 medium, which was supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells (1×10^5/0.2 ml) were cultured in 96-well plates (Falcon, Lincoln Park, NJ) in medium alone or in the presence of T cell mitogen Con A (5 and 10 μg/ml) or B cell mitogen LPS (10 and 20 μg/ml) that were purchased from Sigma. Cells were incubated at 37°C in a 5% CO_2 incubator for 48 hr and were then pulsed with 1 μCi of [^{3}H]thymidine (NEN). After overnight incubation, the cells were harvested onto glass-fiber filters using a microcell harvester, and [^{3}H]thymidine incorporation into the DNA was determined using a liquid scintillation counter (Packard, Downers Grove, IL). Proliferation was expressed as the mean of triplicate counts per minute (cpm) for the samples from each group of rats. Mitogenic responsiveness was calculated as cpm in the stimulated cultures minus cpm in the unstimulated cultures (35).

Assays for IL-2 and IFN-γ The levels of IL-2 and IFN-γ in culture supernatants were measured as we described previously (36). Briefly, an aliquot of 50–100 million of splenocytes was plated in a tissue culture flask and was incubated in the presence or absence of Con A (5 μg/ml) for 24 hr (for IL-2) or 48 hr (for IFN-γ). The culture supernatants were then harvested and stored at −70°C until assay. The levels of IL-2 and IFN-γ in culture supernatants were measured by ELISA techniques using the protocol provided in the Quantikine IL-2 or IFN-γ immunoassay kit (R&D Systems, Minneapolis, MN). Duplicate samples were assayed in each separate experiment and results are expressed as picograms per milliliter.

NK Cell Assay. Splenocytes were cultured in the presence and absence of 1000 U/ml of IL-2 (R&D Systems) for 24 hr. The NK cytotoxic activity against the NK-sensitive cell line (YAC-1 tumor cell line) was measured using fluorescent concentration release assay as described by Proviciali *et al.* (37). Briefly, a stock solution of the fluorescent probe, carboxyfluorescein diacetate (Molecular Probes, Eugene, OR), was prepared and diluted in PBS to give a final concentration of 75 μg/ml (working solution). YAC-1 target cells were washed twice with PBS, resuspended in 1 ml of working solution, and incubated at 37°C for 30–40 min. After washing in PBS, the YAC-1 cells were resuspended in RPMI containing 10% FCS at a concentration of 1×10^5 cells/ml. The carboxyfluorescein diacetate-labeled target cells were incubated with effector cells in 96-well round microtiter plates (Falcon 3077). Effector to target (E:T) ratios were adjusted to 100:1 and 50:1 containing 4×10^4 cells/200 μl. The plate was centrifuged (90*g*) for 2 min to facilitate cell-to-cell interaction. The cells were then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 3 hr. The plate was then centrifuged (700*g*) for 5 min. The supernatant was removed and 100 μl of 1% Triton X100 in 0.5 *M* borate buffer (pH 9.0) was added to each well. The plate was kept for 20 hr at 4°C and the fluorescent release was read with a Titertek Fluoreokan II (Flow Laboratories, McLean, VA). The percentage of specific lysis was calculated according to the formula: $[(F_{med} - F_{exp})/F_{med}] \times 100$ (37), where F represents the fluorescence of the solubilized after the supernatant has been removed; med is fluorescence from target incubated with medium alone; and exp is fluorescence from target incubated with effector cells.

Statistical Analysis. All results are expressed as means ± SEM. Comparison between the mean values obtained in the different groups was performed using Student's *t* test and analysis of variance (ANOVA) using the statistical analysis software GB-STAT (Dynamic Microsystems, Silver Spring, MD). Levels of significance are indicated by an asterisk or $P < 0.05$.

Results and Discussion

MEL plays an important role in synchronizing circadian and circannual rhythms in mammals. Additionally, considerable literature suggests that MEL has antioxidant properties (11–13). There is also evidence that MEL plays a role in regulating the immune response (7–10) and this immunoenhancing role has been correlated with a report of life span extension by MEL (14). Earlier, we found that MEL treatment *in vitro* (ranging from 50–500 pg/ml) was ineffective in modulating mitogen-induced lymphocyte proliferation, and IL-2 or IFN-γ expression in either young or old rats (23). Therefore, we hypothesized that the immuno-

Table I. Plasma Melatonin Concentrations (pg/ml) in AL-Fed, CR and MEL-treated Rats at 6 Months of Age

Time	AL	CR	MEL (4 μg/ml)	MEL (16 μg/ml)
1500 hr	34 ± 7	33 ± 8	2598 ± 560	3176 ± 642
1900 hr	76 ± 19	45 ± 6	2514 ± 701	4006 ± 630
0100 hr	84 ± 18	110 ± 24	2225 ± 520	6032 ± 1641
0400 hr	111 ± 20	117 ± 9	1497 ± 305	3358 ± 808

Note. The values are mean ± SEM of data obtained from six rats per group.

modulatory and anti-immunosenescent effects of MEL that have been reported in mice might only be found *in vivo*.

Our aim in this study was to rigorously examine the effect of chronic MEL treatment on immune parameters using a well-defined *in vivo* model system exposed to doses of MEL that had previously been reported to extend life span and enhance immune function (14). We also included a comparison of the effect of CR on immune parameters as a positive control to ensure that we could detect the effects of a well-established immunoenhancing intervention if negative data were obtained with MEL treatment. Overall, the immunological status of rodents fed a CR diet is superior to the immunological status of non-restricted animals (reviewed in Refs. 28 and 38).

MEL levels in the plasma of the rats at 6 months of age are shown in Table I. AL-fed and CR rats showed a diurnal variation in levels, which peaked during the dark period. MEL levels in the rats treated with MEL at 4 and 16 μg/ml were about 20- and 40-fold higher, respectively, than peak levels in the untreated rats. There was no statistically significant diurnal variation in MEL levels in the MEL-treated rats. This may reflect the pharmacologic levels of MEL that were achieved—exceeding the capacity of the liver and other systems to metabolize MEL. Otherwise, one would have expected MEL levels to be elevated at night when rats consume most of their water. To our knowledge, there are no previous reports of the MEL levels in the plasma of rodents in studies to determine the effect of MEL treatment on immune function, oxidative stress resistance, or life span. Most of those studies, however, used doses of MEL similar to those used in this study (14, 22).

In the first series of experiments, we measured the average number of splenic T cell populations, and helper, cytotoxic, and naive T cell subsets using flow cytometry. The data in Table II show that neither MEL nor CR influenced the number of T cells ($CD3^+$) or helper ($CD4^+$) or cytotoxic ($CD8^+$) T cell subsets. Also, there was no difference between MEL-treated rats, CR rats, and AL rats in the proportion of the cells expressing NK receptor (NKR-P1A). In rats, the differential expression of naive T cell marker by OX22 Mab is thought to discriminate the naive/virgin from the memory phenotype (33). Whereas MEL had no effect on the proportion of naive T cells ($CD3^+OX22^+$) in splenocytes, in comparison with AL control rats, the proportion of $CD3^+OX22^+$ T cells was significantly ($P < 0.05$) higher in lymphocytes from CR rats compared with AL control rats (Table II). This finding supports the recent studies showing that CR maintains a high number of naive/virgin T cells in aging mice (39).

Mitogen-induced lymphocyte proliferation declines with age and this decrease is attenuated by CR (28). To determine if MEL treatment protects against the decline in lymphocyte mitogenesis, splenocytes from rats treated with 4 and 16 μg/ml of MEL and from CR and AL rats were assayed for their response to Con A and LPS. The results in Figure 1 show that the proliferative response of splenocytes from rats treated with 4 or 16 μg/ml of MEL to two different concentrations of Con A (5 and 10 μg/ml) and LPS (10 and 20 μg/ml) was not different from that of the AL rats. However, there was a significant ($P < 0.05$) increase (25%–30%) in proliferative response of splenocytes to Con A, but not to LPS, in CR rats compared with MEL-treated and AL rats.

MEL treatment *in vivo* was reported to increase IL-2 and IFN-γ production in mice (16, 40, 41). IL-2 is a cytokine that is produced by helper T cells and plays a crucial role in lymphocyte proliferation (42). We compared the induction of IL-2 and IFN-γ production by Con A in splenocytes from MEL-treated and CR rats (Fig. 2). Whereas CR potentiated Con A induction of IL-2 and IFN-γ levels relative to AL controls, MEL had no effect on modulating IL-2 or IFN-γ levels.

NK cells mediate cytotoxicity against target cells, particularly against tumor cells that are not restricted by the presence of major histocompatibility antigens. Although NK cells are generally non-specific in their cytotoxic activity, their action is modulated by cytokines such as IL-2 and IFN-γ ((43). To determine if MEL treatment affects NK cell

Table II. Flow Cytometry Analysis of Splenic Lymphocytes from 17-Month-Old MEL-Treated, CR, and AL Rats

Animals	Percentage of positive cells				
	$CD3^+$	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD3^+OX22^+$	$CD3^-NKR\text{-}P1^+$
MEL					
4 μg/ml	34.9 ± 4.8	21.4 ± 4.6	12.5 ± 3.4	42.5 ± 5.4	8.3 ± 1.3
16 μg/ml	35.6 ± 4.7	20.9 ± 4.4	14.1 ± 2.7	46.1 ± 5.7	9.6 ± 1.7
CR	39.2 ± 5.1	22.3 ± 4.0	16.2 ± 3.1	57.2 ± 5.1[a]	9.2 ± 1.4
AL	36.7 ± 4.5	20.9 ± 4.2	14.6 ± 2.8	45.6 ± 5.8	8.4 ± 1.6

Note. The values are means ± SD of data obtained from four rats per group.
[a] The values for CR rats were significantly different than the values for AL- and MEL-treated rats, $P < 0.05$.

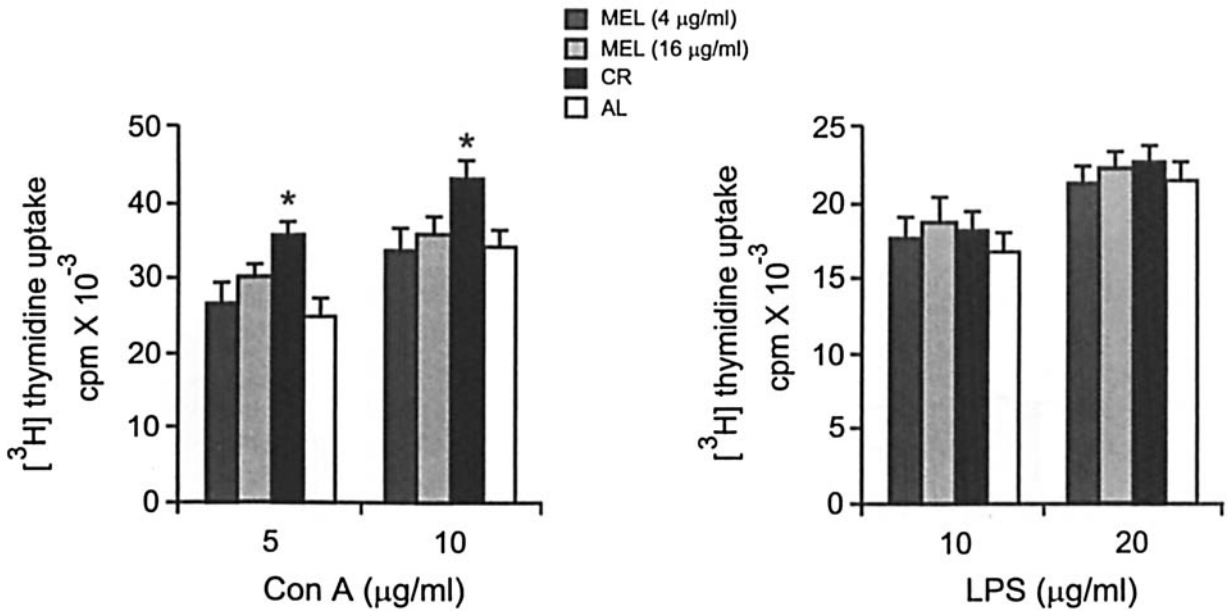

Figure 1. Effect of long-term melatonin treatment and caloric restriction on mitogen-induced lymphocyte proliferation in 17-month-old F344 rats. Splenocytes were isolated from rats that were supplemented with 4 and 16 μg/ml MEL and from CR and AL rats that were cultured in the presence and absence of Con A (5 and 10 μg/ml) or LPS (10 and 20 μg/ml) for 48 hr. The proliferation was determined by [^{3}H]thymidine incorporation using liquid scintillation counting as described in "Materials and Methods." Each point represents the mean ± SE for data obtained from six spleens from each age group. Background values, i.e., [^{3}H]thymidine incorporation of unstimulated cells, were subtracted from the Con A-induced proliferation data. An asterisk indicates that the values for CR rats were significantly different than the values for MEL-treated and AL rats ($P < 0.05$).

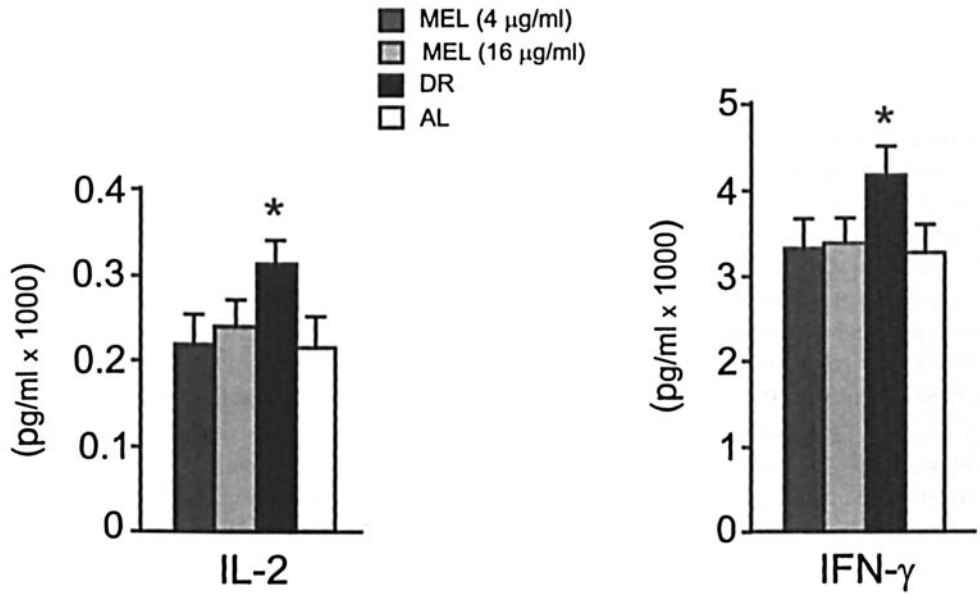

Figure 2. Effect of long-term melatonin treatment and caloric restriction on IL-2 and IFN-γ protein. Splenocytes were isolated and incubated in the presence and absence of Con A for 24 or 48 hr, and IL-2 and IFN-γ protein levels in the culture supernatants was measured as described in "Materials and Methods." Each point represents the mean ± SE for data obtained from six spleens from each group of rats. An asterisk indicates that the values for CR rats were significantly different than the values for MEL-treated and AL rats ($P < 0.05$).

activity, splenocytes from MEL-treated rats, CR rats, and AL rats were assayed for the cytotoxic response against YAC-1 tumor cell line. The results in Figure 3 show that the endogenous and IL-2-induced NK cell activity of splenocytes from rats treated with 4 or 16 μg/ml of MEL did not differ from that of AL rats at either the E:T of 50:1 or 100:1. However, a statistically significant ($P < 0.05$) increase of the IL-2-induced NK cell activity was observed for both E:T cell ratios for the CR group compared with AL control animals. Our data corroborate a recent study that found that long-term MEL treatment had no effect on NK cell number or NK cell activity in old mice (22).

The influence of MEL on the immune system has been under investigation for over a decade. In a mouse model, MEL treatment was reported to increase the immune response against a specific antigen (20), to enhance the pro-

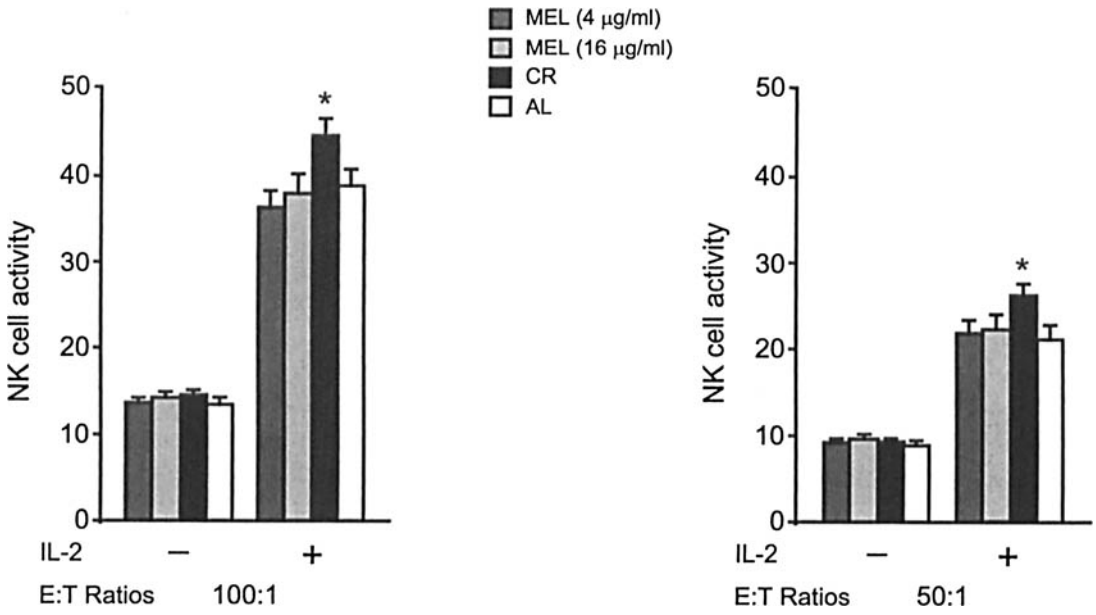

Figure 3. Effect of long-term melatonin treatment and caloric restriction on the endogenous and IL-2-induced NK activity. Splenocytes were cultured in the presence and absence of 1000 U/ml IL-2 for 24 hr. NK activity was assessed against the murine T cell lymphoma, YAC-1, using fluorescent concentration release assay as described in "Materials and Methods." Each points represents the mean ± SE for data obtained from four spleens from each group of rats. An asterisk indicates that the values for CR rats were significantly different than the values for MEL treated and AL rats ($P < 0.05$).

liferative response to Con A and to LPS (44), to increase the levels of IFN-γ (40, 42), and to augment T cell markers as well as Con A-induced proliferation (17). In addition, MEL administration was reported to increase antibody response, T helper activity, and IL-2 production in old mice (16). However, other studies have found that MEL treatment *in vivo* or *in vitro* did not modulate the immune function of mice, rats, and humans. For example, Pawlikoski *et al.* (21) demonstrated that mitogen-induced spleen lymphocyte proliferation was not altered when mouse splenocytes were exposed to exogenous MEL. Another study showed that treatment of human peripheral blood lymphocytes with MEL (at 10^{-3} to 10^{-4} molar concentration) inhibited rather than enhanced phytohemaglutinin (PHA)-induced lymphocyte proliferation (24). Our laboratory previously reported that MEL treatment *in vitro* was ineffective in modulating mitogen-induced lymphocyte proliferation, IL-2, or IFN-γ expression in either young or old rats (23). Direct exposure of mouse spleen lymphocytes to MEL was shown to enhance Con A-induced proliferation, but not IL-2 production (45). Recently, it was demonstrated that long-term MEL treatment (50 μg/day/mouse for 8 months) was ineffective in altering immune parameters such as PHA-induced proliferation, IL-2, NK cell number, or activity in mice (22). Thus, the effects of MEL on lymphocyte function that have been reported by different investigators have been inconsistent. The reasons for these discrepancies are not known. Different species and genotypes might underlie some of these differences.

One concern with negative results, such as those reported for the effect of MEL in this report and others, is that the assays employed may lack sufficient sensitivity to detect differences. A strength of this study is that it included a positive control: namely, CR rats. CR has repeatedly been associated with enhancements of immune function or delay of age-related changes in immune function (reviewed in Refs. 28 and 38). The positive results we see in CR rats give confidence to the lack of effect we observed for MEL. Also, our data are the first to show that MEL treatment was ineffective at either of two doses (4 and 16 μg/ml) in F344 rats. The lower dose is the one reported to reduce mortality in aged mice and rats (14, 15).

One might also argue that the pharmacologic doses of MEL used in these studies could downregulate MEL receptors, and thus abrogate an otherwise enhancing action of MEL on immune function. However, it should be noted that most of the previous reports on immunoenhancing, antioxidant, and life-span extending actions of MEL have used the same range of doses of MEL that we used (i.e., 4–10 μg of MEL/ml of drinking water) for prolonged durations (17, 22). Thus, earlier studies would have been expected to have produced pharmacologic levels of MEL in the circulation. Thus, in those studies where immunoenhancement of MEL was observed, if downregulation of receptors occurred, it is unlikely that it interfered with the action of MEL. Although our results do not preclude the possibility that MEL may have immune potentiating effects in other genotypes or under different treatment regimens, these effects do not appear to be as robust as those of caloric restriction.

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