Exogenous Melatonin Enhances Cell-Mediated, but Not Humoral, Immune Function in Adult Male Deer Mice (*Peromyscus maniculatus*)

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Abstract Many nontropical rodent species display seasonal changes in reproductive physiology and metabolism, as well as in immune function. Field studies of seasonal changes in immune function typically report decreased immune function in the short days of winter compared to summer; presumably, reduced immunity in winter reflects increased glucocorticoid secretion in response to environmental stressors. In contrast, laboratory studies of photoperiodic changes in immunity invariably demonstrate increased immune function in short compared to long days. Although the precise mechanisms regulating short-day enhancement of immune function are not known, it is hypothesized that increased immunity is due to the increased duration of melatonin secretion in short compared to long days. However, melatonin can act both directly (i.e, via melatonin receptors located on lymphatic tissue) and indirectly (i.e., via alterations in gonadal steroids) to affect immune function. The present study examined the effects of exogenous melatonin administration on both cell-mediated and humoral immune function in adult male deer mice (*Peromyscus maniculatus*), as well as the role of gonadal steroid hormones in mediating these effects. Mice either were castrated to remove circulating androgens or received sham operations and were implanted with empty capsules or capsules containing melatonin. Individual mice implanted with melatonin underwent reproductive regression and displayed enhanced splenocyte proliferation to the T-cell mitogen concanavalin A; antigen-specific serum immunoglobulin M production was unaffected by melatonin treatment. Castration had no effect on either cell-mediated or humoral immune function. Taken together, these results suggest that exogenous melatonin enhances cell-mediated, but not humoral, immune function in adult male deer mice and that this effect is independent of gonadal steroid hormones. These results are consistent with a direct effect of melatonin on immunity.

Key words testosterone, seasonal, photoperiod, pineal, corticosterone, disease

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JOURNAL OF BIOLOGICAL RHYTHMS, Vol. 13 No. 3, June 1998 245-252

INTRODUCTION

In addition to marked seasonal changes in reproductive, metabolic, and other physiological functions, many mammalian and nonmammalian species undergo seasonal changes in immune function and disease (John, 1994; Nelson and Demas, 1996; Zapata et al., 1992). Field studies of seasonal changes in immunity typically report reduced immune function and increased disease susceptibility during winter compared to spring and summer (Afoke et al., 1993; Boctor et al., 1989; John, 1994; Lochmiller et al., 1994). Suppression of immune function during winter likely reflects physiological stress of harsh environmental conditions (e.g., low ambient temperatures, reduced food availability) (Demas and Nelson, 1996). Glucocorticoids, primarily corticosterone in rodents, are released from the adrenal gland in response to stress and can suppress immune function (Ader and Cohen, 1993; Black, 1994; Demas et al., 1997). The suppression of immune function typically reported during winter in field studies likely reflects increased glucocorticoid secretion in response to environmental stressors (Nelson and Demas, 1996). In contrast to field studies reporting reduced immune function in the short days of winter, the results of virtually all laboratory studies examining photoperiodic changes in immunity report enhanced immune function in short as compared to long days (Brainard et al., 1988; Champney and McMurray, 1991; Demas and Nelson, 1996; Vaughan et al., 1987). One hypothesis to account for this finding is that individuals of some rodent species appear to have evolved mechanisms to bolster immune function in short days in response to the deterioration of environmental conditions (Demas and Nelson, 1996; Nelson and Demas, 1996).

Although the precise mechanisms mediating photoperiodic changes in immune function are not yet known, it is plausible that increased immunity may be due to the increased duration of melatonin secretion in short days (Brainard et al., 1988; Champney and McMurray, 1991; Demas et al., 1996; Demas and Nelson, 1998). Melatonin can act both directly and indirectly on target tissue within the immune system (Poon et al., 1994; Nelson et al., 1995; Demas and Nelson, 1998). Consistent with a direct effect, treatment of animals with exogenous melatonin enhances both humoral and cell-mediated immunity in mammalian species that are, in general, reproductively nonresponsive to melatonin (i.e., laboratory rats, house mice, humans) (Guerrero and Reiter, 1992; Maestroni, 1993). Furthermore, melatonin receptors have been identified directly on circulating lymphocytes, thymocytes, and splenocytes in these same species (Calvo et al., 1995; Lopez-Gonzales et al., 1992; Rafii-El-Idrissi et al., 1995).

In addition to melatonin, several other hormones are affected by photoperiod, including gonadal steroid hormones (i.e., estrogen, testosterone), prolactin, and corticosterone (Bartness et al., 1993; Gaston and Menaker, 1967; Johnston and Zucker, 1979); all of these hormones can affect immune function (reviewed in Nelson et al., 1995). For example, testosterone typically suppresses immune function (Grossman, 1984, 1985; McCruden and Stimson, 1991). Maintaining animals in short days or treatment with melatonin significantly reduces circulating concentrations of these hormones. Thus, short-day enhancement of immune function compared to long days in male mice may be due to the indirect effect of melatonin on circulating concentrations of androgenic steroid hormones. As discussed above, glucocorticoids are released in response to stress and lead to suppressed immune function (Ader and Cohen, 1993; Black, 1994). However, maintaining animals in short days or treatment with melatonin attenuates the glucocorticoid response and enhances immunity (Demas et al., 1997; Maestroni et al., 1986). These effects are also consistent with an indirect effect of melatonin on immune function.

Previous work in our laboratory supports a direct action of melatonin on cellular immune function (Demas and Nelson, 1998). For example, both male and female deer mice maintained on short day lengths for 10 weeks demonstrated enhanced splenocyte proliferation to the T-cell mitogen concanavalin A (Con A) compared to animals maintained on long day lengths. However, splenocyte proliferation was unaffected by either castration in males or ovariectomy in females. Furthermore, there were no significant differences in circulating corticosterone concentrations in either sex (Demas and Nelson, 1998). Thus, these results suggest that short-day enhancement of cell-mediated immune function is independent of steroid hormones. However, the effects of photoperiod and gonadectomy on humoral immunity (i.e., specific antibody production) were not assessed.

The goal of the present experiment was to examine the effects of exogenous melatonin treatment on both cell-mediated and humoral immunity, as well as the role of gonadal steroid hormones in mediating these

effects. Specifically, if melatonin affects immune function independent of gonadal steroid hormones, then melatonin treatment should increase lymphocyte proliferation and immunoglobulin (Ig) M concentrations in both castrated and intact animals. Castrated and intact mice receiving empty capsules should not display any increase in immune function. However, if the biochemical action of exogenous melatonin is indirect, via a "release" from the immunocompromising effects of testosterone, then intact animals treated with melatonin should not display enhanced immune function relative to intact animals receiving empty capsules.

MATERIALS AND METHOD

Animals

Forty adult (> 60 days of age) male deer mice (*Peromyscus maniculatus bairdii*) were obtained from our laboratory breeding colony. This colony is derived from animals from the Peromyscus Genetic Stock Center at the University of South Carolina. These animals are descendants of animals originally trapped near East Lansing, Michigan (latitude = 42º 51′ N).

Housing Conditions

Deer mice were weaned at 21 days of age and housed with same-sex siblings. Two weeks prior to the initiation of the experiment, all animals were individually housed in polypropylene cages in colony rooms under a cycle of 16 h of light and 8 h of dark (L16:D8; lights on at 0600 h or 0900 h EST). Relative humidity was maintained at 50 ± 5%. Food (Agway Prolab 1000, Syracuse, NY) and tap water were provided ad libitum throughout the experiment.

Experimental Procedure

Mice were randomly selected and assigned to one of four experimental conditions: (1) 10 mice were surgically castrated at 60 days of age and 15-mm Silastic capsules (1.47-mm i.d., 1.95 o.d., Silicone Medical Grade Tubing, American Scientific Product, McGraw Park, IL) filled 10 mm with melatonin crystals (Sigma Chemical Co., St. Louis, MO) were implanted subcutaneously; (2) 10 mice received sham castrations and subcutaneous Silastic melatonin implants; (3) 10 animals were surgically castrated and implanted with empty Silastic capsules; and (4) 10 animals received sham castrations and empty Silastic implants.

Animals were lightly anesthetized with methoxyflurane vapors (Metofane, Pitman-Moore, Inc., Mundelein, IL). A 70% alcohol solution was applied to the interscapular surface, and a 5-mm incision was made perpendicular to midline. Capsules were implanted, and the incision was closed with a 9-mm autoclip (Clay Adams, Parsippany, NJ). Nitrofurazone antibacterial ointment (Phoenix Pharmaceutical, St. Joseph, MI) was applied to the skin surface to prevent infection. Animals were then returned to the colony room.

Animals were maintained individually in their respective experimental conditions for 6 weeks. Animals then received a single subcutaneous injection of 100 µg of the novel antigen keyhole limpet hemocyanin (KLH), suspended in 0.1-ml sterile saline (Day 0) and were then returned to the colony room. KLH is an innocuous respiratory protein derived from the giant keyhole limpet (*Megathura crenulata*). KLH was used because it generates a robust antigenic response in rodents but does not make the animals ill (e.g., inflammation or fever). Blood was drawn at three different sampling periods (Days 5, 10, and 15 postimmunization). These periods were chosen in order to capture peak IgM production during the course of the immune response to KLH. On each sampling day, animals were brought into the surgery room and lightly anesthetized with methoxyflurane vapors. Blood samples (500 µl) were drawn from the retro-orbital sinus between 1000 and 1200 h EST. Handling time was kept consistent and to a minimum; the time from initial removal from the cage to the end of bleeding was less than 3 min. Samples were allowed to clot for 1 h. The clot was removed, and the samples were centrifuged (at 4ºC) for 1 h at 3500 rpm. Serum aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at –80ºC until assayed. On the last day of sampling (Day 15), animals were killed by cervical dislocation and spleens were removed under aseptic conditions and immediately suspended in culture medium (RPMI-1640/Hepes). Paired testes and epididymides, seminal vesicles, epididymal white adipose tissue (EWAT), and brown adipose tissue (BAT) were removed and cleaned of connective tissue. Seminal vesicles were compressed with a glass vial to remove seminal fluid. All organs were weighed by laboratory assistants naive to the experimental hypotheses and treatment assignments.

Cell-Mediated Immunity

Cell-mediated immune function was assessed by measuring splenocyte proliferation in response to the T-cell mitogen Con A using a colormetric assay based on the tetrazolium salt 3-(4,5-demethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). Splenocytes were separated from tissue by compressing the whole spleen between sterile frosted glass slides; separated cells were suspended in 4 ml of culture medium (RPMI-1640 / Hepes supplemented with 1% penicillin [5000 U / ml] / streptomycin [5000 µl / ml], 1% L-glutamine [2 mM / ml], 0.1% 2-mercaptoethanol [5×10^{-2} M / ml], and 10% heat-inactivated fetal bovine serum). Splenocyte counts and viability were determined with a hemacytometer and trypan blue exclusion. Viable cells (which exceed 95%) were adjusted to 2×10^6 cells / ml by dilution with culture medium, and 50-µl aliquots of each cell suspension (i.e., 100,000 cells) were added to the wells of sterile flat-bottom 96-well culture plates. Con A (Sigma Chemical Co., St. Louis, MO) was diluted with culture medium to concentrations of 40, 20, 10, 5, 2.5, 1.25, and 0.60 µg / ml; 50 µl of each mitogen concentration was added to the wells of the plate containing the spleen cell suspensions to yield a final volume of 100 µl / well (each in duplicate). Plates were incubated at 37ºC with 5% CO_2 for 48 h prior to addition of 20 µl of MTS / phenazine methosulphate (PMS) solution (Promega, Madison, WI; 0.92 mg / ml of PMS in sterile Dulbecco's phosphate-buffered saline) per well. Plates were then incubated at 37ºC with 5% CO_2 for an additional 4 h. The optical density (OD) of each well was determined with a microplate reader (model 3550, Bio-Rad, Richmond, CA) equipped with a 490-nm wavelength filter. Mean OD values for each set of duplicates were used in subsequent statistical analyses. Dose response curves were constructed using group means of the OD values at each mitogen concentration and unstimulated cultures.

Humoral Immunity

To assess humoral immunity, serum anti-KLH IgM concentrations were assayed using an enzyme-linked immunosorbant assay (ELISA). Microtiter plates were coated with antigen, incubated overnight at 4ºC with 0.5 mg / ml KLH in sodium bicarbonate buffer (pH 9.6), washed with phosphate-buffered saline (PBS; pH 7.4) containing 0.05% Tween 20 (PBS-T; pH 7.4), then blocked with 5% nonfat dry milk in PBS-T overnight at 4ºC to reduce nonspecific binding and washed again with PBS-T. Thawed serum samples were diluted (1:40) with PBS-T, and 150 µl of each serum dilution was be added in duplicate to the wells of the antigen-coated plates. Positive control samples (pooled sera from deer mice previously determined to have high levels of anti-KLH antibody, similarly diluted with PBS-T) and negative control samples (pooled sera from KLH-naive deer mice, similarly diluted with PBS-T) were also added in duplicate to each plate; plates were sealed, incubated at 37ºC for 3 h, and washed with PBS-T. Secondary antibody (alkaline phosphatase-conjugated-anti-mouse IgM diluted [1:100] with PBS-T; Cappel, Durham, NC) was added to the wells, and the plates were sealed and incubated (1.5 h for IgM and 1 h for IgG) at 37ºC. Plates were washed again with PBS-T, and 150 µl of the enzyme substrate p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO; 1 mg / ml in diethanolamine substrate buffer) was added to each well. Plates were protected from light during the enzyme-substrate reaction and the reaction was terminated after 20 min for by adding 50 µl of 1.5-M NaOH to each well. The OD of each well was determined using a microplate reader equipped with a 405-nm wavelength filter, and the average OD for each set of duplicate wells was calculated. To minimize intra- assay variability, the average OD for each sample was expressed as a percentage of its plate positive control OD for statistical analyses.

Statistical Analyses

Reproductive organ mass data were analyzed using a two-way between-groups analysis of variance (ANOVA). Splenocyte proliferation and IgM data were analyzed using three-way mixed model ANOVAs. Post hoc comparisons between pairwise means were conducted using Tukey honestly significant difference tests, and differences between group means were considered statistically significant at $p < .05$.

RESULTS

Reproductive, Body, and Fat Pad Masses

Paired testes and epididymides, seminal vesicles, and EWAT masses were significantly smaller in melatonin-treated mice compared to control animals receiving empty capsules ($p < .05$ in all cases) (Fig. 1 and Table 1). Castrated mice had significantly smaller

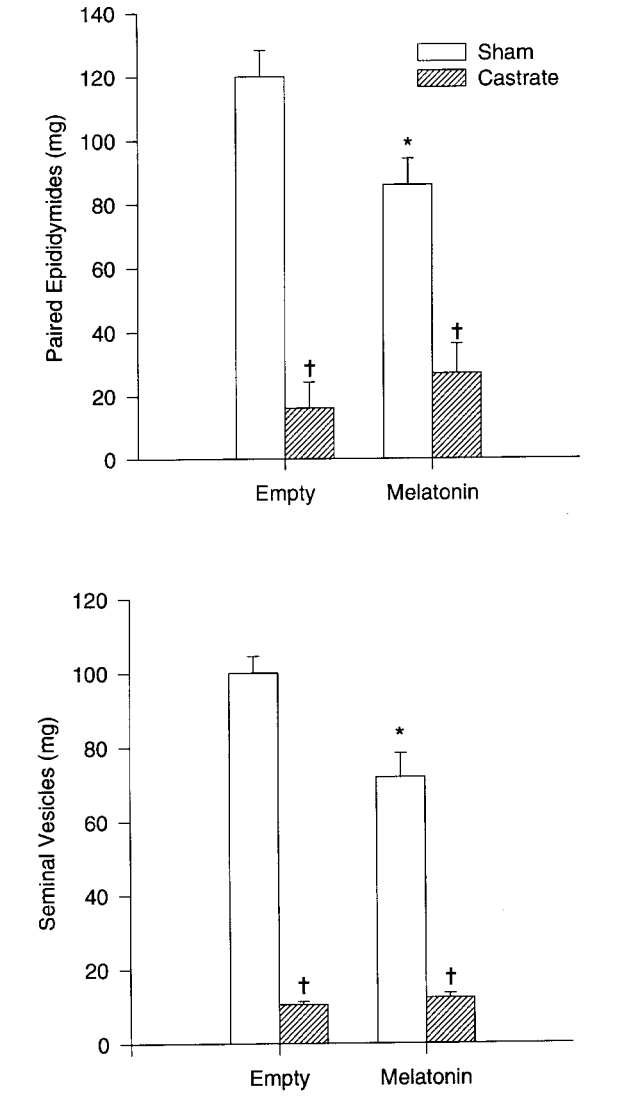

Figure 1. Mean (±SEM) (a) paired epididymal (mg) and (b) seminal vesicle (mg) masses in castrated or sham-operated mice implanted with capsules containing melatonin or empty capsules. Statistically significant differences between pair-wise means are indicated by an asterisk (*).

paired epididymides, seminal vesicles, and EWAT compared to intact animals ($p < .05$ in all cases). Neither BAT nor overall body mass was affected by castration or melatonin treatment (Fig. 1 and Table 1).

Immunologic Measures

Melatonin-treated mice demonstrated greater splenocyte proliferation to Con A compared to animals receiving empty capsules ($p < .05$) (Fig. 2). Castration had no effect on splenocyte proliferation; castrated and sham-operated mice demonstrated comparable splenocyte proliferation ($p > .05$). Serum anti-KLH IgM concentrations were significantly greater on Day 5 compared to Days 10 and 15 in all mice ($p < .05$) (Fig. 3). Neither castration nor melatonin treatment had any significant effect on serum IgM concentrations; all mice displayed comparable IgM concentrations with each sampling period ($p > .05$ in all cases).

Table 1. Mean (± SEM) body (g), reproductive organ, and fat pad masses (mg) in castrated and intact adult male deer mice implanted with empty capsules or capsules containing melatonin.

	Intact		*Castrated*	
	Empty	*Melatonin*	*Empty*	*Melatonin*
Body mass	19.3 ± 0.4	19.4 ± 0.4	18.9 ± 0.7	19.5 ± 0.4
Paired testes	332.4 ± 14.0	254.0 ± 11.0*		
Epididymal white adipose tissue	121.7 ± 6.6	77.6 ± 10.2*	45.2 ± 5.0**	34.7 ± 3.9**
Brown adipose tissue	143.4 ± 9.3	142.8 ± 13.1	135.1 ± 12.3	130.1 ± 11.2

DISCUSSION

Deer mice implanted with melatonin capsules underwent reproductive regression compared to mice implanted with empty capsules. Furthermore, melatonin-treated mice displayed enhanced splenocyte proliferation to Con A compared to mice receiving empty capsules. However, humoral immunity was not affected by melatonin treatment; serum IgM concentrations were similar between mice implanted with empty capsules or mice implanted with capsules filled with melatonin. Castration did not affect immune function. Castrated animals had significantly smaller paired epididymal, seminal vesicle, and EWAT masses compared to intact mice. However, neither splenocyte proliferation nor serum IgM concentrations was affected by removal of the gonads. These results suggest that exogenous melatonin treatment enhances cell-mediated, but not humoral, immune function in deer mice. Furthermore, enhanced immune function after melatonin administration appears to be independent of gonadal steroid hormones in male deer mice.

The pineal gland and its principle secretory product melatonin can affect lymphoid tissue size and morphology, as well immune cell function (Nelson et al., 1995; Vaughan et al., 1987). Melatonin can enhance both humoral and cell-mediated immune function in several species (Guerrero and Reiter, 1992; Maestroni,

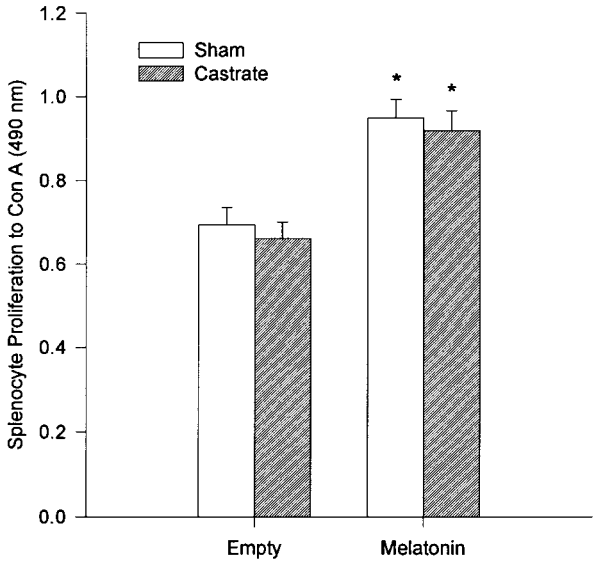

Figure 2. Mean (±SEM) splenocyte proliferation to concanavalin A (Con A) (represented as absorbance units) in castrated or sham-operated mice implanted with capsules containing melatonin or empty capsules. Only the optimal concentration of Con A (10 µg/ml) is graphically represented. Statistically significant differences between pair-wise means are indicated by an asterisk (*).

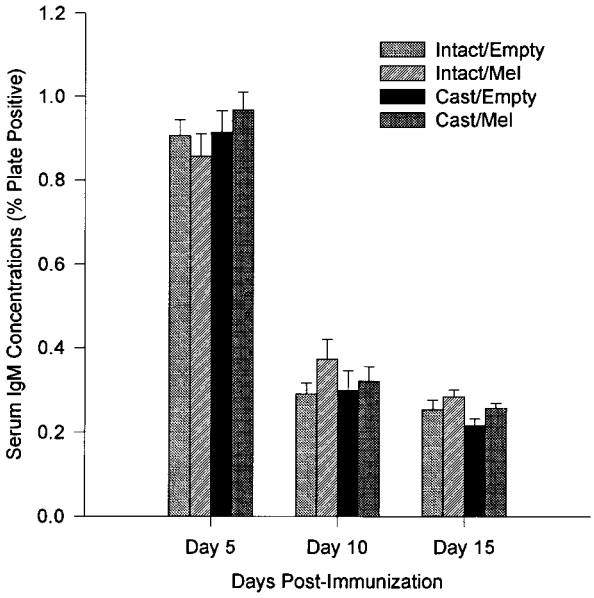

Figure 3. Mean (±SEM) serum immunoglobulin M concentrations (represented as percentage of plate positive) in castrated or sham-operated mice implanted with capsules containing melatonin or empty capsules.

1993). For example, melatonin treatment of both normal and immunocompromised house mice elevates both in vitro and in vivo antibody responses (Caroleo et al., 1992; Maestroni, 1993). Furthermore, antigen presentation by splenic macrophages to T cells is enhanced by melatonin (Pioli et al., 1993). Exogenous melatonin administration also enhances antibody- dependent cellular cytotoxicity (Giordano and Palmero, 1991).

Consistent with a direct action of melatonin on cell-mediated immune function, high-affinity melatonin receptors have been localized on circulating lymphocytes (Calvo et al., 1995; Liu and Pang, 1993; Pang and Pang, 1992), and also on thymocytes and splenocytes (Lopez-Gonzales et al., 1992; Martin-Cacao et al., 1993; Rafii-El-Idrissi et al., 1995). These receptors appear to be high-affinity receptors, with Kd values only slightly higher than those found in the brain (i.e., 1.72-0.34 nM) (Calvo et al., 1995). Based on these findings, the concentrations of melatonin obtained using timed-release Silastic capsules appear sufficient to activate melatonin receptors on lymphatic tissue. Furthermore, the presence of melatonin receptors on lymphatic tissue may explain why exogenous melatonin enhanced cell-mediated, but not humoral, immunity in the present study. It is possible that melatonin binds to receptors localized on splenocytes and increases the general proliferative ability of these cells, leading to enhanced cell-mediated immunity. However, melatonin may not affect the production or release of specific antibodies, leaving humoral immunity unaffected. Further experiments are required to test this hypothesis. Unlike hamsters, in which consistently high concentrations of melatonin can override the reproductive response to the indole-amine, constant release melatonin capsules mimic the effects of short days in *Peromyscus* species. Thus, the present results appear to be physiologically relevant and not simply an artifact of constant melatonin release.

The results of the present study support the hypothesis that melatonin enhances cell-mediated immunity independent of gonadal steroid hormones. Treatment of male deer mice with melatonin enhanced splenocyte proliferation regardless of surgical manipulation. However, it is possible that enhanced immune function with melatonin treatment is due to changes in other hormones such as corticosterone or prolactin. Previous research from our laboratory has examined the role of corticosterone in mediating the immunoenhancing effects of melatonin (Demas and

Nelson, 1998). Intact or gonadectomized male and female deer mice were housed in either long or short day lengths. Short-day mice demonstrated enhanced splenocyte proliferation to Con A compared to long-day animals, regardless of sex. Furthermore, there were no significant differences in circulating corticosterone concentrations among any of the experimental groups (Demas and Nelson, 1998). Thus, enhanced cell-mediated immunity in short days or after melatonin treatment does not appear to be due to changes in glucocorticoid concentrations. These effects may be due to changes in steroid receptor affinity rather than changes in circulating hormone concentrations, but this hypothesis remains to be tested. Exposure to short day lengths or administration of melatonin significantly reduces prolactin concentrations in virtually all rodent species examined (Bartness et al., 1993). Prolactin can also affect immune function (Reber, 1993; Nelson et al., 1995). Generally, prolactin enhances or maintains immunity, but it can inhibit immune function, especially at high or low circulating concentrations (Reber, 1993). The precise role of prolactin in mediating short-day enhancement of immune function requires further study. Taken together, the results of the present study suggest that exogenous melatonin treatment enhances cell-mediated, but not humoral, immune function in adult male deer mice independent of gonadal steroid hormones. These results are consistent with a direct action of melatonin on immune function in deer mice.

ACKNOWLEDGMENTS

The authors thank Brooke Buckley, John Dunlop, Joyce Hairston, Violette Renard, and Sue Yang for technical support and Lance Kriegsfeld and Sabra Klein for valuable comments on the manuscript. The authors are also grateful to Ed Silverman for expert animal care. This study was supported by NICHD Grant MH 57535 and NSF Grant IBN 97-23420, and in part by NICHD Grant P30-HD-06268.

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