- Trabajo realizado por el equipo de la Biblioteca Digital de la Fundación Universitaria San Pablo-CEU
- Me comprometo a utilizar esta copia privada sin finalidad lucrativa, para fines de investigación y docencia, de acuerdo con el art. 37 del T.R.L.P.I. (Texto Refundido de la Ley de Propiedad Intelectual del 12 abril 1996)

Melatonin enhancement of splenocyte proliferation is attenuated by luzindole, a melatonin receptor antagonist

Deborah L. Drazen, Donna Bilu, Staci D. Bilbo and Randy J. Nelson

Am J Physiol Regul Integr Comp Physiol 280:1476-1482, 2001.

The American Journal of Physiology - Regulatory, Integrative and Comparative Physiology publishes original investigations that illuminate normal or abnormal regulation and integration of physiological mechanisms at all levels of biological organization, ranging from molecules to humans, including clinical investigations. It is published 12 times a year (monthly) by the American Physiological Society, 9650 Rockville Pike, Bethesda MD 20814-3991. Copyright © 2005 by the American Physiological Society. ISSN: 0363-6119, ESSN: 1522-1490. Visit our website at http://www.the-aps.org/.

Am J Physiol Regulatory Integrative Comp Physiol
280: R1476–R1482, 2001.

Melatonin enhancement of splenocyte proliferation is attenuated by luzindole, a melatonin receptor antagonist

DEBORAH L. DRAZEN,[1] DONNA BILU,[1] STACI D. BILBO,[2] AND RANDY J. NELSON[2]
[1]*Department of Psychology, The Johns Hopkins University, Baltimore, Maryland 21218-2686; and* [2]*Department of Psychology, Ohio State University, Columbus, Ohio 43210-1222*

Received 11 October 2000; accepted in final form 9 January 2001

Drazen, Deborah L., Donna Bilu, Staci D. Bilbo, and Randy J. Nelson. Melatonin enhancement of splenocyte proliferation is attenuated by luzindole, a melatonin receptor antagonist. *Am J Physiol Regulatory Integrative Comp Physiol* 280: R1476–R1482, 2001.—In addition to marked seasonal changes in reproductive, metabolic, and other physiological functions, many vertebrate species undergo seasonal changes in immune function. Despite growing evidence that photoperiod mediates seasonal changes in immune function, little is known regarding the neuroendocrine mechanisms underlying these changes. Increased immunity in short days is hypothesized to be due to the increase in the duration of nightly melatonin secretion, and recent studies indicate that melatonin acts directly on immune cells to enhance immune parameters. The present study examined the contribution of melatonin receptors in mediating the enhancement of splenocyte proliferation in response to the T cell mitogen Concanavalin A in mice. The administration of luzindole, a high-affinity melatonin receptor antagonist, either in vitro or in vivo significantly attenuated the ability of in vitro melatonin to enhance splenic lymphocyte proliferation during the day or night. In the absence of melatonin or luzindole, splenocyte proliferation was intrinsically higher during the night than during the day. In the absence of melatonin administration, luzindole reduced the ability of spleen cells to proliferate during the night, when endogenous melatonin concentrations are naturally high. This effect was not observed during the day, when melatonin concentrations are low. Taken together, these results suggest that melatonin enhancement of splenocyte proliferation is mediated directly by melatonin receptors on splenocytes and that there is diurnal variation in splenocyte proliferation in mice that is also mediated by splenic melatonin receptors.

immune function; seasonal; pineal; Concanavalin A; diurnal; lymphocyte; circadian

SEASONAL CHANGES IN IMMUNE function have been documented for a wide range of species (39). Although many extrinsic factors influence seasonal changes in physiology and behavior, most laboratory studies examining the effects of environmental influences on immune function have manipulated ambient photoperiod. Several studies have demonstrated that immune status can be affected by exposure to short or long day lengths (e.g., 10, 40, 54). Alterations in specific immune parameters are hypothesized to act in concert with a suite of adaptations that evolved in order for nontropical animals to cope with winter, a time when thermoregulatory demands typically increase and food availability decreases (6, 40).

In virtually all laboratory studies of photoperiod and immune function, short, winterlike photoperiods enhance immune function relative to animals exposed to long, summerlike days (40). Despite the increasing number of studies demonstrating photoperiodic changes in immune function, little is known regarding the neuroendocrine mechanisms underlying these changes. Several hormones that can alter immune function vary on a seasonal basis (and are also affected by photoperiod), such as gonadal steroid hormones, prolactin, and glucocorticoids (40); however, most recent evidence suggests that seasonal changes in immune function are due to changes in the indole-amine melatonin (10, 29, 35). Melatonin is the primary secretory product of the pineal gland, and the duration of its release roughly tracks the annual changes in scotophase (length of night) (4, 9). Melatonin treatment with extended duration (e.g., appropriately timed injections or implantation of melatonin-filled capsules) induces physiological adaptations associated with winter, including reproductive regression (e.g., Syrian hamsters) and enhancement of certain aspects of immune function (e.g., deer mice) (4, 17).

A large body of evidence supports the immunoenhancing role of melatonin (41). For example, exposure of hamsters to short days (<12 h light/day) or daily afternoon injections of melatonin increases splenic mass. Importantly, this increase could be prevented in short-day animals by pinealectomy (52). Pinealectomized C57BL/6 mice have a decreased ability to mount humoral responses against sheep red blood cells (5). Melatonin has been demonstrated to enhance both cell-mediated and humoral immune function in many species (29, 35). Melatonin treatment of both normal and immunocompromised house mice increases in vitro and in vivo antibody responses and T helper cell activity (8, 35). Melatonin administration appears to stimulate humoral immunity by inhibiting apoptosis

Address for reprint requests and other correspondence: D. L. Drazen, Dept. of Psychiatry, Univ. of Cincinnati Med. Ctr. PO Box 670559, Cincinnati, OH 45267-0559 (E-mail: drazen@jhu.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

during early B cell development in mouse bone marrow (55). Suppression of endogenous melatonin secretion in hamsters by the administration of a β-adrenergic antagonist compromises immune function (38).

Mounting evidence indicates that melatonin acts directly on immune tissues to modulate immune function. It remains unspecified, however, which melatonin-receptor subtype(s) mediates the immunoenhancing effect of melatonin on immune function. High-affinity melatonin receptors have been localized on circulating lymphocytes from rodents, chickens, and humans (7, 42) and on thymocytes and splenocytes in humans and a number of rodent and bird species (e.g., 34, 39, 43, 45, 47, 56). Furthermore, in vitro melatonin administration enhances the proliferative ability of splenocytes from prairie voles (*Microtus ochrogaster*) (19, 33) and enhances the mitogenic response of peripheral blood T lymphocytes from chickens (32). Exogenous melatonin enhances cell-mediated immune function in male deer mice independent of gonadal steroid hormones (17). Treatment of animals with exogenous melatonin enhances both cell-mediated and humoral immune function in species that are, in general, considered reproductively unresponsive to melatonin (i.e., rats and house mice) (29, 35).

Melatonin may also act indirectly to alter immune status. Many hormones vary seasonally in response to different photoperiods. Photoperiod-induced changes in hormone concentrations are mediated by melatonin. For example, when animals are maintained on short days or treated with melatonin, glucocorticoid and prolactin secretions are often attenuated and immune function is enhanced (16, 36).

One goal of the present study was to determine the direct contribution of melatonin receptors in mediating enhancement of cell-mediated immunity. Specifically, we administered the high-affinity melatonin receptor antagonist luzindole both in vivo and in vitro to determine the role of the Mel 1b melatonin receptor in changes in cell-mediated immunity. Luzindole is a relatively specific Mel 1b-receptor antagonist, with a 25-fold higher affinity for the Mel 1b receptor than for the Mel 1a-receptor subtype (23). The pharmacokinetics of luzindole have been worked out previously (20), and this substance is effective in a variety of rodent species (e.g., Refs. 23 and 50). If luzindole attenuates the ability of melatonin to enhance splenocyte proliferation, then it can be concluded that melatonin enhancement of splenocyte proliferation is directly mediated by melatonin receptors, specifically, Mel 1b, on splenocytes.

Another goal of the present study was to further investigate the role of melatonin in directly mediating immunity, by examining the effects of changes in both endogenous and exogenous melatonin on splenocyte proliferation. Measurement of splenocyte proliferation in vitro in both untreated cells and cells treated with melatonin allowed the determination of the effects of exogenous melatonin on immune function. Most studies examining the effects of melatonin on immune parameters have either added melatonin (e.g., via injection into the animal or via addition to immune cell cultures) or removed the main source of melatonin completely by pinealectomy (10, 35). Measurement of splenocyte proliferation of untreated cells during both the light and dark phases of the daily light-dark (LD) cycle, however, allowed the assessment of the role of endogenous melatonin in cell-mediated immunity by exploiting the naturally occurring peaks and troughs in endogenous melatonin concentrations. Thus another goal of the study was to investigate how melatonin might play a role in the potential diurnal rhythm in this cell-mediated immune response. If splenocyte proliferation is higher during the dark than during the light phase, then the higher endogenous melatonin concentrations that occur during the dark might be responsible for this enhancement. If this effect is mediated directly by melatonin receptors, specifically, Mel 1b receptors, then in the absence of exogenous melatonin, luzindole should attenuate the enhancement of splenocyte proliferation observed during the dark phase (53).

MATERIALS AND METHODS

Animals

Ninety-five adult (between 60 and 75 days of age) C57BL6/J male mice (*Mus musculus*) were purchased from Charles River (Wilmington, MA) and used in these studies. Animals were individually housed in polycarbonate cages (28 × 17 × 12 cm) in colony rooms with a 16:8-h LD cycle (lights on at 0700 Eastern Standard Time) and were allowed to acclimate for 2 wk before the onset of the experiment. Colony rooms were maintained with an ambient temperature of 21 ± 2°C, and relative humidity was held constant at 50 ± 5%. Food (LabDiet 5001; PMI Nutrition, Brentwood, MO) and tap water were provided ad libitum throughout the course of the study.

Experimental Procedures

Experiment 1. Male house mice ($n = 16$) were brought into the surgery room one at a time and were killed by cervical dislocation. Spleens were removed under aseptic conditions and were immediately suspended in culture medium (RPMI 1640), after which they were used to assess splenocyte proliferation.

Experiment 2. Male house mice were randomly selected and assigned to one of four experimental conditions: *1*) 10 mice received an intraperitoneal injection of luzindole (*N*-acetyl-2-benzyltryptamine) (30 mg/kg), a high-affinity melatonin receptor antagonist (Tocris Cookson, Ballwin, MO), during the middle of their light cycle (1500); *2*) 9 mice received an intraperitoneal injection of PBS vehicle at 1500; *3*) 14 mice received an intraperitoneal injection of luzindole (30 mg/kg) during the middle of their dark cycle (0300); and *4*) 14 mice received an intraperitoneal injection of vehicle at 0300. Luzindole was dissolved in a minimal amount of 95% ethanol and further diluted with PBS, so that the final concentration of ethanol was ~0.1%. Vehicle injections consisted of PBS with 0.1% ethanol. All injections were administered 30 min before cervical dislocation and subsequent spleen removal, which was performed as described for *experiment 1*. The luzindole dose of 30 mg/kg was chosen because this dose has been used successfully to block melatonin receptor function in house mice (11, 22, 26, 50).

Experiment 3. Male house mice were selected randomly and assigned to one of two experimental conditions: *1)* 16 mice were killed during the middle of their light cycle (1500), and the spleens were removed; *2)* 16 mice were killed during the middle of their dark cycle (0300), and the spleens were removed. Splenocytes from each animal were examined in a proliferation assay, except that some of the cell cultures from each animal also received luzindole to determine the ability of the melatonin receptor antagonist to inhibit the ability of the addition of in vitro melatonin to enhance splenocyte proliferation. Luzindole (1 and 10 μM) was added to the wells last, just before the addition of splenocytes.

Splenocyte Proliferation

Cell-mediated immune function was assessed by measuring splenocyte proliferation in response to the T cell mitogen Concanavalin A (Con A) (see Ref. 19 for complete description of assay procedure). Deviations from this protocol are as follows: Con A (Sigma Chemical, St. Louis, MO) was diluted with culture medium to concentrations of 40, 20, 10, and 0 μg/ml for *experiment 1*; for *experiments 2* and *3*, only the optimal dose, 20 μg/ml, in addition to 0 μg were used. Fifty microliters of each mitogen concentration and 100 μl of culture medium were added to the wells of the plate that would contain the spleen cell suspensions to yield a final volume of 200 μl/well (each in duplicate) for those wells to which melatonin was not added. Melatonin (Sigma Chemical) was dissolved in ethanol and diluted in RPMI 1640 medium to a standard stock solution. One-hundred microliters of 500 pg/ml melatonin were added to half of the wells that contained the spleen cell suspensions to yield a final volume of 200 μl/well (each in duplicate); this addition of melatonin led to a final concentration of 250 pg/ml melatonin in these wells. Both Con A and melatonin were added to the plates before the addition of cells.

Statistical Analyses

Proliferative responses were analyzed using a four-way ANOVA with two within-subject variables (mitogen concentration and melatonin treatment) and two between-subject variables (drug treatment and time of day) or a one-way repeated-measures ANOVA. Significant interactions were probed using individual two-way ANOVAs, and all pairwise comparisons of mean differences were conducted using Tukey's honestly significant difference test. Differences between group means were considered statistically significant if $P < 0.05$.

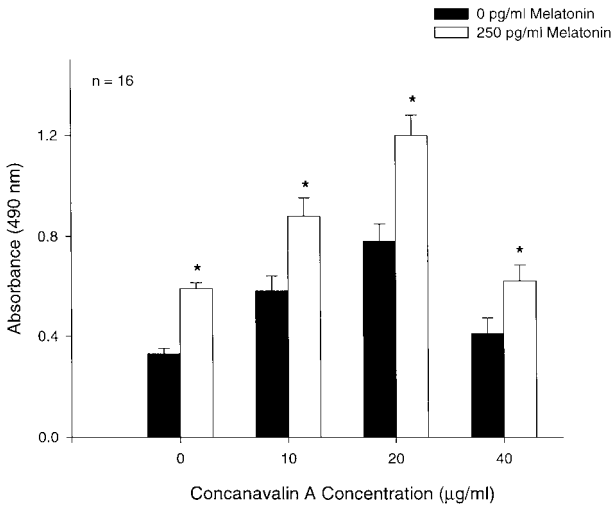

Fig. 1. Mean (±SE) proliferation values (represented as absorbance units) from male C57BL/6 house mice in response to stimulation with Concanavalin A. Closed dark bars represent proliferative values of animals receiving no melatonin; open white bars represent proliferative values of individuals receiving 500 pg/ml melatonin in each assay well (yielding a final well concentration of 250 pg/ml). *Significantly different from paired control ($P < 0.001$).

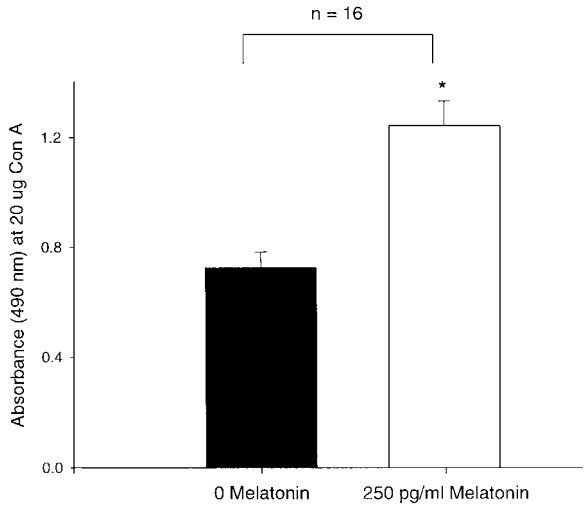

Fig. 2. Mean (±SE) proliferation values (represented as absorbance units) from male C57BL/6 house mice in response to optimal stimulation with Concanavalin A, either with or without the addition of 500 pg/ml melatonin added to each assay well (yielding a final well concentration of 250 pg/ml melatonin). *Significantly different from paired control ($P < 0.001$).

RESULTS

Experiment 1

Splenoctye proliferation was significantly elevated in cultures to which melatonin was added ($P < 0.001$). Proliferative responses varied as a function of mitogen concentration, including basal proliferation, with optimal proliferative responses (i.e., the greatest amount of proliferative response) observed at 20 μg/ml of Con A ($P < 0.001$) (Fig. 1). Melatonin enhanced splenocyte proliferation under basal conditions without stimulation by Con A ($P < 0.001$) (Fig. 1) and when stimulated by the optimal dose of Con A ($P < 0.001$) (Fig. 2). A similar enhancement of proliferation was observed with the addition of melatonin at all Con A doses ($P < 0.001$ in each case) (Fig. 1).

Experiment 2

A diurnal difference was observed in the proliferative response of untreated (i.e., no melatonin added to cultures) spleen cells to Con A; splenocyte proliferation was significantly greater during the night than during the day in saline-injected animals ($P < 0.05$) (Fig. 3). In addition, splenocyte proliferation was significantly enhanced in cultures to which melatonin was added compared with untreated cells both during the day and during the night ($P < 0.05$ in both cases) (Fig. 3). In animals injected with luzindole, however, the melato-

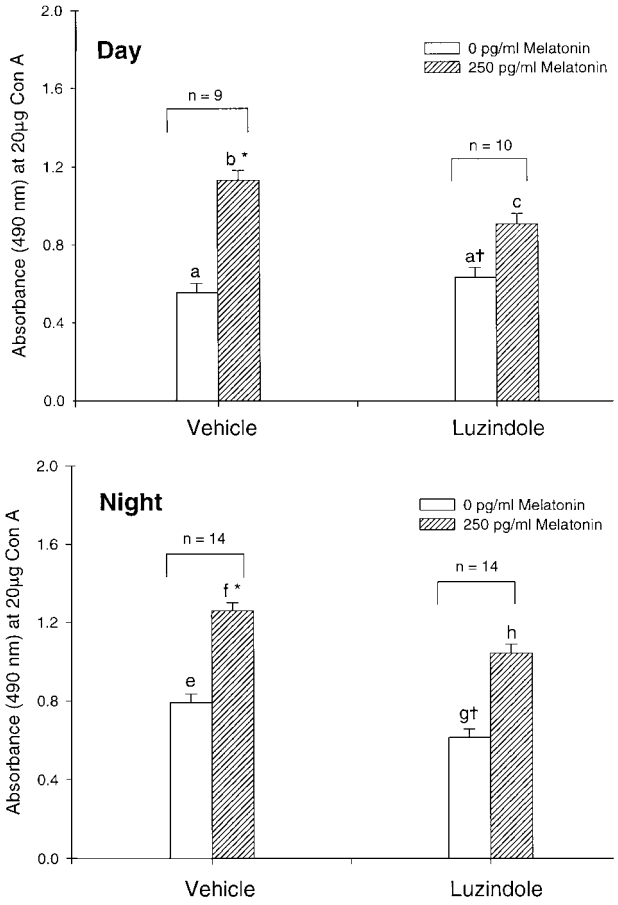

Fig. 3. Mean (±SE) proliferation values (represented as absorbance units) from male C57BL/6 house mice in response to optimal stimulation with Concanavalin A, either with or without the addition of 500 pg/ml melatonin added to each assay well (yielding a final well concentration of 250 pg/ml melatonin). These mice were injected either with 30 mg/kg luzindole or vehicle 30 min before spleen removal. Within graphs, bars that share letters are not significantly different from one another; between graphs, bars that share symbols are not significantly different from one another. Comparisons are statistically significant when $P < 0.05$.

nin enhancement of splenocyte proliferation was significantly attenuated both during the day and the night ($P < 0.05$ in both cases) (Fig. 3). In animals injected with luzindole whose cells were untreated (i.e., no melatonin added to cultures), splenocyte proliferation in response to Con A was significantly reduced during the night ($P < 0.05$) but not during the day ($P > 0.05$) (Fig. 3).

Experiment 3

There was a diurnal difference in the proliferative response of untreated (i.e., no melatonin added to cultures) spleen cells to Con A; basal splenocyte proliferation was significantly higher during the night than during the day ($P < 0.05$) (Fig. 4). In addition, splenocyte proliferation was significantly enhanced in cultures to which melatonin was added compared with untreated cells both during the day and during the night ($P < 0.05$ in both cases) (Fig. 4). Luzindole at a concentration of 1 μM did not significantly affect splenoctye proliferation ($P > 0.05$ in all cases) (data not shown). In cells treated with 10 μM luzindole, however, the melatonin enhancement of splenocyte proliferation was significantly attenuated both during the day and the night ($P < 0.05$ in both cases) (Fig. 4). In wells to which luzindole was added and whose cells were otherwise untreated (i.e., no melatonin added to cultures), splenocyte proliferation in response to Con A was significantly reduced during the night ($P < 0.05$) but not during the day ($P > 0.05$) (Fig. 4).

DISCUSSION

Splenocyte proliferation in response to the T cell mitogen Con A was enhanced in house mice by the addition of melatonin in vitro compared with cultures that did not receive melatonin. These results were consistent regardless of the time of day when cells were obtained and stimulated. Spleen cells to which no melatonin was added had a greater proliferative response to Con A when removed from animals and stimulated

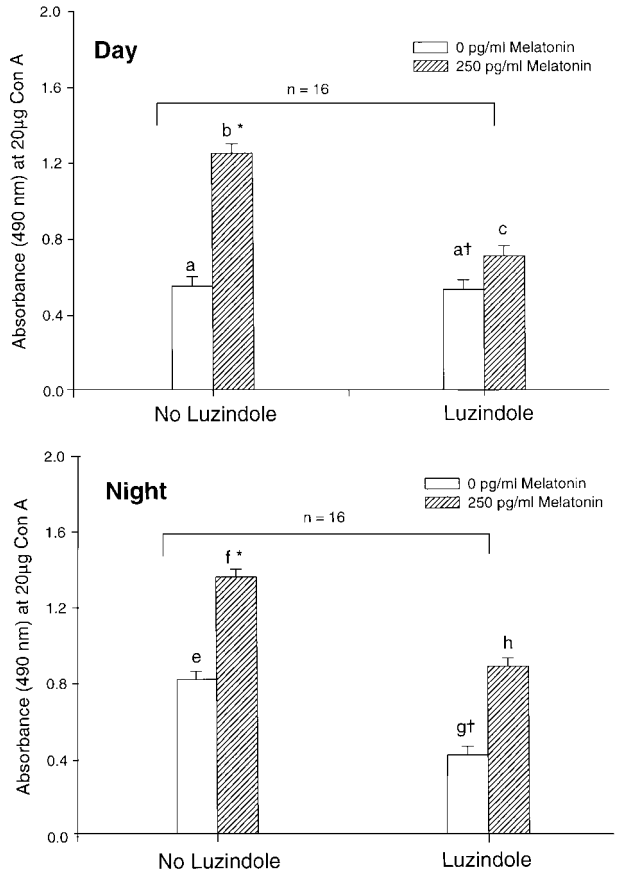

Fig. 4. Mean (±SE) proliferation values (represented as absorbance units) from male C57BL/6 house mice in response to optimal stimulation with Concanavalin A, either with or without the addition of 500 pg/ml melatonin added to each assay well (yielding a final well concentration of 250 pg/ml melatonin), and with or without the addition of 10 μM luzindole. Within graphs, bars that share letters are not significantly different from one another; between graphs, bars that share symbols are not significantly different from one another. Comparisons are statistically significant when $P < 0.05$.

in the middle of the dark cycle compared with untreated cells taken from animals and stimulated during the middle of the light cycle. When the competitive melatonin receptor antagonist luzindole was injected 30 min before spleen removal (and when luzindole was used in vitro), however, the ability of in vitro melatonin to enhance the proliferative ability of spleen cells was reduced both during the day and the night. Luzindole treatment of cells that were not treated with melatonin reduced splenocyte proliferation only during the dark phase when endogenous melatonin concentrations are normally high but not during the light phase.

The present results are consistent with previous findings (13, 19, 41), demonstrating that melatonin enhances immunity in mice. In the present study, when melatonin concentrations were elevated, whether from endogenous sources or resulting from in vitro manipulation, splenocyte proliferation was increased compared with cases in which melatonin concentrations were low. During the night, when the naturally occurring endogenous melatonin peak occurs, splenocyte proliferation was enhanced compared with the naturally low daytime concentrations, confirming data from studies using exogenous melatonin.

The present results support previous work suggesting that melatonin is acting directly on immune tissue to enhance immune function (3, 19, 27, 29, 33). The results of the present study also suggest that melatonin is acting via specific melatonin receptors on spleen cells to enhance their proliferative ability, because luzindole attenuated the ability of both endogenous and exogenous melatonin to enhance splenocyte proliferation. These findings are consistent with the observation of high-affinity melatonin receptors that have been identified on many different tissues within the immune system, including the spleen, of virtually all species studied to date (e.g., 44, 46, 56).

The distribution of melatonin receptor subtypes has not been well characterized. Receptor subtypes Mel 1a and Mel 1b have been identified in mouse, human, and hamster brain (2, 21, 49). To our knowledge, the only characterizations of melatonin receptor subtypes in peripheral tissue are either pharmacological characterizations of, or mRNA expression of either Mel 1a or 1b in vascular smooth muscle, rat tail artery and melanoma cells (18, 48, 51). The present results suggest that the Mel 1b receptors mediate the melatonin-induced enhancement of splenocyte proliferation and strongly suggest that Mel 1b receptors are present on mouse splenocytes. Direct evidence of the presence of these receptors is required in future studies. Luzindole has a 25-fold higher affinity for the Mel 1b receptor than for the Mel 1a receptor; however, both receptors were presumably blocked to a certain extent with the administration of luzindole. The relative contribution, therefore, of receptors Mel 1a and Mel 1b in the melatonin-induced enhancement of splenocyte proliferation was not determined in the present study. As a result, it is possible that some of the observed effects were also due to the partial suppression of the Mel 1a receptor. Use of recently available highly specific melatonin receptor antagonists, in addition to the use of knockout mice missing the gene for specific melatonin receptor subtypes, will further clarify the role of these receptors in the modulation of immune function. Such studies are currently underway in our laboratory. It is also important to note that luzindole attenuated and did not fully block the ability of melatonin to enhance splenocyte proliferation. It is likely, therefore, that other melatonin receptors, such as nuclear melatonin receptors (28), or other membrane-bound receptors that have not yet been identified in mammals (e.g., Mel 1c) contribute to mediating the ability of melatonin to enhance splenocyte proliferation. In addition, given that luzindole is not a powerful Mel 1a antagonist, it is possible that Mel 1a is involved in mediating the immunoenhancing effects of melatonin. These possibilities remain to be tested.

Daily rhythms in immune parameters have been well documented for most species studied to date. Most of these investigations, however, have focused on rhythmicity in the numbers of circulating immune cells, such as circadian fluctuations in the number of circulating and splenic lymphocytes (1, 31), whereas relatively few studies have examined changes in the functional activity of immune cells. In the present study, the ability of splenocytes to proliferate, without any melatonin manipulation, was higher in cultures obtained during the night, compared with cultures obtained during the day. When melatonin was added to the spleen cell cultures in both cases, proliferation was enhanced during both the day and night; luzindole was only effective in attenuating this enhancement at night. Taken together, these results suggest that the diurnal variation observed in splenocyte proliferation is mediated by melatonin. These results are also consistent with recent evidence that melatonin has time-dependent effects on the immune system (10).

The results of the present study suggest that the short-day enhancement of immune function observed for many seasonally breeding species is dependent on the circadian fluctuation in melatonin secretion. It is interesting to note that the melatonin-regulated seasonal changes in the reproductive system are not dependent on such a direct, immediate action of melatonin. In seasonally breeding rodents, short days inhibit reproductive hormones, but these inhibitory effects do not increase in magnitude during the night. The present data suggest a more direct temporal relationship between melatonin amplitude and seasonal adjustments in immune system function, in which the nightly changes in melatonin affect immune function on a daily basis. These cumulative daily changes in melatonin-induced immune function lead to an overall enhancement of immune function for short-day animals that experience a longer nightly duration of melatonin than do long-day animals.

In the last several years, there has been considerable debate as to whether strains of inbred mice derived from *Mus musculus* produce melatonin (24, 25). More recently, HPLC has revealed that there is a short-term peak of melatonin in the middle of the dark period in

C57BL/6 and BALB/c strains of inbred mice (12). These mice appear able to synthesize melatonin; a nighttime peak in the hormone has been confirmed by radioimmunoassay from pineal samples that were obtained every 15 min throughout the day (53). Our results provide further evidence that a nighttime melatonin peak in C57BL/6 mice is functional because in unstimulated spleen cells, luzindole, which acts on melatonin receptors, reduced proliferation during the night but not during the day. It will be interesting in future studies to examine the interaction of melatonin and the immune system in strains that demonstrate a more robust melatonin rhythm.

House mice were chosen for the present studies because they are not seasonal breeders. In seasonally breeding animals, melatonin is only one of many hormones that varies, and many of these hormones affect immune function. Thus it is difficult to study the role of melatonin in mediating changes in immune function independent of changes in other hormones (e.g., prolactin) in seasonal breeders. By studying a nonseasonally breeding animal, we were able to avoid the potentially confounding effects of other changing hormones on immune function. House mice do not respond reproductively or immunologically to photoperiod, making this species an ideal model system in which to manipulate melatonin either exogenously or endogenously, because these animals exhibit a circadian fluctuation in melatonin secretion but not a seasonal melatonin rhythm. An additional advantage of using *Mus musculus* is the widespread acceptance of this species in studies of immunology. Use of this specific strain is consistent with the extensive literature that already exists on melatonin and immune function (14, 15, 30, 37), and it forms the groundwork for future studies on melatonin receptor-knockout mice that use a C57 genetic background.

In summary, the results of the present study support an immunoenhancing role for the pineal hormone melatonin in *Mus musculus*. The addition of melatonin to spleen cell cultures enhanced their ability to divide, and this enhancement was highest at night, when naturally occurring endogenous concentrations of melatonin are at their peak; this also demonstrates that there is diurnal variation in splenocyte proliferation, which appears to be mediated by endogenous melatonin. These results also suggest, when considered with previous studies, that melatonin enhances splenocyte proliferation by acting directly on immune cells. The melatonin receptor antagonist luzindole attenuated the ability of melatonin to enhance splenocyte proliferation both when it was injected into the animal before culturing spleen cells and when it was added along with melatonin, directly to spleen cell cultures. Further studies are necessary to determine more definitively which melatonin receptor subtype(s) is mediating this melatonin-induced enhancement of splenocyte proliferation, in addition to melatonin-mediated enhancement of other immune parameters.

The authors thank L. Kriegsfeld and B. Spar for technical support, and G. Demas, K. Young, and S. Gammie for valuable comments on the manuscript. We are also grateful to Dr. M. Dubocovich for helpful advice regarding the in vitro luzindole doses. We also thank K. Nies for expert animal care.

This study was supported by United States Public Health Service Grant MH-57535 and National Science Foundation Grant IBN-97–23420.

REFERENCES

1. **Abo T, Kawate T, Itoh K, and Kumagai K.** Studies on the bioperiodicity of the immune response. I. Circadian rhythms of human T, B, and K cell traffic in the peripheral blood. *J Immunol* 126: 1360–1363, 1981.
2. **Al-Ghoul WM, Herman MD, and Dubocovich ML.** Melatonin receptor subtype expression in human cerebellum. *Neuroreport* 21: 4063–4068, 1998.
3. **Atre D and Blumenthal EJ.** Melatonin: immune modulation of spleen cells in young, middle-aged and senescent mice. *Mech Ageing Dev* 103: 255–268, 1998.
4. **Bartness TJ, Powers JB, Hastings MH, Bittman EL, and Goldman BD.** The timed infusion paradigm for melatonin delivery: what has it taught us about the melatonin signal, its reception, and the photoperiodic control of seasonal responses? *J Pineal Res* 15: 161–190, 1993.
5. **Becker J, Veit G, Handgretinger R, Atanasio A, Bruchett G, Reuner I, Niethammer D, and Das Gupta TK.** Circadian variations in the immunomodulatory role of the pineal gland. *Neuroendocrinol Lett* 10: 65–72, 1988.
6. **Bronson FH.** Energy balance and ovulation: small cages versus natural habitats. *Reprod Fertil Dev* 10: 127–137, 1998.
7. **Calvo JR, Rafil-El-Idrissi M, Pozo D, and Guerrero JM.** Immunomodulatory role of melatonin: specific binding sites in human and rodent lymphoid cells. *J Pineal Res* 10: 119–126, 1995.
8. **Caroleo MC, Frasca AD, Nistico G, and Doria D.** Melatonin as immunomodulator in immunodeficient mice. *Immunopharmacology* 23: 81–89, 1992.
9. **Carter DS and Goldman BD.** Antigonadal effects of timed melatonin infusion in pinealectomized male Djungarian hamsters (*Phodopus sungorus* sungorus): duration is the critical parameter. *Endocrinology* 113: 1261–1267, 1983.
10. **Champney TH, Allen GC, Zannelli M, and Beausang LA.** Time-dependent effects of melatonin on immune measurements in male Syrian hamsters. *J Pineal Res* 25: 142–146, 1998.
11. **Constantinescu CS, Hillard B, Ventura E, and Rostami A.** Luzindole, a melatonin receptor antagonist, suppresses experimental autoimmune encephalomyelitis. *Pathobiology* 65: 190–194, 1997.
12. **Conti A and Maestroni GJ.** HPLC validation of a circadian melatonin rhythm in the pineal gland of inbred mice. *J Pineal Res* 20: 138–144, 1996.
13. **Currier NL, Sun LZY, and Miller SC.** Exogenous melatonin: quantitative enhancement in vivo of cells mediating non-specific immunity. *J Neuroimmunol* 104: 101–108, 2000.
14. **Del Gobbo V, Libri V, Villani N, Calio R, and Nistico G.** Pinealectomy inhibits interleukin-2 production and natural killer activity in mice. *Int J Immunopharmacol* 11: 567–573, 1989.
15. **Demas GE, Chefer V, Talan MI, and Nelson RJ.** Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. *Am J Physiol Regulatory Integrative Comp Physiol* 273: R1631–R1637, 1997.
16. **Demas GE, Klein SL, and Nelson RJ.** Reproductive and immune responses to photoperiod and melatonin are linked in *Peromyscus* subspecies. *J Comp Physiol [A]* 179: 819–825, 1997.
17. **Demas GE and Nelson RJ.** Short-day enhancement of immune function is independent of steroid hormones in deer mice (*Peromyscus maniculatus*). *J Comp Physiol [B]* 168: 419–426, 1998.
18. **Doolen S, Krause DN, Dubocovich ML, and Duckles SP.** Melatonin mediates two distinct responses in vascular smooth muscle. *Eur J Pharmacol* 345: 67–69, 1998.

19. **Drazen DL, Klein SL, Yellon SM, and Nelson RJ.** In vitro melatonin treatment enhances splenocyte proliferation in prairie voles. *J Pineal Res* 28: 34–40, 2000.
20. **Dubocovich ML.** Luzindole (N-0774): a novel melatonin receptor antagonist. *J Pharmacol Exp Ther* 246: 902–910, 1988.
21. **Dubocovich ML, Benloucif S, and Masana MI.** Melatonin receptors in the mammalian suprachiasmatic nucleus. *Behav Brain Res* 73: 141–147, 1996.
22. **Dubocovich ML, Mogilnicka E, and Areso PM.** Antidepressant-like activity of the melatonin receptor antagonist, luzindole (N-0774), in the mouse behavior despair test. *Eur J Pharmacol* 182: 313–325, 1990.
23. **Dubocovich ML, Yun K, Al-Ghoul WM, Benloucif S, and Masana MI.** Selective MT2 melatonin receptor antagonists block melatonin-mediated phase advances of circadian rhythms. *FASEB J* 12: 1211–1220, 1998.
24. **Ebihara S, Hudson DJ, Marks T, and Menaker M.** Pineal indole metabolism in the mouse. *Brain Res* 416: 136–140, 1987.
25. **Ebihara S, Marks T, Hudson DJ, and Menaker M.** Genetic control of melatonin in the pineal gland of the mouse. *Science* 231: 491–493, 1986.
26. **Fang JM and Dubocovich ML.** Activation of melatonin receptor sites retarded the depletion of norepinepherine following inhibition of synthesis in the C3H/HeN mouse hypothalamus. *J Neurochem* 55: 76–78, 1990.
27. **Fraschini F, Demartini G, Esposti D, and Scaglione F.** Melatonin involvement in immunity and cancer. *Biol Signals Recept* 7: 61–72, 1998.
28. **Guerrero JM, Pozo D, Garcia-Maurino S, Carillo A, Osuna C, Molinero P, and Calvo JR.** Nuclear receptors are involved in the enhanced IL-6 production by melatonin U937 cells. *Biol Signals Recept* 9: 197–202, 2000.
29. **Guerrero JM and Reiter RJ.** A brief survey of pineal gland-immune system interrelationships. *Endocr Res* 18: 91–113, 1992.
30. **Inserra P, Zhang Z, Ardestani SK, Araghi-Niknam M, Liang BL, Jiang SG, Shaw D, Molitor M, Elliott K, and Watson RR.** Modulation of cytokine production by dehydroepiandrosterone (DHEA) plus melatonin (MLT) supplementation of old mice. *Proc Soc Exp Biol Med* 218: 76–82, 1998.
31. **Kawate T, Abo T, Hinuma S, and Kumagai K.** Studies of the biperiodicity of the immune response. II. Co-variations of murine T and B cells and a role of corticosteroid. *J Immunol* 126: 1364–1367, 1981.
32. **Kliger CA, Gehad AE, Hulet RM, Roush WB, Lillehoj HS, and Mashaly MM.** Effects of photoperiod and melatonin on lymphocyte activities in male broiler chickens. *Poult Sci* 79: 18–25, 2000.
33. **Kriegsfeld LJ, Drazen DL, and Nelson RJ.** In vitro melatonin treatment enhances cell-mediated immune function in male prairie voles (*Microtus ochrogaster*). *J Pineal Res.* In press.
34. **Lopez-Gonzales MA, Calvo JR, Osuna C, and Guerrero JM.** Interaction of melatonin with human lymphocytes: evidence for binding sites coupled to potentiation of cyclic AMP stimulated vasoactive intestinal peptide and activation of cyclic GMP. *J Pineal Res* 12: 97–104, 1992.
35. **Maestroni GJ.** The immunoneuroendocrine role of melatonin. *J Pineal Res* 14: 1–10, 1993.
36. **Maestroni GJ, Conti A, and Pierpaoli W.** Role of the pineal gland in immunity. Circadian synthesis and release of melatonin modulates the antibody response and antagonizes the immunosuppressive effect of corticosterone. *J Neuroimmunol* 13: 19–30, 1986.
37. **Maestroni GJ, Conti A, and Pierpaoli W.** Role of the pineal gland in immunity: melatonin enhances the antibody response via an opiatergic mechanism. *Clin Exp Immunol* 68: 384–391, 1987.
38. **Maestroni GJ and Pierpaoli W.** Pharmacologic control of the hormonally mediated immune response. In: *Psychoneuroimmunology*, edited by Ader R. New York: Academic, 1982, p. 405–425.
39. **Martini-Cacao A, Lopez-Gonzalez MA, Reiter RJ, Calvo JR, and Guerrero JM.** Binding of 2-[^{125}I]melatonin by rat thymus membranes during postnatal development. *Immunol Lett* 36: 59–64, 1992.
40. **Nelson RJ and Demas GE.** Seasonal changes in immune function. *Q Rev Biol* 71: 511–548, 1996.
41. **Nelson RJ and Drazen DL.** Melatonin mediates seasonal adjustments in immune function. *Reprod Nutr Dev* 39: 383–398, 1999.
42. **Pang CS and Pang SF.** High affinity specific binding of 2-[^{125}I]iodomelatonin by spleen membrane preparations of chicken. *J Pineal Res* 12: 167–173, 1992.
43. **Pang CS, Tang PL, Pang SF, and Brown GM.** Comparison of the pharmacological characteristics of 2-[^{125}I]iodomelatonin binding sites in the lung, spleen, brain and kidney of chicken. *Biol Signals* 4: 311–324, 1995.
44. **Poon AM, Ayre EA, Song Y, and Pang SF.** Melatonin implant decreases the density of 2[^{125}I]iodomelatonin binding sites in the chicken spleen. *Biol Signals* 3: 278–287, 1994.
45. **Poon AM and Pang SF.** 2[I-125] iodomelatonin binding-sites in spleens of guinea pigs. *Life Sci* 50: 1719–1726, 1992.
46. **Poon AM, Wang XL, and Pang SF.** Characteristics of 2-[^{125}I]iodomelatonin binding sites in the pigeon spleen and modulation of binding by guanine nucleotides. *J Pineal Res* 14: 169–177, 1993.
47. **Rafil-El-Idrissi M, Calvo JR, Pozo D, Harmouch A, and Guerrero JM.** Specific binding of 2-[^{125}I]iodomelatonin by rat splenocytes: characterization and its role on regulation of cyclic AMP production. *J Neuroimmunol* 57: 171–178, 1995.
48. **Roberts JE, Wiechmann AF, and Hu DN.** Melatonin receptors in human uveal melanocytes and melanoma cells. *J Pineal Res* 28: 165–171, 2000.
49. **Song CK, Bartness TJ, Petersen SL, and Bittman EL.** Co-expression of melatonin (MEL 1a) receptors and arginine vasopressin mRNAs in the Siberian hamster suprachiasmatic nucleus. *J Neuroendocrinol* 12: 627–634, 2000.
50. **Sugawara T, Sieving PA, Iuvone PM, and Bush RA.** The melatonin receptor antagonist luzindole protects retinal photoreceptors from light damage in the rat. *Invest Opthalmol Vis Sci* 51: 2458–2465, 1998.
51. **Ting KN, Blaylock NA, Sugden D, Delagrange P, Scalbert E, and Wilson VG.** Molecular and pharmacological evidence for MT1 melatonin receptor subtype in the tail artery of juvenile Wistar rats. *Br J Pharmacol* 127: 987–995, 1999.
52. **Vaughan MK, Hubbard GB, Champney TH, Vaughan GM, Little JC, and Reiter RJ.** Splenic hypertrophy and extramedullary hematopoiesis induced in male Syrian hamsters by short photoperiod or melatonin injections and reversed by melatonin pellets or pinealectomy. *Am J Anat* 179: 131–136, 1987.
53. **Vivien-Roels B, Malan A, Rettori MC, Delagrange P, Jeanniot JP, and Pevet P.** Daily variations in pineal melatonin concentrations in inbred and outbred mice. *J Biol Rhythms* 13: 403–409, 1998.
54. **Yellon SM, Fagoaga OR, and Nehlsen-Cannarella SL.** Influence of photoperiod on immune cell functions in the male Siberian hamster. *Am J Physiol Regulatory Integrative Comp Physiol* 276: R97–R102, 1999.
55. **Yu Q, Miller SC, and Osmond DG.** Melatonin inhibits apoptosis during early B-cell development in mouse bone marrow. *J Pineal Res* 29: 86–93, 2000.
56. **Yu ZH, Yuan H, Lu Y, and Pang SF.** [^{125}I]iodomelatonin binding sites in spleens of birds and mammals. *Neurosci Lett* 125: 175–178, 1991.