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

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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.

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.
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 prolifera-

*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 uM) 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 ug/ml for experiment 1; for experiments 2 and 3, only the optimal dose, 20 ug/ml, in addition to 0 ug were used. Fifty microliters of each mitogen concentration and 100 ul 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 ul/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 ul/well (each in duplicate) for those wells to which melatonin was not added. Melatonin treatment and melatonin treatment and luzindole treatment 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.

RESULTS

Experiment 1

Splenocyte 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 ug/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-
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. 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). Luzindole at a concentration of 1 $\mu$M did not significantly affect splenocyte proliferation ($P > 0.05$ in all cases) (data not shown). In cells treated with 10 $\mu$M 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...
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 nighttime 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 reproducitively 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.

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