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Melatonin modulation of lymphocyte proliferation and Th1/Th2 cytokine expression

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Abstract

Melatonin is hypothesized to play a role in neuroimmunomodulation. This study investigated the in vitro effects of melatonin (10^{-12}–10^{-6} M) on human peripheral blood mononuclear cell (PBMC) proliferation and T helper type 1 and T helper type 2 (Th1/Th2) cytokine expression. In vitro doses of melatonin significantly increased PBMC proliferation ($p < 0.05$) and decreased IL-10 production in culture supernatants ($p < 0.05$). However, there was no effect of melatonin on the stimulated production of IFN-γ or on the intracellular accumulation of the activation antigen CD69, IFN-γ, or IL-10 as measured by flow cytometry. These data support the notion that physiologic doses of melatonin increase lymphocyte proliferation possibly due to decreases in production of the inhibitory cytokine IL-10. Published by Elsevier Science B.V.

Keywords: Melatonin; Immunity; Cytokines; T helper lymphocytes; Humans

1. Introduction

The hormone melatonin is secreted by the pineal gland and is thought to have a role in transducing information about diurnal and seasonal time to the body (Brzezinski, 1997). In aging and in neuropsychiatric disorders such as depression (Rubin et al., 1992; Voderholzer et al., 1997; Wahlund et al., 1998) and alcoholism (Fonzi et al., 1994; Röjdmark et al., 1993; Wikner et al., 1995), abnormalities in the secretory profile of melatonin are reported to occur in association with disturbances of sleep-wake activity (Jean-Louis et al., 1998; Rodenbeck et al., 1998). Furthermore, immune alterations are associated with disordered sleep-wake activity (Redwine et al., 2000). Despite the abundance of these observations, there has been limited effort to evaluate melatonin and its effects on immune function.

Recent observations implicate melatonin as a neuroendocrine mediator of neural-immune interactions. Melatonin receptors are expressed on lymphoid cells and are found on lymphoid tissues throughout the body (Calvo et al., 1995; Cardinali et al., 1997). In vivo supplementation with melatonin is reported to abrogate stress- and glucocorticoid-induced immunosuppression (Maestroni et al., 1988; Mocchegiani et al., 1999), potentiate antigen presentation, antibody production and lymphocyte proliferation (Pioli et al., 1993), improve survival in AIDS and Venezuelan equine encephalomyelitis infection models (Bonilla et al., 1997; Zhang et al., 1999), and alter severity of autoimmune disorders (Constantinescu et al., 1997; Conti and Maestroni, 1996). In contrast, other in vivo studies found no effect of melatonin on natural killer cell activity or lymphocyte proliferation (Provinciali et al., 1997), and in vitro doses of melatonin failed to alter lymphocyte proliferation in mice (Pawlikowski et al., 1988) and rats (Pahlavani and Harris, 1997). Less is known about the effects of melatonin in the clinical setting, although administration of this hormone is reported to improve performance status and immune functions in cancer patients by stimulating Th1-type immune responses (Lissoni et al., 1994; Neri et al., 1998).

The relative balance of T helper 1 vs. T helper 2 (Th1/Th2) cytokine expression is thought to play a critical role in the regulation of cellular immune responses, with impacts on susceptibility to infectious disease and/or progression of inflammatory disorders (Romagnani, 1997).

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Recent evidence suggests that melatonin also influences the expression of Th1/Th2 cytokines, although data are limited and to some extent, contradictory with almost no observations generated using human peripheral blood mononuclear cells (PBMC). However, in animals, administration of melatonin was associated with increased stimulated IL-2 and IFN-γ levels, decreases in Th2 cytokine expression, and improved survival in a murine AIDS infection model (Inserra et al., 1998; Zhang et al., 1999). Similarly, Wichmann et al. (1996) found that melatonin attenuated the decrease in production of IL-2 and IL-3 in mice submitted to traumatic hemorrhage. In contrast, other studies have reported an upregulation of Th2-type cytokines (Shaji et al., 1998) following melatonin with no effects on TNFα and IL-6 (Sacco et al., 1998; Williams et al., 1998). In humans, the data are more limited; one study found that physiologic (10^{-9} M) doses of melatonin inhibited concanavalin A-induced lymphocyte proliferation (Konakchieva et al., 1995), whereas two other studies demonstrated that pharmacologic, micromolar concentrations of melatonin were required to achieve any inhibitory effects (Vijayalxmi et al., 1996; Persengiev and Kyurchiev, 1993). In regards to cytokine production, Garcia-Mauriño et al. (1997) showed that physiologic concentrations of melatonin in vitro increased production of IL-2, IFN-γ and IL-6, whereas Arzt et al. (1988) found decreases of IFN-γ following incubation with melatonin. The evidence of melatonin's influence on monocyte cytokine production also remains contradictory (Barjavel et al., 1998; Di Stefano and Paulesu, 1994; Fjaerli et al., 1999; Morrey et al., 1994). The effects of melatonin on the relative expression of Th1/Th2 cytokines in human PBMC is not known.

The aim of the present study is to evaluate the effects of physiologic doses of melatonin on human PBMC function, as measured by lymphocyte proliferation and the relative expression of Th1 vs. Th2 cytokines. Cytokine production was assayed using two approaches: measurement of IFNγ and IL-10 in supernatants of PBMC cultures, and by use of cytokine flow cytometry with assay of intracellular accumulation of the activation antigen CD69, IFNγ, and IL-10 in CD4 T cell sub-populations.

2. Materials and methods

2.1. Subjects

In this investigation, normal volunteers ($n = 8$) were selected from a group of laboratory controls participating in ongoing studies of sleep and immunity under UCSD IRB protocols #980756 and 000753. The volunteers were screened for medical and psychiatric disorders and had no acute infections and no history of chronic infectious or autoimmune disease. There were four men and four women ranging in age between 18 and 55 years.

2.2. Preparation of blood samples and isolation of PBMCs

Peripheral venous blood was obtained between 7:00 and 11:00 am using EDTA-supplemented vacutainers (Becton Dickinson, BD). In 50-ml conical tubes, blood was diluted 1:1 with phosphate buffered saline (PBS; Gibco) and underlayered with Ficoll-Hypaque (Sigma) for separation of PBMC as described by Boyum (1968). The cells were washed twice in PBS and spun for 30 min at 1400 rpm, and finally resuspended in RPMI 1640 medium (Gibco), supplemented with 10% fetal bovine serum (Gibco), 20 mM Hepes (Omega) and 1% L-glutamine (Omega). The cell concentration was adjusted to 1×10^6 viable cells/ml.

2.3. Proliferation and stimulated cytokine production assays

PBMCs were plated in 96-well round bottom plates in triplicate or in six-well culture plates for proliferation or stimulated cytokine assay, respectively, at the final cell concentration of 5×10^5 cells/ml. The cells were treated with melatonin (Sigma) at molar concentrations of 10^{-6}–10^{-12} M. Melatonin was dissolved in 70% ethanol and diluted in RPMI 1640 immediately prior to use; the final concentration of ethanol was $< 0.002\%$. Additionally, staphylococcal enterotoxin B (SEB; Sigma) was added to some wells at the dose of 1 ng/ml or 1 μg/ml as indicated. For control, media was used instead of melatonin and/or SEB. The cells were then incubated at 37°C and 5% CO_2 in humidified atmosphere for 72 h.

For the proliferation assay, 1 mCi of ^{3}H-thymidine (ICN) was added to each well 18 h before the end of incubation. At the end of incubation, plates were frozen at −80°C until harvested. The radioactivity was counted with a β-radiation counter.

For the determination of stimulated cytokine production, cell-free supernatants were collected at the end of incubation and stored at −80°C. Commercial ELISA kits (R&D) were used, following manufacturer's guidelines, to determine the concentration of IFN-γ and IL-10 in the supernatants.

2.4. Whole blood stimulation and immunofluorescent intracellular staining

For cell preparation and intracellular immunostaining, we adopted a protocol similar to the one decribed by Suni et al. (1998). Heparinised whole blood was treated in polypropylene conicals at 1 ml/conical with varying concentrations of melatonin. Positive controls contained 10 μg/ml SEB, whereas negative controls had neither melatonin nor SEB added. All samples were co-stimulated with anti-CD28 monoclonal antibody (2 mg/ml; PharMingen), followed by incubation for 6 h at 37°C in a 5% CO_2 humidified atmosphere. For the last 4 h of incubation, Brefeldin A (20 μl 0.5 mg/ml, Sigma) was added. At the end of the incubation, cold 20 mM EDTA (100 ml) was

added, and cells were vigorously vortexed and incubated for 15 min at room temperature. After erythrocytes were lysed (10 ml of 1 × lysing solution, BD), the tubes were centrifuged for 8 min at 1600 rpm; cells were resuspended in wash buffer (PBS plus 0.5% BSA and 0.1% NaN3) to be kept overnight at 4°C before assay by flow cytometry.

For flow cytometric analysis, cells were aliquoted into polystyrene staining tubes, and washed in wash buffer before they were permeabilised (500 ml 1 × permeabilising solution, BD) for 10 min, washed and spun again. Following permeabilisation, cells were stained with a titrated monoclonal antibody cocktail consisting of anti-CD4 FITC and anti-CD69, anti-IFN-gamma or anti-IL-10 PE monoclonal antibody (all obtained from BD, except anti-IL10 monoclonal antibody from PharMingen), for 50 min in the dark at room temperature. After staining, cells were washed twice and fixed in 1% paraformaldehyde (Fisher Scientific).

Two-color flow cytometric analysis was performed on a Coulter Elite flow cytometer using Coulter Elite software. Forward and side scatter were used to gate on lymphocytes, which were then represented on a two-color (FITC and PE) flow chart. At least 50,000 CD4 + events were counted to determine the percentage of CD69 positive or cytokine secreting cells within this subpopulation. Background values in the nonstimulated controls were below 1.5% in all samples used in the analyses.

2.5. Statistical analysis

A 3 (SEB doses) × 8 (melatonin concentration) repeated measure ANOVA (SPSS statistical software 10.0) was used to test for main effects of melatonin concentration, SEB, and their interaction on proliferative responses. Paired Student's *t*-tests were performed to identify differences in specifc melatonin doses.

In the instance of stimulated cytokine levels, several data points were below the limits of detection, and this threshold value, rather than zero or missing data, was used in the statistical analyses. Furthermore, since these cytokine data were not normally distributed, stimulated cytokine- and intracellular cytokine values were analysed using nonparametric Wilcoxon signed-ranks tests.

3. Results

3.1. Effect of melatonin on PBMC proliferation

Five separate experiments assessed the effects of physiological and pharmacological doses of melatonin on

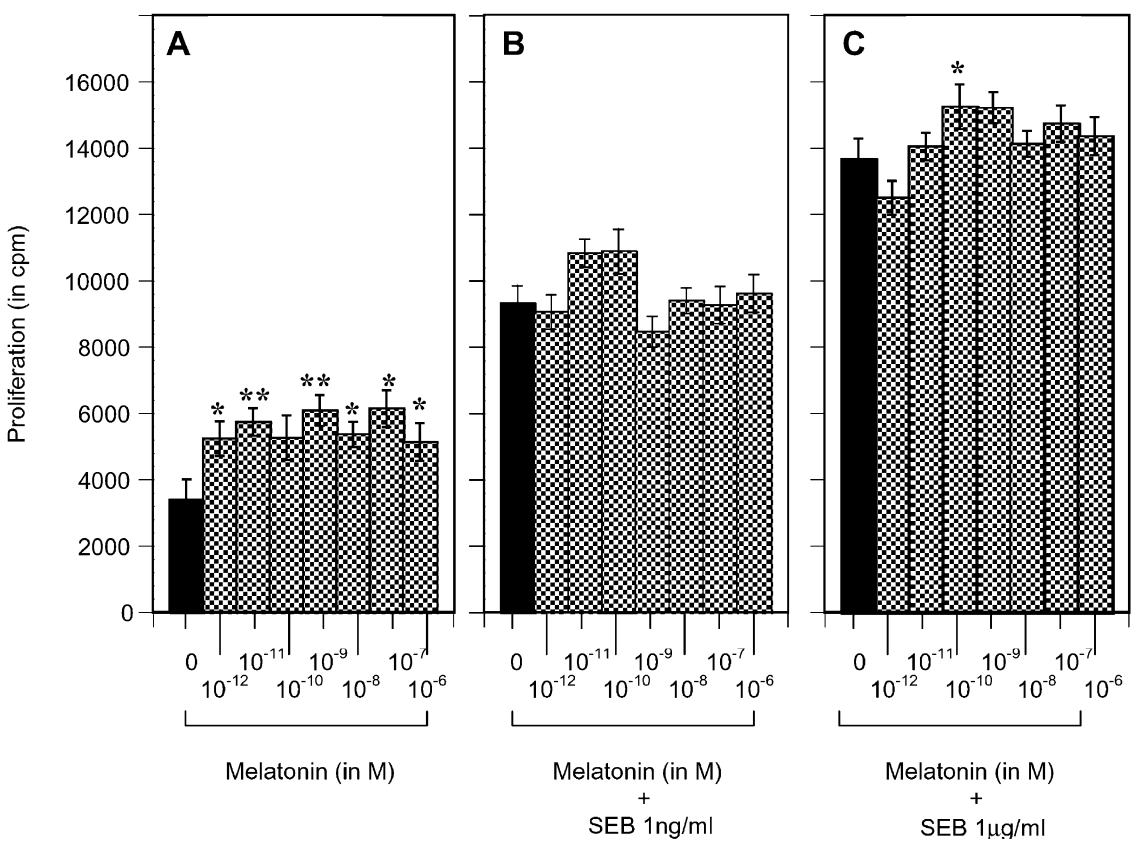

Fig. 1. Peripheral blood mononuclear cell proliferation in response to stimulation with melatonin only (A), and in combination with SEB 1 ng/ml (B) and SEB 1 μg/ml. Data are represented as means ± SEM using triplicate values of five/three (A), two/two (B) and three/two (C) separate experiments/donors. An 8 (melatonin cencentrations) × 3 (SEB doses) repeated-measure ANOVA demonstrated a melatonin effect: $F = 2.25$, $p < 0.03$, and an SEB effect: $F = 65.05$, $p < 0.01$. Student's *t*-tests revealed significant differences: * vs. 0 $p < 0.05$; ** vs. 0 $p < 0.005$.

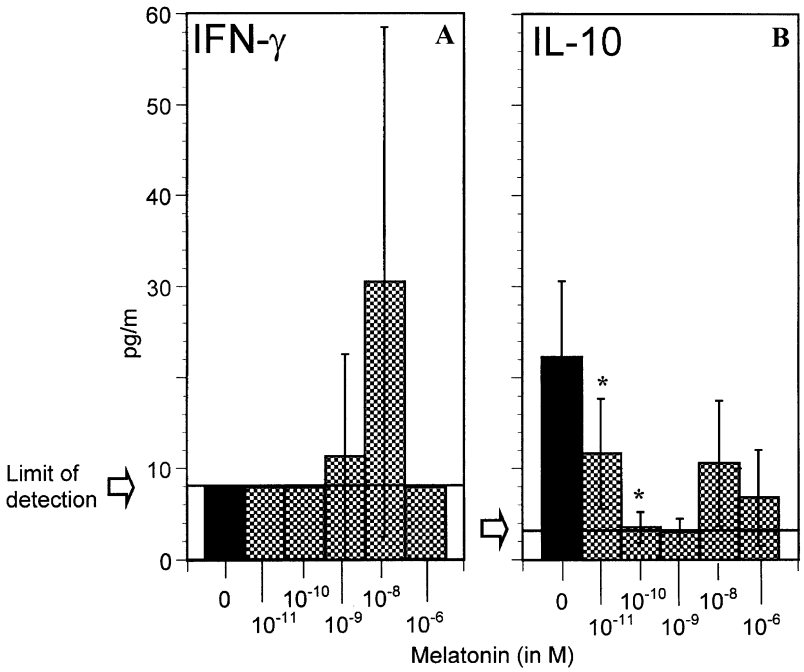

Fig. 2. IFN-γ (A) and IL-10 (B) in supernatants of cultures stimulated with melatonin. Data are represented as means ± SEM using values from eight/seven separate experiments/donors. Wilcoxon signed-ranks test: * vs. 0 $p < 0.05$.

lymphocyte proliferation with and without doses of SEB. As shown in Fig. 1, there were main effects for melatonin ($F = 2.25$, $p < 0.03$) and SEB ($F = 65.05$, $p < 0.01$) on lymphocyte proliferation. Across a range of concentrations (10^{-12}, 10^{-11}, 10^{-9} to 10^{-6} M), melatonin augmented proliferation as compared to controls that contained no

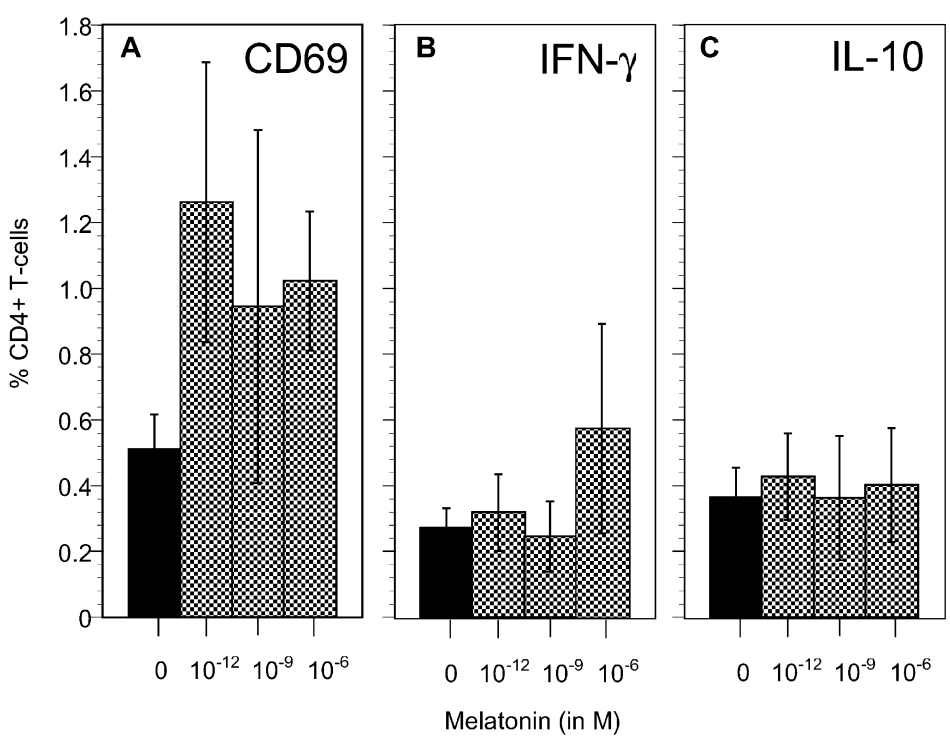

Fig. 3. Intracellular accumulation of CD69 (A), IFN-γ (B) and IL-10 (C) in T-helper cells stimulated with Melatonin. Data are represented as means ± SEM using values from at least four separate experiments and donors. Statistical analysis did not reveal any significant differences.

melatonin (all t's ≥ 2.2, all $p < 0.05$). While SEB was also found to increase proliferative responses as compared to controls (Fig. 1B,C), there was no interaction between melatonin and SEB; melatonin did not further increase lymphocyte proliferation above the level achieved by SEB alone.

3.2. Effect of melatonin on stimulated cytokine levels

To further investigate the effect of melatonin on lymphocyte function, levels of IFN-γ and IL-10 were measured in culture supernatants following eight separate experiments (Fig. 2). Stimulated cytokine production of IL-10 was decreased in association with melatonin. As compared to controls, IL-10 were lower in cultures containing melatonin doses 10^{-11} and 10^{-10} M ($Z = -2.37$, $p < 0.02$; $Z = -2.17$, $p < 0.03$), with a trend for lower levels at melatonin doses 10^{-9} and 10^{-6} M ($Z = -1.68$, $p < 0.09$; $Z = -1.69$, $p < 0.09$). For IFN-γ, levels were similar in control and cultures containing varying doses of melatonin.

3.3. Effect of melatonin on the intracellular accumulation of cytokines

Cytokine levels in supernatants from PBMC cultures are the cumulative result of cytokine expression and degradation by heterogeneous cell populations over 72 h of culture. Thus, to determine whether decreases of IL-10 were due to changes in the production of this cytokine by T helper lymphocytes, four additional experiments were conducted that used flow cytometry, and measured the intracellular accumulation of IL-10 along with the expression of the early activation marker CD69 and IFNγ on a single cell basis in CD4 populations. Although graphical presentation of these data suggested increased expression of CD69 in relation to melatonin treatment consistent with the lymphocyte proliferation data, statistical analyses did not reveal any effect of melatonin on the intracellular accumulation of CD69, IFNγ, or IL-10 (Fig. 3).

4. Discussion

This study found that physiological concentrations of melatonin in vitro were associated with increases in lymphocyte proliferation and with decreases of IL-10 production in culture supernatants. However, melatonin had no significant effect on IFN-γ production and there was no association between melatonin and intracellular accumulation of CD69, IFN-γ and IL-10. Taken together, these data suggest that decreases in the inhibitory cytokine IL-10 may occur in association with melatonin administration, and thus contribute to increases in lymphocytes as measured by proliferative responses.

Melatonin receptors are expressed on immune cells, and these in vitro data support the notion that melatonin binding at these sites has functional consequences. However, intracellular assessment of cytokine expression failed to reveal any substantial effects of melatonin, in contrast with the observed effects on lymphocyte proliferation and stimulated cytokine production. While flow cytometric assessment of cytokine expression has the distinct advantage of examining accumulation of cytokines in unique cell populations shortly after removal from the in vivo milieu, it is possible that the 6-h incubation period was not of sufficient duration for melatonin to exert an effect on cellular activation. In mice or rats treated with melatonin in vivo, at least 5 days lapsed prior to assessment of immune function. Likewise in humans, the in vitro effects of melatonin on human PBMCs have typically been found following culture conditions lasting longer than 24 h, although findings are mixed even with these procedures (Arzt et al., 1988; Di Stefano and Paulesu, 1994; Garcia-Mauriño et al., 1997). Other studies have suggested that the monocyte may be relatively more sensitive to the effects of melatonin than are lymphocytes (Barjavel et al., 1998; Morrey et al., 1994). Alternatively, the differential effects of in vitro administration of melatonin may be due the interaction of melatonin with other neuroendocrine hormones in vivo (Konakchieva et al., 1998; Petrovsky and Harrison, 1997).

There are several limitations of this study that require consideration. First, the study sample is small and the variance in many of the assay procedures is great, despite the use of a single operator to minimize inter-assay differences and systematic control of confounding factors, including time of blood draw and time of year as previously suggested (Di Stefano and Paulesu, 1994; Fjaerli et al., 1999). Second, the incubation intervals were fixed, and it is possible that preincubation conditions would yield different results. In view of these limitations, the negative results in relation to IFN production and intracellular accumulation of cytokine should be cautiously interpreted. Nevertheless, these data suggest that melatonin shows a potential to alter lymphocyte function at the cellular level, and to induce lymphocyte activation possibly via suppression of IL-10 expression.

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