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Melatonin enhances Th2 cell mediated immune responses: Lack of sensitivity to reversal by naltrexone or benzodiazepine receptor antagonists

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Abstract

Chronic administration of melatonin for 5 days to antigen-primed mice increased the production of pro-inflammatory cytokine IL-10 but decreased the secretion of anti-inflammatory cytokine TNF-α. These results further confirm that melatonin activates Th2-like immune response. Whether melatonin-mediated Th2 response is dependent on opioid or central and peripheral benzodiazepine receptors was also examined. Hence, melatonin was administered to antigen-sensitised mice with either naltrexone (a μ opioid receptor antagonist) or flumazenil (a central benzodiazepine receptor antagonist) or PK11195 (a peripheral benzoidiazepine receptor antagonist). No significant difference in melatonin-induced Th2 cell response was observed by naltrexone, flumazenil or PK11195 treatment. These findings suggest that the Th2 cell response induced by melatonin in antigen sensitised mice neither dependent on endogenous opioid system nor is modulated through the central or peripheral benzodiazepine receptors. (Mol Cell Biochem **221**: 57–62, 2001)

Key words: melatonin Th1/Th2 cells, IL-4, IFN-γ, IgG1, IgG2a, endopioids, central/peripheral benzodiazepine receptors

Introduction

Melatonin is the chief secretory product of the pineal gland. Besides its well-known circadian regulation and control of seasonal reproduction, it is considered to play an important role in neuro-immunomodulation [1]. Melatonin enhances humoral and cell mediated immunity [2–5]. However, the functional significance of melatonin in immunomodulation is not yet known. Available evidence suggests that both direct and indirect mechanisms are responsible for the immunomodulatory actions of melatonin [1]. The specific binding sites (both membrane and nuclear) of melatonin on immune cells in higher vertebrates indicate its direct effect on the immune system [6–9]. However, a clear connection between the activation of these binding sites on lymphocyte functions by melatonin has not been established [1]. Indirect mechanism may involve the melatonin-mediated modulation of the endogenous opioid system (EOS) and the benzodiazepine receptor. This inference follows from observations that in antigen-primed mice the concomitant administration of specific μ opioid receptor antagonist naltrexone abolished the immune enhancing actions of melatonin but β-endorphin and dynorphin 1–13 mimicked the action [3, 4, 10]. It has been shown recently that melatonin can modulate the central and peripheral benzodiazepine receptor mediated functions [11–13]. Both the central and peripheral benzodiazepine receptors are expressed on lymphocytes and monocytes, and the ligands for these receptors modulate the immune response [14–16]. Although the role of benzodiazepine receptors in melatonin-induced neuro-pharmacological effects has been extensively studied [11–13], but no study has addressed the possible role of these receptors in melatonin induced-immune response.

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In many *in vivo* studies, modulation of the immune functions by melatonin has been observed at supra-physiological doses [3–5, 17]. At such doses, melatonin may interact and modulate the actions mediated by endogenous opioid or central and peripheral benzodiazepine receptors [11, 12, 18]. Our earlier studies have demonstrated that melatonin enhances the generation of Th2 cells, as shown by the predominant secretion of IL-4 and IgG1-antibody, and decreased the production of IL-2, IFN-γ and IgG2a-subtype [17]. Therefore, in the present study we wish to understand whether the melatonin-induced enhancement of Th2 cell activity in antigen-primed mice is mediated through the involvement of endogenous opioid, central or peripheral benzodiazepine receptors.

Materials and methods

Animals

Inbred and disease-free female Balb/c mice, 8–10 weeks old were obtained from the Institute's Animal House Facility. The animals were housed in groups of 5 for each experiment in a constant temperature (25 ± 1°C) room and subjected to a 12/12 h light cycle (light started at 06.00 h) and they had free access to water and food available *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee.

Drug, antigen, and antibodies

Melatonin (Morepen Laboratories, Parwanoo, India) in 1% ethanol-water, flumazenil (F. Hoffmann-La Roche & Co, Basel, Switzerland) and PK11135 (RBI, Natick, USA) in 1% dimethyl sulfoxide-water, and naltrexone (Sigma, St. Louis, MO, USA.) in water were used in the present study. Ovalbumin and goat anti-mouse IgG1, IgG2a (Sigma, St. Louis, MO, USA), anti-goat horseradish peroxidase (Sera Labs, Crawley Down, UK), recombinant murine IL-2, IL-4, IL-10, IFN-γ, TNF-α (Genzyme, Cambridge, MA, USA) and antibodies to IL-4 (Pharmingen, St. Diego, CA, USA) and anti-IL-2 moAbs (cocktail of TIB222, HB8794 and CRL 1698) were used as a culture supernatants.

Cell lines and hybridomas

The cell lines and hybridomas used in this study, WEHI-164, WEHI-279, HT-2 (CRL-1841), TIB222 (PC61.5.3), CRL 1698 (7D4) and HB 8794 (S4B6) were procured from ATCC (Rockville, MD, USA).

Immunization protocol

The ovalbumin (2 mg/ml) was dissolved in PBS (0.01 M, pH 7.2) and emulsified in complete Freund's adjuvant (CFA). The emulsion (100 µl) was then injected i.p. to the different groups, each comprising of 5 female Balb/c mice. A booster dose of the antigen was administered after 1 week. Five days before bleeding, the mice were injected s.c. daily with melatonin (10 and 20 mg/kg body wt of mice). The control animals were injected intraperitoneally (i.p.) with 100 ml each of placebo (PBS only) and 1% ethanol-PBS (vehicle for melatonin). The selection of melatonin dose was based on previous reports [5, 10, 17]. Blood was drawn from orbital sinus 24 h after the last injection of melatonin, and sera were used for quantification of cytokines and IgG isotypes. To study the possible role of EOS and central or peripheral benzodiazepine receptors in the regulation of melatonin action, naltrexone (2.5 and 5 mg/kg), flumazenil (1 and 2 mg/kg) and PK11195 (0.5 and 1 mg/kg) were administered subcutaneously (s.c.). to all the mice, 5 min prior to melatonin (10 mg/kg) injection.

Bioassay for measuring IL-2 and IL-4

IL-2 and IL-4 concentrations were measured using HT-2 cells as described earlier [19]. The sera were separated from the blood of the animals (both experimental and control), the levels of IL-2 and IL-4 were measured by their abilities to induce the proliferation of HT-2 cells [19]. 1×10^4/well of HT-2 cells were cultured in 96-well microtitre plates containing the medium and different concentrations of serum obtained from the control and the experimental animals. Antibodies were used for the selective inhibition of IL-2 and IL-4 lymphokines. The cells were incubated for 16 h at 37°C, pulsed with 0.5 µCi/well of [^{3}H]-thymidine and harvested 8 h later. The incorporation of [^{3}H]-thymidine was measured by liquid scintillation spectrometry. By comparing with a standard curve using recombinant IL-2 or IL-4 (Genzyme) the concentrations of IL-2 and IL-4 were calculated as pg/ml from triplicate determinations of the mean counts per minute.

Bioassays for measuring IFN-γ and TNF-α

Interferon-γ and TNF-α were assayed by their abilities to inhibit the proliferation of WEHI-279 [19] and WEHI-164 [20] cells respectively. The WEHI-279 or WEHI-164 cells were cultured in 96-well flat-bottomed plates at a density of 1×10^5 cells/ml with different concentrations of sera obtained from the control and the experimental animals. The cells were incubated for 16 h at 37°C and then pulsed with 0.5 µCi/well of [^{3}H]-thymidine and harvested 8 h later. The radioactivity

incorporated was measured using liquid scintillation spectrometry. The levels of IFN-γ or TNF-α (pg/ml) were computed by comparison with a standard curve for recombinant IFN-γ or TNF-α (Genzyme).

Estimation of IL-10

IL-10 was estimated according to the manufacturer's (Genzyme) protocols. Briefly, 50 µl (1.0 µg/ml) of anti-mouse IL-10 moAb was adsorbed overnight at 4°C on polystyrene microtitre plates. After the blocking, sera were added followed by biotinylated goat anti-mouse IL-10 Ab, streptavidin-HRP and tetramethylbenzidine. After each incubation, the plates were washed with PBS-Tween 20. Titration curve of rIL-10 (Genzyme) was used as standards for calculating the concentration (pg/ml) in the test samples tested.

Determination of OVA-specific IgG1and IgG2a isotypes by ELISA

The production of OVA-specific IgG1 and IgG2a antibodies was measured as explained earlier [17]. Ninety-six-well microtiter plates (Costar, Cambridge, MA, USA) were coated overnight with 50 µl of OVA (25 µg/ml) in carbonate-bicarbonate buffer (0.05 M, pH 9.6 at 4°C). After extensive washing with PBS-Tween 20 buffer, 50 µl of blocking buffer (3% skimmed milk in PBS-T) was applied to the wells and these were then incubated at 37°C for 60 min. The blocking buffer was removed and $\log_2$ dilutions of test and control sera were added. The reaction was allowed to proceed for 18 h at 4°C. The microplates then were washed and 50 µl of goat anti-mouse IgG1 or IgG2a were added. The plates were incubated at 37°C for 2 h washed at each step and then 50 µl anti-goat HRP was added to each well and incubated at 37°C for 1 h. The plates were washed thoroughly again and then 50 µl of OPD was added to the plates and incubated at 37°C for 20 min. The reaction was terminated by adding 50 µl of 7% H_2SO_4. Absorbance of light at 492 nm was read with a microplate reader (Eurogenetics, Torino, Italy). The antibody titres were expressed as the highest dilution of serum that yielded a 0.2 optical density (OD).

Statistical analysis

All the values were expressed as means ± S.D. and the data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. A value of $p < 0.05$ was considered statistically significant.

Results

Effect of melatonin on the secretion of IL-2, IL-4, IL-10, IFN-γ and TNF-α in ovalbumin primed mice

We have earlier shown the role of melatonin on IL-2, IL-4 and IFN-γ [17]. In the present study, we wish to evaluate the role of melatonin on anti-inflammatory cytokine IL-10 and pro-inflammatory cytokine TNF-α in mice immunized with the antigen. Chronic administration of melatonin (10 and 20 mg/kg) for 5 days to antigen primed mice showed significant decrease ($p < 0.05$) in the serum levels of TNF-α as compared to the control group. On the other hand, secretion of IL-10 was greatly enhanced ($p < 0.05$). However, an increase in the dose of melatonin from 10–20 mg per kg wt of mice did not have much effect on the levels of any of the cytokines tested (Table 1). Chronic levels of melatonin also resulted in decrease in the production of IL-2 and IFN-γ and an increase in the secretion of IL-4. This further substantiates our earlier findings [17].

Effect of melatonin on OVA specific immunoglobulin production

The impact of melatonin on the production of OVA-specific immunoglobulins was monitored in the sera of the animals. Among the IgG isotypes, i.e. IgG1 and IgG2a analysed, a significant increase in the production of IgG1 antibody was detected in the sera of antigen-primed mice administered with melatonin as compared to the animals treated with OVA alone. Contrary to this, melatonin inhibited the production of IgG2a (Table 1).

Effect of naltrexone, flumazenil and PK11195 on melatonin induced immune response

A dose of 10 mg/kg of melatonin has been shown earlier to be optimum [17]. An increase in this dose to 20 mg/kg did not produce any discernible change (Table 1). Therefore, in subsequent experiments, only a dose of 10 mg/kg body wt of mice was employed. At both doses, neither naltrexone nor central (flumazenil) nor peripheral (PK11195) benzodiazepine receptor antagonists induced any immune response in unprimed mice. These antagonists also failed to induce any effect on the yield of cytokines or immunoglobulins in antigen primed mice (data not shown). This could be due to the competitive antagonistic nature of these ligands at the doses administered. Further, co-administration of these antagonists also failed to reverse the secretion of IL-2, IL-4, IFN-γ and TNF-α induced by melatonin alone in antigen-primed ani-

Table 1. Effect of melatonin (10 and 20 mg/kg) for 5 days on the secretion of IL-2, IL-4, IL-10, IFN-γ and TNF-α and IgG isotypes (IgG1 and IgG2a) in ovalbumin primed Balb/c mice. The control group of mice were injected with PBS alone

	PBS	Ovalbumin	Melatonin (10 mg/kg)	Melatonin (20 mg/kg)
IL-2 (ng/ml)	1.09 ± 0.04	1.35 ± 0.04	0.68 ± 0.03*	0.78 ± 0.02*
IL-4 (ng/ml)	4 ± 0.52	2.90 ± 0.28	14.6 ± 0.8*	13.3 ± 0.9*
IL-10 (pg/ml)	53 ± 3.9	48 ± 0.15	78 ± 2.5*	85 ± 3.4*
IFN-γ (pg/ml)	25 ± 0.09	32 ± 3.0	19 ± 1.5*	17 ± 2.0*
TNF-α (ng/ml)	105 ± 7.8	141 ± 16	61 ± 12*	59 ± 9*
IgG1 (titer volume)	12683 ± 859	8305 ± 984	79367 ± 3097*	59583 ± 2792*
IgG2a (titer volume)	2105 ± 39	2472 ± 347	1099 ± 317*	1367 ± 198*

Values are means ± S.D. *$p < 0.05$ compared to control group (Dunnett's *t*-test).

mals. These antagonists also could not reverse the effect of melatonin on the secretion of OVA specific IgG1 or IgG2a Abs. These observations suggest that endopioids or benzodiazepine receptors do not participate in immunomodulatory actions of melatonin on Th2-like cells (Table 2).

Discussion

The differentiation of naïve $CD4^+$ T cells into Th1 and Th2 cells is important in the outcome of the disease. The factors that influence Th1 and Th2 response include antigen dose, the nature of peptide/MHC complex, exposure to cytokines and co-stimulatory molecules, the kind of adjuvant and the type of antigen presenting cell (APC). The cytokine milieu present during the priming of naïve T cells determines the ultimate pathway of differentiation. Th1 cells are defined by their specific production of IL-2, IFN-γ, TNF-β, TNF-α, etc. and are responsible for the induction of cell-mediated immunity. Th2 cells secrete IL-4, IL-5, IL-10, etc., and are responsible for humoral immunity [19, 21–25].

It has been previously reported that the chronic administration of melatonin to antigen challenged mice enhanced Th2 cell response in terms of the predominant production of IL-4 and IgG1, but it decreased the yield of IL-2, IFN-γ and IgG2a [17]. In the present study, similar experimental conditions enhanced the production of anti-inflammatory cytokine IL-10. Whereas, pro-inflammatory cytokine TNF-α decreased. Our earlier work also reported an inhibition in the secretion of IL-2 and IFN-γ and increase in the production of IL-4. This strengthens our hypothesis that melatonin activates Th2-like immune response while Th1 mediated response is suppressed.

IL-10 is known to have inhibitory activities on monocytes, Th1 cells and NK cells, but stimulates B-lymphocytes to secrete IgG1 and IgG3 antibody [26, 27]. Thus in the present study, a decrease in IL-2, IFN-γ and TNF-α and an increase in IgG1 may be attributed to increased production of IL-10 [27]. It was also observed that melatonin failed to enhance either Th1 or Th2 cell mediated immune response in unprimed mice.

Many of the pharmacological actions of melatonin are attributed to its regulatory control over other neurotransmitters and hormones. The main aim of the present study was to determine whether μ opioid and benzodiazepine receptors play any role in the melatonin induced Th2 cell activity. Since these receptors play a crucial role in circadian regulation, the behavioural properties of animals and anti-stress properties

Table 2. Effect of co-administration of naltrexone (2.5 and 5 mg/kg), flumazenil (1 and 2 mg/kg) or PK11195 (0.5 and 1 mg/kg) with melatonin (10 mg/kg) on secretion of IL-2, 1L-4, IFN-γ, TNF-α and IgG-isotypes (IgG1 and IgG2a) in OVA primed mice

Treatment (mg/kg)	IL-2 (ng/ml)	IL-4 (ng/ml)	IFN-γ (pg/ml)	TNF-α (ng/ml)	IgG1 (titer volume)	IgG2a (titer volume)
Melatonin (10)	0.68 ± 0.03	14.6 ± 0.8	19 ± 1.5	61 ± 12	79367 ± 3097	1099 ± 317
Naltrexone (2.5) + Melatonin (10)	0.70 ± 0.08	14.3 ± 1.3	21 ± 2.3	69 ± 19	71562 ± 9280	1126 ± 298
Naltrexone (5) + Melatonin (10)	0.71 ± 0.02	14.9 ± 0.8	23 ± 3.4	71 ± 16	76473 ± 5218	1434 ± 525
Flumazenil (1) + Melatonin (10)	0.60 ± 0.08	14.9 ± 1.3	17.2 ± 1.4	58 ± 15	76130 ± 2128	1108 ± 316
Flumazenil (2) + Melatonin (10)	0.62 ± 0.06	15.9 ± 1.5	20.1 ± 2.2	64 ± 14	73079 ± 4136	1202 ± 537
PK11195 (0.5) + Melatonin (10)	0.62 ± 0.03	14.7 ± 1.3	19.5 ± 2.0	68 ± 17	81216 ± 6954	1543 ± 481
PK11195 (1.0) + Melatonin (10)	0.65 ± 0.07	13.7 ± 1.4	21.4 ± 2.3	62 ± 18	72659 ± 7864	1496 ± 533

Values are means ± S.D. Comparison of melatonin (10 mg/kg) alone treated group with other groups was done by one way ANOVA. None of the immune response induced by melatonin (10 mg/kg) was sensitive to reversible by naltrexone, flumazenil or PK 11195 (Dunnett's *t*-test).

of melatonin, agonists for these receptors may be expected to modulate immune functions [4, 12]. It has been reported that immune enhancing actions of melatonin in stressed animals was sensitive to reversal by naltrexone, a selective antagonist for μ opioid receptors [3, 10]. It may be clarified at this point that the present study, focussed on the immunomodulatory action of melatonin in unstressed mice. So it could be possible that in unstressed condition melatonin enhance immune functions independent of opioidergic pathway. Several *in vitro* and *in vivo* studies have demonstrated that agonists for μ opioid receptors inhibit T cell proliferation and Th1 cell mediated cellular immunity [28, 29]. Although the present result indicate that melatonin suppresses Th1 cell mediated immune response, naltrexone failed to antagonize this effect. Similarly, melatonin mediated enhancement in Th2 responses were also insensitive to reversal by naltrexone. It has been reported that μ opioid receptor agonists augment natural killer cell cytotoxicity and nitric oxide production, whereas indolamines have the opposite effect [30, 31]. Induction of peripheral and central hyperalgesia by endotoxin involves the activation of proinflammatory cytokines such as IL-1β and TNF-α, which can be antagonized by anti-inflammatory and morphine class of drugs [32, 33]. Recent work from this laboratory has shown that melatonin attenuates the development of lipopolysacharide-induced hyperalgesia, which is not sensitive to reversal by naltrexone [20]. Thus these findings suggest that melatonin-induced immunomodulatory actions in non-stressed animals are mediated independently of opioidergic pathways.

Similar to neuropharmacological activities, immunomodulatory actions of melatonin and central or peripheral benzodiazepine receptor ligands resemble each other. Like melatonin, benzodiazepines have been found to modulate the metabolic functions of immune cells [34, 35]. They enhance humoral immune response to sheep red blood cells and inhibit the release of TNF-α, IL-1 and IL-6 by macrophages [3, 14, 15, 20, 36]. Melatonin also exhibits anti-proliferative action on various cancer cell lines [37–39]. Although the roles of central or peripheral benzodiazepine receptors in the regulation of neuro-pharmacological responses of melatonin have been reported [11–13], their participation in the immune regulatory effect of melatonin has not yet been explored. Our observations have shown for the first time the failure of both central or peripheral benzodiazepine receptor antagonists to reverse the immuno-modulatory action of melatonin in antigen-primed mice.

In conclusion, the present study suggests that melatonin mediated Th2 type of immune response is independent of opioid, central and peripheral benzodiazepine receptors. Further, melatonin enhances pro-inflammatory cytokine IL-10 and inhibits the production of anti-inflammatory cytokine TNF-α in antigen-primed mice.

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