Regulation of T-cell-independent and T-cell-dependent antibody production by circadian rhythm and melatonin

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Abstract

Melatonin is a hormone that has immunomodulatory activity and is believed to influence the production of antibodies in mammals. The aim of the present study was to investigate the effect of suppressed melatonin synthesis on the antibody production. BALB/c mice were immunized with T-cell-dependent (TD) and T-cell-independent (TI) antigens and kept under (i) normal lighting, (ii) constant exposure to light, (iii) exposed to light and treated daily with melatonin. It was revealed that melatonin modulated TD and TI antibody production. Suppressed melatonin synthesis increased the amount of IgM, IgG1, IgG2b and IgG3 antibodies after immunization with TI antigen. The level of TD antibodies IgM, IgG2a, IgG2b and IgG3 also increased, however, the antigen-specific antibodies of IgG1 isotype significantly decreased in mice exposed to light. Daily melatonin treatment brought the antibody level back to normal. The antibody concentration in the sera of mice kept at normal lighting was significantly higher when the immunizations were performed in the evening. The action of melatonin on B cells via MT2 receptor was shown in vitro and in vivo.

Keywords: antibodies, melatonin, MT2 melatonin receptor, T-cell dependent, T-cell independent

Introduction

Melatonin is a hormone mainly produced by the pineal gland (1). The production of melatonin occurs during the night in response to the darkness and is inhibited by the light. Melatonin has been called ‘the hormone of darkness’. The secretion of melatonin reaches maximum level in the middle of the night and gradually decreases by the morning. Melatonin is also synthesized extrapineal in different tissues and cells (2), the cells of immune system, e.g. human lymphocytes (3), resting or PHA-stimulated Jurkat cells (4), mouse, rat and human bone marrow cells (5), thymocytes (6), mast cells, natural killers, eosinophilic leukocytes, platelets and endothelial cells (3).

Melatonin is known as a regulator of the biological clock and also as a potential immunomodulator. It modulates the activity of dendritic cells (7), natural killer cells (8–10), neutrophils (11) and T cells (8). Melatonin modulates the functions of macrophages (12). It increases the expression of MHC class II molecules (thus enhances antigen presentation to T cells) and augments the cytokine [IL-1, IL-6, tumor necrosis factor (TNF) \(\alpha\) and M-CSF] production (13). It is believed that melatonin could provide the third signal for T-cell activation (the first two signals are through the T-cell receptor and CD28 on the T cell) (14). It enhances the growth of T cells and modifies the cytokine secretion (3, 4, 13, 15). The effect of melatonin was observed on human T cells only when cells were not activated or were just slightly activated with PHA (13). The influence of melatonin on T cells is controversial. It can enhance the immune response, acting as a pro-inflammatory agent. It is suggested that melatonin promotes Th1-mediated immune responses in humans by up-regulating IL-12 production by antigen-presenting cells (16). It was also revealed that melatonin can differentially affect murine T cells. It promotes Th2 (17, 18) or Th1 response (19, 20).

The role of melatonin on the function of B cells is not clear enough. According to some studies, treatment with melatonin increases the production of antibodies (17, 18). Regodon et al. showed that melatonin could be used as vaccine agent in sheep. Melatonin implants resulted in the increase of antibody titers that synergized with aluminium hydroxide (21). We investigated the influence of suppressed melatonin synthesis on antibody production in mice. We showed that circadian rhythm and melatonin regulate T-cell-independent (TI) and T-cell-dependent (TD) antibody production.
Melatonin concentration in sera and cell culture

The concentration of melatonin was determined by ELISA using IBL melatonin ELISA kit. The samples were extracted and ELISA performed according to company’s guidelines.

Statistics. The paired two-tailed Student’s t-test was used with P <0.05 taken as the level of significance.

Results

The influence of melatonin on TI and TD antibody production

The effect of melatonin on antibody production was investigated using mice model with suppressed melatonin synthesis. Melatonin secretion is inhibited by light. Therefore, to suppress synthesis of melatonin, BALB/c mice were exposed to constant lighting conditions. Mice kept under constant light but got daily melatonin injections. Experimental mice were kept at certain lighting conditions for 1 week and immunized with TI (TNP–Ficoll and TNP-LPS) or TD (TNP-ovalbumin) antigens as described in Methods. The animals throughout the experiment were kept at the same lighting conditions. The TNP-specific antibodies were measured in the pre-immune sera and sera of immunized animals. TNP-specific antibodies of IgM, IgG1, IgG2b and IgG3 isotypes were detected in the sera after the immunization with TI type II antigen TNP-Ficoll (Fig. 2a). The amount of TNP-specific antibodies was higher in the sera of mice kept under constant light (inhibited melatonin synthesis) as compared with control mice kept under normal light/dark conditions. The amount of antibodies in mice exposed to constant lighting but with daily melatonin treatment was similar to the level of antibodies in mice kept at normal light/dark conditions. Thus, the production of specific antibodies increased when melatonin synthesis was inhibited (constant light). To prove the influence of melatonin on TI antibody production, we immunized the mice with TI type I antigen TNP-LPS. The increased amount of IgG1- and IgG3-specific antibodies (Fig. 2b) was obtained in the mice group that was kept under the constant lighting as compared with the control mice group (living at the normal light/dark conditions). The amount of IgM antibodies in that group varied from experiment to experiment. The IgM anti-TNP antibodies were measured by ELISA.
were increased under the constant lighting or similarly as in the control group (Fig. 2b). It means that melatonin inhibited the secretion of TI antibodies of IgM, IgG1, IgG2b and IgG3 classes.

In order to reveal the differences and similarities between the action of TI and TD antigens, we performed the immunizations with TD antigen TNP-ovalbumin (Fig. 3). Similar to the immunization with TI antigen, we detected increased amounts of IgM, IgG2a, IgG2b and IgG3 TNP-specific antibodies in the sera of mice kept at constant lighting conditions versus control groups. The amount of IgG1 class TNP-specific antibodies, on the contrary, was significantly lower in the sera of this mice group where melatonin synthesis was inhibited by the light. The influence of melatonin on regulation of TD antibody production was different: it stimulated IgG1 and inhibited IgM, IgG2a, IgG2b and IgG3 class antibody production. Similar data of melatonin effect on antibody production were obtained by immunizing BALB/c females and males, also C57BL6 females (data not shown.)

The daytime of immunization affects the amount of specific antibodies

To investigate how circadian rhythm modifies the production of antibodies, we immunized the mice with TD antigen in the morning and in the evening. The amount of antibodies in the sera significantly depends on the time of immunization. The level of antibodies was much higher when the immunizations were performed in the evening as compared with those in the morning (Fig. 4).

Melatonin modulated antibody production through MT2 receptors

The effect of melatonin on antibody production in vitro was also analyzed. Peritoneal or splenic cells were isolated...
1 week after the last immunization with TD antigen and cultivated in vitro without stimulation or stimulated with TNP-ovalbumin for 9–10 days. Unstimulated splenocytes (Fig. 5a) as well as peritoneal cells (data not shown) from mice kept at normal light/dark conditions produced antibodies of IgG1 isotype. Similar as in serum, the IgG1 levels were significantly reduced in cultures from mice kept at constant lighting. The antibody production was restored when mice were treated with melatonin.

The IgG1 levels were not altered significantly when antigen (TNP-ovalbumin) or melatonin alone was added into the cell culture prepared 7 days after the last immunization. Antibody production did not change after addition of MT2-selective inhibitor luzindole into the cell culture with unstimulated splenocytes (data not shown). TNP-ovalbumin in the presence of melatonin up-regulated IgG1 antibody production in splenocyte cultures from mice kept at normal conditions (Fig. 5b). This antibody secretion was inhibited by luzindole. However, melatonin did not induce antibody secretion in TNP-ovalbumin-stimulated splenocytes from mice kept at constant lighting. Unstimulated splenocytes from mice kept at normal conditions did not produce IgM class antibodies but started to produce antibodies after antigen stimulation. The antibody secretion was inhibited by luzindole. Splenocytes from mice kept at constant lighting did not produce IgM class antibodies with or without antigen stimulation in vitro (Fig. 5).

The IgG1 isotype antibody production could be induced in vitro by antigen in the splenocytes isolated about 2–4
and during the immunization with TD antigen. The immunizations and treatments with 4-P-PDOT were performed in the evenings (Fig. 6). The 4-P-PDOT-treated mice produced significantly less IgG1 isotype antibodies and more antibodies of other isotypes. Thus, treatment with MT2 antagonist 4-P-PDOT gave the same effect as the exposure under constant lighting (Fig. 3).

**Discussion**

The secretion of melatonin occurs during the night in response to the darkness. It reaches its maximum in the middle of the night and then decreases until the morning. The light inhibits the synthesis of melatonin.

Maestroni et al. also Vivien-Roels et al. demonstrated that in mice (C57BL/6J, BALB/c, AKR/J, CBA and C57BL/6 strains) the secretion of melatonin follows a clear-cut circadian rhythm. The peak of melatonin in the sera was obtained in the middle of dark phase. The peaks were of different amplitude, with the smallest elevation in melatonin level found in BALB/e mice (22, 23). We investigated how the reduced melatonin synthesis influenced the antibody production. We used the animal model where melatonin synthesis was suppressed. All previously performed investigations on modulation of antibody production by melatonin were performed either with pinealectomized animals or using additional treatment with melatonin (13, 17, 18).

Direct influence of melatonin on B cells is not clear. Some data indicate that melatonin failed to influence the activity of B cells in a TI manner. Melatonin had no influence on LPS-stimulated B cell proliferation or secretion of immunoglobulins (IgA, IgM, IgG1 and IgG2a) (14). Our data demonstrates that melatonin can modulate TI antibody secretion by B cells. The amount of antibodies increased when melatonin synthesis was suppressed (mice kept at constant artificial lighting). Mice kept at constant lighting and treated daily with melatonin produced similar amount of antibodies as the control group. This means that melatonin negatively regulates TI antibody production.

The effect of melatonin on B cells could be indirect due to Th. According to the literature, daily injections of melatonin specifically enhance the production of IgG1 but decrease IgG2a antigen-specific antibody secretion and has no effect on the production of IgG2b- and IgG3-specific antibodies in BALB/c mice (18). The primary and secondary antibody production in response to sheep red blood cell (SRBC) was markedly decreased in mice treated with propranolol (inhibition of the night-time rise of melatonin) in the evening, while the responses of the morning-treated mice were similar to those of the controls. The immunoenhancing effect of melatonin was evident only when melatonin was administered in the afternoon or in the presence of TD antigenic stimulation (13). According to our results, inhibition of melatonin synthesis caused the increase of antibodies of IgG2a, IgG3 and IgM isotype after the immunization with TD antigen. On the other hand, the amount of IgG1 antibodies significantly decreased. The same tendency was observed with primary and secondary immune responses.

Melatonin could mediate effect on B cells through Th. The regulation of IgG isotype switching in vivo will depend on...
Fig. 5. Production of TNP-specific antibodies in vitro by splenocytes from mice with suppressed synthesis of melatonin. (A) Decreased production of IgG1 isotype antibodies in unstimulated splenocyte cell culture. BALB/c mice were divided into three groups and kept at different lighting conditions: at normal light/dark (black circles), constant light (white circles) and constant light with daily administration of melatonin (gray circles). The mice were immunized with TD antigen TNP-ovalbumin. The splenocytes were isolated 7 days after the third immunization and cultured as described in Methods. The secreted antibodies were detected by ELISA after culturing the cells for 9 days. The significant
splenocytes were isolated 23 days after the third immunization, stimulated with TNP-ovalbumin, and incubated either for 24 h in normal lighting conditions. The mice were kept at normal lighting or constant light conditions and immunized with TNP-ovalbumin. The immunizations (indicated by arrows) and daily 4-P-PDOT injections (white circles) were performed in the evening. The blood was collected at days 7, 14, and 21 after the first immunizations. TNP-specific antibodies were detected by ELISA. One circle represents the antibody concentration (arbitrary units) in sera of one mouse. Eight to ten mice were per group. The significant differences (calculated by Student’s t-test) between mice groups kept in normal dark/light conditions and constant light are marked: *P < 0.05, **P < 0.005, ***P < 0.0005.

Fig. 6. MT2-selective inhibitor 4-PDOT influences the amount of TNP-specific antibodies in sera of mice immunized with TD antigen. BALB/c mice (females, 8–9 weeks old) were divided into two groups and kept at normal light/dark conditions. One mouse group was untreated (black circles) and the second one got daily 0.25 mg kg

1 4-P-PDOT injections (white circles). The mice were immunized with TD antigen TNP-ovalbumin. The immunizations (indicated by arrows) and daily 4-P-PDOT injections were performed in the evening. The blood was collected at days 7, 14 and 21 after the first immunizations. TNP-specific antibodies were detected by ELISA. One circle represents the antibody concentration (arbitrary units) in sera of one mouse. Eight to ten mice were per group. The significant differences (calculated by Student’s t-test) between mice groups kept in normal dark/light conditions and constant light are marked: *P < 0.05, **P < 0.005, ***P < 0.0005. (A) The antibody concentration (arbitrary units) in sera of mice with an intact immune system (C3H/He) or with CD4 cells from C57BL/6 mice. Melatonin suppressed the T h2 cytokines and increased antibodies to TNP-ovalbumin (10

1) in the presence or absence of T cell-depleted lymphocytes. (B) Melatonin receptor antagonist luzindole inhibits in vitro-induced IgG1 antibody secretion. The splenocytes were prepared as in (A) and stimulated in vitro with TNP-ovalbumin (10 μg ml

1) and melatonin (10 μg ml

1). Luzindole was added at 10 μM final concentration. (C) Luzindole and 4-P-PDOT inhibited the production of antigen-induced IgG1 antibodies in splenocyte cell culture from mice kept at normal light conditions. The mice were kept at normal lighting and constant light conditions and immunized with TNP-ovalbumin. The splenocytes were isolated 3 days after the third immunization, stimulated in vitro with TNP-ovalbumin (10 μg ml

1) in the presence or absence of MT2 antagonists luzindole and 4-P-PDOT. The amount of IgG1 antibodies was analyzed 9 days later by ELISA. One representative experiment (of three) is shown. (D) Melatonin concentration in the splenocyte cell culture supernatant (from Fig. 4C) was determined by ELISA. According to literature data, melatonin could act differently on T h1 differentiation toward T h1 and T h2. It modulates human CD4 cells to differentiate into T h1 (16, 25). Probably, the effect of melatonin on T cells is indirect, as melatonin enhances IL-12 production by cultured human monocytes. IL-12 drives T-cell differentiation toward T h1 phenotype, causing the enhanced IFN-γ production reported for melatonin (8). Contrary to human system, majority data showed that melatonin activates murine T h2 cells (17, 18). Melatonin could modify cytokine secretion by anti-CD3-stimulated-purified CD4 T cells (BALB/c mice) only in the presence of antigen-presenting cells (14). However, Colombo et al. (19) have shown that melatonin increases IFN-γ production by C57BL6 mice splenocytes. Melatonin suppressed the T h2 immune response in rats infected with Trypanosoma cruzi (26). Information on the role of melatonin on regulation of IL-12 secretion by murine macrophages is limited. Only one publication by Majewska et al. showed that melatonin inhibited the T h1-dependent immune response by suppressing the production of IFN-γ and IL-12 by cells in the lymph node of CBA mice (20).

The experiments showing that melatonin induces T h2 response (also possible class switch to IgG1) were performed using BALB/c mice, e.g. mouse strain that produces more T h2 cytokines. Therefore, we checked how suppressed melatonin synthesis influences the antibody production in BALB/c and also in C57BL6 mice. CD4 cells from C57BL6 as well as CBA/J mice produce more T h1 cytokines (27). We obtained similar results with both strains of mice. Similarly, Lopes et al. (28) showed that melatonin influence on the granuloma-circadian rhythm in mice (C57B1/6 and Swiss) was not strain specific. Strain differences in the formation of 6-sulphatoxymelatonin from melatonin were also not obtained in the mouse strains C3H/He, C57BL6 and BALB/c (29).

Jimenez-Caliani et al. (30) showed that melatonin acts differently on female and male MRL/lpr mice. These mice develop an autoimmune disease spontaneously. Administration of melatonin in drinking water for 1 month reduced symptoms of disease in females and enhanced in males, e.g. in females decreased pro-inflammatory cytokines (IL-2, IL-6, IFN-γ and TNF-γ, IL-1β), increased anti-inflammatory cytokine IL-10 in serum, decreased titers of auto-antibody and improved the histological pattern. The effect of melatonin on males was just the opposite (30). Authors assume that different cytokine pattern and antibody isotypes depend on sex hormones, i.e. estrogen and testosterone. Testosterone and estrogen differently affect T h1 and T h2 cytokine release following trauma-hemorrhage (31). Testosterone suppressed both IgG anti-dsDNA antibody and total IgG production in PBMC from systemic lupus erythematosus patients due to
Reduced IL-6 production in monocytes (32, 33). On the contrary, estrogen induces normal murine CD5+ B cells to produce auto-antibody (34). Elevations in the levels of estrogen can promote the survival and activation of high-affinity autoreactive B cells in systemic lupus erythematosus (35). On the other hand, melatonin inhibits testosterone secretion in the rat (36).

To exclude the influence of estrogen and testosterone, experiments were performed with females and males. No major differences were obtained on antibody secretion and numbers of various immune system cells. Therefore, it could be concluded that obtained effect of suppressed melatonin synthesis on antibody production is due to melatonin.

The quantity of specific antibodies in the blood was dependent on the time of immunization. We compared the amount of specific antibodies in the sera of mice immunized with TD antigen and kept at normal lighting conditions. Higher level of antibodies was found when the immunizations were performed in the evening as compared with the morning ones.

The production of antibodies could be influenced by another hormone—corticosterone. Contrary to melatonin, the peak of corticosterone occurs in rodents at the transition between day and night (37, 38) and reaches the maximum around 4 p.m. (39–42). The influence of corticosterone on antibody production is controversial (suppression or enhancement of antibody production might occur). This could be explained due to different experimental conditions and/or animal strains. Removal of corticosteroids via adrenalectomy (mice have very small amounts of serum corticosterone) results in higher SRBC-induced antibody production in A/J female mice (43). When the adrenalectomized BALB/c mice were treated with corticosterone, the antibody response was significantly suppressed (44). Melatonin administration antagonized the depression of antibody production induced by corticosterone in vivo (45). However, Stanulis et al. (46, 47) showed that corticosterone treatment did not influence the Ti (antigen TNP-Ficoll) antibody production in B6C3F1 mice but enhanced the T-dependent antibody response to SRBC. The same authors showed that corticosterone could produce a shift toward a T2-predominate response, thus increasing TD antibody production (46).

The amount of lymphocytes in human blood depends on the circadian time with the maximum at 22–23 o’clock (48). The amount of monocytes/macrophages in the peritoneal cavity depends also on circadian time. The numbers of those cells increased significantly during the night (V. Černyšov, I. Girkontaitė, unpublished results). The increased number of antigen-presenting cells could be linked to a better immune response.

In summary, the quantity of antibody in the sera of mice immunized with TD antigen depends on the amount of melatonin during daytime of immunization (e.g. decreasing or increasing the melatonin level or suppressed synthesis of melatonin). As higher antibody production was obtained during evening immunization, we could recommend performing the rodent immunization in the late afternoon/evening. It is expected that the titer of antibodies in other species, including humans, also depends on the time of immunization; however, this needs further investigation.

Receptors MT1 and MT2 are the best-characterized melatonin receptors (6). Melatonin receptor MT1 probably is not involved in T- and B-cell function modulation by melatonin in mice because melatonin (melatonin capsule implanted in vivo) enhanced both splenocyte proliferation (Con A stimulation) and anti-keyhole limpet hemocyanin IgG concentrations in both wild-type and mice lacking a functional gene for melatonin receptor MT1 (49). Luzindole is widely used as MT2-selective pharmacological antagonist (50, 51). It shows 25-fold higher affinity for the MT2 than the MT1 receptor (52). Therefore, we used luzindole in cell culture experiments to block MT2 receptors. IgG1 (or sometimes IgM)-specific antibody secretion was induced by antigen or antigen in the presence of exogenous melatonin only in the splenocyte culture from mice kept under normal lighting. This antibody production was inhibited by luzindole, meaning that melatonin modulates antibody production due to MT2 receptor. The antibody production was inhibited by luzindole in cultures with exogenous melatonin and as well as in cultures without addition of melatonin. This means that luzindole in our experiments also inhibited the effect of endogenous melatonin which is synthesized by immune cells in huge amounts (51, 53). Antibody secretion was not induced by antigen in vitro in splenocytes from mice kept under constant lighting. To prove that melatonin acts via MT2 receptor, we tested another melatonin antagonist 4-P-PDOT, which has >300-fold higher affinity for the MT2 versus MT1 (54, 55). We found that 4-P-PDOT inhibits the TNP-ovalbumin-induced IgG1 antibody production in vitro. The action of 4-P-PDOT in vivo was similar to that under constant lighting, thus meaning that melatonin modifies antibody production via MT2 receptor.

In summary, we could state that circadian rhythm and melatonin modulated antibody secretion in mice. Melatonin most likely acts via MT2 receptors.

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References
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49 Drazen, D. L. and Nelson, R. J. 2001. Melatonin receptor subtype MT2 (Mel 1b) and not mt1 (Mel 1a) is associated with melatonin-induced enhancement of cell-mediated and humoral immunity. Neuroendocrinology 74:178.


