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Role of the pineal gland in immunity

III: MELATONIN ANTAGONIZES THE IMMUNOSUPPRESSIVE EFFECT OF ACUTE STRESS VIA AN OPIATERGIC MECHANISM

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SUMMARY

We have recently demonstrated that the pineal neurohormone melatonin exerts important immunoregulatory functions. We now report that exogenous melatonin counteracts completely the effect of acute anxiety–restraint stress on thymus weight and antibody response to sheep red blood cells (SRBC). In addition, administration of melatonin in the evening prevented paralysis and death of mice infected with sublethal doses of encephalomyocarditis virus (EMCV) after acute stress. The anti-stress activity of melatonin was present in mice injected with T-dependent antigens, and it was abolished by the contemporary administration of the specific opioid-antagonist naltrexone. This suggests that melatonin exerts its remarkable anti-stress effect on antigen-activated cells via an opiatergic mechanism. These findings have important implications at both basic and clinical levels. They provide a new approach to a possible physiological 'up-regulation' of the immune response under virus- and/or stress-related immunosuppression.

INTRODUCTION

Psychological and emotional conditions of the most varied nature and origin can affect neuroendocrine and immune functions. It is now widely accepted that these closely interwoven mechanisms change the susceptibility to various diseases, including cancer (Ader, 1981; Fox & Newberry, 1984; Guillemin, Cohn & Melnechuck, 1985; Plotnikoff *et al.*, 1986).

Environmental stimuli, such as light and temperature, are transduced into neuroendocrine signals by the cyclic circadian synthesis and release of melatonin (*N*-acetyl-5-methoxytryptamine) by the pineal gland (Brown & Niles, 1982; Reiter, 1984; Axelrod, Fraschini & Velo, 1982). Psychosocial factors and stress also seem to modify the production of melatonin (Linch, Eng & Wurtman, 1973), and alterations of the circadian rhythm of its release have been associated with anxiety states, affective disorders and cancer (Brown & Niles, 1982; Arendt, Wirz-Justice & Bradtke, 1977; Birau, 1981). Furthermore, the pineal gland and melatonin in particular seem to exert an oncostatic role in a variety of experimental and clinical models (Blask, 1984). We have reported that functional and pharmacological inhibition of melatonin synthesis leads to a significant depression of humoral and cell-mediated immune responses in mice (Maestroni & Pierpaoli, 1981; Maestroni, Conti & Pierpaoli, 1986b). We have demonstrated that when melatonin is administered in a circadian fashion to normal adult mice, the neurohormone possesses powerful immuno-augmenting properties (Maestroni, Conti & Pierpaoli, 1986b, 1987a, b). Evening melatonin can also antagonize the suppression of antibody production induced by pharmacological doses of corticosterone or cyclophosphamide (Maestroni *et al.*, 1986b, 1987a, b). Melatonin was effective only in *vivo* on antigen-primed mice, and its immuno-augmenting effects were antagonized completely by the specific opioid-antagonist naltrexone (Maestroni *et al.*, 1986b, 1987a, b).

Of basic experimental and clinical significance is the evidence in this report that exogenous melatonin can abrogate completely the effects that anxiety stress induced by physical restraint produce on thymus weight, antibody production and on resistance to sublethal inocula of EMCV in mice. In accordance with previous findings, we show here that even under acute stress situations melatonin operates on antigen-activated cells via an opiatergic mechanism.

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MATERIALS AND METHODS

Mice

BALB/cJ inbred female mice from our animal quarter, aged 2–3 months, were used. The mice were maintained under a dark–light cycle of 12 hr (6 a.m., light on; 6 p.m., light off) at $22 \pm 1°$. Great care was taken to avoid environmental stress before and during the course of the experiments (noise, smells, cage crowding and so on).

Drugs

Melatonin (*N*-acetyl-5-methoxytryptamine) was purchased from Biosynth Inc., Staad. Solutions were obtained by dissolving melatonin in a minimal volume of ethanol and diluting with sterile phosphate saline (PBS) to a final 0·2% ethanol-PBS dilution. The same ethanol-PBS dilution was used as vehicle when other drugs were injected (naltrexone) and in control mice. Corticosterone-acetate and naltrexone were purchased from Sigma Co., St Louis, MO, and ICI 174,864 from CRB Ltd., Cambridge, U.K.

Haemolytic plaque-forming cells (PFC) assay

The number of spleen plasma cells producing direct (IgM) plaques after immunization of mice with SRBC was evaluated by the conventional agar haemolytic PFC in petri-dishes. Briefly, 2 ml of 1·2% agarose (Difco Labs, Detroit, MI) in Dulbecco PBS were pipetted in 6-cm petri-dishes to constitute the bottom layer. Agarose, 0·8 ml of 0·75%, in Eagle's MEM (Gibco, BBL, MA), was mixed with 45 μl of 20% SRBC and 45 μl of a 1:10 dilution of the spleen cell suspensions to be assayed for PFC. Direct IgM-mediated plaques were revealed by the addition of 0·4 ml of absorbed guinea-pig complement (Gibco).

Viruses

A preparation of encephalomyocarditis virus (EMCV, 16) was a gift of Professor R. Wyler, Veterinary Medicine, University of Zürich. Twenty microlitres were injected intracranially into the brain of 20 ether-anesthetized BALB/cJ adult male mice. The brains were removed during the acute paralysis–myocarditis stage, and a homogenate was prepared with an all-glass Potter at 1:10 weight/volume concentration in isotonic saline and stored in 0·5-ml aliquots at −30°. The same EMCV preparation was used in all the experiments done. *Vaccinia* virus was purchased from the Serum Institute, Bern.

Restraint stress

The mice were stressed by restraining them in 50-ml plastic tubes, with 10·5 mm-wide ventilation holes and a screw cap. The operation was repeated every day for 4 days from 10 a.m. The restraint produced anxiety but not complete immobilization.

Light microscopy

Thymuses were removed and fixed in Bouin solution. Serial sections in paraffin were stained with haematoxylineosin and examined.

Statistics

The results were evaluated statistically by the analysis of variance.

RESULTS

In earlier work we have suggested that the endogenous opioid system may mediate the immunological effect of melatonin: in fact the opioid-antagonist naltrexone completely abolished its immuno-stimulating activity (Maestroni *et al.*, 1987a, b). In this study, we investigated the possible effect of another opioid antagonist, namely ICI 174,864, on the primary antibody response to SRBC in melatonin-treated mice. At low concentrations (< 2 mg/kg body weight, b.w.) naltrexone binds preferen-

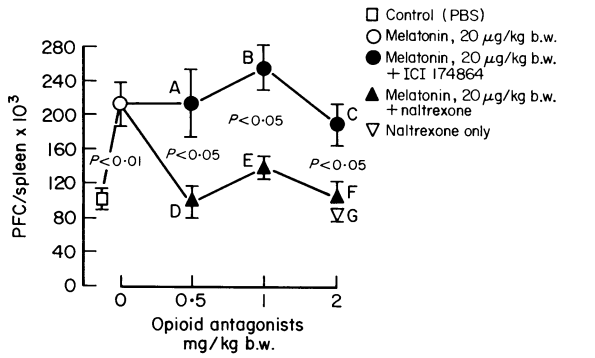

Figure 1. Naltrexone but not ICI 174,864 antagonizes the effect of melatonin on the primary response to SRBC. The mice were randomized in groups and inoculated i.p. with 4×10^8 SRBC at 11 a.m. Two groups ($n = 12$) were treated s.c. either with melatonin (20 μg/kg body weight, b.w.) or with PBS at 4 p.m. (2 hr before onset of darkness) for 4 consecutive days. The dose of melatonin was chosen according to previous dose–response studies (Maestroni *et al.*, 1987a). Further groups ($n = 6$) were also treated with melatonin (A–F). Together with melatonin, groups A, B, C were injected s.c. with the delta-receptor opioid antagonist ICI 174,864 while groups D, E, F were injected s.c. with the μ-receptor antagonist naltrexone at the reported doses. Group G was injected with naltrexone only. Four days after antigen injection the mice were killed and the primary antibody response to SRBC was evaluated by measuring the number of direct (IgM) PFC in the spleen. Variation bars represent the standard error. *P* values were obtained by the analysis of variance of melatonin versus PBS and of ICI 174,864 versus naltrexone compared at equal doses.

Table 1. Melatonin counteracts the effect of immobilization stress on primary antibody response and on thymus weight in mice. This melatonin effect is antagonized by the opioid antagonist naltrexone

Group	(*n*)	Stress	Treatment	PFC/spleen	Thymus weight (mg) / Body weight (g)
A	(15)	+	PBS	99903 ± 34420*	1·67 ± 0·57†
B	(15)	+	Melatonin	199157 ± 62888*	2·35 ± 0·75†
C	(9)	+	Melatonin + Naltrexone	93391 ± 39509	1·61 ± 0·53
D	(5)	+	Naltrexone	84149 ± 26795	1·60 ± 0·48
E	(14)	−	—	182035 ± 65881	2·39 ± 0·63

The mice were restrained, as described, for 2 hr/day for 4 days. One group (E) was not stressed, as control. All mice were injected on the first day at 1 p.m. with 4×10^8 SRBC i.p. Injections of PBS, melatonin (20 μg/kg b.w.) and of naltrexone (1 mg/kg b.w.) were performed s.c. at 4 p.m. for 4 days. Group E was left untreated. On the 4th day, the number of direct PFC and thymus and body weight were measured.

Variations are represented by the standard deviation.

* $P < 0·01$ A versus B and versus E, B versus C and versus D

† $P < 0·05$ A versus B and versus E, B versus C and versus D (analysis of variance).

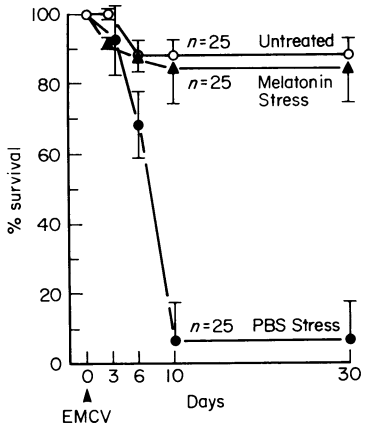

Figure 2. Evening administration of melatonin reverses the impaired immune resistance of stressed mice inoculated with a sublethal dose of EMCV. The mice were inoculated s.c. with 0·2 ml of a 2×10^{-8} dilution of EMCV on Day 0. The mice were divided in groups and two of them were restrained as described in the Materials and Methods. One of these group was treated daily for 10 days with 40 μg/kg b.w. of melatonin i.p. at 4 p.m. The remaining stressed group was treated with PBS only as control. The third group was neither stressed nor treated. Survival of three experiments is recorded as percentage and reported ± SD.

tially μ-type receptors (Paterson, Robson & Kosterlitz, 1984), while ICI 174, 864 has high affinity for δ-type receptors (Cotton *et al.*, 1984). Figure 1 shows that ICI 174,864 does not influence the immuno-augmenting effects of melatonin, thus indicating that the opioid receptors involved in the activity of melatonin do not belong to the δ-type. In contrast, naltrexone confirmed its antagonistic activity. However, in spite of the high affinity of naltrexone for μ-receptors, we cannot exclude a participation of other receptor types. In fact, different sensitivities to naltrexone antagonism have been reported for the effect of various opioid agonists, including the k-agonist dynorphin (Goldstein, 1984). Unfortunately, selective agonists for k receptors are not yet available.

Both melatonin and the endogenous opioid system have been shown to participate in the neuroendocrine response to acute stress (Plotnikoff *et al.*, 1986; Linch & Deng, 1986). It is also well known that acute stress causes thymus involution and depression of immune responses (Kelley, 1980). We tested the effect of exogenous melatonin on thymus weight and primary antibody response to SRBC in mice that had been stressed by physical restraint. Table 1 illustrates the results of these experiments. It shows that melatonin antagonized completely the effect induced by restraint stress on thymus weight and antibody production (group B). The effect of melatonin was completely abolished by 1 mg/kg body weight of naltrexone (group C). This indicates that, even in acutely stressed animals, melatonin operates via the endogenous opioid system.

The same stress model was utilized for evaluating the activity of melatonin in mice injected with sublethal doses of EMCV, a highly pathogenic and aggressive virus in rodents (Lennette & Schmidt, 1969). The results illustrated in Fig. 2 demonstrate that melatonin possesses an astonishing protective capacity in stressed and EMCV-infected mice. Eighty-five percent of the mice treated with evening melatonin survived. The reduction of resistance to the virus produced by acute stress was completely reversed by melatonin. Mortality of non-stressed control groups injected with sublethal doses of EMCV was negligible (Fig. 2).

Table 2. Melatonin antagonizes the effect of immobilization stress on thymus weight only in antigen-primed mice

Group	(*n*)	Stress	Treatment	Thymus weight (mg) Body weight (g)
A	(5)	+	Antigen (SRBC) + melatonin	2·667 ± 0·646*
B	(5)	+	Antigen (SRBC) + PBS	1·525 ± 0·313
C	(18)	+	Melatonin	1·628 ± 0·456
D	(18)	+	PBS	1·555 ± 0·460
E	(8)	−	Normal controls	2·633 ± 0·350†

Restraint was performed as described in the Materials and Methods. The mice were injected (A, B) or not (C, D) with 45×10^8 SRBC i.p. on the first day at 1 p.m. (1 hr after the first restraint session). Group E was neither stressed nor injected with SRBC. Groups A and C were then treated daily with melatonin (20 μg/kg b.w.) at 4 p.m. Groups B and D were injected with PBS at the same time of the day. At the end of the experiment (4 days after SRBC injection) thymus and body weight were measured.

Values are given ± the standard deviation.

* $P < 0{\cdot}01$ A versus B, C, D.

† $P < 0{\cdot}01$ E versus B, C, D (analysis of variance).

We have shown previously that melatonin has immuno-augmenting properties only in antigen-primed mice, an indication that antigen-activated cells may be the targets of melatonin (Maestroni *et al.*, 1986b). We investigated whether or not this antigen-dependent attribute of melatonin was responsible also for the anti-stress effect. We found that this is indeed the case. Table 2 illustrates the effect of melatonin on the thymus weight of stressed mice inoculated or not with SRBC.

The histological structure of the thymuses taken from SRBC-primed and stressed mice treated with melatonin (a) or with PBS (b) and from SRBC-injected non-stressed mice (c) is shown in Fig. 3. It seems evident that acute stress induces a drastic involution of the thymic cortex and that melatonin does not produce a reconstitution of this section of the thymus (a versus b). This indicates that melatonin exerts its activities on the thymic medulla (a versus c), which contains mature T cells. In fact, the *in vitro* response to the T-cell mitogen phytohaemagglutinin of thymocytes from stressed *and* melatonin-treated mice is more elevated than that of stressed and PBS-treated animals (data not shown). However, this important point clearly deserves a deeper analysis.

It is widely accepted that acute stress activates the synthesis and secretion of pro-opiomelanocortin gene products, such as ACTH and endorphins, and that the consequent adrenal response to ACTH mediates the stress-associated atrophy of the lymphatic system (Kelley, 1980). We have reported recently that exogenous melatonin antagonizes the suppression of the primary response to SRBC induced in mice when corticosterone is administered in the drinking water (Maestroni *et al.*, 1986b). We report here on experiments in which we investigated further whether or not melatonin was also able to antagonize the reduction of thymus weight induced by pharmacological doses

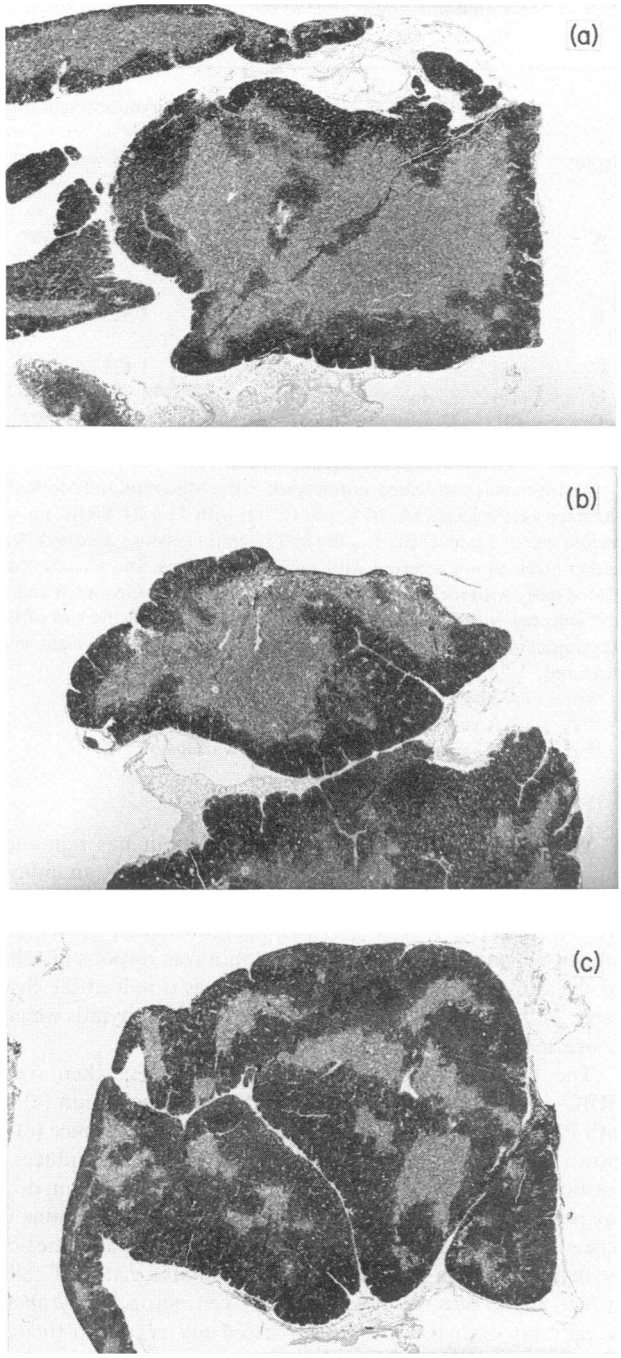

Figure 3. Effect of melatonin treatment on the thymus of stressed mice. The thymuses from some of the mice used in the experiments shown in Table 2 were fixed in Bouin solution and processed for histology: care was taken to compare similar sections of the thymus. The figure shows a representative example of the following groups: (a) SRBC-primed, restrained and melatonin-treated mice; (b) SRBC-primed, restrained and PBS-treated mice; and (c) non-stressed, normal controls. Haematoxilin-eosin, magnification × 5·5.

of corticosterone injected s.c. in normal and antigen-primed mice. The results of these experiments, shown on Table 3, fully confirm those obtained with the restraint-stress model (Tables 1 and 2). In fact, melatonin antagonized the effect of corticosterone on thymus weight only in mice injected i.v. with 8×10^6 pock-forming units (pfu) of *Vaccinia* virus (Table 3). Experiments are now underway to evaluate the effect of melatonin on anti-viral T-cytotoxic responses *in vivo*.

Table 3. Melatonin antagonizes the reduction of thymus weight induced by corticosterone in mice immunized with vaccinia virus

Group	(*n*)	Corti-costerone	Vacc. virus	Treatment	Thymus weight (mg) / body weight (g)
A	(10)	+	+	PBS	1·59 ± 0·48
B	(10)	+	+	Melatonin	2·28 ± 0·17*
C	(10)	+	−	PBS	1·63 ± 0·40
D	(10)	+	−	Melatonin	1·64 ± 0·16
E	(10)	−	−	—	3·01 ± 0·20

Groups A, B, C, D were injected s.c. daily for 5 days at 8 a.m. with a PBS suspension of corticosterone-acetate (0·5 mg/mouse/day). Groups A, B were then inoculated i.v. with 8×10^6 pfu of *Vaccinia* virus at 12 a.m. On the same day and for 5 consecutive days groups B, D were treated s.c. at 4 p.m. with 40 μg/kg b.w. of melatonin. Groups A, C were injected with PBS as control. On the 5th day the mice were killed and thymus and body weight measured.

Values are given ± the standard deviation.

* $P < 0{\cdot}01$ B versus A, C, D (analysis of variance).

DISCUSSION

Our results indicate that endogenous opioid peptides that are assumed to bind to μ or k-type opioid receptors are activated by exogenous melatonin and can counteract the effect of stress and/or of corticosterone on the immune response and on the size of the thymus. This effect does not seem to depend on a direct protection of lymphoid cells from the lytic action of corticosterone (Fig. 3). Therefore, it is improbable that there is a direct melatonin–opioid interference in pituitary–adrenal mechanisms as well as in the recently suggested feedback loops between these neuroendocrine systems and lymphocyte and/or monocyte products (Smith & Blalock, 1986; Besedovsky *et al.*, 1986). On the other hand, normal, non-stressed mice injected with SRBC and treated with melatonin showed, as reported elsewhere (Maestroni *et al.*, 1986b, 1987a), an increase in antibody production but no enlargement of the thymus. Further studies are needed to demonstrate conclusively that melatonin acts via a specific opioid peptide. Endorphins and enkephalins have been reported to exert many, albeit controversial, effects on the immune system (Plotnikoff *et al.*, 1986). In relation to the size of lymphatic organs, large doses of enkephalins injected in rodents can induce marked changes in the size of spleen and thymus (Murgo, Faith & Plotnikoff, 1986). The same enkephalins do not affect the primary antibody response to SRBC in mice (Murgo *et al.*, 1986). It is conceivable that many contradictory findings reported recently can be explained by a complete neglect of a proper hormonal circadian rhythmicity for the expression of an immune response. In fact, we have demonstrated that exogenous melatonin has immuno-augmenting properties only if injected at a time of the day which is synchronous to its endogenous circadian rhythm (Maestroni *et al.*, 1987b).

The melatonin–opioid anti-stress effect on antibody production and thymus weight may reflect a physiological function of

endogenous melatonin. We have reported that mice kept for three generations under constant environmental lighting, a procedure which abrogates pineal function, do not grow normally and display a marked atrophy of the thymolymphatic system (Maestroni & Pierpaoli, 1981). Experiments to elucidate this important point are in progress.

The present results have important implications both at the experimental and clinical level. In a general sense, it is not surprising that melatonin, the pineal neurohormone which co-ordinates the neuroendocrine response of the organism to basic environmental variables such as light and temperature, also exhibits such a dramatic activity on the immune system. Antigens can be certainly considered as fundamental components of the environmental stimuli and to represent special 'stressors' (Smith & Blalock, 1986; Besedovsky *et al.*, 1986; Maestroni *et al.*, 1986a). Antigen-activated lymphocytes have been shown to release stress hormones, such as ACTH and endorphins (Smith & Blalock, 1986), and the monokine IL-1 has been demonstrated to stimulate directly ACTH production in the pituitary (Besedovsky *et al.*, 1986). Melatonin may represent a cardinal molecule which modulates, via the endogenous opioid system, the immunosuppression induced by infectious agents and by stress. On the other hand, the endogenous opioid system seems to play a key role in the co-ordination of the response to stress (Plotnikoff *et al.*, 1986), and there are indications that the negative influence of stress or 'distress' derives from an exhausted endogenous opioid system (Cohen *et al.*, 1986). Most interestingly, unescapable acute anxiety stress such as that generated in this study has been shown to lower the circadian production of melatonin (Linch & Deng, 1986). Thus, melatonin may possess the physiological role of restoring the ability of the endogenous opioid system to co-ordinate the stress response. Finally, the identification of this fundamental neuro-immunoregulatory mechanism can provide the basis for new physiologic prophylactic and immuno-therapeutic interventions.

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