Pineal Melatonin, Its Fundamental Immunoregulatory Role in Aging and Cancer

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INTRODUCTION

A closely interwoven network of immune and neuroendocrine mechanisms protects our organism against a variety of environmental threats. Derangements of neuroendocrine functions such as those associated with or caused by failure to cope with stressful events or "distress" may lead in turn to an impairment of immunologic functions and possibly to an increased incidence of infections, autoimmune diseases and cancer.[1-3] As a matter of fact, functional connections and feedback loops between the immune and the neuroendocrine systems are being increasingly recognized.[4-6] Thus, primary defects in the neuroendocrine system or psychologic disturbances may adversely affect the immunologic machinery. The opposite is also true. In the case of aging, for example, the associated decline of both psychic and immune performance might, therefore, be triggered either by primary psycho-neuroendocrinologic or by immune alterations.

The pineal gland is a fundamental modulator of the entire neuroendocrine system. The pineal gland functions as a true "biologic clock" secreting in a circadian fashion its main neurohormone melatonin or N-acetyl-5-methoxytryptamine. Melatonin synthesis and release is regulated mainly by the light-dark cycle with a peak during the night, darkness hours.[7,8] However, other environmental variables such as temperature, humidity and, perhaps, pheromones and magnetism may influence its rhythm.[7,8] Also various physiopathologic states can affect melatonin rhythms. For example, in man alterations of melatonin production have been associated, amongst other things, with aging and cancer. In particular, low or impaired melatonin production has been described in aging[9] and various modifications of the melatonin rhythm have been found in cancer patients.[10]

We have recently found that the circadian synthesis and release of melatonin exerts an important immunomodulatory role.[4,11] Melatonin appears to be a physiologic "up-regulator" of the immune system and to operate via the endogenous opioid system (EOS) on antigen-activated cells.[12,13] Here we report further on other immunologic

properties of exogenous melatonin in the reversal of the immune impairment and thymus atrophy induced either by restraint stress or by pharmacologic corticosterone. Furthermore, we show that when properly administered in the course of primary immunization, melatonin can specifically and permanently enhance the "memory" immune reactivity against a specific antigen.

MELATONIN AS PHYSIOLOGIC "UP-REGULATOR" OF THE IMMUNE RESPONSE

When administered to mice in the evening, melatonin increased the primary antibody response (IgM + IgG) to T-dependent antigens. On the contrary, when administered in the morning, no effect on the immune response was apparent.[12] This circadian immunoenhancing effect was exerted by a broad range of melatonin doses (10 μg to 10 mg/kg body weight). On the basis of these findings we investigated if treatment with evening melatonin in the course of primary immunization against sheep red blood cells (SRBC) and/or vaccinia virus (VV) leads to any modification of the secondary response against the same antigen. TABLES 1 and 2 describe the results of

TABLE 1. Administration of Melatonin During Primary Immunization Enhances the Secondary Antibody Response to SRBC in Mice[a]

Group	(n)	Treatment during the Primary Immunization	Secondary Response PFC/Spleen (Ig M + Ig G)
A	(14)	Melatonin for 7 days	125413 ± 45271[b]
B	(14)	PBS for 7 days	64706 ± 32964

[a] Two groups of mice were immunized with 4×10^8 SRBC intraperitoneally (i.p.) and were injected subcutaneously (s.c.) for the following 7 days with melatonin (20 μg/kg b.w.) at 4 p.m., 2 hours before onset of darkness. Control mice were inoculated s.c. with 0.5 ml of PBS at the same hour. After this treatment the mice were left undisturbed for a further 3 weeks and then boostered i.p. with 4×10^8 SRBC. *Neither melatonin nor PBS* was inoculated during this second immunization. The secondary antibody response to SRBC was evaluated 3 days after the booster injection.

[b] $p \leq 0.01$: A vs B.

these experiments in which mice were immunized with SRBC or VV and treated by evening administration of melatonin for 6 days. The mice were then left undisturbed for 4 weeks and then boostered in order to evaluate the secondary antibody response to SRBC and the secondary T-cytotoxic response to VV, *without any further melatonin injection.* As is shown on TABLES 1 and 2, melatonin treatment during primary immunization against T-dependent antigens permanently enhanced the humoral and cellular immune reactivity against that given antigen. The mechanism of this remarkable action of melatonin is still unknown. However, it is possible that melatonin stimulates the differentiation and/or proliferation of memory cells via the EOS.

IS MELATONIN THE ANTI-STRESS HORMONE?

We have already reported that melatonin has the interesting property of reversing the depression of antibody production induced by corticosterone given to mice in the drinking water.[11] Here we report on experiments in which we investigated whether melatonin is able to counteract the effect of restraint-anxiety stress on antibody production, on thymus weight and on resistance against lethal infections with the very aggressive murine encephalomyocarditis virus (EMCV). TABLE 3 shows that evening administration of melatonin could completely buffer the depression of antibody production and thymus weight induced by the acute restraint stress in mice inoculated with SRBC (group B vs A). This effect of melatonin appeared to be dependent on

TABLE 2.[a]

Effector to Target (Ratio)	% of Specific ^{51}Cr Release ± S.D.[b] PBS (n = 8)	Melatonin (n = 8)	% Variation (Melatonin vs PBS)
50 : 1	40.0 ± 4.6	52.3 ± 4.1[c]	+ 30
25 : 1	31.7 ± 6.1	44.8 ± 3.8[c]	+ 41
12 : 1	23.5 ± 4.6	32.7 ± 3.0[c]	+ 39
6 : 1	14.4 ± 3.8	23.2 ± 3.5[c]	+ 64

[a] C3H/He female mice, two months old, were injected i.v. with 1×10^6 PFU of vaccinia virus (VV) at 12 noon. In the following 6 days the mice were injected s.c. with melatonin (40 μg/kg b.w.) at 4 p.m., 2 hours before onset of darkness. Control mice were inoculated s.c. with 0.5 ml of PBS at the same hour. After this treatment the animals were kept undisturbed for 7 weeks with free access to food and water and under a 12-hour light cycle. A booster injection of 8×10^6 PFU of VV i.v. was then performed. *Neither melatonin nor PBS* was inoculated during this second immunization. After 4 days the antiviral cytotoxic T cell response was tested by the ^{51}Cr release assay. Effector spleen cells were diluted with L929 target cells at the following ratios: 50:1, 25:1, 12:1 and 6:1.

[b] % of specific ^{51}Cr release ± standard deviation (S.D.) was calculated according to the formula: $\frac{\text{Exp. release-Spontaneous release}}{\text{Total cpm}} \cdot 100 = \%$ spec. rel.

[c] $p < 0.01$.

antigen-activated T cells, because unprimed mice (groups G and H) or mice injected with T-independent antigens (LPS, groups E and F) did not show any melatonin effect. The anti-stress action of melatonin appeared also to be antagonized completely by the contemporary administration of the specific-opioid antagonist naltrexone. This suggested that even in stress situations, melatonin operates via the EOS (group C vs B). On the other hand, naltrexone alone had no effect either on antibody production or on thymus (group D). This showed that no direct naltrexone-sensitive opiatergic mechanisms are involved in the immunologic effect of acute stress. Most interesting, evening melatonin was able to confer resistance against lethal doses of EMCV in infected and stressed mice. TABLE 4 reports the survival of mice that were stressed

TABLE 3. Melatonin Counteracts the Effect of Restraint-Anxiety Stress on Thymus Weight and Antibody Production of Mice Injected with T-dependent Antigens[a]

Group	(n)	Stress	LPS	SRBC	Treatment	PFC/Spleen	mg Thymus Weight / g Body Weight
A	(15)	+	−	+	PBS	99903 ± 34230[b]	1.67 ± 0.57[c]
B	(15)	+	−	+	melatonin	199157 ± 62888[b]	2.35 ± 0.75[c]
C	(9)	+	−	+	melatonin + naltrexone	93391 ± 39509	1.61 ± 0.53
D	(5)	+	−	+	naltrexone	84149 ± 26795	1.60 ± 0.48
E	(10)	+	+	−	PBS	3773 ± 1187	0.76 ± 0.14
F	(10)	+	+	−	melatonin	1786 ± 392	0.76 ± 0.065
G	(18)	+	−	−	PBS	—	1.55 ± 0.46
H	(18)	+	−	−	melatonin	—	1.62 ± 0.45
I	(14)	−	−	+	—	182035 ± 65881	2.39 ± 0.63
L	(14)	−	−	−	—	—	2.67 ± 0.42

[a] Female, 2-3-months-old BALB/cJ mice kept under a 12-hour light cycle at 23 ± 1°C, with free access to food and water, were randomized in groups. Some groups were then stressed by restraining the mice in 50 ml plastic tubes with ventilation holes, for 2 hours/day for 4 days, starting from the day of antigen injection. Sheep red blood cells (SRBC) were injected i.p. (4×10^8/mouse) at 1 p.m., 1 hour after the first stress session. *Escherichia coli* lipopolysaccharide (LPS, type 055:B5, Sigma Co., St. Louis, USA) was also injected i.p. (100 μg/mouse) at 1 p.m. From the day of antigen injection, the mice were injected each evening at 4 p.m. (2 hours before onset of darkness) with melatonin (40 μg/kg body weight, s.c., Biosynth. AG, Staad, Switzerland), phosphate saline (PBS, 0.5 ml s.c.) and/or with naltrexone (1 mg/kg body weight, Sigma Co., St. Louis, USA). The number of spleen plasma cells producing direct (IgM-mediated) plaques after immunization of mice with SRBC or LPS was evaluated by the conventional hemolytic plaque-forming cells (PFC) assay in petri dishes. Anti-LPS, IgM-producing cells were detected after coating the SRBC by incubation with 2 mg/ml LPS 1 hour at 37°C. The values are expressed ± the standard deviation and the differences have been evaluated by the analysis of variance.

[b] $p < 0.01$: A vs I; B vs A, C, D.

[c] $p < 0.05$: A vs I, L; B vs A, C, D, G, H.

TABLE 4. Evening Administration of Melatonin Reverses the Impaired Immune Resistance of Mice Stressed Acutely by Physical Restraint and Inoculated with a Sublethal Dose of Encephalomyocarditis Virus (EMCV)[a]

(n)	Stress	EMCV	Treatment	% Survival ± S.D. 3 Days	6 Days	10 Days	30 Days
(25)	+	+	PBS only	92.3 ± 10.9	68.3 ± 9.5	7.7 ± 10.9	7.7 ± 10.9
(25)	+	+	melatonin	91.9 ± 0.45	88.1 ± 4.9	84.3 ± 10.4	84.3 ± 10.4
(25)	−	+	untreated	100.0 ± 0.0	88.2 + 4.9	88.2 ± 4.9	88.2 ± 4.9

[a] *Female, 2-3-months-old BALB/cJ mice were inoculated with 0.2 ml of 2 × 10^8 dilutions of EMCV in saline on day 0. The mice were divided in groups and two of them were restrained two hours per day for 4 days as described (see* TABLE 3). One of these groups was treated daily for 10 days with 1 μg of melatonin i.p. at 4 p.m. The remaining stressed group was treated with saline and served as control. The third group was neither stressed nor treated. Survival of 3 experiments is recorded as percentage and reported ± the standard deviation (S.D.).

by physical restraint and treated with melatonin or PBS. Other mice were inoculated with EMCV, received no melatonin and served as a nonstressed control group. It is evident that melatonin administered in the evening during 10 days after EMCV infection exerted a striking protective activity against EMCV and reversed completely the immunosuppressive influence of restraint stress (TABLE 4).

CONCLUSIONS AND PERSPECTIVES

The findings presented here, together with those previously reported,[9,11,13] point to a general immunoaugmenting effect of endogenous and/or exogenous melatonin. In particular, the present report shows that when administered in the course of primary immunization, melatonin possesses the remarkable property of permanently stimulating the immunologic reactivity against a given antigen. This attribute of melatonin holds promising obvious implications for vaccination procedures.

It seems clear that melatonin does not act directly but rather via the EOS.[12,13] Also the astonishing anti-stress properties of melatonin are, in fact, abrogated by the specific-opioid antagonist naltrexone. Furthermore, melatonin seems to exert its effect only in mice inoculated with T-dependent antigens. This indicates that antigen-activated T lymphocytes are possible targets for the melatonin-opioid action, and that final effector molecules could be products of these activated T cells. The identification of the specific opioid peptide involved in the melatonin action will allow a deeper analysis of the mechanisms involved. Experiments in this direction are in progress.

Another important aspect to be studied concerns the nature of the protection exerted by melatonin on the thymus of stressed and/or corticosterone-treated mice. We have performed preliminary studies by comparing histologic sections of thymuses from stressed and melatonin-treated mice with similar sections of thymuses from stressed and saline-treated mice. Although we did not perform any quantitative morphometric analysis, it is evident that melatonin did not protect the thymus cortex from the thymolitic action of corticosteroids, but rather produced a striking enlargement of the thymic medulla.[14] Obviously, this important point needs further investigation. The fact that exogenous, evening melatonin exerts this astonishing anti-stress effect might reflect a physiologic role of the neurohormone. Along this line, a prerequisite to any successful coping with stressful events would be an optimal synthesis and/or release of pineal melatonin. As a matter of fact, it has been suggested that failure to cope with "negative" events or distress (*e.g.*, during aging) may depend on an exhausted EOS,[13] whose function seems also to be the coordination of the neuroendocrine response to a variety of nonspecific stressors.[3] In this regard, melatonin may have the physiologic function of restoring the EOS and thus the capacity of the organism to respond and adapt to environmental variables (*e.g.*, temperature or antigens) and to psychosocial stress.

Many immunologic effects of stress closely resemble those associated with aging. For example, in both situations the thymus gland becomes atrophic and immune functions are impaired or imbalanced. As reported above, aging has been associated with impaired melatonin production.[9] It is thus reasonable that exogenous melatonin, *i.e.*, the pineal neurohormone that tunes the entire neuroendocrine system according to basic environmental variables, might be able to correct the immune impairment associated with aging.

As far as neoplasia is concerned, the mechanism of the widely reported oncostatic

activities of melatonin is still obscure. On the basis of our findings, the obvious suggestion would be that such activity depends on immunologic mechanisms. Experiments are now being carried out to help elucidate this important point.

All together, the present findings delineate a fundamental neuroimmunomodulatory mechanism that may open new physiologic prophylactic and immunotherapeutic interventions.

PROLONGATION OF LIFE IN MICE BY MELATONIN

The variety of the neuroendocrine mechanisms affected by melatonin, combined with our first clear findings on its immunoenhancing and anti-stress activity, guided us to the idea that the pineal gland could be considered a sort of "homeostatic headquarters," *i.e.*, the organ in which afferent information of the most diverse nature and origin (light-darkness cycle, temperature, stress, antigens) is integrated, elaborated and finally transmitted as efferent signals able to coordinate and to modulate the neuroendocrine and immune adaptive responses of the organism to the external and/or internal environment. On the other hand, the aging process can be synthetically and generally defined as a progressive decline and finally the loss of the capacity to cope with the environmental challenges. In addition, it is known that aging is associated with a progressive impairment of a most basic pineal function, namely the circadian production of melatonin.[9]

The duration of life of an organism is the result of a series of biologic processes in which any event is conditioned and determined by the preceding one. However, nature has provided all living creatures with rhythmic mechanisms able to reset the homeostatic capacity or, in other words, the adaptive functions of the organism. In this context, the pineal gland and in particular the circadian rhythm of melatonin synthesis seem to play a central role, both as a primary biologic clock which tunes the entire organism according to the basic light-darkness cycle, and as a pacemaker, via the rhythmic melatonin signal to the whole neuroendocrine system.[7,8]

On the basis of these considerations we thought of investigating the effect of chronic, exogenous melatonin on the life span of aging mice kept under a 12-hour light cycle at 23 $\pm$ 1°C with free access to food.

Starting in November 1985, 10 male C57BL/6J inbred mice were given melatonin (10 μg/ml) in the drinking water every day. Another group of 10 mice were given tap water containing the same concentration of ethanol (0.01%) used to dissolve the neurohormone for the treated group. In order to avoid irregular drinking habits and to guarantee that the mice would ingest melatonin only during the night (darkness) hours, all the bottles with or without melatonin were always removed in the morning at 8:30 a.m. and put back in the evening at 6:00 p.m. The drinking water with or without melatonin was changed twice weekly. When the experiment started (November 1985) all the mice were 575 days (about 19 months) old and perfectly healthy.

To our surprise chronic, circadian, night administration of melatonin resulted in a progressive, striking improvement of the general state of the mice and, most important, in a remarkable prolongation of their life.

In fact, starting at 5 months from the initiation of melatonin administration, the body weight of the untreated mice still surviving started to decrease rapidly, and also astonishing differences in the fur and in the general conditions of the two groups (vigor, activity, posture) became increasingly evident. Melatonin treatment preserved

completely optimal pelage conditions and the body weight was maintained at the original values (about 36 grams). Furthermore, the mean survival time ± standard deviation was 931 ± 80 days in the melatonin-treated group versus 752 ± 81 days in the untreated controls. This difference is significant with $p < 0.01$ (analysis of variance).

It would be premature to speculate on the significance of our preliminary results and the mechanism by which night, chronic administration of melatonin in aging mice prolongs their life span by approximately 20% (6 months) and, most important, greatly improves their general body conditions. Also, these preliminary findings need to be confirmed and extended in other strains of mice and other species. However, as proposed above, the melatonin-dependent circadian resetting of neuroendocrine and immune functions might account for this astonishing prolongation of the life span in melatonin-treated mice.

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