Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. 82, Suppl. II: 75-79, 1987.
Intern. Symp. on Immunomodulators

THE PINEAL NEUROHORMONE MELATONIN AND ITS PHYSIOLOGIC OPIATERGIC IMMUNOREGULATORY ROLE

GEORGES J.M. MAESTRONI*/ +, ARIO CONTI* & WALTER PIERPAOLI**

*Instituto Cantonale di Patologia, 6604 Locarno, Switzerland **Institute for Integrative Biomedical Research, Ebmatingen, Switzerland

The pineal gland functions as a neuroendocrine transducer that coordinate the organism response to changing environmental stimuli such as light and temperature. The main and best known pineal neurohormone is melatonin that is synthesized and released in a circadian fashion with a peak during the night darkness hours. We have recently reported that melatonin exerts important immunoregulatory functions. Here we describe the astonishing property of exogenous melatonin which is able to counteract completely the depressive effect of anxiety-restraint stress and/or of corticosterone on thymus weight, antibody production and antiviral responses. This effect seems to be mediated by antigen-activated T cells via an opiatergic mechanism.

Immune and neuroendocrine functions cooperate in a closely interwoven network to protect the organism from various environmental attacks. For example, antigens and/or pathogenic microorganisms that may be considered a special type of environmental challenge, can induce synthesis and secretion of stress hormones such as proopiomelanocortin gene products both centrally and peripherally (Maestroni & Pierpaoli, 1981; Smith & Blalock, 1986; Besedovsky et al., 1986). On the other hand, psychogenic and emotional stress can deeply affect the immune system in part via the very same proopiomelanocortin derivatives (Kelley, 1980). It is widely held that the immune-neuroendocrine network affect the susceptibility to various diseases, cancer included (Ader, 1981; Fox & Newberry, 1984; Guillemin et al., 1985; Plotnikoff et al., 1986).

Basic environmental informations such as light cycle and temperature are transduced into signals capable of modulating most neuroendocrine mechanisms by the pineal gland. In fact, the indoleamine melatonin (N-acetyl-5-methoxytryptamine) synthesized and secreted in a circadian fashion by the pineal gland upon the nocturnal activation of beta-adrenergic receptors (Deguchi & Axelrod, 1973) displays a very large variety of activities (Brown & Niles, 1982; Reiter, 1984). This activity is especially evident in seasonally breeding animals on hormones of the reproductive, adrenal and thyroid systems (Reiter, 1984). In man, variations of the circadian rhythm in plasma melatonin have been associated with puberty, menstrual cycle, affective disorders (anxiety, depression), psychosomatic diseases and cancer (Arendt et al., 1977; Wettenberg, 1978; Birau, 1981). In regard to cancer, the pineal gland and its neurohormone melatonin have been widely described to exert and important oncostatic activity (Blask, 1984). We have recently reported the functional and pharmacologic inhibition of melatonin synthesis leads to a significant reduction of humoral and cell-mediated immune responses (Maestroni & Pierpaoli, 1981; Maestroni et al., 1986a).

Furthermore, we demonstrated that melatonin administered in a circadian fashion to normal mice exerts powerful immunoaugmenting effects (Maestroni, et al., 1986a, b; 1987a, b). Melatonin proved also to antagonize the immunosuppression induced by cyclophosphamide and corticosterone (Maestroni et al., 1986a; 1987a). Melatonin was effective only *in vivo* on antigen primed animals and its action was completely abolished by the specific opioid antagonist naltrexone (Maestroni, et al., 1986b; 1987a, b).

Of basic and clinical relevance is the present report that melatonin injected in the evening can counteract completely the effect of restraint stress and of pharmacologic corticosterone on thymus weight and the immune response to T-dependent antigens via an opiatergic mechanism.

Exogenous melatonin also protected acutely stressed mice inoculated with lethal doses of encephalomyocarditis virus (EMCV).

This work has been supported in part by the Swiss Nationalfonds, grant no. 3.267.0.85 and by the Roche Research Foundation.

+ To whom correspondence must be sent.

MATERIALS AND METHODS

Animals – BALB/cJ, inbred female mice, aged 2-3 months, were used. The mice were maintained under a dark-light cycle of 12 hours at 22 ± 1 C. 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, Switzerland. Solutions were obtained by dissolving melatonin in a minimal volume of absolute ethanol and diluting with sterile phosphate saline (PBS) to a final 0,2% ethanol-PBS dilution. Controls of melatonin injected mice were always injected with 0,2% ethanol-PBS. Corticosterone-acetate, Escherichia Coli lipolysaccharide (LPS, Type 055:B5) and naltrexone were purchased from Sigma. Co., St. Louis, USA.

Haemolytic plaque forming cell (PFC) assay – The number of spleen plasma cells producing direct (IgM) plaques after immunization of mice with sheep red blood cells (SRBC) was evaluated by the conventional agar haemolytic PFC assay in petri dishes (Jerne test).

PHA blastogenesis – Thymocyte suspensions were obtained by teasing the thymuses in culture medium constituted by RPMI 1640, 5% foetal calf serum 2 mM L-glutamine, 50 μg/ml streptomycin, with the aid of a loose-fitting teflon pestle. Cells were counted and adjusted to the desired concentration. $6x10^5$ thymocytes in 200 ul of culture medium were incubated for 72 hours at 37°C, 5% CO_2, with or without 10 μg/ml of phytohemagglutinin (PHA, Fakola, Inc., Basel, Switzerland) in U-bottomed, 96 wells microplates. 20 hours before harvesting, cultures were pulsed with 0.5 uCi ^{3}H-thymidine (NEN Research Products, Zürich, Switzerland). Cells were then harvested by an automatic cell harvester and the incorporated activity measured by liquid scintillation and expressed by counts per minute (cpm).

Viruses – A preparation of encephalomyocarditis virus (EMCV, Lennette & Schmidt, 1969) was a gift of Prof. R. Wyler, Veterinary Medicine, University Of Zürich, 0,02ml 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 0.5ml aliquots at -30°C. The same EMCV preparation was used for all the experiments done. Vaccinia virus was purchased from The Serum Institute, Bern, Switzerland.

Restraint stress – The mice were stressed by restraining them in 50 ml plastic tubes with 5 mm-wide ventilation holes. The operation was repeated every day for 4 days from 10 a.m. to 12 a.m. The restraint produced anxiety but not complete immobilization.

Statistics – The results were evaluated statistically by the analysis of variance (Fisher test).

RESULTS

Table I shows that melatonin injected s.c. in the evening counteracted completely the effect of restraint-anxiety stress on thymus weight and on the primary immune response to SRBC (group B vs A). The specific opioid antagonist naltrexone (group C) abolished the protective effect of melatonin that, on the other hand, was exerted only in antigen-primed mice (groups F vs E). This suggested that melatonin acts via activation of the endogenous opioid system (EOS) on antigen-activated cells. Also, melatonin appeared unable to protect mice injected with the T-independent antigen LPS from the effect of restraint stress (Table II). Furthermore, direct PFC against LPS-coated SRBC were not affected by melatonin either in stressed or in normal mice (data not shown) suggesting that targets of the melatonin-opioid action are probably T-lymphocytes. The same antigen-dependent protective attribute of evening melatonin was apparent in corticosterone treated and Vaccinia-virus-injected mice (Table III) confirming the data obtained in the restraint -stress model. However, the histologic appearance of thymuses either from stressed or corticosterone injected mice that were treated with melatonin showed that melatonin did not protect the thymus cortex from the lytic action of adrenal steroids but rather exerted action enlarging the thymic medulla (pictures not shown). Consistent with the histologic pictures is the response to the T-cell mitogen PHA of thymocytes from stressed and melatonin treated mice versus that of stressed and PBS-treated mice (Table IV). It is in fact well known that medullary thymocytes are mature cells and respond better to PHA than cortical cells.

Finally, Figure shows that exogenous melatonin has the astonishing property of protecting

TABLE I

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 and occurs only in antigen (SRBC) injected mice

Group	(n)	Stress	SRBC	Treatment	PFC / Spleen	mg Thymus weight / g Body weight
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	(18)	+	–	PBS	———	1.55 ± 0.46
F	(18)	+	–	Melatonin	———	1.62 ± 0.45
G	(14)	–	+	Controls	182035 ± 65881	2.39 ± 0.63
H	(8)	–	–	Controls	———	2.63 ± 0.35

The mice were restrained as described in materials and methods and injected at 1 p.m. (1 hour after the first stress session) on day 0 with 4 x 10^8 sheep red blood cells (SRBC) i.p. Melatonin (40 μg/kg body weight, b.w.), naltrexone (1 mg/kg b.w.) and PBS (0.5 ml) were injected s.c. at 4.00 p.m. each day for 4 consecutive days. These dose were chosen according to previous dose-response studies (Maestroni, et al., 1987 a,b). Direct PFC, thymus and body weight were evaluated on day 4 and are expressed ± the standard deviation.

*$p < 0.05$ A vs B, G, D and B vs C, D, E, F.

**$p < 0.01$ A vs B, G and B vs C, D.

TABLE II

Melatonin counteracts the effect of restraint stress on thymus weight only in mice injected with T-dependent antigens

Group	(n)	LPS	SRBC	Stress	Treatment	Thymus weight (mg) / Body weight (g)
A	(10)		+	+	Melatonin	2.667 ± 0.646*
B	(10)		+	+	PBS	1.525 ± 0.313
C	(10)	+		+	Melatonin	0.763 ± 0.065
D	(10)	+		+	PBS	0.762 ± 0.137
E	(10)				Normal controls	2.721 ± 0.466

The mice were stressed as usual (materials and methods) and injcted i.p. at 1 p.m. with 4 x 10^8 and/or 200 μgLPS on day 0. Melatonin (40 μg/ kg b.w.) or PBS were injected at 4 p.m. each day for 4 days. At the end of the experiment thymus and body weight were measured.

*$p < 0.01$ A vs B, C, D.

TABLE III

Melatonin antagonizes the corticosterone induced atrophy of the thymus only in mice immunized with vaccinia virus (T-dependent antigen)

Group	(n)	Corticosterone	Vacc. Virus	PBS	Melatonin	mg Thymus weight / g Body weight
A	(10)	+	+	+		1.59 ± 0.48
B	(10)	+	+		+	2.28 ± 0.17*
C	(10)	+		+		1.63 ± 0.40
D	(10)	+			+	1.64 ± 0.16
E	(10)			Normal controls		3.01 ± 0.20

The mice were injected s.c. daily at 8 a.m. with 0.5 mg of corticosterone-acetate suspended in PBS. 8 x 10^6 pock forming units of Vaccinia virus were injected in groups A and B at 1 p.m. Melatonin (40 μg/kg b.w.) and PBS were injected for 5 consecutive days at 4 p.m. Thymus and body weight were measured on day 5.

*$p < 0.01$ B vs A, C, D.

TABLE IV

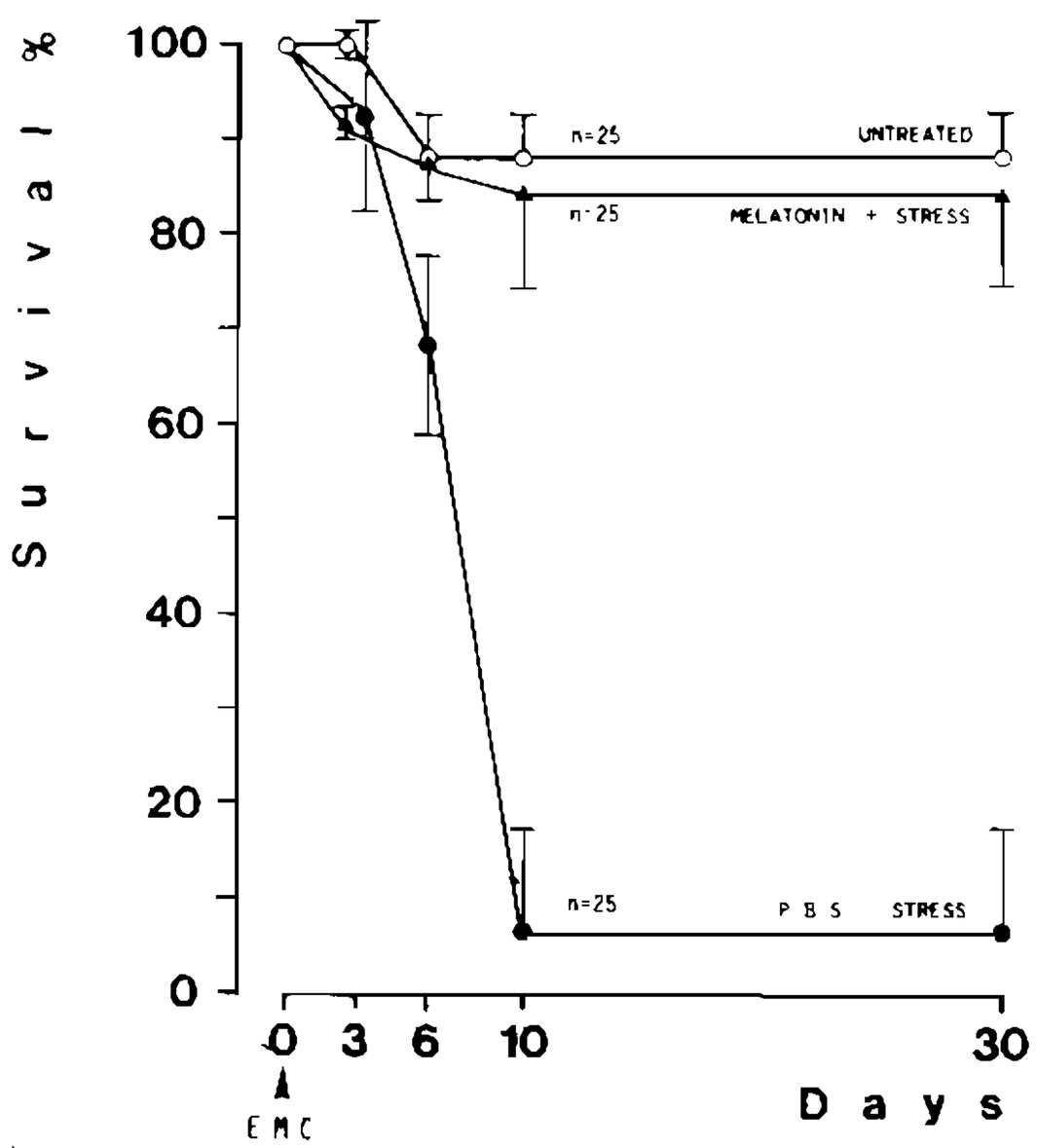
Thymocytes from stressed and melatonin treated mice respond better to PHA than thymocytes from stressed and PBS treated mice

(n)	Stress	SRBC	Treatment	mg thymus weight / g body weight	PHA cpm ± s.d.
(6)	+	+	Melatonin	2.47 ± 0.30	13310 ± 5623*
(6)	+	+	PBS	1.22 ± 0.28	3655 ± 1325

* $p < 0.01$

Thymocytes were obtained from some of the mice used in the experiments shown in Table I (group A and B). The ^{3}H-thymidine incorporation values are reported as net counts per minute (cpm) ± the standard deviation (PHA values – background).

restrained-stressed mice from sub-lethal inocula of EMCV. Almost the totality of stressed and virus inoculated mice died within 10 days from the infection while treatment with evening melatonin protected the great majority of equally stressed and virus infected mice. Percent survival of stressed and melatonin treated mice was, in fact, very similar to that of unstressed and EMCV injected mice (Figure).



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). Female 2-3 months old BALBc/J were inoculated s.c. with 0.2 ml of 2×10^{-8} dilution of EMCV in saline on day 0. The mice were divided in groups and two of them restrained two hours per day for 4 days as described (see materials and methods). One of these group 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 only as control. The third group was neither stressed nor treated.
Survival of 3 experiments is recorded as percentage and reported ± the standard deviation.

DISCUSSION

The present results show that melatonin has a powerful antistress action via the activation of the EOS and a T-cell-dependent mechanism. Together with previously reported findings (Maestroni et al., 1986a, b; 1987, a, b) these results suggest that melatonin can be considered a physiologic "up-regulator" of the immune system. The pharmacologic effect of melatonin might reflect a physiologic role. In fact, melatonin is effective at rather low doses and also functional and pharmacological inhibition of melatonin synthesis and release result in depression of immune responses (Maestroni & Pierpaoli, 1981; Maestroni et al., 1986 a; 1987 a). This important point deserves, however, a deeper analysis. The melatonin-opioid anti-stress action is clearly exerted only in T-dependent immune responses. This may mean that the melatonin effect is ultimately mediated by products of activated T-lymphocytes.

The cyclic circadian release of melatonin is known to coordinate the neuroendocrine responce of the organism to changing environmental conditions (Reiter, 1984). Thus, in a general sense, the anti-stress action of melatonin is not surprising also because antigens may be considered amongst environmental stimuli, a special kind of stressors (Maestroni, et al., 1986 b; Smith & Blalock, 1986).

The naltrexone effect on the melatonin action suggest an involvement of the EOS. Although the demonstration that melatonin acts via a specific endogenous opioid peptide awaits further studies, this seems quite interesting. It has, in fact, been suggested that the EOS coordinate the organism response to stress (Plotnikoff et al., 1986) and there are indications that the negative influence of stress or "distress" derives from an exhausted EOS (Cohen et al., 1986).

On the other hand, the EOS have been widely shown to exert important although controversial immunoregulatory effects (Plotnikoff et al., 1986). Most interesting, unescapable acute stress such as that used in this study has been shown to lower circadian melatonin synthesis (Linch & Deng, 1986). Thus, melatonin may possess the physiologic role of restoring the EOS ability to drive the organism response to stress. These findings have obvious and important implications for immunotherapeutic interventions.

ACKNOWLEDGMENTS

We thank Mrs. E. Hertens for skillful technical assistance and Miss T. Bacciarini for secretarial help.

REFERENCES

ADER, R., ed., 1981. *Psychoneuroimmunology"*, Academic Press, New York. ARENDT, J.; WIRZ-JUSTICE, A. & BRADTKE, J., 1977. Annual rhythm of serum melatonin in man. *Neurosci. Letter, 7* :327-330.

BESEDOWSKY, H.; DEL REY, A.; SORKIN, E. & DINARELLO, A., 1986. Immunoregulatory feedback between interleukin 1 and glucocorticoid hormones. *Science, 233* :652-654.

BIRAU, N.; 1981. Melatonin in human serum: progress in screening investigation and clinic, p. 297-328. In *"Melatonin: Current Status and Perspectives"*, Proc. Int. Symp. on Melatonin, Bremen, Germany, (N. Birau & W. Schlott, eds.), Pergamon Press, Oxford.

BLASK, D.E., 1984. The Pineal: an Oncostatic Gland? p. 253-284. In: *The Pineal Gland*, (R.J. Reiter, ed.). Raven Press, New York.

BROWN, G.M. & NILES, L.P., 1982. Studies on melatonin and other pineal factors. p. 205-253. In: *Clinical Neuroendocrinology, vol. II*, (G.M. Besser & L. Martini eds.). Academic Press, New York.

COHEN, M.R.; PICKAR, D.; DUBOIS, M. & COHEN, R.M., 1986. Studies of the endogenous opioid system in the human stress response. p. 35-47. In: *Enkephalins and Endorphins: Stress and the Immune system*, (N.P. Plotnikoff; R.E. Faith; A.J. Murgo & R.A. Good, eds.), Plenum Press, New York.

DEGUCHI, R. & AXELROD, J., 1973. Control of circadian change of serotonin N-acetyltransferase in the pineal organ by beta-adrenergic receptors. *Proc. Natl. Acad. Sci. USA, 70* :2411-2413.

FOX, B.H. & NEWBERRY, R.H., eds., 1984, *Impact of Psychoneuroendocrine System in Cancer and Immunity*, C.J. Hogrefe, Inc., New York.

GUILLEMIN, R.; COHN, M. & MELNECHUCK, T., eds., 1985. *Neural Modulation of Immunity*, Raven Press, New York.

KELLEY, K.W., 1980. Stress and the immune function. A Bibliographic review., *Ann. Rech. Vet., 11* :445-478.

LINCH, H.J. & DENG, M.H., 1986. Pineal responses to stress., *J. Neural Transm. Suppl., 21* :1-8.

MAESTRONI, G.J.M. & PIERPAOLI, W., 1981. Pharmacologic control of the hormonally mediated immune response. p. 405-425. In: *"Psychoneuroimmunology"*, (R. Ader, ed.), Academic Press, New York.

MAESTRONI, G.J.M.; CONTI, A. & PIERPAOLI, W., 1986a. Role of the pineal gland in immunity. Circadian synthesis and release of melatonin modulates the antibody response and antagonizes the immunosuppressive effect of corticosterone. *J. Neuroimmunol., 13* :19-30.

MAESTRONI. G.J.M.; CONTI, A. & PIERPAOLI, W., 1986b. Melatonin regulates immunity via an opiatergic mechanism., *Clin. Neuropharmacol., 9 Suppl. 4* :479-481.

MAESTRONI, G.J.M.; CONTI, A. & PIERPAOLI, W., 1987a. Role of the pineal gland in immunity. II. Melatonin enhances the antibody response via an opiatergic mechanism., *Clin. Exp. Immunol., 68* :384-391.

MAESTRONI, G.J.M.; CONTI, A. & PIERPAOLI, W., 1987b. The pineal gland and the circadian, opiatergic, immunoregulatory role of melatonin., *Ann. NY Acad. Sci., 469* :67-78.

PLOTNIKOFF, N.P.; FAITH, R.E.; MURGO, A.J. & GOOD, R.A., 1986. *Enkephalins and Endorphins: Stress and the Immune System*, Plenum Press, New York.

SMITH, E.M. & BLALOCK, J.E., 1986. A complete regulatory loop between the Immune and neuroendocrine systems operate through common signal molecules (hormones) and receptors. p. 119-129. In: *Enkephalins and Endorphins: Stress and the Immune system"*, (N.P. Plotnikoff et al., eds.), Plenum Press, New York.

WETTEMBERG, L., 1978. Melatonin in humans. Physiological and clinical studies., *J. Neural Transm., Suppl. 13* :289-310.