ELSEVIER

PII S0024-3205(98)00326-9

Life Sciences, Vol. 63, No. 9, pp. 711-719, 1998

0024-3205/98 $19.00 + .00

SEASONAL EFFECTS OF ROTATIONAL STRESS ON LEWIS LUNG CARCINOMA METASTASIS AND T-LYMPHOCYTE SUBSETS IN MICE

Laura Perissin*, Sonia Zorzet§, Valentina Rapozzi*, Renato Carignola°, Alberto Angeli° and Tullio Giraldi*§.

*: Department of Biomedical Sciences and Technologies, Section of Pharmacology, University of Udine, Udine, Italy.
§: Department of Biomedical Sciences, University of Trieste, Trieste, Italy.
°: Department of Clinical and Biological Sciences, Clinica Medica Generale, University of Torino, Orbassano (Torino), Italy.

(Received in final form June 9, 1998)

Summary

Rotational stress specifically increases the formation of spontaneous lung metastasis in mice bearing Lewis lung carcinoma, without significantly modifying the growth of primary tumor. The increase in metastasis number and volume caused by rotational stress varies in magnitude with a highly significant circannual rhythm; the acrophase approximately coincides with summer solstice. Rotational stress causes a significant reduction in the number of CD3+ and CD4+ T-lymphocyte subsets in summer, whereas in winter the number of CD3+ subset is significantly increased; the CD4+/CD8+ ratio and the number of NK 1.1 antigen positive cells are not significantly modified by rotational stress in both periods considered. The increase in metastasis formation by rotational stress thus appears to negatively correlate with the number of splenic CD3+ and CD4+ T-lymphocyte subsets. This seasonal behavior occurs in spite of the control of light cycle, temperature and humidity in the animal housing, suggesting the existence in the host of an endogenous oscillator with a circannual period. These data indicate the opportunity to consider endogenous rhythms within the host, as well as seasonal factors, in studies on stress and neuro-immunomodulation in experimental oncology.

Key Words: seasonality, circannual rhythm, tumor metastasis, rotational stress, splenic T-lymphocyte, mice

Animal models are a valuable tool for investigating the effects of stress on cancer. The literature on the effects of experimental stressors on tumor initiation and progression in laboratory animals is abundant, and relevant reviews are available (1). Exposure to stress paradigms has in general

Corresponding author: Tullio Giraldi
Department of Biomedical Sciences, University of Trieste, I-34100 Trieste, Italy.
Phone: +39 40 6763539 FAX: +3940577435 Email: giraldi@univ.trieste.it

been associated with variations in tumor incidence and growth in rodents. However, the data reported indicate that variable results were obtained, and that stress may facilitate or inhibit tumor growth, or else may have no discernible effect, depending on the type of stressor and timing of its administration, as well as on the characteristics of the tumor-host system employed (1).

The specific effects of stress on the process of tumor metastasis appear to have been marginally investigated, in spite of the clinical relevance of this phenomenon (2). Several experimental stress paradigms, including rotational and restraint stress, electric foot shock and early weaning, caused a significant and specific increase of pulmonary metastasis formation in mice bearing Lewis lung carcinoma (3, 4). In mice subjected to rotational stress tumor metastatic dissemination was found to be sensitive also to housing, as well as handling stress (3); the latter finding is an accord with the results obtained by other Authors (5).

The systematic repetition of the experiments with rotational stress and Lewis lung carcinoma over several years in our laboratory indicated that the magnitude of the increase in metastasis caused by this stressor is highly variable, and appeared to display a seasonal trend. The aim of this paper has been consequently that to retrospectively submit the data collected over a span of 4 years to a rhythmometric analysis.

The mechanism by which stress may influence tumor incidence and progression, including metastatic dissemination, has been proposed to consist in the modulation of antitumor immune responses of the host via neuro-endocrine circuits (6, 7). T-lymphocyte and NK cell mediated responses significantly participate in the control of the metastatic process (5-7) and display rhythmic characteristics (8-12). Splenic CD3+, CD4+ and CD8+ antigen positive T-lymphocyte subsets, as well as NK 1.1 positive cells, have been consequently determined in mice bearing Lewis lung carcinoma, as a function of rotational stress application, in two different seasonal periods. The results obtained are hereafter reported.

Methods

Animals, tumor transplantation and measurement. The animals used were female C57BL/6 and C57BL/6 X DBA/2 F1 (hereafter called BD2F1) mice weighing 18-20 g, purchased from Charles River, Calco, Como, Italy. Lewis lung carcinoma, originally provided by the National Cancer Institute, Bethesda, MD, USA, is maintained in C57BL/6 mice by subcutaneous injection in the axillary region of 50 mm^3 of minced tumor tissue aseptically prepared from donors similarly inoculated 2 weeks before. For experimental purposes, the tumor is propagated in BD2F1 mice by intramuscular injection of a tumor cell suspension containing 10^6 viable tumor cells (3). Tumor transplantation, as well as primary tumor volume and lung metastasis measurement (on day 14 and at sacrifice on day 21 from tumor inoculation, respectively) were routinely performed as described in detail elsewhere (13).

Animal housing and rotational stress. To reduce the uncontrolled stress from shipment and housing, animals were kept in a protected environment for 2 weeks before each experiment and throughout its duration (14, 15). To avoid overcrowding or isolation effects on tumor progression (14-15), the animals were kept in groups of 5 in plastic cages measuring 27×21×14 cm with a stainless steel grid cover (Techniplast Gazzada Srl, Buguggiate Varese, Italy). To minimize acoustic, olfactory and visual communication the cages were kept in cabinets allowing laminar airflow in between and placed in a room remote from the animals rooms; staff entrance was limited to the supply of water and food. The light-dark cycle in the room was 12-12 hr, with an intensity in the cages of approximately 5 lux. Temperature and relative humidity were constant at 20°C and

60% respectively. Further details on the experimental setting have been reported in detail elsewhere (3). Rotational stress was applied to the animals in the low stress environment by spinning the cages at 45 rpm for 10 min. every hour from time of tumor inoculation until sacrifice on day 21.

Rhythmometric analysis. The variables were analyzed for the effect of time in the year by one-way ANOVA across 12 months consisting of 12 equal division of the year beginning January 1 (rather than calendar months of unequal days). The least-squares fit of a 1-year cosine was used to quantify circannual periodicity and determine rhythm characteristics, using January 1 as the acrophase reference. The rhythm characteristics estimated by the single and population-mean cosinor procedures included the mesor (middle value of the fitted cosine representing a rhythm adjusted mean), the amplitude (the distance from the minimum and maximum of the fitted cosine function), and the acrophase (time of peak value in the fitted cosine function). A p value for rejection of the zero-amplitude assumption was determined for each data series, indicating whether or not the cosine model accounted for a significantly greater proportion of the variability in the time series when compared with the total variability around a flat line (the mean). Rhythm detection was considered statistically significant when $p<0.05$. While the cosinor method involving a single fitted period may not accurately represent the true characteristics of the actual time-dependent variations if asymmetries exist in a time series, the procedure is nevertheless useful for objectively assessing and quantifying periodicity selected *a priori*, in our case the year (16).

Measurement of splenic lymphocyte sub-populations. Tumor bearing mice were subjected to rotational stress and were sacrificed as indicated in Table 2. Individual spleens were processed immediately after sacrifice by cervical dislocation; each spleen was disaggregated and then passed through a double layer of gauze to obtain a single cell suspension. The cells were washed and T-lymphocytes were separated from red blood cells by Ficoll-Hypaque centrifugation (Sigma, St. Louis, MO) (17). The final suspension of splenic T-lymphocytes was labeled with anti-mouse monoclonal antibodies (moAb) using as staining medium PBS, pH 7.4, containing 0.5% BSA and 0.1% NaN_3. Aliquots of 10^6 viable cells in 0.5 ml of staining medium, counted by trypan-blue exclusion test, were incubated in the dark for 30 min at 4°C with 50 µl of rat anti-mouse moAb, CD3 (0.5 µg), CD4 (1 µg), CD8 (1 µg), NK 1.1 (2µg) (Pharmingen, San Diego, CA). Stained cells were examined using an EPICS flow cytometer (Coulter, Miami, FL), each analysis consisted of 10,000 events counted (18). Results for single color analysis are expressed as total number of positive cells collected in the spleen of each animal.

Statistical analysis. Tabled values are group means ± SD. Data were subjected to the appropriate factorial ANOVAs assessing significance against an alfa-level $p<0.05$. When the individual effect of the treatments and the interaction between the independent variables in a 2x2 Anova was significant, the data were subjected to *post hoc* Tukey test for significance of the differences in the mean values. All analyses were performed using standard procedures implemented in the Systat package (SYSTAT Inc., Evanston, IL) (19).

Results

The data on the effects of rotational stress on the progression of Lewis lung carcinoma in mice reported in Table 1 refer to a total of 19 separate experiments performed in different periods of the year over a total of 4 years; the interval between successive time points along a single 1-year scale is less than 2 months. The total population was composed of 126 non-stressed and 137 stressed mice. The analysis by conventional statistics of the pooled data did not reveal significant effects of rotational stress upon the growth of the primary tumor. On the contrary, in the stressed animals

there is a highly significant increase in the number and volume of spontaneous pulmonary metastasis. The dispersion of data is large, as indicated by their standard deviation and range, and even larger in stressed mice than in non-stressed controls (Table 1). The cosinor analysis of the data concerning the primary tumor volume and the formation of lung metastasis, expressed in terms of their actual number or volume, does not reveal the existence of a significant circannual rhythm, either in controls or in the animals subjected to rotational stress. However, when the effects of the application of rotational stress are expressed as fractional increase of metastasis number in stressed mice over non-stressed controls, a highly significant time dependent cyclic variation can be detected (p=0.00019). The rhythm adjusted mean corresponds to an increase of 128.5%, with an amplitude of 48.7 %. The acrophase (maximum percent change) was computed at day 201, approximately coinciding with the summer solstice (Figure 1). Similar results were obtained when the effects of rotational stress on metastasis volume were expressed as fractional increase over non-stressed controls and were analyzed (circannual cosine function, p=0.00018). The mesor corresponds to an increase over the relevant value of controls of 144.5%, with an amplitude of 64.7%; the computed acrophase was at day 214.

The examination of T-lymphocyte subsets in the spleen of the animals show a significant effect of stress, season and of their interaction on CD3+ subset as indicated by separate 2 x 2 ANOVAs, $F_{(1,16)}$=4.38, p=0.05; $F_{(1,16)}$=6.64, p=0.02 and $F_{(1,16)}$=40.8, p<0.0001, respectively. The effects of stress, season and of their interaction are significant also on CD4+ subset, $F_{(1,16)}$=8.03, p=0.01; $F_{(1,16)}$=9.86, p=0.006 and $F_{(1,16)}$=26.7, p<0.0001, respectively. On CD8+ subset, the interaction of stress with season is significant, $F_{(1,16)}$=20.0, p<0.0001, whereas the effects of stress and season are significant on CD4+ / CD8+ ratio, $F_{(1,16)}$=6.04, p=0.03 and $F_{(1,16)}$=21.5, p<0.0001, respectively. For CD3+ T-lymphocyte subset the *post hoc* Tukey test indicates a significant reduction by stress in summer, and a significant increase in winter, whereas for CD4+ a significant reduction by stress in summer, accompanied by a non significant tendency to increase are observed in winter. The 2 x 2 ANOVA shows an effect on NK cell number which is significant for season only, $F_{(1,16)}$=4.66, p=0.05 (see Table 2).

TABLE 1.
Effects of Rotational Stress on Primary Tumor Growth and on the Formation of Spontaneous Lung Metastasis in Mice Bearing Lewis Lung Carcinoma.

RS	n	Primary tumor Volume (cm^3)		Metastasis Number		Metastasis Volume (mm^3)	
		Mean±SD	Median Range	Mean±SD	Median Range	Mean±SD	Median Range
-	126	2.53±1.49	2.34 0.032-7.28	28±20	23 0-99	99.3±71.6	82.0 0-294
+	137	2.74±1.37	2.36 0.21-7.55	36±26[a]	30 2-132	137±104[b]	114 1-495

RS: rotational stress
a, b: means significantly different from non-stressed controls, Student's t test
a: p=0.012, b: p=0.002

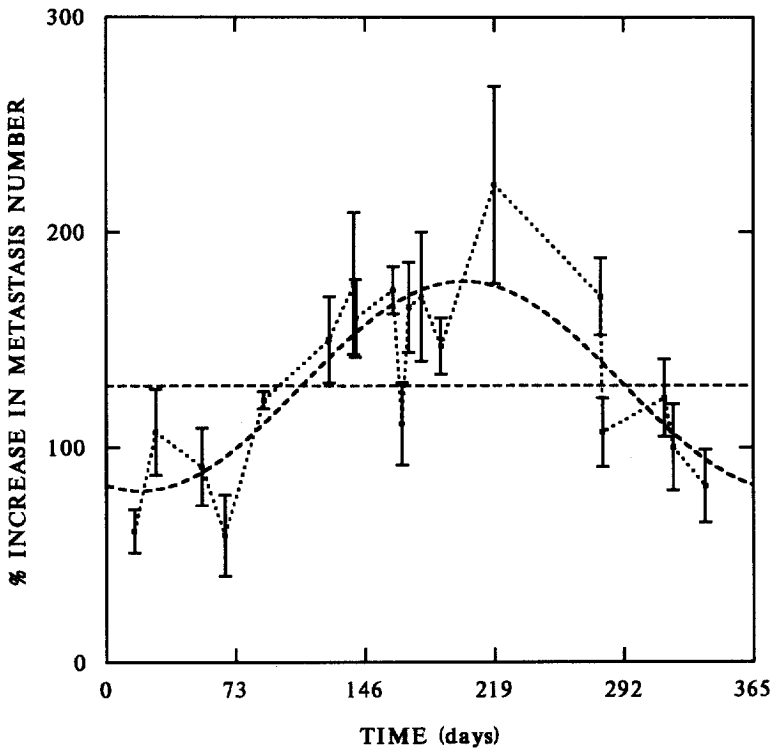

Fig. 1

Chronogram of circannual variations in the mean (±SE) percent increase of metastasis number in mice subjected to rotational stress in comparison with non stressed controls. The time of each experiment is expressed in days starting from January 1st. The sinusoidal dotted line corresponds to the function best fitting the experimental data according to cosinor computation; the horizontal dotted line corresponds to Mesor (mean value of the computed function. Rhythmometric parameters were: Mesor 128.5±6.2, Amplitude 48.7±8.9, Acrophase –201.4±10.6, p=0.00019.

Discussion

Rotational stress, as originally devised by Riley et al., is a well defined experimental paradigm for the application of a relatively mild stress to laboratory mice (15). In addition to the careful characterization and standardization of this experimental procedure, it is worthy to note that its use involves the control and reduction of housing stress (20), which is a neglected factor poten tially contributing to the variability of the results obtained applying experimental stress paradigms to laboratory animals (21). Rotational stress has been shown to facilitate the growth of 6C3HED lymphosarcoma in C3H/He mice (20). More recently, it has been shown to specifically increase the formation of lung metastasis in mice bearing Lewis lung carcinoma, having at the same time negligible effects on the primary tumor (3).

TABLE 2.
Effects of Rotational Stress on Splenic T-Lymphocyte Subpopulations in Mice Bearing Lewis lung Carcinoma

Month	RS	CD3+	CD4+	CD8+	CD4+/CD8+	NK
July	-	50.2±4.8[a]	36.2±4.7[a]	13.8±2.2	2.7±0.7	4.0±1.1
	+	30.8±3.7[a]	20.9±1.4[a]	9.1±0.9	2.3±0.1	3.4±0.5
December	-	41.4±7.3[b]	32.4±6.6	8.1±2.4	4.1±0.8	4.3±0.5
	+	51.3±3.8[b]	36.8±2.4	11.6±2.4	3.3±0.5	4.6±0.7

Each value is the mean (±SD) obtained using groups of 10 mice which were implanted s.c. with Lewis lung carcinoma on day 0, and were subjected to rotational stress (RS) on days 0-21. The animals were sacrificed on day 21, and the number ($x10^6$) of splenic T-lymphocytes positive for CD3, CD4 and CD8 antigens, and of NK cells positive for NK1.1 antigen, was determined by cytofluorimetric analysis. The experiment has been performed in July and in December as indicated. The data were subjected to Anova, whose results are indicated in the Results section. Means marked with the same letter are significantly different, Tukey test, $p<0.05$.

The data presently reported, besides confirming the previous observations showing that rotational stress increases the formation of spontaneous lung metastasis in mice bearing Lewis lung carcinoma, additionally show that the magnitude of the increase in tumor pulmonary dissemination varies sinusoidally following a circannual periodicity, being appreciably greater during summer and smaller during winter.

The seasonality of the effects of rotational stress on metastasis is complemented by an analogous seasonality of action of the stressor upon the number of splenic T-lymphocyte subsets. Indeed, the larger increase in metastasis caused by rotational stress in July is accompanied by a significant reduction in CD3+ and CD4+ T-lymphocyte subpopulations. Conversely, the reduction in metastasis caused by rotational stress in December is concomitant with a significant increase in the CD3+ subset.

This finding is consistent with the view that experimental stress may influence tumor metastatic spread in mice by means of neuroendocrine modulation of antitumor immune responses of the host involving T-lymphocyte effectors (5, 22). This finding is also in agreement with the reported existence of circannual variations in immune functions of the host, as observed for many species including mouse and man (8-12), and with the general enhancement observed during winter conditions, and the ensuing short photoperiod, in several parameters of cell mediated immunity (10).

Evidence has been provided that the pineal gland is essential for the regulation of photoperiodic responses, and that its indolic hormone melatonin is responsible for transmitting day length information (23). The amplitude of the nocturnal peak of melatonin is variable according to the length of the night, and a clear night/day difference in fall and winter but not in spring and early summer was correspondingly observed in the European hamster (24). It is interesting to note that during the experiments reported here, the mice were continuously kept with a 12/12 light/dark illumination cycle and with a constant temperature and humidity, starting with an acclimatization

to these housing conditions for two weeks before undergoing any experimental procedure. In spite of the acclimatization to, and of the stability of, the housing conditions, the animals showed significant seasonal differences in the effects of rotational stress on the parameters considered. The results here reported are thus consistent with the existence of endogenous, genetically programmed rhythms in the circannual domain (25, 26). Moreover, the nocturnal urinary excretion of melatonin was found to be sensitive to the application of rotational stress in BD2F1 mice (27). Preliminary experiments suggest that the effects of rotational stress on nocturnal melatonin urinary secretion may display seasonal effects, but require further examination and confirmation. As far as a possible relationship between immune functions and melatonin is concerned, it is noteworthy that melatonin activates monocytes, inducing cytotoxicity and IL-1 secretion after LPS treatment (28). Moreover, melatonin also activates Th-1 lymphocytes, as observed for CD4+ but not CD8+ cells, via a possible nuclear-receptor mediated mechanism (29).

Glucocorticoids might also be responsible for the seasonality of the presently reported effects. Corticosterone is the most representative glucocorticoid hormone of mice, and has well documented immunoregulatory properties (20) together with seasonal changes of serum concentration observed in male laboratory rats (30). Rotational stress indeed causes a greater increase in plasma corticosterone in winter as compared with summer (unreported results). However, it has to be noted that the administration of mitotane, an inhibitor of corticosterone synthesis, did suppress the expected rise of serum steroid levels by rotational stress above base level (which was not modified by the treatment), but did not significantly modify the effects of the stressor on lung metastasis (31).

In conclusion, the results presently reported appear of interest for the seasonality encountered in the effects of rotational stress on spontaneous metastasis of Lewis lung carcinoma in mice, for the negative correlation found between metastasis formation and the number of splenic T-lymphocyte subsets. The underlying mechanism(s) are not fully clarified at present and appear to require a deeper elucidation. Moreover, the results obtained appear of value for their experimental implications, indicating that usually neglected seasonal factors may be responsible for the inconsistencies appearing in the literature on stress and cancer. Additionally, they may also be pertinent for studies regarding in more general terms stress and neuroimmunomodulation, suggesting the opportunity to standardize the experiments with regard to seasonal or endogenous neuroendocrine rhythms. The seasonal effects on tumor metastasis presently reported do not appear to be limited to Lewis lung carcinoma, and preliminary observations indicate that seasonal dependent responses to stress occur also in mice bearing TLX5 lymphoma or MCa mammary carcinoma subjected to rotation or immobilization procedures. Further experimental work on the effects of different stress paradigms on various tumor-host systems, in relation to neuroimmunomodulation and seasonal factors appears of interest and needed, and is partially in progress in the laboratory of the authors.

Acknowledgements

This work was supported by the Italian National Research Council (CNR) Special Project "ACRO" contract n° 96.00574.PF39, and by MURST 40 to Prof. T. Giraldi, U.S.U. 1995. The generosity of Fondazione C. & D. Callerio in providing the protected animal rooms is gratefully appreciated. The authors wish to thank Dr. Alberta Bergamo for her skillful technical assistance during flow cytometric analysis.

References

1. A. JUSTICE, Psychol. Bull. **98** 18-138 (1985).
2. K. BAMMER, Stress and Cancer .K. Bammer and B.H. Newberry (Eds), 137-163, C.J. Hogrefe, Toronto (1981).
3. T. GIRALDI, L. PERISSIN, S. ZORZET, P. PICCINI and V. RAPOZZI, Eur. J. Cancer Clin. Oncol. **25** 1583-1588 (1989).
4. T. GIRALDI, L. PERISSIN, S. ZORZET, V. RAPOZZI and M.G. RODANI, Intern. J. Neuroscience **74** 265-278 (1994).
5. J.A. MOYNAHAN, G.J. BRENNER, R. COCKE, J.D. KARP, S.M. BRENEMAN, J.M. DOPP, R. ADER, N. COHEN, L.J. GROTA and S.Y. FELTEN, Handbook of human stress and immunity R. Glaser and J. K. Kiecolt-Glaser (Eds), 1-22, Academic Press, San Diego, (1994).
6. M.M. MAREEL, P. DE BAETSERLIER and F.M. VAN ROY (Eds.), Mechanisms of invasion and metastasis , 419-485, CRC Press, Boca Raton, (1991).
7. D.L. BELLINGER, K.S. MADDEN, S.Y FELTEN and D.L. FELTEN, The Psychoimmunology of cancer, C.E. LEWIS, C. O'SULLIVAN and J. BARRACLOUGH (Eds.), 55-106, Oxford University Press, (1994).
8. M.A. BROCK, J. Immunol. **130** 2586-2589 (1983).
9. F.A. LEVI, C. CANON, Y. TOUITOU, A. REINBERG and G. MATHÉ, J. Clin. Invest. **81** 407-413 (1988).
10. R.J. NELSON, G.E. DEMAS, S.L. KLEIN and L.J. KRIEGSFELD, J. Pineal Res. **19** 149-165 (1995).
11. A.I. ESQUINO, L. SELGAS, A. ARCE, V. DELLA MAGGIORE and D.P. CARDINALI, Brain Behav. Immun. **10** 92-102 (1996).
12. A. ANGELI, G. GATTI, M.L. SARTORI and R.G. MASERA, Chronobiologia **19** 93-110 (1992).
13. L. PERISSIN, S. ZORZET, P. PICCINI, V. RAPOZZI and T. GIRALDI, Clin. Expl. Metastasis **9** 541-549 (1991).
14. R.C. LABARBA, Psychosom. Med. **32** 258-276 (1970).
15. V. RILEY, M.A. FITZMAURICE and D.H. SPACKMAN, Perspectives on behavioral medicine, S.M. Weiss, J.A. Herd and B.H. Fox (Eds), 371-400, Academic Press, New York (1981).
16. W. NELSON, Y.L. TONG, J.K. LEE and F. HALBERG, Chronobiologia 6, 305-323 (1979).
17. S.V. HUNT, Lymphocytes a practical approach, C.G.B. Klaus (Ed), 1-34, IRL Press, Oxford (1987).
18. G. DASIC, S. PACOR, A. BERGAMO, G. SALERNO, B. VRANESIC, R. JUKIC, I. TOMASIC and G. SAVA, Int. J. Oncol. **5** 275-284 (1994).
19. SYSTAT for DOS: Quick Reference, Version 6 Edition. Evanston, IL: SYSTAT, Inc., (1994).
20. V. RILEY, Science **212** 1100-1109 (1981).
21. Z. STEPLEWSKI, P. ROBINSON-GOLDMAN and W.H. VOGEL, Cancer Lett. **34** 257-261 (1987).
22. W.H. VOGEL and D.B. BOWER, N. Plotnikoff, A. Murgo, R. Faith and J. Wybran (Eds), Stress and Immunity, 493-507, CRC Press, Boca Raton (1991).
23. R.J. REITER, Endocr. Rev. **12** 151-180 (1991).
24. P. PEVET, B. VIVIEN-ROELS and M. MASSON-PEVET, Role of melatonin and pineal peptides in neuroimmunomodulation, F. Fraschini and R. J. Reiter (Eds), 147-156, Plenum Press, London-New York (1991).
25. F. HALBERG, J.M. LAGOGUET and A. REINBERG, Int. J. Chronobiol. **8**, 225-268 (1983).
26. H.V. RATAJCZAK, P.T. THOMAS, R.B. SOTHERN, T. VOLLMUTH and J.D. HECK,

Chronobiol. Intern. **10** 383-394 (1993).
27. L. PERISSIN, S. ZORZET, V. RAPOZZI and T. GIRALDI, J. Pineal Res. **15** 138-140 (1993).
28. K.M. MORREY, J.A. MCLACHLAN, C.D. SERKIN and O. BAKOUCHE, J. Immunol. **153** 2671-2680 (1994)
29. S. GARCIA-MAURINO, M.G. GONZALES-HABA, J.R. CALVO, M. RAFII-EL-IDRISSI, V. SANCHEZ-MARGALET, R. GOBERNA and J.M. GUERRERO, J. Immunol. **159** 574-581 (1997)
30. WONG, K.D. DOHLER, M.J. ATKINSON, M. GEERTINGS, R.D. HESCH and A. VON ZUR MUHLEN, (1983). J. Endocrinol. **97** 179-185 (1983).
31. L. PERISSIN, S. ZORZET, P. PICCINI, V. RAPOZZI and T. GIRALDI, Pharmacol. Res. **21** 461-462 (1989).