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Melatonin administration in tumor-bearing mice (intact and pinealectomized) in relation to stress, zinc, thymulin and IL-2

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Abstract

Melatonin (MEL) may counteract tumors through a direct oncostatic role. MEL is also an antistress agent with immunoenhancing properties against tumors due to a suppressive role of MEL on corticosterone release. Rotational stress (RS) (spatial disorientation) facilitates metastasis progression in mice. Also, MEL counteracts tumors because of its influence on immune responses via the metabolic zinc pool, which, is reduced in tumors and stress. Zinc is required for normal thymic endocrine activity (i.e. thymulin) and interleukin-2 (IL-2) production. Because in vivo data is still controversial, exogenous MEL treatment (22 days in drinking water) in both intact and pinealectomized (px) mice bearing Lewis lung carcinoma leads to significant decrements of metastasis volume, restoration of the negative crude zinc balance, recovery of thymulin activity and increment of IL-2 exclusively in intact and px tumor bearing mice subjected to RS. Significant inverse correlations are found in both stressed intact and px tumor bearing mice after MEL treatment between zinc and corticosterone ($r = 0.78$, $P < 0.01$; $r = 0.80$, $P < 0.01$, respectively). Positive correlations between zinc and IL-2 ($r = 0.75$, $P < 0.01$; $r = 0.73$, $P < 0.01$, respectively) or thymulin ($r = 0.75$, $P < 0.01$; $r = 0.82$, $P < 0.01$, respectively) are observed in same models of mice. These findings suggest a MEL action to decrease metastasis mediated by a possible interplay between zinc and MEL, via corticosterone, with consequent restoration of thymic efficiency and IL-2 production. MEL as an antistress agent with immunoenhancing properties in cancer deserves further consideration. © 1999 International Society for Immunopharmacology. Published by Elsevier Science Ltd.

Key words: Zinc; Melatonin; Stress; Metastasis; Thymulin; Corticosterone; IL-2

Abbreviations: AAS, atomic absorption spectrophotometry; ELISA, enzyme-linked immunoabsorbent assay; FTS, inactive zinc-unbound thymulin; I, intact mice; IL-2, interleukin-2; MEL, melatonin; MP, metabolic period; NF-kB,

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nuclear factor-kb; POMC, proopiomelanocortin; Px, pinealectomized mice; RIA, radioimmunoassay; RS, rotational stress; SDI, stressed intact mice; SDPx, stressed pinealectomized mice; TNF-α, tumor necrosis factor-α; ZnFTS, active zinc-bound thymulin; ZnFTS + FTS, total thymulin.

1. Introduction

Melatonin (MEL) counteracts tumors with a direct oncostatic role or as an antistress agent with immunoenhancing properties. In vitro MEL inhibits tumor growth (Shellard, Whelan & Hill, 1989; Cos & Blask, 1994; Cos, Fernandez & Sanchez-Barcelo 1996). Leukemia bearing mice have shown an antistress role of MEL with immunoenhancing properties against tumors because of a suppressive action of MEL on corticosterone (Maestroni & Conti, 1990). Rotational Stress (RS) facilitates the progression of tumors in mice, via neuroendocrine-immune modulation effectors of the host (Justice, 1985; Vogel & Bower, 1991; Giraldi, Perissin, Zorzet, Piccini & Rapozzi, 1989). In turn, RS affects MEL production in BD2F1 mice (Giraldi, Perissin, Zorzet & Rapozzi 1994a; Giraldi, Perissin, Zorzet, Rapozzi & Rodani, 1994b; Perissin, Zorzet, Rapozzi, Paoletti & Giraldi, 1994), enhancing circulating MEL in hybrid BD2F1 mice (Perissin, Zorzet, Rapozzi & Giraldi, 1993), despite no MEL induction has been reported in C57BL/6 mice (Ebihara, Marks, Hudson & Menaker, 1986). MEL action against tumors also occurs because of MEL's immunomodulatory role (Caroleo, Frasca, Nistico' & Doria, 1992; Maestroni, 1993; Pierpaoli & Regelson, 1994; Conti & Maestroni 1995). In vivo and in vitro data have recently shown MEL to affect thymic endocrine activity (i.e. thymulin) and interleukin-2 (IL-2), in young pinealectomized (px) and old mice by means of metabolic zinc pool turnover, via glucocorticoids pathway. The interplay between zinc and MEL, via corticosterone, for normal immune efficiency has assigned to MEL a role as an immunomodulating agent (Mocchegiani et al., 1994a, 1996, 1998). Zinc deficiency, impaired thymulin activity and altered serum IL-2 occur both in cancer and stress (Fabris & Mocchegiani, 1995). Zinc is required both for thymulin activity which regulates T-cell mediated immunity (Dardenne et al., 1982) and for IL-2 production by lymphocytes (Tanaka, Shiozawa, Moromoto & Fujita, 1990). Circulating IL-2 is also a good marker to test immune impairment in zinc-deficient cancer patients (Prasad, Beck, Grabowski, Kaplan & Plathog, 1997a). With these premises, MEL administration was carried out in px and intact mice bearing Lewis lung carcinoma subjected or not to RS, and metastasis volume, metabolic zinc pool, thymulin activity, corticosterone and IL-2 plasma levels were measured in order to further verify MEL's antistress role with immunoenhancing properties (Maestroni & Conti, 1990) and/or its direct oncostatic role (Shellard et al., 1989; Cos & Blask, 1994; Cos et al., 1996). MEL action against tumors is still controversial and undefined (Stanberry, Das Gupta, & Beattie, 1983; Blask, 1984; Slominski & Pruski, 1993; Giraldi et al., 1994a,b; Karasek, 1997) and requires urgent clarification.

2. Experimental procedures

2.1. Animals, tumor transplantation and measurements

Female BD2F1 mice (C57BL/6 × DBA/2 F1) (2 months of age) (Charles River, Calco, Como, Italy) weighing 18–21 g, were used. Lewis lung carcinoma was provided by the National Cancer

Institute, Bethesda MD, U.S.A. The subcutaneous propagation of tumor in BD2F1 mice, as well as the measure of primary tumor volume and lung metastasis were performed as described elsewhere (Giraldi et al., 1989; Giraldi et al., 1994a,b). All animals were weighed before the experiment and at sacrifice to determine whether the effects seen were related to dietary intake (Mocchegiani et al., 1998). Hybrid BD2F1 mice were chosen because the syngenic implantation of Lewis lung carcinoma, whose malignant progression is sensitive to RS, works well in this strain (Giraldi et al., 1989).

2.2. Animal housing and rotational stress

Mice, with drinking water and pellet food (Nossan, Italy) ad libitum, were kept 5 per cage in order to avoid the effects of overcrowding or isolation on tumor progression (Riley, Fitzmaurice & Spackman, 1981). The cages were placed in a low housing stress (protected) environment for 2 weeks before tumor inoculation, in order to allow the animals to recover from the stress of shipment (Riley et al., 1981). As such, mice become adapted to the new housing conditions. Temperature and humidity were constant (20°C and 60%, respectively). The cycle of illumination was 12/12 h. (lights on from 0800–2000 h) with an intensity of 2000 lux in the cages. RS (spatial disorientation) 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, as described elsewhere (Giraldi et al., 1989; 1994b). RS is a good model for stress and tumor progression studies in animals (Riley et al., 1981; Justice, 1985; Giraldi et al., 1989; 1994a,b).

2.3. Pinealectomy and melatonin administration

Surgical pinealectomy was performed using a procedure described previously (Moechegiani et al., 1996). A post mortem inspection of the brain was performed at sacrifice in order to exclude in px animals pineal gland presence. The pinealectomy was performed on mice at 2 months of age. Tumour inoculation and melatonin treatment were performed 2 months later because 2 months is sufficient time to allow for the disappearance of endogenous nocturnal peak of MEL in inbred mice (Conti & Maestroni 1996).

MEL (1.25 mg/Kg/night) was administered into the drinking water for 22 days in order to see MEL effects on metastasis volume in relation to RS in tumor bearing BD2F1 mice (Giraldi et al., 1994a,b). MEL (6.2 mg/l) was dissolved in water containing 0.2% ethanol. The MEL-water was administered during the dark/night period (2000–08:00 h), from tumor inoculation until sacrifice. MEL-containing bottles were removed from 0800–2000 h. Normal tap water was administered during this light/day period. MEL treatment, tumor inoculation and rotational stress were carried out in mice at the age of 4 months. Primary tumor volume was determined on day 14 and lung metastasis were measured at sacrifice on day 22, respectively, from tumor inoculation. The age of mice (experimental groups and controls) averaged 4.7 months at sacrifice. The experimental model was carried out in May–June because of seasonal variations of MEL and the effect of RS on metastasis volume were more evident in spring and summer (Perissin et al., 1994)

2.4. Metabolic zinc assessment

The metabolic period (MP) for zinc assessment was performed for 7 days before the sacrifice (Mocchegiani et al., 1994a). The measurements of food and water intake and of urinary and faecal

excretion were performed every day for each mouse. Zinc was measured in food and water, and the zinc intake was calculated on the basis of food and water consumption per day. Zinc was measured in urine and faeces and the zinc excretion was calculated on the basis of urine and faeces excretion per day. The difference between zinc intake and zinc excretion is the crude zinc balance (metabolic zinc pool) (Mocchegiani et al., 1994a). In order to avoid the stress of placing the mice in metabolic cages, urine and faeces over 24 h were collected on Wathman 3MM chromatographic paper placed below a stainless steel grid and covering the bottom of the cages (Perissin et al., 1993).

2.5. Zinc determination

2.5.1. Blood

Heparinized blood samples for zinc measurements were collected in fluorinated tubes (No. 115317, LP, Italy) and centrifuged 20 min later at 3000 ***g*** for 10 min. Zinc was determined by A.A.S. according to the method and zinc references standard of Fernandez & Khan (1972).

2.5.2. Urine

Zinc determination in urine samples was carried out over 24 h by A.A.S. using a dry-washing technique and 5 ml of HCl 6N for zinc extraction, according to the method and zinc references standard, as suggested by Chapman & Pratt (1961).

2.5.3. Faeces

The determination of zinc in faeces was carried out by A.A.S. according to the method of Lodefoged (1980). Briefly, 2 g of faeces was boiled for 30 min in 15 ml of a mixture of HCl-HNO_3 and deionized water in the proportion 27 : 3 : 20. After boiling, the mixture was diluted to 50 ml with deionized water, and then a reading was taken at A.A.S. using zinc references standard (Lodefoged, 1980).

2.5.4. Food

The method of Lodefoged (1980) was used to analyse zinc in food. Briefly, 25 ml of hydrochloric acid solution was added to 5 g of food sample, brought to boiling point and filtered through No. 1 Whatman filter paper. The filtrate was directly analysed against the zinc references standard (Lodefoged, 1980).

2.5.5. Water

The determination of zinc in the drinking (tap) water was performed by A.A.S. using the method and zinc references standard of Fishman & Downs (1966).

2.6. Thymulin determination

This technique, originally developed by Bach, Dardenne, Pleau and Bach (1975) and described elsewhere (Mocchegiani et al., 1994a,b), is specific for zinc-bound active thymulin (ZnFTS), because the assay is unaffected by other thymic hormones, and the rosette-inducing activity is completely removed by passing plasma samples through an antithymulin immunoadsorbent. The sensitivity of the bioassay makes it possible to detect 1 pg/ml of synthetic thymulin (Sigma, U.S.A.).

The assay is reliable; in two consecutive blind assays a difference no greater than $1/\log_2$ was found in all samples. In order to avoid possible interference by zinc bioavailability, thymulin measurements were performed concomitantly with the in vitro addition of zinc sulphate to the plasma samples at a final concentration of 200 nM, which is the optimal concentration for unmasking zinc-unbound inactive thymulin (FTS). As such, the total amount of thymulin produced (zinc-bound ZnFTS + zinc-unbound FTS) can be evaluated in the circulation (Mocchegiani et al., 1994a,b). Thymulin activity is expressed as $1/\log_2$. The apparently low molar concentration of zinc required may be explained by the fact that the bioavailable free zinc is no more than 2–3% of total plasma zinc, the majority being bound to proteins which are retained by the 50,000 mol wt cut-off membranes (Mocchegiani et al., 1994a,b). This bioassay is still required because thymulin radioimmunoassay developed recently is unable to discriminate between zinc-bound and zinc-unbound thymulin (Safieh et al., 1990).

2.7. Corticosterone determination

Plasma corticosterone was tested in blood samples collected between 0900 and 1000 h by intracardiac puncture immediately after sacrifice by cervical dislocation, with the greatest care being taken not to cause further stress by these procedures. Moreover, mice were sacrificied in a separate room from others in the sequence: control mice, tumor-bearing mice, and tumor-bearing mice + MEL, in order to avoid the possibility of stress caused by the sacrifice (Mocchegiani et al., 1998). The analysis was determined by using RIA rat-corticosterone-^{3}H kit (ICN Biomedicals, Inc, CA, U.S.A.) which used a specific anti-corticosterone antibody. The data obtained (expressed in ng/ml) were referred against a standard curve. The percentage of cross reaction with other steroids was <0.01. The sensitivity was of 0.05 ng/ml of corticosterone.

2.8. Interleukin-2 determination

Interleukin 2 (IL-2) was measured in plasma samples by means of an enzyme-linked immunoabsorbent assay (Inter Test 2X Mouse IL-2 ELISA kit, Genzyme, U.S.A.) based on a sandwich principle using monoclonal anti-mouse(m)IL-2 in conjunction with biotinylated polyclonal anti-mIL-2 antibody. A peroxidase-conjugate, streptavidin, which binds to biotin-tagged immune complexes captured in the plate, was used. The results, expressed in pg/ml, correspond to the average of two separate assays. The specificity of the results was confirmed using an Inter Test-2X kit with other mouse or rat recombinant cytokines that did not produce absorbances above the 0 pg/ml standard. A standard curve was obtained by plotting the concentrations of mIL-2 standards vs their resulting absorbances. The mIL-2 concentrations in experimental samples were determined from the standard curve. The sensitivity of the ELISA mIL-2 kit ranged between 15 and 960 pg/ml.

2.9. Statistical analysis

The significance of the differences between means was assessed using paired Student's *t*-tests, Mann–Whitney tests and ANOVA tests (one-way). Correlations were determined via linear regression analysis by the least-square method. The difference between the various regression lines was evaluated by analysis of covariance. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Pinealectomy, melatonin, tumor progression and rotational stress

Pinealectomy, melatonin treatment or RS do not cause any significant effect on primary tumor growth in mice bearing Lewis lung carcinoma (Table 1). In contrast, RS increases the volume of lung metastasis in intact mice as compared to respective controls (146.8 vs 69.6) ($P < 0.05$) (Table 1). Surgical pinealectomy does not modify metastasis volume in non-stressed mice (126.6 vs 69.6). Significant increments of metastasis volume are observed in px stressed mice, as compared to respective controls (386.0 vs 126.6) or to intact stressed controls (386.0 vs 146.8) ($P < 0.05$) (Table 1). MEL treatment is ineffective in reducing metastasis volume in non-stressed mice, with or without pinealectomy (175.6 vs 69.6; 222.4 vs 126.6, respectively) (Table 1). MEL treatment is effective in reducing metastasis volume in intact stressed (114.8 vs 146.8) and in px stressed mice (116 vs 386) ($P < 0.05$) as compared to respective controls (Table 1). Influences of dietary intake as well as of RS on body weight are avoided because of no body weight differences between treated and untreated MEL tumor bearing mice (intact and px mice with or without RS) at sacrifice as compared to values observed at the beginning of experiment (data not shown). The night-MEL drinking water uptake is 4.8–5 ml/night/mouse. No differences are found for this parameter between stressed and non-stressed mice (intact and px) and respective controls. No significant

Table 1
Effect of pinealectomy, exogenous melatonin administration and rotational stress in primary tumor growth and lung metastasis formation

Mice	Mel	RS	Primary tumor volume (cm^3)	Metastasis volume (mm^3)
Intact	−	−	3.5±0.42	69.6±15.2
Px	−	−	3.6±0.37	126.6±31.4
Intact	+	−	2.9±0.16	175.6±46.3
Px	+	−	2.9±0.26	222.4±49.5
Intact	−	+	3.5±0.36	146.8±24.5*
Px	−	+	2.9±0.24	386.0±13.2**
Intact	+	+	3.1±0.29	114.8±16.6***
Px	+	+	3.1±0.23	116.0±17.0***
Sham–px	−	−	3.5±0.21	74.2±9.2+
Sham–px	−	+	3.2±0.19	162.6±15.2

10 animals for each experimental group: 5 animals for cage. RS = Rotational stress Mean±SD.
* $P < 0.05$ when compared to the values of non-stressed controls (146.8 vs 69.6) (Mann–Whitney test).
** $P < 0.05$ when compared to values of Px alone (386.0 vs 126.6) or to RS alone (386.0 vs 146.8) (Mann–Whitney test).
*** $P < 0.05$ when compared to Px stressed mice (116 vs 386.0) or to intact stressed mice (114.8 or 105.0 vs 146.8) (Mann–Whitney test).
No significant differences exist in primary tumor growth and in metastasis volume between no stressed, sham–px and intact mice (74.2 vs 69.6) and between stressed sham–px and intact mice (162.6 vs 146.8) after two months from pinealectomy, whereas significant differences exist between non-stressed and stressed sham–px mice (+ $P < 0.01$) (74.2 vs 162.6) (Mann–Whitney test).

Table 2
Pinealectomy, melatonin treatment and neoplasia: zinc metabolism

	Crude zinc balance (μg/day/mouse)	Zincaemia (μg/dl)
A		
Intact	$+1.6 \pm 0.5$	170.2 ± 2.8
Intact + tumor	-2.2 ± 0.8	60.1 ± 9.4*
Intact + tumor + MEL	-1.05 ± 0.7	56.7 ± 9.7
B		
Sham–px	$+1.3 \pm 0.6$	158.4 ± 3.7
Sham–px + tumor	-2.0 ± 0.9	63.4 ± 7.7*
Px	-1.9 ± 0.8	88.7 ± 5.0
Px + tumor	-4.0 ± 1.0	67.5 ± 11.0*
Px + tumor + MEL	-3.0 ± 0.8	56.2 ± 9.7

10 animals for each experimental group.
Mean + SD.
* $P < 0.001$ when compared to intact, Px control, sham–px, respectively.

differences in volume metastasis are observed between intact and sham–px tumor bearing mice subjected or not subjected to RS (Table 1). Significant differences are observed in sham–px tumor bearing mice subjected to RS as compared to non RS subjected mice (162.6 vs 74.2) ($P < 0.01$), as it occurs in intact stressed mice (146.8 vs 69.6) ($P < 0.05$) (Table 11).

3.2. Pinealectomy, MEL and crude zinc balance

Crude zinc balance is positive in intact mice (Table 2A). The tumor induces a negative crude zinc balance with reduced zincaemia in intact mice as compared to respective controls. The crude zinc balance is negative both in px controls and in px tumor bearing mice (Table 2B). MEL treatment is ineffective in restoring a crude zinc balance and plasma zinc levels in both tumor bearing intact and px mice (Table 2A,B). More marked negative crude zinc balance and more reduced zincaemia are observed in presence of tumor (px and intact) (Table 2A,B). The crude zinc balance is negative despite the zinc content in Nossan (Como, Italy) food being 208 pp/m. No differences exist between intact and sham–px mice in presence/absence of tumor, respectively (Table 2A,B).

3.3. Pinealectomy, MEL, crude zinc balance and rotational stress

The crude zinc balance is negative in stressed intact controls whereas plasma zinc levels are in the normal range (Table 3A). The tumor induces negative crude zinc balance associated with low plasma zinc levels (Table 3A). MEL treatment restores crude zinc balance as well as plasma zinc levels (Table 3A). Px stressed mice show a negative crude zinc balance which is more marked in

Table 3
Pinealectomy, melatonin treatment, neoplasia and rotational stress: zinc metabolism

	Crude zinc balance (μg/day/mouse)	Zincaemia (μg/dl)
A		
Stressed intact controls	-1.9 ± 0.7	130.0 ± 10.0
Stressed intact + tumor	-2.8 ± 0.8	58.4 ± 4.6*
Stressed intact + tumor + MEL	$+0.2 \pm 0.7$	95.0 ± 4.3
B		
Stressed Sham–px	-1.8 ± 0.7	120.7 ± 12.0
Stressed Sham–px + tumor	-2.5 ± 0.6	63.0 ± 6.7*
Stressed Px	-2.4 ± 0.8	85.0 ± 4.8
Stressed Px + tumor	-4.7 ± 0.9	68.0 ± 5.6
Stressed Px + tumor + MEL	$+2.0 \pm 0.8$	109.8 ± 5.9

10 animals for each experimental group.
Mean $\pm$ SD.
* $P < 0.001$ when compared to stressed intact, stressed Px control, sham–px, respectively.

the presence of tumor (Table 3B). MEL treatment restores crude zinc balance as well as plasma zinc levels in stressed px tumor bearing mice (Table 3B). No differences exist in negative crude zinc balance between stressed sham–px and intact mice in presence/absence of tumor, respectively (Table 3A,B).

3.4. Pinealectomy, MEL, and thymic endocrine activity

Active thymulin (ZnFTS) and total thymulin (active ZnFTS + inactive FTS) plasma levels are in the normal range for the age in intact (I) mice (Fig. 1A). The tumor leads to a significant reduction of ZnFTS as compared to intact controls ($P < 0.001$), whereas total thymulin levels are not affected by the tumor (Fig. 1A). MEL treatment is ineffective in restoring ZnFTS in tumor bearing mice (Fig. 1A). Plasma ZnFTS levels are low in px mice as compared to intact controls ($P < 0.001$) (Fig. 1B). Tumour and MEL treatment do not affect reduced ZnFTS levels in px mice (Fig. IB). Total thymulin levels are in the normal range for the age in px mice with no modifications by tumor or MEL treatment (Fig. 1B). No significant differences exist in active and total thymulin between intact and sham–px mice in presence/absence of tumor, respectively (Fig. 1A,B)

3.5. Pinealectomy, MEL, thymic endocrine activity and rotational stress

Stressed intact mice without tumor (SDI) show no significant modifications of ZnFTS and total thymulin plasma levels as compared to intact controls (I) (Fig. 2A). The tumor causes a marked reduction of ZnFTS ($P < 0.001$), whereas total thymulin is not modified (Fig. 2A). MEL treatment restores ZnFTS (Fig. 2A). Px stressed mice (SDPx) show decreased ZnFTS as compared to SDI

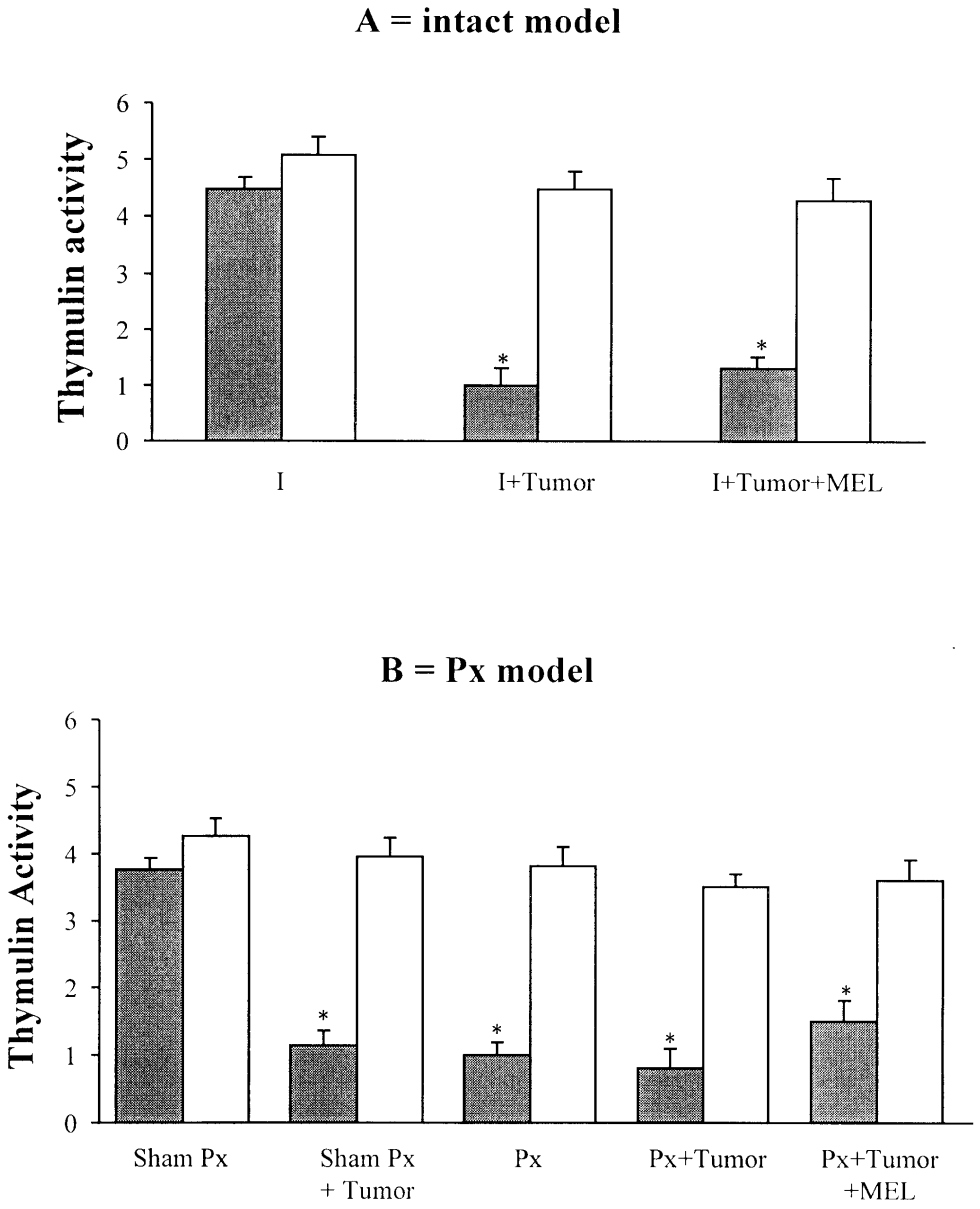

Fig. 1. Plasma active thymulin (ZnFTS) (■) and total thymulin (ZnFTS+FTS) (□) in intact (I) mice (Fig. 1A) and in pinealectomized (Px) mice (Fig. 1B) after injections of Lewis lung carcinoma and the effect of melatonin treatment in both models of tumor bearing mice. Thymulin activity is expressed in $1/\log_2$. * $P < 0.001$ when compared to active thymulin of intact or sham–px controls (I) Mean+SD.

mice ($P < 0.05$). More marked reduction of ZnFTS is induced by the tumor in SDPx mice as compared to SDPx controls ($P < 0.05$) (Fig. 2B). MEL treatment increases ZnFTS in SDPx tumor bearing mice (Fig 2B). No differences in total thymulin are observed in these three px mice groups

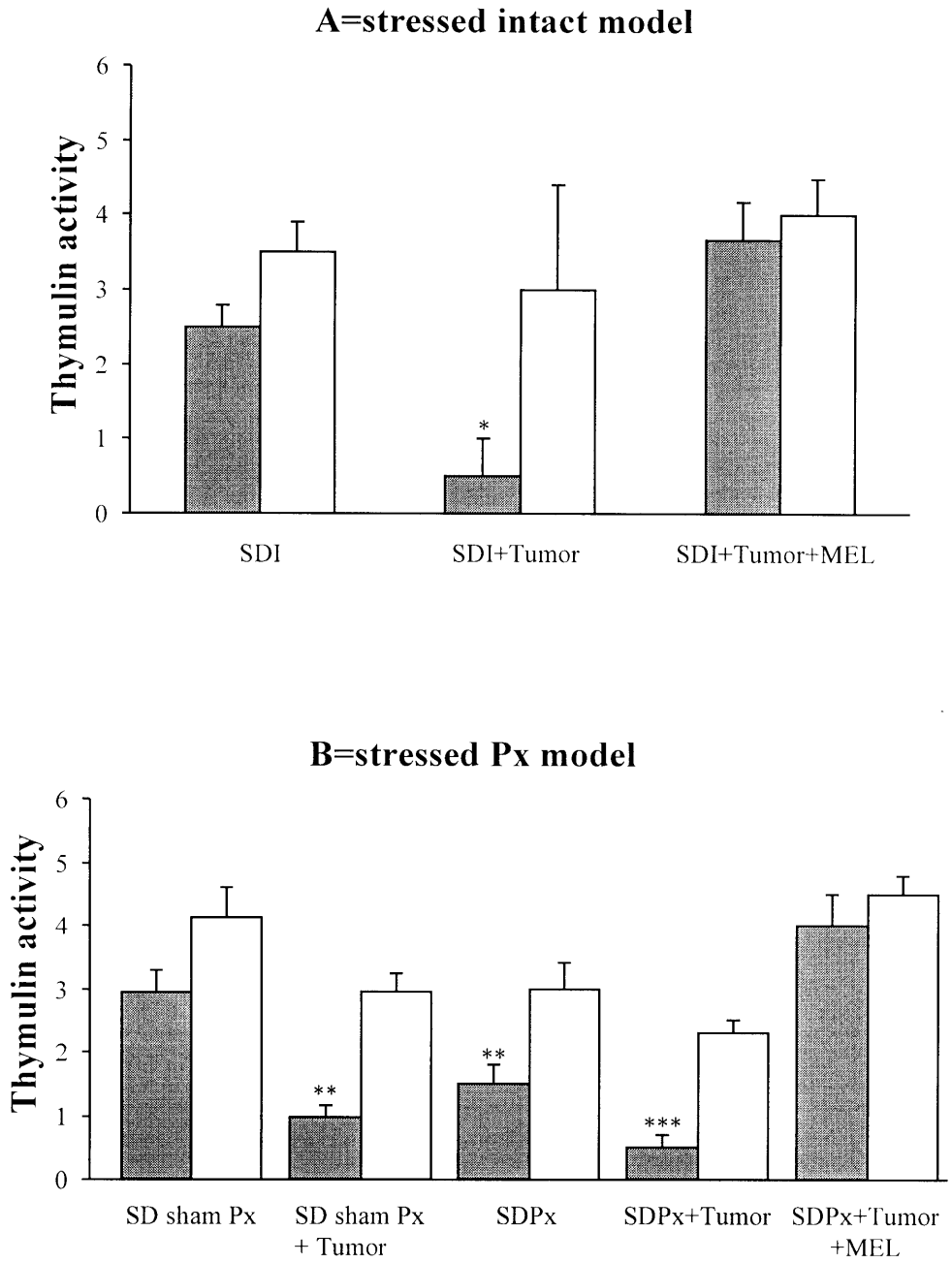

Fig. 2. Plasma active thymulin (ZnFTS) (■) and total thymulin (ZnFTS+FTS) (□) in stressed intact mice (SDI) (Fig. 2A) and in stressed pinealectomized mice (SDPX) (Fig. 2B) after injections of Lewis lung carcinoma and the effect of melatonin treatment in both models of stressed tumor bearing mice. Thymulin activity is expressed in $1/\log_2$. * $P < 0.001$ when compared to active thymulin of SDI or SD sham–px controls; ** $P < 0.05$ when compared to active thymulin of SDI or SD sham–px controls; *** $P < 0.05$ when compared to active thymulin of SDPx controls. Mean+SD.

(Fig. 2B). Significant positive correlations are found between ZnFTS and zinc in both stressed tumor bearing intact and px mice treated with MEL ($r = 0.75$, $P < 0.01$; $r = 0.82$, $P < 0.01$, respectively), whereas they are absent in non-stressed tumor bearing mice treated with MEL (intact and px). No significant differences exist in active and total thymulin between SDI and stressed (SD) sham–px mice in presence/absence of tumor, respectively (Fig. 2A,B).

3.6. Corticosterone

Corticosterone is increased in px mice as compared to intact mice (230 vs 135) ($P < 0.001$) (Table 4A,B). Tumour and MEL treatment do not lead to any significant modifications of corticosterone in intact and px mice as compared to respective controls (Table 4A,B). RS induces significant increments of corticosterone in intact mice as compared to intact controls (177 vs 135) ($P < 0.05$), whereas the increased levels of corticosterone in px mice are not affected by RS (240 vs 230) (Tables 4 and 5A,B). The tumor does not affect corticosterone in stressed intact mice (173.9 vs 177), whereas it leads to significant increments exclusively in px stressed mice as compared to px stressed controls (330.7 vs 240) ($P < 0.01$) (Table 5A,B). MEL treatment reduces corticosterone in both stressed tumor bearing intact and px mice (109.4 vs 173,9; and 277 vs 330.7, respectively) ($P < 0.01$) (Table 5A,B). A trend to corticosterone reduction is also observed after MEL treatment in both non-stressed tumor bearing intact and px mice (128 vs 136 and 288.4 vs 311.6, respectively) (Table 4A,B). Significant inverse correlations are found between corticosterone and zinc in both stressed tumor bearing intact and px mice treated with MEL ($r = -0.78$, $P < 0.01$; $r = -0.80$, $P < 0.01$, respectively), whereas they are absent in non-stressed tumor bearing mice treated with MEL (intact and px). The stress interference due to pinealectomy is avoided because of no significant corticosterone differences between sham–px and intact mice, and between stressed sham–px and

Table 4
Pinealectomy, melatonin treatment and neoplasia: corticosterone plasma levels

	Corticosterone (ng/ml)
A	
Intact	135 ± 3.5
Intact + tumor	136 ± 4.4
Intact + tumor + MEL	128 ± 3.5
B	
Sham–px	128 ± 3.8
Sham–px + tumor	138 ± 11.9
Px	230 ± 26*
Px + tumor	311.6 ± 25.1
Px + tumor + MEL	288.4 ± 47.2

* $P < 0.001$ when compared to intact control and sham–px mice.

Table 5
Pinealectomy, melatonin treatment, neoplasia and rotational stress: corticosterone plasma levels

	Corticosterone (ng/ml)
A	
Stressed intact	177.0 ± 28*
Stressed intact + tumor	173.9 ± 27
Stressed intact + tumor + MEL	109.4 ± 18.2***
B	
Stressed Sham–px	181.0 ± 29
Stressed Sham–px + tumor	182.1 ± 31
Stressed Px	240.0 ± 26
Stressed Px + tumor	330.7 ± 24.2**
Stressed Px + tumor + MEL	277.2 ± 21***

* $P < 0.05$ when compared to non-stressed intact control (Table 5A).
** $P < 0.01$ when compared to Px controls and sham–px + tumor.
*** $P < 0.01$ when compared to stressed intact, stressed sham–px or stressed Px controls, respectively.

intact mice in presence/absence of tumor, respectively, as well as between px and px stressed mice (Table 4 and 5).

3.7. Interleukin-2 (IL-2)

IL-2 plasma levels are reduced in both intact and stressed tumor bearing mice as compared to respective controls ($P < 0.001$) (Fig. 3A, panels 1 and 2) with no modifications after MEL treatment (Fig. 3A, panel 1). Significant increments are observed in stressed tumor bearing intact mice (SDI + tum.) after MEL treatment ($P < 0.05$) (Fig. 3A, panel 2). The same recovery is also achieved in stressed tumor px mice (SDPx + tum + MEL) as compared to respective controls (SDPx or SDPx + tum) ($P < 0.05$) (Fig. 3B, panel 4). Tumour and MEL treatment do not affect reduced IL-2 plasma levels present in px mice (Fig. 3B, panel 3). Significant positive correlations are found between zinc and IL-2 in both stressed tumor bearing intact and px mice treated with MEL ($r = 0.75$, $P < 0.01$; $r = 0.73$, $P < 0.01$, respectively). Significant inverse correlations are found between corticosterone and IL-2 in both stressed tumor bearing intact and px mice treated with MEL ($r = -0.70$; $P < 0.05$; $r = -0.68$; $P < 0.05$, respectively), whereas they are absent in non-stressed tumor bearing mice treated with MEL (intact and px). No significant differences exist in IL-2 between intact and sham–px mice (Fig. 3A,B, panels 1 and 3) as well as between SDI and SD sham–px mice (Fig. 3A,B, panels 2 and 4) in presence/absence of tumor, respectively.

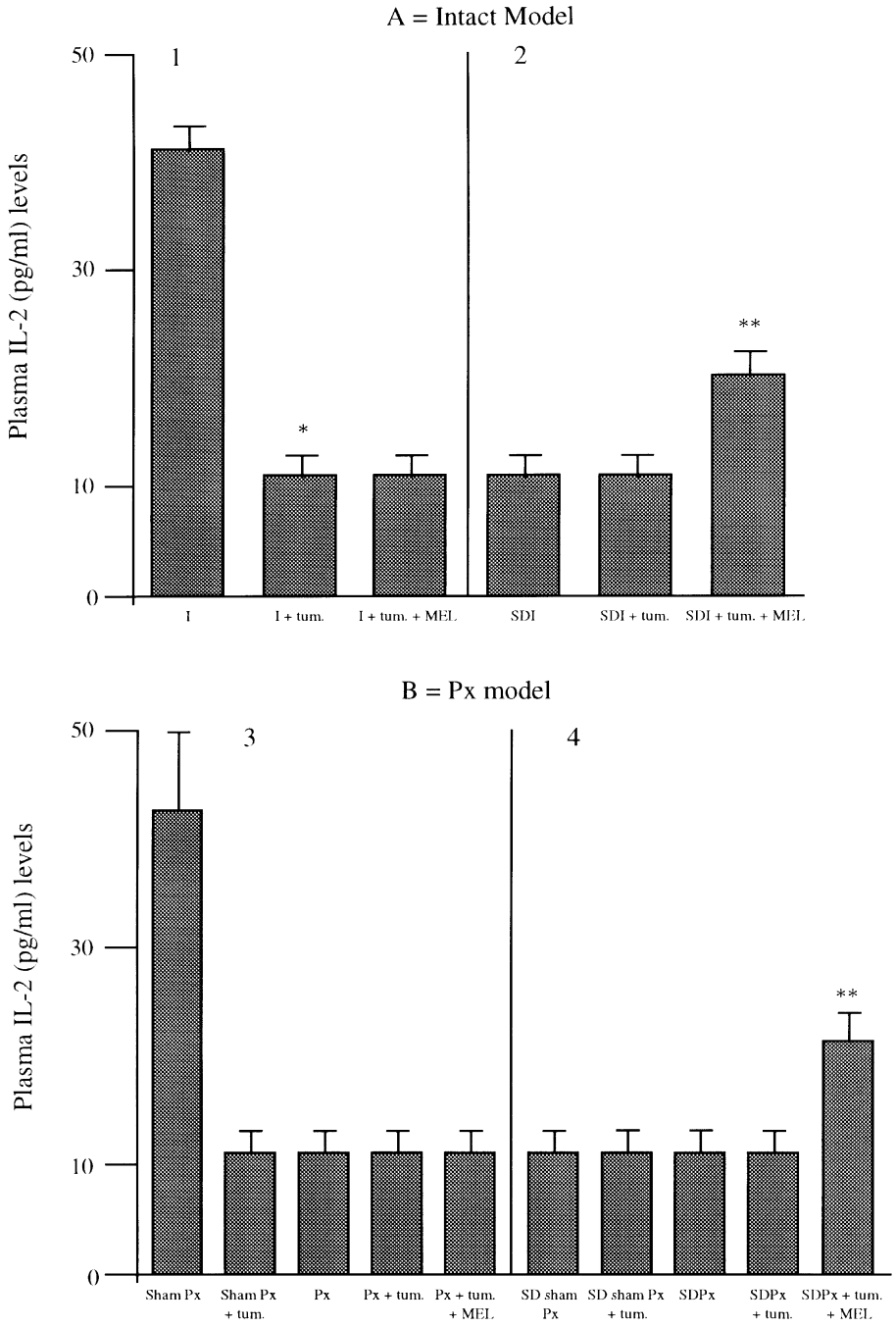

Fig. 3. A) Plasma IL-2 levels in intact mice (I) (panel 1) and in stressed intact mice (SDI) (panel 2) after injections of Lewis lung carcinoma and the effect of melatonin treatment in both intact tumor bearing mice (no stressed and stressed mice) (panels 1 and 2). B) Plasma IL-2 levels in pinealectomized (Px) (panel 3) and in stressed pinealectomized mice (SDPx) (panel 4) after injections of Lewis lung carcinoma and the effect of melatonin treatment in both px tumor bearing mice (no stressed and stressed px mice) (panel 3 and 4). * $P < 0.001$ when compared to intact controls (I); ** $P < 0.05$ when compared to SDI or SDPx control mice, respectively. Mean + S.D.

4. Discussion

RS leads to significant increments of metastasis volume associated with impaired ZnFTS and decreased IL-2 production which is more evident in px stressed tumor bearing mice. MEL decreases metastasis volume and restores ZnFTS and IL-2 exclusively in stressed intact and px tumor bearing mice. The use of MEL as an anti-stress agent with immunoenhancing properties against tumors deserves more consideration.

RS facilitates tumor progression in mice, via neuroendocrine-immune modulation effectors of the host (Justice, 1985; Giraidi et al., 1989). MEL as an antistress agent with immunoenhancing properties, particularly on $Th1^+$ cells, via opiatergic mechanism, has been documented in leukemia bearing mice (Maestroni & Conti, 1990), because of suppressive role of MEL on corticosterone release (Gupta, 1990; Arendt, 1994). In vitro models show MEL's direct oncostatic role because it inhibits DNA synthesis in cancer cell lines (Hill & Blask, 1988; Shellard et al., 1989; Helton & Kane, 1991; Stankov et al., 1991; Pickering & Niles, 1992; Cos & Blask, 1994; Cos et al., 1996) In vivo experiments are, by contrast, controversial and undefined (Stanberry et al., 1983; Blask, 1984; Slominski & Pruski, 1993; Giraldi et al., 1994a).

MEL's oncostatic action may be due to its immunomodulatory role (Caroleo et al., 1992; Maestroni, 1993; Pierpaoli & Regelson, 1994; Skwarlosonta, 1996). Direct and indirect mechanisms acting on the immune system have been proposed. MEL receptors on lymphocytes (Lopez-Gonzales, Calvo, Osuna & Guerrero, 1992), in the thymus (Martin-Cacao et al., 1993; Pozo, Delgado, Calvo, Garcia-Perganeda & Guerrero, 1997) and at the nuclear level (Calberg, Weinsemberg & Schrader, 1997) have been found. Indirect mechanisms involving the neuroendocrine-immune interactions (Maestroni 1993; Gupta, 1990) or the proopiomelanocortin (POMC) gene expression (Gupta, Ghose & Revskoy, 1994), may be added. Among the latter, the metabolic zinc pool has also been proposed (Mocchegiani et al., 1994a, 1996, 1998), because of the relevance of zinc for normal immune response (Chandra, 1985), including thymic endocrine activity. Indeed zinc is required to confer biological activity to thymulin (ZnFTS), which in turn regulates T-cell mediated immunity (Dardenne et al., 1982). The zinc-unbound form (FTS) is inactive with an inhibitory action on the active form (ZnFTS) (Mocchegiani et al., 1994b). The in vitro addition of zinc to plasma samples containing FTS unmasks the inactive form showing the total amount (active ZnFTS + inactive FTS) of thymulin molecules produced (Mocchegiani et al., 1994b). This occurs in marginal zinc deficiencies, including cancer (Moechegiani et al., 1994b). Supplementing zinc restores central and peripheral immune deficits, suggesting that the thymic failure is not intrinsic but largely dependent on low peripheral zinc bioavailability to saturate all thymulin molecules produced (Fabris & Mocchegiani, 1995). Zinc is also crucial for the glucocorticoids pathway (Prasad, 1985). Because of MEL's suppressive effect on corticosterone (Maestroni & Conti 1990, Gupta, 1990, Arendt, 1994), and the action of MEL on the metabolic zinc pool is mediated by glucocorticoids, an interplay between zinc and MEL, via glucocorticoids, has been proposed during development and aging for normal immune efficiency with the role of MEL as an immunomodulating agent (Mocchegiani et al., 1994a, 1996, 1998).

Lewis lung carcinoma leads to negative crude zinc balance in both models of mice (intact and px), despite the zinc content in Nossan food (208 ppm), perhaps because of an intestinal malabsorption in cancer (Duncan & Dreosti, 1975). ZnFTS levels are reduced with no modifications after MEL treatment. Total thymulin level is not affected by the tumor or by MEL treatment.

Thus, the thymulin defect is triggered because of low zinc peripheral bioavailabilty to saturate all thymulin molecules produced in tumor bearing mice, as in human cancer (Mocchegiani et al., 1994b). In contrast, MEL treatment restores crude zinc balance, ZnFTS and corticosterone in stressed intact and px mice independent of the absence/presence of tumor. Although MEL-water dose is pharmacological (Perissin et al., 1993), it is not toxic (Maestroni & Conti, 1990; Mocchegiani et al., 1996), but may mimic physiological control mechanisms of the pineal gland because of MEL's short half-life (Lang, Aubert, Conne, Bradtke & Sizonenko, 1983).

In this context, three important questions arise: firstly the presence of circulating MEL in hybrid BD2F1 mice because of no circulating MEL in C57BL/6 mice (Ebihara et al., 1986); secondly the effective amount of MEL-water intake during the dark period either because the half-life of MEL is short (12–40 min.) (Lang et al., 1983), or because mice drink normal tap water during the light period. Third the real effects on metastasis volume, corticosterone, zinc and immune parameters due to surgical stress (pinealectomy) and/or RS (Prasad, 1985; Giraldi et al., 1994a,b). The observed effects are only related to RS because of no differences in corticosterone after 2 months from pinealectomy between sham–px and intact mice and between px and px stressed mice. Circulating MEL has been clearly demonstrated in young inbred C57BL/6 mice (Conti & Maestroni, 1996), including young-adult hybrid BD2F1 mice (Perissin et al., 1993, Giraidi et al., 1994a,b) used here. Indeed undetectable night urinary MEL secretion is observed in young hybrid BD2F1 mice even after functional pinealectomy (<20 vs 10^7 pg/mouse/12 h in young control) (Perissin et al., 1993). Finally, total MEL-water uptake during the dark period is 4.8–5 ml/night/mouse, corresponding to MEL-water amount uptaken by old mice (5 ml/night/mouse) when normal tap water is deprived during the light period (Pierpaoli & Regelson, 1994; Mocchegiani et al., 1994a, 1998). Thus, MEL-water uptake occurs in hybrid BD2F1 mice independently by a possible major consumption of water during the light period and despite the bitter taste of MEL (Mocchegiani et al., 1998). MEL treatment was not performed on sham–px tumor bearing mice because of no differences in volume metastasis, and in immunological and nutritional parameters with intact tumor bearing mice, as previously shown between intact and sham–px + MEL mice (Mocchegiani et al., 1996). No influence of dietary intake because body weights are similar in treated and control mice (data not shown).

Metastasis volume reduction only occurs in stressed intact and px tumor bearing mice. The restoration of metabolic zinc pool and thymic functions by MEL, via corticosterone, may be, thereby, more involved for three reasons. (1) High corticosterone levels and the reduced metabolic zinc pool in old and px mice are restored by MEL treatment (Mocchegiani et al., 1994a, 1996), (2) MEL nocturnal peak restoration in old, MEL treated mice is strictly related with normalization of nocturnal peaks of zinc and ZnFTS, via corticosterone (Mocchegiani et al., 1998), and (3) In vitro experiments from old thymic explants show a direct action of zinc, rather than MEL, on restoring thymic functions (Mocchegiani et al., 1998). The presence of significant correlations (negative or positive) between zinc and corticosterone or ZnFTS exclusively in stressed intact and px tumor bearing mice after MEL treatment, lends further support to this interpretation.

MEL counteracts α-2 adrenergic immunosuppression on peripheral blood lymphocytes (Liebmann, Hofer, Felsner, Wolfer & Schauenstein, 1996). Adrenergic system is controlled by glucocorticoids in stress (Mobley, Manier & Sulser, 1983) and involved on cytokine production by activated lymphocytes (Besedovsky & Del Rey, 1992). These findings, together with the interplay between zinc and MEL, via glucocorticoids, (Mocchegiani et al., 1994a, 1996, 1998), which is, in

turn, involved in adrenergic receptors modulation (Viticchi, Bulian, Pierpaoli & Piantanelli, 1994; McLeod & Cairncross, 1995), and the role of zinc, MEL and glucocorticoids for IL-2 activity (Tanaka et al., 1990; Caroleo et al., 1992; Hadden, 1995; Mocchegiani et al., 1996), suggest the presence of an immunoregulatory circuit where MEL, via glucocorticoids and the adrenergic system, may be relevant for major zinc bioavailability in MEL stressed, tumor bearing mice. As such, increments of IL-2 from activated lymphocytes may occur. In vitro; studies showing zinc to increase IL-2 production from activated lymphocytes of cancer patients (Prasad et al., 1997a), confirm this assumption. Thus IL-2 increments due to major zinc bioavailability in tumor stressed mice is not an epiphenomenon but a specific signal against tumors, as suggested in human cancer (Prasad et al., 1997a,b). This confirms the relevance also of circulating IL-2 as a good immunological marker in zinc-deficiency cancer condition, as previously suggested (Prasad et al., 1997a). The presence of significant positive or inverse correlations between zinc or corticosterone and IL-2, respectively, in intact and px stressed mice showing metastasis reduction, support this interpretation. Because mRNA level of IL-2 is decreased when Jurkat cells (malignant T-lymphoid cells) grow in zinc-deficient medium (Prasad, 1993), is a further indirect evidence of that. Thus, an action of MEL as antistress agent may be supported in order to induce major zinc biovailability for thymic efficiency and IL-2 production, which is, in turn, involved in zinc-uptake by thymulin-epithelial cells and consequently in the production of ZnFTS molecules (Saha, Hadden & Hadden 1995, Hadden, 1995). The major zinc bioavailability may occur by the suppressive effect of MEL on glucocorticoid pathway (Gupta, 1990), which, in turn, induces a zinc-depleting effect (Prasad, 1985; Fabris, Mocchegiani & Provinciali, 1997). Alternatively, the presence of MEL receptors in gut cells may be involved (Lee & Pang, 1991). Zinc-finger proteins affect IL-2 gene expression in T lymphocytes (both circulating and into the tumor) (Owaki et al., 1993; Skerka, Decker & Zipfel, 1995; Nagashima et al., 1997). This finding with our data are suggestive to assign peculiar role to zinc for IL-2 production, which, in turn, may reactivate anergic natural killer cells against tumor (Agrawal, Kranz, Reddish & Longenecker, 1998). Zinc induces also apoptosis of tumor cells (Provinciali, Di Stefano, Stronati & Fabris, 1996a), whereas MEL is less effective both in vivo and in vitro models (Sainz et al., 1995; Provinciali, Di Stefano, Bulian, Tibaldi & Fabris, 1996b). Possible MEL apoptotic role against tumor may occur by means of a decrement of NF-kB DNA binding activity (Chuang, Mohan, Metz & Reiter, 1996), which is, in turn, under the control of TNF-α (Wang, Mayo & Baldwin, 1996). Because TNF-α is reduced in MEL treated mice (Ben-Nathan, Maestroni & Conti, 1997), the apoptotic role of MEL is still unclear. Thus, without discrediting MEL's direct oncostatic role, MEL is more an immunomodulating agent, via zinc, against tumors, this warrants further work. In conclusion, the interplay between zinc and MEL, via corticosterone, for normal immune response, including IL-2, already proposed during development and aging (Mocchegiani et al., 1994a, 1996, 1998), may be also suggested in cancer with this possible physiological cascade: MEL treatment $\rightarrow$ reduction of corticosterone release $\rightarrow$ major zinc bioavailability $\rightarrow$ consequent normal immune response. Because of no differences in increased survival between old zinc and old MEL treated mice (Mocchegiani et al., 1998), zinc treatment with chemotherapy in cancer patients, other than MEL (Lissoni et al., 1995), may be taken into account. MEL is an antioxidant (Reiter, 1994) with a protective role on stem cells against toxicity by antiblastic drugs (Maestroni et al., 1994). Because also zinc is an antioxidant (Willson, 1977), with a protective role against chemotherapic toxicity by means of zinc-bound metallothioneins (Lazo et al., 1991), the proposal of physiological zinc treatment with chemotherapy

in cancer (Hadden, 1995; Fabris & Mocchegiani, 1995, Prasad et al., 1997a,b) might find further rationale.

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