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MELATONIN DECREASES BONE MARROW AND LYMPHATIC TOXICITY OF ADRIAMYCIN IN MICE BEARING TLX5 LYMPHOMA

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Summary

When CBA male mice bearing TLX5 lymphoma were treated in the evening with a single i.v. dose of adriamycin (20-40 mg/Kg), the administration of a single pharmacological dose of melatonin (10 mg/kg s.c.) 1 hr earlier reduced the acute mortality from 10/24 to 2/24. The increase in survival time caused by adriamycin over drug untreated controls was not reduced by melatonin. The administration of melatonin alone did not cause any antitumor or evident toxic effect. Melatonin also attenuated the reduction caused by adriamycin in the number of bone marrow GM-CFU, and of CD3+, CD4+ and CD8+ splenic T-lymphocyte subsets. Reduced and total glutathione levels were decreased in the bone marrow and in the liver cells of the animals treated with adriamycin, and were significantly restored by melatonin. Moreover, lipid peroxidation by adriamycin was reduced by melatonin, as indicated by malondialdehyde measurement in the liver of the treated animals. These data indicate that the protective effects of melatonin against the host toxicity of the prooxidant antitumor drug, adriamycin, might be attributed at least partially to its antioxidant properties. These findings appear of interest in relation to the physiological rhythmic levels of endogenous melatonin and to the chronotoxicology of anthracyclines.

Key Words: melatonin, adriamycin toxicity, bone marrow, splenic T-lymphocyte subsets, adriamycin antitumor activity

The pineal gland and its indole hormone, melatonin, have been shown in numerous experimental studies to be involved in cancer progression. In the 30's Engel suggested a link between the pineal gland and cancer, on the basis of the observation that an inhibition of Ehrlich carcinoma growth was obtained in albino mice upon administration of a pineal gland extract (1, 2). Cancer

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treatment with pineal extracts has been performed later in the clinic, resulting in a reported retardation in the progression of the disease and in an improvement of the quality of life of the patients (3). The role of pineal gland and of melatonin for cancer growth has been investigated rather extensively in laboratory animals. Surgical pinealectomy resulted in the increased growth *in vivo* of different types of experimental tumors, such as Walker 256 carcinoma (4, 5), melanotic melanoma N°1 (6), Yoshida sarcoma (7), and polyoma virus induced tumors (8). Tumor growth was correspondingly attenuated in pinealectomized animals by the administration of exogenous melatonin, as reported for malignant melanoma in hamsters (9, 10) and for a mammary tumor induced by the chemical carcinogen 7,12-DMBA in rats (11). Tumor growth inhibition *in vivo* and *in vitro* following treatment with melatonin in non pinealectomized animals has been described in some instances (12-16), although contrasting reports, also showing a stimulation of tumor growth, are available in the literature (17, 18).

Immunoregulatory properties of melatonin, capable to favorably modify host-tumor relationships and hence to reduce tumor growth were also observed (19-22). In human patients with advanced lung or colorectal cancer, melatonin has been reported to induce an apparent control of the neoplastic growth and an improvement of the quality of life in a substantial number of cases (23), to increase the therapeutic effect of low doses of interleukin-2 (IL-2) (24, 25), and to attenuate the adverse effects of IL-2 treatment in patients with non-small cell lung cancer (26). Webb and Puig-Domingo recently reviewed the possible clinical relevance of melatonin in neoplastic disease (27).

In mice bearing Lewis lung carcinoma melatonin has been shown to protect the bone marrow stem cells from the apoptosis induced by cyclophosphamide and etoposide, while it does not reduce the antitumor action of these drugs. This effect of melatonin was suggested to occur via interaction with its receptors on T-helper lymphocytes in the bone marrow (28), leading to the stimulation of the production of a Th cell factor constituted of two cytokines named MIO (melatonin induced opioids). This factor would act in its turn on bone marrow stromal cells inducing the release of hematopoietic growth factors (29). On the other hand, melatonin has been shown to cause potent direct antioxidant effects, rapidly scavenging hydroxyl (30, 31) and peroxyl radicals (32). Additionally, melatonin can also upregulate endogenous antioxidant defenses, as shown for glutathione peroxidase activity in the brain (33) and in the liver (34). The antioxidant action of melatonin is also supported by experiments showing that it decreases the DNA damage caused by ionizing radiation in cultured cells (35), the *in vivo* cataract formation induced by buthionine sulfoximine (BSO) in rats (36, 37), the DNA damage caused by chemical carcinogen safrole (38), as well as the kainate excitotoxicity in cerebellar granular neurons (39). The anthracycline antitumor drug, adriamycin, is widely used in the clinic. The most serious adverse effects limiting the tolerable dose intensity are myelosuppression, gastrointestinal toxicity and acute cardiac toxicity eventually leading to cumulative late cardiomyopathy (40). Numerous studies investigated the underlying mechanisms, and an oxidative damage to membrane lipids and to other cellular components is believed to be a major factor in the cardiac toxicity of adriamycin and other anthracyclines (41-45).

The aim of this work has been therefore that to examine the effects of the administration of adriamycin, of exogenous melatonin, and of their combination, in terms of toxicity for the host and antitumor activity in mice implanted with TLX5 lymphoma. The acute toxicity for the host has been evaluated in terms of lethality, as well as of the effects on bone marrow stem cells and splenic T-lymphocyte subsets. The possible relevance of oxidative damage caused by adriamycin, and its prevention by melatonin, has been determined measuring glutathione levels in bone marrow cells and in the liver, and lipid peroxidation in terms of malondialdehyde levels in the liver. The results obtained are hereafter reported.

Materials and methods

Reagents. Melatonin was a kind gift of Prof. Fraschini, University of Milano, Italy. Adriamycin was obtained from Pharmacia S.p.A. Milano, Italy, and the other reagents used were purchased from Sigma Chemical Co, Sigma Chimica Divisione della Sigma-Aldrich S.r.l., Milano, Italy.

Animals and tumor transplantation. The animals used are male CBA/LAC mice weighing 22-25 g, belonging to a conventional local breeding colony. The animals were provided food and water *ad libitum*, and were kept constantly with a 12/12 light/dark cycle (lights on from 8 a.m. to 8 p.m.). TLX5 lymphoma was originally provided by the Chester Beatty Research Institute, London, England. Tumor implantation was performed injecting each mouse i.p. with 0.1 ml of a suspension containing 10^5 viable tumor cells. The tumor cells were obtained from donors inoculated 8 days before, were washed by centrifugation at 500xg and were resuspended in PBS after counting for trypan blue exclusion.

Drug treatment. Melatonin was dissolved in 0.9% NaCl saline containing 4% ethanol, and was administered s.c. in a volume of 0.05 ml/10 g of body weight. Adriamycin was dissolved in 0.9% NaCl solution, and was administered i.v. in 0.05 ml/10 g of body weight or i.p. in 0.1 ml/10 g of body weight, as indicated. The treatment with melatonin was performed at 8 p.m. (light off), whereas the treatment with adriamycin was made at 9 p.m..

GM-CFU assay. The number of granulocyte/macrophage-colony forming units (GM-CFU) was determined after *in vivo* treatment with the tested drugs. Following sacrifice, 10^5 viable bone marrow cells were incubated in 0.3% semisolid agar in RPMI-1640 medium containing 10% fetal calf serum and 10% lung conditioned medium (LCM) as source of stimulating factors. LCM was prepared by mincing the lungs from 2 mice into small pieces and incubating the pieces at 37° C with 5% CO_2 for 3 days in RPMI-1640 medium containing 10% fetal calf serum. The cultures were kept for 7 days at 37°C in humidified air and then examined by phase contrast microscopy; colonies containing more then 50 cells were counted as GM-CFU.

Measurement of splenic T-lymphocyte subpopulations. Spleens were removed immediately following sacrifice by cervical dislocation. They were disaggregated and then passed through a double layer of gauze to obtain single cell suspensions. The cells were washed and lymphocytes were separated from red blood cells by Ficoll-Hypaque centrifugation [Sigma, St. Louis, MO] (46). The final suspension of splenic lymphocytes was labeled with antimouse monoclonal antibodies using a PBS staining medium, 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 antimouse monoclonal antibodies to CD3 (0.5 µg), CD4 (1 µg) or CD8 (1 µg) [Pharmingen, San Diego, CA]. Stained cells were examined using an EPICS flow cytometer (Coulter, Miami, FL); each analysis consisted of 10,000 events counted (47). Results for single color analysis are expressed as total number of positive cells collected from the spleen of each animal.

Glutathione assay. The reduced (GSH) and oxidized (GSSG) glutathione levels were measured in bone marrow and in liver cells by a high-performance liquid chromatography (HPLC) technique. After sacrifice, the livers were removed, and were kept frozen at -80°C until assay. The liver of each mouse was homogenized after thawing with a teflon pestle potter glass homogenizer (1 g liver in 5 ml of 1.15 % KCl) at 0°C. Bone marrow samples and liver homogenates were processed following the method of Reed *et al.* (48). Briefly, 1 ml of liver homogenate in 1.15 % KCl solution (20-30 mg/ml protein) or 1 ml of bone marrow cells (10^6 cells) was added with 0.05 ml of 70% perchloric acid. After protein precipitation, 0.5 ml of the supernatant was treated

immediately with 50 μl of 0.08M fresh aqueous solution of iodoacetic acid and then neutralized with an excess of $NaHCO_3$. After 60 min in the dark at room temperature, 0.5 ml of an alcoholic solution of 1-fluoro-2,4-dinitrobenzene (1.5 ml/ 98.5 ml absolute ethanol) was added, and the reaction allowed proceeding for 4 hours in the dark. The samples were then chromatographed using a reverse-phase ion exchange column microbondapak NH_2 3.9x300 mm (Waters). Glutathione levels were related to protein content in the samples, which was determined by the method of Lowry *et al.* (49).

Lipid peroxidation assay. Liver homogenates prepared as described for glutathione assay were subjected to MDA (malondialdehyde) measurement. The assay consists in the hydrolysis of liver lipoperoxides to form MDA which reacts with thiobarbituric acid (TBA) to yield a red MDA-TBA adduct, which is measured by spectrophotometry after extraction with butanol, following the method of Uchiyama and Mihara (50). Briefly, 0.5 ml of liver homogenate in 1.15% KCl solution, was added with 3 ml of 0.5% TCA and 1 ml of 0.6% TBA aqueous solution. The mixture was heated for 45 min on a boiling water bath; after cooling, 4 ml of n-butanol was added and mixed vigorously. The butanol phase was separated by centrifugation (4000 r.p.m. x 10 min.) and absorbance was measured at 532 nm. Liver peroxide concentrations were computed by reference to a calibration curve prepared by standards of tetraethoxypropane (TEP), which undergoes hydrolysis to liberate stoichiometric amounts of MDA (51). The values of MDA were expressed as nmol/mg of protein, determined by the method of Lowry *et al.* (49).

Statistical analysis. Tabled values are group means ± S.D. Data were subjected to the appropriate factorial ANOVAs assessing significance against an alpha-level $P < 0.05$, or to Kruskall-Wallis analysis of variance, as well as Kaplan Meier, logrank and Cox proportional hazard analysis as appropriate. All analyses were performed using standard procedures implemented in the Systat package (SYSTAT Inc., Evanston, IL)(52).

Results

Antitumor activity and toxicity of melatonin and adriamycin. Data reported in Table 1 show that the administration of a single s.c. dose of melatonin (10 mg/Kg) does not significantly modify the survival time of CBA mice bearing TLX5 lymphoma, as indicated by Kaplan-Meier and log-rank statistics. In the same animal tumor system, adriamycin displays a significant antitumor action when administered i.v. as a single dose in the range 14-28 mg/Kg, the survival time of the treated mice being significantly increased over drug untreated controls, as determined by Kaplan-Meier analysis. The stratification of the data for adriamycin indicates a significant effect (log-rank chi-square = 42.1, DF = 2, $P < 0.0001$; mean survival time = 15.7 vs 10.1), and a not significant effect for 10 mg/Kg s.c. melatonin (log-rank chi-square = 2.4, DF = 1, $P = 0.122$; mean survival time = 15.9 vs 13.9). Bivariate Cox proportional hazard analysis further indicates that adriamycin constitutes a significant negative risk factor (HR = 0.252, 95 % CL 0.512 - 0.124), whereas the effects of melatonin are insignificant (HR = 0.849, 95 % CL 2.799 - 0.258). On the contrary, the toxicity of adriamycin, as indicated by the number of toxic deaths occurring before day 9, is significantly reduced by melatonin. Indeed, the total number of such toxic deaths in the dose range of 20-40 mg/Kg of adriamycin is 10/24; when the treatment with adriamycin is combined with melatonin administration the number of toxic deaths is significantly reduced to 2/24, Yates corrected chi-square = 5.444, DF = 1, $P = 0.02$ (see Table 2).

Effects of melatonin and adriamycin on bone marrow granulocyte/macrophage-colony forming units. Separate 2 x 2 ANOVAs indicate that the treatment with 28 mg/Kg i.v. adriamycin causes a significant effect on the number of granulocyte/macrophage-colony forming units (GM-CFU) ($F_{(1,19)} = 20.3$, $P < 0.0001$). The effects of 20 mg/Kg melatonin s.c. are not significant, whereas

TABLE 1.

Effects of Melatonin on Survival Time of Mice Implanted with TLX5 Lymphoma.

Melatonin	Toxic Deaths	Mean Survival Time (days)	
-	0/8	9.4	p=0.462
+	0/7	9.6	

Groups of 8 CBA male mice were implanted on day 0 with 10^5 TLX5 lymphoma cells. On day 1 they were treated as indicated at 8 p.m. with melatonin (10 mg/Kg s.c.). Mean survival times and their difference were determined by Kaplan Meier and logrank analysis.

TABLE 2.

Toxic Deaths and Increase in Survival Time of CBA Mice Implanted with TLX5 Lymphoma Treated with Adriamycin and Melatonin.

Adriamycin (mg/Kg)	Melatonin	Toxic Deaths	Mean Survival Time (days)
-	-	0/9	10.1
40	-	7/8	9
40	+	2/8	14.8
28	-	2/8	18.5
28	+	0/8	17.3
20	-	1/8	16.6
20	+	0/8	17.1
14	-	0/8	13.4
14	+	0/8	13.8

Groups of 8 CBA male mice were implanted on day 0 with 10^5 TLX5 lymphoma cells. On day 1 they were treated at 8 p.m. with melatonin (10 mg/Kg s.c.) and at 9 p.m. with adriamycin (i.v.) as indicated. Acute toxic deaths were considering those occurring before day 9. Mean survival time was determined using Kaplan Meier statistics (for the results of statistical analysis see the Results section).

TABLE 3.

Granulocyte/Macrophage Colony Forming Units in the Bone Marrow of Normal CBA Mice Treated with Adriamycin and Melatonin.

Adriamycin	Melatonin	GM-CFU
-	-	67.2±24.1
-	+	42.3±10.3[a]
+	-	15.0±2.2[ab]
+	+	41.3±8.1[b]

Each value is the mean ± S.D. obtained using groups of 6 non tumor bearing CBA male mice. The animals were treated on day 1 with melatonin (20 mg/kg s.c.) at 8 p.m. and with adriamycin (28 mg/Kg i.v.) at 9 p.m. as indicated. The animals were sacrificed on day 5, and the number of granulocyte/macrophage colony forming units (GM-CFU) in 10^5 bone marrow cells was determined. The data were subjected to ANOVA analysis, whose results are illustrated in the Results section. Means marked with the same letters are significantly different, Tukey test, $P<.05$.

TABLE 4.

Splenic T-Lymphocyte Subpopulations in Mice Bearing TLX5 Lymphoma Treated with Adriamycin and Melatonin.

ADRIAMYCIN	MELATONIN	CD3+	CD4+	CD8+	CD4+/CD8+
-	-	23.3±4.5	14.7±4.0	8.6±1.2	1.7±0.5
-	+	16.9±2.9	10.7±1.8	6.2±1.6	1.8±0.4
+	-	3.6±0.2	1.7±0.2	1.9±0.1	0.9±0.1
+	+	7.1±0.4	3.3±0.2	3.8±0.6	0.9±0.2

Each value is the mean ± S.D. obtained using groups of 6 CBA male mice which were implanted on day 0 with 10^5 TLX5 lymphoma cells. On day 1 they were treated with melatonin (20 mg/Kg s.c.) at 8 p.m. and with adriamycin (28 mg/Kg i.v.) at 9 p.m. as indicated. The animals were sacrificed on day 5, and the number (x 10^6) of CD3+, CD4+, CD8+ and CD4+/CD8+ lymphocytes in spleen was determined. The data were subjected to ANOVA analysis, whose results are illustrated in the Results section.

those of the interaction of both treatments are significant ($F_{(1,19)} = 18.8$, $P < 0.0001$). Tukey test indicates that the reduction caused by adriamycin is significantly reversed by the concurrent administration of melatonin (see Table 3).

Effects of melatonin and adriamycin on splenic T-lymphocyte subsets. Separate 2 x 2 ANOVAs indicate a significant effect of adriamycin on CD3+ ($F_{(1,16)}$=150.1, P<0.0001), CD4+ ($F_{(1,16)}$=107.9, P<0.0001) CD8+ subsets ($F_{(1,16)}$=93.3, P<0.0001), as well as on CD4+/CD8+ ratio ($F_{(1,16)}$=33.5, P<0.0001). The interactions of the treatments with adriamycin and melatonin are also significant on CD3+ ($F_{(1,16)}$=16.7, P=0.001), CD4+ ($F_{(1,16)}$=8.2, P=0.011) and CD8+ subsets ($F_{(1,16)}$=20.1, P<0.0001) (see Table 4).

Effects of melatonin and adriamycin on glutathione levels and lipid peroxidation. When the animals are treated with a single dose of 28 mg/Kg adriamycin i.v. and with a single dose of 20 mg/Kg melatonin s.c. and their livers are examined, separate 2 x 2 ANOVAs indicate a significant effect of adriamycin, melatonin and of the interaction of the treatments on reduced glutathione ($F_{(1,17)}$ = 145.7, P < 0.0001, $F_{(1,17)}$ = 16.7, P = 0.001 and $F_{(1,17)}$ = 23.1, P < 0.0001, respectively). A significant effect of the treatments and of their interaction is observed on total glutathione concentration ($F_{(1,17)}$ = 132.4, P < 0.0001, $F_{(1,17)}$ = 15.3, P = 0.001 and $F_{(1,17)}$ = 23.0, P < 0.0001, respectively), and also on MDA ($F_{(1,19)}$ = 40.0, P < 0.0001, $F_{(1,19)}$ = 26.6, P < 0.0001 and $F_{(1,19)}$ = 11.8, P = 0.003, respectively). On oxidized glutathione, the interaction of the treatments with adriamycin and melatonin is marginally significant ($F_{(1,17)}$ = 4.2, P = 0.056), whereas on GSH/GSSG ratio only the effect of adriamycin is significant ($F_{(1,17)}$ = 26.0, P < 0.0001). Tukey test indicates that the reduction caused by adriamycin on reduced and total glutathione is significantly reverted by the concurrent administration of melatonin; on MDA, the increase observed after adriamycin is conversely attenuated significantly (see Table 5). The measurement of glutathione levels in bone marrow cells after treatment with 3 weekly adminis-

TABLE 5.

Lipid Peroxidation and Glutathione Levels in the Liver of CBA Mice Treated with Adriamycin and Melatonin.

Adriamycin	Melatonin	GSH	GSSG	GSH/GSSG	tGSH	MDA
-	-	56.6±4.7^{ab}	3.1±0.9	19.4±4.8	59.7±5.0^{ab}	1.8±0.2^{a}
-	+	55.3±4.6^{cd}	2.7±0.3	20.0±1.2	58.0±4.8^{cd}	1.3±0.5^{b}
+	-	26.4±3.8^{ace}	2.2±0.4	11.8±1.4	28.6±4.1^{ace}	4.6±0.3^{abc}
+	+	42.3±2.8^{bde}	2.9±0.4	14.7±1.8	45.2±3.0^{bde}	2.1±1.2^{c}

Each value is the mean ± S.D. obtained using groups of 6 CBA male mice which were treated on day 1 at 8 p.m. with melatonin (20 mg/Kg s.c.) and with adriamycin (28 mg/Kg i.v.) at 9 p.m. as indicated. The animals were sacrificed on day 5. The values for malonaldehyde (MDA), reduced glutathione (GSH) and oxidized glutathione (GSSG) are expressed as nmol/mg protein. The data were subjected to ANOVA analysis, whose results are illustrated in the Results section. Means marked with the same letters are significantly different, Tukey test, P<.05.

TABLE 6.

Glutathione Levels in the Bone Marrow Cells of Normal CBA Mice Treated with Adriamycin and Melatonin.

Adriamycin	Melatonin	GSH	GSSG	GSH/GSSG	tGSH
-	-	5.3±0.61	0.20±0.04	21.5±1.95	5.5±0.65
+	-	3.4±0.23	0.14±0.01	24.6±0.82	3.6±0.25
+	+	6.6±1.15	0.27±0.07	23.9±1.71	6.9±1.59

Each value is the mean ± S.D. obtained using groups of 4 non tumor bearing CBA male mice. The animals were treated once a week for 3 weeks with melatonin (10 mg/Kg s.c.) at 8 p.m. and with adriamycin (5 mg/Kg i.p.) at 9 p.m. as indicated. Glutathione levels were determined in the bone marrow cells of animals sacrificed 3 days after the last treatment. The values of reduced glutathione (GSH), oxidized glutathione (GSSG) and total glutathione (tGSH) are expressed as nmol/mg protein. The experiment was performed in duplicate, and the data were subjected to Kruskal-Wallis analysis whose results are illustrated in the Results section.

trations of 5 mg/Kg adriamycin i.p. preceded by melatonin 10 mg/Kg s.c. indicates a significant restoration by melatonin of the levels of reduced, oxidized and total glutathione after adriamycin (Kruskall-Wallis analysis of variance, chi-square = 3.87, DF = 1, P = 0.049), (see Table 6).

Discussion

The results presented show that in male CBA mice bearing TLX5 lymphoma the administration of a single subcutaneous dose of 10 mg/kg melatonin is devoid of antitumor effects, as indicated by the absence of a significant modification of the life span of the treated animals. This finding indicates that the tumor line presently employed, TLX5 lymphoma, is not sensitive to direct cytotoxic antitumor and/or oncostatic effects of melatonin at the dosage and with the treatment schedule employed. However, other human and animal tumors have been reported to respond to a direct antitumor action for melatonin (12, 13, 53-55). At the same time, no evident toxic effect of melatonin at the dosages used was observed, nor was observed in normal mice with dosages up to 20 mg/Kg. The observed lack of host toxicity of melatonin is in accord with published reports indicating that melatonin is tolerated also at dosages much higher than those presently employed (56).

On the other hand, the data reported show that the administration in the evening of 10 mg/Kg melatonin to mice bearing TLX5 lymphoma significantly reduces the host toxicity caused by the administration 1 hour later of adriamycin in the dose range 20 - 40 mg/Kg, as indicated by the reduction in the occurrence of early acute toxic deaths. At the same time, adriamycin administered in the dose range 14 - 28 mg/Kg significantly increases the survival time of the mice bearing TLX5 lymphoma which were not affected by the acute toxicity of the treatment with the anthracycline. The concurrent administration of melatonin does not reduce the magnitude of the antitumor effects of adriamycin. The reduction in the proportion of acute toxic

deaths caused by adriamycin is accompanied by a significant reduction in the number of GM-CFU in the treated animals, and this reduction is significantly attenuated by melatonin, which is devoid of significant effects by itself. Furthermore, melatonin also attenuates the toxicity of adriamycin on lymphatic organs. In fact, the number of CD3+, CD4+ and CD8+ splenic T-lymphocyte subsets, which is significantly reduced by adriamycin, is significantly restored by melatonin. This finding is in agreement with the view that melatonin possesses immunoregulatory properties, which are relevant when the functions of the immune system are depressed by stress (19) or by antitumor drugs (21).

The presently observed attenuation of bone marrow toxicity of adriamycin by melatonin is matching that obtained by Maestroni *et al.* examining the effects of melatonin on the toxicity and antitumor action of cyclophosphamide and etoposide. In mice bearing Lewis lung carcinoma, the antitumor action of both drugs was retained after melatonin treatment, whereas their hematological toxicity was significantly reduced (57, 58). The mechanism by which melatonin attenuated the hematological toxicity of cyclophosphamide and etoposide has been studied in detail by the same Authors (28, 59, 60). Their results indicate that melatonin binds to helper T-lymphocytes, inducing the release of a Th cell factor constituted of two cytokines named MIO (melatonin induced opioids). In its turn, this factor acts on bone marrow stromal cells inducing the release of hematopoietic growth factors, such as GM-CSF (29, 60). The reduction of bone marrow toxicity of adriamycin by melatonin might be caused by a mechanism alternative to that involving MIO, consisting of the antioxidant action of melatonin exerted against the prooxidant effects of adriamycin. In fact, melatonin significantly restores to control values the levels of reduced, oxidized and total glutathione which are lowered by adriamycin in bone marrow cells. The view that the protective action of melatonin is exerted by means of its antioxidant properties is supported by the measurement of glutathione levels and lipid peroxidation in liver cells. Indeed, adriamycin significantly lowers the concentration of reduced and total glutathione in the liver, as already observed by other investigators (61-64); the concurrent administration of melatonin significantly restores these levels and marginally corrects the concentration of its oxidized form. Adriamycin measurably causes lipid peroxidation in liver cells, as indicated by elevated MDA levels, whose increase is almost totally reverted by melatonin. These results indicate that melatonin may attenuate the oxidative damage caused by adriamycin at least in bone marrow and liver cells. Although melatonin has been shown to directly scavenge oxygen reactive species (65, 66), an indirect mechanism appears to be operative, consisting in the restoration of the cellular total levels of glutathione. The data here presented do not allow to clarify whether glutathione peroxidase and/or glutathione reductase, whose levels are sensitive to both adriamycin (67-69) and melatonin (33, 34 65), participate in determining the reported effects. Moreover, glutathione depletion by adriamycin can be due also to inhibition of glutathione synthesis and specifically of γ-glutamyl cisteine synthase (69), as well as to an increase in glutathione-S-transferase activity (70) and/or in lipid peroxidation byproducts, requiring conjugation and detoxification by glutathione-S-transferases. Although we have no data at present regarding the effects of melatonin on these enzymes, the protection from MDA accumulation supports the hypothesis that glutathione conjugation may be involved in the mechanism of depletion by adriamycin and restoration by melatonin. More in general, this hypothesis is also supported by the findings of Montilla et al., showing that the nephropathy induced in the rat by adriamycin is suppressed by melatonin, concomitantly with a restoration of gluathione levels which are reduced by the treatment with the anthracycline (71). As far as the protective effects of melatonin on adriamycin toxicity are concerned, it has to be noted that a large proportion of the studies which examined the antioxidant action of melatonin were performed using male animals, whereas female mice were used in the experiments of the group of Conti and Maestroni (28, 29, 57, 59, 60). The relative importance of the antioxidant mechanism presently proposed for the effects of melatonin on adriamycin toxicity, in relation to

the mechanism involving MIO forwarded by Conti and Maestroni for cyclophosphamide and etoposide (29, 60), can not be presently assessed, and the possible role played factors related to the sex of the experimental animals used should also be taken into consideration for future experimental investigations.

In conclusion, the results presented show that the administration of a pharmacological dose of melatonin to CBA mice bearing TLX5 lymphoma is devoid of evident toxic and of antitumor effects. At the same time, melatonin does not decrease the antitumor action of adriamycin, and significantly reduces the acute host toxicity of this drug, suggesting that an enhancement in the dose intensity of adriamycin can be achieved by its combined administration with exogenous melatonin. Bone marrow and splenic T-lymphocyte toxicity of adriamycin is attenuated by melatonin, with a mechanism consistent with an antioxidant action of melatonin which is effective against the prooxidant action of adriamycin. These data appear to encourage the examination of the role and mechanism of the antioxidant action of endogenous melatonin and of the administration of exogenous melatonin in pharmacological doses, in relation to the therapeutic and toxic action of prooxidant antitumor drugs, as well as of antineoplastic agents generating nitrogen and carbon centered radicals. Other organ directed toxic effects of anthracyclines, such as early and late cardiac toxicity (72-75), have been attributed to their prooxidant properties and seem to deserve investigation, since they are of crucial importance as factors clinically limiting the tolerable dose intensity of these drugs. Moreover, the data reported also appear to encourage the study of the endogenous melatonin concentration and its rhythmic variations in relation to the chronotoxicological data available for anthracyclines in laboratory animals (76) and in clinical antitumor chemotherapy (77).

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