Stress and Chemotherapy

Combined Effects on Tumor Progression and Immunity in Animal Models

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ABSTRACT: **In mice bearing Lewis lung carcinoma, rotational and restraint stress specifically increases the formation of lung metastasis, and restraint stress markedly attenuates the antitumor effects of cyclophosphamide. The aim of this investigation was therefore to examine the effects of restraint stress on tumor metastasis in mice bearing MCa mammary carcinoma, and on the effectiveness of CCNU and DTIC. Restraint stress increases MCa mammary carcinoma metastasis, causes a marked reduction in cyclophosphamide activity, and a minor attenuation of the effects of CCNU and DTIC. The possible occurrence of seasonal factors, observed for the increase by rotational stress of Lewis lung carcinoma metastasis, was also determined for cyclophosphamide effectiveness. The survival time of control mice is longer in February than in June, and is not appreciably modified by rotational stress. The effects of cyclophosphamide are similar in both seasonal periods, and are similarly attenuated by rotational stress. The seasonal effects of rotational stress, and the reduction of the effects of cyclophosphamide caused by rotational stress, are accompanied by corresponding variations in the number of CD3+ and CD4+ splenic T-lymphocyte subsets and in the CD4+/CD8+ ratio, respectively. The reported effects of stress on tumor progression and on the effectiveness of cyclophosphamide thus appear to occur via modulation of immune responses of the host directed against the tumor. These data appear of interest for their experimental implications, and suggest the opportunity to consider the role that the stress during treatment may play in determining the effectiveness of clinical antitumor chemotherapy.**

INTRODUCTION

Clinical oncologists are giving increased attention to the quality of life of their cancer patients, and investigations focused on the role that psychosocial factors may play in the progression of clinical cancers by means of neuroendocrine modulation of immune functions of the host continues to be carried on with encouraging results.[1,2] In this connection, model systems consisting of laboratory animals bearing

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transplanted tumors and subjected to stress paradigms offered a valuable experimental tool and provided useful results.[3] Studies are available showing that the exposure to stress causes specific effects on the immune system of the host and concomitantly influences tumor growth.[4] Yet, the specific effects of stress on malignant metastatic dissemination in experimental systems received limited attention, in spite of the clinical relevance of tumor metastatic spread.[5] Moreover, although immune responses of the host may participate in determining the efficacy of antitumor drugs,[6] little attention appears to have been given to the neuroimmunomodulation induced by stress on the outcome of antineoplastic chemotherapy. Indeed, the latter aspect may deserve investigation, since low dosages of cyclophosphamide and melphalan were equally or more effective in causing cures in mice bearing MOPC-315 plasmacytoma[7–9] than higher dose levels of the same drugs. Mice cured with the low-dosage treatment schedules showed a strong acquired immune resistance to further tumor challenges that did not occur using high dosages of both antitumor drugs,[7,8,10,11] and that was shown to depend on the appearance of Lyt 2+ T cells.[10,12] Findings consistent with a dosage-dependent immunoregulatory action of melphalan and cyclophosphamide, crucial in curing MOPC-315 plasmocytoma in mice, also were obtained in rats bearing KMT-17 fibrosarcoma upon treatment with bleomycin.[13]

As far as metastasis is concerned, several paradigms, including rotational and restraint stress, have been shown to specifically increase the metastatic spread of Lewis lung carcinoma in syngeneic mice, in a way that is independent of the effects of the stressor on the growth of the primary tumor.[14,15] The increase in metastasis formation caused by rotational stress was shown to vary in magnitude with a highly significant circannual rhythm, with the acrophase coinciding with the summer solstice; the magnitude of the increase in metastasis formation negatively correlated with the number of splenic CD3+ and CD4+ T-lymphocyte subsets.[16] On the other hand, in mice bearing Lewis lung carcinoma, the application of restraint stress caused the attenuation of the cytotoxic effects of cyclophosphamide, reducing the increase in life span and the number of long-term survivors caused by the drug. Physical restraint and cyclophosphamide concomitantly reduced the CD4+/CD8+ ratio in an additive way.[17]

On the basis of these findings and considerations, the aim of the present investigation has been to examine the effects of restraint and rotational stress on the effectiveness of cyclophosphamide, CCNU, and DTIC in mice bearing MCa mammary carcinoma, and to relate them to the numerosity of CD3+, CD4+, CD8+ splenic T-lymphocyte subpopulations and NK cells. The results obtained are reported below.

MATERIALS AND METHODS

Animals and Tumor Transplantation

For the experiments with Lewis lung carcinoma, the animals used are female C57BL/6 and C57BL/6 X DBA/2F_1 (BD2F1) mice weighing 18–20 g, purchased from Charles River, Calco, Como, Italy. Lewis lung carcinoma, originally provided by the National Cancer Institute, Bethesda, Maryland, 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 two weeks before. For

experimental purposes, the tumor is propagated in BD2F1 mice by intramuscolar injection of a tumor cell suspension containing 10^6 viable tumor cells.[14]

For the experiments with MCa mammary carcinoma, the animals used are female CBA/Lac mice weighing 18–20 g, obtained from a locally established breeding colony, grown according to the standard procedure for inbred strains. MCa mammary carcinoma, originally obtained from the Department of Experimental Biology and Medicine of the Rudjer Boskovic Institute (Zagreb, Croatia) is maintained and propagated, for experimental purposes, in CBA/Lac mice by intramuscular injection of a tumor cell suspension containing 10^6 viable tumor cells.[18]

Measurement of Tumor Growth and Metastasis Formation

Primary tumor volume was determined 14 days after tumor inoculation by caliper measurements of its short and long axis. The number and volume of metastases were determined at necroscopy after sacrifice on day 21 from tumor inoculation by examining the surface of the lungs with a low-power stereo microscope. The details of the procedures used have been reported in detail elsewhere.[14]

Experimental Stress Paradigms

In order to reduce the variable stress resulting from shipment and housing,[19–21] the animals were kept in a protected environment for 2 weeks preceding each experiment and throughout the duration of the experiment. The protected environment consisted of cabinets containing the animal cages with laminar air flow, minimizing acoustic, olfactory, and visual communication among the cages. The cabinets were placed in a room remote from the animals' rooms, where staff entered only once every 5 days to check the animals for water and flood supplies, which were available *ad libitum.* The light–dark cycle in the room was 12–12 h, with an intensity in the cages of approximately 5 lux. Temperature and relative humidity were constant at 20°C and 60%, respectively. The animals were kept 5 per cage in order to avoid the effects of overcrowding or isolation on tumor progression.[19,20] Restraint stress consisted of tying the animals' legs with string fixed to small plastic boards; daily sessions lasting 1 h were repeated on days 1–6 from tumor inoculation. 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 the time of tumor inoculation until sacrifice on day 21.

Drug Treatment

Cyclophosphamide (CYCLO, CY) was kindly provided by Schering, while CCNU and DTIC were obtained from Rhone-Poulenc and Roner, respectively. To avoid the stress of repeated handling and intraperitoneal injections,[4,22] the drug was administered orally admixed in powdered food on days 1–6 from tumor implantation. Drug concentration was selected to provide the daily dosage indicated in the tables, on the basis of a measured average daily food consumption of 5.0 ± 0.1 g per mouse. The dosage used for each drug is the optimal one for the treatment schedule employed, as previously determined in separate experiments.

Measurement of Splenic T-Lymphocyte Subpopulations and NK Cells

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).[23] 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 the 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), CD8 (1 µg), or NK 1.1 (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.[24] Results for single-color analysis are expressed as total number of positive cells collected from the spleen of each animal.

Statistical Analysis

Data were subjected to the appropriate factorial ANOVAs assessing significance against an alpha-level $p < 0.05$; the significance of the effects of individual experimental variables and of their interaction(s) is indicated in the tables. Survival was analyzed using Kaplan-Meyer and Cox proportional hazard methods, and the results

TABLE 1. Effects of Cyclophosphamide and rotational stress on the survival time of mice bearing Lewis lung carcinoma

CYCLO	Rotational stress	Seasonal period	Mean survival time (days)	Long-term survivors
–	–	June	26.8	0/10
–	+		25.8	0/10
+	–		34.4	4/10[a]
+	+		27.8	0/10
–	–	February	47.8	0/10
–	+		42.9	0/10
+	–		56.3	6/10[a]
+	+		38.2	0/10
HR = 0.206 (0.066–0.064)[b]	HR = 8.24 (2.51–27.8)[b]	HR = 0.083 (0.026–0.265)[b]		

NOTE: Groups of 10 mice were implanted s.c. with Lewis lung carcinoma on day 0. The animals received cyclophosphamide (240 mg/kg/day orally) on days 1–6, and were subjected to rotational stress on days 1–21, as indicated. The survival time was recorded and analyzed using the Kaplan-Meier method. Values are reported as mean survival time and as the fraction of mice with a survival time > 90 days.

[a]Different from the other experimental groups, Pearson chi-square $p < 0.05$.

[b]Multivariate Cox proportional hazard analysis, $p < 0.0001$ (95% CI).

are indicated in TABLE 1. Standard procedures, implemented in the Systat package (SYSTAT Inc., Evanston, IL), were used.

RESULTS

The first series of experiments examined the effects of the treatments in terms of primary tumor volume measured 2 weeks after implantation, and of lung metastasis volume 1 week later at necroscopy after sacrifice. Data reported in TABLE 2 illustrate the results obtained in mice bearing MCa mammary carcinoma. ANOVA indicates that in drug-untreated controls, restraint stress causes a significant increase in lung metastasis formation, although its effects on the primary tumor are insignificant. The effects of cyclophosphamide are significant and similarly pronounced on primary tumor and metastasis volume; the reduction in metastasis volume and in the number of mice with metastasis is significantly attenuated upon the combination of restraint stress with drug treatment. CCNU and DTIC do not cause any significant effect on the primary tumor; CCNU significantly decreases metastasis incidence and volume, whereas DTIC reduces metastasis incidence only. The number of mice with metastasis after treatment with CCNU and DTIC is increased by restraint stress to values that do not differ significantly from those of drug-untreated controls.

In mice bearing Lewis lung carcinoma, cyclophosphamide is highly effective on primary tumor and metastasis; the further application of rotational stress significantly attenuates the effectiveness of cyclophosphamide. The effects of rotational stress on the growth of the primary tumor and on the formation of lung metastasis in drug-untreated controls significantly depend on the seasonal period, and consist of an increase in summer and a decrease in winter. After treatment with cyclophosphamide, the fraction of mice without primary tumor or metastasis is markedly reduced, and the effectiveness of cyclophosphamide is significantly attenuated by rotational stress; these effects do not significantly depend on the seasonal period (see TABLE 3).

Further experiments were performed in mice bearing Lewis lung carcinoma, in order to determine the effects of cyclophosphamide and its combination with rotational stress on the survival time of the animals. Kaplan-Meier analysis and log-rank test indicate a significant effect of the treatments on survival when the data are stratified on the eight experimental groups (Mantel chi-square = 51.18, $p < 0.0001$). Univariate Cox proportional hazard estimation confirms a significant effect ($p < 0.008$) for cyclophosphamide treatment (HR = 0.232, 95% C.I. 0.077–0.697), for rotational stress (HR = 6.84, 95% C.I. 2.16–21.6), and for the seasonal period (HR = 0.160, 95% C.I. 0.053–0.479). Multivariate Cox proportional hazard analysis also significantly confirms ($p < 0.0001$) that cyclophosphamide treatment is a negative risk factor (HR = 0.206, 95% C.I. 0.066–0.640), that rotational stress is a significant positive risk factor (HR = 8.24, 95% C.I. 2.51–27.8), and that the seasonal period has a significant relevance for survival (HR = 0.083, 95% C.I. 0.026–0.265). The mean survival time of the untreated animals appears to be greater in winter than in summer, and to be prolonged by cyclophosphamide; rotational stress lacks effects by itself, but attenuates the increase in survival caused by cyclophosphamide. The significant fraction of long-term survivors in the cyclophosphamide group (4/10 in June and 6/10 in February, Pearson chi-square $p < 0.05$) is reduced to 0/10 in both

TABLE 2. Effects of restraint stress, and cyclophosphamide, CCNU, and DTIC on primary-tumor growth and metastasis formation in mice bearing MCa mammary carcinoma

Drug	Drug treatment	Restraint stress	Primary tumor volume (cm^3)	Number of mice with tumor/total number	Metastasis volume (mm^3)	Number of mice with metastasis/total number
CYCLO	–	–	0.51 ± 0.21	9/9	49.8 ± 30.6	9/9
	–	+	0.85 ± 0.72	9/9	116.3 ± 39.4	9/9
	+	–	0.04 ± 0.02	6/9[a]	14.0 ± 0.0	2/9[a,b]
	+	+	0.24 ± 0.19	9/9	26.6 ± 10.3	8/9
ANOVA			CY		Stress, CY, stress*CY	
CCNU	–	–	1.70 ± 0.45	9/9	37.8 ± 21.4	9/9
	–	+	1.86 ± 0.28	9/9	90.4 ± 55.1	9/9
	+	–	1.54 ± 0.36	9/9	4.5 ± 0.7	2/9[a]
	+	+	1.60 ± 0.36	9/9	9.2 ± 6.4	5/9
ANOVA					CCNU	
DTIC	–	–	1.69 ± 0.45	9/9	37.7 ± 21.4	9/9
	–	+	1.86 ± 0.28	9/9	90.4 ± 55.1	9/9
	+	–	1.61 ± 0.32	2/9	37.7 ± 37.6	3/9[a]
	+	+	1.56 ± 0.37	5/9	24.3 ± 23.8	6/9
ANOVA					DTIC, stress*DTIC	

NOTE: Each value is the mean ± SD obtained using groups of 9 mice that were implanted i.m. with MCa mammary carcinoma on day 0. The animals received orally cyclophosphamide (240 mg/kg/day on days 1–6, CYCLO), CCNU (9.5 mg/kg/day on days 1–14), or DTIC (60 mg/kg/day on days 1–14), and were subjected to restraint stress on days 1–6, as indicated.

[a] Different from drug untreated controls, Pearson chi-square $p < 0.05$.

[b] CYCLO different from CYCLO + stress, Pearson chi-square $p < 0.05$.

TABLE 3. Effects of Cyclophosphamide and rotational stress on primary-tumor growth and lung metastasis formation in mice bearing Lewis lung carcinoma

CYCLO	Rotationl stress	Seasonal period	Primary tumor volume (cm^3)	Number of mice with tumor/total number	Metastasis volume (mm^3)	Number of mice with metastasis/total number
−	−	June	2.03 ± 0.20	10/10	27.5 ± 8.42	10/10
−	+		2.43 ± 1.29	10/10	82.5 ± 25.7	10/10
+	−		0	0/10[a]	0	0/10[a]
+	+		0.69 ± 0.24	10/10	14.7 ± 4.55	10/10
−	−	February	2.28 ± 0.49	10/10	32.4 ± 19.6	10/10
−	+		1.68 ± 0.45	10/10	10.5 ± 5.46	10/10
+	−		0.34 ± 0.26	4/10[a]	0	0/10[a]
+	+		0.81 ± 0.45	10/10	1.47 ± 0.15	6/10
ANOVA			Stress, CY, seas.*stress, seas.*CY, seas*CY*stress		Seas., CY, Stress Seas.*stress Seas.*CY Seas.*CY*stress	

NOTE: Each value is the mean ± SD obtained using groups of 10 mice that were implanted s.c. with Lewis lung carcinoma on day 0. The animals received cyclophosphamide (240 mg/kg/day orally, CYCLO) on days 1–6, and were subjected to rotational stress (RS) on days 1–21, as indicated.

[a] Different from the other groups, Pearson chi-square $p < 0.05$.

TABLE 4. Effects of Cyclophosphamide and rotational stress on splenic T-lymphocyte subpopulations and NK cells

Rotational stress	CYCLO	Seasonal period	CD3+	CD4+	CD8+	CD4+/CD8+ ratio	NK
−	−	June	23.5 ± 3.14	13.6 ± 1.81	10.7 ± 1.63	1.27 ± 0.08	2.66 ± 0.73
+	−		13.9 ± 1.27	7.32 ± 1.11	5.92 ± 0.74	1.26 ± 0.29	2.30 ± 1.01
−	+		10.6 ± 2.12	6.61 ± 1.09	4.11 ± 1.13	1.68 ± 0.43	1.41 ± 0.32
+	+		6.99 ± 1.30	4.08 ± 0.40	2.65 ± 0.71	1.61 ± 0.34	1.04 ± 0.22
−	−	February	21.0 ± 3.45	15.7 ± 3.42	9.12 ± 0.90	1.71 ± 0.24	3.32 ± 1.80
+	−		31.0 ± 6.67	23.1 ± 2.77	7.50 ± 2.15	3.25 ± 0.81	4.25 ± 2.02
−	+		13.1 ± 2.47	10.0 ± 0.99	3.38 ± 1.30	3.37 ± 1.44	2.01 ± 1.49
+	+		10.2 ± 1.10	7.40 ± 0.85	1.59 ± 0.32	4.84 ± 1.39	1.91 ± 0.41
ANOVA			Seas.,CY Seas.*stress Seas.*CY Seas.*stress*CY	Seas.,CY Seas.*stress Seas.*CY Stress*CY Seas.*stress*CY	Stress,Cy Seas.*stress*CY	Seas.,stress,CY Seas.*stress Seas.*CY	Seas., CY

NOTE: Each value is the mean ± SD ($\times 10^6$) obtained using groups of five mice that were subjected to rotational stress (RS) and were treated with cyclophosphamide (CYCLO), as indicated.

seasonal periods. The pronounced effects of cyclophosphamide in terms of survival thus appear to be sharply reduced by rotational stress, and not to depend on the seasonal period (TABLE 1).

Finally, data in TABLE 4 illustrate the effects of the treatments on splenic T-lymphocyte subsets and on NK cells in normal mice. ANOVA indicates a significant interaction of rotational stress, cyclophosphamide, and seasonal period on CD3+ and CD4+ subsets. A reduction in these subsets is caused by rotational stress in June, whereas an increase is observed in February. In both seasonal periods cyclophosphamide causes a decrease in these subsets, which are further reduced by the combination of drug treatment with rotational stress.

DISCUSSION

The application of stress paradigms, such as rotational and restraint stress, has been previously shown in the authors' laboratory to specifically increase the formation of spontaneous lung metastasis in mice bearing Lewis lung carcinoma.[14,15] Restraint stress also has been shown in the same animal-tumor system to markedly attenuate the antitumor effects of cyclophosphamide, both in terms of tumor and metastasis size at sacrifice, and also more meaningfully of the animals' survival.[17] The aim of the present investigation therefore has been to examine the effects of restraint and rotational stress on tumor metastasis in mice bearing a different tumor, and on the effectiveness of the treatment with different antitumor drugs.

Indeed, when tumor and metastasis size are determined at the end of treatment in mice bearing MCa mammary carcinoma, rotational stress increases metastasis formation and markedly attenuates the pronounced antitumor effects of cyclophosphamide, as observed in mice bearing Lewis lung carcinoma. In these experimental conditions, CCNU displays a similarly effective antitumor action, whereas the effects of DTIC are less pronounced; for the latter drugs, the application of rotational stress causes a tendency toward the reduction of their effects, which does not reach statistical significance.

Rotational stress has been shown to increase metastasis formation in mice bearing Lewis lung carcinoma, displaying a circannual rhythm; metastasis was increased in summer and decreased in winter.[16] The data currently reported confirm this finding, and further indicate that the effects of cyclophosphamide on tumor and metastasis size are similarly attenuated by rotational stress in both seasonal periods. The examination of the survival time of the animals provides additional information, which is particularly relevant in a therapeutic perspective. Multivariate Cox proportional hazard analysis indicates a significant effect for the seasonal period and rotational stress, as well as for cyclophosphamide treatment. The survival time of control mice is approximately twice as long in February as compared with June, and is not appreciably modified by rotational stress. On the other hand, cyclophosphamide causes an increase in survival time that is substantially equivalent in both seasonal periods; the combined application of cyclophosphamide and rotational stress reduces the mean survival time of the animals by a similar extent. A similar proportion of cures caused by cyclophosphamide (4/10 and 6/10 long-term survivors in June and February, respectively), is reduced to 0/10 upon application of rotational stress in both seasonal periods.

The seasonal effects of rotational stress in mice bearing Lewis lung carcinoma, and the reduction of the effects of cyclophosphamide caused by rotational stress in mice bearing the same tumor, were accompanied by corresponding variations in the number of CD3+ and CD4+ splenic T-lymphocyte subsets[16] and in the CD4+/CD8+ ratio,[17] respectively. The data presented reveal a significant effect of rotational stress and cyclophosphamide on the number of CD3+ and CD4+ subsets, which is additive when the treatments are combined. These results support the view that the reported effects of the stress paradigms on tumor metastasis and on the effectiveness of the tested antitumor drugs are caused via modulation of immune responses of the host directed against the tumor.

In conclusion, these data appear of interest for their experimental implications, showing that tumor metastasis in laboratory mice can be influenced by stress paradigms in a way that may also display significant seasonal factors. Moreover, stress may also influence the effectiveness of antitumor drugs, although seasonal factors appear of little relevance for these effects. The effects of stress on tumor progression and response to chemotherapy appear to occur via modulation of T-lymphocyte immune functions of the host. In a clinical perspective, psychosocial factors have been shown to significantly influence tumor progression in cancer patients.[1,2,25,26] However, these investigations concerned patients who had already completed their treatment, including chemotherapy. The data presented suggest the opportunity to also consider in future investigations the role that the stress during the period of acute treatment, deriving from life events as well as from the treatments themselves, may play in determining the effectiveness of clinical antitumor chemotherapy.

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