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Neuroimmunotherapy of Advanced Solid Neoplasms with Single Evening Subcutaneous Injection of Low-dose Interleukin-2 and Melatonin: Preliminary Results

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On the basis of the demonstrated existence of immunoneuroendocrine interactions and on the previously observed synergistic action betwen the pineal hormone melatonin (MLT) and interleukin-2 (IL-2), we have designed a neuroimmunotherapeutic combination consisting of low-dose IL-2 and MLT in the treatment of advanced solid neoplasms. The study included 24 patients with advanced solid tumours (non-small cell lung cancer 9; colorectal cancer 7; gastric cancer 3; breast cancer 2; cancer of pancreas 1; hepatocarcinoma 1; unknown primary tumour 1), 21 of whom showed distant organ metastases. Not all patients responded to previous chemotherapies, or had tumours for which no standard therapy was available. Moreover, not all patients were able to tolerate IL-2 immunotherapy at the conventional doses. IL-2 was given subcutaneously at a dose of 3×10^6 U/day at 8.00 p.m. for 6 days/week for 4 weeks. MLT was given orally at a dose of 50 mg at 8.00 p.m. every day, starting 7 days before IL-2 injection. In non-progressed patients, a second cycle was given after a 21-day rest period. A partial response was seen in 3/24 patients (lung 2; stomach 1; duration: 11, 4, 4 months, respectively). Moreover, a minimal response (duration: 8+ months) was seen in 1 lung cancer patient. Stable disease was obtained in 14/24 patients (median duration: 6+ months), while the remaining 6 patients progressed. An improvement in performance status was seen in 7/24 patients. No important toxicity was observed. Mean eosinophil and lymphocyte levels significantly increased during the immunotherapy, and their rise was significantly higher in patients with response or stable disease than in those with progressive disease. These preliminary results show that neuroimmunotherapy with low-dose IL-2 and the pineal hormone MLT is a biologically active and well tolerated strategy, capable of determining an apparent control of tumour growth in patients with advanced solid neoplasms, for whom no standard effective therapy is available.
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INTRODUCTION

SEVERAL CLINICAL studies have documented that interleukin-2 (IL-2) represents one of the most active immune substances in the treatment of metastatic renal cancer and melanoma [1–5]. On the contrary, other solid neoplasms seem to be less responsive to IL-2; in particular, IL-2 has appeared to induce only a low tumour regression rate in the most frequent cancer histotypes, such as breast carcinoma and lung cancer [1, 6, 7]. Therefore, several combined strategies have been proposed to enhance the anti-tumour efficacy of IL-2, by its combination with chemotherapeutic drugs [8, 9] or with other cytokines [10–13]. The association with doxorubicin seems to be one of the most promising chemoimmunotherapeutic regimen [14], capable of determining objective tumour regressions in some patients with breast or lung cancer. As far as the association with cytokines is concerned, IL-2 has been given in combination with tumour necrosis factor (TNF) [15] or with interferons [16], without, however, any evident improvement in the efficacy of the immunotherapy.

The reduction of IL-2 toxicity, achieved by a subcutaneous rather than intravenous injection, allowed an extension of the clinical use of IL-2 in cancer immunotherapy [17]. However, it has been remarked that almost all combinations containing IL-2 have been elaborated without taking into consideration the interactions between immune and neuroendocrine systems [18–20]. In fact, it has been shown that the immune reactions, including the IL-2-induced activation of host antitumour immune defenses, depend not only on immune factors, but also on the neuroendocrine regulation of the immune system [18–20]. Lymphocytes may produce hormones, and also, neuropeptides and neurohormones produced by the psychoneuroendocrine system may influence the immune responses themselves [18–20]. Therefore, the neuroendocrine variations, which occur during IL-2 immunotherapy [21, 22], may influence the clinical efficacy of IL-2 treatment. Moreover, the neuroendocrine control of the immunity would be responsible for the existence of circadian variations in immune cell number and activity [23]. Finally, preliminary results would suggest that IL-2 receptors on immune cells may be modulated by neurohormones; the regulation of

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IL-2 receptors on immune cells could constitute an important mechanism responsible for the neuroendocrine regulation of the immune responses [24].

Despite the well-documented existence of feed-back mechanisms operating between cytokines and neurohormones [18–20], at present no clinical study has been performed to evaluate the efficacy of IL-2 in association with neurohormones. Taking into consideration the interactions existing between cytokines and the neuroendocrine system, we have elaborated a neuroimmunotherapeutic strategy of tumours, by defining the cancer neuroimmunotherapy as a concomitant administration of IL-2 and neurohormones provided by immunomodulating properties. Within the great number of immunomodulating neurohormones, we have investigated the pineal hormone melatonin (MLT) [24–26]. MLT has appeared to stimulate the immune system [25] and to potentiate the antitumour efficacy of IL-2 itself in several experimental conditions [26]. Our preliminary results in humans [24] have shown that MLT may be associated with IL-2 to inhibit macrophage-mediated suppressive events. Moreover, we have observed that a single subcutaneous injection of low-dose IL-2 in the evening, when the immune system is more active [23], suffices to activate the immune system when it is associated with pharmacological doses of MLT, by suggesting that the pineal hormone may be successfully used to reduce the dose of IL-2 required to induce an effective antitumour response. The present study shows the preliminary biological and clinical results of a combination of IL-2 and MLT in the treatment of advanced solid tumours.

MATERIALS AND METHODS

The study included 24 consecutive cancer patients with locally advanced or metastatic solid neoplasms. All patients were resistant to previous chemotherapies or had tumours for whom no effective standard therapy was available. Moreover, no patient was eligible for IL-2 immunotherapy at the conventional high intravenous doses [1–5] because of low performance status (PS), age, presence of brain metastases and/or important cardiovascular or bronchopulmonary diseases. Tumour histotypes were as follows: non-small cell lung cancer 9 (adenocarcinoma 5; epidermoid cancer 3; large cell cancer 1); colorectal carcinoma 7; gastric cancer 3; breast cancer 2; cancer of pancreas 1; hepatocarcinoma 1; unknown primary tumour 1. Visceral metastasis sites were present in 21/24 patients, while the remaining 3, all affected by lung cancer, had a locally advanced unresectable disease. 11 patients had been previously treated with chemotherapy, while the other 13 received the neuroimmunotherapy as a first line therapy for their advanced disease. All patients had rapidly progressed, as documented by the increase of at least 30% of the neoplastic lesions every 2 months and/or by the appearance of new lesions within the last 2 months prior to the neuroimmunotherapy. Clinic characteristics of patients are shown in Table 1.

Human recombinant IL-2 was supplied by Euro-Cetus (Amsterdam, The Netherlands). MLT was supplied by Helsinn Chemicals (Breganzona, Switzerland). The experimental protocol was explained to each patient and informed consent was obtained. IL-2 was given subcutaneously into different parts of the abdominal wall at a dose of 3×10^6 U/day at 8.00 p.m. for 6 days/week for 4 consecutive weeks, corresponding to one immunotherapeutic cycle. MLT was orally given at a daily dose of 50 mg at 8.00 p.m. every day, starting 7 days before the first IL-2 injection. IL-2 was given in the evening because of the documented increase in lymphocyte proliferation during the dark period of the day [23]. MLT was also given in the evening, since it has been demonstrated that the biological sensitivity to the exogenous administration of the pineal hormone is more pronounced in this period of the day [27]. In responder patients or in those with a stabilisation of the disease, a second neuroimmunotherapeutic cycle was given after a 21-day rest period; after that, patients underwent a maintenance therapy consisting of IL-2 plus MLT 1 week/month until the progression of disease.

Table 1. Clinical data of 24 advanced solid tumour patients treated with IL-2 plus the pineal hormone MLT

Male/female	12/12
Median age (years) (range)	58 (36–72)
Tumour histotype	
Non-small cell lung cancer	9
Adenocarcinoma	5
Epidermoid carcinoma	3
Large cell carcinoma	1
Colorectal carcinoma	7
Gastric carcinoma	3
Breast carcinoma	2
Cancer of pancreas	1
Hepatocarcinoma	1
Unknown primary adenocarcinoma	1
Visceral metastases	21/24
Lung	5
Liver	9
Lung + liver	1
Lung + liver + brain	3
Serouses	3

Clinical response was graded as follows: complete response (CR) was the disappearance of all neoplastic lesions for at least 1 month; partial response (PR) was a reduction of more than 50% in the sum of the products of the perpendicular diameters of all measurable lesions for at least 1 month; minimal response (MR) was a reduction more than 25% but less than 50%; stable disease (SD) was no decrease or increase more than 25% in tumour measurements; progressive disease (PD) was an increase of more than 25% in any single neoplastic measurable lesion, or the appearance of new neoplastic lesions. The performance status (PS) was evaluated in patients according to Karnofsky's score.

To evaluate the neoplastic lesions, radiological examinations were repeated every month. Routine laboratory tests were made every week. Moreover, in 9 patients, we also determined serum levels of neopterin and TNF at weekly intervals, by using a radioimmunoassay method and commercially available kits.

Results were statistically analysed by the Student's *t*-test, analysis of variance and coefficient of correlation, as appropriate.

RESULTS

Individual characteristics of patients and their clinical response to the immunotherapy are reported in Table 2. No CR was observed. A PR was achieved in 3/24 (12.5%) patients, the first with lung adenocarcinoma, the second with epidermoid cell lung cancer and the third with gastric adenocarcinoma (duration: 11, 4 and 4 months, respectively). Moreover, a MR was seen in 1 patient with lung adenocarcinoma (duration: 8+ months). A SD was observed in 14/24 (58%) patients, with a median duration of 6+ months (range 4–10 months). The remaining 6/24 (25%) patients progressed during the first cycle of immunotheapy. An

Table 2. Clinical data and response to therapy in 24 advanced solid neoplasms with IL-2 plus MLT

No.	Sex	Age (years)	Tumour site	Metastasis sites	Previous chemotherapy	Clinical response	Response duration (months)	Progression sites	Performance status Before	Performance status After	Survival (months)
1	F	51	Lung (E)	Lung	—	PD	—	Lung	50	50	3
2	M	63	Colon (A)	Liver, lung	FU	PD	—	Liver	40	40	2
3	F	38	Lung (A)	—	—	PR	11	Lung	20	50	13+
4	M	48	Unknown (A)	Peritoneum	—	PD	—	Peritoneum	70	70	3
5	M	58	Lung (A)	—	—	SD	10+	—	90	100	10+
6	F	36	Lung (A)	Liver, brain, bone	—	SD	4	Bone	40	40	6
7	F	46	Rectum (A)	Liver	FU	PD	—	Lung	80	80	5
8	M	61	Lung (LG)	Lung, liver, brain	PE	PD	—	Brain	60	50	4
9	M	58	Lung (A)	Lung	—	MR	8+	—	70	100	8+
10	M	39	Rectum (A)	Peritoneum	FU	SD	4	Peritoneum	20	60	8
11	F	54	Breast (D)	Lung, liver, brain	CMF, FEC	SD	2	Liver	20	20	4
12	F	66	Stomach (A)	Liver	—	SD	8+	—	70	90	8+
13	F	42	Colon (A)	Liver	FU	PD	—	Liver	20	40	3
14	M	72	Lung (E)	Liver	—	SD	4	Liver	90	90	7+
15	M	64	Stomach (A)	Liver	—	PR	4	Liver	40	80	7
16	F	62	Colon (A)	Lung	FU	SD	7+	—	80	80	7+
17	M	70	Lung (E)	—	—	PR	4+	Lung	60	90	7
18	F	52	Pancreas (A)	Liver	—	SD	6+	—	50	50	6+
19	M	58	Stomach (A)	Liver	FU	SD	6+	—	100	100	6+
20	F	48	Colon (A)	Liver	FU	SD	6+	—	90	90	6+
21	M	58	Lung (A)	Lung	—	SD	6+	—	60	60	6+
22	F	39	Colon (A)	Liver	FU	SD	5+	—	90	90	5+
23	M	70	Liver (H)	Pleura	—	SD	5+	—	70	70	5+
24	F	37	Breast (IC)	Lung, nodes	FEC, CMF	SD	4+	—	80	80	4+

M: Male; F: female; E: epidermoid carcinoma; A: adenocarcinoma; D: ductal carcinoma; LG: large cell carcinoma; H: hepatocarcinoma; IC: inflammatory carcinoma.

FU: Fluorouracil; PE: cisplatin, etoposide; CMF: cyclophosphamide, methotrexate, fluorouracil; FEC: fluorouracil, epirubicin, cyclophosphamide. PR: partial response; MR: minimal response; SD: stable disease; PD: progressive disease.

evident improvement in the PS was observed in 7/24 (29%) patients, and in particular it was achieved in 4/7 patients with a PS less than 50% before the start of the immunotherapy.

Episodes of fever higher than 38°C were sporadically observed only in 4/24 (17%) patients. No other toxicity was observed, and in particular no cardiovascular complication occurred. A worsening of the neurological symptomatology was seen in 1/3 patients with brain metastases, and it was controlled by low doses of steroids. No patient had thrombocytopenia during the study; on the contrary, platelet mean number increased during the administration of IL-2 plus MLT, without, however, significant differences in respect to the pretreatment values (360 000 ± 70 000 vs. 230 000 ± 60 000/mm^3). The overall survival time was 6+ months; the mean survival time was significantly higher in patients with response or SD than in the progressed ones (6.7 ± 0.5 vs. 3.3 ± 0.4 months; $P < 0.005$).

The variations in lymphocyte and eosinophil mean number and in serum levels of neopterin and TNF are illustrated in Figs 1 and 2, respectively. The mean number of both lymphocytes and eosinophils significantly increased during IL-2 administration in respect to the values seen before the onset of the immunotherapy. Serum mean concentrations of neopterin and TNF were also significantly enhanced under treatment, with a peak after the second week of IL-2 injection. Neopterin rise was significantly correlated to that of TNF ($r = 0.8$). Lymphocyte and eosinophil variations observed in progressed patients or in those with response or SD are illustrated in Fig. 3. The increase

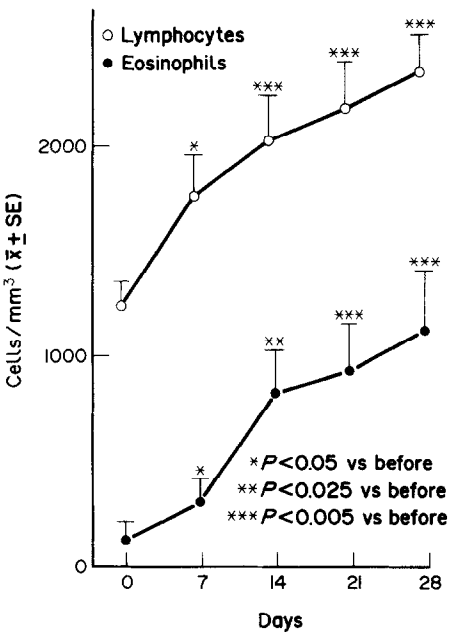

Fig. 1. Lymphocyte and eosinophil mean number during the first neuroimmunotherapeutic cycle with IL-2 plus MLT in 24 advanced solid tumour patients.

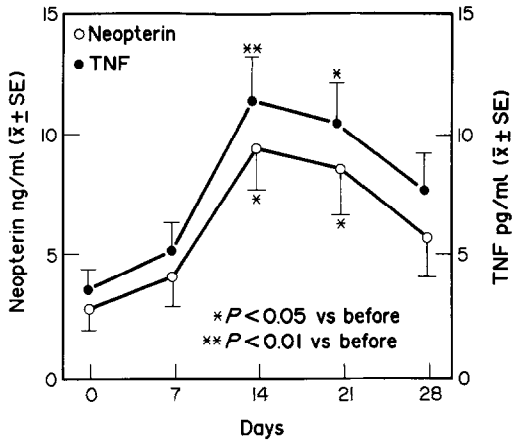

Fig. 2. Mean serum levels of neopterin and TNF in 9 advanced solid tumours patients treated with IL-2 plus MLT.

in lymphocyte and eosinophil mean number was significantly higher in patients with response or SD than in progressed patients. On the contrary, no difference was seen between patients with PR or MR and those with SD in the increase in eosinophils (1846 ± 259 vs. 1436 ± 118) and lymphocytes 1451 ± 225 vs. 1172 ± 149/mm^3).

DISCUSSION

The results of this preliminary study show that low-dose IL-2 given once daily in the evening, which would represent the period of the day during which lymphocyte proliferation spontaneously enhances [23], is able to activate the immune system and to determine at least an apparent control of tumour growth

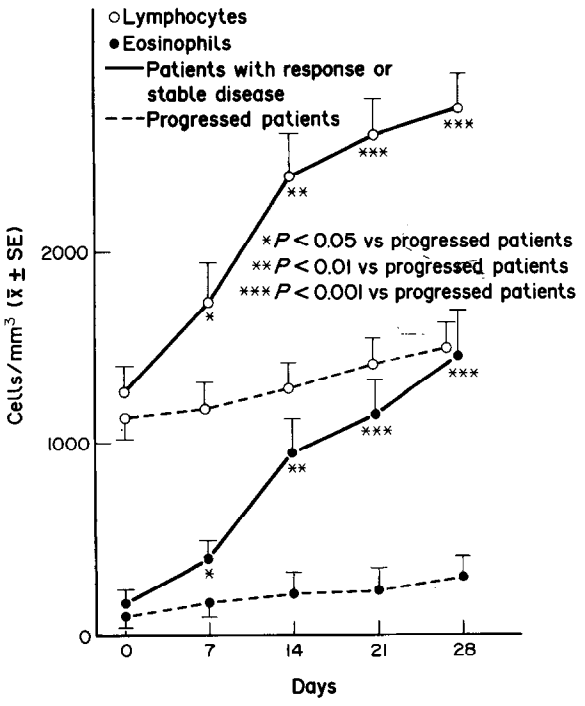

Fig. 3. Lymphocyte and eosinophil mean number during immunotherapy with IL-2 plus MLT in 6 progressed patients and in 18 patients with response or stable disease.

in rapidly progressed patients when it is associated with the pineal hormone MLT. These effects have been obtained in solid tumours, which generally do not respond to IL-2 alone [1, 6, 7]. The stabilisation of the neoplastic disease has appeared to be associated with an evident increase in eosinophil and lymphocyte number, and with an apparent longer survival. Therefore, as previously observed in animals [24–26], this preliminary clinical investigation would confirm in humans that the neurohormone MLT may potentiate the action of IL-2.

The mechanisms by which MLT would synergise with IL-2 are complex and still unknown. The pineal hormone could potentiate the efficacy of IL-2 by counteracting the concomitant generation of suppresive events [23], mainly mediated by macrophages [27], as suggested by the low increase in neopterin, which is a specific marker of macrophage activation, in respect to its rise previously seen during IL-2 alone [27]; in particular, MLT could inhibit the macrophagic stimulatory role on the release of soluble IL-2 receptor [28], which may compete for IL-2 with IL-2 cell surface receptor [29]. The prevention of thrombocytopenia, observed in this study, would also document an inhibition of macrophages, which are responsible for platelet destruction [30]; moreover, the rise in platelet number under treatment with IL-2 plus MLT would suggest a synergistic action between the pineal hormone and cytokines involved in platelet generation, such as interleukin-3 and interleukin-11.

Another interesting observation may be drawn from the eosinophil pattern during the neuroimmunotherapy; eosinophilia observed in this study with the association between IL-2 and MLT would seem to be greater than that described in the literature with IL-2 alone [1–5, 17]. This finding would suggest that MLT may be a growth factor for eosinophils; however, since no eosinophilia was observed with MLT alone in our previous studies [31], it is more probable that the pineal hormone may act by amplifying the response of eosinophils to IL-2. Alternatively, MLT could act on T-helper lymphocytes type 2 by enhancing their response to IL-2, with a following increased production of interleukin-5 [32], which is the main growth factor for eosinophil generation. In any case, since both eosinophils [17, 33] and lymphocytes [1–5] are involved in the antitumour response, the association between IL-2 and MLT might allow a more effective host anticancer defense. Our previous experimental studies had shown that MLT exerted immunostimulating properties only after immune activation induced by antigen injection [34], while no stimulatory effect was found in physiological conditions. The mechanism by which MLT may display immunomodulating effects only after immune activation are still unknown; the results of this study could suggest that MLT-induced immune stimulation may require the presence of IL-2, whose secretion is enhanced after immune activation [35]. The synergistic action between IL-2 and MLT in the amplification of the immune responses might constitute an important physiological neuroendocrine–immune circuit, from whose interactions would depend the efficacy of the immune response itself; the progressive decline in the pineal function with age [36] might explain the decrease in the immune performance in the elderly humans. A more detailed knowledge of the physiological connections between the immune and nervous systems will allow more effective immunotherapeutic strategies of cancer.

In conclusion, this study shows that the neuroimmunotherapeutic combination with low-dose IL-2 and the pineal hormone MLT represents a new effective and well-tolerated oncologic therapy of tumours resistant to conventional treatments, that may be administered also in patients with a low performance

status. Therefore, because of its capacity of inducing an evident biological response, the neuroimmunotherapy of cancer with IL-2 and MLT deserves further investigations to better define its therapeutic activity in relation to the different tumour histotypes; in particular, randomised studies with IL-2 alone vs. IL-2 plus MLT will be required to establish which is the role of the pineal hormone in cancer immunotherapy.

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