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IMMUNOMODULATION IN A TREATMENT PROGRAM INCLUDING PRE- AND POST-OPERATIVE INTERLEUKIN-2 AND CHEMOTHERAPY FOR CHILDHOOD OSTEOSARCOMA

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Aims and background: The treatment applied in our Institution to children with localized osteosarcoma between 1991 and 1999 consisted of four interleukin 2 (IL-2) courses (9 x 10^6 IU/mL/daily x 4), alternated with pre- and post-operative polichemotherapy. The aims of the present study were to quantify the modifications of some immunological parameters induced by IL-2 and to verify whether polychemotherapy could reduce them. An additional aim was to assess whether any correlation between the immune modifications and the clinical outcome could be found.
Patients and methods: We evaluated in 18 consecutive patients the following changes, induced in blood by each IL-2 course: number of lymphocyte subpopulations and natural killer (NK) cells, lymphokine activated killer (LAK) and NK activities.
Results: Chemotherapy did not influence the modifications of the number of NK and CD4+ cells and of the LAK and NK activities, induced by each of the four courses of IL-2. The magnitudo of the NK activity and the peak of the NK absolute counts significantly correlated with the clinical outcome.
Conclusions: The results show that the use of IL-2 permitted a repeated immune activation despite the intensive chemotherapy. Furthermore, although the limited number of cases precludes any definitive conclusion, the results suggest a possible role of the NK cells in the control of osteosarcoma.

Key words: interleukin-2, NK activity, NK cells, Osteosarcoma.

Introduction

Different studies had shown that multidrug-resistant human cancer cells are susceptible to adoptive cellular immunotherapy, for example to LAK cells or activated phagocytes[1,2]. These findings led to the idea that adoptive immunotherapy could be associated with chemotherapy (CT) to kill multidrug-resistant clones. Nevertheless, when a complementary immuno- and chemo-therapy are associated, a possible interference of CT in the immune functions has to be taken into account. Numerous studies considering the effects of CT immunoreactivity in humans demonstrated immunosuppressive effects of some drugs, including methotrexate[3-5]. Other drugs, as cisplatinum, could have a positive influence on immune functions[7-10]. As to other drugs, such as adriamycin, the results concerning the effects on immune activities are controversial[9,11]. Furthermore, the dynamics of the immunologic functions induced during treatment with combined polychemotherapy and IL-2 are still not well clarified. The rationale of the present study was to look for possible interferences between CT and the IL2-induced activation of the immune system in a setting of children with osteosarcoma, receiving a treatment plan which consisted of sequential infusions of IL-2 and polychemotherapy before and after surgical resection of the primary tumor.

The study focused on the quantification of the changes in the peripheral blood mononuclear cell subpopulations (PBMC) and LAK/NK activities induced by the initial course with IL-2, and on the evaluation whether these changes could be reproduced with the subsequent courses with IL-2, given after CT. Additionally, we looked in this cohort of patients for any correlation between the modifications of the immunological parameters induced by IL-2 and the clinical outcome.

Material and methods

Study design

All children with biopsy-proven and previously untreated non metastatic osteosarcoma admitted at the Istituto Nazionale Tumori of Milan between 1991 and 1999 were treated according to the program shown in Table 1, which was approved by the local Ethic and Scientific Board.

Interleukin 2 was provided by EuroCetus BV, Amsterdam, The Netherlands. Leukapheresis was performed 36 hours after the end of the first course with IL-2 to

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Table 1 - Modifications of absolute cell count/mm³ induced by 1 course of IL-2

	Baseline	Day 4	Day 6
T cells			
CD3+CD4+	950 ± 325	710 ± 422	2408 ± 1633[1]
CD3+CD8+	707 ± 143	413 ± 169	1006 ± 592
CD4+CD25+	95 ± 54	21 ± 18	365 ± 219[1]
CD4+CD122+	0	12 ± 9	101 ± 78[1]
CD8+CD25+	0	14 ± 8	43 ± 27[1]
CD8+CD122+	7 ± 5	7 ± 4	63 ± 41[1]
NK cells			
CD3-CD56+	210 ± 113	260 ± 93	802 ± 328[1]
CD16+CD56+	197 ± 93	239 ± 142	775 ± 325[1]
CD16+CD122+	102 ± 62	141 ± 97	620 ± 349[1]
CD16+CD25+	22 ± 15	34 ± 27	79 ± 32[1]

[1] P <0.05 compared to the value before the course.

collect at least 20 x 10^9 PBMC/m². PBMC were then pulsed with IL-2 *in vitro* with a short-term method[12] and cryopreserved.

For the purposes of the present study, we evaluated a cohort of 18 consecutive patients treated according to this program. The immunological parameters tested in blood were: absolute count of NK cells and of lymphocytes subpopulations, LAK and NK activities. These parameters were evaluated immediately before (day 0), immediately after (day 4), 48 hours after (day 6) and 96 hours (day 8) after each IL-2 course. The LAK and NK activities on apheresed cells were evaluated immediately after pulsing, before the cryopreservation.

Immunophenotype of PBMC subpopulations

The analysis of cell surface antigens on PBMC was performed by two-colour immunofluorescent flow cytometry assay, using a panel of monoclonal antibodies with a standard direct staining method. The panel of monoclonal antibodies included CD3 (leu-4), CD4 (leu-3a), CD8 (Leu2a), CD16 (Leu11a), CD25 (Anti-IL-2R), CD56 (NKH-1) and CD122 (IL-2Rp75). Non-reactive isotype control mAb were murine IgG1-PE and IgG1-FITC.

NK and LAK activities

To evaluate the NK and LAK activities of the PBMC in blood and in apheresed cells, K562 and Daudi cell lines were used respectively. The target cells were labelled with 100μCi/L x 10^6 cells ^{51}Cr (NEN, Florence, Italy) for 1 hour at 37 °C; then, cells were washed, resuspended in a density of 5 x 10^4 cells/mL, dispensed into 96-well conical bottom microtiter plates and then effector cells were added to effector at the target ratios of 60 : 1, 20 : 1, 6 : 1 and 2 : 1. After a 4-hr incubation at 37 °C, aliquots of the supernatants were collected and their radioactivity was measured in a γ scintillation counter. Total cpm were obtained by adding 100 mL of 1% NP40 detergent (BDH, Poole, UK) to the wells containing target and effector cells, while spontaneous release was evaluated in wells containing target cells alone. All experiments were performed in triplicates and data are expressed as a percentage of specific release according to the following formula: specific release (%) = (cpm release in the presence of effector cells – cpm spontaneously released by target cells incubated with medium alone)/(cpm maximum release by addition of NP40 detergent – cpm spontaneously released by target cells incubated with medium alone).

Each determination represents an average of triplicate assays; the data are reported as the mean with the effector at target ratio 20 : 1.

Correlation of immunological parameters with outcome

To evaluate whether the IL-2 courses could have a clinical impact, we correlated the magnification of the immunological parameters above described with the outcome. For our purpose, two categories ("high" and "low") were arbitrarily assigned to each immunological parameter, as indicated in Table 3. The cut-off value to assign each patient to the category "high" or "low" was the median value measured at day 6 of the 72 IL-2 courses given to the entire study population.

Statistical analyses

The Student's t test, the Mann-Whitney test and the chi-square test were used for the correlation between the immunological parameters and their connection with the clinical outcome. The event-free survival curves were estimated using the Kaplan-Meier method and differences between groups were tested using the log-rank test. Values of P <0.05 were considered significant.

Results

The study population consisted of 9 females and 9 males, with a median age of 13 years (range, 6-17). Sixteen of them received a limb-sparing surgery, while in the remaining 2 patients a limb amputation was performed. The histological examination of the resected bone documented a tumor necrosis ≥90% in 12 cases and <90% in 6. According to the protocol, after surgery, 12 patients received the same chemotherapy as in the presurgical phase, whereas 6 were given adriamycin. All patients completed the entire treatment program within the scheduled time. The median follow-up was 5.8 years; the 5-year disease-free survival and overall survival probability were 0.61 and 0.71, respectively.

The absolute cell count showed an increasing number of lymphocytes after the end of the first course of IL-2: the peak was reached within 48 hours after the end of the IL-2 infusion (ie day 6). This phenomenon was repeated also after each of the other three IL-2 courses. The analysis of the PBMC subpopulations showed a significant increase of CD4+ lymphocytes, NK cells, LAK and NK activities after each course of IL-2 in a similar pattern in all patients, with a broad interpatient variability (Figure 1 and Table 2). The LAK and NK activities, as expected, were magnified after the 1 hr-pulsing *in vitro* on the apheresed PBMC. At day 8 of each course, the number of PBMC, the LAK and the NK activities returned to levels superimposable to those of the baseline (data not shown).

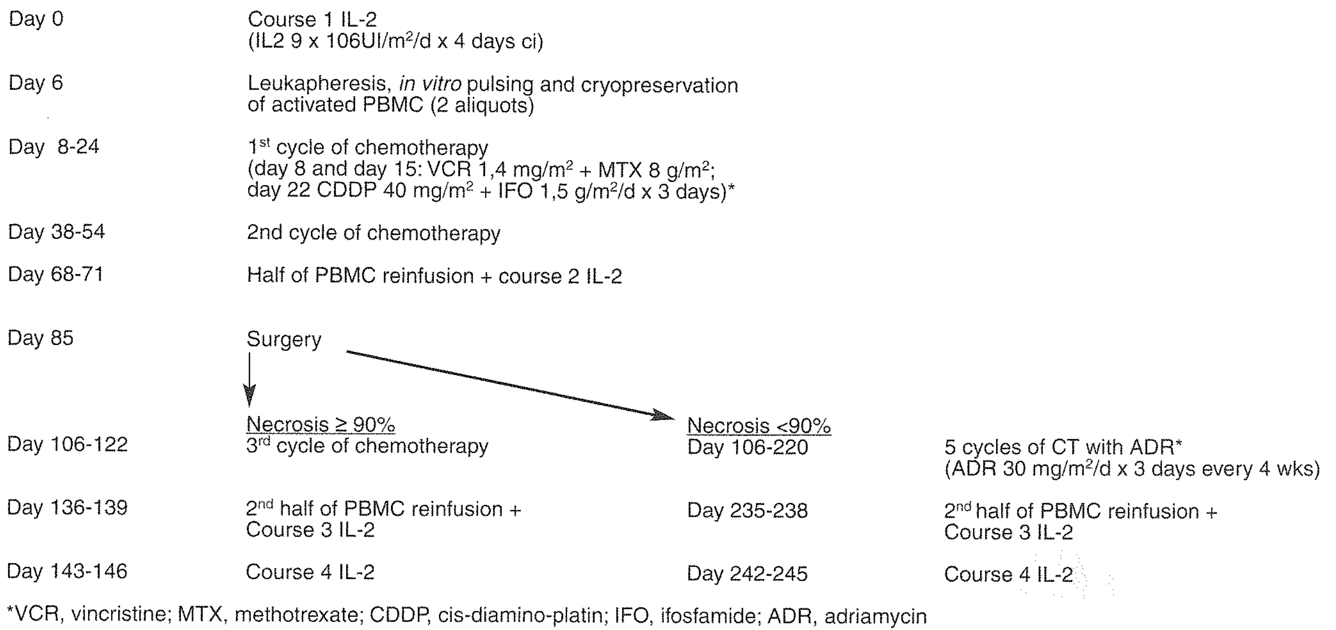

Figure 1 - Treatment plan for nonmetastatic osteosarcoma, Istituto Nazionale Tumori, Milan, 1991-1999.

Table 2 - Absolute cell count/mm³ after each following course of IL-2, at day 6

	Course 2	Course 3	Course 4
T cells			
CD3+CD4+	1804 ± 894[1]	1734 ± 622[1]	2173 ± 1820[1]
CD3+CD8+	1271 ± 744[1]	990 ± 422	1029 ± 622
CD4+CD25+	246 ± 152[1]	205 ± 192[1]	745 ± 635[1,2]
CD4+CD122+	140 ± 69[1]	139 ± 83[1]	196 ± 133[1]
CD8+CD25+	31 ± 16[1]	37 ± 19[1]	110 ± 93[1,2]
CD8+CD122+	39 ± 21[1]	81 ± 62[1]	245 ± 112[1,2]
NK cells			
CD3-CD56+	872 ± 389[1]	723 ± 289[1]	1121 ± 441[1,2]
CD16+CD56+	578 ± 229[1]	600 ± 491[1]	1189 ± 228[1,2]
CD16+CD122+	530 ± 299[1]	573 ± 388[1]	802 ± 642[1,2]
CD16+CD25+	83 ± 46[1]	41 ± 24[1]	125 ± 102[1,2]

[1]$P < 0.05$ compared to the value before the course; [2]$P < 0.05$ compared to the other courses.

Table 3 - Correlation between immunologic parameters and outcome

Variable	Category[1]	Frequency	Relapse-free	Relapsed
CD3+CD4+	High (≥1604)	9	3	6
	Low (<1604)	9	5	4
CD3+CD8+	High (≥1100)	9	5	4
	Low (<1100)	9	7	2
CD3-CD56+	High (≥828)	9	8	1
	Low (<828)	9	3	6
NK activity	High (≥24)	9	7	2
	Low (<24)	9	6	3
LAK activity	High (≥31)	9	5	4
	Low (<31)	9	6	3

[1]Mean value of the 4 IL-2 courses, measured at the peak (day 6 after each IL-2 course). Values represent the number of circulating cells/mm³ (CD3+CD4+, CD3+CD8+, CD3-CD56+) and the % of specific cytotoxicity (NK and LAK activities).

The different drugs administered in the post-operative phase did not influence the immune modifications induced by IL-2. In fact, the magnitudo of the changes in the immunological parameters was similar in both groups of patients who received vincristine + methotrexate/cisplatinum + ifosfamide and adriamycin, respectively (data not shown).

When looking for any clinical benefit from the observed immunological changes, we found a strong correlation between the NK absolute count and outcome. In fact, among the 7 patients who experienced a relapse, 6/7 had a low peak and 1/7 had a high peak of NK cells respectively. The other immunological parameters did not show significant correlation with the clinical outcome (Table 3 and Figure 2).

Discussion

In the past fifteen years, there has been a considerable interest in the use of IL-2 to elicit the immune response in different cancer types, and many trials included the combination of immunotherapy with IL-2 and CT. The alternation of IL-2 and dacarbazine in melanoma patients was one of the first combination treatments of this type. This association gave transient favourable results, and showed acceptable toxicity[13]. In successive studies IL-2 was administered in combination with other cytokines (IL-4, IL-12, rIFN alpha), with or without the association with CT, or even employed in programs including also ormonotherapy in patients with advanced solid tumors, mostly represented by melanoma and renal cell carcinoma[11,14-18].

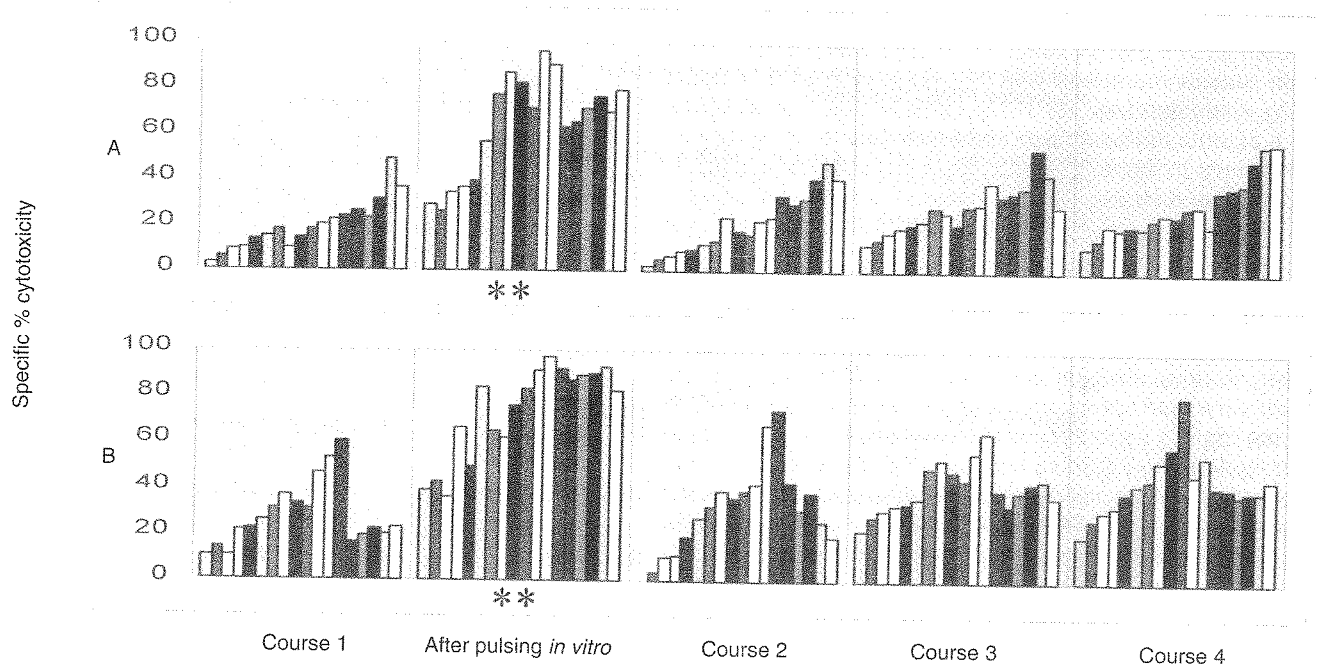

Figure 2 - NK activity (A) and LAK activity (B) after IL-2 courses. Each bar represents an individual patient, and each patient is indicated in the same relative position in each panel of the figure. The results are expressed as percentage specific cytotoxic activity at an E/T ratio of 20 : 1. The data are derived from three experiments and expressed as the mean; the SD of each data point was less than 10%. **Significantly increased (P <0.05) in all cases, compared to the percentage specific cytotoxicity measured at day 6 in the blood, before leukapheresis.

When a combination of CT and immunotherapy is planned, a possible interference between these two treatment modalities has to be taken into account. In fact, some chemotherapeutic agents show synergistic activity with immunotherapy. This phenomenon could be linked to the inhibition of mechanisms of immunosuppression or could be related to the alteration of neoplastic cells that enhances immunogenicity. Cisplatinum was one of the most promising drugs with these properties, since different studies showed *in vitro* and *in vivo* that it was able to enhance endogenous natural killer activity, without impairment of generation of IL-2- induced LAK cells[1,7-10]. Nevertheless, there are many evidences that the majority of chemotherapeutic agents can limit the activity of the immune system and could frustrate the effects of immunostimulating treatments[3-6]. In fact, although the cancer itself could be immunosuppressive, CT is the primary contributor to immunodeficiency, which is primarily related to T-cell depletion[5,19].

In order to look for a possible modulation of the immune system by a polichemotherapy using both drugs with stimulating or inhibiting activity, we monitored some immunological functions during a treatment combining alternating IL-2 and CT courses. The results suggested that – with the schedule adopted – the immunomodulatory effects of the IL-2 courses were not affected by the repeated cycles of CT. In particular, the expansion of both NK and T cell subpopulations, with the exception of CD3+CD8+ cells, as well as the priming of LAK and NK activities induced by the first course with IL-2, were elicitable with a similar magnitudo also after the following IL-2 courses.These results are only partially comparable with those of other studies, where IL-2 was given concomitantly or immediately close to cytotoxic drug administration and in various combinations: in the present study the IL-2 infusions were given two weeks after the chemotherapy, when the myelotoxicity was overcome. This could explain the effectiveness of IL-2 in stimulating the immune response, despite the use of myelotoxic and immunosuppressive drugs. For this reason, we suggest that immunotherapy with IL-2 can be repeated cyclically in treatment programs combining IL-2 and intensive CT, and an optimal schedule should use the cytokine after recovery of myelotoxicity from the previous CT cycle. Furthermore, the peak of NK cells in the present study was more evident after the two consecutive courses of IL-2 at the end of the treatment program, thus suggesting an higher efficacy of closely repeated IL-2 administrations.

The expansion of NK cells we observed after the two close IL-2 courses resembles the results of other studies, which demonstrated that a pulsing with IL-2 during a continuous intravenous infusion or daily repeated subcutaneous IL-2 led to an *in vivo* expansion and activation of NK cells[20,21].

The treatment program applied to the patients who are object of the present study also provided for the use of LAK cells reinfusions. The rationale for using LAK cells in this setting consists in the peculiar trophism of LAK cells for the interstitium of the lung[22], which is the most frequent site of relapse of osteosarcoma[23]. The activation of PBMC collected with apheresis after the first

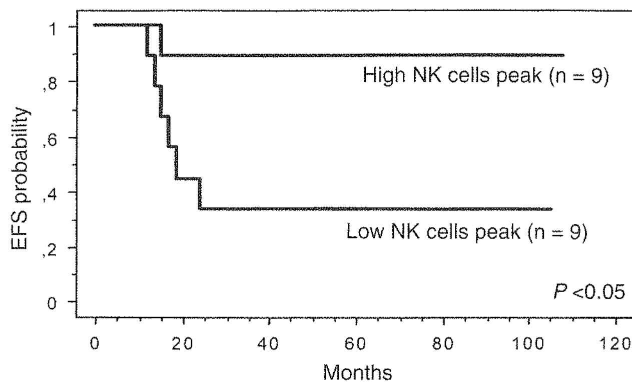

Figure 3 - Event-free survival probability according to the mean peak of NK cells. The number of CD3-CD56+ NK cells was measured at the peak, at day 6 of each of the four IL-2 courses, in all patients.
The categories "high" and "low" are defined by the criteria described in the Material and methods section.

IL-2 course was performed with the method described by Horton[12], and consisted in a short-term pulsation *in vitro* with IL-2 and rapid freezing and storage of activated cells. This method permitted to obtain large amounts of cells with magnified LAK and NK activities, and to maintain them avaliable for the reinfusion at the planned time.

An additional aim of this study was to identify a possible direct clinical contribution of the immunotherapy. For this reason we evaluated the correlation between the individual immune activation and the outcome, and we found that the high expansion of NK cells after the IL-2 infusions correlated with a good outcome. Even if the small series hinders any definitive conclusion, the association we found between favourable clinical outcome and peak of NK absolute count in this series suggest a role for the NK cells in the tumor control. The specific NK cells activity against osteosarcoma has been already demonstrated in osteosarcoma cell lines[24,25], while the present study represents the first *in vivo* indication of a possible effect of the NK cell compartment against osteosarcoma. To clarify the issue of the role of NK cells and of other immunocompetent cells in inducing tumor necrosis and the growth control in osteosarcoma, a new immunohystochemical evaluation has been started in the entire patient population of children treated between 1991 and 1999 with the program illustrated in the present study; the endpoint of this evaluation will be the identification of mononuclear cell subpopulations in the specimens of the resected tumor in these patients. At our knowledge, the treatment plan that was object of the present study was the first one employing IL-2 in combination with CT in osteosarcoma. As a matter of fact, a prospective study will be necessary to prove the therapeutic value of IL-2 and LAK cell infusions in the treatment of osteosarcoma.

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