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Immunological Reconstitution After High-dose Chemotherapy and Autologous Blood Stem Cell Transplantation for Advanced Ovarian Cancer

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We evaluated the immunological reconstitution of patients who underwent high-dose chemotherapy and autologous blood stem cell transplantation (ABSCT) for advanced ovarian cancer. Sixty days after transplantation a complete reconstitution of lymphocytes and of the CD3, CD4, CD8, CD19, and CD16/56 subsets was observed in this series. A significant increase in the count of interleukin-2 receptor expressing lymphocyte (CD25) was found on day +60 after transplantation compared to that obtained at diagnosis and before transplantation. A significantly higher lymphokine-activated killer (LAK) precursor activity was seen on day +60 compared to the values obtained at diagnosis and before transplantation while natural killer activity did not show any significant variation. We conclude that ABSCT gives prompt and complete immunohaematopoietic reconstitution after high-dose treatment. Moreover, our data support the feasibility of interleukin-2/LAK therapy as consolidative therapy after ABSCT.
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INTRODUCTION

HIGH DOSE chemotherapy and autologous bone marrow transplantation (ABMT) have now become an important treatment modality for patients with haematological and solid tumours [1–5].

More recently, the utilisation of autologous blood stem cell transplantation (ABSCT) [6–8] has significantly shortened the drug-induced myelosuppression compared to ABMT [9, 10]. Several reports described the kinetics of the immunological recovery after intense chemotherapy without stem cell rescue [11] or high dose chemotherapy followed by ABMT [12–15]. On the other hand, only preliminary data are available for patients receiving ABSCT after high dose chemotherapy [16]. Generally, following ABMT, a delayed lymphocyte recovery mainly due to a decrease in the $CD4^+$ subset is observed; in contrast an increase is observed in $CD8^+$ and in activated T and natural killer (NK) subsets [12–15]. Furthermore, Higuchi *et al.* [17] have recently indicated that lymphokine-activated killer (LAK) precursor

Table 1. Autologous blood stem cell transplantation in 6 patients with advanced ovarian cancer

Patients	Cell doses NC × 10^9/kg	MNC × 10^9/kg	CFU-GM × 10^4/kg	Peripheral blood recovery (day) WBC> 1000/μl	PMN > 500/μl	PLT > 50 000/μl	Lymph > 1000/μl
01	1.06	0.90	28.60	+ 11	+ 13	+ 26	+ 20
02	0.77	0.60	16.00	+ 12	+ 15	+ 12	+ 50
03	0.82	0.72	10.90	+ 10	+ 15	+ 13	+ 33
04	1.08	0.76	37.00	+ 11	+ 11	+ 10	+ 65
05	0.87	0.72	11.30	+ 14	+ 15	+ 14	+ 66
06	0.84	0.70	15.60	+ 11	+ 14	+ 15	+ 28
Mean	0.90	0.73	19.90	+ 11.5	+ 13.8	+ 15.0	+ 43.6
Range	0.77–1.08	0.60–0.90	10.9–37.0	10–14	11–15	10–26	20–66

NC, nucleated cells; MNC, mononuclear cells; CFU-GM, colony forming unit granulocyte–macrophage; WBC, white blood cells; PMN, polymorphonuclear leucocytes; PLT, platelets; Lymph, lymphocytes.

activity is present in the peripheral blood mononuclear cells of patients who underwent ABMT at 17–83 days after transplant, and such activity is mediated by the $CD56^+$ cells including a subset of $CD8^+$ cells. In the present paper we evaluated the immunological reconstitution of 6 patients with ovarian cancer receiving high-dose chemotherapy and ABSCT. Results concerning lymphocyte subset analysis, quantitative serum immunoglobulins, NK activity and LAK precursor activity are discussed.

PATIENTS AND METHODS

Patient samples

The study population included 6 patients with advanced ovarian cancer (FIGO stage III and IV) who entered a phase II study of high-dose chemotherapy and ABSCT. The protocol of this study has been previously described [8]. Briefly, untreated advanced ovarian cancer patients with a residual tumour greater than 0.5 cm after cytoreductive surgery underwent two courses of induction chemotherapy consisting of cisplatin 40 mg/m^2 intravenously (i.v.) from day 1 to day 4 and cyclophosphamide 1.5 g/m^2 i.v. given over 2 h on day 5. The second course was administered 4 weeks later. All patients responding to induction courses received high-dose chemotherapy including cisplatin 100 mg/m^2 i.v. over 2 h on day −5, etoposide 650 mg/m^2 i.v. over 2 h on day −4 and carboplatin 1.8 g/m^2 i.v. as continuous infusion over 24 h on day −3. Peripheral blood stem cells (PBSC), harvested after 2–3 weeks from the start of each course of induction chemotherapy and cryopreserved as previously described [18], were infused on day 0. Table 1 summarises the infusion data and the engraftment time. Patients were nursed in conventional single-bed rooms until complete haematopoietic recovery. Antibacterial and antifungal prophylaxis was given according to current investigational protocols. Broad-spectrum antibiotics were given when fever exceeded 38°C. In this series the number of days with fever > 38°C were 1.8 (range 0–3) and we did not identify any microbial sources of infections. After appropriate informed consent peripheral blood samples were collected prior and at 10 day intervals over a 2 month period after PBSC infusion.

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Cell counts and lymphocyte subset analysis

Blood cell counts and leucocyte differential were performed on the day of venesection using the Technicon H*1 System (Technicon, Tarrytown, New York) as previously described [19]. Lymphocyte subset analysis was performed using a FACScan flow cytometer (Becton-Dickinson, Mountain View, California) with an argon laser operating at 488 nm adjusted at 0.5 W and the following monoclonal antibodies (Mab) conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): anti-Leu-4 (CD3), anti-Leu-3a (CD4), anti-Leu-2a (CD8), anti-Leu-12 (CD19), anti-Leu-11a (CD16), anti-Leu-19 (CD56), anti-interleukin 2 receptor (CD25) and anti-HLA-DR (HLA-DR) (Becton-Dickinson). A small aliquot of heparinised whole blood (100 ml) was mixed with 10 μl of FITC-conjugated Mab and/or 10 μl of PE-conjugated Mab and then incubated for 25 min at 4°C. After incubation, erythrocytes were lysed with NH_4Cl buffer (NH_4Cl 8.29 g/l, $KHCO_3$ 1 g/l, 4 × NaEDTA 0.037 g/l, pH 7.4) for 10 min at room temperature, washed twice with 0.1% sodium azide in phosphate-buffered saline and then analysed. The acquisition gate excluded occasional cell aggregates and debris and was set on the lymphocyte region. The gate was adjusted on the basis of cell forward light and side scatter characteristics and on the basis of the expression of CD14 and CD45 surface antigens on gated cell population. CD14+/45+ monocytic cell population was consistently excluded from gated cells. A minimum of 15 000 events were acquired for each antigen determination. Fluorochrome-conjugated mouse IgG1 and IgG2a monoclonal antibody controls were used to assess the degree of non specific binding to each leucocyte sample.

Assessment of NK and LAK precursor activities

On the day of venesection collected and heparinised blood samples were subjected to Ficoll-Hipaque (density 1.077 g/ml; Pharmacia LKB, Uppsala, Sweden) density sedimentation at 400 *g* for 30 min at 20°C. Isolated mononuclear cells (MNC) were tested for NK activity on the same day by a 4 h ^{51}Cr release assay using the NK-sensitive cell line K562 as target cells. Preliminary experiments were performed to determine culture conditions under which LAK precursor activity could be detected in isolated MNC. A variety of interleukin-2 (IL-2; Genzyme, Boston, Massachusetts) concentrations (100–10 000 units/ml) and a variety of culture durations (1–7 days) were tested. One thousand units/ml of IL-2 were found to be optimal for LAK precursor activity induction (data not shown) and this concentration was used in all subsequent cultures. Since 3 or 4 days were found to be optimal for LAK precursor activity

induction, the culture duration was 3 days. After culture LAK precursor activity was measured by a 4-h ^{51}Cr release assay, using the OVRS 1000 ovarian carcinoma cell line (NK-resistant ovarian carcinoma cell line) as target cells. The percentage of lysis was determined according to the formula:

$$\% \text{ of lysis} = \frac{\text{Experimental cpm - spontaneous cpm}}{\text{Maximal cpm - spontaneous cpm}} \times 100\% .$$

Spontaneous counts per min (cpm) were determined by measuring the amount of ^{51}Cr released from 10^4 target cells incubated in the absence of effector cells. Maximal cpm were determined by resuspending 10^4 target cells into the assay supernatant. A variety of effector/target ratios, ranging from 5:1 to 100:1, were tested for LAK precursor and NK activities.

Quantitative serum immunoglobulins

Serum immunoglobulin concentrations were measured by the nephelometric method (Beckman Assay Protein System, Beckman Instruments Inc., Palo Alto, California).

Statistical analysis

Serum immunoglobulin concentrations, lymphocyte count, lymphocyte subsets, LAK precursor activity and NK activity at diagnosis, before and after transplantation were compared using analysis of variance (ANOVA).

RESULTS

Quantitative serum immunoglobulins

In this series of patients serum levels of IgG, IgM and IgA were in the normal range at diagnosis and their concentrations did not differ significantly before and after ABSCT (data not shown).

Lymphocyte recovery

The lymphocyte count reached an average of 1000 per μl on day +44 (range 20–66) after transplantation (Table 1). The lymphocyte count on day +60 was not significantly different from that obtained at diagnosis and on day −10 (Fig. 1). Lymphocyte subset analysis demonstrated that on day +60 a complete reconstitution in lymphocyte subsets was obtained in this series. In fact ANOVA did not reveal significant differences in the peripheral count of CD3, CD4, CD8, CD19 subsets evaluated at diagnosis on day −10 and on day +60 after transplantation (Fig. 1). In contrast ANOVA revealed a significant increase in the count of interleukin-2 receptor expressing lymphocytes (CD25) on day +60 compared with those obtained at diagnosis and on day −10, while the CD16/56 lymphocyte subset count did not show any significant variation (Fig. 2). Figure 2 shows that the CD3/DR lymphocyte subset count was significantly increased on day −10 and on day +60 compared with diagnosis. Figure 1 shows that the reconstitution of the CD4 subset was prompt and complete from day +10 to day

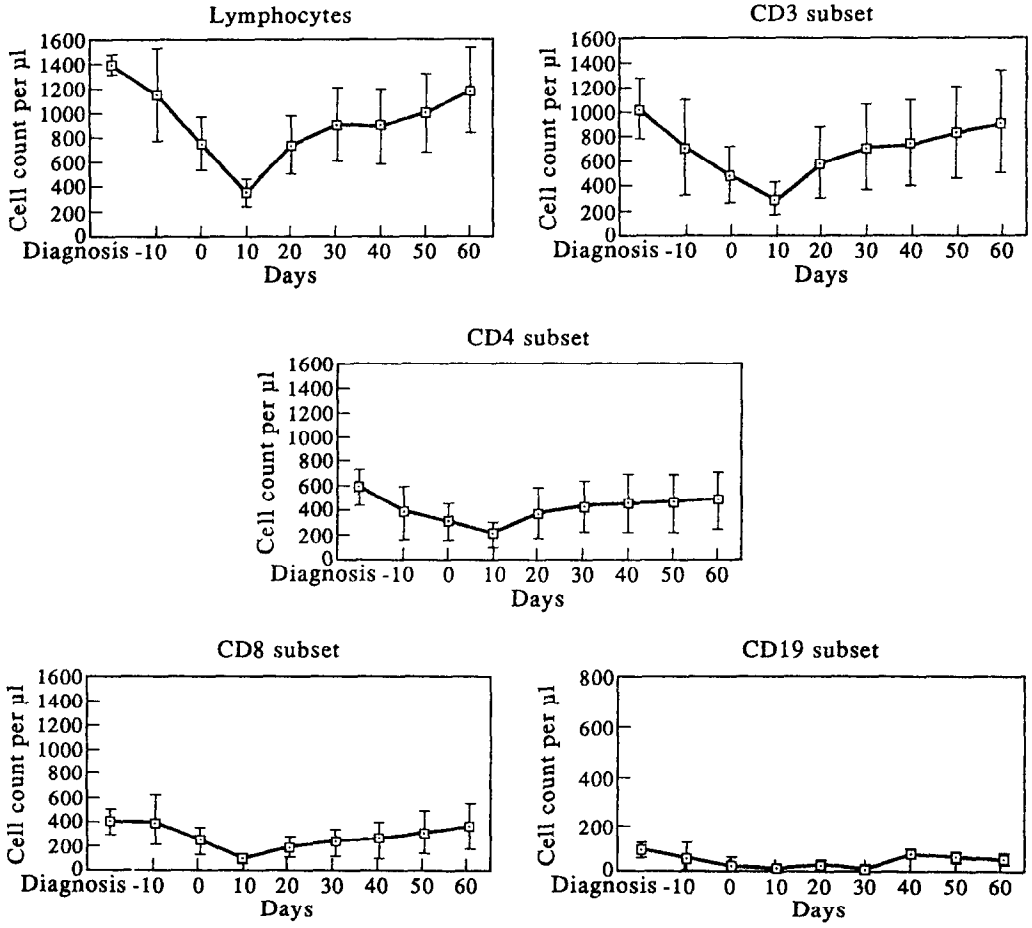

Fig. 1. Kinetics of the recovery of lymphocytes and of CD3, CD4 CD8 and CD19 subsets in the peripheral blood during autologous blood stem cell transplantation. Recoveries were plotted on the basis of the average values +/− S.D. obtained in 6 patients. The lymphocyte count and CD3, CD4, CD8, and CD19 subset counts observed at diagnosis, on day −10 and on day +60 did not differ significantly ($P > 0.05$ in ANOVA).

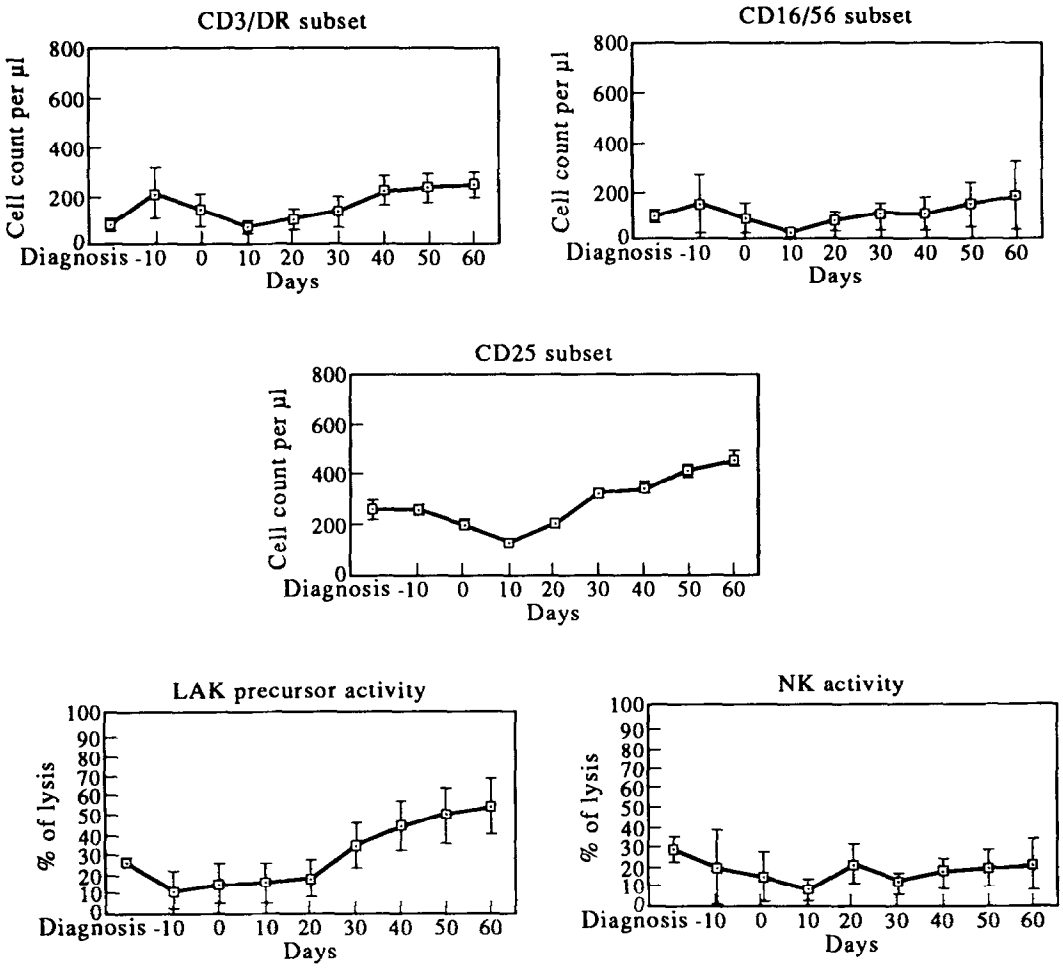

Fig. 2. Kinetics of the recovery of CD3/DR, CD16/56, CD25 lymphocyte subsets and of lymphokine-activated killer (LAK) precursor and natural killer (NK) activities in the peripheral blood during autologous blood stem cell transplantation. Recoveries were plotted on the basis of the average values +/− S.D. obtained in 6 patients. Values for LAK precursor and NK activities were expressed for an effector/target cell ratio of 50:1. NK activity and the count of CD16/56 subset did not differ significantly at diagnosis, on day −10 and on day +60. CD3/DR subset: $P = 0.0045$ in ANOVA; $F = 5.637$ ($P < 0.05$) for diagnosis vs. −10, $F = 6.252$ ($P < 0.05$) for diagnosis vs. +60, $F = 0.016$ ($P > 0.05$) for −10 vs. +60 on Scheffe's F-test. CD25 subset: $P = 0.0001$ in ANOVA; $F = 0.088$ ($P > 0.05$) for diagnosis vs. −10, $F = 58.19$ ($P < 0.05$) for diagnosis vs. +60, $F = 62.81$ ($P < 0.05$) for −10 vs. +60 on Scheffe's F-test. LAK precursor activity: $P = 0.0001$ in ANOVA; $F = 3.178$ ($P > 0.05$) for diagnosis vs. −10, $F = 9.053$ ($P < 0.05$) for diagnosis vs. +60, $F = 22.96$ ($P < 0.05$) for −10 vs. +60 on Scheffe's F-test.

+20, and the CD4 count did not increase greatly from day +20 to day +60. In contrast the reconstitution of the CD8 subset was slower, and the CD8 count reached the pre-transplant value on day +60. After transplantation the CD4/CD8 ratio ranged from 1.27 to 2.28 with the lowest value (1.27) on day +60.

LAK precursor and NK activities

A significant increase in LAK precursor activity was found on day +60 compared to that obtained at diagnosis and on day −10 ($P = 0.0001$ in ANOVA; Fig. 2). The differences were significant for all effector/target cell ratios studied (data not shown). In contrast, NK activity did not appear significantly different at diagnosis, before and after transplantation.

Patients' outcome

All 6 patients are alive with functional bone marrow and normal blood count with a median follow-up of 24 months (range 11–31). 2 and 4 of these cases had pathological complete and partial response, respectively.

DISCUSSION

The present experience shows that patients with advanced ovarian cancer, who underwent high-dose chemotherapy and ABSCT, had a complete lymphocyte subset reconstitution 60 days after transplantation and did not experience significant variations in serum immunoglobulins concentrations. In this series of patients the kinetics of lymphocyte recovery were in line with the recovery of PMN and platelets (data not shown) and suggest a model in which the reconstitution of the immune-haematopoietic system is due to a common biological event, at least during the early phase of reconstitution.

The curves of lymphocyte subset reconstitution show an early increase of the CD4 subset count between day +10 and day +20 and a late recovery of the CD8 subset. We did not find a delayed CD4 subset recovery as described in several clinical experiences in allogeneic or ABMT [12–15] confirming the hypothesis of To *et al.* [16] that the pattern of immune reconstitution may be different during ABSCT from that seen during allogeneic or ABMT. Moreover, we did not find significant changes in CD3, CD4, CD8 lymphocyte subsets as

described after intense chemotherapy without stem cell rescue [11].

Our experience confirms previous studies which indicate that LAK precursor activity is rapidly reconstituted after autologous stem cell transplantation [17]. In addition, we observed a statistically significant increase of LAK precursor activity on day +60 compared to the average values obtained 10 days before transplantation and at diagnosis. Moreover, a concomitant increase in the count of lymphocytes expressing the IL-2 receptor was observed 60 days after transplantation. These results suggest that between day +30 and day +60 our patients experienced a substantial amplification of their LAK precursor activity as a consequence of the combination of high-dose treatment followed by the immuno-haematopoietic reconstitution. In the present experience we have not found a significant increase of NK activity after the completion of immuno-haematopoietic reconstitution. In conclusion, ABSCT gave prompt and complete immuno-haematopoietic reconstitution after high-dose treatment, and this experience further supports the feasibility of IL-2/LAK therapy as consolidative therapy after autologous transplantation. In fact the systemic administration of IL-2 to patients with advanced cancer results in significant immunomodulatory effects [20–23], and tumour regressions have been documented in patients treated with IL-2/LAK therapy [24, 25]. The use of IL-2/LAK therapy after maximal cytoreduction and during late immuno-haematopoietic recovery after ABSCT when LAK precursor activity appears to be significantly enhanced could be most effective in killing residual tumour cells. Two recent clinical experiences demonstrated its own feasibility after ABMT [26, 27].

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