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Interleukin-2 dose, blood monocyte and CD25+ lymphocyte counts as predictors of clinical response to interleukin-2 therapy in patients with renal cell carcinoma

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Summary. The purpose of this study was to determine immunological parameters in the peripheral blood that correlate with the clinical effect of interleukin-2 (IL-2) in patients with metastatic renal cell cancer. A group of 26 patients with metastatic renal cell cancer underwent IL-2 treatment using a 36-day schedule with continuous intravenous IL-2 infusion (3×10^6 units m^{-2} day^{-1}) administered from days 1 to 5 and days 12 to 16. The white blood cell count and the absolute and relative number of neutrophils, lymphocytes, eosinophils and monocytes were recorded six times in peripheral blood during the treatment. Also the blood counts of T cell and NK cell subsets and cells expressing the T cell activation markers IL-2Rα and VLA-1 were measured. The lymphokine-activated killer (LAK) cell cytotoxicity was measured either with or without additional in vitro stimulation by IL-2. Multivariate statistical analysis showed that the clinical responses were related to the administered dose of IL-2, to a low number of blood cells expressing IL-2 receptors and to a reduction in the blood monocyte count ($P < 0.05$).

Key words: Renal cell carcinoma – IL-2 – Response prediction – $CD25^+$ lymphocytes

Introduction

The response rate to interleukin-2(IL-2)-based immunotherapy depends upon treatment schedule and cancer type, varying from 0% to 30% with renal cell cancer and malignant melanoma patients achieving the highest response rates [19]. Despite extensive attempts to assess parameters in the peripheral blood that can predict the outcome of this treatment, only the rebound lymphocytosis after cessation the IL-2 infusion [22] and the lymphokine-activated killer (LAK) cell cytotoxicity induced by IL-2 in vivo [15, 17] have been found to relate to the clinical response. The present study was carried out to determine predictors of the clinical effect of IL-2 therapy in metastatic renal cell cancer by measuring a higher number of immunological blood parameters at shorter intervals during IL-2 therapy than investigated in previous studies.

The patients develop side-effects during IL-2 therapy that can only be reversed by reducing or interrupting the infusion rate of IL-2. Thus, many patients will not receive the scheduled amount of IL-2 or receive IL-2 during all scheduled periods. This varying IL-2 administration may cause different distributions of peripheral blood cells in the patients and weaken the statistical relation between the blood parameters and the clinical response. Therefore, the intensity of the IL-2 treatment in each patient was also taken into account in the statistical analyses.

Materials and methods

Patients and therapeutic protocol. A group of 26 consecutive patients with metastatic renal cell carcinoma receiving IL-2 therapy entered the study. The median age was 58 years (range = 37–73) and the female/male ratio was 14/12. Treatment was performed in a 36-day treatment cycle, during which recombinant IL-2 (Proleukin; EuroCetus Corporation, Amsterdam, The Netherlands) was administered continuously and intravenously in a dose of 3×10^6 Cetus units m^{-2} day^{-1} from days 0 to 5 and again from days 12 to 16. Response evaluation was carried out on day 36 of treatment according to the WHO criteria [16]. Detailed description of the patient population and the clinical course of these patients will be reported elsewhere. Blood samples for immunological evaluation were obtained before starting and ending each IL-2 infusion (days 0, 5, 12 and 15), between the two IL-2 infusion periods (day 8) and at the end of the treatment cycles (day 36). Blood samples drawn three times with 2-week intervals from 5 healthy subjects, who did not receive IL-2 therapy, served as controls.

The effect of dose reduction or interruption of IL-2 infusion on blood parameters was evaluated by dividing the patients into three groups according to how much of the scheduled IL-2 dose they had received and how often the IL-2 infusion had been interrupted (Table 1). Reducing the scheduled dose without interrupting the IL-2 infusion had no significant effect upon the blood parameters, which showed similar patterns of alterations in patients of groups A and B (Table 1). In contrast, the

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Table 1. Intensity of interleukin-2(IL-2) treatment[a]

Parameter	Group A	Group B	Group C
CR	2	0	0
PR	2	2	0
NC/PD	5	5	10
Total	9	7	10
$10^{-6}\times$ administered IL-2 dose (units)	46 (36–52)	40 (28–52)	36 (19–54)
Percentage of scheduled IL-2 dose	97 (95–100)	76 (62–91)	67 (40–90)
No. interruptions of IL-2 infusion	0.5 (0–1)	3.0 (1–5)	3.3 (1–7)

[a] Patients were divided into three groups according to the administered dose and the number of interruptions of IL-2 infusion during the first cycle. Group A: therapy given according to the scheduled time course and dose, eventually with minor dose modifications. Group B: IL-2 dose reduced, but mainly administered during the scheduled periods. Group C: both dose and infusion time differed from schedule. Figures give mean values and range. CR, complete response; PR, partial response; NC, no change; PD, progressive disease

patients in group C had interruptions of the IL-2 infusion of several hours duration, which resulted in substantial changes in the lymphocyte counts.

Leukocyte determination. The white blood cell count and the percentage of neutrophils, lymphocytes, eosinophils and monocytes were measured in whole blood using a Technicon H.1 automated haematology counter (Technicon, Belgium). The absolute numbers of neutrophils, lymphocytes, eosinophils and monocytes were calculated by multiplying the percentage of each cell type with the white blood cell count.

Purification of peripheral blood mononuclear cells (PBMC). PBMC were purified from whole blood mixed with calcium heparinate (5000 IU) by Ficoll/Hypaque density gradient centrifugation [3]. The PBMC were washed three times and resuspended in RPMI-1640 tissue-culture medium supplemented with 10% fetal calf serum for use in ^{51}Cr-release assays, or resuspended in phosphate-buffered saline + 2.5% fetal calf serum for immunofluorescence staining.

Immunofluorescence and flow cytometry. The subpopulations of PBMC were examined by dual-colour immunofluorescence experiments analysed in a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif. USA) with monoclonal antibodies (mAbs) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or biotin. The panel of mAbs comprised 21 different combinations of the following: CD3 (Leu4, Pan-T), CD4 (Leu3, T_h), CD8 (Leu2, $T_{c/s}$), T cell receptor (TCR-1, α/β chain), CD16 (Leu11, NK cells), CD56 (Leu19, NK cells), CD57 (Leu7, NK cells), CD25 (IL-2Rα, low-affinity IL-2 receptor, activated T cells), CD14 (LeuM3, monocytes), TCR-δ-1 (T cell receptor, γ/δ chain) and VLA-1 (very late activation antigen, late T cell activation in PBMC). TCR-δ-1 and VLA-1 mAbs were purchased from T Cell Sciences Inc. Cambridge, USA. All other mAbs were purchased from Becton Dickinson (Mountain View, Calif., USA). Labelling of PBMC was performed as previously described [13]. A total of 5000 cells were analysed in each experiment. PBMC incubated with avidin-FITC-, PE- or FITC-conjugated IgG and unstained PBMC served as controls in the FACS analysis. Trypan blue staining was used to estimate the percentage of dead cells in the samples, which never exceeded 10%, and usually was between 0% and 3%.

Some of the mAbs used bind unspecifically to F_c receptors on monocytes. The contribution of this unspecific binding to the total binding of mAb (specific + unspecific) varies during treatment, because IL-2 induces substantial changes in the ratios between monocytes and other PBMC. To eliminate this unspecific binding to monocytes, the gate function of the FACScan software was set to limit measurements to the lymphocyte population. In unstimulated PBMC, lymphocytes and monocytes were seen as two well-separated populations when displayed in a cell-granularity (side scatter) versus cell-size (forward scatter) dot plot. In vivo stimulation with IL-2 increased the size and granularity of some lymphocytes resulting in a poor separation of the lymphocyte and monocyte populations. To include all lymphocytes and exclude monocytes from the gated lymphocytes, the following procedure was used: PBMC were labelled with LeuM3 and the LeuM3$^+$ cells were displayed against cell granularity. Then a gate was set to include only the LeuM3$^-$ cells, which represent lymphocytes and granulocytes. Using only recordings from these cells, the exact distribution of the lymphocytes was displayed in a cell-granularity versus cell-size dot plot. Finally, a gate was set around this population and only registrations from these cells were recorded. This population usually contained 0%–2% LeuM3$^+$ monocytes, and so excluded the possibility of significant unspecific binding of mAbs to F_c receptors on the monocytes. In this gating procedure, only a few, if any, lymphocytes were lost, since 98%–100% of the cells in the excluded monocyte population were positive for the LeuM3 marker, which is only expressed on monocytes [7]. These findings were supported by double-fluorescence experiments using CD45 (leucocyte common antigen, HLe-1, 2D1 clone, Becton Dickinson) and LeuM3 (CD14), showing only 0%–3% CD14$^-$CD45$^+$ cells in the excluded monocyte population. The absolute number of positive cells in each subpopulation was calculated by multiplying the percentage of positive cells with the absolute lymphocyte count in the peripheral blood. Variations in antigen density were evaluated by comparing the mean channel of fluorescence intensity of the positive populations.

Assessment of cytotoxic activity. The cytotoxic activity of PBMC was assayed by 4-h ^{51}Cr-release assays using the T24 cell line derived from a transitional cell carcinoma [4, 5, 21] as target cell. The T24 cell line is NK-insensitive but LAK-sensitive. The LAK-cell-mediated cytotoxicity against T24 cells is about 75% of the lysis of Daudi cells, which is the classic LAK target cell line [13]. The two cell lines are lysed by phenotypically similar PBMC and share surface structures that are recognized by LAK cells [14]. The ^{51}Cr-release assay was performed as previously described [13] using 10^4 target cells and 5×10^5 PBMC in 1 ml RPMI-1640 medium alone (in vivo stimulation) or supplemented with 1000 units IL-2/ml (in vivo + in vitro stimulation). IL-2 alone did not lyse the target cells.

Statistical analysis. Multivariate analysis was performed, which allows all of the variables to act together to make it possible to determine the relative importance of each variable. A stepwise logistic regression model was used [1], which is included in the BMDP statistical software program. A significance level at 5% was used.

Results

Of the 26 patients, 6 responded to the treatment; 2 had a complete and 4 a partial response. Tumour regression occurred in those patients who received the highest cumulative dose of IL-2 during the first treatment cycle and developed a reduction in the number of blood monocytes during treatment (from day 0 to day 8) as well as exhibiting a low CD25$^+$ PBMC count on day 12 ($P<0.05$) (Fig. 1). No other parameters were found to be significantly related to the clinical response. These relationships were independent of dose reductions and interruptions of the IL-2 infusions, since the statistical analyses based upon data from patients receiving IL-2 therapy of almost similar intensity (groups A and B, Table 1) gave similar conclusions.

In contrast to the results from IL-2-treated patients, the numbers of blood monocytes and CD25$^+$ PBMC in untreated controls remained constant with time, showing a mean variance in the monocyte counts of 2% (SD $\pm$2%) and in the CD25$^+$ PBMC counts of 1% (SD $\pm$1%).

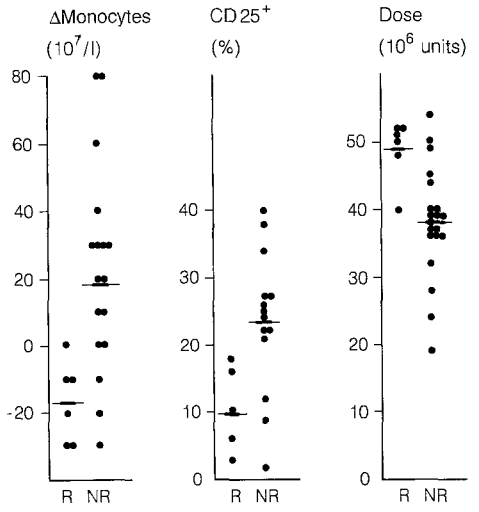

Fig. 1. Parameters related to clinical response. Reduction in monocyte counts from treatment day 0 to day 8, low percentage of CD25+ peripheral blood mononuclear cells on day 12 and high cumulative dose of IL-2 administered in the first cycle of IL-2 treatment correlated with clinical response. *R*, complete or partial response; *NR*, no response (stable or progressive disease)

IL-2 infusion induced neutrophilia, lymphopenia and eosinophilia in all patients and cessation of IL-2 infusion was followed by a rebound leukocytosis and lymphocytosis as reported by others (data not shown) [8, 9].

Figure 2 shows the distribution of lymphocyte subsets during therapy. The numbers of both NK cells and T cells increased, and as the number of NK cells increased relatively more than that of T cells, NK cells even became the predominant cell type in a few patients. The alterations in numbers of cells positive for the T cell activation markers, VLA-1 and CD25, showed similar patterns even though VLA-1 mainly was expressed on CD8+ cells and CD25 on CD4+ T cells.

During therapy, the cytotoxicity of PBMC induced in vivo was increased by 150% from pretreatment levels and the cytotoxicity induced in vivo and in vitro increased by 260% (data not shown).

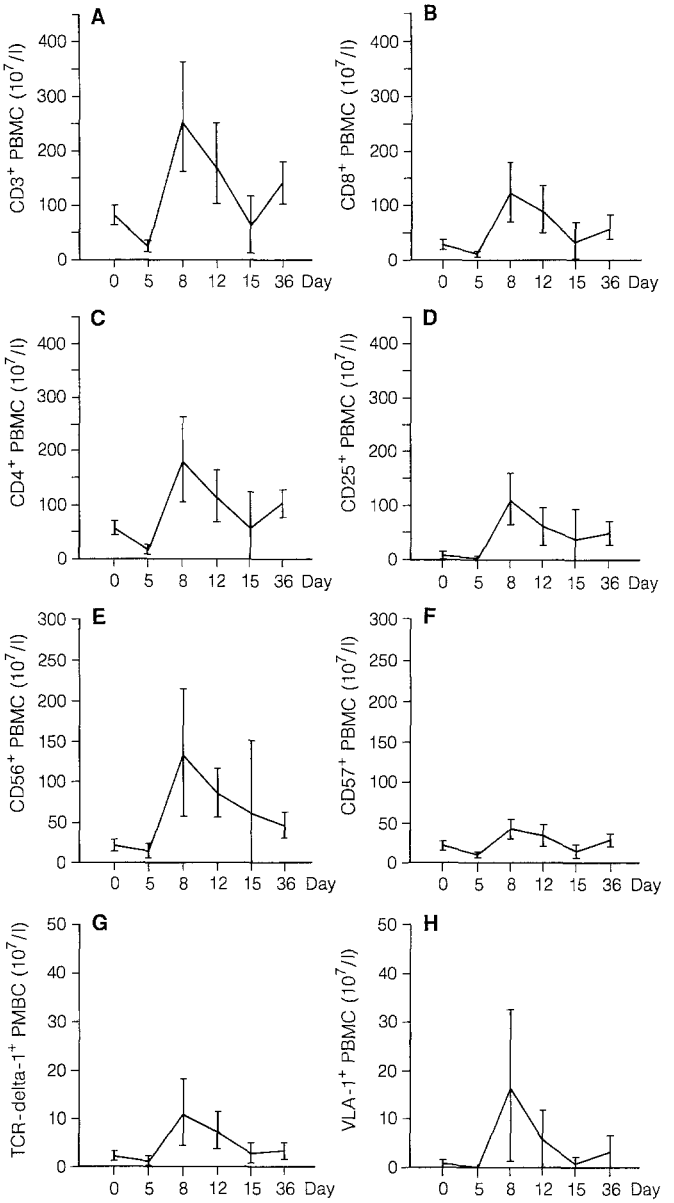

Fig. 2 A – H. Interleukin-2(IL-2)-induced changes in lymphocyte subsets of all patients ($n = 26$). Absolute number of lymphocyte subsets during the first cycle of IL-2 therapy (mean and 95% confidence limits); *PBMC*, peripheral blood mononuclear cells. **A, B, C, G**: T cells; **D**: IL-2 receptors; **E, F**: NK cells; **H**: VLA-1

Discussion

The present study demonstrates a statistically significant relation between clinical response to IL-2 therapy and the combination of (a) a reduction in the blood monocyte counts, (b) a low number of CD25+ PBMC, and (c) a high dose of IL-2.

The alterations in the monocyte counts may appear small, but constitute up to a 100% change, which is significantly higher than the slight random variations of about 2% observed in the untreated controls. The reduction in the blood monocyte counts of responding patients may result from extravascular cell migration. A substantial tumour infiltration of monocytes/macrophages and T cells has been reported during IL-2 treatment of patients with malignant melanoma and breast cancer, which correlated with the clinical effect [6, 20]. We have observed a similar infiltration of these cells associated with an infiltration of CD25+ lymphoid cells in bladder tumours of patients treated with IL-2 and LAK cells (G. G. Hermann, et al., in preparation). These results suggest that IL-2, either directly or indirectly, stimulates the migration of monocytes and lymphocytes from the peripheral blood to other sites, which may explain the observed reduction in blood monocyte counts as well as the low numbers of CD25+ PBMC in the present responding patients.

To our knowledge, this is the first study that shows a direct relation between the IL-2 dose and the clinical response in man. In mouse models, a clear dose/response relationship has been demonstrated [18]. A similar relationship in humans has only been suggested indirectly by a significant relation (a) between the administered IL-2 dose

and rebound lymphocytosis [2], and (b) between the rebound lymphocytosis and the clinical response [22].

The expression of VLA-1 on PBMC during IL-2 therapy has not been reported previously. VLA-1 is an adhesion molecule belonging to the β_1 subfamily of integrins and is, in peripheral blood, restricted to activated T cells like the CD25 marker [11]. In contrast to the CD25 marker, which is expressed only a few hours after cell activation, it has been reported that VLA-1 is not highly expressed until 2–3 weeks following cell activation, i.e. after the time when the number of $CD25^+$ PBMC has diminished [12]. However, we observed increased expression of VLA-1 on PBMC even after 1 week of IL-2 therapy and this expression showed similar patterns of fluctuation to that of the CD25 marker. Despite this finding, only the CD25 marker was related to the clinical response, which may be because of the expression of these markers on different PBMC cell subsets. The LAK cytotoxicity determined in this study varied as described in other studies using IL-2 alone, and was not related to the clinical response [2, 10]. Only two previous reports have indicated a correlation between LAK cytotoxicity and the clinical response. In these studies, IL-2 was administered in combination with either IFNβ [15] or cyclophosphamide [17], both of which interfere with the LAK-inducing effect of IL-2 and may influence the relationship with the clinical response.

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