**ABSTRACT**

In this study 15 consecutive melanoma patients were treated with two courses of bolus recombinant interleukin 2 (rIL2) and rIL2 plus *in vitro*-generated lymphokine-activated killers (LAK), respectively. The immunological monitoring performed after 4 days of rIL2 or rIL2 plus LAK, indicate that the *in vivo* peripheral blood lymphocyte (PBL), activation (spontaneous proliferation, tumor cytotoxicity, number of NK*+* PBL, obtained after the second cycle of rIL2 plus LAK is significantly higher than after the first cycle of rIL2 alone. During the 5-day interval between the two courses, PBL activation returns to baseline levels and no evidence for increased sensitivity of PBL to rIL2 is present. To further confirm this, two additional patients were studied, in whom rIL2 was administered by continuous i.v. infusion. In these two patients the *in vivo versus in vitro* PBL activation could be directly and simultaneously compared by using *in vitro* the same concentration of rIL2 reached and maintained in the patients' sera. The PBL activation induced *in vivo* by a cycle of rIL2 alone was significantly less (about 10 times) than that obtained *in vitro* with a comparable rIL2 concentration. Thus, the infusion of *in vitro* highly activated PBL could explain the increased *in vivo* lymphocyte activation of the second cycle of rIL2 plus LAK over the first cycle of rIL2 alone.

**INTRODUCTION**

The lymphocyte growth factor, IL2, is able to activate *in vitro* PBL to exert potent antitumor cytotoxicity against autologous and allogeneic tumor cells (1). These PBL are operationally defined as "lymphokine-activated killers" or LAK cells (2).

*In vitro*-generated LAK cells have been administered together with IL2 to tumor-bearing animals and to cancer patients, and have been shown to induce tumor regression in several AI studies (3–7).

Although animal models of AI clearly indicated that the administration of *in vitro* activated LAK is important in achieving a successful therapy, clinical responses have also been obtained with IL2 alone given at high doses or for prolonged periods of time (8–10). The need for the use of "ex vivo" generated LAK cells thus remains an unsettled issue (11).

In that study (13) a direct comparison between the *in vivo* and *in vitro* PBL activation in the same patients indicated that a significantly higher *in vitro* PBL activation in terms of spontaneous proliferation, tumor cytotoxicity and IL-2 receptor-positive cell expansion was obtained (13). In the present report we studied 15 metastatic melanoma patients who received two separate courses of bolus rIL2 and rIL2 plus *in vitro*-generated LAK. rIL2 was also administered by continuous infusion in two additional patients, thus allowing us to compare the *in vivo versus in vitro* PBL activation exerted by IL2 once the steady blood level of serum IL2 in each patient was known.

**MATERIALS AND METHODS**

rIL2. rIL2 was provided by Glaxo IMB SA (Geneva, Switzerland) for the bolus injection study and by Eurocetus BV (Amsterdam, Holland) for the continuous infusion study. The ratio between the Company Unit and the Biological Response Modifier Program (BRMP) Unit is 1/1 for Glaxo and 2.3/1 for Eurocetus.

Patients and Treatments. Fifteen melanoma patients (code 5201–5215) entered the bolus injection study between January 1987 and February 1988. All had histologically proven malignant melanoma with disseminated (Stage III) disease and had measurable lesions in the lungs, liver, s.c. tissue, or lymph nodes. No brain metastases were present in these patients. No other treatment was administered during the 4 weeks preceding rIL2 administration. Patients were treated with bolus i.v. injections of 1.4 x 10⁶ units/m² rIL2 for 5–6 consecutive days. Patients were then subjected to four consecutive leukaphereses, beginning 18–24 h after rIL2 discontinuation. Leukapheresed lymphocytes were processed as described (14) and cultured for 3–4 days in Nunc Cell Factory (Nunc, Roskilde, Denmark). Cells were subsequently harvested and infused back into the patient; infused cells ranged between 1.5 and 6 x 10⁷ cells/patient (median 3.5 x 10⁷). With the first LAK cell infusion, the patients received again rIL2 at the same dosage used in the first part of the treatment for 3–6 days, unless toxicity required dose reduction. More detailed clinical data are reported elsewhere (15).

One patient (code 5101) with pulmonary and bone metastases from Ewing's sarcoma 1 year after completion of first line chemotherapy, was treated with the same scheme but with rIL2 administered by continuous i.v. infusion at a dose of 3 x 10⁶ units/m²/day. An additional patient (code 1501) with metastatic renal cell carcinoma was treated with continuous i.v. infusion of rIL2 for 5 days at the dose of 3 x 10⁶ units/m². Acetaminophen, indomethacin, furosemide, and i.v. fluids were routinely administered to manage side-effects.

Immunological Monitoring. Heparinized blood samples were obtained on Days –3, –1, 5–7, and 15–17 from the beginning of rIL2 administration and 7–15 days after termination of treatment.

Spontaneous Proliferation of PBL. Mononuclear cells were seeded in 96-well plates (10⁴ cells/well, Costar, Cambridge, MA) and incubated in RPMI 1640 (MA Bioproducts, Walkersville, MD) plus 2 mM glutamine (Flow, Irvine, UK), 20 mM HEPES buffer (MA Bioproducts), 100 units/ml penicillin G (Farmitalia, Milan, Italy), 100 µg/ml Streptomycin (Farmitalia), and 10% human serum (complete medium, CM) in the presence of [3H]dThd (NEN, Florence, Italy, 6.7 Ci/mM, 1 µCi/ well) for 6 h at 37°C in a humidified CO₂ atmosphere. Cells were subsequently harvested and radioactivity counted in a beta counter.

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2. To whom requests for reprints should be addressed, at Division of Experimental Oncology D, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milano, Italy.

3. The abbreviations used are: IL2, interleukin 2; rIL2, recombinant interleukin 2; (Pt)PBL, (patient) peripheral blood lymphocytes; LAK(s), medium; MAAb, monoclonal antibody; dThd, thymidine; E/T, ratio between Effector lymphocytes and Target tumor cells; AI, adoptive immunotherapy; TR, total release; SR, spontaneous release.
Control PBL of normal donors were used; their radioactivity uptake was always in the range of pretreatment PtPBL values (200–1000 cpm).

Phenotype of PtPBL. An indirect immunofluorescence method was used to evaluate the immunological phenotype of PtPBL. Mabs OKT3, OKT4, OKT8 (Ortho Diagnostic, Cologna, Italy), TEC-IL2-R (anti-IL2 receptor, Technogenetics, Turin, Italy), Leu 19 (Becton-Dickinson, Milan, Italy) were used. Mabs B73.1 (anti-CD16) and D1-12 (anti-DR) were a gift from Dr. B. Perussia (Philadelphia) and Dr. R. Accolla (Lausanne), respectively. The percentage of fluorescence positive cells was evaluated by FACS analysis.

Cytotoxicity of PtPBL. A 6-h ³H-cr release assay (16) was employed. The results were expressed as percentage specific ³H-cr release (cpm sample – cpm SR/cpm TR – cpm SR × 100); SR were less than 30%.

The overall results from these 15 consecutive patients are presented in Table 1. Both proliferation and cytotoxicity against K562, Daudi, and Me665/2 melanoma line were significant. The lysis of autologous melanoma cells, which could be studied in four patients, increased after IL2 + LAK administration without reaching statistical significance (P = 0.078).

The rebound in the number of PBL after IL2 discontinuation was higher when the patients received LAK cells (Table 1).

In one patient (Patient 5207) cultures of the LAK cells became accidentally contaminated and two out of three scheduled LAK infusions were cancelled, with the patient receiving 1.5 × 10⁶ cells only, but a rIL2 dose similar to that administered in the first phase. In this patient no additional activation was seen following LAK cell administration (data not shown).

In Vivo PBL Activation after Continuous Infusion of rIL2 ± LAKs. We previously showed that bolus rIL2 treatment determines the in vivo activation of PtPBL, but that the in vitro culture of PtPBL with rIL2 generates a much higher activation (13). Thus the higher in vivo activation seen in patients receiving rIL2 plus LAK could be due to the infusion of ex vivo-activated cells which result in the enrichment of the pool of in vivo-circulating LAK cells.

Our previous study, however, might be biased by the fact that patients received bolus rIL2 injections and thus constant IL2 levels in their sera could not be maintained as it occurs in the in vitro system. To further analyze this aspect, we studied a patient (Patient 5101) treated by continuous rIL2 infusion.

Fig. 2 shows the results obtained in Patient 5101. A high in vivo activation of PtPBL in terms of proliferation and tumor cytotoxicity was reached in this case too, following the combined administration of rIL2 plus LAK, even if in the second phase (rIL2 plus LAK), rIL2 could be administered to the patient for only 3.5 days because of side-effects. It can also be noted that the in vivo activation exerted by rIL2 alone disappeared by Day 11 and thus no residual activation was present at the first day of rIL2 plus LAK treatment. In this patient and in Patient 1501 PBL were obtained before rIL2 and 5.5 days after rIL2 discontinuation. PBL were then cultured for 5 days in the presence of 20 units/ml of rIL2 and subsequently tested for proliferation and cytotoxicity against K562, Daudi, and the melanoma line Me 665/2. The results of the cytotoxicity assay are presented in Table 2 and show no increased in vitro-induced LAK activity in PBL obtained after rIL2 treatment. Similar results were obtained for proliferation (data not shown).

Comparison of In Vivo versus In Vitro PBL Activation in Patients Receiving Continuous Infusion of rIL2. While patient 5101 was receiving the first 5-day course of rIL2 alone, her PBL (obtained immediately before rIL2 treatment) were stimulated in vitro for the same time with 5, 20, and 50 units/ml of rIL2, to compare the in vivo rIL2-induced PBL activation with that obtained by culturing them in vitro in the presence of the same rIL2 concentration measured in the patient’s serum. Multiple concentrations of rIL2 had to be used in vitro because at the time the assay was run the serum IL2 concentrations achieved by treatment in this patient were unknown.

Fig. 3, A and B, shows the PBL cytotoxicity against the Daudi and the Me665/2 lines generated after in vitro or in vivo exposure to rIL2. In vivo rIL2 treatment resulted in a level of PBL cytotoxicity comparable with that obtained in vitro at 5 units/ml of rIL2. Conversely, after rIL2 plus LAK, the in vivo cytotoxicity reached the level obtained in vitro with 50 units/ml. Similar results were obtained for spontaneous proliferation of LAK cells (Fig. 4A).

When the serum derived from the patient during rIL2 administration was assayed on the CTLL line, a value of 77 units/ml of IL2 was measured (Fig. 5). Thus, in Patient 5101 the admini
IN VIVO VERSUS IN VITRO rIL-2-INDUCED PBL ACTIVATION

Fig. 1. Effect of bolus rIL2 and rIL2 plus LAK administration on spontaneous PBL proliferation and cytotoxicity in patients 5209 (left) and 5214 (right). Pt, patient.

Table 1 In vivo PBL activation in 15 consecutive melanoma patients following treatment with LAKs + rIL2 or with rIL2 alone

<table>
<thead>
<tr>
<th>Assay</th>
<th>Pretreatment</th>
<th>rIL2 alone</th>
<th>rIL2 + LAKs</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation*</td>
<td>446 ± 91</td>
<td>2,796 ± 723</td>
<td>10,634 ± 4,014</td>
<td>0.017</td>
</tr>
<tr>
<td>Lysis of</td>
<td></td>
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<tr>
<td>K562</td>
<td>9 ± 2</td>
<td>17 ± 4</td>
<td>39 ± 8</td>
<td>0.007</td>
</tr>
<tr>
<td>Daudi</td>
<td>5 ± 2</td>
<td>14 ± 3</td>
<td>31 ± 7</td>
<td>0.026</td>
</tr>
<tr>
<td>Allogeneic Me</td>
<td>8 ± 2</td>
<td>17 ± 3</td>
<td>40 ± 11</td>
<td>0.013</td>
</tr>
<tr>
<td>Autologous Me</td>
<td>8 ± 3</td>
<td>10 ± 3</td>
<td>25 ± 7</td>
<td>0.078</td>
</tr>
<tr>
<td>% DR* PBL*</td>
<td>18 ± 3</td>
<td>22 ± 3</td>
<td>40 ± 7</td>
<td>0.013</td>
</tr>
<tr>
<td>Nr of PBLS</td>
<td>1,765 ± 147</td>
<td>3,381 ± 343</td>
<td>9,223 ± 1,686</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* One-way variance analysis between rIL2 alone and rIL2 + LAK groups.
* Evaluated by [3H]TdR uptake (10^5 cells/well); values are expressed as cpm (mean ± SE).
* Evaluated by 51Cr release assay at E/T ratio of 50/1; values are expressed as percentage of specific 51Cr release (mean ± SE).
* Evaluated by indirect immunofluorescence with D1-12 MAb and FACS analysis (mean ± SE).
* Values are expressed as number of PBL/cmm of blood (mean ± SE).

istration of rIL2 resulted in a serum IL2 level of 77 units/ml but the resulting in vivo PBL activation was only comparable with that occurring in vitro with ~5 units/ml. The analysis of cytotoxicity curves, obtained with different E/T ratio values, permits to estimate that the PBL activation produced in vivo by rIL2 alone is 4–10 times lower than that obtained in vitro with the same IL2 concentration or after rIL2 plus LAK. In a second patient treated by continuous 5-day i.v. infusion of IL2 only (Figs. 3, C and D, and 4B), a serum level of 59 units/ml of IL2 was obtained. In this case an in vivo PBL activation corresponding to that obtained in vitro with an IL2 concentration of ~5 units/ml was detected, confirming the results of the previous patient. The scheduled LAK cell infusions were cancelled in this patient, due to treatment toxicity; the patient thus received only one course of rIL2 alone.

DISCUSSION

In this paper the degree of in vivo PBL activation was monitored in a series of 15 consecutive melanoma patients undergoing bolus rIL2 treatment followed, 5.5 days later, by a second course of rIL2 plus LAK. Interindividual variability was avoided since the same group of patients was treated with rIL2 or rIL2 plus LAK.

The results indicate that the second part of the treatment is followed by a higher in vivo PBL proliferation and tumor cytotoxicity. These data are in agreement with the recently reported results of a controlled randomized trial comparing rIL2 versus rIL2 plus LAK treatment in metastatic cancer patients, and showing a significantly higher complete response rate in patients receiving rIL2 plus LAK than in those treated.
Table 2  In vitro rIL2 induced cytotoxicity of PrPBL obtained before and after rIL2 administration

<table>
<thead>
<tr>
<th>Pt</th>
<th>E/T ratio</th>
<th>Target</th>
<th>Before rIL2</th>
<th>After rIL2</th>
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<tbody>
<tr>
<td>5101</td>
<td>100</td>
<td>K562</td>
<td>49</td>
<td>43</td>
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<tr>
<td></td>
<td>50</td>
<td></td>
<td>56</td>
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<td>11</td>
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<tr>
<td></td>
<td>100</td>
<td>Me665/2</td>
<td>55</td>
<td>58</td>
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<td>50</td>
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<td>12</td>
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<tr>
<td></td>
<td>100</td>
<td>Daudi</td>
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<td>50</td>
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<td>39</td>
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<td>12</td>
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<td>27</td>
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</table>

Fig. 3. Comparison between in vitro PBL cytotoxicity obtained with three different rIL2 concentrations, and in vivo PBL activation after 5 days of rIL2 or rIL2 plus LAK in Patients 5101 (A, B) and after 5 days of rIL2 alone in Patient 1501 (C, D). Open and dashed bars refer to percentage of specific 51Cr release at E/T ratios of 50/1 and 12/1, respectively. Patients were treated by continuous i.v. rIL2 infusion.

Fig. 4. In vitro versus in vivo effects of rIL2 on spontaneous proliferation in Patients 5101 (A) and 1501 (B). Values refer to mean [3H]dThd uptake cpm. See legend of Fig. 3 for patients' treatment.

with rIL2 alone (18). Gemlo et al. (19) also show that the infusion of LAK plus rIL2 is associated with increased serum levels of γ-interferon, tumor necrosis factor-α and -β over those obtained with rIL2 alone. It must be considered, however, that the only patient who displayed spontaneous cytotoxicity against the autologous tumor before therapy (Patient 5213), lost this activity from the blood after rIL2 treatment. It is possible that this specific activity could have been diluted in the total number of PBL by the massive PBL activation generated by rIL2. Our study did not include patients treated by two courses of rIL2 alone and thus it cannot be completely ruled out that the activation induced during the first part of the treatment might have increased the effect of the second phase. Only a controlled trial of rIL2 versus rIL2 plus LAK could definitely answer this question. Three lines of evidence argue against such a possibility: (a) in our previous study (13) no residual PBL activation was present 6–7 days after rIL2 treatment; (b) the monitoring of the patients immediately before the administration of LAK failed to show any residual activation (Figs. 1 and 2 and data not shown), and no increased sensitivity to rIL2 was noted in PBL obtained after rIL2 treatment; (c) in the patient who accidentally received the lowest amount of LAK along with a total dose of rIL2 comparable to that of the other patients, no increased activation ensued. Sondel et al. (20) have reported that a progressive increase in total PBL lytic activity takes place in patients treated by repeated rIL2 cycles. In their study, however, the interval between two cycles was of 3 days only, a time which may leave residual PBL activation; moreover the progressive increase of total PBL lytic activity observed by these authors, appears to be due to the increase in the total number of PBL, whereas no significant increase in cytotoxicity was found on a per cell basis between the first and fourth cycle. It should be noted that in our study 5.5 days elapsed between the two cycles of rIL2.

Another study on multiple cycles of IL2 infusion (21), in which the interval between two cycles was longer than in the Sondel's study, failed to reveal a consistent increase in the number of PBL or in the spontaneous cytotoxicity of PBL after the fourth cycle of treatment over the first one.

The administration of rIL2 by continuous infusion permitted a proper comparison of the in vitro versus in vivo PBL activation. In fact it was possible to culture PrPBL with rIL2 concentrations similar to those achieved in vivo and to keep constant IL2 plasma levels. This comparison indicates that the same rIL2 concentration, tested on the lymphocytes of the same patient and over the same period of time, gives an in vivo PBL activation
much lower than that obtained in vitro. It is worth noting that rIL2 concentrations commonly used to activate PBL in vitro (100–1000 units/ml) are higher than those achievable in vivo; this may lead to a further increment in the difference between the in vitro and the in vivo effect of rIL2 on PBL activation.

It is interesting to note that the subsequent administration of LAK cells in Patient 5101, was associated with an in vivo PBL activation close to that reached in vitro using the IL2 levels achieved in vivo. Whether this might be due to the presence in the blood of the infused LAK cells or caused by a greater stimulation of resident lymphocytes by LAKs [or by cytokines released by LAKs (19)], is presently unknown.

The reduced in vivo PBL activation raises an interesting issue about the presence of immunosuppressive substances in cancer patients (22–24). These factors could be produced by the tumor itself and be present before rIL2 administration, thus reducing the stimulatory effect of rIL2 and lowering the in vivo PBL activation. Alternatively the administration of rIL2 may induce the release in the serum of soluble IL2R which can compete with the cellular receptors for the available IL2. Augmented IL2R have been described in the sera of advanced cancer patients (25, 26) and they increase up to 100 times the pretreatment values in patients receiving rIL2 (27). Interestingly, IL2R values are about two times higher in patients receiving IL2 plus LAK than in those treated with IL2 alone (27); infused LAK cells may shed IL2R in the sera of patients thereby causing this further increment. Increased IL2R levels were found in most of these patients following treatment. The possibility that sera collected from patients after rIL2 treatment can inhibit PBL response to IL2 through elevated levels of IL2R is under investigation in our laboratory.

The presence of these immunosuppressive factors may explain the better activation of PnPBL seen in vitro where patient serum is not present.

On the whole, our data indicate an impaired in vivo rIL2-induced lymphocyte activation and emphasize the need for testing the biological activity and clinical efficacy of in vitro-activated lymphocytes in controlled trials.

ACKNOWLEDGMENTS

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Differences between in Vivo and in Vitro Activation of Cancer Patient Lymphocytes by Recombinant Interleukin 2: Possible Role for Lymphokine-activated Killer Cell Infusion in the in Vivo -induced Activation

Carlo Gambacorti-Passerini, Licia Rivoltini, Marina Radrizzani, et al.