Exogenous Interleukin 2 Recruits in Vitro Lymphokine-activated Killer Activity by in Vivo Activated Lymphocytes

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ABSTRACT

Cryopreserved and thawed lymphocytes can be used instead of fresh lymphocytes to avoid test-to-test variability in studies of fluctuations of natural killer (NK) and lymphokine-activated killer (LAK) activities as a function of time. We investigated the effects of 1-h versus 18-h resting of lymphocytes on their lytic activities, because the process of cryopreservation and thawing decreases NK and LAK activities. Lymphocytes from renal cell cancer patients receiving adoptive immunotherapy were studied. An 18-h versus 1-h resting period led to a significant increase in NK activity but had no significant effect on LAK activity. The presence of 1200 IU/ml interleukin 2 (IL-2) in the medium 1 h prior to and during the cytotoxicity (CTX) assay increased in vitro and in vivo IL-2-induced LAK activities. This phenomenon has been interpreted as IL-2 dependency of effector lymphocytes (J. A. Hank, P. C. Kohler, G. Weil-Hillman, N. Rosenthal, K. H. Moore, B. Storer, D. Minkoff, J. Bradshaw, R. Bechhofer, and P. M. Sondel. Cancer Res., 48: 1965–1971, 1988). We performed kinetic studies to assess the role of effector lymphocyte recruitment in these experiments. LAK activity was tested in the presence or absence of IL-2 during preincubations and CTX assays varying between 0 and 120 min. These kinetic studies showed that effector lymphocyte recruitment indeed contributed to the increased level of LAK activity when IL-2 was added to the CTX assay. A minimal incubation period of 30 min was required to detect recruitment of lymphocytes. Effector lymphocytes could be recruited for periods varying between 90 and >240 min, depending on the lymphocyte donor. We conclude that: (a) in vitro, IL-2-mediated recruitment of lymphocytes due to presence of IL-2 in the CTX assay may lead to an overestimate of the actual LAK activity; and (b) in vivo, prolonged IL-2 infusion after the administration of activated lymphocytes seems warranted in order to recruit maximal levels of effector lymphocytes with LAK activity.

INTRODUCTION

Biological response modifiers can induce freshly obtained or cloned lymphocytes to kill fresh tumor cells and/or a variety of cultured cells (1–7). This phenomenon is called lymphokine-activated killing. In descending order of the magnitude of the effect, LAK activity is exerted by CD3+, CD16- NK cells, T-cell receptor γδ+, CD3+, CD16+/- T-cells, and T-cell receptor αβ+, CD3+ T-lymphocytes (1, 7, 8). Activated lymphocyte killing is, like NK cell activity, major histocompatibility complex unrestricted (for a review, see Refs. 1 and 8). Clinical applications for the induction of LAK activity by infusion of IL-2 alone or in combination with in vitro IL-2-activated lymphocytes have been reported as salvage therapy for various types of cancer (9–14).

Immune parameters such as the phenotype of peripheral blood lymphocytes, their in vitro proliferative capacities, and their cytolytic activities have been monitored in such patients.

No significant correlations between fluctuations of these parameters and antitumor responses have been reported thus far (13, 15, 16). However, it is relevant to monitor cytolytic activities in order to ascertain that the administration of IL-2 and/or IL-2-activated lymphocytes indeed has resulted in the desired increment of these activities in vivo. Therefore, the optimal assay conditions for detection of LAK activity must be established.

In order to avoid test-to-test variability in longitudinal studies, cryopreserved lymphocytes obtained on different occasions can be tested simultaneously. Cryopreservation and thawing decrease cytolytic activity of lymphocytes. An 18-h preincubation at 37°C of the thawed lymphocytes ("resting") has been shown to restore NK activity (17, 18). In this study, we determined the optimal resting conditions for lymphocytes obtained from cancer patients before, during, and after treatment with IL-2 and for in vitro IL-2-activated lymphocytes.

Recently, Hank et al. (16) reported that in vivo IL-2-activated lymphocytes become dependent on IL-2 to exert in vitro cytotoxic activity. Additionally, we conclude that the increased level of tumor cell lysis in the presence of IL-2 results from rapid in vitro recruitment of LAK activity. In order to discriminate between these two phenomena, we performed kinetic analyses of IL-2-induced modulations of the cytolytic process.

MATERIALS AND METHODS

Patients. Patients with advanced renal cell cancer were treated in a Phase II trial with IL-2 and IL-2-activated lymphocytes. The therapeutic protocol is summarized in Fig. 1; the clinical results have been reported elsewhere (14). Patients received an initial cycle of IL-2 (EuroCetus, Amsterdam, The Netherlands) at 18 × 106 IU/m2/day as a continuous infusion for 5 days. After a 2-day rest, they underwent leukapheresis using a CS 3000 (Fenwal, Deerfield, IL) for 5 h/day on the subsequent 4 days. The lymphocytes were activated in vitro at 3 × 106 cells/ml with 6000 IU IL-2/ml for 4 to 5 days in 3-liter polyolefin culture bags (PL732; Fenwal). Thereafter, the activated lymphocytes were reinfused on days 12, 13, and 15 of the treatment cycle coupled with a continuous i.v. IL-2 infusion. The activated lymphocytes from the third and fourth leukaphereses were pooled for the third reinfusion. After 3 weeks of rest, this treatment cycle was repeated once. We studied the lymphocytes obtained during 16 treatment cycles administered to 10 patients.

Monitoring Schedule. In Fig. 1, the monitoring points are indicated. Sample 1, peripheral blood obtained 1–14 days before in vivo IL-2 administration; Sample 2, lymphocytes from the first leukapheresis, i.e., 2 days after completion of the 5-day IL-2 infusion; Sample 3, in vitro activated lymphocytes from the first leukapheresis; Samples 4 and 5, lymphocytes obtained from peripheral blood at 2 and 8 days, respectively, after the last infusion of in vitro activated lymphocytes.

Isolation and Cryopreservation of Lymphocytes. Lymphocytes were isolated from heparinized peripheral blood samples by density centrifugation (Ficoll-Isopaque). Cryopreservation in liquid nitrogen and thawing were performed as previously described (17). After thawing, lymphocytes were resuspended at 2 × 106 cells/ml and incubated at 3°C and 5% CO2 for 1 or 18 h in medium containing 20% fetal calf serum before testing.
Fig. 1. Treatment protocol of renal cell cancer with IL-2 plus in vivo activated lymphocytes in relation to monitoring schedule. Sample 1, peripheral blood, 1 to 14 days before in vivo IL-2 administration. Sample 2, first leukapheresis. Sample 3, first batch of in vitro IL-2-activated lymphocytes. Samples 4 and 5, peripheral blood obtained 2 or 8 days after infusion of the last batch of in vitro activated lymphocytes.

Table 1 Recruitment of LAK activity by preincubation with IL-2 and/or the presence of IL-2 in the CTX assay.

<table>
<thead>
<tr>
<th>Lymphocyte sample</th>
<th>IL-2 incubation before CTX (min)</th>
<th>IL-2 during CTX assay</th>
<th>CTX assay (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After in vivo IL-2 treatment (Sample 2)</td>
<td>0</td>
<td>-</td>
<td>21*</td>
</tr>
<tr>
<td>30</td>
<td>+</td>
<td>5.3</td>
<td>9.5</td>
</tr>
<tr>
<td>60</td>
<td>+</td>
<td>6.1</td>
<td>9.4</td>
</tr>
<tr>
<td>Day 2 after infusion of in vivo activated lymphocytes (Sample 4)</td>
<td>0</td>
<td>-</td>
<td>23.5</td>
</tr>
<tr>
<td>30</td>
<td>+</td>
<td>34.0</td>
<td>58.9</td>
</tr>
<tr>
<td>60</td>
<td>+</td>
<td>38.6</td>
<td>57.2</td>
</tr>
</tbody>
</table>

* Percentage weighted mean of specific lysis for E:T ratio 22.4:1.

Cytotoxicity Assays. Cytotoxic activity was determined in a standard 3-h 51Cr release assay (6). Briefly, varying numbers of lymphocytes were seeded in triplicate in 96-well, round-bottomed microtiter plates (150 μl/well). Target cells labeled with 51Cr (100 μCi/0.5 × 106 cells) were added in a volume of 100 μl/well. At the end of the incubation period (37°C and 5% CO2), supernatants were collected, and 51Cr release was measured.

The effect of IL-2 on cytolytic activity was determined as follows. Effector lymphocytes were incubated in medium with or without IL-2 (1200 IU/ml) in the microtiter plate for 1 h at 37°C before adding target cells. IL-2 concentration during the cytotoxicity assay was 0 or 1200 IU IL-2/ml, and the assay was terminated at 30, 60, or 120 min.

Statistics. The matched pair t test was used to assess the effect of IL-2 on NK and LAK activity separately for the two incubation time conditions. Analyses of variance were carried out to assess the influence of cell donor (patient) and sampling time (Samples 1 to 5) in these experiments.

RESULTS

Resting of Lymphocytes for 18 h versus 1 h Significantly Increases Their NK but not LAK Activity in Medium without IL-2. Cryopreservation and thawing decrease lymphocytic NK (K562) and LAK (Daudi) activities (15, 16, 18). We studied whether resting for 1 h or 18 h would best restore NK and LAK activities. Lymphocytes obtained at five occasions prior to and during immunotherapy were studied because cryopreservation, thawing, and/or resting may have different effects on the lytic activities of these lymphocytes.

Fig. 2 shows the comparison of NK and LAK activities of lymphocytes rested for 1 h (abscissa) or for 18 h (ordinate). WMSL of individual lymphocyte samples after 18 h resting are plotted against WMSL after 1 h resting (WMSL at effector:target ratio 12.5:1). The dots above the line y = x represent increased WMSL after 18 h resting as compared to 1 h resting. The cytotoxicity assays were performed in medium without IL-2. In vitro IL-2-activated lymphocytes (Sample 3) showed high levels of NK (K562) and LAK (Daudi) activities. Peripheral blood lymphocytes obtained after reinfusion of in vitro IL-2-activated lymphocytes (Samples 4 and 5) still showed increased levels of NK and, to a much lesser extent, LAK activities, as compared to preinfusion levels (Samples 1 and 2). These differences were most evident when the resting period was extended from 1 h to 18 h. After pooling of the results of the five sampling points, the effect of 18 h versus 1 h resting was significant for NK activity [mean ΔWMSL (1 SEM) = 8.0% (2.8%) (n = 41); P = 0.01] but not for LAK activity [1.2% (2.1%) (n = 41)]. The effects of 18 h versus 1 h resting on NK and LAK activities were not significant when IL-2 was present during the cytotoxicity assay [NK: mean Δ WMSL (1 SEM) = 4.2% (2.5%) (n = 39); LAK: 3.9% (2.2%) (n = 41)].

IL-2-enhanced In Vitro Cytolysis of Lymphocytes Is Due to Rapid Recruitment of LAK Activity. In vitro major histocompatibility complex-unrestricted cytotoxicity generated in vivo by administration of IL-2 has been suggested to be dependent on the presence of IL-2 during the CTX assay (16). We studied whether an alternative mechanism could also be operational, i.e., whether the IL-2-enhanced cytolysis is due to recruitment of LAK activity.

First, we determined the effects of a 1-h preincubation step with 1200 IU/ml IL-2 on 18-h rested lymphocytes immediately prior to the assay followed by the presence of IL-2 during the assay. Lymphocytes preincubated and tested in medium without IL-2 served as controls. Fig. 3 shows the comparison of NK and LAK activities of lymphocytes in the presence versus the absence of IL-2. As expected, in vitro IL-2-activated lympo-
LAK RECRUITMENT BY IL-2 IN THE CYTOTOXIC ASSAY

Target cell: K562

Fig. 2. Resting frozen and thawed lymphocytes for 18 h enhances NK (K562) but not LAK (Daudi) activity. NK activity was studied on 38 lymphocyte samples obtained from 17 patients, and LAK activity was studied on 39 lymphocyte samples of 7 patients. Samples 1 to 5 are explained in "Materials and Methods." Cryopreserved lymphocytes were thawed and subsequently rested for 1 h or 18 h (see "Materials and Methods"). CTX assays were performed in medium without IL-2. The percentages of specific lysis obtained at the E:T ratios 50:1, 25:1, 12.5:1, and 6.25:1 were transformed to weighted mean of specific lysis % (see "Materials and Methods"). The % WMSL obtained for individual samples after 18 h resting are plotted against % WMSL after 1 h. The lines represent [% WMSL 18 h] = [% WMSL 1 h].

Fig. 3. Addition of IL-2 to the medium 1 h prior to and during the CTX assay increases LAK (Daudi) but not NK (K562) activities. NK assays were performed on 39 lymphocyte samples from 7 patients and LAK assays on 67 lymphocyte samples from 10 patients. Cryopreserved lymphocytes were thawed and rested for 18 h. Thereafter, the lymphocytes were incubated in medium with or without 1200 IU IL-2/ml for 1 h at 37°C before adding target cells. The percentages of specific lysis obtained at the E:T ratios 50:1, 25:1, 12.5:1, and 6.25:1 were transformed to % WMSL (see "Materials and Methods"). The % WMSL obtained for individual samples in the presence of IL-2 are plotted against % WMSL without IL-2. The lines represent [% WMSL with IL-2] = [% WMSL without IL-2].

cyties show high levels of NK and LAK activity (Sample 3). The dots above the line y = x represent increased lytic activities resulting from preincubation with IL-2 and presence of IL-2 in the assay. After pooling of the results of the five sampling points, preincubation in the presence of IL-2 had no significant effect on NK activity [mean ΔWMSL (1 SEM) = -0.3% (1.5%) (n = 53)]. In contrast, LAK activity was significantly increased [mean ΔWMSL (1 SEM) = 6.1% (0.9%) (n = 79; P = 0.001)]. Analysis of variance showed that this IL-2-mediated increase in LAK activity was dependent on the individual patient from whom the lymphocytes had been obtained (P = 0.003), and the blood sample type (1 through 5) (P = 0.04). For the eight patients with stable or progressing disease, a wide variation of IL-2-mediated increase of LAK activity was observed (mean ΔWMSL computed over all five samples ranging from 0.4 to 14.2%); the two patients showing complete remission had a mean ΔWMSL of 7.3 and 10.6%, respectively (not significant). The average increase was lowest for Samples 1 and 5. We also studied whether the IL-2-mediated increase in LAK activity could be achieved using concentrations <1200 IU/ml. Lymphocyte samples from six patients which showed a clear increase in LAK activity at 1200 IU/ml were chosen. An increase in LAK activity was observed at IL-2 concentrations as low as 30 IU/ml; this effect increased proportionally with the IL-2 concentration used (data not shown).

Second, we studied the kinetics of the IL-2-induced increase in LAK activity. Lymphocytes were obtained after in vivo IL-2 treatment (Sample 2) and at 2 days after the last infusion of in vitro IL-2-activated lymphocytes (Sample 4). LAK activity of these lymphocytes was tested in the absence or presence of 1200 IU/ml IL-2 during the CTX assay after a preincubation step with 1200 IU/ml IL-2 for 0, 30, or 60 min. The duration of the cytotoxicity assays in these experiments also varied (i.e., 30, 60, or 120 min) (Table 1). Sample 2 lymphocytes showed low levels of LAK activity (<10%) in the absence of IL-2 at any time, whereas Sample 4 lymphocytes showed significant LAK activity (>20%) under these conditions. Importantly, lymphocytes that had not been preincubated with IL-2 exerted similar levels of LAK activity in 30-min cytotoxicity assays irrespective of the presence of IL-2. In 30-min CTX assays, IL-2-mediated increase in LAK activity (Sample 2, 2.5% → 6.1%; Sample 4, 22.6% → 38.6%) was positively correlated with the duration of preincubations (0 min → 60 min). The levels of LAK activities also increased proportionally with the duration of the CTX assay. However, in CTX assays of both 60- and 120-min duration, the effect of preincubation with IL-2 on the level of LAK activity reached its maximum at 30 min. These results demonstrated that in this patient, (a) in vivo activated lymphocytes...
LAK RECRUITMENT BY IL-2 IN THE CYTOTOXIC ASSAY

Fig. 4. Kinetics of IL-2-induced increase in LAK activity. Peripheral blood lymphocytes from 4 patients obtained 2 days after the last infusion of in vitro activated lymphocytes (Sample 4) were incubated for 0 to 120 min in 1200 IU IL-2/ml, washed, and subsequently tested for LAK activity in the presence (closed symbols) or absence (open symbols) of 1200 IU IL-2/ml in 30- and 120-min CTX assays. LAK activity expressed at % WMSL at E/T ratio of 14:1 is plotted as a function of the total time of IL-2 incubation (i.e., IL-2 preincubation + duration of the assay).

did not require exogenous IL-2 to exert LAK activity in vitro; and (b) IL-2-mediated recruitment of LAK activity required at least 30 min and reached its maximum at 90 min.

We extended these kinetic studies by investigating in four patients the effect of exogenous IL-2 on LAK activities in CTX assays of 30 and 120 min duration using lymphocytes obtained 2 days after the last infusion of in vitro activated lymphocytes (Sample 4). Again, incubation times of lymphocytes with IL-2 prior to the CTX assay were varied as well (i.e., 0, 30, 60, or 120 min). The results are set out in Fig. 4. All levels of LAK activities in both 30- and 120-min CTX assays were relatively high in Patient 4, intermediate in Patient 1, and relatively low in Patients 2 and 3. Incubation of the effector lymphocytes with IL-2 led to a time-dependent increase in LAK activity in all patients irrespective of the presence or absence of IL-2 in the CTX assay. The magnitudes of these increments varied among the patients. In Patients 1 and 4, preincubation with IL-2 led to a relatively large increase in LAK activity, whereas these increments were relatively small in Patients 2 and 3. The IL-2-induced increase in LAK activity in the 120-min CTX assay did not yet reach maximal levels after 120 min preincubation, irrespective of the absence or presence of IL-2 in the assay, in all patients except Patient 4. These results confirmed those presented in Table 1 in that exogenous IL-2 was not required to sustain LAK activity. In addition, IL-2-induced recruitment of effector lymphocytes could continue for >120 min.

The addition of IL-2 to either 30- or 120-min CTX assays did not lead to large increments in LAK activities of effector lymphocytes, with the exception of those of Patient 4. In this patient, recruitment of effector lymphocytes had already reached its plateau at 60 min preincubation (120 min CTX assay); addition of IL-2 to the CTX assay led to a further increase (10% WMSL). We conclude that this latter increment represented IL-2 dependency of effector lymphocytes. Such dependency might also contribute to the relatively strong effect of IL-2 addition to the 30-min CTX assay observed in Patient 4 as compared to the other three patients.

DISCUSSION

An appropriate comparison of NK and LAK activities of lymphocytes from cancer patients before, during, and after immunotherapy requires cryopreservation and batchwise testing in order to avoid interassay variability. Ideally, NK and LAK activities should not be impaired by cryopreservation and thawing. However, cryopreservation and thawing do decrease NK and LAK activities (17–20). In the present study, we have shown that resting of lymphocytes for 18 h versus 1 h increases their NK but not their LAK activity. These effects of resting were independent of patient or type of lymphocyte sample (i.e., prior to immunotherapy, after IL-2 infusion, after in vitro IL-2 activation, or after reinfusion of activated lymphocytes). Thus, the direct use of frozen and thawed lymphocytes without resting may result in an underestimate of LAK activity as compared to the use of fresh lymphocytes.

Hank et al. (16) reported that addition of IL-2 to the CTX assay significantly increases NK and LAK activities of both fresh and cryopreserved and thawed lymphocytes obtained after IL-2 therapy, whereas lymphocytes obtained prior to IL-2 therapy showed similar NK and LAK activities in the presence or absence of IL-2. In the present study, addition of IL-2 to the CTX assay increased LAK activity but not NK activity. The absence of the enhancing effect of IL-2 on NK activity, observed on frozen and thawed lymphocytes in our study, suggests either that this function has not yet recovered after 18 h resting, or that the lymphocytes mediating this function have been selectively lost by the cryopreservation and thawing procedure. Hank et al. (16) concluded that the lymphocytes mediating LAK activity had become dependent on IL-2 for their in vitro cytotoxic function. We conclude from our kinetic analysis that IL-2-induced increase of LAK activity also results from recruitment of effector lymphocytes. First, we showed that the addition of IL-2 to the CTX assay is not required for the effector lymphocytes to exert LAK activity, although it increased their level of LAK activity (Table 1; Fig. 4). Second, we found that the duration of preincubation of lymphocytes with exogenous IL-2 (0–120 min) correlates positively with the overall level of LAK activity in 30- and 120-min CTX assays in the absence of exogenous IL-2 (Fig. 4). Lymphocytes require the presence of IL-2 for at least 30 min for recruitment of LAK activity. Therefore, the IL-2-mediated increase in LAK activity is only seen in assays lasting longer than 30 min. The kinetics of IL-2-mediated recruitment of effector cells varies among the patients (Table 1; Fig. 4). However, these observations leave the possibility of IL-2 dependency of a subpopulation of effector cells still open. This phenomenon may have occurred in Patient 4,
whose lymphocytes showed an increment in LAK activity upon addition of IL-2 to the 120-min CTX assay, which extended beyond the plateau reached by IL-2 preincubation. It remains to be studied whether the occurrence of IL-2 dependency of effector lymphocytes is restricted to samples with high LAK activity, as observed in Patient 4.

Our findings have two important implications: (a) for assessment of LAK activity: exposure of lymphocytes to IL-2 for periods exceeding 30 min (prior to and/or during cytolysis) may lead to an overestimate of the actual lytic activity in the peripheral blood because of the recruitment of LAK activity; and (b) for immunotherapy: prolonged in vivo administration of IL-2 seems warranted in order to induce and maintain maximal levels of in vivo LAK activity through continuous effector cell recruitment. In this context, it is relevant that the serum IL-2 concentrations obtained in our therapeutic protocol (i.e., around 60 IU/ml) are similar to those necessary to recruit in vitro LAK activity. Our suggestion is supported by the demonstration of an improved response rate (up to 50%) among renal cell cancer and melanoma patients receiving 4 to 5 days of IL-2 administration after infusion of in vitro activated lymphocytes (21), instead of 1 to 2 days in other studies (9–12, 14).

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