Defective Lymphokine-Activated Killer Cell Generation and Activity in Acute Leukemia Patients With Active Disease

By Robert Foa, Maria Teresa Fierro, Alessandra Cesano, Anna Guarini, Margherita Bonferroni, Donatella Raspadori, Roberto Miniero, Francesco Lauria, and Felice Gavosto

In 26 myeloid and lymphoid acute leukemia patients at presentation the capacity to generate interleukin-2 (IL-2)-induced lymphokine-activated killer (LAK) cells effective against the natural killer (NK)-resistant Raji cell line, as well as the susceptibility of the blasts to normal peripheral blood (PB) LAK cells and to autologous LAK effectors was analyzed. The overall PB LAK activity against Raji cells was significantly lower in acute leukemia patients compared with normal controls (mean, 1,473 ± 971 SD LU/10⁶ LAK effectors v 3,540 ± 1,862; P < .001). The sensitivity of the blasts to autologous LAK cells was also significantly lower than to normal LAK effectors (517 ± 593 LU/10⁶ LAK effectors v 1,304 ± 1,086; P < .01). When the data were analyzed independently, four patterns of behavior could be recognized. The relatively largest group (9 of 26) included patients in whom effective LAK cells could be generated against the Raji line, but in whom the blasts were resistant to autologous PB-LAK effectors while being susceptible to normal LAK cells (defective specific LAK activity). In 5 of 26 cases, an incapacity to generate LAK activity against both allogeneic and autologous target cells was observed (defective LAK generation). In six further cases, the blasts were resistant to both allogeneic and autologous LAK populations, though the latter were effective against the Raji line (resistant blasts). The same defects could also be shown with bone marrow-derived LAK cells. Only in six cases did the leukemic blasts appear susceptible to autologous and allogeneic LAK cells. In four patients the analysis could be repeated at remission, and in three a restoration of the LAK function against the primary blasts was recorded. In the 10 cases studied at relapse, the blasts were resistant to autologous LAK effectors in nine and to normal LAK in seven. These data demonstrate that in most acute leukemia patients with active disease, a defect of the LAK machinery, either a deficient generation of LAK cells or the resistance of the blasts to LAK effectors, may be documented, pointing therefore to a possible contributory role of the LAK system in the control of leukemic cell growth. In view of the frequent normalization of the autologous LAK activity at the time of remission, immunotherapy with IL-2/LAK cells should be primarily aimed to patients with minimal residual disease.

THE ROLE OF NATURAL killer (NK) cells in the immunosurveillance against tumor growth is well documented. In the field of hematologic malignancies, many reports have shown a defective NK activity in preleukemic and leukemic patients at diagnosis, while in the remission phase this activity seems restored. Recently, the potential use of adoptive immunotherapy in the management of cancer has received new impulse from the identification of a form of cytotoxicity mediated by previously unrecognized lymphokine-activated killer (LAK) cells. These effectors, generated in vitro in the presence of interleukin-2 (IL-2), are capable of killing a wide variety of fresh solid tumors in vitro (as assessed in a 4-hour ⁵¹Cr release assay) and in animal models. The infusion of recombinant IL-2 (rIL-2) and LAK cells or of rIL-2 alone has been successfully used in the treatment of human solid tumors, particularly renal cell carcinoma and melanoma. We and other investigators have shown that leukemic blasts, both of myeloid and lymphoid origin, are also susceptible to the lytic action of LAK effectors, and that LAK activity can be generated from peripheral blood (PB) lymphocytes of leukemic patients in remission. On the basis of these preclinical studies, immunotherapy with rIL-2/LAK cells is being currently used in the management of acute leukemia patients, particularly in an attempt to eradicate minimal residual disease.

In the present study we evaluated whether the LAK machinery could be impaired in acute leukemia patients at presentation and at relapse in analogy with what occurs for the NK activity, and, should this be the case, which step of the lytic activity was affected. For this goal we investigated the inducibility of LAK effectors from PB and bone marrow (BM) samples infiltrated with a variable proportion of blasts. These effectors, as well as normal LAK cells, were then challenged both with the Raji cell line and with autologous blasts, to distinguish between a defective production of LAK effectors, a defect in the direct lytic action, and an intrinsic insensitivity of the leukemic cells.

MATERIALS AND METHODS

Subjects. Twenty-six acute leukemia samples collected at diagnosis were studied. Eleven were acute myeloid leukemias (AML), 11 common acute lymphoblastic leukemias (CALL), two pre-B-ALL (positive for intracytoplasmic immunoglobulins), one B-ALL, and one a T-ALL. The diagnosis was established by morphologic, cytochemical, and immunologic examination of PB and BM preparations.

In 11 cases the percentage of blasts in the PB was ≥ 80%, in 11 it was between 6% and 50%, and in the remaining four the PB was 0-5%.

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not contaminated with blasts. The percentage of blasts in the BM was always greater than 80%. In four cases the study could be repeated in the remission phase of the disease and in one also at the time of relapse. Nine further cases (8 AML and 1 cALL) were studied only at relapse.

Isolation of PB and BM cells. PB and BM samples from patients and normal individuals were collected in tubes containing preservative-free heparin. Mononuclear cells were recovered from the interface of a Lymphoprep (Nycomed AS, Oslo, Norway) gradient. After two washings, the blast populations were either used as fresh targets or, most often, cryopreserved in liquid nitrogen. Effector cell populations were suspended in RPMI 1640 medium (Flow Laboratories, Opera, Italy) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Flow) and incubated in plastic flasks at 37°C for 1 hour in a humidified incubator. Nonadherent cells were collected, washed once in RPMI medium, and used as effector cells.

Induction of LAK activity. PB or BM mononuclear cells from patients and normal individuals were suspended at a concentration of 1 x 10^6/mL in culture medium (RPMI plus 10% FBS) containing 100 U/mL of rIL-2 (kindly provided by Glaxo Imb, Geneva, Switzerland) and cultured in 25-cm^2 flasks (Flow) in upright position. In a previous study we reported that no major differences in the generation of LAK cells were encountered with 100 and 1,000 U of Glaxo rIL-2/mL. Cells were counted every 3 days, refed with fresh culture medium and rIL-2, and the cell concentration adjusted to 1 x 10^6/mL. Normal PB LAK cells were studied after 3 to 5 days of incubation at 37°C in a 5% CO2 humidified incubator. LAK function from the patients' PB and BM was tested after 7 days incubation, to allow the activation of the residual LAK effectors diluted within the leukemic population. In another study we documented that a shorter incubation time did not allow the generation of effective LAK cells from leukemic samples containing a high proportion of pathologic cells. In several cases the incubation period with rIL-2 was prolonged up to 14 days; because the results did not vary, all reported results will refer to data obtained after 7 days of incubation. The PB LAK activity was evaluated in all leukemic cases. In selected cases this activity was also assessed on BM lymphocytes.

Cytotoxic assay. The Raji cell line and primary leukemic blasts were used as targets of LAK activity in a 4-hour ^5^Cr release assay. For primary samples, either BM or PB (when the percent of blasts was >80%) leukemic cells were used. The target cells were incubated for 1 hour (Raji) or for 2.5 hours (primary cells) at 37°C with 100 μL Na^1^CrO_4_ (New England Nuclear, Dupont, Italy) washed twice with RPMI, and then mixed with various numbers of effectors at final effector:target (E:T) ratios of 100:1, 50:1, 25:1, and 12.5:1 into round-bottom microtitre plates (Flow). For primary cells the incubation time prolonged to 2.5 hours allows a better ^5^Cr incorporation. The plates were incubated at 37°C for 4 hours and centrifuged at 1,200 rpm for 10 minutes. An aliquot (100 μL) of the supernatant was collected and counted in a gamma scintillation counter. All experiments were performed in triplicate and the percentage of ^5^Cr release was calculated according to the following formula: (E - S)/(M - S) x 100 (where E is the mean cpm release in the presence of effector cells; S is the mean cpm spontaneously released by the target cells incubated with medium alone; and M is the mean cpm of 100 μL aliquot of resuspended target cells). Only cases in which the ratio between spontaneous and maximum ^5^Cr release was lower than 25% were included in the study. The cytotoxic activity was determined by plotting the percentage of ^5^Cr release against the E:T ratio and expressed as lytic units (LU) per 10^6_ lymphocytes. An LU was defined as the number of effectors required to produce 40% specific cytotoxicity of 5 x 10^6_ target cells.

RESULTS

PB mononuclear cells from 26 acute leukemia patients at diagnosis containing variable percentages of blasts were examined for their ability to generate LAK effectors in a classic ^5^Cr release assay against the Raji cell line and against autologous leukemic blasts. For each experiment, LAK effectors generated from normal PB lymphocytes were also tested against Raji cells and the leukemic blasts. The overall LAK capacity of the patients' PB to lyse the Raji line was significantly lower (P < .001; Student's t-test), compared with that of normal PB LAK cells (1,473 ± 971 SD LU vs 3,340 ± 1,862). When the susceptibility of the leukemic blasts was investigated, autologous PB LAK cells generated 517 ± 593 LU compared with 1,304 ± 1,066 with normal PB LAK effectors. Again, this difference was statistically significant (P < .01).

When the data were analyzed individually, four different patterns of behavior, which were independent of the lymphoid or myeloid origin of the blasts, could be identified. These are summarized in Fig 1, and detailed in the following paragraphs.

Defective production of LAK effectors. In 5 of 26 cases (19.2%), a very low generation of PB LAK effectors active
against both autologous blasts and the Raji cell line was recorded. This finding was confirmed in one case (no. 3, Table 1) by the failure to generate LAK activity also from BM lymphocytes. On the other hand, in all cases the blasts were susceptible to the lytic action of normal LAK cells.

**Lack of specific lytic activity.** In 9 of the 26 cases tested (34.6%), the patients' LAK cells showed a lytic action towards the Raji line, but, when challenged against their own blasts, they displayed a low autologous lysis. The lack of specific lytic activity was documented by the good sensitivity of the same blasts to the cytotoxic action of normal allogeneic LAK effectors. The behavior of LAK effectors generated in two of these patients from BM lymphocytes (nos. 2 and 5, Table 2) was similar to that of PB LAK cells, the only difference being that of lower, though detectable, cytotoxic values against Raji cells. In three cases we had the possibility of testing the patients' PB lymphocytes on different occasions that corresponded to different clinical situations; at diagnosis a defective specific activity was detected (nos. 1a, 2a, and 3a, Table 2), while in complete remission the PB LAK effectors recovered their capability of lysing their own blasts (nos. 1b, 2b, and 3b, Table 2). Case no. 1 could be further retested at the time of relapse and, once again, the PB LAK cells showed a decreased specific lytic machinery (no. 1c, Table 2).

**Blasts resistant to normal and leukemic LAK effectors.** In six cases (23.1%), despite the generation of a good LAK activity from the patients' PB lymphocytes against Raji cells, the blasts showed a low susceptibility to the lytic action of both autologous and allogeneic LAK effectors. In case no. 3 (Table 3) this held true also for BM LAK cells. Case no. 6 could be retested at the time of remission; although the lytic activity against autologous blasts had improved compared to diagnosis, the LU/10^6 LAK effectors still remained low.

**Blasts sensitive to normal and leukemic LAK effectors.** Only in 6 of the 26 cases tested (23.1%), the leukemic blasts were susceptible to the cytotoxic action of both autologous and allogeneic LAK cells. In two additional cases (nos. 5 and 6, Table 4) the blasts appeared more sensitive to autologous LAK effectors than to normal allogeneic LAK cells. Nine further cases were studied only at the time of relapse. As shown in Table 5, in eight the blasts were almost resistant to autologous LAK effectors, despite displaying in
most cases a good lytic activity against Raji cells. In six cases the blasts showed a low susceptibility also to normal allogeneic LAK cells.

**DISCUSSION**

The results of this study demonstrate that a defective LAK generation or a reduced susceptibility of the blasts to autologous LAK effectors can be documented in the majority of acute leukemia patients, of both myeloid and lymphoid origin, at diagnosis. A detailed analysis of the behavior of each individual acute leukemia case allowed us to recognize four groups of patients that showed distinctive features on the basis of the different patterns of behavior. Patients belonging to group I were unable to produce effective LAK cells against both the Raji line and autologous blasts, despite a normal sensitivity of the blasts to normal allogeneic LAK effectors. These findings indicate that either a global failure of LAK cell generation, or, more interestingly, a selective impairment of the autologous lysis, occur overall in 53.8% of the acute leukemia cases studied at diagnosis.

An additional group of patients (group III) included cases in which the neoplastic cells were resistant to the lytic action of both normal and autologous LAK effectors, though the patients' LAK cells showed a good LAK activity against the Raji line. The final outcome is again an impairment of the LAK system that renders this immune machinery potentially inactive in controlling the leukemia, though via an indirect mechanism. Overall, only in six patients (23.1%) was the LAK function effective in the allogeneic and/or autologous system.

Taken together, the results of this study indicate that in 76.9% of acute leukemia patients studied at diagnosis the overall LAK activity was impaired through different mechanisms, either a specific or a generalized defective generation of LAK effectors, or, less frequently, an inherent resistance of the leukemic blasts to LAK-induced lysis. The possibility that a decrease in the immunodefenses of the host, including both NK and LAK functions, may play a role in the onset and expansion of the leukemic process is intriguing. This possibility has been suggested by the demonstration that a higher NK activity may be found in acute leukemia patients who experience the longest survival.

### Table 3. Defective Susceptibility of Leukemic Blasts to Both Allogeneic and Autologous LAK Effectors

<table>
<thead>
<tr>
<th>Case</th>
<th>Material</th>
<th>Percent of Blasts</th>
<th>Normal LAK v Raji</th>
<th>Normal LAK v Blasts</th>
<th>LAK Leukemia v Raji</th>
<th>LAK Leukemia v Autologous Blasts</th>
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<tr>
<td>1 cALL</td>
<td>PB</td>
<td>14</td>
<td>2,343</td>
<td>451</td>
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<tr>
<td>2 cALL</td>
<td>PB</td>
<td>80</td>
<td>5,588</td>
<td>535</td>
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<td>80</td>
<td>3,063</td>
<td>44</td>
<td>1,475</td>
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<tr>
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<td>BM</td>
<td>90</td>
<td>3,093</td>
<td>295</td>
<td>2,520</td>
<td>750</td>
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<tr>
<td>4 Pre-B-ALL</td>
<td>PB</td>
<td>20</td>
<td>3,765</td>
<td>59</td>
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<tr>
<td>5 T-ALL</td>
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<td>2,062</td>
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<td>5,990</td>
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<td>1,680</td>
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<td>0</td>
<td>4,820</td>
<td>ND</td>
<td>2,102</td>
<td>302</td>
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Mean ± SD (of PB at diagnosis) 3,821 ± 1,634 250 ± 234 1,901 ± 327 255 ± 187

**Table 4. Leukemic Blasts Sensitive to Allogeneic and Autologous LAK Effectors**

<table>
<thead>
<tr>
<th>Case</th>
<th>Material</th>
<th>Percent of Blasts</th>
<th>Normal LAK v Raji</th>
<th>Normal LAK v Blasts</th>
<th>LAK Leukemia v Raji</th>
<th>LAK Leukemia v Autologous Blasts</th>
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<tr>
<td>1 AML</td>
<td>PB</td>
<td>80</td>
<td>3,122</td>
<td>1,240</td>
<td>502</td>
<td>1,319</td>
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<td>PB</td>
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<td>2,957</td>
<td>1,083</td>
<td>682</td>
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<td>PB</td>
<td>41</td>
<td>4,183</td>
<td>4,480</td>
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<td>1,940</td>
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<td>3,824</td>
<td>1,542</td>
<td>2,962</td>
<td>1,220</td>
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<td>1,242</td>
<td>527</td>
<td>1,744</td>
<td>1,021</td>
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Mean ± SD (of PB) 3,269 ± 1,132 1,564 ± 1,485 1,566 ± 1,009 1,499 ± 374
higher in chronic myeloid leukemia patients after BM transplantation who fail to generate IL-2-mediated lytic activity against the leukemic cells. Furthermore, the beneficial effect of viral hepatitis and the apparent anti-leukemic effect of graft-versus-host disease (GVHD) strengthen the potential role of immunosurveillance in the control of leukemia. The possibility that an impairment of one of the steps of the LAK system may play a role in the onset and dissemination of the disease is underlined by the demonstration that, in the remission phase of the disease, the specific lytic activity against autologous blasts is often restored. Furthermore, a deficient autologous killing was documented in all but one of the 10 cases studied at relapse, even in samples devoid of circulating blasts. It is tempting to suggest that a specific lytic defect may occur more frequently at relapse than at diagnosis, stressing the potential role of the cytotoxic compartment in controlling disease progression. A regulatory role of LAK cells is further suggested by the evidence that, following in vivo administration of IL-2 to acute leukemia patients, a marked amplification of the NK and LAK compartment takes place. In addition, in two AML patients who showed a different response to IL-2 treatment, in the responsive one the blasts were lysed by autologous LAK cells, while in the unresponsive one the blasts were resistant (unpublished). Long-term studies are necessary to establish whether the few patients that, at diagnosis, display a good LAK function directed against their own autologous blasts may show a more favorable outcome. Furthermore, horizontal monitoring of individual patients will tell us whether and to what extent the LAK function (both allogeneic and autologous) can be restored following treatment and whether repeated assessments of LAK activity may represent a useful marker of disease. Finally, the demonstration of a defective LAK system in acute leukemias at diagnosis and in relapse argues against the use of an immunotherapeutic approach in patients with active disease, both in terms of in vivo IL-2 administration and of the optimal timing for ex vivo generation and collection of autologous LAK effectors. This theory is corroborated on clinical grounds by the recent demonstration that rIL-2 alone is of little value in AML patients with a high marrow blastosis. On the other hand, in agreement with the demonstration that in remission the LAK function may be restored, in three of five patients with a small proportion of residual marrow blasts a complete remission has been documented with IL-2 alone. Thus, the evaluation in an autologous setting of the susceptibility of the leukemic population in a given patient in different phases of the disease may be crucial for selecting the optimal timing for a potentially effective immunotherapeutic intervention.

ACKNOWLEDGMENT

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REFERENCES


Table 5. LAK Scenery in Acute Leukemia Patients at Relapse

<table>
<thead>
<tr>
<th>Case</th>
<th>Material</th>
<th>Percent of Blasts</th>
<th>Normal LAK v Raji</th>
<th>Normal LAK v Blasts</th>
<th>LAK Leukemia v Raji</th>
<th>LAK Leukemia v Autologous Blasts</th>
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<td>1 AML</td>
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<td>4,566</td>
<td>1,225</td>
<td>1,322</td>
<td>1,432</td>
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</tbody>
</table>

Mean ± SD 2,639 ± 1,242 463 ± 537 1,809 ± 1,878 280 ± 446


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